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TITLE

THYROID HORMONE RECEPTOR AND ALBUMIN GENE
EXPRESSION IN XENOPUS LAEVIS OVARY
AND LIVER.

AUTHOR

Alan R. Brooks.

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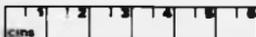
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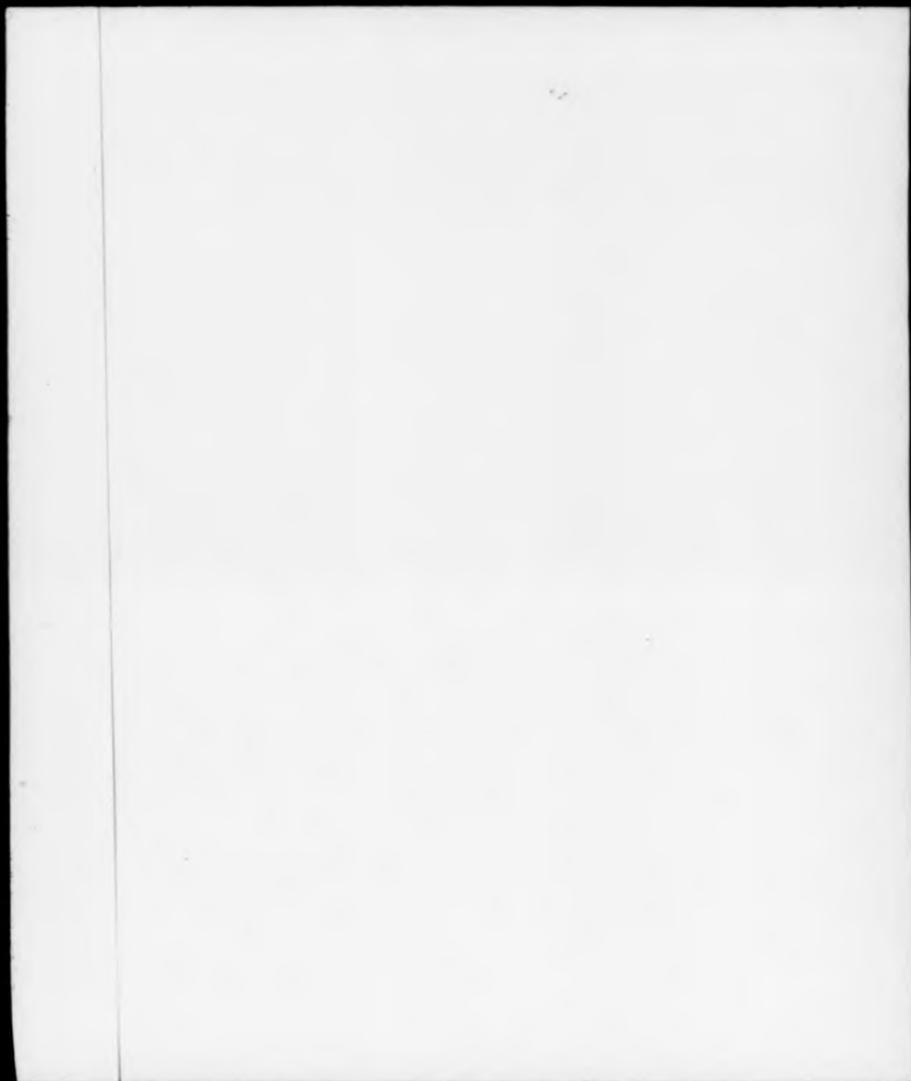


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THYROID HORMONE RECEPTOR AND ALBUMIN GENE

EXPRESSION IN XENOPUS LAEVIS OVARY

AND LIVER.

A thesis submitted for the degree of doctor
of philosophy.

Alan R. Brooks.

Animal molecular genetics group,
Department of Biological Sciences
University of Warwick.

November, 1989.

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Acknowledgements.

I gratefully acknowledge the help of my supervisor Bob Old throughout the course of this project. My thanks are also due to Glen Sweeney for his advice and encouragement when things were going badly, and for supplying various materials. I also thank Darrin Smith for proofreading the manuscript.

I also acknowledge John Shuttleworth for the kind gift of the Xenopus laevis mature oocyte cDNA library, David Moore for providing the plasmid pTK28mult, Mary Weiss for the plasmid pUMSΔE1, and Gerald Crabtree for the plasmids B5400 and B120.

Financial support was provided by the Medical Research Council.

Declaration.

All the results presented in this thesis were obtained by the author, apart from those that are specifically indicated in the text. All the oocyte injections were performed by Bob Old.

All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been used for any previous application for a degree.

Dedication.

To my parents, without whom

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SUMMARY.

I have isolated a full length cDNA clone encoding a thyroid hormone receptor from a Xenopus laevis mature oocyte cDNA library. This receptor binds thyroid hormone with high affinity when expressed in COS cells. Expression from the thymidine kinase promoter containing an artificial thyroid hormone response element could be induced by thyroid hormone in the presence (but not in the absence) of this receptor. Thyroid hormone receptors isolated from other organisms are known to act directly upon gene expression at the level of transcription by binding to regulatory sequences within thyroid hormone responsive genes. My results with the Xenopus thyroid hormone receptor are consistent with this mode of action.

There appear to be several rare transcripts in X.laevis ovary which hybridised to the thyroid hormone receptor cDNA clone. These transcripts could not be detected in eggs, suggesting that they are located in the follicle cells rather than the oocyte itself.

Several rare transcripts which hybridised to the cDNA clone are also present in early embryonic stages. These differently sized but clearly related transcripts are suggestive of differential splicing. Synthetic RNA prepared from the entire cDNA translates very poorly *in vitro* and this is almost certainly due to the presence of several AUG codons upstream of the long open reading frame.

Thyroid hormone is known to play a vital role in inducing the many biochemical and physiological changes that occur during amphibian metamorphosis. It is almost certain that some of these changes are the result of the activation and repression of specific genes at a transcriptional level. I have demonstrated that transcription of the Xenopus albumin gene is activated at the onset of metamorphosis. The albumin gene are therefore candidates for genes whose expression is activated by thyroid hormone. I have tested the response of a cloned X.laevis 68Kda albumin gene to thyroid hormone in a transient transfection assay. From this experiment I tentatively conclude that thyroid hormone does increase expression of the 68Kda albumin gene, at the level of the messenger RNA, and that this increase requires the presence of the co-transfected (Xenopus) thyroid hormone receptor.

The liver-specific expression of albumin genes from Xenopus and other organisms is known to be controlled at the level of transcription by liver-specific trans-acting factors which interact with regulatory sequences upstream of these genes. *In vivo*, in the developing liver, transcription of the Xenopus albumin genes might require the presence of both tissue-specific trans-acting factors and the thyroid hormone receptor complex.

Abbreviations.

ATP, dATP,	adenosine triphosphate, deoxyadenosine
ddATP	triphosphate, dideoxyadenosine triphosphate.
AR	androgen receptor.
bp	base pair.
cDNA	complementary DNA.
Ci	Curie.
CIAP	calf intestinal alkaline phosphatase.
CTP, dCTP,	cytidine triphosphate, deoxycytidine
ddCTP	triphosphate, dideoxycytidine triphosphate.
DNA	deoxyribonucleic acid.
DTT	dithiothreitol.
dTTP,	Thymidine triphosphate, deoxythymidine
ddTTP	triphosphate.
EDTA	Ethylenediaminetetra acetic acid.
ER	estrogen receptor.
GR	glucocorticoid receptor.
GTP, dGTP,	guanosine triphosphate, deoxyguanosine
ddGTP	triphosphate, dideoxyguanosine triphosphate.
HSV	herpes simplex virus.
Kb	kilobase pairs.
Kda	kilodaltons.
l	litre.
ml	millilitre.
Mops	3-(N-morpholino) propanesulfonic acid.

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MR	mineralocorticoid receptor.
ng	nanogram.
nt	nucleotide.
ORF	open reading frame.
pfu	plaque forming units.
PG	picogram.
rATP	Adenosine triphosphate.
rCTP	Cytidine triphosphate.
rGTP	Guanosine triphosphate.
rTTP	Thymidine triphosphate.
PR	progesterone receptor.
RAR	retinoic acid receptor.
rGH gene	rat growth hormone gene.
RNA	ribonucleic acid.
rpm	revolutions per minute.
rRNA	ribosomal RNA.
rTR	rat thyroid hormone receptor.
rUTP	uridine triphosphate.
S	Svedberg.
SDS	sodium dodecyl sulfate.
SV40	simian virus 40.
T3	triiodothyronine.
TK	thymidine kinase.
TR	thyroid hormone receptor.
Tris	Tris(hydroxymethyl)aminomethane.
tRNA	transfer RNA.

μg	microgram.
μl	microlitre.
v/v	volume/volume.
w/v	weight/volume.
αMHC	alpha myosin heavy chain.

Introduction

CHAPTER 1

INTRODUCTION

Determining the mechanisms by which the expression of eukaryotic genes are controlled is of prime importance if we are to understand how a single cell, the fertilised egg develops into a complex multicellular organism. The identity and function of a differentiated cell ultimately depends upon the pattern of gene expression within that cell. From a simplified point of view, the process of development and differentiation is the result of a specific temporal and spatial pattern of differential gene expression. Thus, a lot of work has been focused upon determining the mechanisms by which particular genes are expressed in one cell type but not in others.

The transmission of information from gene to protein is extremely complex, involving many steps, the precise details of which are only now being elucidated. Therefore there are many steps at which gene expression may be regulated. It is now becoming clear that the expression of significant numbers of genes is at least partially controlled post-transcriptionally. There are many examples in the literature where differential gene expression is controlled by stabilisation of the mRNA. The discovery that the primary transcripts of some genes are subject to alternative splicing provides an additional point for the control of gene expression. There is at least one example

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of the developmental regulation of alternative splicing (Troponin T in the chicken embryo; Cooper and Ordahl, 1985). There are also examples of translational control (for a recent example see Miller and Hinnebusch, 1989).

However, it is now becoming clear that for the majority of genes the primary control point is at the level of transcription. As yet very little is known about the nature of the signals and biochemical pathways which bring about transcriptional changes. However, significant progress has been made towards understanding the mechanisms of transcription initiation. It has become clear that the frequency of transcription initiation depends upon protein factors that interact with DNA sequence elements within genes. An increasing number of these trans-acting factors are being identified and the genes for some of them have been cloned. The next step will be to try and understand how the expression and/or activity of the transcription factors themselves is regulated.

1.1 The control of eukaryotic mRNA synthesis.

A variety of methods have led to the identification of DNA sequences required for the transcription of genes by RNA polymerase II (for more details see the reviews by Dynan and Tjian, 1985, Maniatis et al, 1987). It is now clear that these sequence elements can be broadly divided into two groups; promoter elements and enhancers.

Introduction

Promoters are located immediately upstream of the start of transcription and are typically no more than 150bp in length. The promoter is required for accurate and efficient initiation of transcription and contains a binding site for RNA polymerase. Promoters have a modular structure, being composed of a variable number of clearly defined sequence elements (see the review by Dynan, 1989). The majority of RNA polymerase II promoters contain an AT rich motif known as a TATA box located about 30 base pairs upstream of the transcription initiation site. The primary function of the TATA box appears to be to ensure that transcripts are initiated at the correct start site. In addition to a TATA box, promoters may also contain one or more sequence elements that are required for the maximal activity of the promoter. Some of these such as the CAAT box and SP1 box are found in the promoters of many genes and confer a basal level of transcription (see the review by Dynan and Tjian, 1985). Other sequence elements are required for correct tissue-specific transcription (for example the octamer motif found in the promoter of immunoglobulin heavy chain genes (Schaffner, 1989), and the HPI element found in the promoters of several liver-specific genes (Courtois *et al.*, 1987; Kugler *et al.*, 1988; Hardon *et al.*, 1988; Courtois *et al.*, 1988). Some promoter elements are responsible for conferring inducible transcription (for example the AP1 site confers inducibility by phorbol esters (see the review by Lenardo

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and Baltimore, 1989)).

Enhancers are distinct from promoters in that they can affect transcription at great distances from the transcription start site. They are also characterised by the ability to function in both orientations and when placed upstream or downstream of the transcription start site. Enhancers often play crucial roles in tissue-specific transcription whilst others are responsible for inducible transcription, for example by steroid hormones (see Maniatis et al, 1987 for a review).

In almost all cases both enhancers and the conserved sequence elements within promoters have been shown to specifically interact with proteins called transcription factors. This interaction leads to the enhancement or repression of the rate of transcription initiation. Based upon work with the prokaryotic transcription factors lambda and cro, it is thought that eukaryotic transcription factors activate transcription via protein-protein interactions (see the review by Ptashne, 1988). However, the exact details of how protein factors, especially those bound to sites distant from the promoter (enhancers), activate transcription remain to be elucidated. Only a small number of negatively acting transcription factors have been identified but there is some evidence that they may function by blocking the binding sites on DNA for other positive transcription factors (for example; Barberis et al, 1987).

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An increasing number of transcription factors are being purified and a number have been cloned. Structural and functional analysis has shown that the different transcription factors are usually composed of a variety of separable DNA binding and trans-activation domains.

Several distinct types of DNA binding domain have now been identified in eukaryotic transcription factors (for a review see Mitchell and Tjian, 1989). Zinc finger motifs were first identified as being important for the DNA binding activity of the RNA polymerase III transcription factor TFIIIA (Miller *et al.*, 1985). Since then at least two distinct classes of zinc finger motif have been identified in RNA polymerase II transcription factors (see Evans and Hollenberg, 1988). In the first class, typified by SP1, two cysteine and two histidine residues stabilise the domain by tetrahedrally coordinating a zinc ion. A second class is typified by the DNA binding domains of the steroid hormone receptors. Here a zinc ion is tetrahedrally coordinated by two pairs of cysteine residues rather than the histidine-cysteine arrangement found in SP1. Both SP1 and the glucocorticoid receptor have been shown to require zinc for DNA binding activity (Kadonga *et al.*, 1987; Freedman *et al.*, 1988), and mutational analysis has confirmed the importance of the conserved cysteine residues in the DNA binding domain of the glucocorticoid receptor (Giguere *et al.*, 1986; Hollenberg and Evans, 1988). The steroid hormone type DNA

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binding domain will be discussed in more detail in section 1.4.4.

A second type of DNA binding domain is the so called homeodomain (for a review see Levine and Hoey, 1988). This domain was initially identified as a conserved region in a number of *Drosophila* proteins that have crucial roles in embryogenesis. Since then the homeodomain has also been found in vertebrate genes. The homeodomain is distantly related to the helix turn helix motif of prokaryotic transcriptional activators.

The cloning of three mammalian transcription factors oct-1, oct-2 and pit-1 and unc-86, a regulatory protein from *Caenorhabditis elegans* lead to the discovery of a new class of transcription factors related to the homeodomain containing proteins (for a review see Levine and Hoey, 1988). These four proteins all contain a bi-partite domain termed the POU domain which consists of the homeodomain and a second region the POU box. More recently, several other POU domain containing genes have been isolated. The distinct tissue distributions of their mRNAs suggest that they may play a role in brain development and differentiation (He et al, 1989).

A third type of DNA binding domain has been identified in the mammalian transcription factors jun, fos, C/EBP and CREB (for a review see Mitchell and Tjian, 1989). This consists of a conserved stretch of 30 amino acids which have a net negative charge, followed by four leucine

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residues at intervals of seven amino acids. The leucine region (known as the leucine zipper) mediates dimerisation (between identical or different monomers) and this is essential for DNA binding. It is thought that the DNA binding domain is created by dimerisation and that both the basic region and the leucine region contribute to the DNA binding domain.

The number and types of DNA binding domain is certainly not limited to these four because several transcription factors do not fit in to any of these categories.

The regions of transcription factors responsible for activation of transcription have been less well defined than those responsible for DNA binding. Activation domains have been identified in only a small number of transcription factors and so far several types have been identified. One of these consists of a short stretch of amino acids the only significant features of which are an overall negative charge and the ability to form an amphipathic helix (see Ptashne, 1988). This so called acidic domain has been functionally identified only in GCN4 and GAL4. However, one of the activation domains of the glucocorticoid receptor resembles this type of structure (Hollenberg and Evans, 1988). It has been proposed that acidic activation domains could make contacts with other components of the transcriptional machinery, such as TFIID, and thereby activate transcription (Ptashne, 1988).

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Another type of activation domain was identified in the transcription factor SP1. The most notable feature of this domain is that it is rich in glutamine residues. A third type of activating domain consisting of a stretch of amino acids rich in proline has been identified in the CAAT box transcription factor, CTF (see the review by Mitchell and Tjian, 1989).

A big question which remains to be answered is how transcription factors themselves are regulated. Many transcription factors are phosphoproteins so their activity could be regulated by phosphorylation/dephosphorylation. This provides a possible link between transcription factors and the second messenger system present in many cells. The activity of one transcription factor, NF κ B, is known to be regulated by a second protein; I κ B. I κ B binds to NF κ B in the cytoplasm and prevents it from activating transcription (for a review see Lenardo and Baltimore, 1987). Treatment with phorbol esters results in the dissociation of I κ B and the subsequent transcriptional activation of various lymphoid-specific genes. For steroid and thyroid hormone receptors, transcriptional activation can only occur when the receptor has formed a complex with its cognate hormone. There is now at least one example of the transcriptional control of the expression of a transcription factor (Xanthopoulos *et al.*, 1989). The transcription factor C/EBP

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is an enhancer binding protein thought to be involved in the liver-specific transcription of a number of genes. The mRNA encoding this protein is found at higher concentrations in liver cells than in most other cell types. Nuclear run-on experiments indicated that the restricted distribution of the mRNA was due to transcriptional regulation of the gene.

1.2 The involvement of chromatin structure in gene regulation.

Any model for the control of eukaryotic gene expression must take into account the fact that inside cells the DNA is complexed with a variety of proteins in the form of chromatin. Most genes that are being expressed (as well as the non-expressed ones) are organized into nucleosomes. However, the chromatin structure of expressed genes has often been found to be significantly different from those which are transcriptionally inactive (for a review see Gross and Garrard, 1987). Transcriptionally active genes often contain nuclease hypersensitive sites. These sites are now known to be caused by the binding of trans-acting factors to cis-acting regulatory elements (for example; Emerson *et al.*, 1985). Histone H1 is responsible for the compaction of nucleosomes into the 300A⁰ fibre, and active genes may also be organised into a decondensed chromatin

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structure via the depletion of histone H1. There is some evidence that some active genes may be under torsional stress and that this maybe necessary for transcriptional activation. Active genes may also be undermethylated. Since the state of methylation is maintained through semi-conservative DNA replication it has been proposed that this might provide a mechanism for the stable maintainance of patterns of gene expression (for example; Groudine and Conkin, 1985).

The establishment of this active chromatin state is believed to be initiated by the binding of transcription factors to cis-acting regulatory sequences (see Gross and Garrard, 1987). This may require DNA replication since nucleosomes have been shown to prevent binding of some transcription factors (Emerson et al, 1985). Several lines of evidence suggest that the interactions between transcription factors and cis-acting DNA elements may only be required to establish transcriptionally active chromatin and not to maintain or propagate it (for example: Wang and Calame, 1986). This provides a possible mechanism for the maintainance of patterns of gene expression through cell division.

The DNA in both mitotic and interphase nuclei is organised into loops of 5 to 100Kb that are anchored to what is called the nuclear matrix. A class of sequences termed matrix association regions have been defined that may mediate loop attachment (Cockerill and Garrard, 1986).

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Interestingly, these sequences are sometimes found close to transcriptional enhancers. It has been suggested that genes within each loop could be coordinately regulated by the conversion of a whole loop from an inactive to an active chromatin configuration (see Gross and Garrard, 1987). This provides an explanation of how enhancers are able to effect transcription over such large distances. Binding of trans-acting factors to an enhancer, that may be located at the base of a loop, could lead to the induction of an active chromatin domain that spans the whole loop. This in turn would lead to the activation of genes many thousands of base pairs away from the enhancer.

1.3 Liver-specific gene expression.

When I began this project very little was known about the mechanisms underlying liver-specific gene expression. In the past three years the development of new techniques for analysing the interactions between proteins and DNA has lead to tremendous progress in this field. In the section which follows I will describe what was known when I began this project, and then go on to describe in sections 1.3.2 and 1.3.3 the current state of research into liver-specific gene expression.

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1.3.1 Liver-specific gene expression is controlled mainly at the level of transcription.

In 1981 Darnell and his co-workers (Derman et al., 1981) isolated several cDNA clones encoding mRNAs that were enriched in mouse liver. cDNA was prepared from unfractionated mouse liver RNA, thus selecting for those mRNAs that were most abundant. The resulting cDNA clones were screened with polyA⁺ RNA prepared from mouse liver and mouse L-cells. In this way several cDNA clones specific to liver and several common to both liver and L-cells were isolated. Both groups of cDNA clones were used to determine the rate of transcription of their corresponding genes in mouse liver and brain. This was done by pulse labelling of the RNA being synthesised in isolated nuclei. The results showed that the nuclei from brain cells did not transcribe the liver-specific genes at all or only at a very low level. The common genes were transcribed at similar rates in both cell types. This suggested that transcriptional control was responsible for the liver-specific expression of these moderately abundant genes. Tilghman and Belayew (1982) showed that the accumulation of alpha fetoprotein mRNA in the liver of the developing mouse foetus and its decline at birth was the result of alterations in the rate of transcription.

The finding that the tissue-specific expression of many liver-specific genes was controlled at the level of

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transcription lead to the search for the *cis*-acting DNA sequences responsible. Much progress has been made in this area and in the identification of the *trans*-acting factors that interact with them, and which may be ultimately responsible for liver-specific expression. This work is outlined in the following section.

1.3.2 The control of liver-specific transcription.

Several liver-specific genes have been studied in great detail with respect to the *cis*-acting DNA sequences required for their tissue-specific transcription and the factors that interact with them. These include the rat and mouse serum albumin genes, the human alpha-1 antitrypsin (α 1AT) gene and the rat alpha and beta fibrinogen genes.

The rat albumin gene has been studied by Yaniv and co-workers (Cereghini *et al.*, 1987; Heard *et al.*, 1987; Cereghini *et al.*, 1988). Classical deletion analysis of the 5' flanking region by transfection into hepatoma cell lines indicated that the first 150bp upstream of the transcription start site contained all of the critical sequences necessary for tissue-specific expression. DNaseI footprinting experiments with nuclear proteins prepared from cell lines which express albumin showed that at least six distinct proteins were bound to this region. One of these covers the TATA box and a second covers the CAAT box. Three of the remaining footprints lie in the region

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upstream of the CAAT box and will be referred to as distal elements (DE) I, II and III. DEII appears to bind a protein related to the transcription factor NF-1. The sixth footprint lay in a region between the CAAT box and the TATA box and was designated the proximal element (PE). When footprinting was done with proteins prepared from de-differentiated hepatoma cells that do not express the albumin gene a subtle difference was found. The pattern of DNaseI protection on the three distal elements was identical to that seen with extracts from differentiated (albumin expressing) cell lines. However there was a clear difference in the interaction at the proximal element. The footprint in this region was significantly shorter with nuclear proteins prepared from albumin negative hepatoma cell lines. When footprinting was carried out with a mixture of extracts from albumin expressing and non-expressing cell lines, the non-expressing pattern dominated. This suggested that albumin negative cells contain a dominant, negatively acting factor.

Further characterisation of the protein interactions occurring on the proximal element showed that two mutually exclusive proteins were interacting with this region. One of these, designated albumin promoter factor (APF) is found only in liver or differentiated hepatoma cell lines. The other protein termed variant APF (vAPF) is present only in those cell lines not expressing albumin. Both proteins have identical sequence specificities but differ

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in molecular weight and thermostability. It has been known for some time that when somatic hybrids are formed between differentiated hepatoma cells and cells not expressing liver-specific functions, the liver-specific functions are lost. This phenomenon, known as extinction, could reflect the presence of a dominant, negatively acting transcription factor in those cells not expressing liver-specific functions. In one such somatic hybrid it was shown that the APF protein was lost and that vAPF appeared. This suggests that vAPF is the negatively acting factor responsible for extinction of liver-specific functions.

In vitro transcription assays have shown that APF is essential for efficient transcription from the rat albumin promoter. In addition, both APF and vAPF will interact with homologous sequence elements present in the promoters of several other liver-specific genes (this will be discussed in more detail in section 1.3.3). The simple model prompted by these results is that vAPF is a negative transcription factor that is present in cells of non-hepatic lineage. By binding to the proximal element of the albumin promoter and similar elements in other liver-specific genes, it prevents the formation of an active transcription complex. In hepatic cells, vAPF is absent and APF binds to the PE. This is the crucial step which allows binding of all the other proteins in a productive way to form an active transcription complex. However, it

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cannot be as simple as this because extinction in somatic hybrids often involves the loss of only one liver-specific function. vAPP is known to interact with the promoters of several liver-specific genes so the simple model would predict that expression of all these would be extinguished. Also, extinction is seen in fusions with fibroblasts that do not have vAPP.

Deletion analysis of the human α 1AT gene showed that sequences between 37 and 137bp upstream of the CAP site were crucial for liver-specific expression (Simone et al., 1987; Monaci et al., 1988). Within this region two domains were identified by DNaseI footprinting and site directed mutagenesis (Monaci et al., 1988). These have been designated the A and B domains. In addition there are at least two other regulatory elements located upstream of the A and B domains that are required for maximal transcription. However, these two elements are active in both liver and non-liver cells. The protein which binds to the A domain has been called LFA1 and the protein which binds to the B domain was called LFB1. Both proteins have been partially purified. In an in vitro transcription system double stranded oligonucleotides corresponding to either the A or B domain inhibit transcription from the wild type promoter, indicating that binding of LFA1 and LFB1 is essential for transcription. When an oligonucleotide containing the B domain was used as a probe in gel retardation assays it was found that two

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distinct proteins found in liver nuclear extract were binding in this region. These two proteins were designated LFB1 and LFB2. By using shorter oligonucleotides derived from the B domain it was shown that that these two proteins had different but overlapping sequence specificities. LFB1 bound to a region on the right side of the B domain whilst LFB2 bound to a region on the left of the B domain. Further experiments indicated that the binding of LFB1 and LFB2 to the B domain was mutually exclusive. In an in vitro transcription reaction in liver nuclear extract, an oligonucleotide containing only the LFB1 binding site was as effective as the whole B domain at reducing transcription. This indicated that LFB1 was required for efficient transcription. In contrast, an oligonucleotide containing just the LFB2 binding site did not reduce transcription at all, indicating that binding of LFB2 to the B domain was not required for transcription in liver nuclear extract. Nuclear extracts prepared from spleen, where the α 1AT gene is not transcribed, did not generate DNaseI footprints in either the A domain or on the TATA box. However, spleen extract did protect the B domain from digestion with DNaseI. This footprint was very similar (but not identical) to that seen with partially purified LFB2 on the B domain, and was specifically competed by an oligonucleotide containing the LFB2 binding site. The protein responsible for this footprint must therefore be very similar (if not identical) to the LFB2

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protein found in liver. This LFB2-like protein is more abundant in spleen than LFB2 in liver.

In summary, LFA1 and LFB1 are both required for efficient transcription in liver nuclear extract, and both are absent from spleen. LFB2 is found in both liver and spleen extracts, but is more abundant in spleen, and is not required for transcription. In liver extract, binding of LFB1 and LFB2 to the B domain is mutually exclusive. This suggests that LFB2 might be a repressor which acts by preventing the binding of the activator, LFB1. It could be that a fine balance between the concentrations of LFB1 and LFB2 is responsible for liver-specific transcription. In liver, where the concentration of LFB1 is high and LFB2 is low, transcription of the α 1AT gene is activated. In spleen the concentration of LFB2 is high, and LFB1 is virtually non-existent resulting in repression. The sequence of the B domain from the human α 1AT promoter shows a high level of homology with the proximal element of the rat albumin promoter defined by Yaniv and co-workers. The PE was also shown to bind two mutually exclusive proteins (called APF and vAPF) one of which could be a repressor.

Li et al (1988) also reported that two regions within the human α 1AT promoter were protected from DNaseI digestion by liver nuclear extract. These regions correspond exactly to the A and B domains identified by Monaci et al (1988). Li et al (1988) showed that proteins

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binding to these domains are positive transcription factors. In extracts from various cells and tissues (including spleen) that do not express the $\alpha 1$ AI gene, they did not detect any proteins binding to either of these domains. This is in contrast to the results reported by Monaci et al (1988) who reported that in spleen extracts the B domain did bind a protein, LFB2 that could be a repressor. This apparent contradiction must reflect slight differences in experimental technique.

By transfection into liver cell lines, Courtois et al (1987) showed that sequences between 117 and 78bp upstream of the CAP site of the rat β -fibrinogen promoter were essential for liver-specific transcription. However, sequences outside of this region were required for maximal transcription. Extracts from differentiated liver cell lines produced two DNaseI footprints in the first 300bp upstream of the CAP site. The regions covered by these footprints will be referred to as the distal element (centred around -135) and the proximal element (centred around -90). The proximal element (PE) thus corresponds to the region defined as being important for transcription in transfected liver cell lines. The footprint on the distal element was also generated with extracts from non-liver cell lines whilst the footprint on the PE was specific to liver cell nuclear extracts. The liver-specificity of the protein interacting with the PE was confirmed in gel retardation assays. This protein was designated HNF-1

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(hepatocyte nuclear factor 1). In further experiments (Baumhueter et al., 1988) it was shown that a de-differentiated variant of the hepatoma cell line Fao had lost HNF-1 but gained a related protein, vHNF-1 (v for variant). Reversion of this de-differentiated cell line results in the re-expression of many liver-specific functions and is associated with the re-appearance of HNF-1. In somatic cell hybrids between liver cell lines and fibroblasts, which show extinction of liver-specific functions, HNF-1 is lost and vHNF-1 appears. Thus the presence of HNF-1 and the absence of vHNF-1 correlates with liver-specific transcription. vHNF-1 was shown to have the same or similar sequence specificity to HNF-1. These authors suggested that vHNF-1 may act as a repressor of liver-specific gene transcription by binding to the PE and preventing binding of HNF-1. It is tempting to speculate that since vHNF-1 is 20kd smaller than HNF-1, it may lack a domain essential for the activation of transcription. These findings are analogous to those reported for the rat albumin promoter (APF/vAPF) and the human α 1AT promoter (LFB1/LFB2). Indeed, the sequence elements recognised by these three pairs of factors in the three promoters show significant sequence similarity, and are almost certainly the same proteins. This will be discussed in more detail in section 1.3.3.

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The interaction of nuclear proteins with the mouse albumin promoter has been studied in great detail by Schibler and co-workers (Lichtsteiner et al., 1987; Gorski et al., 1986); Maire et al., 1989). Deletion analysis indicated that 170bp upstream of the CAP site were sufficient for efficient liver-specific in vitro transcription (Gorski et al., 1986). In contrast to these in vitro experiments, Pinkert et al. (1987) reported that in transgenic mice a region located between 10.4 and 8.5Kb upstream of the CAP site of the mouse albumin gene was essential for high level expression in adult liver. This far upstream region behaved like an enhancer in that its position and orientation relative to the albumin gene were not critical. Sequences between -8.5Kb and -0.3Kb were not required for efficient expression in adult liver. Thus it would seem that this enhancer is required in vivo but is not necessary for efficient transcription in vitro. This could be interpreted as a requirement for an enhancer to generate an active chromatin configuration in the vicinity of the albumin gene, which is then accessible to other trans-acting factors closer to the CAP site.

Footprinting experiments with liver nuclear extracts (Lichtsteiner et al., 1987) indicated that there were at least six binding sites (A to F) for specific DNA binding proteins between the TATA box and nucleotide position -172. Deletion of site F had very little effect upon the rate of transcription (Gorski et al., 1986). The identities

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of the proteins interacting with the six sites have been determined (Lichtstainer and Schibler, 1989; in press). A comprehensive mutational analysis of the six binding sites has been carried out (Maire et al., 1989). Mutations within sites D and C (which bind C/EBP and NF-Y respectively) reduced in vitro transcription in both liver and spleen nuclear extracts. These sites must therefore bind proteins which are present in both spleen and liver. Mutation of site E which binds an NF-1 like protein, resulted in only a two fold reduction in transcription in liver nuclear extract. Site B had been shown to bind HNF-1 (hepatocyte nuclear factor 1), a protein first identified as being crucial for the liver-specific transcription of the rat β -fibrinogen gene (Courtois et al., 1987). Substitutions within site B had the greatest effect upon transcription in vitro in liver nuclear extract (about a 10 fold reduction). These mutations had no effect upon transcription in spleen nuclear extract. Gel retardation assays showed that the protein binding to site B (HNF-1) was indeed confined to liver cells (i.e. absent from spleen). In a second type of analysis each binding site was placed alone, upstream of the "core" albumin promoter (contains just the TATA box). Site B stimulated transcription more efficiently than any of the other sites in liver nuclear extract, but was completely silent in spleen nuclear extract. Site D (binds C/EBP) also stimulated transcription in liver nuclear extract but less

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so than site B. Site D was also able to stimulate transcription in spleen NE but to a much lesser extent. Site C was equally active in both spleen and liver nuclear extracts. Sites E and F increased transcription only slightly in liver nuclear extract and not at all in spleen NE. Clearly, site B which binds HNF-1, is the most important of the six binding sites in controlling liver-specific transcription. Site D and its cognate protein C/EBP are also important for liver-specific transcription. A number of the other sites are active in both spleen and liver NE and presumably are required to obtain maximum transcription in vivo. In fact there is some evidence that the various proteins may function cooperatively.

The liver-specific expression of the Xenopus 68Kd albumin gene promoter has also been investigated using similar approaches (Schorpp et al, 1988b). The 5' flanking region of the 68Kd albumin gene from +19 to -4200 was linked to the reporter gene CAT. Various deletions of the 5' flanking DNA were prepared and assayed for CAT activity after transfection into one liver cell line and two non-liver cell lines. Surprisingly, deletion of sequences between -4200 and -662 resulted in a large increase in CAT activity in the liver cell line. Sequences between +19 and -77 were sufficient for high expression in transfected hepatoma cells, and produced only low CAT activity in non-liver cell lines. However, deletion down to position -62

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reduced CAT activity by five to ten fold. These results were confirmed at the RNA level. This deletion analysis defined a region between -77 and -62 as being crucial for efficient transcription in transfected liver cells, and this was designated the HPI element (for hepatocyte promoter element 1). A series of point mutations within this region confirmed the importance of the HPI element. An oligonucleotide corresponding to this sequence was used in gel retardation assays with nuclear extracts from various cell lines. The HPI oligonucleotide bound a specific protein in nuclear extract from hepatoma cell lines but no specific binding occurred with extracts from non-liver cell lines. The HPI element was also necessary for in vitro transcription in rat liver nuclear extract. The sequence of the HPI element shows good homology with the binding sites for HNF-1 in the promoters of other liver-specific genes (see section 1.3.3 for more detail).

In the work described so far, sequence elements close to (within 200 bp of) the transcription start site have been shown to play the major role in determining liver-specific transcription. However, it is clear that sequences upstream of this are required for maximum transcription in liver nuclear extracts or transfected cell lines. Darnell and his co-workers (Grayson et al., 1988a; Grayson et al., 1988b; Costa et al., 1988) have identified liver-specific enhancers in both the mouse transthyretin (TTR) gene and

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the mouse α 1AT gene. The enhancer in the TTR gene is located between 1.6 and 2Kb upstream of the CAP site and stimulates transcription ten to thirty fold in transfected liver cell lines. The α 1AT enhancer is located between -190 and -500 and could stimulate transcription to a similar degree. Sequences downstream of -160 were sufficient for only a low level of liver-specific transcription. Thus, for these genes at least, sequence elements upstream of the proximal promoter elements (within 200bp of the CAP site) are required for efficient liver-specific transcription. Both enhancers appear to consist of three distinct binding sites for trans-acting factors. Oligonucleotides corresponding to these binding sites were able to cross-compete for binding in gel retardation assays. These experiments indicated that one site in each of the enhancers interacted with a similar protein that was present in many cell types. The sequence of the binding site suggests that this protein is probably AP1. The remaining four sites (two in each enhancer) appeared to bind the same liver-specific factor. It was therefore suggested that this liver-specific factor could play a major role in the coordinate expression of these two liver-specific genes (and perhaps others as well). Surprisingly, the four binding sites for this factor showed only weak sequence homology. The consensus sequence that was derived seemed to be significant because mutations at conserved residues severely reduced binding

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of the factor. This consensus sequence does not resemble the binding sites for other liver-specific transcription factors that have been identified (APF, HNF-1, LFB1, HP1)

1.3.3 Evidence for a common liver-specific promoter element.

As described in section 1.3.2, the liver-specific transcription of several liver-specific genes has been analysed in some detail. From this research it is clear that several of these genes all contain a related DNA sequence element that plays a crucial role in liver-specific transcription. These sequence elements are shown aligned for maximum conservation in figure 1. The five genes in which this element was initially identified are shown at the top of this figure. Also indicated are the names of the trans-acting factors which interact with these elements. In addition, this sequence element can be found in many other liver-specific genes and these are also shown in figure 1. Interestingly, the alpha fetoprotein gene promoters all have two copies of this element within 150bp of the CAP site. The human hepatitis B virus infects the liver, which explains the presence of this conserved element upstream of the gene coding for the surface antigen protein of this virus. It is not surprising to find such a conserved sequence in, for example the rat and mouse albumin genes, given that the

Figure 1 The "HP1 element" is present in many liver-specific genes.

GENE	PROTEIN	CONSENSUS:
Human alpha-1 antitrypsin	LF81/LFB2	-74 TGGTAAATAATTNACG
Rat albumin	APF/vAPF	-61 TGGTAAATGATGTACG
Rat beta ₂ -fibrinogen	HNF 1/vHNF 1	-81 TAGTAAATATTEACG
Xenopus 68kd albumin	HP 1 BP	-67 AGGTTAAATAATTTCCA
Mouse albumin	HNF 1	-66 TGGTAAATGATCTACG
Human albumin		-66 TAGTAAATAATCTACAA
Human alpha-fetoprotein (-60)		-60 AGGTACTAGTTACAG
Human haptoglobin		-141 CT GTTAAGAGTTGAGCT
Human beta ₂ -fibrinogen		-90 ATTAATATTAC
Human alpha-fetoprotein (-131)		-131 CT GTTAATATTGGC
Human transthyretin		-129 GTTACTTATTCTC
Mouse albumin		-66 TGGTAAATGATCTACG
Mouse alpha-fetoprotein		-65 AGGTACTAGTTACAG
Mouse alpha-fetoprotein		-130 CT GTTAATATTGGC
Mouse transthyretin		-131 GTTACTTATTCTC
Mouse alpha-1 antitrypsin		-74 GTAAT-ATT CAT
Rat alpha-fetoprotein		-65 AGGTACTAGTTACAG
Rat alpha-fibrinogen		-43 AGGTAAATCATCACCT
Rat pyruvate kinase		-91 TGGTAACTTTAACCA
Rat alpha-fetoprotein		-132 CT GTTAAT ATTGG C
Rat transthyretin		-129 GTTACTT ATT CT C
Xenopus 74kd albumin		-66 AGGTAATCATTTT CCA
Chicken albumin		-65 CAGGTAATGTTTT ACAG
Hepatitis B virus		-88 GTTAATCATT- ACT
Human alpha-fetoprotein (enhancer)		-3500 TGATTAATAAATT- ACA

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whole of the proximal promoter region is well conserved. The same is true of the rat and mouse alpha fetoprotein (AFP) gene promoters. However, this element has clearly been conserved in the promoters of unrelated genes which resemble each other only in respect of the fact that they are expressed only or predominantly in liver. This sequence element has also been conserved during evolution between Xenopus and humans. From now on I will refer to this element as the HP1 element. From the comparison shown in figure 1 a consensus sequence can be derived as shown. This element appears to have a degree of dyad symmetry, which is a characteristic of the binding sites of many (although not all) transcription factors.

As outlined in section 1.3.2, four research groups have independently identified a trans-acting factor that interacts with the HP1 element of four different liver-specific genes. These factors have been called APF, LFB1, HNF1 and HP1 binding protein (HP1 BP). In almost all cases the source of this trans-acting factor was the rat (either rat liver nuclear extract or nuclear extracts from hepatoma cell lines). In the case of the rat albumin promoter it was demonstrated that this factor (APF) was also present in mouse and human liver cell lines. These trans-acting factors were found to be absent in tissues or cell lines not expressing liver-specific functions, although the number of tissues and cell lines tested was far from exhaustive. This would indicate that these trans-

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acting factors are restricted to cells or tissues that express liver-specific functions.

Several groups have noticed the presence of the HP1 element in the promoters of several liver-specific genes. Prompted by this conservation they have tested these elements from the different genes in competition experiments for binding of their liver-specific factor (Kugler et al., 1988; Lichtsteiner and Schibler, 1989 in press; Courtois et al., 1987; Courtois et al., 1988; Hardon et al., 1988; Cereghini et al., 1988). Generally, these competition experiments have taken the form of gel retardation assays. The HP1 element from one gene is used as a probe and then the corresponding element from another liver-specific gene is used as a competitor. If competition occurs the two elements must be able to specifically bind the same factor. The results of all these experiments are summarised in figure 2. It should be noted that in no instance have the HP1 elements from two different genes been reported not to compete. The gaps in the table represent situations where cross-competition has not been tested. The results strongly suggest that APF, LFB1, HNF1 and HP1 BP are identical proteins (or at least proteins with identical sequence specificities).

In conclusion, it seems likely that a large number of liver-specific genes contain a common promoter element, the HP1 element, which is important for liver-specific expression. This provides for the coordinate regulation of

Figure 2.

A summary of the binding of four liver-specific transcription factors to the conserved HP1 element present in many liver-specific genes.

The results are those of Courtois et al., 1987; Kugler et al., 1988; Courtois et al., 1988; Hardon et al., 1988; Careghini et al., 1988; Lichtsteiner and Schibler, 1989 in press). In most cases binding has been tested by gel retardation and competition with oligonucleotides containing the HP1 element from various liver-specific genes. There are no reports of any of these proteins not binding to a HP1 homologue.

Figure 2

Cross reaction of liver-specific transcription factors with the conserved HP1 element of various liver-specific genes.

GENE	FACTOR			
	LFBI	APF	HNF 1	HP 1 BP
Rat alpha fibrinogen			✓	
Human alpha-1 antitrypsin	✓	✓	✓	
Rat beta fibrinogen	✓	✓	✓	✓
Mouse albumin		✓	✓	✓
Human albumin	✓			
<u>Xenopus</u> 68kd albumin		✓		✓
Rat alpha-fetoprotein (-65)		✓	✓	
Rat alpha-fetoprotein (-132)			✓	
Rat albumin		✓	✓	
Human alpha-fetoprotein (-60)	✓			
Mouse transthyretin			✓	
Mouse alpha-1 antitrypsin			✓	
Hepatitis B virus			✓	

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liver-specific transcription. Within a single species these elements interact with the same positive transcription factor that is present in liver cells, but not in non-liver cells. Non-liver cells may contain a negative factor (vAPF/vHNF1/LFB2), that is related to this positive factor in that it binds to the same DNA sequence, the function of which is to repress transcription. The HPI element and its cognate factor are certainly not entirely responsible for determining liver-specific transcription. For example, both the mouse α 1AT genes and the mouse TTR genes contain this element within 100bp of the CAP site. Darnell and his co-workers (Costa et al., 1986; Grayson et al., 1988a; Grayson et al., 1988b) have shown that the first 160bp of the mouse α 1AT promoter and the first 202bp of the mouse TTR promoter direct only a low level of liver-specific transcription; tissue-specific enhancers are required for maximal transcription.

Watanabe et al. (1987) identified a cell-specific enhancer in the human α 1AT gene. Domain B of this enhancer is located 3.5Kb upstream of the CAP site and has been shown to bind a nuclear factor from hepatoma cells (Sawadaishi et al., 1988). The binding site for this protein bears strong sequence similarity to the HPI element (see figure 1). Indeed, in gel retardation assays, the HPI element from the promoters of the human albumin gene and the human α 1AT gene itself could compete for binding of this protein to the enhancer element (Sawadaishi

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et al, 1988). This suggests that the same protein which interacts with the HP1 element in the promoter of many liver-specific genes also interacts with a liver-specific enhancer. Thus, in the case of the human α 1AT gene, a common sequence element, HP1, interacting with the same trans-acting factor is present in both the promoter and the enhancer. This is similar to the situation of the octamer motif in immunoglobulin genes.

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1.4 GENE REGULATION BY STEROID AND THYROID HORMONES.

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Steroid hormones are a group of structurally related molecules that have many diverse effects upon the physiology, development and differentiation of eukaryotes (see the review by Evans, 1988). Included in this family of molecules are the sex hormones estrogen, progesterone, and testosterone, glucocorticoids, androgens, mineralocorticoids, α -2,5 dihydroxyvitamin D₃, and retinoic acid. Thyroid hormone, although not strictly a steroid hormone, is grouped with this family for reasons which will become clear later.

Thyroid hormone is synthesised in the thyroid gland by the iodination of tyrosine. In mammals, two forms of thyroid hormone are secreted by the thyroid gland; thyroxine (L-3,5,3',5'-tetraiodothyronine) and triiodothyronine (L-3,5,3'-triiodothyronine). Triiodothyronine (T₃) is more active than thyroxine (T₄). One of the major effects of thyroid hormone in mammals is to increase the basal metabolic rate. Almost all organs are affected, the major exception being the brain. Both excessive secretion of thyroid hormone and deficiency in thyroid hormone result in disease states in humans.

The use of radiolabelled ligands showed that steroid hormones associated with specific intracellular receptors. Upon treatment with hormone these receptors

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became tightly bound to chromatin. This was associated with the induction and repression of specific genes (Ivarie and O'Farrell, 1978). It is now clear that at least some of the effects of steroid and thyroid hormones are the result of direct alterations in gene expression. Gene regulation by steroid hormones has been intensively researched over the last ten years and will be the subject of the rest of this chapter.

1.4.1 A simple model for the action of steroid hormones.

A widely accepted, general model exists for the mechanism of action of steroid hormones. Steroid hormones, being small hydrophobic molecules can pass freely across the cell membrane. Once inside the cell the hormone binds to a specific intracellular receptor protein. This binding causes a conformational change in the receptor, a process referred to as receptor "activation". The activated form of the receptor has an increased affinity for specific DNA sequences termed hormone response elements (HREs), found near to genes that are regulated by that particular hormone. By binding to these sites the hormone-receptor complex is able to influence the rate of transcription of the linked gene.

Recent research has, in general, supported this model. However, there is some confusion as to how the receptor becomes activated by the hormone. There have been some

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contradictory results published in regard to whether hormone binding is strictly necessary for DNA binding. This will be discussed in a later part of this chapter.

1.4.2 The structure of steroid and thyroid hormone receptors.

Major support for the model of steroid hormone action has come from the isolation and characterisation of the specific intracellular receptors. The receptors for nearly all the known steroid hormones have now been cloned. The human glucocorticoid receptor was isolated from a cDNA expression library using an antibody raised against the purified receptor (Hollenberg et al., 1985). The human estrogen receptor was isolated using antibodies against the purified receptor or synthetic oligonucleotides corresponding to ER peptide sequences (Green et al., 1986; Greene et al., 1986). The human mineralocorticoid (Arriza et al., 1987) and androgen receptors (Chang et al., 1988) were isolated by low stringency hybridisation with the highly conserved DNA binding domain from the GR. Homologues from other species have been isolated by cross-hybridisation with the human receptor clones.

When the human estrogen receptor was sequenced (Green et al., 1986) it was found that it contained a segment that was closely related to the erb-A oncogene of avian erythroblastosis virus (AEV). Similar homology was found

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in the chicken estrogen receptor and the human glucocorticoid receptor (Krust et al., 1986 and Weinberger et al., 1985). The cloning of the chicken and human cellular homologues of the v-erb-A gene (Sap et al., 1986; Weinberger et al., 1986) lead to the discovery that they encoded receptors for thyroid hormone. Since then thyroid hormone receptor cDNA clones have also been isolated from rat and mouse either by homology to v-erb-A or to other previously isolated thyroid hormone receptors.

Comparison of the deduced amino acid sequences of different hormone receptors has indicated that they are structurally organised into distinct domains (Weinberger et al., 1985; Krust et al., 1986; Kumar et al., 1986; Hollenberg et al., 1987; Rusconi and Yamamoto, 1987). The sizes of the various receptors exhibit considerable diversity; from 410 amino acids for the rat thyroid hormone receptor alpha 1 to 984 amino acids for the human mineralocorticoid receptor. However, all of the receptors show significant sequence homology and can be divided up into three main domains (see the reviews by Green and Chambon, 1988; Beato, 1989; Evans, 1988; and figure 3). The amino terminal domain, or A/B domain is the most variable in both sequence and size, and is primarily responsible for the large variation in the size of the different receptors. The function of this domain remains unclear, but it may be involved in modulating transcriptional activation. All steroid hormone receptors

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possess a short (66 or 68 amino acids), well conserved central domain which is rich in cysteine residues. This domain is responsible for the DNA binding function of the receptor. The c-terminal region beyond the DNA binding domain is also relatively well conserved between different hormone receptors, and contains a large number of hydrophobic residues. This domain is known to be responsible for ligand binding. Since the structures of the various steroid hormones show considerable diversity, the conservation of this domain presumably reflects a common overall structure required for hormone binding. Evidence suggests that this domain also contains regions required for receptor dimerisation, nuclear translocation and transcriptional activation.

There is conclusive evidence that steroid hormone receptors are phosphoproteins, and it appears that phosphorylation can modulate the affinity of the receptors for their cognate hormone (Beato, 1989).

1.4.3 The steroid hormone receptor superfamily.

The finding that steroid and thyroid hormone receptors have a common structure has led to the proposal that they are part of a superfamily of genes that encode ligand dependent transcription factors (see Evans, 1988). Figure 3 shows the structures of the human steroid hormone receptor family of proteins for which the ligands are

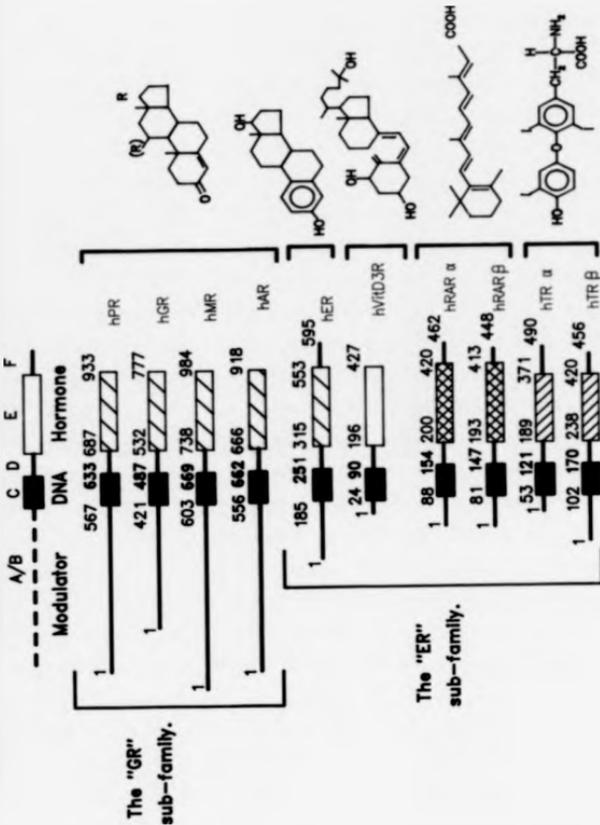
Figure 3.

The human nuclear receptor family of proteins and their ligands.

The structures of the various members of the human steroid hormone receptor superfamily are drawn schematically to show the locations of the different domains. The proteins have been aligned according to the position of the highly conserved DNA-binding domain. The sizes of the different proteins, in amino acids, and the positions at which the domains within them begin and end are also shown above each protein. The receptor superfamily can be divided in to two sub-groups, the "ER sub-family" and the "GR sub-family", based upon overall sequence similarity and DNA sequence specificity. The chemical structures of the ligands which are bound by the various receptors are also shown. The ligands of the PR, GR, MR and AR have a common structure that differs only in the identities of the side chain groups (R). Abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; GR glucocorticoid receptor; MR, mineralocorticoid receptor; AR, androgen receptor; VitD3R, vitamin D3 receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor.

This figure is adapted from Green and Chambon (1988).

Figure 3 Structures of the human nuclear receptor family of proteins and their ligands.



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known. Based upon overall sequence homology the different receptors can be divided into two sub-families. The receptors for progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR) fall into one sub-family. The receptors for estrogen (ER), vitamin D3 (VitD3), retinoic acid (RAR) and thyroid hormone (TR) are more related to each other than to other members of the receptor superfamily, and make up the second sub-family.. Humans have at least two genes encoding slightly different retinoic acid receptors designated alpha and beta. Similarly, humans like the rat also have two genes encoding thyroid hormone receptors.

The presence of the well conserved DNA binding domain has promoted searches for other members of this superfamily. Using low stringency hybridisation at least six new members of this family have been isolated, for which the ligands are unknown. The estrogen ^{receptor} related genes 1 and 2 (ERR1 and ERR2) from human are more closely related to the steroid hormone receptors than the thyroid hormone receptors (Giguere et al, 1988). The nur77 cDNA clone was originally isolated as one of a group of genes expressed during the G₀/G₁ transition in mouse fibroblasts. One of them, nur77, turned out to be a member of the steroid receptor superfamily (Hazel et al, 1988). Miyajima et al (1988) isolated four human cDNA clones designated ear1, ear2, ear3, and ear7 which encode proteins that are members of the steroid hormone receptor superfamily. Three

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of these genes have been classified as novel members of the superfamily. Interestingly, one of these unidentified receptors (*ear1*) and the thyroid hormone receptor alpha gene (*ear7*) are transcribed from opposite strands of the same genetic locus (Miyajima *et al.*, 1989). Recently, it has been reported that the gap gene *knirps* of *D.melanogaster* has significant homology to the steroid hormone receptor superfamily (Nauber *et al.*, 1988). This gene is known to be involved in organising the posterior segmentation pattern of the embryo. The homology between *knirps* and the steroid hormone receptor superfamily lies only in the DNA binding domain. The homology is about 50% (depending upon which of the steroid hormone receptors it is compared to). Importantly, the cysteine residues which are invariant in the DNA binding domain of all members of the receptor superfamily, are also conserved in *knirps*. Unlike the steroid hormone receptors, *knirps* does not have an amino terminal domain; the DNA binding domain spans amino acids 5 to 67. The authors suggest that like the steroid hormone receptors, *knirps* may also be a ligand dependent transcription factor, although there is no evidence for this. It is tempting to speculate that the putative ligand of the *knirps* protein might be a morphogen, and that the function of *knirps* is to "read" a gradient of this morphogen within the abdominal region of the embryo. A second *Drosophila* gene, *knirps-related*, has also been shown to be a member of the steroid receptor

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superfamily (Oro et al., 1988). This gene shows 85% homology to knirps within the DNA binding domain and 92% homology to knirps in a stretch of 25 amino acids immediately to the C-terminal side of the DNA binding domain. The C-terminal regions of the two proteins are otherwise unrelated, and the C-terminal region of knirps-related shows no homology to members of the steroid receptor superfamily. A function has not yet been assigned to knirps-related.

The production of cholesterol is regulated by feedback mechanisms. Apparently, some of this regulation occurs at a transcriptional level (Sudhof et al., 1987). Cholesterol is structurally related to steroid hormones so it seems likely that these effects upon transcription could be mediated by a cholesterol receptor which is a member of the steroid receptor superfamily. The structure of the herbicide dioxin is closely related to that of thyroid hormones and some of its effects are caused by changes in gene expression. It seems likely that dioxin may also act via a receptor protein related to the steroid hormone receptors. The investigation of novel receptors may lead to the discovery of new hormone response systems.

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1.4.4 The DNA binding domain of steroid and thyroid hormone receptors.

The central cysteine rich domain is the most well conserved region within the steroid hormone receptor superfamily. The degree of homology within this 66 amino acid domain varies from a minimum of about 40% to a maximum of 94% at the amino acid level. Several features of this region suggest that it might be responsible for DNA binding. Firstly it contains a lot of basic amino acids likely to interact with DNA. Secondly, the arrangement of cysteine residues is similar to that found in the DNA binding domain of the RNA polymerase III transcription factor TFIID (Weinberger et al, 1986). When mutations were made specifically within this region of the GR, DNA binding was prevented (Giguere et al, 1986; Hollenberg and Evans, 1988). The ability of these mutated receptors to bind hormone was unaffected, indicating that the overall structure of the receptor was intact. Absolute proof that this region was responsible for DNA binding came from exchanging the putative DNA binding domains of different receptors. When the putative DNA binding domain from the human ER was swapped with that of the human GR, the resulting protein activated transcription from glucocorticoid responsive promoters in response to estrogen (Green and Chambon, 1987). For the majority of steroid hormone receptors it has not been directly

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demonstrated that the central cysteine rich region is responsible for DNA binding. However, given the strong conservation within this region this is a fair assumption. The assignment of the DNA binding domain has recently been shown to be functionally correct for the rat TR type β 1 (Thompson and Evans, 1989).

The most striking feature of the homology within the DNA binding domain of different receptors is the conservation of the cysteine residues (see figure 9). The nine cysteine residues are in fact invariant in all the steroid and thyroid hormone receptors that have been analysed. The positioning of the cysteine residues is reminiscent of a motif first identified in the 5S gene transcription factor TFIIIA (Miller *et al.*, 1985). TFIIIA contains a series of repeating units rich in cysteine and histidine. Each unit is thought to be able to fold into a finger like structure supported by the tetrahedral coordination of a zinc ion. Each zinc ion would be coordinated by two histidine and two cysteine residues. This zinc finger motif has since been recognised in a number of DNA binding proteins (see the review by Evans and Hollenberg, 1988). The actual existence of zinc fingers has only been demonstrated for TFIIIA itself (Diakun *et al.*, 1986; Lee *et al.*, 1989). Although related to TFIIIA, the DNA binding domain of the steroid hormone receptors forms a distinct class of zinc finger motifs. Each zinc ion would be coordinated by two pairs of cysteine residues rather than the histidine-

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cysteine arrangement found in TFIIIA. Also, the DNA binding domain of the steroid hormone receptor family contains only two zinc fingers whilst TFIIIA has nine. The proposal that the DNA binding domain of the steroid hormone receptors form a zinc finger structure similar to that of TFIIIA is supported by the finding that zinc is necessary for the ER to bind to DNA (Sabbah et al., 1987). Also, site directed mutagenesis of the conserved cysteine residues prevents DNA binding (Hollenberg and Evans, 1988). Nuclease and chemical protection experiments with purified TFIIIA indicated that each finger binds in the major groove of the DNA double helix and interacts with five nucleotides (Fairall et al., 1986). The binding sites for steroid hormone receptors contain dyad symmetry (see the review by Evans, 1988), implying that the receptors bind as dimers. Thus each binding site may be contacted by four zinc fingers. Specific residues within the DNA binding domain have been implicated in determining the sequence specificity of the interaction. This will be discussed in more detail in section 1.4.13.

1.4.5 The ligand binding domain.

Sequence comparisons between different steroid hormone receptors, for example between the human and chicken ERs (Krust et al., 1986), suggested that the carboxy terminal region might be responsible for hormone binding.

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The functional assignment of the ligand binding domain to the carboxy terminal domain is based largely upon work with the human glucocorticoid and estrogen receptors. Giguere et al (1986) generated a random set of mutations covering the whole of the human GR. These were then expressed in cultured cells and assayed for their ability to bind hormone and to activate transcription from the mouse mammary tumour virus (MMTV) long terminal repeat (which is responsive to glucocorticoids). One set of mutations confirmed the importance of the DNA binding domain; receptors with mutations in this region could bind hormone but were unable to activate transcription. A second group of mutations resulted in the loss of hormone binding. These mutations defined a region spanning the last 200 amino acids of the protein as being crucial for hormone binding. This region was therefore designated the hormone binding domain. Rusconi and Yamamoto (1987) came to similar conclusions regarding the rat GR. The location of the ligand binding domain in the human ER has been determined by a similar mutational analysis (Kumar et al, 1986), and corresponds to the position of the ligand binding domain in the GR. The location of the ligand binding domain in the majority of the other steroid hormone receptors has been assigned by homology to the human GR. This assignment was supported by experiments in which hybrid thyroid hormone/glucocorticoid receptors were created (Thompson and Evans, 1989). All possible

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combinations of the amino terminus, DNA binding domain, and ligand binding domain were created. The hybrid receptors were then expressed in tissue culture cells and tested for their ability to activate either a glucocorticoid or a thyroid hormone responsive test gene in response to either glucocorticoid or thyroid hormone. Hybrid receptors containing the putative ligand binding domain of the thyroid hormone receptor always activated transcription in response to thyroid hormone, irrespective of the identity of the DNA binding domain or the amino terminal domain.

1.4.6 Cis-acting DNA sequences responsible for activation of gene expression by steroid hormones; hormone response elements.

A moderate number of genes whose expression is regulated by steroid hormones have been cloned. Gene transfer techniques have been used to identify DNA sequences responsible for induction by steroid hormones (for example; Klein-Hitpab et al, 1986; Ye et al, 1988; Kerner et al, 1989; for a review see Yamamoto, 1985). In a number of cases the DNA sequences defined as being necessary for hormonal regulation have been shown to specifically bind the partially purified hormone receptor (for example; Klein-Hitpass et al, 1989; see review by Beato, 1989 and references therein). The sequence elements defined in this

Figure 4.

Consensus steroid hormone response elements.

Numbers refer to the number of response elements that match the consensus sequence at each position.

Glucocorticoid

response
element
(GRE)

G₆ G₈ T₆ A₇ C₈ A₆ N N N T₉ G₁₀ T₁₀ T₇ C₁₀ T₁₀

(Ten sequences compared)

Estrogen
response
element
(ERE)

G₄ G₅ T₅ C₄ A₅ N N N T₅ G₅ A₂ T₃ C₄ C₄

(Five sequences compared)

TRE half site
(Brent et al)

A G G T C
A A

TRE half site
(Norman
et al, 1989)

G G G A C G
T C

VitD3 response
element from
the osteocalcin
gene

(Kerner et al, 1989)

← →
GTGACTCACCGGGTGAACGGG

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way have often been shown to confer hormone inducibility on a heterologous promoter (for example; Klein-Hitpab et al., 1986; Brent et al., 1989a). The sequence elements defined by these approaches are known as hormone response elements (HRE). By comparing the HREs from different hormone responsive genes it has been possible to define consensus sequences (see reviews by Beato, 1989; Green and Chambon, 1988; Evans, 1988). The consensus for the glucocorticoid response element (GRE) is a 15-mer, partially palindromic sequence. This consensus is perhaps the most meaningful in that it has been derived from the largest number of responsive genes (see the review by Evans, 1988). The consensus estrogen response element (ERE) has been derived from five genes and consists of a 13-mer also exhibiting partial dyad symmetry. These two consensus sequences are shown in figure 4. A consensus sequence has not yet been defined for the progesterone and androgen response elements. The DNA sequences responsible for induction of the human osteocalcin gene by vitamin D3 have very recently been defined (Kerner et al., 1989). This consists of a 21bp element from the 5' flanking region of the gene which was shown to confer vitamin D3 responsiveness on the viral TK promoter. Since no other vitamin D3 response elements (VDRE) have been identified it is not possible to derive a consensus sequence. However the VDRE from the osteocalcin gene does resemble both the ERE and elements that mediate response to thyroid hormone

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(see figure 4).

The precise DNA sequences that confer induction by thyroid hormone have only been determined for one T3 responsive gene; the rat growth hormone (GH) gene (Glass et al., 1987; Brent et al., 1989a; Norman et al., 1989). The induction of the rat growth hormone gene by thyroid hormone has been studied in some detail by a number of groups and there is still some controversy as to the exact location of the TRE, and whether there is more than one.

Glass et al. (1987) carried out a deletion analysis of the rat growth hormone gene promoter to determine sequences necessary for induction by T3. Their results indicate that sequences between -235 and -145bp were sufficient to confer a response to T3. They then used an avidin-biotin DNA binding assay (ABCD assay; see Glass et al., 1987 for details) to detect binding of thyroid hormone receptor (TR) to this region of the rat GH promoter. This assay indicated specific binding of both rat and human TR to sequences between -186 and -158bp. Furthermore, DNase I footprinting with nuclear extract (from cells that express the TR) showed that a protein was binding between -178 and -163bp. When this region was deleted from the rat GH promoter no induction by T3 occurred. An oligonucleotide containing the sequence between -178 and -163bp of the rat GH promoter could confer T3 induction upon the TK promoter. These results are in agreement with the prior identification of a T3 inducible DNase I hypersensitive

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site 170bp upstream of the CAP site of the rat GH gene (Nyborg and Spindler, 1986). Moore and his co-workers (Brent et al., 1989a) carried out a similar deletion analysis of the rat GH promoter. They reported that sequences between 183 and 137bp upstream of the CAP site were required for induction by T3. However it is clear that when sequences between -237 and -183bp were included the response to T3 was much greater. A fragment from the rat GH promoter spanning nucleotides -200 to -157 was able to confer induction by T3 upon the TK promoter in an orientation independent manner (Brent et al., 1989a). These results are in agreement with those of Glass et al. (1987). When this sequence element was narrowed down further to a region spanning -190 to -167 T3 induction still occurred but it was reduced by two fold. This region contains an imperfect direct repeat, designated the A and B domains (Brent et al., 1989a) and an imperfect inverted repeat termed the C domain (Brent et al., 1989b). Point mutations and deletions within the A and B domains were shown to significantly reduce the response to T3. The C domain on the other hand did not appear to be strictly necessary for T3 induction. An oligonucleotide containing the complete A and B domains and only the first 3 bases of the C domain could confer induction by T3 although the level of induction was small (see figure 5). A 17-mer oligonucleotide that contained just the A and B domains could not confer a response to T3 (see figure 5).

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Interestingly, two point mutations which converted the B domain to a perfect direct repeat of the A domain resulted in a significant increase in the level of induction by T3. For example, the 17-mer oligonucleotide discussed above (containing just the A and B domains) conferred an eleven fold induction when it was altered so that it contained a perfect direct repeat (see figure 5). The corresponding sequence from the wild type rat GH promoter could not confer a response to T3 (see figure 5). This suggests that the TR has a higher affinity for the mutated element containing a perfect direct repeat than for the wild type element. These findings are in slight disagreement with those of Glass et al (1987) previously described. As shown in figure 5 the DNaseI footprint detected by this group covers the B and C domains rather than the A and B domains identified as being the important regions by Brent et al (1989a). Izumo and Madhavi (1988) have localized sequences necessary for the thyroid hormone induction of the rat alpha myosin heavy chain (α MHC) gene to a region spanning -161 to -71bp. Within this is a 13bp element that matches a region of the rat GH promoter covering the B and C domains in 10 out of 13 positions (see figure 5). A region of the α MHC gene that contains this 13bp element (-151 to -122) could specifically bind the rat thyroid hormone receptor (type alpha 1) that had been synthesised in vitro (Izumo and Madhavi, 1988). This would tend to emphasise the importance of the B and C domains for T3 induction.

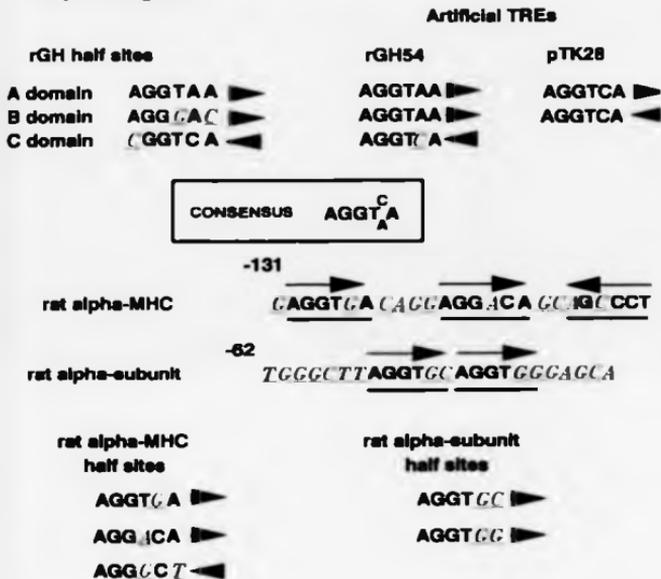
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Further experiments carried out by Moore and his co-workers (Brent et al, 1989b) indicate that all three of the domains (A, B and C) are important for induction by T3. A whole series of artificial constructs were made in which various combinations of these domains and mutated versions of them were placed in front of the TK promoter, and these were tested for T3 inducibility. An oligonucleotide containing all three domains from the rat GH promoter could confer a 3.6 fold induction by T3. When this sequence was altered such that the A and B domains were perfect direct repeats and the C domain matched the A domain in five out of six positions (rGH54) the induction by T3 was increased dramatically to 43 fold (see figure 5). When two copies of the C domain as present in rGH54 were placed as an inverted repeat in front of the TK promoter (TK28) the induction by T3 was 26 fold. With two copies of TK28 (pTK28mult) the induction was 125 fold (see figure 5). We have obtained pTK28mult from Dr. Moore and it was used in some of the experiments that will be described later.

Based upon these sort\$ of experiments Brent et al (1989b) have proposed a consensus "half site" for the T3 response element. This half site is equivalent to either of the A, B or C domains of the rat GH promoter. The sequence of this consensus half site is AGGT(^{C/A})A and at least two copies of this element in close proximity are needed for a T3 response. It is unclear whether these half sites can

Figure 6

Thyroid hormone response element "half sites" in the rat growth hormone gene and other thyroid hormone responsive genes.



Arrows () indicate orientation of the half sites within the promoter.

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function in either direct repeat or inverted repeat orientation, or both. The rat GH promoter has two copies of the half site in direct repeat orientation (the A and B domains) and a third (the C domain) is an inverted repeat of both of the others. The rat GH half sites are shown in figure 6 along with the half sites present in the artificial TREs rGH54 and pTK28mult. That this consensus half site is meaningful in the context of other promoters is supported by the analysis of two other T3 responsive genes; the rat alpha myosin heavy chain gene (rat α MHC) and the rat glycoprotein hormone alpha subunit gene (rat α -subunit gene). As discussed earlier, the sequences responsible for the T3 induction of the rat α MHC gene have been narrowed down to a region between -151 and -122bp. Within this 70bp region three "half sites" can be identified in an identical arrangement to those in the rat GH promoter (see figure 6). The sequences responsible for the T3 induction of the rat α -subunit gene have been localized to a region between -80 and +33bp and the binding site for pure thyroid hormone receptor (synthesised in vitro from a cDNA clone) was localized to a region between -74 and -38 (Burnside et al., 1989). Within this region (-74 to -38) two "half sites" in direct repeat orientation can be identified (see figure 6).

In conclusion, the experiments carried out by Glass et al. (1987) and by Moore and his colleagues (Brent et al., 1989a and 1989b) indicate that the TRE consists of a rather

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poorly conserved 6bp element with the consensus sequence AGGT(C/A)A. At least two copies of this half site in close proximity are required to generate a functional TRE. Both direct repeat and inverted repeats of the half site appear to be functional. The half site consensus sequence is similar to the consensus for the ERE, and the significance of this will be discussed in more detail in section 1.4.12.

Ye et al (1988) reported that sequences between -208 and -178 from the rat GH promoter were sufficient to confer T3 induction upon the minimal rat GH promoter (-104 to +7). This minimal promoter is not responsive to thyroid hormone, but does contain a binding site for a cell-specific transcription factor (Ye et al, 1988). Thus, the TRE that they have defined (see figure 5) contains only one of the half sites (the A domain) identified by Moore and his co-workers (Brent et al, 1989a and 1989b). This region of the rat GH promoter (-208 to -178) identified by Ye et al (1988) does contain an imperfect inverted repeat. However, this element could only confer induction by T3 when it was placed close to the rat GH promoter. Further experiments with the enhancerless Rous sarcoma virus (RSV) promoter suggested that sequences between -236 and -146 of the rat GH promoter (which spans the whole of the thyroid hormone responsive region shown in figure 5) could only function as a TRE in association with other cis-acting sequences located between -146 and -47 of the rat

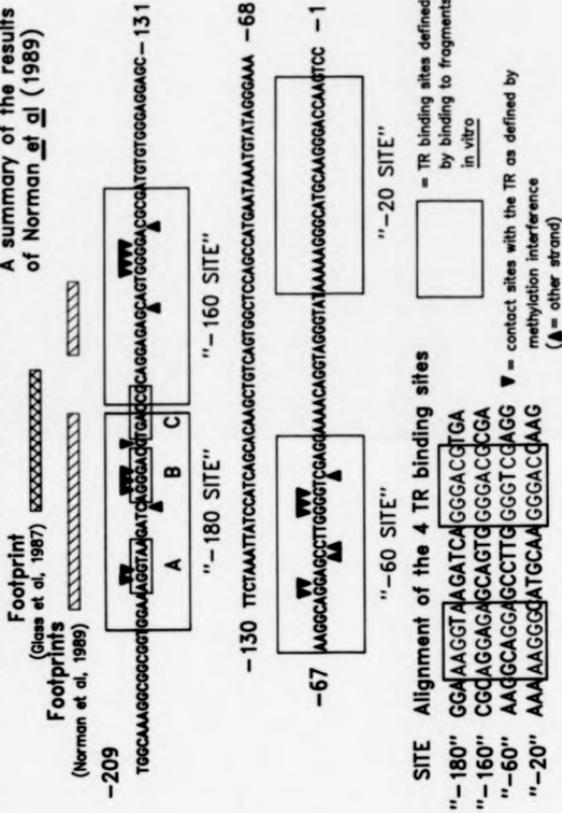
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GH promoter (Ye et al., 1988). This suggests that interaction between the TR bound at the TRE and other trans-acting factors is essential for induction by thyroid hormone (see section 1.4.8 for further discussion of this). This does not necessarily contradict Brent et al. (1989a) who found that various fragments from within this -236 to -146 region could alone confer T3 inducibility on a heterologous promoter since they used the TK promoter whilst Ye et al. (1989) used the RSV promoter. The TK promoter might well contain a binding site(s) for a trans-acting factor with which the TR can interact. Clearly, how you define a TRE depends upon the environment in which it is tested.

Finally, it should be noted that the TR appears to bind to multiple sites within the 5' flanking region of the rat GH gene (Lain et al., 1988). One of these binding sites lies between -181 and -149 which correlates with the work described so far. However, binding was observed at three additional sites; -1730 to -1230, -530 to -230 and -149 to +12. In a very recent paper by the same group (Norman et al., 1989) a comprehensive analysis of the region of the rat GH gene spanning -237 to +12 has been described. These workers have used gel retardation, footprinting assays, and mutational analysis to localise four TR binding sites centred at -180, -160, -60 and -20 within this region. This alters the previous view that there was only one TR binding site in this region (Brent et al.

Figure 7 Thyroid hormone receptor (TR) binding sites on the rat growth hormone gene promoter (-237 to -1).

A summary of the results of Norman et al (1989)



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1989a and 1989b; Glass et al., 1987). However these findings do not essentially contradict the previous work. Highly purified rat liver TR was used in the determination of these four binding sites, and it was confirmed that the sites really were binding TR (and not some other protein) by labelling with [¹²⁵I] T3 (Norman et al., 1989). I have summarised the binding data (gel retardation and footprinting) of Norman et al. (1989) in figure 7. The mutational analysis was consistent with the binding data, and competition experiments indicated that all four sites were binding the same TR (there is more than one TR protein in rat liver) with similar affinities. The functional importance of these sites was tested by fusing the region of the rat GH gene from -237 to +11 containing various mutations, to the reporter gene CAT. The T3 inducibility of these constructs was tested by transient expression in rat GC cells (which contain functional TRs). The results indicated that all four binding sites contributed to the T3 responsiveness of the promoter. However, it is clear that the two distal binding sites; "site -180" and "site -160" are the most important. A mutation in the "-160 site" reduced T3 induction to 69%, whilst a mutation in the "-180" site reduced T3 induction to 31%. When both of these sites were mutated simultaneously, T3 induction was eliminated completely. Thus, of these two sites the "-180" site is more important. Mutations in the "-60" and "-20" sites reduced

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T3 induction to 59% and 74% respectively. Furthermore, when sequences spanning -141 to +11 of the rat GH gene, which contain only the "-60" and "-20" sites, were fused to CAT and tested in the same way no induction by T3 occurred. This suggests that the two most proximal TR binding sites are functional only if they can interact with proteins bound to sequences within the -142 to -237 region of the promoter.

The results of Norman et al (1989) tend to emphasize the importance of the "-180 site" which corresponds to the A and B half sites identified by Brent et al (1989a). When the sequences of the four TR binding sites were compared (Norman et al, 1989) they were able to identify a conserved motif; $GCG^A/T^G/C$ (see figure 7). However they do admit that these four binding sites are very purine rich so multiple alignments are possible. The position of this conserved element differs by only one nucleotide from the A and B half sites. Perhaps Norman et al (1989) derive a different consensus from Moores' group because theirs is derived by comparing different TR binding sites within the same gene, the rat GH gene. The way in which the consensus sequence proposed by Brent et al (1989b) was derived is subtly different; it was based upon mutations that generate the most efficient TRE in the context of a heterologous promoter (TK). Since interactions between TR bound at a TRE and other trans-acting factors (including other TRs) is clearly important in determining the level

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of transcriptional activation, the context in which a putative TRE is tested will have a major effect upon the level of induction. Because of this the results of Norman et al (1989) are more likely to be meaningful in the context of the rat GH gene. The consensus proposed by Brent et al (1989b) is perhaps likely to be more generally applicable. Clearly, many related sequences can function as a TRE. It may not be possible to define a consensus TRE that is meaningful in the context of different genes from different organisms.

A number of genes are known to be regulated by retinoic acid at the level of transcription (Wang et al, 1985), but the sequences responsible for this regulation have not been determined. Therefore nothing is known at present about the characteristics of natural retinoic acid response elements (assuming they do exist, which is likely). However, it has been shown that retinoic acid can induce gene expression through an artificial, "consensus" thyroid hormone response element (Umesono et al, 1988). Evidence suggests that this is mediated by binding of the retinoic acid receptor to the TRE (Umesono et al, 1988). Bedo et al (1989) demonstrated that treatment of a rat pituitary cell line with retinoic acid induced an increase in the rate of transcription of the chromosomal rat GH gene. Thus, transcription of the rat GH gene can be induced by retinoic acid as well as by T3. The results of

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Umesono et al (1988) described above suggest that this induction by RA may be mediated by the same cis-acting-DNA sequences previously identified as a TRE. Interestingly, treatment of cells with both T3 and RA produced a larger induction than either hormone alone, indicating that these hormones were acting synergistically. Similar results were obtained with a transiently transfected rat GH reporter gene containing 1.8Kb of 5' flanking DNA. The significance of these findings for the regulation of the rat GH gene in vivo remain unclear.

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1.4.7 Steroid hormone receptors bind to hormone response elements as dimers.

Several lines of evidence suggest that a receptor dimer binds to a hormone response element. Many of the hormone response elements that have been identified show a degree of dyad symmetry which suggests that one monomer may contact each half site (see figure 4). Analysis of the contact sites between the receptor and DNA using methylation interference have supported this idea. For example, analysis of three of the four binding sites for thyroid hormone on the rat growth hormone promoter showed symmetrical contact sites on either side of the centre of dyad symmetry (Norman et al., 1989; and figure 7). Moreover, the positions of the contact sites are consistent with the hypothesis that the receptor contacts the same face of the DNA on both halves of the palindrome (Norman et al., 1989). A similar symmetrical methylation interference pattern was found on the ERE (Kumar and Chambon, 1988). Mutations in one half of a palindromic ERE decreased the formation of stable complexes with the ER, suggesting that both halves of the palindrome were necessary for tight binding (Kumar and Chambon, 1988). It has now been shown directly that the ER does indeed bind as a dimer to a ERE (Kumar and Chambon, 1988). Furthermore, dimerisation was induced by estrogen. Experiments with various truncated receptors suggested

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that as well as the hormone inducible dimerisation function associated with the hormone binding domain, there was also a weak constitutive dimerisation function present in the DNA binding domain (Kumar and Chambon, 1988). Monomeric ERs did not bind to the ERE indicating that dimerisation was essential for DNA binding. By analogy, it seems likely that other steroid hormone receptors also bind to HREs as a hormone induced dimer, although this has not been directly demonstrated.

1.4.8 Multiple regions are important for activation of transcription.

Very little is known about the exact mechanism by which "activated" hormone-receptor complexes regulate the rate of transcription initiation. Most of the hormone responsive genes that have been cloned are activated by steroid hormones and so most work has concentrated on regions of the receptor responsible for activation. However, it should be remembered that some genes are repressed by steroid hormones (discussed in detail in section 1.4.9).

No clear picture has emerged regarding the locations of those regions of the receptor responsible for activation. It is now clear that multiple regions are involved and that there is considerable variation between different receptors. Indeed, the relative importance of different

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regions varies according to the target gene.

Mutational analysis of the human GR identified two regions distinct from the DNA and hormone binding domains that were necessary for the receptor to activate transcription (Giguere et al, 1986). One of these domains, designated Tau 1 (T1), is about 100 amino acids long and is located within the amino terminal (A/B) domain. Mutations within this region completely abolished transcriptional activation without affecting hormone binding. The second domain, Tau 2 (T2) is only about 20 amino acids long and is located between the DNA binding domain and the hormone binding domain. Mutations in T2 reduced transcriptional activation by 90%, again without affecting hormone binding. Thus, both these domains appear to be essential for activation of transcription. Both T1 and T2 could function independently of their position within the receptor and multimerisation caused an increase in the level of transcriptional activation (Hollenberg and Evans, 1988). Conclusive evidence that the T1 domain was an entirely independent transcription activation domain came from an experiment in which the T1 domain was inserted into the amino terminal region of the rat TR type alpha 1 (Thompson and Evans, 1989). This resulted in a greater than ten fold increase in the level of transcriptional activation. When two copies of the T1 domain were inserted activation was increased by fifty fold. The T1 and T2 domains have no obvious sequence

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similarity with the exception that both are acidic in character. Thus they bear some resemblance to the activation domains identified in the yeast transcription factors GAL4 and GCN4. Indeed, a synthetic amphipathic alpha helix based upon the activation domain of GAL4 could function in the GR in place of the T2 domain (Hollenberg and Evans, 1988).

Removal of the ligand binding domain from the PR, GR and ER was found to generate a constitutively active transcription factor, i.e. hormone was not needed to activate transcription from a responsive promoter. In the case of the rat GR the constitutive activity was close to that observed with the wild type receptor (Godowski et al., 1987). This result showed that the hormone binding domain itself is not absolutely required for activation of transcription. This led to the proposal that the function of the hormone binding domain is to suppress either DNA binding or transcriptional activation or both. In this model hormone binding would result in the un-masking of pre-existing DNA binding and/or transactivation domains. However, removal of the ligand binding domain from the human GR produced a constitutive receptor with only 20% of the activity of the wild type receptor (Hollenberg and Evans, 1988). Similarly, deletion of the hormone binding domain from the human ER did produce a constitutive receptor, but one with only 5% of the activity of the wild type receptor (Kumar et al., 1987). The ability of this

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mutant to bind to an ERE was unaffected. These results indicate that regions within the hormone binding domain do contribute to the activation of transcription. The DNA binding domain of the yeast transcription factor GAL4 when isolated from the rest of the protein can bind to its recognition site in DNA but fails to activate transcription. Chimeric receptors consisting of this DNA binding domain linked to the ligand binding domains of either the human GR or ER can stimulate transcription from GAL4 responsive reporter genes only in the presence of the appropriate hormone (Webster *et al.*, 1988). This confirms that there is a transcriptional activation function in the hormone binding domain and indicates that its activity is dependent upon hormone binding. Interestingly, either hormone or antihormone were necessary for binding of these chimeras to response elements in DNA but only the hormone caused transcriptional activation. Thus, in the absence of hormone or antihormone the hormone binding domain appears to mask the DNA binding domain. This suggests that the binding of hormone or antihormone first results in a transformation in the receptor which allows binding to the response element in DNA. The hormone but not the antihormone can induce a transcription activation function present in the hormone binding domain. Thus, antihormones could function by competing for binding to the receptor.

In conclusion, the human GR appears to have at least three separate activation domains. One of these is in the

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hormone binding domain and is entirely dependent upon hormone binding. The second (T1) is in the A/B domain, whilst the third (T2) is in the "spacer region". Both T1 and T2 appear to be constitutively active.

Interestingly, the requirement for the amino terminal domain appears to be gene specific. Activation of the estrogen responsive gene PS2 is severely reduced by removal of the A/B domain from the ER, whilst activation of the HSV TK promoter and a reporter gene containing the estrogen response element from the vitellogenin gene was unaffected (Kumar et al., 1987). Two forms of the human PR have been isolated which differ only in the amino terminus. Both forms can activate transcription from the MMTV promoter but only the A form can activate the ovalbumin promoter (Tora et al., 1988). This suggests that the A/B domain may interact with other factors on the promoters of target genes. In this way the A/B domain could modulate the degree of transcriptional activation.

Steroid hormone response elements are often found clustered with binding sites for other trans-acting factors (see Schule et al., 1988 for references). There is now clear evidence that steroid hormone receptor complexes bound at response elements in DNA do interact with other transcription factors. Schule et al. (1988) constructed a series of artificial promoters in which various combinations of transcription factor binding sites were placed in front of the TK promoter linked to CAT. These

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constructs were transfected into HeLa cells, which possess all the the appropriate trans-acting factors, along with a plasmid expressing a cloned GR. Induction of CAT activity by glucocorticoid treatment was then measured. None of the binding sites tested (except for the GRE) conferred any induction by glucocorticoids when placed alone in front of the TK promoter. A single GRE alone showed only a low level of induction by glucocorticoids. This induction was dramatically increased when the binding site for another transcription factor, or indeed a second GRE, was placed close to the GRE. All of the binding sites tested (sites for NF1, CP1, SP1, OTF and the CACCC box factor) had this strong synergistic effect. The degree of synergism varied between the different binding sites and was also altered by changing the spacing between the binding sites. It would be interesting to use this system to determine which regions of the receptor were required for this synergism. This would presumably define regions of the receptor which interact with the various trans-acting factors.

Almost nothing is known about the location of regions required for transcriptional activation in receptors other than the ER and GR. Deletion of the whole of the A/B domain of the rat TR type β 1 had no effect upon its ability to activate transcription from the MMTV promoter into which a thyroid hormone response element had been inserted (Thompson and Evans, 1989). This shows that the A/B domain is not required for transcriptional activation

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from this promoter. It might, however be required for activation of other promoters.

1.4.9 Repression of genes by steroid hormones.

Glucocorticoids have been shown to negatively regulate the transcription of a number of genes, including the bovine prolactin gene (Camper et al., 1985), the pro-opiomelanocortin gene (Israel and Cohen, 1985), the rat prolactin gene (Adler et al., 1988), and the gene encoding the alpha subunit of the glycoprotein hormones (Akerblom et al., 1988).

Camper et al. (1985) showed that 1Kb of 5' flanking DNA from the bovine prolactin gene was sufficient to mediate glucocorticoid repression of the reporter gene CAT in rat pituitary cells. DNaseI footprinting with purified GR indicated that the receptor bound at seven distinct sites between -50 and -562bp (Sakai et al., 1988). These footprints define a negative glucocorticoid response element (nGRE). The seven nGREs show only limited sequence similarity and the consensus is poorly related to the consensus for a positive GRE. A single nGRE from the prolactin gene was able to confer glucocorticoid repression upon various heterologous promoters. In cells which lack an endogenous GR the repression by glucocorticoids was dependent upon co-transfection of a cloned GR. The nGRE resembled enhancer elements in that it

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could function in both orientations and when placed at a distance from the CAP site. Importantly, the nGRE dramatically increased expression from the TK promoter in the absence of glucocorticoids and in the absence of the co-transfected GR. In contrast, the positive GRE from the MMTV LTR did not have this stimulatory effect in the absence of glucocorticoids or the GR. It was therefore suggested that the nGRE is also a binding site for a positive transcription factor that enhances basal transcription. The repression by glucocorticoids could then be caused by the hormone-receptor complex either competing with and/or inactivating this positive factor. The question remains as to how the GR/hormone complex can activate transcription when bound at a GRE but fails to do so when bound at a nGRE. The authors proposed that the nGRE sequences might alter the structure of the bound receptor such that it did not act as a positive regulator when bound at these sites.

Glucocorticoid repression of the gene encoding the alpha subunit of the glycoprotein hormones (α subunit) appears to be the result of competition between the GR and the cAMP-mediator protein for overlapping DNA sequences (Akerblom *et al.*, 1988). The inhibition is mediated by a region of the α subunit gene promoter that contains two cAMP response elements (CREs) that bind the cAMP-mediator protein and thereby mediate activation by cAMP. Footprinting with purified GR indicated that each of the

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CREs overlapped with binding sites for GR. The sequence of these nGREs show only modest homology to the consensus for the positive GRE. The inhibition by glucocorticoids is only seen in situations when the CREs are acting as enhancers, i.e. in the presence of raised levels of cAMP. Inhibition was clearly mediated by the same receptor that causes transcriptional activation of other genes, and was not observed when the DNA binding domain of the GR was mutated. This and other evidence (Akerblom et al., 1988) suggests that the negative regulation by glucocorticoids is the result of competition between the GR and the cAMP-mediator protein for binding to their respective, overlapping sites. Once again it is not clear why binding of the receptor to a nGRE fails to result in transcriptional activation. Possibly it is the context of the GRE with respect to the binding sites for other transcription factors which determines whether it mediates activation of transcription. As discussed earlier a single GRE alone confers only a small stimulation by glucocorticoids and a much bigger stimulation is generated by the synergistic interaction with other transcription factor binding sites (Schule et al., 1988).

A similar competition mechanism has been proposed by Drouin et al. (1987) to explain the glucocorticoid repression of the pro-opiomelanocortin gene. Here the GR could prevent the binding of the CAAT box transcription factor to its target sequence.

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Transcription of the mouse proliferin gene is induced by phorbol esters and repressed by glucocorticoids (Mordacq and Linzer, 1989 and references therein). Phorbol ester induction is mediated by a 31bp region of the 5' flanking DNA that contains a binding site for the transcription factor AP1, and a number of sites similar to the SV40 SpbI element. Both sites are necessary for phorbol ester induction (Mordacq and Linzer, 1989). Repression by glucocorticoids requires the presence of the GR, and this was shown to bind to a region of the proliferin gene promoter that overlaps with the AP1 site. This 31bp region was sufficient for glucocorticoid repression. These results suggest, once again, that glucocorticoid repression of transcription is the result of the receptor preventing the functioning of a positive transcription factor. Gel retardation with extracts from mouse cells showed that a different complex formed on the 31bp element when the cells had been treated with glucocorticoid (Mordacq and Linzer, 1989). This is consistent with several mechanisms of glucocorticoid repression, including competition with AP1 for binding to overlapping sites, protein-protein interaction between AP1 and the GR, and glucocorticoid induction of a gene encoding another factor that binds at or close to the AP1 site.

The rat prolactin gene is negatively regulated by glucocorticoids but is also positively regulated by estrogen (see Adler et al, 1988 for references). The ERE

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responsible for the positive regulation is located in a distal enhancer element. Expression of the prolactin gene is confined to the anterior pituitary gland. This tissue-specific transcription is controlled by a series of related cis-acting sequences located in both the distal enhancer and in a region proximal to the CAP site (Nelson et al., 1988). These elements bind the positive transcription factor Pit-1 (a member of the POU class of transcription factors). As expected, deletion of the ERE in the distal enhancer eliminated the stimulation of expression by estrogen (Adler et al., 1988). However, in the presence of a high concentration of ER supplied by a co-transfected ER, expression from the prolactin promoter from which the ERE had been removed, was repressed by estrogen. The evidence presented (Adler et al., 1988) suggests that two estrogen dependent processes are regulating the prolactin gene; classical activation which occurs via the ERE, and repression. Normally, the positive regulation masks the repression so that repression can only be observed when the ERE is inactivated. When the ERE in the prolactin enhancer was converted to a GRE the inhibitory effect of glucocorticoids was altered to a significant stimulation of expression. This same construct was repressed by estrogen. The authors then went on to determine which regions of the ER were required for the inhibitory effect. Surprisingly, the DNA binding domain of the receptor was not required for inhibition but was

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necessary for activation. Clearly this means that the inhibition by estrogen cannot be mediated by the classical binding of the hormone-receptor complex to a cis-acting element. The region required for inhibition was narrowed down to the so called hinge domain that lies between the DNA and hormone binding domains. The corresponding region from the GR could restore the inhibitory function to an ER from which the hinge region had been deleted.

Deletion analysis of the rat prolactin gene 5' flanking DNA (Adler et al., 1988) showed that multiple regions, each of which contains a binding site for Pit-1 could confer estrogen and glucocorticoid dependent inhibition. Indeed, three copies of the Pit-1 binding site could confer estrogen and glucocorticoid dependent inhibition on the TK promoter. This suggests a model for steroid hormone-mediated inhibition in which the hinge region of the GR/ER interacts in some way with the Pit-1 protein either bound to the promoter or free in solution. This interaction is dependent upon binding of the hormone to the receptor and would prevent Pit-1 from activating transcription from the prolactin promoter. The significance of the estrogen dependent inhibition in vivo is uncertain given that it is only observed when the ERE is inactivated and in the presence of high concentrations of ER. However, the glucocorticoid dependent inhibition is observed in vivo and clearly occurs by the same mechanism.

Thus the inhibition by glucocorticoids of the rat and

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bovine prolactin genes appear to occur by two distinct mechanisms. This is not unlikely given that the 5' flanking regions of the two genes differ considerably.

In all the systems described above inhibition of gene expression by glucocorticoids appears to be mediated by interference with the function of a positive transcription factor. In three cases binding of the GR to so called nGREs competes with the binding of positive trans-acting factors to an overlapping site. The situation with the rat prolactin gene is more intriguing since it does not appear to involve binding of the GR to DNA.

1.4.10 The thyroid hormone receptor family.

As mentioned earlier, the cellular homologues of the v-erb-A gene are known to encode receptors for thyroid hormone (Sap et al, 1986; Weinberger et al, 1986). It is now clear that the human genome contains multiple genes homologous to v-erb-A (Dayton et al, 1984; Miyajima et al, 1988; Miyajima et al, 1989; Drabkin et al, 1988), not all of which necessarily encode thyroid hormone receptors. Accumulated evidence indicates that a number of these genes are clustered together on chromosome 17 (q21-25) and chromosome 3 (p21-25) (Green and Chambon, 1988; Miyajima et al, 1988; Benbrook and Pfahl, 1987; Miyajima et al, 1989). Miyajima et al (1988) isolated four human cDNA clones by homology to the v-erb-A gene. These were

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designated ear1, ear2, ear3 and ear7. The genes encoding ear2 and ear3 are located on chromosomes 19 and 5 respectively and are clearly members of the steroid receptor superfamily. However, the proteins encoded by these two cDNAs are distinct from any of the steroid or thyroid hormone receptors previously isolated. The ligands for these two novel receptors have not been determined. Both ear1 and ear7 are located on chromosome 17 at the same locus; 17q21. The predicted sequence of the protein encoded by ear7 is almost identical to that of two independently isolated thyroid hormone receptor cDNA clones; erba-T-1 (Benbrook and Pfahl, 1987) and hTRC/2 (Nakai et al., 1988). The predicted protein sequences of these three receptors contain several isolated differences which are probably the result of sequencing errors. Therefore it has been assumed that these clones all originated from the same mRNA species (Miyajima et al., 1989). However, there are conflicting reports regarding the ability of the proteins encoded by these three clones to bind thyroid hormone. The erba-T-1 and hTRC2 proteins were reported to bind T3 with high affinity (Benbrook and Pfahl, 1987; Nakai et al., 1988). In contrast, the protein encoded by the ear7 cDNA did not bind to T3 (Miyajima et al., 1989). This apparent paradox remains unresolved at present.

Comparison of the sequence of the ear1 and ear7 cDNA clones indicated that they could be encoded by opposite

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strands of the same genetic locus. This was confirmed by the cloning of the corresponding genomic fragment (Miyajima et al., 1989). Analysis of this genomic fragment suggested that the primary transcript of the ear7 gene (the thyroid hormone receptor alpha gene) might undergo differential splicing to generate two thyroid hormone receptor proteins, designated ear71 and ear72. This was confirmed by northern blot analysis; a probe taken from a region common to both proteins hybridised to two mRNA species of 5 and 2.5Kb. A probe taken from a region specific to ear71 hybridised to only the 5Kb mRNA whilst a probe specific to ear72 detected only the 2.5Kb mRNA. The predicted ear72 protein is 490 amino acids long and corresponds to the ear7/erbA-T-1/hTRW2 cDNA clones. The ear71 protein is predicted to be 410 amino acids long, and differs from ear72 only in the carboxy terminus (the hormone binding domain). As yet no cDNA clones corresponding to ear71 have been isolated. In order to test the T3 binding capability of the ear71 protein an artificial construct was prepared that contained all the sequences predicted to be present in ear71. This was used to synthesise ear71 protein in vitro, and this was shown to bind T3 with high affinity ($K_d=10^{-12}M$). The ear1 cDNA clone hybridised to a single mRNA species of 2.9Kb. The protein encoded by ear1 is also a member of the steroid receptor superfamily and the putative ligand binding domain shows 40% homology to the corresponding region in

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the chicken TR. The earl protein did show specific T3 binding, in that binding was competed by excess unlabelled T3. However, the affinity of this binding was too low to calculate a dissociation constant. Thus the true ligand for the earl protein remains to be identified.

A second distinct human thyroid hormone receptor has been isolated by Weinberger et al (1986). This cDNA clone encodes a protein of 450 amino acids that is more distantly related to the viral v-erb-A gene than the alpha type TR isolated by Benbrook and Pfahl (1987), Nakai et al (1988), and Miyajima et al (1988). This receptor has been designated as a beta type (hTRB) and is located on chromosome 3 (p21-25). The hTRB protein binds thyroid hormone with high affinity.

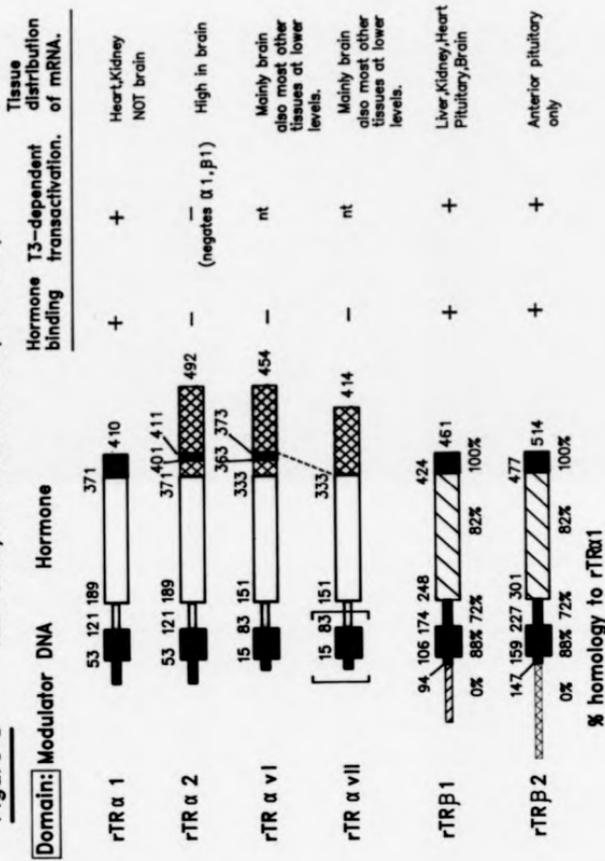
Two distinct human retinoic receptor cDNA clones have also been isolated (Petkovich et al, 1987; Brand et al, 1988; Benbrook et al, 1988). The clone isolated by Petkovich et al (1987) has been designated hRAR α and the gene encoding it is located on chromosome 17 q21.1 (see Brand et al, 1988 for refs.). The second retinoic acid receptor designated hRAR β , was originally identified as being at the site of integration of hepatitis B virus in a hepatocellular carcinoma (Dejean et al, 1986). The gene encoding this receptor is located on chromosome 3 p24 (see Brand et al, 1988 for refs.). In conclusion, these two loci at 17q21-25 and 3p21-25 both contain one gene encoding a retinoic acid receptor and another encoding a

Figure 8.

The rat thyroid hormone receptor family.

The structures of the six rat c-erbA-related proteins that have been isolated to date are drawn schematically to show the different domains. All the proteins are drawn with the amino terminus to the left of the page and have been aligned according to the location of the DNA-binding domain (blue box). Numbers above each region indicate the amino acid residue at which the region begins or ends. The receptors are divided into two groups; the alpha types and the beta types (which are encoded by two genes). Within each of these sub-groups (but not between), regions drawn in the same colour or shading have identical amino acid sequences. The column titled "T3-dependent trans-activation" refers to experiments in which the ability of the cloned receptor to activate expression of a thyroid hormone-responsive promoter in response to T3 has been tested in co-transfected cell lines. It has been shown that the rTR α 2 will inhibit the T3-dependent activation mediated by rTR α 1 or rTR β 1 (Koenig et al, 1989). The brackets around the amino terminal and DNA binding domains of rTR α VII denotes the fact that the sequence of this region has not been determined, but has been assumed to be the same as that of rTR α VI. Abbreviations used are T3, triiodothyronine; nt, not tested. References to the structures (nucleotide sequences and predicted amino acid sequences) of the various rat thyroid hormone receptors are: rTR α 1, Thompson et al (1987); rTR α 2, Izumo and Mahdavi (1988); rTR α VI and rTR α VII, Mitsuhashi et al (1988); rTR β 1, Koenig et al (1988); rTR β 2, Hodin et al (1989).

Figure 8 The rat thyroid hormone receptor family.



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thyroid hormone receptor. This suggests that the two loci have evolved after a chromosome duplication event. In addition, a novel steroid hormone receptor is encoded on the opposite strand to the hTR α gene at 17q21. The primary transcript of the hTR α gene is alternately spliced to generate two slightly different receptor proteins that (according to the results of Miyajima *et al.*, 1989) differ in their ability to bind thyroid hormone.

A total of six distinct thyroid hormone receptor cDNA clones have been isolated from various rat tissues (see the legend to figure 8 for references). Comparison of the predicted amino acid sequences of these six receptors indicates that they are probably derived from two distinct genes designated alpha and beta. A genomic clone encompassing the 3' end of the rat TR α gene has been isolated (Mitsuhashi *et al.*, 1988). The alpha gene gives rise to at least four isoforms of the receptor by alternate splicing (see figure 8). In this figure identical shading or colour indicates identical amino acid sequences but only within the alpha and beta families. The four alpha proteins are identical from the amino terminus through to a position 182 amino acids into the hormone binding domain, except that rTR α vI and rTR α vII are 38 amino acids shorter at the amino terminus. It is not known how the difference at the amino terminus is generated. The sequences of the four isoforms diverge at corresponding positions within the hormone binding domain. The splicing

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pathway that generates the $\text{TR}\alpha 1$, $\text{TR}\alpha\text{VI}$ and $\text{TR}\alpha\text{VII}$ mRNAs has been deduced from the DNA sequence of the 3' end of the rat TR gene by Mitsuhashi et al (1988). However they do not take into account the existence of the $\text{rTR}\alpha 2$ cDNA clone (Izumo and Mahdavi, 1988), which is clearly a product of the same gene. However, Mitsuhashi and Nikodem (1989) mention that they have isolated another rat alpha type TR cDNA clone that is generated by alternative splicing at the carboxy terminus. This clone which they call $\text{rTR}\alpha\text{VIII}$ could be the same as $\text{rTR}\alpha 2$. The three isoforms $\text{rTR}\alpha 2$, $\text{rTR}\alpha\text{VI}$ and $\text{rTR}\alpha\text{VII}$ all have a common 81 amino acid c-terminal region. The $\text{rTR}\alpha 2$ and $\text{rTR}\alpha\text{VI}$ forms also have in common a stretch of 30 amino acids (indicated by the blue hatched box in figure 8). In addition, $\text{rTR}\alpha\text{VI}$ contains a block of 10 amino acids identical to the c-terminal 10 amino acids of $\text{rTR}\alpha 1$ (indicated by the red box in figure 8), whilst the corresponding region of $\text{rTR}\alpha 2$ appears to be unique.

Of the four alpha type isoforms, only $\text{TR}\alpha 1$ actually binds thyroid hormone with high affinity. This suggests that sequences within the c-terminal 40 amino acids of $\text{rTR}\alpha 1$ are crucial for hormone binding. The in vitro translation products of $\text{rTR}\alpha 1$, $\text{rTR}\beta 1$ and $\text{rTR}\alpha 2$ will bind specifically to a thyroid hormone response element in vitro (Izumo and Mahdavi, 1988; Lazar et al, 1988; Koenig et al, 1989 (references therein)). It is not known whether hormone is required for binding of $\text{rTR}\beta 1$ and $\text{rTR}\alpha 1$ to a

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response element. Presumably the non-hormone binding rTR α 2 binds to response elements "constitutively" (i.e. in the absence of hormone) since it is unable to bind T3. It has been demonstrated that rTR α 1 can activate transcription from the thyroid hormone responsive α -myosin heavy chain (α -MHC) gene promoter in response to T3 (Izumo and Mahdavi, 1988). Thus, rTR α 1 appears to be a conventional hormone dependent transcription factor. In contrast, rTR α 2 did not activate transcription from the α -MHC gene promoter (Izumo and Mahdavi, 1988).

The alpha type rat TRs are expressed with differing abundance in virtually all the rat tissues that have been tested, including brain, heart, liver, spleen and kidney (Murray et al., 1988; Thompson et al., 1987; Mitsuhashi et al., 1988; Mitsuhashi and Nikodem, 1989). The existence of several differentially spliced mRNAs makes slot of the earlier data difficult to interpret with respect to which mRNA species is actually being detected. However it is clear that in the brain, the variant (non-hormone binding) receptor mRNAs are much more abundant than the functional receptor mRNA (Mitsuhashi and Nikodem, 1989). These authors reported that the ratio of variant (all types) to functional receptor mRNA in rat brain was 6:1. The non-binding variants were also more abundant in kidney (ratio 5:1), and liver and heart (ratio 2:1). The abundance of the alpha type TR mRNAs in the liver but not in the brain was affected by the thyroid state of the animal

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(Mitsuhashi and Nikodem, 1989). In hypothyroid rats the levels of both the variant and functional receptor mRNAs were increased by 50% compared to normal rats. Similarly, Lazar and Chin (1988) found that the level of the rTR α 2 mRNA in a pituitary cell line decreased by 50% following T3 treatment. The significance of these changes is unclear at present.

The function of one of these non-hormone binding isoforms has been tested in a co-transfection experiment (Koenig *et al.*, 1989). The rTR α 2 protein was expressed in a cell line that is functionally defective in T3 response either alone or in combination with the rTR α 1 and rTR β 1 proteins (see figure 8). Both rTR α 1 and rTR β 1 are functional receptors in that they can bind hormone and activate transcription from a responsive promoter. By co-transfection with an artificial T3 responsive test gene the ability of the cells to respond to T3 was assayed. In cells transfected with rTR α 1 or rTR β 1 alone, expression of the test gene was strongly induced by T3 treatment. However, when the cells were also co-transfected with a five fold excess of the rTR α 2 plasmid (in a triple co-transfection) the test gene was not induced by T3. In addition, this repression of the test gene by rTR α 2 was shown to be dose dependent, i.e. cells transfected with more rTR α 2 relative to rTR α 1 or rTR β 1, showed a greater repression of the test gene. Furthermore, the activation mediated by rTR β 1 was more sensitive to repression by

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rTR α 2 than the activation mediated by rTR α 1. There are several explanations for these results. It is possible that the rTR α 2 receptor could compete with the functional receptors for binding to the TRE. Alternatively, rTR α 2 could form inactive heterodimers with the functional receptor. The authors speculate that the inhibitory effect of rTR α 2 could be modulated by the binding of an as yet unidentified ligand to this protein. These findings provide an explanation for the long standing paradox that the brain (of mammals) contains significant amounts of hormone binding TRs but does not exhibit an increased metabolic rate in response to T3. Northern blot analysis indicates that high levels of the rTR α 2 mRNA (Lazar *et al.*, 1988) and the VI and VII mRNAs (Mitsuhashi *et al.*, 1988) are present in rat brain. It seems likely that the non-hormone binding variants repress the functional TRs in the brain so that it does not show the classical response to T3. Why this should be the case is unclear. Presumably the non-hormone binding variants must be involved in regulating the response of the brain to T3.

A recent paper (Lazar *et al.*, 1989) reports that a novel member of the steroid hormone receptor superfamily is encoded on the opposite strand of the rat thyroid hormone receptor alpha transcriptional unit. This receptor, designated Rev-ErbA α , was initially identified as a cDNA clone. The predicted amino acid sequence of the 56Kda Rev-ErbA α protein is similar to the receptors for thyroid

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hormone and retinoic acid, but the protein does not bind either of these ligands in vitro. The coding regions of Rev-ErbA α and rTR α 2 overlap by only 269bp within a single exon that encodes part of the carboxy terminus (hormone binding domain) of both proteins. The mRNA encoding Rev-ErbA α is present in many tissues, being particularly abundant in brown fat and skeletal muscle. Thus, in both rat and humans, a novel steroid hormone receptor is encoded by the opposite strand of the thyroid hormone receptor alpha gene.

The two rat beta type thyroid hormone receptor cDNA clones are closely related to the alpha type clones just described. However, the predicted protein sequences differ from the alpha types throughout the length of the protein, indicating that they are derived from a distinct gene. The rTR β 1 (β 1) and rTR β 2 (β 2) proteins are identical from a position 12 amino acids before the start of the DNA binding domain through to the c-terminus of the protein (see Hodin et al, 1989 and figure 8). The amino terminal domains are however entirely unrelated. This strongly suggests (but does not prove) that the β 1 and β 2 mRNAs are generated by alternative splicing. The DNA binding domain of the β type proteins is 88% identical at the amino acid level to the DNA binding domain of the α type rat TRs. The first 176 amino acids of the hormone binding domain of the β types show 82% amino acid identity to the corresponding region of the α type TRs. The c-terminal 37 amino acids

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of the beta types are identical to the corresponding region of rTRM1 (marked in red in figure 8). Both $\beta 1$ - and $\beta 2$ have been shown to bind thyroid hormone with high affinity in vitro (Koenig et al., 1988; Hodin et al., 1989). Both of the beta type TRs can activate transcription from a T3 responsive reporter gene in response to T3 (Koenig et al., 1988; Hodin et al., 1989).

Northern blot analysis of RNA from various rat tissues using probes specific for either the TR $\beta 1$ or TR $\beta 2$ mRNAs indicated that the B2 mRNA is expressed only in the anterior pituitary gland (Hodin et al., 1989). In contrast, the $\beta 1$ mRNA was present at different levels in all the tissues analysed, highest levels being in the brain, heart and brown fat. The rat pituitary tumour cell line GH₃ contains equivalent levels of $\beta 1$ and $\beta 2$ transcripts. Treatment of these cells with T3 reduced the level of $\beta 2$ transcripts by 85% whilst the level of $\beta 1$ transcripts actually increased slightly (Hodin et al., 1989). The significance of this differential effect of T3 on the two beta type TRs for thyroid hormone physiology is not clear. It is possible that the two forms of the beta type TR ($\beta 1$ and $\beta 2$) are transcribed from the same gene but from alternative promoters. Differential regulation of these promoters could then be responsible for both the distinct tissue distribution of the two mRNAs and the differential effect of thyroid hormone. Alternatively, the pituitary specific expression of the $\beta 2$ mRNA could be the result of

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tissue-specific differential splicing.

Presumably, the different tissue distributions of the $\beta 1$ and $\beta 2$ mRNAs reflect a role for the two receptors in the tissue-specific regulation of thyroid hormone responsive genes. With this in mind it is interesting that the only difference between the $\beta 1$ and $\beta 2$ proteins lies in their amino terminal (A/B) domains. As mentioned earlier in this chapter the A/B domain of steroid hormone receptors has been implicated in modulating the transactivation of target genes. Receptors with different A/B domains may differ in their ability to activate various target genes.

1.4.11 Differential expression of the alpha and beta retinoic acid receptors.

Two human retinoic acid receptors (RARs) designated alpha and beta have been characterised (Petkovich et al., 1987; Benbrook et al., 1988; Brand et al., 1988) and these are encoded by two genes (see section 1.4.10). These two receptors (designated hRAR α and hRAR β) have almost identical DNA and hormone binding domains but differ considerably in their n-terminal (A/B) domains. Retinoic acid (RA) is a vitamin A derivative that is believed to have an important role in the differentiation and development of vertebrates. For example, a gradient of RA is thought to act as a morphogen to supply positional information to the developing chicken limb bud (see The et

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al., 1989 for references). The two human RARs appear to have different affinities for retinoic acid in that different concentrations of RA are required to get similar levels of activation of a CAT linked reporter gene (Brand et al., 1988). The mRNAs encoding the alpha and beta hRARs show different tissue distributions (The et al., 1989). Also, treatment of human hepatoma cells with RA caused a 10 to 50 fold increase in the level of RAR α mRNA without affecting the level of RAR β mRNA (The et al., 1989). The induction of RAR α mRNA occurs at the level of transcription and does not require protein synthesis (The et al., 1989). Therefore RA directly regulates transcription of the hRAR α gene, possibly by the binding of a retinoic acid receptor/ligand complex to a response element in the promoter of this gene. The significance of this regulation is not known. This phenomenon is similar to the differential regulation of the rat TRs β 1 and β 2 by thyroid hormone (Hodin et al., 1989), although here the levels of β 2 mRNA are reduced by T3 treatment.

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1.4.12 Promiscuous recognition of hormone response elements by steroid hormone receptors.

In section 1.4.6 I described the sequences in DNA called hormone response elements (HREs) that are the binding sites for steroid hormone receptors and mediate transcriptional activation of a linked gene. There has been an un-stated assumption that the target gene specificity of steroid hormone receptors would be entirely determined by the specificity of binding to distinct HREs. Recently this assumption has been challenged by the finding that a number of the steroid hormone receptors have the capability to bind to common HREs.

It has been known for some time that the MMTV-LTR is responsive to a number of steroid hormones, including glucocorticoids, progesterone, androgens and mineralocorticoids (see review by Beato, 1989). The HRE within the MMTV-LTR is a rather large sequence element of approximately 70 to 100bp (Scheidereit and Beato, 1984; Cato et al., 1986; Chalepakis et al., 1988). The binding sites for PR, GR, AR and MR do overlap within this region but it is clear that there are distinct differences in the interaction of the different receptors within this region (Chalepakis et al., 1988). In addition, mutations within the HRE indicated that there were differences in the importance of particular residues for the binding of the PR and GR (Chalepakis et al., 1988). It would therefore

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appear that the various hormone receptors are binding to overlapping but not identical sequences within the MMTV HRE.

A much clearer indication of the promiscuous recognition of HREs by steroid hormone receptors comes from the finding that a consensus GRE can mediate transcriptional activation by progesterone, androgens and mineralocorticoids as well as glucocorticoids (Strahle et al. 1987; Ham et al. 1988). As mentioned earlier, no natural response elements for PR, AR or MR have been identified.

Glass et al. (1988) investigated the binding of the thyroid hormone receptor to various synthetic hormone response elements. They found that the receptor could bind with high affinity to both the ERE from the vitellogenin gene (vitERE) and the GRE from the metallothionein gene. The affinity of the TR for the vitERE was equivalent to that of the ER for this element. The affinity of the TR for the GRE was lower, but still significant. It was noticed that the sequence of the TRE from the rat GH gene (in this case the TRE they are referring to is the B and C domain half sites; see figure 5) resembled the vitERE (Glass et al., 1988). The homology between the two response elements was greatly increased by introducing a gap of 3bp in to rat GH TRE at the centre of the dyad symmetry. Thus the sequence of an ERE is much like that of a TRE except that the ERE has an extra 3bp at the centre of the palindrome. Furthermore, methylation interference

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experiments indicated that the TR made similar contacts with the vitERE and a perfectly palindromic TRE (Glass et al., 1988). In transfection experiments the rat GH TRE could confer induction by T3 upon the TK promoter. When the TRE was mutated to the sequence of the vitERE, expression became responsive to estrogen. However, treatment with T3 actually reduced the induction by estrogen. This negative effect was also found when 3bp were inserted into the TRE at the centre of dyad symmetry. This suggests a model in which the TR binds to an ERE in a transcriptionally inactive form. In this way it competes with the ER for binding to the ERE and reduces induction by estrogen.

In the rat GH TRE the gap between the two half sites that are in inverted repeat orientation (the B and C domains) is 1 bp (see figure 5). The affinity of the TR for the rat GH TRE into which between 1 and 6 bp had been inserted at the centre of the half sites, was not significantly reduced (Glass et al., 1988). It is generally accepted that steroid hormone receptors bind to HREs as dimers, with each monomer contacting one half site of the palindrome. Inserting extra nucleotides between the half sites not only increases the distance between the half sites, but also rotates them relative to each other. The TR dimer must therefore be astoundingly flexible to be able to bind to a palindrome separated by anything between 1 and 6bp. Clearly, when an extra 3bp are inserted into

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the TRE it stops functioning (although it can still bind the TR), and instead can confer induction by estrogen. This finding has interesting implications for the organisation of TRE half sites in T3 responsive genes (see figure 6). The A and B half sites of the rat GH TRE are separated by 4bp and might therefore be expected to function as an ERE and not as a TRE. However, the experiments performed by Brent *et al* (1989a) showed that the A and B half sites (containing two point mutations that create a perfect palindrome) could confer induction by T3. In the rat α MHC gene, the two TRE half sites in inverted repeat orientation have a 2bp gap (see figure 6). The physiological relevance of the finding that the TR can (in the presence of thyroid hormone) inhibit transcriptional activation from an ERE by the ER remains unproven.

I have already mentioned that the retinoic acid receptor can activate transcription via a TRE. This is further evidence that HREs are not as distinct as we might have expected, but form a family of related sequences. A comparison of all the response elements indicates that they are all distantly related (see review by Beato, 1989; and figure 4). As mentioned earlier in this chapter, the steroid hormone receptors can be sub-divided into two groups based upon overall sequence similarity (see figure 3). One group contains the GR, PR, AR and MR, whilst the second group consists of the ER, TR, RAR, and VitD3R.

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Interestingly, all the members of the first group can activate transcription through the MMTV-LTR. A comparison of the DNA binding domains of all these receptors shows that the members of the first group always have the amino acid sequence Gly-Ser between the two cysteine residues that form the C-terminal half of the first zinc finger (figure 9). Among members of the second group the sequence here is always Glu-Gly (figure 9). This suggests that these residues play an important role in determining DNA-binding specificity, and recently it has been demonstrated that this is the case (see the review by Berg, 1989; and section 1.4.13).

The research described in this section suggests unexpected connections between the actions of different steroid hormones. However, different hormones do have distinct biological effects which raises the question of how specificity is determined when different receptors can recognise the same HRE. There are a number of explanations, all of which may contribute to determining specificity. Firstly, steroid hormone receptors are not expressed ubiquitously thus precluding some cells from responding to particular hormones. Also, intracellular levels of hormones do not necessarily follow the level of circulating hormone because some cells have the ability to convert some hormones to inactive or more active derivatives. In the case of thyroid hormone, deiodination of circulating thyroxine to the much more active analogue

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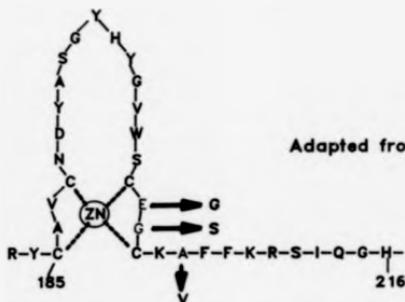
T3 can occur in some tissues. It has also been suggested that small differences in the affinity of different receptors for the same HRE could be significant in vivo. Throughout this chapter experiments have been described which indicate that steroid hormone receptors modulate the rate of transcription by interacting with other transcription factors. It seems likely that this interaction plays a major role in determining which genes can be activated by a particular hormone receptor. Many experiments have indicated that hormone receptors can bind to a HRE without necessarily activating transcription (discussed in detail in section 1.4.14). Thus, several receptors may be able to bind to a HRE but only one (the "correct" one) may be able to interact productively with other (cell-specific or basal) transcription factors bound at nearby sites. The finding that different receptors are able to bind to the same DNA sequence with, in at least one case, opposite transcriptional effects does not contradict the simple model but indicates that gene regulation by steroid hormones is much more complicated than had been envisaged.

1.4.13 Receptor determinants of DNA binding specificity.

Investigations into the DNA sequence requirements for binding of different steroid hormone receptors has lead to

Figure 9

A schematic representation of the first zinc finger of the estrogen receptors' DNA binding domain showing the "discriminating" amino acids. Also shown is an amino acid sequence comparison of sub-region CI from various members of the human steroid receptor family.



Discriminating amino acids are shown in red. Arrows show changes which alter specificity of the ER to that of the GR.

		C	L	I	C	G	D	E	A	S	G	C	H	Y	G	V	L	T	C	G	S	C	K	V	F	F	K	R	A	M	E	G	
"GR sub-family"	hPR	C	L	I	C	G	D	E	A	S	G	C	H	Y	G	V	L	T	C	G	S	C	K	V	F	F	K	R	A	M	E	G	
	hMR	C	L	V	C	G	D	E	A	S	G	C	H	Y	G	V	V	T	C	G	S	C	K	V	F	F	K	R	A	V	E	G	
	hAR	C	L	I	C	G	D	E	A	S	G	C	H	Y	G	A	L	T	C	G	S	C	K	V	F	F	K	R	A	E	G		
	hGR	C	L	V	C	G	D	E	A	S	G	C	H	Y	G	V	L	T	C	G	S	C	K	V	F	F	K	R	A	V	E	G	
"ER sub-family"	hER	C	A	V	C	N	D	Y	A	S	G	Y	H	Y	G	V	S		C	E	G	C	K	A	F	F	K	R	S	I	Q	G	
	hRAR	C	F	V	C	Q	D	K	S	S	G	Y	H	Y	G	V	S	A		C	E	G	C	K	G	F	F	R	R	S	I	Q	K
	hTR	C	V	V	C	G	D	K	A	T	G	Y	M	Y	R	C	I	T		C	E	G	C	K	G	F	F	R	T	I	Q	K	
	hVD3R	C	G	V	C	G	D	R	A	T	G	F	N	F	N	A	M	T		C	E	G	C	K	G	F	F	R	S	M	K	R	

The cysteine residues that are believed to coordinate with zinc are shown blue. Amino acids which are common to only one of the sub-families are shown red.

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the conclusion that HREs are a family of related sequences. Some progress is now being made towards understanding the other half of the equation; i.e. determining which amino acid residues within the receptors determine target gene specificity. By combining these two approaches we can begin to unravel the molecular interactions that underlie target site recognition by steroid receptors.

As described in section 1.4.4, the DNA binding domain is believed to form two zinc finger-like structures. By swapping each of these fingers individually between two different receptors it was shown that the C-terminal finger (CI) and not the second finger (CII) determined target gene specificity (Green et al, 1988). In the context of this finding it is interesting from an evolutionary point of view that the two fingers are encoded by separate exons (Green and Chambon, 1988 and references therein). This analysis has been extended further by altering specific amino acids within the CI sub-region and testing the effect upon target gene specificity (Mader et al, 1989; Umesono and Evans, 1989; Danielsen et al, 1989). Analysis of the human estrogen receptor in this way (Mader et al, 1989) showed that the identity of three amino acid residues were crucial for determining target gene specificity (Figure 9). Two of these lie between the two cysteine residues on the c-terminal side of the first finger that are thought to

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coordinate with zinc. The third "discriminating" residue is at the base of the first finger, again on its c-terminal side (figure 9). When the two residues between the cysteines were altered from Glu-Gly to Gly-Ser (as found in the GR) the receptor could weakly activate transcription from a reporter gene containing a GRE but not at all from one containing an ERE. When the third discriminating residue was also altered from Ala to Val (as found in the GR) the receptor could activate transcription from the GRE containing reporter gene more strongly, still without activating transcription from the ERE containing reporter gene. Similar results were obtained when essentially the same experiments were carried out on the glucocorticoid receptor (Umesono and Evans, 1989; Danielsen et al, 1989). When the Gly-Ser sequence between the two cysteines was altered to Glu-Gly (as found in the ER) the resulting receptor strongly activated transcription from an ERE containing reporter gene and only weakly activated a GRE containing reporter gene. In addition, when only the Glu to Gly change was made the resulting receptor could activate transcription from both reporter genes. Although the identity of these three residues is clearly sufficient to discriminate between a GRE and a ERE the data does not exclude the possibility that other residues play a role. It is perhaps surprising that residues at the base of the fingers rather than at the tips are apparently responsible for

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interacting with specific bases in the HRE.

As discussed previously, the steroid hormone receptor superfamily can be sub-divided into two sub-families both structurally and according to their ability to activate transcription from the MMTV LTR (see sections 1.4.12 and 1.4.3). The three discriminating amino acids identified in these studies are conserved within (but not between) the two sub-families (figure 9). Thus the "GR sub-family" has Gly-Ser and Val whilst the "ER sub-family" has Glu-Gly and Gly/Ala (figure 9). Furthermore, all four members of the "GR sub-family" can activate transcription of a reporter gene containing a consensus GRE (Strahle et al., 1987; Ham et al., 1988). Similarly, three of the four members of the "ER sub-family", namely the ER, TR, and RAR can bind to a common responsive element (a "TRE") (see section 1.4.12). This leads to the conclusion that these three discriminating residues are involved in recognising bases that differ between the consensus GRE and the consensus ERE/TRE. The half sites for these are: TGTTCT for the GRE and: TGACCA for the ERE/TRE. These differ by three bases (shown underlined) so it may be that it is these that are recognised by the discriminating amino acids in the receptor.

The identification of these discriminating amino acids also helps attempts to understand structural aspects of the interaction between steroid receptors and DNA. It has been predicted that the ten amino acids that follow the C-

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terminal cysteines of the CI finger could form an amphipathic alpha helix (see the review by Berg, 1989). This is an alpha helix in which one side is hydrophobic and the other hydrophilic. This may be analogous to the "recognition helix" identified in the helix-turn-helix motif of prokaryotic transcription factors. The hydrophilic side of this helix is thought to interact with DNA, recognising specific bases. In the predicted structure of the alpha helix for the human ER the discriminating Ala residue lies on the hydrophilic face where it could contact DNA. The second zinc finger (CII) does not determine target gene specificity so it may interact with DNA in a non sequence-specific manner, helping to stabilise binding. Alternatively, it has been proposed that its sole function is in dimerisation by making protein-protein contacts with another receptor monomer (Berg et al, 1989)

1.4.14 The role of the hormone in "activating" the receptor.

The simple model for the action of steroid hormones states that the unbound receptor is in a conformation that precludes DNA binding. Binding of hormone to the receptor in the cytoplasm alters its conformation such that it is able to bind to DNA. This is accompanied by transfer of the receptor/hormone complex to the nucleus, possibly in

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an active process. Several lines of evidence indicate that the process of receptor activation is not as straightforward as this model suggests.

The sub-cellular localisation of steroid hormone receptors has been studied for many years, and it was initially thought that in the absence of ligand the receptor was localised to the cytoplasm. However, when monoclonal antibodies became available it was found that the estrogen and progesterone receptors were intranuclear in the absence of hormone where they were relatively weakly bound (King and Greene, 1984; Welshons et al., 1984; Kumar et al., 1986). Following hormone treatment the estrogen receptor became tightly bound to chromatin (Jensen and DeSombra, 1973; Welshons et al., 1984; King and Greene, 1984; Kumar et al., 1986). The TR and VitD3R are also mainly nuclear in the absence of hormone, but were reported to be tightly bound to chromatin (Samuels and Tsai, 1973; Charles et al., 1975; Walters et al., 1981). Although there has been some controversy in the literature, it is now agreed that in contrast to the ER, PR, TR and VitD3R, the GR is mainly cytoplasmic in the absence of hormone and nuclear after hormone treatment (Picard and Yamamoto, 1987; Guiochon-Mantel et al., 1989 and references therein). Thus for the ER, PR, TR and VitD3R hormone binding is not necessary for nuclear localisation.

The exclusion limit for passive diffusion of proteins

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through nuclear pores is thought to be about 70Kd (Lang et al., 1986). The human GR, PR, MR, and one form of the AR all exceed this limit so one might expect that an active nuclear translocation process would be required for them to enter the nucleus. The ER, TR, RAR and VitD3R are all smaller than the exclusion limit, so might be expected to diffuse freely into the nucleus.

The hormone dependent nuclear localisation of the GR is dependent upon specific regions of the receptor (Picard and Yamamoto, 1987). These workers transiently expressed various mutated versions of the rat GR in tissue culture cells and monitored their sub-cellular localisation with antibody. One region required for nuclear localisation was mapped to a region between the DNA binding and the hormone binding domains. This domain, designated nuclear localisation signal 1 (NL1), is close to but distinct from the Tau 1 activation domain identified in the human GR (Hollenberg and Evans, 1988). The NL1 domain has 50% sequence similarity to a nuclear localisation signal identified in SV40 (Picard and Yamamoto, 1987). This sequence is absolutely conserved in the human, mouse and rat GRs. Furthermore, when other steroid hormone receptors are aligned according to the position of the DNA binding domain, this eight amino acid sequence is identical in the corresponding regions of the rabbit and human PRs and there is only one change in the chicken PR (Guiochon-Mantel et al., 1989). Significant homology is also seen

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with the AR and MR, whilst homology to the various ERs is poor. This region has also been shown to function as a nuclear localisation signal in the rabbit PR (Guiochon-Mantel, 1989; and see next paragraph). When the hormone binding domain was removed from the rat GR leaving NL1 intact, the truncated receptor was localised in the nucleus in the absence of hormone (Picard and Yamamoto, 1987). Thus, NL1 is a constitutively active nuclear localisation domain (in that it functions in the absence of hormone) which is normally masked by the hormone binding domain. A second nuclear localisation domain (NL2) was coincident with the hormone binding domain, and was entirely dependent upon hormone binding.

The PR is mainly nuclear in the absence of hormone, and a recent report (Guiochon-Mantel *et al*, 1989) shows that two major mechanisms are responsible for its nuclear localisation. A nuclear localisation signal was located between the DNA and hormone binding domains. This region of the PR is exactly homologous to the NL1 domain in the GR, but in contrast to NL1 it is constitutively active in the intact protein. This sequence is responsible for the observed nuclear localisation of the PR in the absence of hormone. When this sequence is deleted the receptor is entirely cytoplasmic in the absence of hormone, but can be shifted to the nucleus by hormone treatment. Thus, there is a second nuclear localisation function that is entirely hormone dependent. Removal of both the hormone binding

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domain and the constitutive NLI domain from the PR produced a protein that was nuclear in the absence of hormone. Therefore, the hormone dependent nuclear localisation function must be located in the DNA binding domain itself. When the receptor was truncated further so that the DNA binding domain was also removed, the protein was entirely cytoplasmic, showing that nuclear localisation of the truncated receptors was not simply due to reduction in size resulting in passive diffusion. Thus there are clearly two nuclear localisation functions, one of which is independent of hormone. The other is located in the DNA binding domain (perhaps it is the DNA binding domain per se), and is only functional in the presence of hormone. This suggests that the hormone binding domain masks the DNA binding domain, and hormone binding causes a conformational change that unmasks the DNA binding domain and results in nuclear localisation. Some elegant experiments (Guiochon-Mantel et al, 1989) showed that the hormone independent nuclear localisation of the PR involved movement of receptor monomers into the nucleus. In contrast, the hormone induced nuclear localisation appeared to involve the movement of receptor oligomers (the number of receptor subunits interacting could not be determined). What significance this has to the in vivo situation where most of the PR molecules are nuclear in the absence of hormone is unclear.

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Nothing is known about the nuclear localisation of other steroid hormone receptors.

There is now some evidence that in vitro, purified steroid hormone receptors can bind to their specific binding sites on DNA (hormone response elements) in the absence of hormone. For example, using DNaseI footprinting it was shown that GR prepared from rat liver could bind to the GRE of the MMTV-LTR in the absence as well as the presence of hormone (Willmann and Beato, 1986). Furthermore, binding to the GRE was also observed when the GR was bound to a glucocorticoid antagonist. The DNaseI footprints were indistinguishable in all three situations (Willmann and Beato, 1986). This indicates that hormone is not strictly required for binding of the receptor to the GRE. Whether binding of unliganded receptor to a GRE actually results in transcriptional activation is an entirely different question. It was also shown that in order to obtain binding of the receptor, either free or bound to hormone, to the GRE, it was essential to treat the receptor preparation at 25°C for 30 minutes. Without this heat "activation" of the receptor, no binding occurred (Willmann and Beato, 1986). The requirement for heat treatment to "activate" binding of the GR to a GRE has been confirmed (Denis et al., 1988). At physiological body temperatures receptor activation in vivo should be spontaneous. This is an important point which I will

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return to later. Affinity purified PR has also been used to examine whether hormone is required for binding of the receptor to the HRE (Bailly et al., 1986). Once again it was reported that the receptor could bind to the HRE in the presence of hormone and hormone antagonist, and in the absence of hormone. However, binding was hormone dependent when "crude cell extracts" were used (Bailly et al., 1986 and references therein).

In dramatic contrast to these in vitro results, genomic footprinting showed that the GR will bind to a GRE (in the rat tyrosine amino transferase gene) in vivo only after hormone treatment (Becker et al., 1986). A possible explanation of the difference between the in vitro and in vivo results is that in vivo, in the absence of hormone, the receptor is complexed with some cellular component that prevents it from binding to the HRE. The hypothesis would be that hormone binding induces dissociation of the receptor from this complex. Partially purified receptor preparations could have dissociated from this component during the extraction or purification procedure resulting in a constitutively binding receptor. This would account for the finding that "crude cell extracts" in which dissociation had not occurred were dependent upon hormone for DNA binding. There is now considerable evidence that suggests (but does not prove) that ligand-free steroid receptors are complexed with a heat shock protein. The evidence for this is outlined below.

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In "cytosolic preparations" (a high speed supernatant of a tissue homogenate) the hormone-free progesterone and glucocorticoid receptors have a sedimentation coefficient of about 8 Svedbergs (S) (Joab et al., 1984; Sullivan et al., 1985; Catelli et al., 1985). The way in which these cytosolic preparations are made does not preclude nuclear components, so both nuclear and cytoplasmic receptors are present. The size of pure steroid receptor is only 4 to 5S, indicating that PR and GR prepared in this way are complexed with another entity. This complex is stabilised by molybdate and peroxide (Tienrunroj et al., 1987) but is dissociated by heat treatment (25°C for 30 minutes), dilution of the extract and increased ionic strength (0.25M KCl) (Joab et al., 1984; Groyer et al., 1987). In most reports this 8S receptor complex has been isolated in the presence of molybdate. However, it is also possible to isolate it in the absence of molybdate, indicating that it is not just an artefact of the molybdate treatment (Denis et al., 1988). Various biochemical and immunological experiments have shown that the 8S receptor complex is unable to bind to both non-specific DNA (Mendel et al., 1986; Housley et al., 1985; Groyer et al., 1987) and hormone response elements (Denis et al., 1988). Upon binding of hormone or heat treatment the 8S complex is converted to the 4S receptor which is now able to bind to non-specific DNA (Mendel et al., 1986; Housley et al., 1985; Groyer et al., 1987). However, there have been apparently conflicting

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reports regarding the conversion of the 8S receptor complex to a form which can bind specifically to hormone response elements. Binding of the partially purified PR to a GRE was reported to occur independently of the hormone and in the presence of an antagonist (Bailly et al., 1986). Heat treatment was not required to "activate" the receptor. However, in these experiments it is not clear whether the receptor preparation used was in the form of the 8S complex or not. Willmann and Beato (1986) found that the 8S GR complex in crude cytosol that had not been stabilised with molybdate would bind specifically to the MMTV GRE only if "heat activated" by incubation at 25°C for 30 minutes prior to the binding reaction. Binding of the "heat activated" GR to the GRE occurred in both the presence and the absence of hormone, and also in the presence of a glucocorticoid antagonist. In contrast to these results it was reported (Denis et al., 1988) that using molybdate stabilised 8S GR complexes, binding of GR to the MMTV GRE only occurred if the receptor preparation was first treated with the hormone and then incubated at elevated temperature (25°C for 30 minutes). No binding was detected if the complexes were heat treated first and then incubated with hormone, or when treated with either hormone or elevated temperature alone (Denis et al., 1988).

When a crude extract, prepared from HeLa cells that had been transiently transfected with a plasmid expressing the human ER, was used in a gel retardation assay, binding of

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the ER to the vitellogenin ERE was hormone dependent (Kumar and Chambon, 1988). No binding of the ER to the ERE was detectable unless the cells had been treated with estrogen before preparing the cell extract. Interestingly, binding also occurred with extracts prepared from cells that had been treated with the anti-estrogen tamoxifen, suggesting that this antagonist acts by inhibiting the activation function of the receptor. These whole cell extracts were prepared by homogenisation or freeze-thawing in a simple buffer that did not contain peroxide or molybdate. Binding reactions were at 20°C for 15 minutes, but otherwise no "heat activation" was carried out. Binding to the ERE was also hormone dependent when the hormone was added after preparing the extracts (Kumar and Chambon, 1988).

Despite these worrying discrepancies it is clear that the GR and the PR in the 8S complex cannot bind to a HRE. When it is converted to the 4S form it can bind specifically to a HRE. The conversion from the 8S to the 4S form was found to be accompanied by the liberation of a 90Kd, non-steroid binding phosphoprotein (Joab et al., 1984). There is now overwhelming evidence that this non-steroid binding protein is the heat shock protein HSP90 (Catelli et al., 1985; Sanchez et al., 1985; Mendel et al., 1986). It has been shown that this same heat shock protein is also associated with the chicken estrogen and androgen receptors in a similar 8S complex in cytosolic

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preparations (Joab et al., 1984).

It has been proposed (Catelli et al., 1985; Sanchez et al., 1985; Mendel et al., 1986) that in the absence of hormone, steroid hormone receptors are bound to HSP90 in an 8S complex, and that this prevents the receptor from binding to DNA. Possibly the heat shock protein blocks the DNA binding domain of the receptor. Upon hormone binding, HSP90 would be displaced thereby unmasking the DNA binding domain. This provides an attractive explanation for why the purified (i.e. non-complexed) receptor can bind to HREs in vitro in the absence of hormone, whilst in vivo, and with crude extracts that were not heat treated, binding was hormone dependent. Thus the role of hormone in receptor activation may simply be to dissociate the receptor from HSP90; the receptor itself is constitutively active unless complexed to HSP90. Although this mechanism is attractive it has not been proven unambiguously that the HSP90 receptor complexes detected in cytosolic preparations actually exist in vivo and are not an artefact of the isolation procedure. If the HSP90 theory is correct then the HSP90-receptor complex must be stable under physiological conditions. Clearly, the complexes isolated from cells using procedures that are as undistruptive as possible are remarkably unstable. In particular, heat treatment at 25°C causes the dissociation of HSP90. So, at face value, one would not expect the complex to exist inside the cell. Therefore, the HSP90-

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receptor complex would have to be somehow stabilised in vivo if it is to play its proposed role in receptor activation. Furthermore, since most steroid hormone receptors are mainly nuclear in the absence of hormone, HSP90 would have to be present in the nucleus. As far as is known, HSP90 is mainly cytoplasmic (see Bailly et al. 1986). However this does not mean that a small amount of HSP90, sufficient to complex all the steroid receptors is present in the nucleus. As yet it has not been possible to reproduce the interaction between HSP90 and the receptor in vitro. (see Bresnick et al., 1989). Conditions which disrupt the receptor-HSP90 complex have been found to inactivate the ability of cytosols to bind steroid hormones, suggesting that the complex may be necessary for hormone binding (Bresnick et al., 1988). Further evidence that the receptor must be complexed to HSP90 in order to bind hormone has recently been published (Bresnick et al. 1989). Partially purified GR was subjected to various treatments and then assayed for its ability to bind hormone. Under no conditions was an HSP90-free receptor able to bind hormone. Moreover, receptors that had been inactivated could not be reactivated, once again suggesting that the dissociation of HSP90 is irreversible. If the conclusion from this work is correct it raises the question of how various steroid hormone receptors synthesised in vitro from cDNA clones are able to bind hormone. As far as I know, all hormone binding assays have

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been done with protein synthesized in rabbit reticulocyte lysate, and this is known to contain HSP90 (Bresnick et al, 1989).

The human ER expressed in yeast is able to mediate activation of a co-transfected gene containing an ERE in response to estrogen (Metzger et al, 1988). Furthermore, in vitro, binding of ER prepared from yeast expressing the human ER, to an ERE, was shown to be hormone dependent (Metzger et al, 1988). Thus, if HSP90 is necessary for the inactivity of the unliganded ER, then the analogous yeast heat shock protein must also be able to interact with the ER.

Finally, two recent papers (Damm et al, 1989; Graupner et al, 1989) appear to show that thyroid hormone receptor can bind to a TRE in the the absence of hormone in vivo, which is not consistent with the HSP90 theory. When a reporter gene (TK promoter linked to CAT) containing an artificial TRE was transfected into CV1 cells a high basal level of CAT activity was detected, and this was only marginally stimulated by T3 treatment (Damm et al, 1989). When this reporter gene was co-transfected with a plasmid which expresses the rat TR α 1 under control of the RSV promoter, CAT activity was reduced by 80% in the absence of hormone compared to that seen without the receptor (Damm et al, 1989). Hormone treatment caused a 20 fold stimulation of CAT activity. This suggests that the receptor functions as a repressor in the absence of

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hormone. The TR was then expressed in COS cells from a high copy number SV40 vector in order to ensure high intracellular levels of the receptor. Whole cell extracts from the transfected COS cells were used in a gel retardation assay with the artificial TRE. This showed that the TR could bind to the TRE even when the cells had not been treated with T3 (Damm et al., 1989). This result appears to suggest that, at least for TR, hormone is not required in vivo for binding to the response element. However, activation of transcription does not occur in the absence of hormone so presumably T3 binding is required to "induce" a trans-activation function in the TR. This is consistent with the report that in the absence of hormone, the TR, like the VitD3 receptor is tightly bound to chromatin in the nucleus (Walters et al., 1981). It might also be argued that the ligand independent binding observed in these experiments is the result of the high intracellular concentration of the TR. Under these conditions the cells might not have sufficient HSP90 to complex all of the TR, leaving some uncomplexed TR to bind to the TRE.

In similar transfection experiments it was shown that a reporter gene containing the TRE of the rat α -myosin heavy chain gene could be induced by retinoic acid in the presence of the co-transfected retinoic acid receptor beta (Graupner et al., 1989). It has already been demonstrated that RAR could act through a TRE (Umesono et al., 1988;

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Bedo et al, 1989). The unexpected finding was that when a thyroid hormone receptor (hTR α or hTR β) was also expressed in these cells (in triple transfections), induction of CAT by retinoic acid was repressed in the absence of T3. In contrast, CAT activity was induced by T3 in the presence of the TR. Importantly, this induction by T3 was not repressed by the presence of the unliganded RAR. This again suggests that the unliganded TR can bind to a TRE and that in doing so it can repress activation by the RAR. This was confirmed by gel retardation assays. Furthermore, the use of hybrid receptors showed that the inhibitory effect of the TR was mediated through the DNA binding domain of the RAR. This strongly suggests that the unliganded TR competes with the liganded RAR for binding to the TRE. Once bound at the TRE the unliganded TR is unable to activate transcription. In contrast, the unliganded RAR cannot compete with the liganded TR for binding to the TRE. This suggests an essential difference between the mode of action of TR and RAR; TR can bind to a TRE in the absence of hormone whilst the RAR cannot. However it is possible that the unliganded RAR is able to bind to a genuine retinoic acid response element (none have yet been identified), but not to a TRE. Another aspect of these experiments is that the RAR and the TR are behaving differently in the same cell. Assuming that HSP90 is responsible for preventing the RAR from binding to the TRE in the absence of hormone, then there is clearly

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sufficient HSP90 available to accomplish this. Yet, the TR is still able to bind to the TRE in the absence of hormone. This strongly suggests that HSP90 does not form a complex with the TR which prevents it binding to the TRE in the absence of T3. Of course, this does not mean that HSP90 is not involved in the activation by hormone of other steroid receptors, particularly those that are only loosely bound to chromatin in the absence of hormone or the GR which is cytoplasmic in the absence of hormone. It is looking more as if the mechanism of receptor activation by hormone may be different for different receptors.

1.5 The role of thyroid hormone in amphibian metamorphosis.

Amphibian metamorphosis is a rapid and dramatic developmental process that involves many biochemical, cellular and morphological changes. Presumably this involves changes in the pattern of gene expression. Unlike many of the changes in gene expression that occur in adult organisms which are often transient, it is generally believed that many of the changes that occur at metamorphosis are irreversible (see Morris, 1987; Lyman and White, 1987).

It has been known for many years that thyroid hormone plays a major role in initiating amphibian metamorphosis. Removal of the thyroid gland from pre-metamorphic larvae prevents metamorphosis indefinitely, and this can be

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relieved by very low concentrations ($10^{-9}M$) of thyroid hormone (T3). Also, inhibitors of thyroid hormone biosynthesis (thiourea and thiouracil) prevent metamorphosis (Gordon et al., 1943). Furthermore, metamorphosis can be induced prematurely by incubating Xenopus laevis larvae in thyroxine (May and Knowland, 1980). By analogy with other vertebrate systems it seems likely that thyroid hormone exerts its effects upon amphibian metamorphosis by modulating the expression of specific genes at a transcriptional level. However it is clear that the changes that occur during metamorphosis are so complex that they cannot all be regulated directly by thyroid hormone alone. It seems more likely that thyroid hormone is the trigger which sets in motion a complex developmental program. As yet it has not been demonstrated that any genes are regulated directly by thyroid hormone at the level of transcription during metamorphosis.

Both spontaneous and thyroxine induced metamorphosis is associated with a switch from ammonotelic to ureotelic excretion of amino-group nitrogen. This switch is made possible by increases in the levels of several enzymes in the liver which are essential for the formation of urea. One of these is carbamoyl phosphate synthetase (Metzenberg et al., 1961; Wixom et al., 1972; Mori et al., 1979). It has been reported that significant structural and biochemical changes occur in the liver of amphibia during metamorphosis (see Lyman and White, 1987 for references).

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For example, there is an increase in the synthesis of secreted liver proteins such as albumin and transferrin. The increase in the level of carbamoyl phosphate synthetase that is associated with thyroid hormone treatment was shown to be due to an increase in the level of the mRNA encoding this enzyme (Mori et al., 1979). More recently, Morris (1987) has assayed the levels of two urea cycle enzymes during the thyroxine induced metamorphosis of Rana catesbeiana using cross-hybridisation with mammalian cDNA clones. Over a three day period following the start of treatment with T3 the levels of mRNA in the liver encoding the enzymes carbamoyl phosphate synthetase and arginosuccinate synthetase increased coordinately by ten fold. The level of liver mRNA encoding another enzyme, phosphoenolpyruvate carboxykinase (PEPCK) fell by 90% following T3 treatment. This is in contrast to what has been observed in rat liver, where the level of PEPCK mRNA is increased by T3. These results suggest that T3 may cause changes in the levels of specific gene products by altering the level of their mRNAs. However, because these experiments were done with whole animals it is possible that the effects of T3 are indirect. In fact Morris (1987) suspected that the reduction in the level of PEPCK mRNA was the result of the T3 induced release of insulin. Lyman and White (1987) employed differential screening of a liver cDNA library prepared from T3 treated Rana catesbeiana to isolate cDNA clones corresponding to mRNAs

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whose levels are altered by thyroid hormone. Three clones corresponding to mRNAs whose levels are increased after T3 treatment were isolated, and the levels of two of these mRNAs were found to increase at metamorphic climax. Two clones corresponding to mRNAs that are repressed by T3 were also isolated, and the level of one of these mRNAs was found to decline to undetectable levels during metamorphic climax. Once again it is not possible to say that the level of any of these mRNAs is directly regulated by thyroid hormone. Clearly these changes are occurring at a pre-translational level, but this could be the result of post-transcriptional events or regulation of transcription or both.

Another change that occurs at metamorphosis is the formation of the adult skin, the composition of which is entirely different from that of the larva. This is associated with the appearance of adult keratins. In Xenopus laevis, adult keratins first appear between stages 48 and 52 (see Mathisen and Miller, 1989). Thyroid hormone has been shown to induce the synthesis of adult keratins both in vivo, and in skin explants cultured in vitro (Mathisen and Miller, 1989). Their results suggest that two steps are involved in the induction of adult keratins. The first step results in the synthesis of low levels of keratins and does not require thyroid hormone. After addition of T3 there was a lag period of two days before levels of adult keratin mRNA started to increase. During

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normal metamorphosis there was a 1000 fold increase in the level of adult keratin mRNA between stages 48 and 52. It is not known whether this induction is the result of an increased rate of transcription or post-transcriptional events such as mRNA stabilisation. In mammalian systems induction of transcription by thyroid hormone is a very rapid process; induction can often be detected within a few hours of administering the hormone (for example see Bedo et al, 1989). The lag period observed by Mathisen and Miller (1989) suggests that perhaps T3 is not activating keratin gene expression in a similar way. Perhaps the action of T3 is indirect so that a lag period occurs while the hormone is acting on some other process. For example, T3 might directly regulate a small number of genes, the products of which are involved in activating (and repressing) structural genes such as the adult keratins. Once the adult keratin genes had been induced thyroid hormone was not required to maintain expression, indicating that, unlike the action of steroid hormones in adult mammals, induction was irreversible.

It has been demonstrated that most tadpole tissues, including the liver, contain receptors for thyroid hormone (Kistler et al, 1975; Galton and St. Germain, 1985). Based upon various biochemical changes it was shown that X.laevis larvae first acquire competence to respond to exogenous thyroid hormone between stages 36 and 41 (Tata, 1968). This presumably corresponds to the appearance of

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the receptor. Indeed, by using radiolabelled T3 it was shown that high affinity binding sites for T3 first appeared in whole Xenopus larvae between stages 31 and 34 (Tata, 1970). During this period there was a large increase in the binding of labelled T3 by the larvae (Tata, 1970). Thyroid hormone first appears in the blood of X.laevis larvae at about stage 50 (see Mathisen and Miller, 1989), which is some considerable time after the appearance of the receptor.

As noted above, it has not yet been demonstrated that any of the genes whose expression increases (or decreases) during metamorphosis are directly regulated by thyroid hormone. Perhaps the only unambiguous way to demonstrate this is to test prospective T3 responsive genes in transfected cell lines or in in vitro transcription systems. It would be a major step forward if the specific nuclear receptor for thyroid hormone was cloned. This would enable the involvement of the receptor in the regulation of genes that are activated or repressed by thyroid hormone during amphibian metamorphosis to be investigated.

1.6 Evidence that the Xenopus albumin genes are regulated by thyroid hormone.

In adult liver, expression of albumin is repressed by estrogen (May et al., 1982b; Wolffe et al., 1985). This

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repression has been reported to be the result of a decrease in the levels of albumin mRNA in the liver (May et al., 1982b; Wolffe et al., 1985), although another group (Philipp et al., 1982) found that levels of albumin mRNA were unaffected by estrogen. There is also disagreement as to whether the proposed reduction in the level of albumin mRNA is the result of purely post-transcriptional events (Kazmaier et al., 1985), or a combination of a decreased transcription rate and post-transcriptional events (Wolffe et al., 1985).

Albumin is the major protein in the blood of vertebrates. Very early work on amphibian metamorphosis showed that the levels of serum proteins in the blood of pre-metamorphic Rana catesbeiana were remarkably low, and that the amounts increased dramatically during metamorphosis (Herner and Frieden, 1960; Frieden et al., 1975). Further studies showed that the major change in serum proteins associated with either spontaneous or T3 induced metamorphosis was a dramatic increase in the level of albumin (Frieden et al., 1975). However, it should be noted that albumin was only identified by its mobility in paper chromatography. Some of these experiments were on Xenopus laevis, and the data indicate a 40 fold difference in the level of albumin protein in the blood of pre-metamorphic larvae and adults. However, the exact time course of the induction of albumin synthesis (and secretion) during metamorphosis was not investigated. When

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pieces of adult Xenopus liver were maintained in tissue culture, synthesis of liver-specific proteins decreased over a period of time (Wangh, 1982). Albumin synthesis could be restored with various hormones. Dexamethasone (a glucocorticoid) and thyroid hormone appeared to act together to re-introduce albumin synthesis, suggesting that albumin expression may be up-regulated by thyroid hormone (Wangh, 1982). Obviously, this might not be a direct effect, and it was not known at which level albumin synthesis was being affected.

Investigations into the function of thyroid hormone in amphibian metamorphosis would be significantly advanced by the cloning of the thyroid hormone receptor. The deduced structure might lend support to the proposal that some of the effects of thyroid hormone are the result of direct modulation of transcription of specific genes. Also, the ability to express the receptor in a more defined system such as cultured cells might enable the effect of thyroid hormone on specific hormone responsive genes to be studied. Our laboratory has been studying the Xenopus laevis 68Kda albumin gene with respect to its tissue-specific expression in adult liver. The albumin genes of amphibians are unique in that as well as being expressed in a tissue-specific manner they also appear to be activated at metamorphosis. I have shown that the X.laevis albumin genes are first transcribed at the onset of metamorphosis, and I have isolated a full length clone

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from a X.laevis cDNA library that encodes a high affinity receptor for thyroid hormone. This receptor mediates the induction by T3 of the expression of a reporter gene containing an artificial thyroid hormone response element. This shows that the Xenopus receptor functions in an analogous way to the mammalian thyroid hormone receptors, lending support to the idea that thyroid hormone regulates the transcription of specific genes during metamorphosis. Furthermore, preliminary experiments suggest that thyroid hormone may directly induce the expression of the Xenopus albumin genes at a pre-translational level, by a mechanism that requires the thyroid hormone receptor.

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CHAPTER 2

MATERIALS.

2.1 General materials.

Restriction enzymes were from Amersham International (U.K.), Northumbria biologicals limited (NBL) and Bethesda research laboratories, Maryland U.S.A. (BRL). E.coli DNA polymerase I and T4 DNA ligase were obtained from Amersham International, T3 and T7 RNA polymerases were from BRL, sequencing grade DNA polymerase Klenow fragment was from NBL and AMV reverse transcriptase was from Life sciences Inc. (U.S.A.).

The lambda in vitro packaging kit (Giga Pack Gold) was from Stratagene (La Jolla, CA, U.S.A) supplied through NBL.

All radioisotopes were supplied by Amersham International at the following specific activities; α [³²P] dGTP and α [³²P] dCTP; 3000 Ci/mmol, γ [³²P] ATP; 5000 Ci/mmol, α [³²P] rUTP; 3000 Ci/mmol, [³⁵S] dATP; 1000 Ci/mmol, [¹²⁵I] triiodothyronine; >1200 μ Ci/ μ g, [³⁵S] methionine; >1000 Ci/mmol, [³H] rTTP; 30Ci/mmol.

Nitrocellulose sheets (Hybond-C) and Nylon filters (Hybond-N) were obtained from Amersham. Type II agarose (medium EEO) was supplied by Sigma chemical company and low melting point agarose was from FMC Bioproducts or ICN Biomedicals Inc. Materials for bacteriological media were from Difco laboratories (Michigan, U.S.A.) and Oxoid limited (England).

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E.coli transfer RNA (type XX) was supplied by Sigma.

X-ray film was from Fuji photo company limited (Japan)

All other chemicals and reagents were from BDH ("Analar" grade) or from Sigma chemical company unless otherwise stated.

2.2 Stock Solutions.

Deionised formamide - Formamide was stirred with Amberlite monobed resin MB-3 or MB-4 (5g per 100 ml of formamide) until the pH was 7, filtered through Whatman No. 1 filter paper and then stored at -20°C.

Filtered formaldehyde - A 40% (w/v) solution of formaldehyde was filtered through Whatman No. 1 paper.

TE - 10mM Tris.HCl (pH7.5), 1mM EDTA (pH8)

10*TBE - 108 g/l Tris base, 55 g/l boric acid, 9.5 g/l EDTA, pH8.

20*SSC - 3M NaCl, 0.3M Na Citrate, pH7

50 * Denhardtts - 1% each of Ficoll, polyvinylpyrrolidone, bovine serum albumin.

10*MOPS - 0.2M 3-(N-morpholino) propanesulphonic acid, 50mM sodium acetate, 10mM EDTA, pH7

2.3 Bacteriological Media.

LB was prepared by dissolving 10g of NaCl, 10g of bactotryptone and 5g of yeast extract in 1 litre of distilled water. For LB plates this was supplemented with 15g/litre of bacto agar.

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2*TY was prepared by dissolving 8g of NaCl, 16g of bactotryptone and 10g of yeast extract in 1 litre of distilled water.

H-Agar was made by adding 8g of NaCl, 10g of bactotryptone and 15g of bacto agar to 1 litre of distilled water

NZY broth was prepared by dissolving 5g of NaCl, 2g of $MgSO_4 \cdot 7H_2O$, 5g of yeast extract and 10g of NZ amine in 1 litre of water. For NZY plates this was supplemented with 15g per litre of bacto agar. NZY top agarose was made by adding 0.7g per 100 ml of agarose (type II) to NZY broth.

H-top agar was prepared by dissolving 10g of bactotryptone, 8g of NaCl and 6g of agar (or 6g of agarose for H-top agarose) in 1 litre of distilled water.

Terrific broth (TB) was occasionally used to grow bacteria for large scale plasmid isolation. It was prepared by dissolving 12g of bactotryptone and 24g of yeast extract in 800 ml of distilled water, adding 3ml of glycerol and then autoclaving. Just before innoculating this with bacteria, 100ml of 0.17M KH_2PO_4 and 100ml of 0.72M K_2HPO_4 (both previously sterilized by autoclaving) were added.

All media were autoclaved before use.

Antibiotics were used in plates and media at the following final concentrations. Ampicillin; 100 μ g/ml, tetracycline; 15 μ g/ml.

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2.4 Bacterial, plasmid and phage genotypes.

2.4.1 E. coli strains.

JM101 - supE, thi, Δ (lac-proAB), [F', traD36, pro AB, LACIqZDMI5] 17-18 ditto, not tra D36.

MC1061 - F⁻, ara D139, Δ (ara, leu)7696, Δ lac Y74, gal U⁻, gal K⁻, har⁻, hsm⁺, strA.

DH1 - F⁻, endA1, hsdR17(rk⁻, mk⁺), supE44, thi-1, λ ⁻, recA1, gyrA96, relA1.

Y1090 - [Δ Lac U169, proA⁺, lon, araD139, strA, supF, trpC22:Tn10(pMC9)].

BB4 - supF58, supE44, hsdR514 (rk⁻, mk⁻), galK2, galT22, trpR55, metB1, tonA, lambda⁻, D(arg-lac)U169 [F', proAB, lacIqZDMI5, Tn10(tet^r)].

XL1-blue - endA1, hsdR17(rk⁻, mk⁺), supE44, thi-1, lambda⁻, recA1, gyrA96, relA1, (lac⁻) [F', proAB, lacIqZDMI5, Tn10(tet^r)].

2.4.2 Plasmid vectors.

pBR322 - General purpose cloning vector.

pSP64/pSP65 - SP6 in vitro transcription vectors (Promega Biotech, Madison U.S.A)

pGEM1/pGEM2 - SP6/T7 in vitro transcription vectors (Promega Biotech, Madison U.S.A)

pGEM3Z/pGEM4Z - SP6/T7 in vitro transcription vectors also incorporating the LacZ colour selection system (Promega Biotech, Madison U.S.A)

pSVL - an SV40 late expression vector designed for high

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level transient expression in eukaryotic cells (Pharmacia ,U.S.A.) The "poison" sequence of pBR322 is not present resulting in an increased plasmid copy number and a higher level of transient expression in COS cells.

pBluescript SK⁻ - The plasmid generated by automatic excision from λ ZAP. Has an extensive polylinker, the lacZ colour selection system and promoters for both T3 and T7 RNA polymerases (Stratagene, La Jolla, CA U.S.A)

2.4.3 Bacteriophage vectors.

λ ZAP - a bacteriophage lambda insertion vector (Stratagene, La Jolla, CA, U.S.A). Cloned inserts can be automatically excised from the phage vector and converted to the plasmid vector pBluescript for ease of handling (Short et al, 1988). This was purchased in the form of DNA that was pre-digested with EcoRI and de-phosphorylated.

M13mp18 and M13mp19 - bacteriophage M13 vectors designed for sequencing by the dideoxy chain termination method (Messing et al, 1981)

M13mp10 and M13mp11 - earlier versions of the mp18/19 vectors differing only in the polylinker (Messing et al, 1981).

2.4.4 Plasmid and Bacteriophage Recombinants.

λ x68a206 - a Xenopus laevis genomic clone in the lambda vector charon 4A containing the complete 68KDa albumin gene kindly provided by Dr. G. Ryffel (May et al, 1982a).

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pSVL-8 - a DNA fragment from a Xenopus laevis histone gene cluster containing a functional H3 gene inserted in the SV40 vector pSVL2

pTK28mult - A plasmid in which two copies of an oligonucleotide containing an idealised thyroid hormone response element have been inserted just upstream of the TK promoter linked to the reporter gene CAT (Brent et al., 1989b). This was kindly provided by D. Moore.

pUMSAE1 - a rat albumin promoter/CAT fusion construct kindly donated by M. Weiss. (Heard et al. (1987)).

B5400 and B120 - Plasmids containing the rat β -fibrinogen promoter (numbers refer to the length of DNA upstream of the transcription start site in nucleotides), linked to the reporter gene CAT (Courtois et al., 1987). These plasmids were kindly provided by G. Crabtree.

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CHAPTER 3.

METHODS.

3.1 Oocytes, Eggs And Embryos.

3.1.1 Oocytes

Oocytes were obtained by anaesthetising a female Xenopus laevis with MS222 and carrying out a partial ovariectomy. The oocytes were manually stripped from the ovary, washed and maintained in full strength Barth-X.

3.1.2 Eggs and embryos.

Female X. laevis were induced to ovulate by subcutaneous injection with 250 units of serum gonadotrophin (Intervet U.K. Ltd) 48 hours before laying followed by 250 units of chorionic gonadotrophin B (Intervet U.K. Ltd) 16 hours before laying. Male X. laevis were injected with 250 units of chorionic gonadotrophin 8 hours before laying.

For in vitro fertilisation a male X. laevis was killed by injection with 0.5ml of 250mg/ml phenobarbitone (euthatal, May and Baker), and the testis removed into full strength Barth-X on ice. 50 to 100 eggs layed in full strength Barth-X were transferred to a petri dish and dredged with pieces of teased testis for 30 seconds. After leaving for 1 minute a small amount of distilled water was added to activate the sperm. Two minutes later the dish was flooded with distilled water.

Fertilised eggs were de-jellied in 2% (w/v) cysteine (pH

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adjusted to 8 with NaOH) and then washed at least 4 times in full strength barth-X or one tenth barth-X.

Prior to gastrulation embryos were transferred to one tenth barth-X to avoid exogastrulation. After hatching larvae were fed on vegetable soup added to the water.

Embryos were staged according to Nieuwkoop and Faber (1956).

3.2 Microinjection Of Xenopus Oocytes.

Oocytes that had been obtained as described in section 3.1 were microinjected using a fine, drawn out glass capillary mounted on a micro manipulator. The needle was linked via a thin gauge pipe to a syringe driven by a vernier, and the system partially filled with oil. Oocytes were transferred to a piece of moist filter paper placed on a microscope slide and injected with about 10nl of DNA or RNA dissolved in sterile water, under a binocular microscope. For nuclear injections the needle was inserted a small distance into the animal pole where the germinal vesicle usually lies. Cytoplasmic injections were into the vegetal half of the oocyte.

3.3 Microinjection Of Fertilized Eggs.

Batches of eggs were fertilized in vitro and de-jellied soon after rotation. Embryos just beginning the first cleavage were transferred to 1*BX supplemented with 5% (w/v) ficoll (m.w 40,000) and injected in the animal pole

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with about 10nl of DNA dissolved in 80mM NaCl. Embryos were injected only between the 2 cell and 4 cell stages. Immediately after injection embryos were transferred to a 14°C incubator. They were kept in 5% ficoll at 14°C until they had reached stage 6 to 8 since this was found to reduce leakage of cytoplasmic material from the hole made by microinjection. However, continued incubation in 5% ficoll was found to be toxic. The embryos were then washed several times in $1/10^{\text{th}}$ barth-X and incubated in $1/10^{\text{th}}$ barth-X at 14°C for several days. Dead or abnormal embryos were discarded regularly. When the embryos reached stage 45 they were transferred to tanks of frog water and reared as normal.

3.4 Tissue Culture Techniques (COS cells).

COS cells (a monkey kidney cell line) were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), glutamine, penicillin, and streptomycin at 37°C in 5% carbon dioxide. This fibroblast cell line grows as a monolayer and was divided (1 in 3) about every four days using the following procedure. The media was removed and replaced with 2.5ml of versene which was left in place for about 2 minutes. This was removed and replaced with 2.5ml of a versene:trypsin mix (1:1). The flask was agitated several times over a period of about 5 minutes to help release the cells from the surface of the flask. 60ml of DMEM + FCS was then added

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and the cells dispersed in to a fine suspension by shaking vigorously and pipetting the media up and down several times. It was important to do this in order to obtain an even monolayer of cells. When growing up a number of flasks for a single experiment the trypsinized cells were pooled at this stage so that the flasks would be as identical as possible. 20ml aliquots of the cell suspension were then transferred to fresh flasks and grown for at least 2 days before transfection.

3.5 Isolation Of RNA.

3.5.1 Isolation of liver RNA

RNA was isolated from larval and adult Xenopus liver using the guanidinium thiocyanate/caesium chloride method of Chirgwin et al (1979). The liver was removed from an animal that had been killed with euthatal and cut into pieces in phosphate buffered saline (PBS). The pieces were washed several times in PBS and then homogenised in five tissue volumes of lysis buffer (6 M guanidinium thiocyanate, 10mM Tris.HCl pH7.5, 1mM β -mercaptoethanol) that had been prepared one day earlier. After adding 0.1 volumes of sarcosyl NL the homogenate was made 15% (w/v) with caesium chloride and centrifuged at 2000 rpm in a bench centrifuge to pellet the debris. The supernatant was carefully layered on to 2ml of 5.7M caesium chloride, 100mM EDTA (pH8) in an ultracentrifuge tube. The tube was centrifuged for 12 to 18 hours in a swing out rotor at 36,000 rpm at 20°C. The

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following day the supernatant was aspirated off until about 1ml remained, which was then very gently poured off. The tube was left inverted on a piece of tissue to allow the remaining fluid to drain away. The RNA pellet was resuspended in sterile water or DEPC treated water and then extracted with an equal volume of neutral phenol. The aqueous phase was extracted with an equal volume of water saturated ether and then precipitated with 2.5 volumes of ethanol in the presence of 0.3M sodium acetate (pH6.5) at -20°C for at least 2 hours. The RNA was recovered by centrifugation at 10,000 rpm for 15 minutes, dried under vacuum and resuspended in sterile water or DEPC treated water. The RNA concentration was determined by measuring the absorbance at 260 nanometers assuming a reading of 1 for a 40µg/ml solution. Finally, the RNA was ethanol precipitated as before and stored (in 70% ethanol) at -20°C until required.

3.5.2 Isolation of RNA from Xenopus ovary, eggs and embryos.

Groups of eggs or embryos or pieces of dissected ovary were transferred to a glass homogeniser and homogenised in approximately 4 volumes of Kressmans buffer (10mM Tris.HCl pH7.5, 1.5mM MgCl₂, 10mM NaCl, 1mg/ml proteinase k (Boehringer)). After adding SDS to a final concentration of 2% (w/v) the homogenate was centrifuged at 10,000 rpm for 1 minute and then either incubated at 37°C for 30 minutes or

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extracted immediately with an equal volume of neutral phenol. Several more phenol extractions were performed as required. For latter stage embryos it was necessary to shear the DNA using a syringe fitted with a fine gauge needle. The aqueous phase was extracted at least once with chloroform and then nucleic acids precipitated with 2.5 volumes of ethanol in the presence of 0.3M sodium acetate (pH6.5).

3.5.3 Isolation of RNA from microinjected oocytes.

Groups of 10 to 20 healthy oocytes were transferred to eppendorf tubes and excess medium removed. 0.5ml of NaE (0.3M sodium acetate pH6.5, 1mM EDTA pH8) was added and the oocytes homogenised by squirting them up down a yellow eppendorf tip. SDS was then added to a final concentration of 2% (w/v) and the homogenate extracted immediately with an equal volume of neutral phenol. The aqueous phase was taken and extracted with an equal volume of chloroform and then nucleic acids precipitated by adding 2 volumes of ethanol.

3.5.4 Isolation of RNA from cultured COS cells.

The attached cells were washed twice with 10ml of ice cold PBS and then scraped off in 10ml of PBS using a rubber policeman. The cell suspension was transferred to a sterile plastic universal and centrifuged at 2000 rpm for 4 minutes at room temperature. The cell pellet was resuspended in 1

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ml (per 75cm² tissue culture flask) of lysis buffer (140mM NaCl, 10mM Tris.HCl pH8.6, 1.5mM MgCl₂, 0.5% [v/v] NP40), vortexed briefly and then left on ice for five minutes. The suspension was then transferred to an eppendorf tube and centrifuged in a minifuge at 12,000 g for 1 minute to pellet the nuclei. The supernatant was transferred to a fresh tube and an equal volume of 2*PK buffer (200mM Tris.HCl pH7.5, 25mM EDTA pH8, 300mM NaCl, 2% (w/v) SDS) was added. Proteinase K was added to a final concentration of 0.2mg/ml and the tubes incubated at 37°C for 30 minutes. The solution was then extracted once with neutral phenol and the aqueous phase re-extracted with water saturated ether. After allowing any remaining ether to evaporate, RNA was precipitated by adding 0.1 volumes of 3M NaAc (pH 6.5) and 2.5 volumes of ethanol and leaving at -20°C for at least 2 hours. RNA was recovered by centrifugation at 12000 g for 15 minutes, dried under vacuum and resuspended in DEPC treated water. The concentration was determined by measuring the absorbance at 260 nanometers in a Beckman mini-cell quartz cuvette.

3.6. Isolation Of Genomic DNA From Adult Xenopus Blood.

An adult female was anaesthetised by immersion in 0.2% (w/v) MS222 for 20 to 30 minutes. The thorax was then opened on the ventral side to expose the heart. A 10 ml syringe was filled with 3ml of 1mg/ml heparin in NMT (100mM NaCl, 10mM Tris.HCl pH7.4, 3mM MgCl₂) and the needle

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inserted into the still beating heart. Blood was gently collected into the syringe and transferred to -cold universals which were centrifuged at 2000 rpm for 5 minutes. The cell pellet was resuspended in 10ml of cold NMT and re-centrifuged. This was repeated until the supernatant was no longer pink. The cells were lysed by resuspending in 10ml of distilled water and leaving at room temperature for 5 minutes. 100ml of neutral phenol was transferred to a glass bottle and the lysed cell suspension added to this. After mixing vigorously the DNA was sheared by twice taking the emulsion in to a syringe through a coarse grade needle. The emulsion was transferred to oakridge tubes and centrifuged at 8000 rpm for 20 minutes at room temperature. The aqueous phase was carefully transferred to a fresh tube and re-extracted with an equal volume of neutral phenol as before. The aqueous phase was transferred to a glass beaker and approximately two volumes of ethanol was added to precipitate the DNA. High molecular weight DNA was spooled on to the end of a pasteur pipette and transferred to a plastic universal. The DNA was dissolved in 10ml of TE (10mM Tris.HCl pH7.5, 1mM EDTA) by shaking gently at room temperature for several hours. Ribonuclease A was added to a final concentration of 5µg/ml and the solution incubated at 37°C for 30 minutes. An equal volume of 2*PK buffer was added along with 0.1 volumes of 2mg/ml proteinase K and the solution incubated at 37°C for 1 hour. The solution was then extracted with an equal

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volume of neutral phenol and the phases separated by centrifugation at 8000 rpm for 20 minutes. The aqueous phase was transferred to corex tubes and the DNA precipitated with ethanol and recovered by centrifuging at 10 000 rpm for 20 minutes. The tubes were allowed to drain on a piece of tissue and the pellets resuspended in 5ml of TE. The DNA concentration was determined by measuring the absorbance at 260 nanometers assuming that a 50 μ g/ml solution has an A_{260} of 1 and the DNA solution stored at 4°C.

3.7. Selection Of Polyadenylated RNA By Oligo dT Cellulose Chromatography

This was carried out essentially as described by Maniatis et al (1982). A 2ml oligo (dT) cellulose column was poured in a disposable plastic column and equilibrated with loading buffer (20mM Tris.HCl pH7.6, 0.5M NaCl, 1mM EDTA, 0.1% SDS in DEPC treated water). Such a column was used several times and stored at 4°C. RNA was dissolved in DEPC treated water and an equal volume of 2* loading buffer (40mM Tris.HCl pH7.6, 1M NaCl, 2mM EDTA, 0.2% SDS) added. This solution was applied to the column, warming the column with a heating lamp as necessary. The column was washed with 5 to 10 column volumes of loading buffer and then with 5 column volumes of wash buffer (20mM Tris.HCl pH7.6, 0.1M NaCl, 1mM EDTA, 0.1% SDS), collecting the flow through in 1 ml aliquots throughout. The A_{260} of these fractions was

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measured as they were collected so that the amount of RNA coming off the column could be assessed. The column was washed with loading buffer and then with wash buffer until the A_{260} was less than 0.1. Polyadenylated RNA annealed to the column was then eluted with 2 to 3 column volumes of elution buffer (10mM Tris.HCl pH7.6, 1mM EDTA (pH8), 0.05% SDS). These eluted fractions were pooled and the RNA concentration determined by measuring the A_{260} . Finally the RNA was split in to aliquots, precipitated with ethanol and stored at -20°C until required.

3.8 Formaldehyde Agarose RNA Gels.

RNA samples (up to $20\mu\text{g}$) were resuspended in $10\mu\text{l}$ of DEPC treated water to which was added $20\mu\text{l}$ of denaturing mix (66% deionised formamide pH7, 1.3% MOPS, 20% formaldehyde) and $3\mu\text{l}$ of loading mix (50% glycerol, 0.2% bromophenol blue). The samples were loaded immediately on a 2% (w/v) agarose, 15% formaldehyde gel made in 1% MOPS buffer (1% MOPS is 20mM Mops, 5mM sodium acetate, 1mM EDTA). The samples were electrophoresed at 40mA (100 volts) for 5 to 6 hours and the gel stained by first soaking in 250ml of 10% glycine for 10 minutes then adding $100\mu\text{l}$ of 10mg/ml ethidium bromide. After 10 minutes the gel was destained by washing twice, for 15 minutes each, in distilled water and then photographed on an ultraviolet light box.

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3.9 Northern Blotting.

After staining with ethidium bromide, RNA gels were soaked in 250ml of 20*SSC for 20 minutes and the RNA transferred to nitrocellulose (Hybond-C, Amersham) as described by Thomas (1980). Four sheets of filter paper (3MM, Whatman) were placed in the bottom of a large plastic tray and moistened with 20*SSC avoiding the formation of bubbles. The gel was placed on the paper and the exposed 3MM paper between the gel and the edge of the tray covered with cling film. The cling film overlapped the edge of the gel by at least 3mm all the way round. The surface of the gel was dried and a piece of nitrocellulose, cut to the same size as the gel, lowered carefully on to the gel, again ensuring that no air bubbles formed. A piece of 3MM the same size as the gel was soaked in 20*SSC and layed on top of the nitrocellulose. Tissue paper was stacked on top of this to a depth of about 5cm and weighed down with a house brick. The gel was left to blot overnight after which the nitrocellulose filter was briefly dried at room temperature and then baked at 80°C under vacuum for 2 hours.

Filters were prehybridised and hybridised in heat sealable plastic bags containing the appropriate solution, weighed down in a water bath set to the required temperature. Prehybridisation was in 20 to 50ml of 5*SSC, 5 * Denhardt's, 50% deionised formamide (pH7), 21mM sodium phosphate (pH6.5), 100µg/ml E.coli tRNA, 50µg/ml denatured, boiled herring testis DNA (Sigma) for 4 to 48 hours. The

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prehybridisation solution was discarded and replaced with 5 to 12ml of the same solution to which radiolabelled probe had been added and the filters hybridised for 16 to 48 hours.

For DNA probes prehybridisation and hybridisation was at 42°C whilst for single stranded RNA probes it was at 60°C. Filters were initially washed twice in 2*SSC, 0.1% SDS for 5 minutes each at room temperature and then twice for 30 minutes each in the final wash conditions (which depended upon the stringency required). After the final wash filters were blotted on 3MM, and while still damp, wrapped in cling film and exposed to X-ray film with one or two intensifying screens at -70°C.

3.10 Non-denaturing Agarose Gels.

DNA samples, to which 0.1 volumes of loading buffer (50% glycerol, 1*TBE, 0.1% bromophenol blue) had been added, were separated in 0.5 to 2% (w/v) agarose gels containing 0.2µg/ml ethidium bromide made in 1*TBE buffer. Gels were run in 1*TBE buffer supplemented with 0.5µg/ml ethidium bromide and examined and photographed on an ultraviolet light box.

3.11 Southern Blotting.

DNA samples separated in non-denaturing agarose gels as described in section 3.10 were transferred to nitrocellulose or nylon membranes using the method of

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Southern (1975). To increase the efficiency of transfer of high molecular weight DNA (especially with genomic southern blots) the gel was first treated with 0.25M HCl for 15 minutes at room temperature on a shaker. This partially hydrolyses the DNA, generating smaller fragments which transfer more efficiently. After a brief wash with distilled water the gel was incubated in a large volume of denaturing solution (1.5M NaCl, 0.5M NaOH) with gentle shaking for 40 minutes. This was repeated with fresh denaturing solution, the gel rinsed with distilled water and then neutralised by gently shaking in a large volume of neutralising solution (3M NaCl, 0.5M Tris-HCl, 1mM EDTA, pH 7) for 40 minutes. This neutralising step was repeated with fresh neutralising solution and the gel was then capillary blotted using 20*SSC as the transfer buffer, exactly as described for northern blotting (see section 3.9).

DNA was fixed to nitrocellulose filters by baking at 80°C under vacuum for 2 hours. Nylon membranes were wrapped in cling film and exposed to ultraviolet light DNA side down on a trans-illuminator for 3 minutes, which cross-links the DNA to the membrane.

Prehybridisation and hybridisation was carried out in heat sealed plastic bags weighed down in a water bath set to the appropriate temperature. Filters were prehybridised in 10 to 30ml (depending upon the size of filter) of 5 * Denhardtts, 6*SSC, 0.1% SDS, 100µg/ml E. coli tRNA at 65 to 68°C for 3 to 48 hours. The prehybridisation solution was

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discarded and replaced with 5 to 12ml of the same buffer containing radiolabelled nucleic acid probe. The bag was re-sealed and hybridised at the same temperature for 16 to 48 hours. Alternatively, prehybridisation and hybridisation were carried out in the same buffer supplemented with 50% deionised formamide (pH7) at 42°C.

Filters were first washed twice in 2*SSC, 0.1% SDS at room temperature for 5 minutes each and then twice for 30 minutes each in the final washing conditions (which depended upon the stringency desired). Finally, the filters were blotted on 3MM and while still damp, wrapped in cling film and exposed to X-ray film with one or two intensifying screens at -70°C.

3.12 Low Melting Point Agarose Gels.

These gels were used in the isolation of DNA fragments which were subsequently radioactively labelled and used as probes, or used for sub-cloning purposes.

DNA samples to which 0.1 volumes of agarose gel loading buffer had been added, were loaded on to a 0.4% (w/v) low melting point agarose gel made in 0.5*TBE buffer and containing 0.2µg/ml ethidium bromide. Gels were run in 0.5*TBE buffer containing 0.5µg/ml ethidium bromide at a maximum of 40 mA. The gel was examined under U.V. light and the required bands excised in as small a gel slice as possible. Exposure of the gel to U.V. light was kept to a minimum to avoid U.V. induced DNA damage. Gel slices were

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transferred to 1.5ml eppendorf tubes and weighed to calculate the volume of the slice (assuming 1g = 1ml). 0.4 volumes of water was added and the slices melted by heating at 65°C for 10 to 40 minutes. After cooling to 37°C an equal volume of neutral phenol was added, the solution whirlmixed vigorously and left on ice for 10 minutes. The tubes were then centrifuged at 12 000 rpm for 5 minutes and the aqueous phase transferred to a fresh tube, carefully avoiding the white precipitate at the interphase. The volume of the aqueous phase was reduced to about 300 μ l by several extractions with butan-1-ol and the DNA precipitated by adding 0.1 volumes of 3M NaAc (pH6.5) and 2.5 volumes of ethanol.

The effectiveness of this procedure was found to depend very much upon the quality of the low melting point agerose, with some batches failing to melt or giving very low recoveries of DNA.

3.13 Primer Extension Analysis Of RNA.

The single stranded oligonucleotide to be used as the primer was end labelled with γ [³²P] ATP (see section 3.37.2) and finally resuspended in 3*PEB (1*PEB is 0.4M NaCl, 10mM PIPES (pH6.4), 0.5mM EDTA (pH8)). Hybridisation reactions were set up by mixing the RNA sample (40 μ l volume in water), 5 μ l of the labelled primer and 7.5 μ l of 5*PEB in a 0.5ml eppendorf tube. The tubes were closed tightly and incubated overnight at the desired temperature. For the

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albumin and albina oligonucleotide primers (which are both 17 mers) 45°C was found to be optimal.

After hybridisation 110 μ l of ethanol was added and the tubes were left at -20°C for 30 minutes. Nuclaic acids were recovered by centrifugation for 12 minutes in a minifuge and dissolved in 20 μ l of reverse transcriptase reaction buffer (50mM Tris.HCl pH8.3, 6mM MgCl₂, 10mM DTT, 1mM each of dATP, dCTP, dTTP, dGTP). To this was added 5 units of AMV reverse transcriptase and the tubes incubated at 37°C for 30 minutes. Ribonuclease A was added to a final concentration of 2 μ g/ μ l and the tubes incubated for a further 10 minutes at 37°C after which DNA was precipitated by adding an equal volume of isopropanol. The pellet was resuspended in 4 μ l of sequencing gel loading dye (90% deionised formamide, 10mM EDTA (pH8), 0.01% xylene cyanol, 0.01% bromophenol blue), heated at 100°C for 5 minutes and then loaded on to a 8% polyacrylamide sequencing gel. The gel was run at 38 watts until the bromophenol blue was about 2 inches from the bottom of the plate. The plates were prised apart and the gel covered with cling film and exposed to x-ray film with one intensifying screen at -70°C.

3.14 S1 Nuclease Protection Assays.

The RNA samples to be analysed were precipitated from ethanol, dried thoroughly under vacuum and resuspended in 10 μ l of hybridisation buffer (40mM PIPES pH6.4, 1mM EDTA

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(pH8), 0.4M NaCl, 80% deionized formamide). The duplex DNA probe was also precipitated with ethanol, dried and resuspended in an appropriate volume of the same buffer. Hybridisation reactions were set up by mixing 10 μ l of RNA solution and 5 μ l of probe and transferring this to a capillary tube which was then heat sealed. The tubes were immersed in a water bath at 80°C for 5 minutes to denature the probe and RNA, and then transferred to a second water bath at the hybridisation temperature. Hybridisation was carried out overnight, after which the contents of the capillary tubes were expelled into 1.5ml eppendorf tubes containing 150 μ l of S1 buffer (0.28M NaCl, 50mM NaAc pH6.4, 4.5mM ZnSO₄, 20 μ g/ml sonicated, denatured herring testis DNA.) and 100 units of S1 nuclease. The reactions were incubated at 30°C for 30 minutes and then stopped by adding 3 μ l of 0.4M EDTA (pH8). The solution was then extracted with an equal volume of neutral phenol and the aqueous phase precipitated with ethanol. After centrifuging in a minifuge for 12 minutes the pellet was dried under vacuum, dissolved in 3 μ l of sequencing gel loading dye, heated at 100°C for 5 minutes and analysed on an 8% sequencing gel.

3.15 Transient Transfection Of COS Cells.

COS cells grown in medium (75 cm²) tissue culture flasks were transfected using DEAE-dextran (Sussman and Milman, 1984). Cells which had been split 3 to 4 days earlier (cells near to confluence) were washed twice with 10ml of

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serum-free DMEM. To each flask was added 5ml of transfection mix that contained 0.2mg/ml DEAE-dextran and up to 12 μ g of caesium chloride purified plasmid DNA, in serum-free DMEM. The flasks were then returned to the incubator for 3 hours. During this period they were gently tilted several times, about every 30 minutes, to try and keep the bottom of the flask covered with the transfection mix. The cells were then washed twice with 10ml of serum-free DMEM. For the receptor binding experiments the cells were then incubated in DMEM containing serum for 48 hours before harvesting. It is almost certain that fetal calf serum contains thyroid hormone, and so it was decided to incubate in serum-free DMEM when testing the effect of exogenously added thyroid hormone upon transactivation of transcription. In initial experiments cells were incubated in serum-free DMEM (+ or - triiodothyronine) immediately after transfection. Under these conditions the cells began to come off the bottom of the flask within 24 hours. To try and overcome this, transfected cells were initially incubated in DMEM containing serum for 24 hours, then washed twice with 10ml of serum-free DMEM (incubating for 40 minutes in serum-free DMEM after the first wash). They were then cultured in serum-free DMEM (+ or - triiodothyronine) for a further 24 hours and harvested.

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3.16 Preparation Of Whole Cell Extracts From Transfected COS Cells For Use In Thyroid Hormone Binding Assays.

The cells were washed twice in situ with 10ml of ice cold PBS and then harvested by scraping them off with a rubber policeman in 10ml of ice cold PBS. The suspension was transferred to a plastic universal and the cells were pelleted by centrifuging at 2000 rpm for 4 minutes at room temperature. The supernatant was discarded and the tubes left to drain, inverted on tissue paper for up to 5 minutes. The pellet was then resuspended in 160 μ l per ^{75cm²} flask of ice cold T3 binding buffer (20mM Tris.HCl pH7.6, 50mM NaCl, 2mM EDTA (pH8), 17% glycerol, 0.5mM PMSF, 5mM β -mercaptoethanol), transferred to a fresh universal and pooled with cell suspensions from other flasks as appropriate. The cells were lysed by freezing in liquid nitrogen (20 seconds to 1 minute depending upon volume of cells) and then thawing immediately in a water bath at 37°C. This freeze thaw process was repeated twice more and the suspension pipetted up and down vigorously using a gilson P200. After transferring to 1.5ml eppendorf tubes the lysed cells were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was transferred to fresh tubes on ice and then either used immediately or stored at -70°C. The extract remained active for several weeks and through several freeze/thaw cycles. Before use the "concentration" of the extract was determined by measuring

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the absorbance at 280 nanometers, and equal A_{280} amounts used in each binding assay.

3.17 Thyroid Hormone Receptor Binding Assays On Extracts From Transfected COS Cells.

Whole cell extracts prepared as described in section 3.16 and stored at -70°C , were thawed on ice and assayed for triiodothyronine binding activity essentially as described by Sap et al (1986). Standard reactions were in T3 binding buffer in a final volume of 250 μl containing 0 to 8 μl of [^{125}I] triiodothyronine (25nM, in solution in 3:1 ethanol: water) and 0 to 100 μl of extract. For competition experiments the reactions also contained 2.5 μl of the appropriate analogue. The reactions were assembled at room temperature, always adding the extract last to the other, pre-mixed components. The tubes were then incubated at 4°C for 6 to 7 hours. The amount of bound [^{125}I] triiodothyronine was then determined using the nitrocellulose filter binding assay of Inoue et al (1983). This procedure was carried out at 4°C in a cold room using pre-chilled T3 binding buffer that lacked PMSF. 1ml of T3 binding buffer was added to the reaction, which was mixed by pipetting and then filtered under vacuum through a millipore nitrocellulose filter that had been pre-washed with 3ml of T3 binding buffer. The rate of flow was previously adjusted to be about 5ml in 15 seconds. The filter was immediately washed 3 times with 5ml of T3

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binding buffer. The filters were dried at room temperature and then counted in a gamma counter for 10 minutes each.

Unlabelled analogues for competition experiments were all obtained from Sigma and dissolved as follows. Triiodothyronine was dissolved and diluted in sterile water, triiodoacetic acid (TRIAc) was dissolved and diluted in 50% DMSO. Reverse T3 was found to dissolve in 10mM NaOH at a maximum concentration of 3mM, and was then diluted as appropriate in 10mM NaOH. All these analogues were stored at -20°C but it was found that TRIAc at concentrations greater than 1mM irreversibly precipitated out of solution after overnight storage.

3.18 Preparation Of "CAT" Extracts From Transfected COS

Cells

The flasks of cells were washed twice with 10ml of cold PBS and then scrapped off the surface of the flask using a rubber policeman into 10ml of cold PBS. The suspension was transferred to a sterile plastic universal and for most experiments $\frac{1}{3}$ rd of the cells (from a single ^{75cm²} flask) used to prepare "CAT" extracts whilst RNA was isolated from the remainder. The cells were first pelleted by centrifuging at 2000 rpm for 3 minutes at room temperature. The supernatant was discarded and the tubes drained for several minutes on tissue paper. The pellet was resuspended in 65 μ l (per ^{75cm²} $\frac{1}{3}$ rd of a flask) of cold 0.25M Tris.HCl (pH7.5) by pipetting up and down in a gilson P200 and transferred to 1.5ml

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ependorf tubes. These were frozen in liquid nitrogen (about 30 seconds) and immediately thawed in a 37°C water bath. This freeze thaw procedure was repeated 3 times to ensure that the cells had lysed and the tubes were then centrifuged at 4°C in a minifuge for 10 minutes. The clear supernatant was transferred to fresh tubes on ice and the "concentration" determined by measuring the absorbance at 280 nanometers. Equal A₂₈₀ amounts were used in each assay. The extracts were either used immediately or stored at -70°C until required.

3.19 CAT Assays On Extracts From Transfected COS Cells.

Chloramphenicol acetyltransferase activity in extracts prepared as described in section 3.18 was determined essentially as described by Gorman et al (1982). Reactions contained (in a final volume of 100µl) 0.125M Tris.HCl (pH7.5), 0 to 60µl of cell extract, and 5µl of [¹⁴C] chloramphenicol. Controls contained no extract or 1µl of chloramphenicol acetyltransferase (10 u/µl, Pharmacia). A 1mg aliquot of solid acetyl coenzyme A (Sigma) which had been stored at -20°C for not more than 2 months was dissolved in 1.5ml of sterile water to give a 0.66mg/ml stock solution. A sufficient volume for use in the current assays was transferred to an ependorf tube and the rest stored at -70°C. This frozen stock solution was only used within one week of initially dissolving the solid. Acetyl CoA deteriorates rapidly on longer storage resulting in

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very insensitive CAT assays (N. Lakin, pers. comm.). The acetyl CoA solution and the reaction mixes were then incubated separately at 37°C for 5 minutes, after which the reactions were started by addition of 5 μ l of acetyl CoA to each reaction mix. The reaction was allowed to proceed at 37°C for 1 hour and then terminated by the addition of 90 μ l of 0.25M Tris.HCl (pH7.5) and 1ml of ice cold ethyl acetate. The solutions were mixed extensively by vortexing several times which extracts the chloramphenicol in to the ethyl acetate. The tubes were centrifuged for 5 minutes in a minifuge and the upper (ethyl acetate) phase transferred to fresh tubes. These were dried in a vacuum desiccator until all the solvent had evaporated (about 1 hour). The chloramphenicol was then dissolved in 20 μ l of ice cold ethyl acetate, vortexing the tubes several times. The samples were then spotted on to a thin layer chromatography plate (aluminium backed silica gel 60, 0.2 mm thick, E. Merck, FRG) using drawn out glass capillaries. Approximately 1 to 2 μ l were applied at a time allowing the spots to air dry between applications. The TLC plate was then placed in a chromatography tank containing 200ml of 95% chloroform, 5% methanol, and a vertical piece of 3MM which helps to saturate the chamber with solvent. This tank was filled with solvent and allowed to equilibrate for at least 1 hour before use. The TLC plate was left in place until the solvent front was about 2 inches from the top of

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the plate, then removed and thoroughly air dried. The plate was then autoradiographed at room temperature.

3.20 Preparation Of CAT Extracts From Microinjected Oocytes.

This method was obtained from Nick Lakin. Groups of 5 to 15 oocytes were transferred to eppendorf tubes and any remaining media carefully removed. They were then homogenised in 50 μ l per oocyte of 0.25M Tris.HCl (pH7.5) by pipetting up and down in a gilson P1000. The homogenate was kept on ice when possible and then centrifuged in a minifuge at 4°C for 10 minutes. This produced a pellet which is mainly yolk, and a clear supernatant with a layer of white coloured lipid floating on top. At 4°C the lid of the tube was opened and a syringe needle used to make a small hole in the side of the tube just above the pellet. The clear supernatant was drained from this hole into a fresh eppendorf tube taking care to avoid the layer of lipid (touching the hole against the side of the tube helps to get the flow started). The extract was frozen at -70°C until required and appeared to be stable for several weeks.

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3.21 Assays For Chloramphenicol Acetyltransferase In Extracts From Xenopus Oocytes.

Extracts prepared as described in section 3.20 were assayed for CAT activity exactly as described in section 3.19 except that 50 μ l of extract (1 oocytes worth) was used in each assay.

3.22 Large Scale Preparation Of Plasmid DNA And Purification By Caesium Chloride/Ethidium Bromide Centrifugation.

The method used was the alkaline lysis method as described by Maniatis *et al* (1982). A single colony was inoculated in to 10ml of sterile LB containing the appropriate antibiotic, and grown overnight at 37°C. The next day 0.25ml of this overnight culture was used to inoculate 25ml of LB, and this culture grown on a shaker at 37°C for 2 hours or until the absorbance at 550 nanometers was about 0.5. This 25ml culture was tipped into a 2 litre flask containing 500ml of LB or TB which was then shaken (200 rpm) overnight at 37°C. The next day, the cells were pelleted by centrifugation at 5000 rpm for 10 minutes. In the description which follows all volumes relate to a single 500ml bacterial culture. The pellets were resuspended in a total of 18ml of ice cold solution I (50mM glucose, 25mM Tris.HCl (pH8), 10mM EDTA (pH8), 5mg/ml lysozyme) and equal volumes transferred to two oakridge centrifuge tubes. After incubating at room temperature for

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five minutes 2 volumes of freshly prepared solution II (0.2M NaOH, 1% SDS) was added and the contents of the tubes mixed by inverting several times. The tubes were left on ice for 10 minutes after which 9ml of ice cold 5M potassium acetate (pH6) was added to each tube. The tubes were mixed by inverting several times and left on ice for 10 minutes. The pH of the solution was then tested with pH paper to ensure that the sodium hydroxide had been completely neutralized, adding more potassium acetate if necessary. Bacterial debris and chromosomal DNA were then pelleted by centrifuging at 13 000 rpm for 30 minutes. The supernatant was transferred to 30ml corex tubes and nucleic acids precipitated by adding 0.6 volumes of isopropanol. After 15 minutes at room temperature the tubes were centrifuged at 10 000 rpm for 30 minutes at 20°C and the resultant pellets washed with 70% ethanol. After briefly drying under vacuum, the pellets were resuspended in a total of 33ml of TE. Exactly 33 g of caesium chloride was dissolved in this and 0.6ml of ethidium bromide (10mg/ml) added. Using a syringe, this solution was transferred to a 40ml, Beckmann, polyMoner, self-sealing centrifuge tube. The tubes were then balanced to within 10mg and the tops heat sealed. They were then centrifuged at 45 000 rpm in a vertical rotor for 18 hours at 20°C.

The tubes were carefully removed and viewed under U.V. light. The lower band, which is supercoiled plasmid was removed from the gradient using a syringe, and extracted at

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least three times with water saturated butan-1-ol to remove the ethidium bromide. The solution was then dialysed against 2 litres of TE for at least 5 hours at room temperature, transferred to corex tubes and the DNA precipitated with ethanol. The DNA was recovered by centrifuging at 10,000 rpm for 30 minutes at 4°C, dried briefly under vacuum and resuspended in 1ml of TE. The solution was then extracted with neutral phenol, and precipitated once more with ethanol. The DNA was pelleted by centrifugation in a minifuge for 12 minutes, dried under vacuum and finally dissolved in 1ml of TE. The DNA concentration was determined by measuring the A_{260} .

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3.23 Standard Sub-Cloning Techniques.

3.23.1 Restriction enzyme digests.

These were carried out according to the manufacturers instructions. Plasmid DNA's were generally digested for 1 hour, whilst genomic DNA for southern blotting was digested for 4 hours, adding a second aliquot of enzyme after 2 hours.

3.23.2 Preparation of plasmid vectors for sub-cloning.

Vectors that had been digested with two enzymes were run on a low melting point agarose gel and the linear vector fragment recovered. Vectors cut with a single restriction enzyme were treated with calf intestinal alkaline phosphatase (CIAP) by adding 1 or 2 units directly to the the restriction enzyme digest 1 hour after the start of the reaction and incubating for a further 40 minutes at 37°C. For enzymes that generate 5' overhangs this was followed by an incubation at 60°C for 30 minutes. After adding 200µl of NaE the reactions were extracted with neutral phenol, and then with ether and finally precipitated with ethanol.

3.23.3 Ligations.

These were carried out in a 10µl reaction containing 1°C buffer (66mM Tris.HCl pH7.6, 6.6mM MgCl₂, 10mM DTT), 1mM rATP, vector DNA, target DNA and T4 DNA ligase. Normally, 20ng of vector and a range of target concentrations was

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used. A control that contained vector alone was always included. For sticky end ligations, 0.1 units of T4 DNA ligase was used in each ligation, and the reactions were incubated at 14°C overnight. Blunt end ligations contained 1 unit of T4 DNA ligase and were incubated at 4°C overnight. When doing ligations with dephosphorylated vectors it was often found useful to include an additional control in which the vector was ligated in the absence of target but in the presence of 5 units of T4 polynucleotide kinase.

3.24 E. coli Plasmid Transformation.

3.24.1 MgCl₂/CaCl₂ Mediated Transformation.

This method was used when a high transformation efficiency was not required or when using the E. coli strain MC1061. Efficiencies of up to 3×10^6 transformants per μg of DNA were obtained using this method with MC1061. An appropriate volume of 2*TY or LB was inoculated with a $1/100$ th volume of an overnight culture of the host bacteria and incubated at 37°C with vigorous shaking until the A₅₅₀ reached 0.5. The culture was then cooled on ice for 10 minutes and the cells pelleted by centrifugation at 2000 rpm for 5 minutes. The tubes were drained briefly on tissue paper and the pellet resuspended in a half volume of ice cold 0.1M MgCl₂. The cells were re-pelleted immediately and resuspended in a half volume of ice cold 0.1M CaCl₂. The tubes were centrifuged immediately and the

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cells resuspended in a $1/20$ th volume of ice cold 0.1M CaCl_2 . After incubating the cell suspension on ice for 90 minutes or longer, 0.2ml aliquots were transferred to eppendorf tubes on ice. To each of these was added half of the appropriate ligation (5 μ l), and after mixing the tubes were incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes, quenched on ice for 5 minutes, and spread on to dried L-agar plates containing the appropriate antibiotic using an ethanol sterilized glass spreader. The plates were incubated upside down in a 37°C incubator overnight.

Competent cells prepared by this method were stored by adding glycerol to 15% (v/v) and freezing 0.2ml aliquots in liquid nitrogen. These were then stored at -70°C for several months with a gradual drop in transformation efficiency over time. When required the tubes were thawed slowly on ice and the DNA added. The tubes were then simply left on ice for 40 minutes, heat shocked and the cells plated as above.

3.24.2 High Efficiency Plasmid Transformation.

This method was routinely used for the transformation of JM101 with M13 vectors and is essentially as described by Hanahan (1983). An appropriately sized culture of host cells was grown as described in section 3.24.1, and when the A_{550} reached between 0.3 and 0.4 the culture was cooled on ice for 15 minutes. The cells were then pelleted

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by centrifugation at 2000 rpm for 5 minutes at room temperature and resuspended in $\frac{1}{3}$ rd the original culture volume of transformation buffer (TFB) (TFB is 10mM KMES pH6.3, 100mM KCl, 45mM MnCl \cdot 4H $_2$ O, 10mM CaCl $_2$, 3mM HAcOCl $_3$). The suspension was left on ice for 15 minutes after which the cells were pelleted as before and resuspended in $\frac{1}{12.5}$ th of the original culture volume of TFB. At this point the cell suspension was transferred to a polypropylene tube and left on ice for 10 minutes after which dimethylformamide (DMF) was added to a final concentration of 4.75% (v/v). The suspension was well mixed immediately after adding the DMF and then left on ice for 10 minutes. A solution of 2.25M DTT in 10mM potassium acetate was added to 4.1% (v/v), mixing immediately after addition, and the suspension left on ice for a further 10 minutes. A second aliquot of DMF was then added as before so that the final concentration of DMF was 9.5% (v/v). The suspension was incubated on ice for 20 minutes and then 0.2ml aliquots transferred to eppendorf tubes. Half of the appropriate ligation mix was added to each tube, the contents mixed well and the tubes left on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes, quenched on ice and either spread on dried agar plates or in the case of M13 transformations plated in top agarose (see section 3.25).

If was not possible to store

A competent cells prepared using this method

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by adding glycerol to 15% (v/v), freezing in liquid nitrogen and storing at -70°C.

3.25 Plating E.coli Transformed Or Infected With Bacteriophage M13.

For each transformation or infection, 3ml of molten H-top agar, that had been cooled to 60°C, was placed in a sterile glass tube in a heating block at 45°C. After allowing the top agar to equilibrate to 45°C, 50µl of X-gal (20mg/ml in DMF), 25µl of IPTG (24mg/ml in water) and 200µl of exponential host cells were added in this order. The transformed or infected host cells were then added to each tube in turn, the contents mixed by covering the top with nesco film and inverting several times, and poured on to a dried H-agar plate. The plate was tilted gently to get an even covering of top agar and then allowed to set for 15 minutes at room temperature, before incubating upside down at 37°C overnight.

3.26 Small Scale Isolation Of Plasmid DNA And M13 RF DNA.

Single bacterial colonies were inoculated into 2ml of 2*TY containing the appropriate antibiotic using a sterile toothpick, and grown in a shaking incubator at 37°C for 6 hours. For preparing M13 RF DNA 2ml of 2*TY was inoculated with 20µl of an overnight culture of the host cells. A single plaque was stabbed with a sterile toothpick and this toothpick placed in the tube of innoculated media.

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Alternatively 10 μ l of a stock of phage particles (in 2*TY) was added to the tube of media and host cells. M13 cultures were grown at 37°C with vigorous shaking (300 rpm) for 5 to 7 hours. The cultures were then transferred to 1.5ml eppendorf tubes and centrifuged in a minifuge for 5 minutes. In the case of M13 infected cells, an aliquot of the supernatant (which contains bacteriophage particles) was removed and kept frozen at -20°C. This supernatant was also used for the preparation of single stranded bacteriophage DNA (see section 3.27). The pellets were resuspended in 0.2ml of STET (8% sucrose (w/v), 0.5% triton x-100 (v/v), 50mM EDTA (pH8), 10mM Tris.HCl (pH8)) and 10 μ l of a 10mg/ml solution of lysozyme was added. The tubes were mixed, incubated on ice for 10 minutes, and then heated at 100°C for 40 seconds. Bacterial debris and chromosomal DNA was then pelleted by centrifuging in a minifuge for 10 minutes. This viscous pellet was removed using a toothpick and nucleic acids in the supernatant precipitated by adding 0.1 volumes of 3M NaAc (pH6.5) and 1 volume of isopropanol. The tubes were left at -20°C for at least 1 hour and then centrifuged in a minifuge for 10 minutes. The pellet was resuspended in 0.2ml of NaE and the solution extracted with an equal volume of neutral phenol. The aqueous phase was extracted with ether and nucleic acids precipitated by adding 2 volumes of ethanol. After at least 1 hour at -20°C, nucleic acids were recovered by centrifuging in a minifuge for 10 minutes.

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The pellet was dried under vacuum and dissolved in 40 μ l of TE. If necessary, RNA was removed by treatment with ribonuclease A at a final concentration 0.2 μ g/ml for 15 minutes at 37°C.

3.27 Preparation Of Single Stranded M13 Template DNA.

Glass tubes containing 2ml of 2*TY were inoculated with 20 μ l of an overnight culture of the host strain, usually JM101. Phage particles from a single plaque were transferred to the media using a sterile toothpick. Alternatively, 10 μ l of a frozen phage stock was added to each tube. The tubes were then incubated at 37°C with vigorous shaking (300 rpm) for 5 to 6 hours. The bacteria were pelleted by centrifugation in a minifuge for 5 minutes and 1.1ml of the supernatant transferred to a fresh tube. This was centrifuged as before and 1ml of the supernatant removed to a fresh tube, avoiding any debris at the bottom of the tube. Bacteriophage particles were then precipitated by adding 200 μ l of 20% (w/v) polyethylene glycol in 2.5 M NaCl and leaving the tubes at room temperature for 20 minutes. The phage were pelleted by centrifuging in a minifuge for 10 minutes and the supernatant removed using a gilson P1000. The tubes were re-centrifuged for 10 seconds to bring down the remaining liquid which was carefully removed using a gilson P20. After another 10 second centrifugation any remaining supernatant was removed using a drawn out glass capillary.

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The white pellet was then resuspended in 0.2ml of NaE by vortexing the tube vigorously, and the suspension extracted with an equal volume of neutral phenol. The aqueous phase was extracted twice with an equal volume of chloroform and the DNA precipitated by adding 2.2 volumes of ethanol. After at least 1 hour at -20°C the DNA was recovered by centrifuging in a minifuge for 10 minutes, dried under vacuum and dissolved in 10 μl of TE. Template DNA was always checked on a 1% agarose gel prior to sequencing.

3.28 Complementation Tests On Single Stranded M13 DNA

This test was used to determine the orientation of DNA fragments inserted in to M13 vectors. 10 μl of phage supernatant from each of the two clones to be tested was mixed with 2 μl of 1% SDS and heated at 65°C for 1 hour. After adding 2 μl of loading dye the samples were analysed on a 1% agarose gel. The presence of a slower migrating band (not present in either phage supernatant alone) indicated that the two clones contained the same insert in opposite orientations.

3.29 Colony Lifts Onto Nitrocellulose.

A piece of nitrocellulose was cut to just fit a 10cm petri dish, marked with a grid and autoclaved. This grid was duplicated on the bottom of an L-amp plate, which was used as the master plate. The nitrocellulose disc was

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lowered on to another L-amp plate and then sterile toothpicks were used to streak out individual colonies (independent transformants) on to the nitrocellulose. One colony was transferred to each square on the grid and then replica plated in the corresponding position on the master plate. The plates were incubated at 37°C for 16 hours after which the master plate was stored at 4°C. The nitrocellulose disc on which the bacterial colonies had grown was then layed, colony side up, on a tray containing two sheets of 3MM that had been moistened with denaturing solution (0.5M NaOH, 1.5M NaCl). After 2 minutes the nitrocellulose disc was transferred to two sheets of 3MM moistened with neutralising solution (3M NaCl, 0.5M Tris.HCl pH7), and left colony side up for 5 minutes or until the NaOH had been completly neutralised. The disc was then placed, colony side up, on a dry piece of 3MM and a second piece of dry 3MM carefully placed on top of the disc. Firm pressure was applied, avoiding any sideways movement, blotting the colonies on to the nitrocellulose. The filter paper was peeled off, and this blotting procedure repeated with a fresh piece of 3MM. The nitrocellulose disc was then placed, colony side up, on two sheets of 3MM moistened with 2*SSC for several minutes. After air drying for one hour the disc was baked at 80°C under vacuum for 2 hours and then prehybridised and hybridised in 3*SSC, 0.1% SDS, 1*Denhardtts, 100µg/ml E.coli tRNA.

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3.30 Synthesis of complementary DNA (cDNA) from mRNA.

3.30.1 Synthesis of double stranded cDNA.

The method described here is based upon the original method of Gubler and Hoffman (1983) which uses a combination of reverse transcriptase, ribonuclease H, and DNA polymerase I to generate double stranded cDNA. This was subsequently modified so that all the reactions could be carried out in a single tube (Gubler, 1988), and this protocol is outlined here. The first strand was synthesised in a reaction containing 50mM Tris.HCl (pH8.3), 10mM MgCl₂, 10mM DTT, 0.1M NaCl, 0.7mM each of dATP, dGTP, dCTP, dTTP, 1 to 10µg of poly A⁺ RNA, 4µg of oligo dT₍₁₂₋₁₈₎, 4µl of α[³²P] dGTP, 80 units of pancreatic ribonuclease inhibitor, and 60 units of AMV reverse transcriptase in a final volume of 60µl. The reaction was allowed to proceed at 37°C for 90 minutes and then stopped by putting the tube on ice. At this point two 1µl aliquots were removed for analysis of the first strand products. To the remaining 58µl was added 125µl of 2*2nd strand buffer (40mM Tris.HCl pH7.5, 10mM MgCl₂, 20mM ammonium sulphate, 200mM KCl, 100µg/ml bovine serum albumin), 50µl of water, 10 units of ribonuclease H, and 140 units of DNA polymerase I, making the final volume 250µl. The reaction was incubated at 14°C for 1 hour and then at 22°C for a further 1 hour. The reaction was stopped by heating at 70°C for 10 minutes and the tube cooled on ice. The ends of the double stranded cDNA were

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then polished by adding 20 units of T4 DNA polymerase and incubating at 37°C for 10 minutes. The reaction was terminated by the addition of EDTA (pH8) to a final concentration of 20mM and SDS to a final concentration of 1 % (w/v). The solution was extracted twice with an equal volume of neutral phenol, and the aqueous phase run down a sepharose CL4B column in NaE, that was set up in a 1ml pipette. The eluate was collected in 0.5ml fractions and monitored with a geiger counter to assess the progress of the cDNA down the column. The fractions containing the first peak of radioactivity (the excluded peak) were kept separate and precipitated with 2.2 volumes of ethanol. Fractions representing the first third of the excluded peak (the larger cDNA molecules) were pooled as were fractions from the second third and the remaining third. Aliquots of these three sets of pooled fractions were analysed on an alkaline agarose gel to assess the size of the cDNA molecules synthesised.

3.30.2 Methylation of cDNA and addition of EcoRI linkers.

The first set of pooled fractions (the largest cDNA molecules) was protected from restriction at internal EcoRI sites by treatment with EcoRI methylase. The cDNA was incubated in 1^xmethylase buffer (50mM Tris.HCl (pH7.5), 1mM EDTA (pH8), 5mM DTT) and 8 μ M s-adenosyl methionine, with 40 units of EcoRI methylase in a final volume of 60 μ l for 15 minutes at 37°C. The reaction was

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terminated by phenol extraction and DNA precipitated with ethanol. The methylated cDNA was resuspended in sterile water and then mixed with 0.7 μ g of EcoRI linkers, that had previously been kinased in the presence of 10 μ Ci of γ [³²P] ATP and a large excess of unlabelled rATP. After adding 24 units of T4 DNA ligase the reaction was incubated at 4°C overnight. The ligase was then inactivated by heating at 65°C for 10 minutes and the reaction made 100mM with NaCl. 25 units of EcoRI were added and the tube incubated at 37°C for 1 hour. The digestion was terminated by extracting with phenol and the aqueous phase applied to a sepharose CL4B column. Fractions were collected and counted using a geiger counter to generate a column profile. The first peak of radioactivity which corresponds to high molecular weight linked cDNA was taken and precipitated with ethanol.

3.31 cDNA Cloning In λ ZAP.

3.31.1 Ligation of linked cDNA into λ ZAP.

The EcoRI linked cDNA was dissolved in sterile water and aliquots ligated with 0.5 μ g of λ ZAP DNA that was pre-digested with EcoRI and dephosphorylated. The ligation was carried out overnight at 4°C in 1*C buffer, 1mM rATP in a final volume of 5 μ l with 6 units of T4 DNA ligase.

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3.31.2 In vitro packaging.

In vitro packaging of ligated λ ZAP was carried out exactly as directed by the manufacturer (Stratagene). After the packaging reaction was completed, 0.5 ml of SM buffer (5.8g/l NaCl, 2g/l $MgSO_4 \cdot 7H_2O$, 50mM Tris.HCl (pH7.5), 0.1g/l gelatin) and 20 μ l of chloroform was added, and the solution stored at 4°C.

3.32 Plating Of Bacteriophage Lambda.

Host cells (BB4, Y1090 or XL1-blue) from an overnight culture were inoculated into an appropriate volume of 2*TY and grown on a shaker at 37°C until the A_{550} reached 0.5. The cells were then pelleted by centrifugation at 2000 rpm for 5 minutes and resuspended in $1/10$ th the original culture volume of 10mM $MgSO_4$. Bacteriophage particles (from a packaging reaction or a phage stock in SM buffer) were then mixed with an appropriate volume of these host cells and allowed to adsorb for 15 minutes at 37°C. Up to 20,000 phage were adsorbed with 0.2ml of host cells. For the circular, 10cm diameter petri dishes 0.2ml of infected host cells were plated on each plate. For larger plates this was scaled up accordingly. Molten top agarose was aliquoted in to sterile tubes and cooled to 45°C. The appropriate volume of infected cells was added to the top agarose and this was then poured onto dried NZY plates and allowed to set on a level surface. 3ml of top agarose was used on 10cm diameter petri dishes, 6.5ml on 15cm diameter

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on 15cm diameter petri dishes and 5ml on 10cm square plates. With λ ZAP it is possible to use the lacZ colour reaction to identify plaques in which the bacteriophage contain insertions within the polylinker. To make use of this facility 50 μ l of 0.5M IPTG and 50 μ l of 250mg/ml X-gal was added to each 3ml of top agar prior to plating. The plates were then incubated upside down at 37°C for the appropriate length of time.

3.33 Amplification Of λ ZAP Libraries And Long Term Storage.

After determining the titre of the packaged phage, aliquots of 50,000 pfu were plated on BB4 on 15cm diameter petri dishes as described in section 3.32. After 6 hours growth at 37°C (when the plaques had not become too large), each plate was overlaid with 10ml of SM buffer and incubated for 12 hours at 4°C with gentle rotary shaking. The SM was then removed to sterile oakridge tubes (polypropylene) and the plates rinsed with a further 2ml of SM buffer. This was pooled with the first 10ml of SM buffer and chloroform was added to a final concentration of 5% (v/v). The tubes were mixed well and left at room temperature for 15 minutes. They were then centrifuged at 5,000 rpm for 10 minutes to pellet the debris and the supernatants transferred to a sterile glass bottle. Chloroform was added to 0.3% (v/v) and the phage stock stored at 4°C.

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3.34 The Benton And Davis Plaque Lift Procedure.

This procedure was used to transfer bacteriophage-DNA from plates to nitrocellulose filters and is essentially as described by Benton and Davis (1975). The host strains BB4 or Y1090 were used in all cases, and these support rapid plaque formation. Bacteriophage lambda were plated in the morning, always using top agarose, and then incubated at 37°C for 5 to 6 hours during the day. Plaques became visible about 4 hours after plating and were allowed to grow for a further 1 to 2 hours. When doing the primary screen of a library, care was taken not to allow the plaques to get too large. The plates were then transferred to the fridge and left overnight. The next day the plates were removed from the fridge, several at a time, and pieces of nitrocellulose cut slightly smaller than the plates and labelled appropriately, were carefully lowered on to the surface of the top agarose. Great care was taken not to allow any air bubbles to form. These were left in place for 1 minute during which time the position of the filters was marked by making three asymmetrical holes through the filter and into the agar with a syringe needle. The position of the holes was also marked on the base of the plates using a marker pen. The filters were carefully removed with a pair of forceps and layed, DNA side up, onto a tray containing several sheets of 3MM that had been moistened with denaturing solution (0.5M NaOH, 1.5M NaCl). The filters were allowed to denature for 1

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1 minute during which time a second nitrocellulose filter was applied to each plate. This replica filter was left in place for 2 minutes, and orientated by making holes in the identical positions used for the first filter. After denaturation the first filter was transferred to a tray containing several sheets of 3MM moistened with neutralising solution (3M NaCl, 0.5M Tris.HCl pH7, 1mM EDTA) and left in place for 4 minutes. It was then moved to a second tray of neutralising solution for a further 5 minutes and finally onto a tray of 3MM soaked in 2*SSC for 5 to 10 minutes. The filters were then allowed to air dry for at least 30 minutes. The same denaturation and neutralisation procedure was followed for the replica filters.

The filters were then baked at 80°C under vacuum for 2 hours and either pre-hybridised immediately or stored at room temperature for a short period.

3.35 Automatic Excision Of Phagemids From λ ZAP Clones.

This was carried out exactly as described by the manufacturers. A culture of BB4 was grown until the A₅₅₀ was between 0.8 and 1, and 0.2ml mixed with 100 μ l of λ ZAP phage stock and 10 μ l of R408 helper phage (7×10^{10} pfu/ml, supplied by Stratagene) in a sterile plastic universal. The cultures were incubated in a 37°C shaker at 200 rpm for 4 to 6 hours. During this time there was very variable growth of the bacteria, but this did not seem to effect

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the yield of phagemid. The cultures were heated at 70°C for 20 minutes and then centrifuged at 2,000 rpm for 5 minutes. The supernatant, which contains the phagemid particles, was transferred to a fresh tube and stored at 4°C. The phagemid was converted to colony form by mixing 0.2ml of a fresh BB4 culture (A₅₅₀ about 0.5) with 0.1 to 100µl of the phagemid stock. After incubating at 37°C for 15 minutes to allow adsorption of the phage, 10 and 100µl aliquots were spread on dried L-amp plates and incubated overnight at 37°C.

3.36 In Vitro Translation.

3.36.1 Translation in rabbit reticulocyte lysate.

RNA was translated in rabbit reticulocyte lysate by mixing 10µl of the lysate (Amersham) with 0.75µl of RNA and 1.75µl of [³⁵S] methionine, and incubating at 30°C for 45 minutes. After adding 30µl of dye mix and heating at 100°C for 3 minutes the sample was analysed on an SDS polyacrylamide protein gel (see section 3.40)

3.36.2 Translation in wheat germ extract.

This was carried out essentially as described by Roberts and Patterson (1973). 3.75µl of wheat germ extract was combined with 2.4µl of energy mix, 1µl of RNA and 1µl of [³⁵S] methionine and incubated at 30°C for 45 minutes. The products were then analysed on an SDS polyacrylamide protein gel (see section 3.40).

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3.37 Methods For Radiolabelling DNA And RNA.

3.37.1 Nick Translation.

DNA fragments isolated from agarose gels or undigested plasmids were labelled by nick translation using the following protocol. A 20 μ l reaction was assembled containing 1*NTB (50mM Tris.HCl (pH7.2), 10mM MgSO₄, 0.1mM DTT, 50 μ g/ml BSA), 100 ng of DNA, 1mM each of dATP, dCTP, dTTP, 2 to 4 μ l of α [³²P] dGTP, 2.3*10⁻³ units of deoxyribonuclease I and 10 units of E.coli DNA polymerase I. The reaction was allowed to proceed at 14°C for 1 hour after which 0.2ml of NaE was added. The solution was extracted with an equal volume of neutral phenol and then with ether. This was then applied to a 10 ml sephadex G-50 column using TE as the buffer. The high molecular weight DNA passes through this column much faster than unincorporated nucleotides, thus allowing the efficiency of incorporation of label into the DNA to be assessed. The first peak of radioactivity was collected, leaving the unincorporated label on the column, and precipitated with ethanol. The labelled DNA was recovered by centrifugation, resuspended in water, heated at 100°C for 5 minutes and then cooled on ice before adding to the hybridisation buffer.

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3.37.2 End-labelling of oligonucleotides with γ [³²P] ATP and polynucleotide kinase.

A 15 μ l reaction was assembled containing 1**C* buffer (66mM Tris.HCl (pH7.6), 6.6mM MgCl₂, 10mM DTT), 4 ng of oligonucleotide (these are already dephosphorylated), 2 to 4 μ l of γ [³²P] ATP and 20 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 1 hour after which 0.2ml of NaE was added, and the solution extracted with an equal volume of neutral phenol. The aqueous phase was extracted with ether and applied to a sephadex G-50 column in order to remove the majority of the unincorporated label. Being relatively small molecules, oligonucleotides tend not to form a distinct peak on a G-50 column, but run just ahead of the unincorporated label. Generally the first 7 radioactive 0.5 ml fractions were taken and precipitated with ethanol.

3.37.3 Preparation of radiolabelled size markers.

Lambda DNA (CI857) that had been digested with both HindIII and EcoRI, was end-labelled by mixing 1 μ g of restricted DNA, 1 μ l of 1mM dATP, 2 μ l of α [³²P] dGTP, 1 μ l of 10*TM buffer (100mM Tris.HCl (pH7.5), 50mM MgCl₂) and 10 units of E.coli DNA polymerase Klenow fragment in a final volume of 10 μ l. The reaction was allowed to proceed at 37°C for 30 minutes and terminated by adding 0.2ml of NaE followed by extraction with an equal volume of neutral phenol. Unincorporated label was removed by running the

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aqueous phase down a sephadex G-50 column, and the labelled DNA recovered by ethanol precipitation.

Essentially the same protocol was used to end-label HpaII digested pBR322 DNA, which was used as molecular weight markers on sequencing gels.

3.37.4 Preparation of mixed cDNA probes from poly A⁺ RNA.

A 25 μ l reaction was assembled which contained 1*TDM buffer (0.1M Tris.HCl (pH8.3), 10mM MgCl₂, 20mM β -mercaptoethanol), 100mM KCl, 1.5 μ g of oligo dT, 1mM each of dATP, dCTP, dTTP, 100 ng of poly A⁺ RNA, 5 μ l of α [³²P] dGTP, 90 units of pancreatic ribonuclease inhibitor and 40 units of AMV reverse transcriptase. The reaction was allowed to proceed at 42°C for 2 hours and then chased by adding dGTP to a final concentration of 2mM, and incubating for a further 15 minutes at 42°C. The RNA:DNA hybrids were denatured by adding NaOH to a final concentration of 0.3M and heating at 65°C for 15 minutes. After cooling rapidly on ice, 0.2ml of NaE was added and the solution applied to a sephadex G-50 column. The excluded peak was recovered by ethanol precipitation.

3.37.5 End-labelling of mRNA with polynucleotide kinase.

The RNA to be labelled was made up to 10 μ l with water and then heated at 100°C for 5 minutes, and allowed to cool at room temperature. This breaks the RNA in such a way that 5' OH termini are generated (Spradling et al.

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1980), and these can be labelled with polynucleotide kinase as described below. To the heated RNA was added 2 μ l of 10*PNK buffer (0.5M Tris.HCl (pH7.6), 0.1M MgCl₂, 50mM DTT, 1mM EDTA (pH8), 1mM spermidine), 3 μ l of water, 3 μ l of γ [³²P] ATP and 2 μ l (20 units) of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes and terminated by adding 0.2ml of NaE and extracting with neutral phenol. The aqueous phase was applied to a sephadex G-50 column, and the excluded peak recovered by ethanol precipitation.

3.37.6 Single stranded M13 "prime cut" probes.

2 μ l of the appropriate M13 template DNA (0.5 to 0.7 μ g) was combined with 3 μ l of water, 2 μ l of 10*TM and 1 μ l of the M13 17 mer sequencing primer (2ng/ μ l) in a 0.5ml eppendorf tube. The tube was floated in a shallow tray of water at 80°C and left to cool for about 40 minutes. 1 μ l of 0.5mM dATP/dCTP/dTTP, 4 μ l of α [³²P] dGTP and 2 units of DNA polymerase klenow fragment were added and the tube incubated at room temperature for 20 minutes. The reaction was then chased by adding 1 μ l of 0.5mM dGTP and incubating at room temperature for a further 20 minutes. 1 μ l of the appropriate restriction enzyme buffer and 10 units of the appropriate restriction enzyme was then added and the reaction incubated at 37°C for 1 hour. The reaction was then heated at 100°C for 5 minutes, cooled on ice and after adding 2 μ l of gel loading dye fractionated on a low

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melting point agarose gel. The single stranded probe fragment was located by autoradiography for 5 minutes at room temperature, excised from the gel and melted in 1ml of hybridisation buffer at 65°C. The melted gel slice was then added directly to the hybridisation buffer.

3.37.7 [³²P] labelled Synthetic RNA probes.

High specific activity single stranded RNA probes are often the best probes for use on northern blots. However they can occasionally hybridise very strongly and non-specifically to ribosomal RNA, something which must be borne in mind when using a probe for the first time. Synthetic RNA probes were prepared by transcribing DNA fragments, cloned in the appropriate vector, with either SP6, T7 or T3 RNA polymerase using the following protocol. The template DNA was first linearised at an appropriate polylinker restriction site, and after phenol extraction and ethanol precipitation dissolved in sterile water. A transcription reaction with a final volume of 25 μ l was then assembled at room temperature (to prevent precipitation of the spermidine). The reaction contained transcription buffer (40mM Tris.HCl (pH7.5), 6mM MgCl₂, 2mM spermidine), 0.5mM each of rATP, rGTP, rCTP, 10mM DTT, 0.5mg/ml BSA, 2 to 5 μ g of template DNA, 60 units of ribonuclease inhibitor, 1 to 4 μ l of α [³²P] rUTP, and 5 units of the appropriate RNA polymerase. This reaction was incubated at 37°C for 1 hour and then terminated by phenol

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extraction. The aqueous phase was applied to a sephadex G-50 column, and the excluded peak collected and precipitated. Typically 40 to 60% of the labelled rUTP was incorporated. The labelled RNA was recovered by centrifugation, dissolved in hybridisation buffer and added directly to the hybridisation bag.

3.37.8 Preparation of [³H] labelled synthetic RNA.

In order to quantitate the results of primer extension analysis with the albumin primer, synthetic RNA was prepared which would hybridise to this primer. In order to calculate the mass of synthetic RNA produced, the RNA was labelled with [³H] rUTP and then aliquots precipitated with TCA and counted in a scintillation counter. The RNA was prepared using essentially the same reaction described in section 3.37.7, except that 4 μ l of [³H] rUTP replaced the [³²P] rUTP, and after incubating for an hour at 37°C the RNA was recovered by a single ethanol precipitation

3.38 Transcription Of Cloned DNA In Vitro For Use In In Vitro Translations.

Unlabelled RNA was prepared in vitro using essentially the same procedure outlined in section 3.37.7 except that the reaction contained all 4 unlabelled ribonucleotides. The reaction was terminated by phenol extraction and RNA recovered by ethanol precipitation.

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3.39 DNA Sequencing Using The M13 Dideoxy Chain

Termination Method.

3.39.1 Standard M13 dideoxy sequencing.

This is a method for rapid and accurate sequencing of DNA fragments cloned in bacteriophage M13 vectors that was first described by Sanger et al (1977).

A set of deoxynucleotide and dideoxynucleotide mixes were assembled as shown below using 0.5mM stocks of each dNTP.

	A ^o	C ^o	G ^o	T ^o
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
TE buffer	20µl	20µl	20µl	20µl

Dideoxy NTP working solutions; 0.1mM ddATP, 0.1mM ddCTP, 0.3mM ddGTP, 0.5mM ddTTP

These concentrations were altered as necessary to fine tune the sequencing reaction.

Chase mix; 0.5mM of all 4 cold dNTPs.

For each clone to be sequenced an annealing reaction was set up by mixing 1µl of single stranded template DNA, 6.5µl of sterile water, 1.5µl of freshly prepared klenow reaction buffer (9µl of 10*core buffer [100mM Tris.HCl (pH8), 50mM MgCl₂], 1µl of 700mM β-mercaptoethanol), and 1µl of M13 17mer sequencing primer (2ng/µl). The tube was

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placed in a large shallow tray containing water at 80 to 85°C and left to cool at room temperature for about 40 minutes. For each template, four 0.5ml eppendorf tubes were labelled A, C, G, and T. In to the bottom of each of the "A" tubes was placed 1 μ l of the A⁰ nucleotide mix and 1 μ l of the ddATP nucleotide. This was repeated for the "C", "G", and "T" tubes using the appropriate nucleotide mixes. The annealed template/primer mix was centrifuged briefly to bring down any condensation and 0.75 μ l of [³⁵S] dATP added. The solution was mixed, and then 0.5 μ l of sequencing grade DNA polymerase Klenow fragment (5 units/ μ l) was added. After mixing briefly 2.5 μ l of this annealed template/primer/label/enzyme mix was transferred to the side of each of the tubes containing the nucleotide mixes. The tubes were closed and then centrifuged in a minifuge for 10 seconds to start the reaction. The reaction was allowed to proceed at 30°C for 15 to 20 minutes after which 2 μ l of chase mix was added and the tubes incubated for a further 15 to 20 minutes at 30°C. The reaction was terminated by pipetting 4 μ l of sequencing dye mix on to the side of each tube and then centrifuging briefly to mix the contents. Each reaction was then split into two aliquots by removing 5 μ l to a fresh tube. These samples were either stored at -20°C overnight or analysed immediately on a 6% polyacrylamide (w/v), urea sequencing gel in 1*TBE buffer, utilising sharks tooth combs throughout. One aliquot of each sample was heated at 100°C for 5 minutes and then

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loaded using a gilson P20. The gel was then run at 38 watts for 2 to 2.5 hours after which the second aliquot of each sample was heated and electrophoresed on the same gel until the bromophenol blue had almost reached the end of the plate. The gel was then fixed in 10% (v/v) glacial acetic acid/10% (v/v) ethanol for 20 minutes. After transferring to a sheet of 3MM, the gel was dried under vacuum at 80°C and exposed to X-ray film at room temperature.

3.39.2 Extended M13 dideoxy sequencing.

This is an adaptation of the standard M13 dideoxy sequencing protocol which enables one to read sequence at greater distances from the primer. This method was obtained from the BRL M13 cloning/dideoxy sequencing manual. With the greater resolving power of 1 meter gel systems this method enables up to 800 bases to be read from a single set of reactions. However, with the gel system available it was only possible to read a maximum of 650 bases from the primer.

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The following termination mixes were prepared;

	A	C	G	T
dATP	25 μ M	250 μ M	250 μ M	250 μ M
dTTP	250 μ M	250 μ M	250 μ M	25 μ M
dCTP	250 μ M	25 μ M	250 μ M	250 μ M
dGTP	250 μ M	250 μ M	25 μ M	250 μ M
ddATP	300 μ M	-	-	-
ddTTP	-	-	-	500 μ M
ddCTP	-	100 μ M	-	-
ddGTP	-	-	150 μ M	-

The template and the primer were annealed by mixing 1 μ l of template DNA, 2 μ l of 5*sequencing buffer (200mM Tris.HCl (pH7.5), 50mM MgCl₂, 250mM NaCl) and 4ng of primer in a final volume of 10 μ l. The tube was then placed in a shallow tray of water at 80°C and left to cool at room temperature for about 40 minutes. For each template, four tubes were labelled A, C, G and T, and 2.5 μ l of the appropriate termination mix was placed in the bottom of each of these tubes. To the annealed primer/template mix was added 2 μ l of extension mix (1.5 μ M dCTP, 1.5 μ M dGTP, 1.5 μ M dTTP), 1 μ l of [³⁵S] dATP, 1 μ l of 0.1M DTT, 1 μ l of water and 2 units of DNA polymerase I Klenow fragment. The contents of the tube were mixed and the tube incubated at room temperature for 5 minutes. 3.5 μ l aliquots of this labelling/extension

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reaction were pipetted onto the sides of each of the tubes containing the termination mixes. The tubes were centrifuged briefly to mix the contents and then incubated at 37°C for 5 minutes. The reactions were then terminated by addition of 4 μ l of sequencing gel loading dye, heated at 100°C for 5 minutes and analysed on a 6% polyacrylamide/urea sequencing gel using two loadings. These gels contained only 0.5*TBE and were run with 0.5*TBE in the top buffer tank and 1*TBE in the lower buffer tank. The first set of samples were run at 24mA for 1.5 hours after which the second set of duplicate samples were loaded on the same gel. The gel was then run at 18mA for a further 4.5 to 5 hours. The gel was then fixed, dried down and autoradiographed.

3.40 SDS Polyacrylamide Protein Gels.

A denaturing protein gel was prepared in two stages. First, the separating gel which consisted of 18% acrylamide, 0.48% bis-acrylamide, 0.375M Tris.HCl (pH8.8), and 0.1% SDS, was poured. After this had set it was overlaid with with a stacking gel which contained 3% acrylamide, 0.15% bis-acrylamide, 0.12M Tris.HCl (pH6.8), and 0.1% SDS into which the well former was inserted. The gel was run in a buffer containing 6g/l tris base, 29g/l glycine, and 0.1% SDS. Before loading, an equal volume of sample loading buffer (62mM Tris.HCl (pH6.8), 10% glycerol, 2% SDS, 0.73M β -mercaptoethanol, 0.001% bromophenol blue)

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was added to each of the samples, which were then heated at 100°C for 3 minutes. The gel was run at 10mA overnight or at 40mA during the day. The gel was then stained in 45% methanol, 10% acetic acid, 0.1% coomassie blue for 30 minutes and destained in 45% methanol, 10% acetic acid. In order to visualise labelled proteins the gel was then placed on a sheet of 3MM, dried down on a vacuum drier, and autoradiographed.

Results and Discussion

RESULTS AND DISCUSSION

CHAPTER 4

Characterisation Of The *Xenopus laevis* 68Kd Serum

Albumin Genomic Clone Ax68a206.

Introduction

The blood of adult *Xenopus laevis* contains two distinct serum albumin proteins that have molecular weights of 68Kda and 74Kda, and are encoded by two distinct but closely related mRNAs (Westley et al., 1981; Schoenberg, 1981). It has been reported that *X.laevis* has a single 68Kda albumin gene and one or perhaps two 74Kda genes (May et al., 1982a; May et al., 1983). The albumin genes are transcribed primarily in the liver of adult frogs, where the resulting mRNA constitutes approximately 10% of the total mRNA population (Westley et al., 1981). The differences in the molecular weights of the two albumins is largely due to the 74Kda, but not the 68Kda albumin being glycosylated (May et al., 1983). In serum, the 74Kda albumin is approximately twice as abundant as the 68Kda albumin, and this is reflected in the abundance of their respective mRNAs.

Our main aim upon receiving the 68Kda albumin genomic clone (May et al., 1982a) was to develop an oocyte assay for identifying promoter sequences and trans-acting factors required for the liver-specific transcription of this gene. At this time the restriction map of this clone

Results and Discussion

had been published (May et al, 1982a), and the approximate positions of the exons determined by R loop mapping (May et al, 1983). Our first goal was to identify the transcription start site. Initial attempts were made using S1 mapping with probes based upon the published maps, but without success. As we discovered later, these failed to work because of inaccuracies in the published maps (May et al, 1982a; May et al, 1983). This chapter describes some of the work which eventually led to the determination of the transcription start site.

4.1 A probe taken from the predicted 5' end of the coding region of the 68Kd albumin gene fails to detect albumin mRNA.

Introduction.

It was decided that it would be interesting to determine the profile of albumin gene transcription in Xenopus development. Initially this was attempted using northern blot hybridisation.

Results

5 μ g of ovary polyA⁺ RNA and 1 μ g of liver polyA⁺ RNA were separated on a formaldehyde agarose gel and transferred to nitrocellulose as described in the Materials and Methods. An M13 prime cut probe was prepared from the KpnI-HindIII M13 sub-clone of λ x68a206 designated mp18KH2.5 (figure 10). Prehybridisation and hybridisation was carried out

Figure 10.

The published restriction map (May et al., 1982a) of the 68Kda albumin genomic clone λ x68a206 with the approximate positions of the exons superimposed. Also shown are two M13 sub-clones of λ x68a206.

The albumin genomic clone λ x68a206 is a 19.6kb genomic EcoRI fragment from X.laevis (open box) encompassing the complete 68Kda albumin gene, inserted in the lambda vector Charon 4A (thin line) (May et al., 1982a). Numbers within the boxes indicate the sizes in Kb of the EcoRI fragments. Those restriction sites within the lambda arms that are relevant to the mapping experiments detailed in chapter 4.2 are shown, as are the sizes of some relevant restriction fragments. A more detailed map of the albumin genomic fragment is shown enlarged below the complete map. The exon/intron structure of the 68Kda albumin gene as determined by analysis of RNA/DNA hybrids (May et al., 1983) is shown superimposed upon the restriction map. Also shown are two M13 sub-clones of λ x68a206a that are relevant to experiments described in chapters 4.1 and 4.3.

Results and Discussion

exactly as described in Materials and Methods. Final wash conditions were 0.1*SSC, 0.1% SDS at 50°C. Even on a long exposure, no hybridisation to the liver polyA⁺ RNA (or to the ovary polyA⁺ RNA) was visible (data not shown). This northern blot was repeated with 2.5µg of freshly prepared liver polyA⁺ RNA and again no hybridisation was detectable. However, when the whole genomic clone λx68a206 was nick translated and used to probe a northern blot, it hybridised very strongly to a transcript of about 2Kb in both polyA⁺ and total liver RNA (data not shown).

Conclusions.

From these results I concluded that the KpnI-HindIII fragment present in mp18KH2.5 was upstream of the first exon, indicating that the published restriction map was unreliable.

4.2 Crude mapping of the exons within λx68a206.

Introduction.

Having discovered that the published map was inaccurate I decided to try and determine the degree of inaccuracy. We needed to know the approximate location of the first exon in order to map the transcription start site. Because albumin mRNA is so abundant in liver (about 10% of polyA⁺ RNA), it should be possible to use a mixed probe prepared from liver message to detect exons within λx68a206.

Results and Discussion

Results.

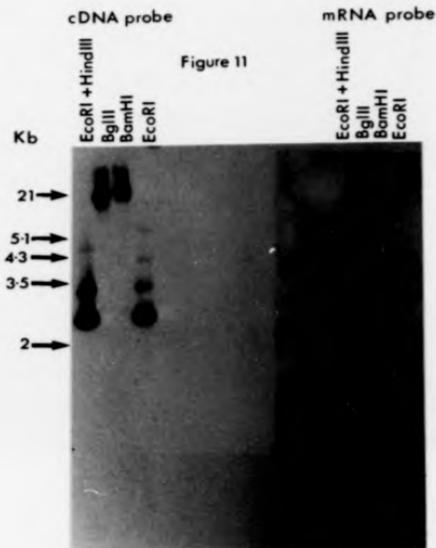
λ x68a206 DNA was digested with several restriction enzymes (as indicated in figure 11), and then each digest was split into two aliquots. Each aliquot was heated at 65°C for 10 minutes to ensure that the cohesive ends of lambda had not annealed, and then separated on a 1% agarose gel. After photographing the gel under U.V. light, the DNA was transferred to nitrocellulose using the Southern blotting protocol described in Materials and Methods. A calibration curve was drawn for this gel and used to accurately determine the sizes of all the fragments. This showed up several inaccuracies in the restriction map of λ x68a206 and the map was modified accordingly. After baking, the filter was cut in half to generate two identical filters. One was hybridised with 1 μ g of adult male liver polyA⁺ RNA that had been end-labelled with polynucleotide kinase as described in Materials and Methods (section 3.37.5). The second filter was hybridised to a probe that was prepared by reverse transcription of adult male liver polyA⁺ RNA primed from oligo d(T) annealed to the poly(A) tail (Materials and Methods section 3.37.4). After hybridisation, both filters were washed in 2*SCC, 0.1% SDS at 60°C and exposed to X-ray film.

As can be seen in figure 11, both the cDNA probe and the kinased mRNA probe hybridised to the same restriction fragments. The EcoRI digest of λ x68a206 produced 9

Figure 11.

Crude mapping of the coding regions within λ x68a206.

λ x68a206 DNA was digested with various restriction enzymes as indicated above each track. Equal aliquots of each digest were separated on an agarose gel and transferred to nitrocellulose using the procedure of Southern (1975). The filter was cut in half to generate two identical filters, one of which was hybridised with radiolabelled single stranded cDNA prepared from male X.laevis liver mRNA ("cDNA probe": see chapter 3.37.4). The second filter was hybridised with male X.laevis mRNA that had been end-labelled with polynucleotide kinase ("mRNA probe": see chapter 3.37.5). Prehybridisation and hybridisation was in 3*SSC, 1* Denhardt's, 100 μ g/ml E.coli tRNA, 0.25% SDS at 65°C. After hybridisation the filters were washed in 2*SSC at 60°C and then exposed to X-ray film for 4 hours.



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fragments, 2 of which are the arms of the vector. (the 0.5Kb fragment predicted by the map was not visible on the gel). Four of these EcoRI fragments; the 5.1Kb, 3.6Kb, 2.7Kb, and 2.2Kb fragments hybridised to both probes. All these fragments are predicted by the published map (figure 10) to contain exons. In agreement with this map, neither the 1.8Kb doublet or the 5' most 1.7Kb EcoRI fragments hybridised to the probes. It is quite clear that with the cDNA probe, but not with the mRNA probe, the ladder of EcoRI fragments shows a steady increase in hybridisation intensity going down the gel. This reflects the inability of most of the reverse transcriptase molecules to reach the 5' end of the albumin mRNA during synthesis of the probe. Therefore, strongest hybridisation occurred to the 3' most exon-containing fragment. The relative intensities of the hybridisation to the EcoRI fragments in figure 11 does correspond to the order of the fragments in the published map (see figure 10). This indicates that the map is essentially correct, at least in respect to the EcoRI sites, and the rough positions of the exons.

In the HindIII/EcoRI double digest, the 2.2Kb and 2.7Kb EcoRI fragments are the same fragments as in the EcoRI digest and hybridised to the probes as before. The 5.1Kb EcoRI fragment is cut by HindIII to give a 4.3Kb fragment which hybridised weakly to the cDNA probe. The 3.6kb EcoRI fragment contains two HindIII sites, giving rise to a fragment of 2.2Kb which ran as a doublet with the 2.2Kb

Results and Discussion

EcoRI fragment. It is therefore not possible to say whether or not this fragment hybridised to the probes. In the BamHI digest 5 fragments were produced, of which the three smaller fragments all come from the vector. Only the two largest fragments (20.3Kb and 18.8Kb), which were only just resolved on the gel, contain any albumin sequence. Although it is not particularly clear on the photograph, the probes hybridised to both these fragments. This means that there must be at least one exon on the 5' side of the BamHI site within the 5.1Kb EcoRI fragment.

BglII digestion of λ x68a206 generated 6 fragments larger than 1.3Kb. In addition, a number of very small fragments were produced, that all come from the vector and can therefore be ignored in the context of this experiment. Three of the 6 larger fragments are also derived entirely from the vector (see figure 10). This leaves a 1.95Kb fragment that comes from within the 5.1Kb EcoRI fragment plus two large fragments (23Kb and 16Kb). The 1.95Kb fragment hybridised only weakly to both probes, and in fact this was only visible on a longer exposure than that shown in figure 11. This suggests that this fragment contains only a single exon, or even only a part of one exon. The 16Kb BglII fragment contains the majority of the insert of λ x68a206 and hybridised strongly to both probes. However, the 23Kb BglII fragment did not hybridise to either probe. The cDNA probe is not particularly informative in this situation since I only expect this

Results and Discussion

fragment to have at most the first two exons. The kinased RNA probe appeared to detect 5' and 3' coding regions with equal sensitivity. Given the fact that the 1.95Kb BglII fragment, which must contain at least one exon, gave only a faint signal I can only tentatively conclude that there is at most one exon (exon one) on the 5' side of the 5' most BglII site. As discussed earlier, a fragment which extends as far as the next BamHI site does hybridise to the mRNA probe.

Conclusions.

This mapping data supports the northern blot result which had suggested that the first exon was further to the 3' side of the 5.1Kb EcoRI fragment than was indicated by the published map. This data in fact suggests that the first exon may well lie on the 3' side of the 5' most BglII site.

4.3 Subcloning the 5.1Kb EcoRI fragment of λ x68a206 (containing exons one to six) into pBR322.

Introduction.

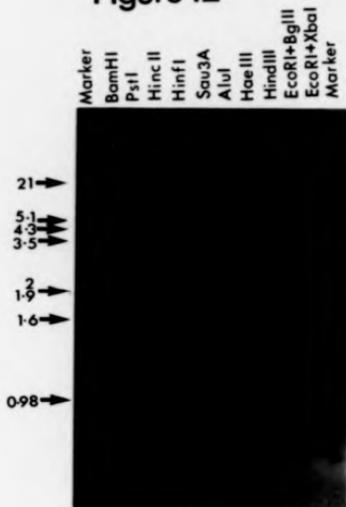
In order to map more accurately the coding regions at the 5' end of the albumin genomic clone it was decided to subclone the 5.1Kb EcoRI fragment into pBR322.

Figure 12.

Mapping of the coding regions within the albumin sub-clone p682F.

The 68Kda albumin sub-clone p682F was digested with various restriction enzymes as shown above each track. The digested DNA was separated on an agarose gel and then transferred to nitrocellulose using the procedure of Southern (1975). The filter was hybridised to male X.laevis mRNA that had been end-labelled with polynucleotide kinase as described in chapter 3.37.5. Prehybridisation, hybridisation and washing conditions were exactly as described in the legend to figure 11. The markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII. Marker sizes are in kilobase pairs (Kb).

Figure 12



Results and Discussion

Results.

pBR322 was linearised with EcoRI, dephosphorylated and ligated with the mixture of DNA fragments from an EcoRI digest of λ x68a206. The E.coli strain MC1061 was transformed with this ligation and plated on L-amp plates. The resultant clones were therefore a mixture of non-recombinants and recombinants potentially containing all 8 EcoRI fragments of λ x68a206. Clones containing the 5.1Kb fragment were selected by colony hybridisation with an M13 sub-clone of λ x68a206, designated mp18HE1.0 (see figure 10). One positive colony was selected, and its identity confirmed by restriction enzyme digests. This plasmid was designated p682F.

4.4 Crude mapping of the coding regions within p682F.

Results.

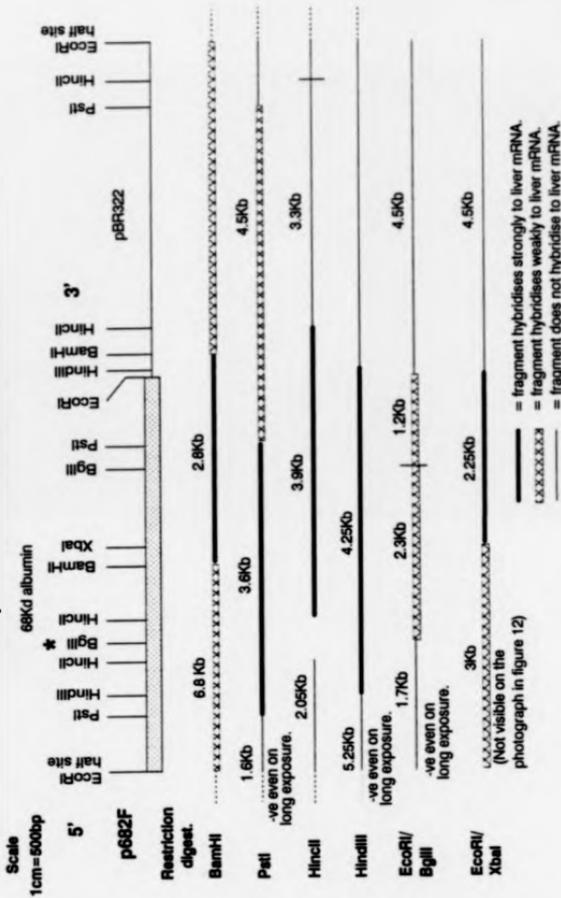
p682F DNA was digested with a number of restriction enzymes as indicated in figure 12, and the fragments separated on a 1% agarose gel. After photographing under U.V. light the gel was Southern blotted and the filter hybridised to male Xenopus liver polyA⁺ RNA that had been end-labelled using polynucleotide kinase. Figure 12 shows the resulting autoradiograph, and the data is summarised in figure 13. The sizes of the various fragments produced by digestion of p682F with the various restriction enzymes was used to update the restriction map. This gave rise to the map shown in figure 13. This figure indicates the

Figure 13.

The restriction enzyme map of the albumin sub-clone p682F and summary of the exon mapping data from figure 12.

p682F consists of the 5.1Kb EcoRI fragment from the albumin insert in λ x68a206, containing exons one to six, cloned into the EcoRI site of pBR322. Restriction enzyme sites within pBR322 which are relevant to the mapping experiments described in chapter 4.4 are also shown. Below the restriction map is a summary of the exon mapping data from figure 12. The fragments produced by the various restriction enzyme digests of p682F are shown. Those fragments which hybridised strongly to male X.laevis liver mRNA are represented by thick lines, whilst those which hybridised only weakly are represented by the cross-hatched boxes. Fragments represented by the thin black lines did not hybridise to this probe.

Figure 13 Restriction enzyme map of the albumin sub-clone p682F and summary of the exon mapping data from figure 12.



Results and Discussion

restriction fragments of p682F which hybridised to the probe, and perhaps more importantly, those which did not.

Conclusions.

From the hybridisation data summarised in figures 13, it can be concluded that no significant length of sequence complementary to albumin mRNA is present on the 5' side of the 5' most BglII site (marked with a *). The transcription start site must therefore lie on the 3' side of this BglII site, almost certainly between this BglII site and next BamHI site.

4.5 Identification of the probable 5' end of the albumin transcript by sequencing and comparison to the 5' ends of other albumin mRNAs.

Introduction.

It was decided to determine the DNA sequence of p682F in the region I had identified as containing the transcription start site. This work was carried out by R. W. Old and P. Day, and will not be discussed in any detail here.

Results.

Both Maxam Gilbert (Maxam and Gilbert, 1980) and M13 dideoxy sequencing was used to generate DNA sequence spanning the region of the 68Kda albumin gene from 650bp upstream of the transcription start site through to the

Figure 14.

The DNA sequence around the transcription start site of the Xenopus 68Kda serum albumin gene.

The DNA sequence of the X.laevis 68Kda serum albumin gene spanning the region from 199 nucleotides upstream of the transcription start site to 136 nucleotides downstream of the transcription start site (Sweeney et al., 1987) is shown. The positions of the transcription start site used in liver, the TATA box, and the predicted amino acid sequence of the first exon are also shown. The hatched rectangle indicates the binding site for the albumin primer which was used to identify the exact transcription start site. Also shown are the conserved sequences that could theoretically base pair to form a stem loop structure in the the 5' untranslated leader of the 68Kda albumin mRNA.

Figure 14

DNA Sequence Around The Transcription Start Site Of
The *Xenopus* 68 Kda Serum Albumin Gene.

-199 AATTTAAAAG GTTTCTAAAA TTCAGAAAAC CGAATATAGA GCAACAGCAA

-149 TACGTTATTT GACCTTAAAA GTTGATTGAC ATTAGGAAAT TCCACAAAAG

-99 TAAAACAACT GCAAACAGCA ACAATTTGAT AGGTTTCAAT AAGTTTCCA...

BgIII

"TATA"

Transcription
start site

-49 GATCTCTCTG AGCAATAGTA TAAAACAAGA GGTATCACTC ATTCAGATC

"conserved"

"conserved"

+2 AGGCTTCTCA GAGGTCCCCA CCCAATACAT CTCCAGTC ATG AAG TGG
Met lys trp

Albumin primer

+49 ATC ACC CTC ATT TGT CTG TTA ATT AGC TCC ACT TTA ATA GGA
Ile thr leu Ile cys leu leu Ile ser ser thr leu Ile glu

+99 TCA AGA ATA ATT TTC AAA AGA GAT ACA G/GTAAGCCTT
ser arg Ile Ile phe lys arg asp thr 1st intron

XXXXXXXXXX = binding site for the albumin primer

Results and Discussion

first exon/intron boundary (Sweeney et al., 1987). It has been reported that the untranslated leaders of albumin mRNA's contain several conserved motifs (Hache et al., 1983). By examining the sequence we had determined for the Xenopus albumin gene, it was possible to identify these conserved motifs. Figure 14 shows the sequence around the transcription start site of the 68Kda albumin gene, and these conserved motifs have been marked "conserved". It has been proposed that these conserved sequences (CCCCAC and GTGG in the case of the Xenopus albumin gene) base pair to form the stem of a stem loop structure in the mRNA (Hache et al., 1983). In the case of the Xenopus mRNA the stem would consist of only 4 nucleotides rather than the 5 predicted for human and the 6 predicted for rat. The first five encoded amino acids of exon one of the Xenopus albumin gene were identified as being identical to those predicted for the albumin proteins of rat (Sargent et al., 1981), human (Minghetti et al., 1986), and chicken (Hache et al., 1983) and the alpha-fetoprotein of mouse (Scott and Tilghman, 1983), except for the substitution of isoleucine for valine. This helped to confirm that we had indeed located the 5' end of the Xenopus albumin coding region. The first intron/exon boundary on figure 14 has been confirmed by S1 mapping (G. Sweeney, pers. comm.), and its position relative to the transcription start site is identical to that of the human albumin gene. Also indicated on figure 14 is the actual transcription start

Results and Discussion

site used in vivo in adult liver. This was determined by primer extension analysis of liver RNA as described in the next section. Almost exactly 30bp upstream of the transcription start site is a good consensus TATA box. The transcription start site lies about 50bp downstream of the BglII site, which is consistent with the mapping data described in sections 4.2 and 4.4. The map published by May et al (1983) positioned the first exon about 2Kb too far to the 5' end of the albumin genomic fragment.

4.6 Identification of the exact transcription start site by primer extension with the "albumin" primer.

Introduction.

Based upon sequence homology to the 5' ends of other albumin mRNA's we had identified the probable transcription start site. In order to determine this unambiguously an oligonucleotide called the albumin primer was designed. This 17mer oligonucleotide is complementary to the sequence of the mRNA just downstream of the putative transcription start site, as indicated in figure 14. This oligonucleotide was used in a primer extension analysis of adult male Xenopus liver RNA.

Results.

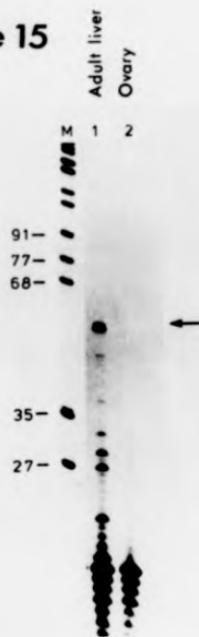
The albumin primer was end-labelled with polynucleotide kinase and hybridised to 2 μ g of total liver RNA and 4 μ g of ovary polyA⁺ RNA. The liver RNA gave rise to two primer

Figure 15.

Identification of the exact transcription start site of the 68Kda albumin gene.

End-labelled albumin primer (GAGGGTGATCCACTTCA) was annealed to 4 μ g of X.laevis ovary polyA⁺ RNA (lane 2) or 2 μ g of adult male X.laevis total liver RNA (lane 1). Annealed primers were extended with reverse transcriptase and the products fractionated on an 8% sequencing gel and located by autoradiography after exposure for 18 hours. Markers (M) were end-labelled fragments from a digest of pBR322 DNA with HpaII. The arrow indicates the position of the band corresponding to correctly initiated transcripts.

Figure 15



Results and Discussion

extension products of 56 and 57 nucleotides (figure 15), and the largest of these corresponds to the 5' end of albumin mRNA. This doublet of bands is often observed with primer extension back to the 5' end of eukaryotic mRNA's in general. This is thought to be due to the inability of some of the reverse transcriptase molecules to traverse the final nucleotide, because in many eukaryotic mRNAs it is capped. This gives rise to a second band 1 nucleotide shorter than the full length product. Even after a longer exposure of the gel shown in figure 15, no primer extended products were detected with the ovary polyA⁺ RNA, indicating that the albumin genes are not transcribed at detectable levels in oocytes.

The sequence of the Xenopus 74Kda serum albumin gene (Schorpp et al., 1988a) is identical to that of the 68Kda gene within the region to which the albumin primer binds. Furthermore, the distance from the albumin primer binding site to the transcription start site is identical in the two genes. We can therefore be confident that the albumin primer will detect both mRNA's and give identically sized primer extended products.

Results and Discussion

CHAPTER 5

The profile of albumin gene transcription during Xenopus development.

Introduction.

One approach to identifying sequences within the albumin promoter required for liver-specific transcription is to inject albumin promoter constructs into fertilised eggs and then analyse transcription from the injected promoter as development proceeds. Before such an experiment could be attempted it was necessary to know when the endogenous albumin promoter first becomes activated so that I could tell whether the injected promoter was being correctly regulated. The developmental profile of albumin transcription would also be of interest in itself.

5.1 Development of an RNA probe to detect albumin mRNA.

Introduction.

Single stranded RNA probes synthesised in vitro and labelled to a high specific activity make the most sensitive hybridisation probes for use on northern blots. Being single stranded, the RNA probe cannot hybridise to itself, and RNA:RNA hybrids formed between the probe and the RNA on the filter are more stable than RNA:DNA hybrids.

Results.

I decided to subclone the 3' most XbaI-EcoRI fragment of

Figure 16.

Construction of the albumin sub-clones pSP65/EX2.2 and pGEM1/XE2.2.

p682F DNA was digested with XbaI (for which there are no sites in pBR322) and EcoRI and the resulting 2.2Kb fragment isolated from a low melting point agarose gel. This fragment was cloned into both pSP65 and pGEM1 that had been digested with XbaI and EcoRI. This gave rise to the plasmids designated pSP65/EX2.2 and pGEM1/XE2.2 respectively. In vitro transcription of pSP65/EX2.2 with SP6 RNA polymerase gives rise to RNA complementary to albumin mRNA (anti-sense). In vitro transcription of pGEM1/XE2.2 with SP6 RNA polymerase generates an RNA molecule containing sequences the same as those in albumin mRNA (sense).

Figure 16 Construction of the albumin subclones pSP65/EX2.2 and pGEM1/XE2.2

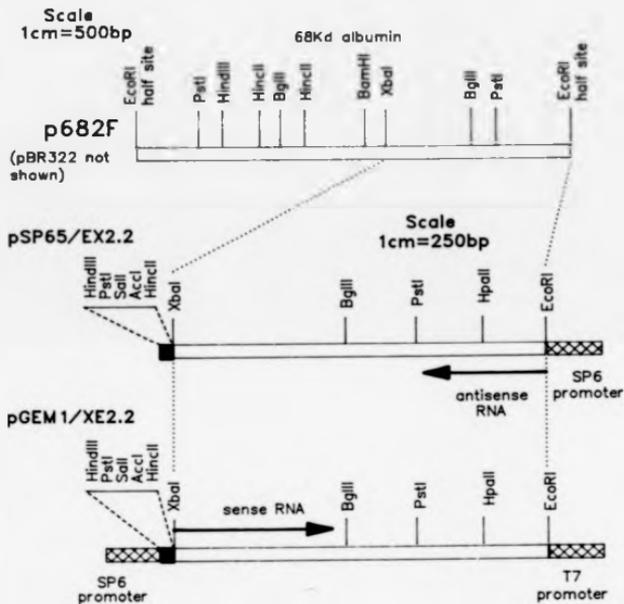
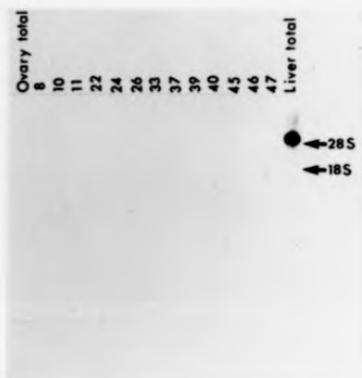


Figure 17.

Northern blot analysis for albumin mRNA using single stranded SP6 RNA prepared from pSP65/EX2.2 as a hybridisation probe.

One embryos worth of total RNA prepared from X.laevis larvae at various stages of development (stages 8 to 47 as indicated above each track), 2.5 μ g of total ovary RNA, and 3 μ g of total adult liver RNA were fractionated on a formaldehyde agarose gel. The RNA was then transferred to nitrocellulose and hybridised to a ³²P labelled single stranded RNA probe prepared by transcription of pSP65/EX2.2 in vitro. Prehybridisation and hybridisation was in 50% formamide, 5*SSC, 1* Denhardtts, 23mM sodium phosphate (pH6.5), 100 μ g/ml E.coli tRNA at 60°C, and final wash conditions were 0.1*SSC at 60°C. A 20 hour exposure of the filter is presented. The arrows indicate the positions of the 28S and 18S rRNAs as detected by ethidium bromide staining of the formaldehyde agarose gel.

Figure 17



Results and Discussion

p682F into the SP6 transcription vector pSP65 (see figure 16). This vector has a polylinker into which restriction fragments can conveniently be inserted. By using pSP65 (rather than pSP64 in which the polylinker is inverted) I could ensure that transcription from the SP6 promoter would generate RNA antisense to albumin mRNA. This clone was designated pSP65/EX2.2 (see figure 16). In order to quantitate the signal obtained with this probe on northern blots, the same fragment was subcloned into the transcription vector pGEM-1. This generated a clone, designated pGEM1/XE2.2, in which transcription from the SP6 promoter produces RNA which is complementary to that produced from pSP65/EX2.2. This sense RNA was synthesised in a reaction containing [^3H] rUTP (see Materials and Methods section 37.8), and the quantity of RNA produced determined by scintillation counting.

5.2 An RNA probe fails to detect albumin mRNA in all stages of Xenopus embryogenesis up to and including stage 47.

Results.

Figure 17 shows a 20 hour exposure of a northern blot probed with a single stranded "antisense" RNA probe prepared from pSP65/EX2.2. The probe has detected albumin mRNA in adult liver total RNA with great sensitivity, but failed to detect albumin mRNA in any of the embryo samples. Even after a 10 day exposure (data not shown) no

Results and Discussion

signal was detected in these samples. Using known amounts of synthetic RNA prepared from pGEM1/XE2.2 it was shown that this probe can detect as little as 5pg of RNA on a northern blot (data not shown).

Conclusions.

The albumin genes are not transcribed at detectable levels in embryonic stages up to and including stage 47. This was perhaps not surprising given that previous workers had reported that serum albumin was undetectable in the blood of pre-metamorphic X. laevis larvae, and that levels of the protein increased dramatically during metamorphic climax (Herner and Frieden, 1960). I therefore decided to look for albumin mRNA in later stages of development, during metamorphic climax (stages 50-65).

5.3 The profile of albumin gene transcription throughout Xenopus development as analysed by primer extension.

Introduction.

A very sensitive method for detecting albumin mRNA is primer extension with the albumin primer. I have used this method to determine the levels of albumin mRNA throughout the development of Xenopus laevis.

Results.

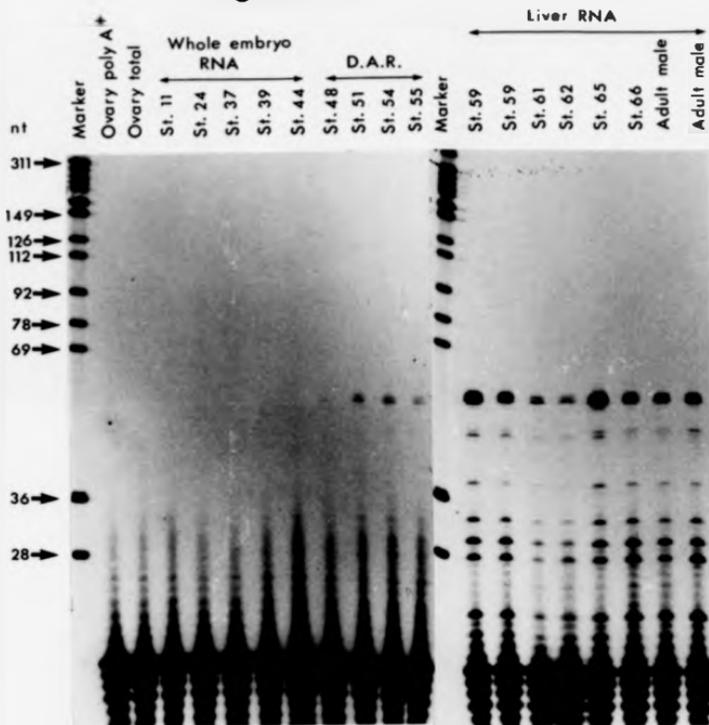
The albumin primer was end-labelled and hybridised to 4µg aliquots of various RNA samples as indicated in figure

Figure 18.

Primer extension analysis for albumin mRNA throughout Xenopus development.

The albumin primer was end-labelled and annealed to 4 μ g aliquots of ovary polyA⁺ RNA, ovary total RNA, whole embryo total RNA prepared from the stages indicated, total RNA prepared from pooled dissected abdominal regions (D.A.R.) of embryos at the stages indicated, total RNA prepared from the livers of single larvae at the stages indicated, and total adult liver RNA. The annealed primers were extended with reverse transcriptase and the products fractionated on 8% sequencing gels, and detected by autoradiography for 18 hours. The markers were end-labelled fragments from a digest of pBR322 DNA with HpaII.

Figure 18



Results and Discussion

18. After extension with reverse transcriptase the products were analysed on an 8% sequencing gel, and the results are presented in figure 18. This figure is a combination of two gels on which the samples from a single analysis had been electrophoresed. Both gels were exposed for 18 hours.

The first point to make is that albumin mRNA first becomes detectable in RNA prepared from the abdominal regions of stage 48 tadpoles (the band is barely visible on this photograph, but was clear on the autoradiograph). Given that the liver only makes up a small percentage of the tissue in these dissected abdominal regions (probably about 10%), the level of albumin transcripts in the liver of these embryos was at least 10 fold higher than is seen in this figure. This means that the albumin genes are in fact being transcribed fairly efficiently at stage 48. I cannot rule out the possibility that low levels of albumin transcripts are present in the livers of larvae before this stage since I have not analysed RNA from the abdominal regions of earlier stage larvae. However, on a longer exposure of this gel (data not shown), when the signal from the stage 48 RNA is quite strong, nothing is visible in the adjacent track (stage 44). Also, albumin transcripts could not be detected in total embryo RNA prepared from any stage up to and including stage 47 (figure 17). By stage 51 the level of albumin transcripts is significantly higher than at stage 48.

Results and Discussion

The variation in the level of albumin mRNA detected in the RNA prepared from the livers of individual larvae (tracks 14 to 19) is almost certainly due to RNA degradation in some of the samples. Primer extension analysis of other larval liver RNA samples has shown no consistent variation in the level of albumin mRNA during metamorphosis (data not shown). Clearly, the level of albumin transcripts in some of these larval liver samples is significantly higher than the level in the same amount of adult liver RNA. This indicates that albumin gene expression is more active in the livers of metamorphosing larvae than in the adult liver. This could of course be either a transcriptional effect or a post-transcriptional effect, or a combination of the two.

Conclusions.

The Xenopus albumin genes are first transcribed in the liver of metamorphosing larvae at about stage 48 of development. This corresponds to the start of the metamorphic climax and is therefore consistent with the results reported by Herner and Frieden (1960). The level of albumin transcripts in the livers of larvae undergoing metamorphic climax (stages 59 to 62) is significantly higher than in adult liver.

Results and Discussion

CHAPTER 6

Cloning of albumin cDNA's from *Xenopus laevis*.

Introduction.

At the time I began this work, four groups had reported the isolation of albumin cDNA clones from *Xenopus laevis* (Westley et al., 1981; Schoenberg, 1981; Phillip et al., 1982; Wolfe et al., 1985). The restriction maps of these albumin cDNAs show considerable diversity, and we were therefore suspicious that some of them were not albumin clones at all. It is known that *X.laevis* has one 68Kda albumin gene and one or possibly two 74Kda genes (May et al., 1982a). One might therefore expect to find 2 or possibly 3 classes of albumin cDNAs. I therefore decided to isolate my own albumin cDNAs with the aim of resolving the paradox of the inconsistent restriction maps.

6.1 Preparation of double stranded cDNA and cloning in M13mp11.

Introduction.

Since albumin mRNA is very abundant in liver RNA it should be necessary to screen only a small number of liver cDNA clones in order to obtain albumin clones. For this reason it was decided to use an M13 vector rather than a lambda vector which would be much less convenient.

Double stranded cDNA was prepared from adult male liver mRNA essentially as described by Gubler and Hoffman

Results and Discussion

(1983). In this method oligo d(T) is annealed to the polyA tail and then extended back towards the 5' end of the mRNA with reverse transcriptase. The second strand is synthesised by DNA polymerase I in the presence of ribonuclease H. Ribonuclease H introduces nicks into the RNA strand of the RNA:DNA hybrid, and DNA synthesis by DNA polymerase I is primed from these nicks.

Results.

Double stranded cDNA was synthesised essentially as described in Materials and Methods (section 3.30.1) except that the first and second strands were synthesised in separate reactions rather than in the same tube.

The double stranded, blunt-ended cDNA (20 to 160ng) was then ligated with 50ng of SmaI cut, dephosphorylated M13mp11 RF DNA. The ligations were transformed into JM101 using the high efficiency procedure of Hanahan (1983). The transformed bacteria were plated in top agarose containing X-gal and IPTG, and a total of about 300 white plaques were obtained. Two of these plates (about 220 white plaques) were screened for albumin clones.

6.2 Screening the liver cDNA clones for albumin.

Results.

M13 plaques on the two plates selected were transferred to nylon (Hybond-N) discs exactly as described for phage lambda in Materials and Methods except that the DNA was

Results and Discussion

fixed to the filter by ultra violet illumination. The hybridisation probe used was an albumin cDNA clone designated pX1A14 (Schoenberg, 1981) which we had independently sequenced and confirmed as an albumin sequence. After hybridisation the filters were washed in 0.1*SSC at 55°C. Two plaques hybridised strongly to the probe, and these were designated mp11X1a1 and mp11X1a2.

6.3 Restriction enzyme mapping of the albumin cDNA clone

mp11X1a2 indicates that it belongs to the 74Kda class.

Results.

Both mp11X1a1 and mp11X1a2 were mapped with the restriction enzymes EcoRI, HindIII, PstI, KpnI, XbaI, XhoI, and BamHI. mp11X1a1 was found to have an insert of just 650bp and contained no sites for these restriction enzymes. mp11X1a2 contained an insert of just under 2Kb and the restriction map is shown in figure 19, along with the published maps of the other albumin cDNA clones which align with that of mp11X1a2. The characteristic bunching of an EcoRI site and a HindIII site at one end of the clone and an EcoRI site and a PstI site at the other end indicates that mp11X1a2 is a 74Kda albumin cDNA clone. The map of mp11X1a2 fits well with that of the 74Kda albumin cDNA clones isolated by Westley et al (1981), and of the class I albumin clones isolated by Schoenberg (1981). Interestingly, there would appear to be two types of 74Kda albumin cDNAs based upon the restriction maps (see figure

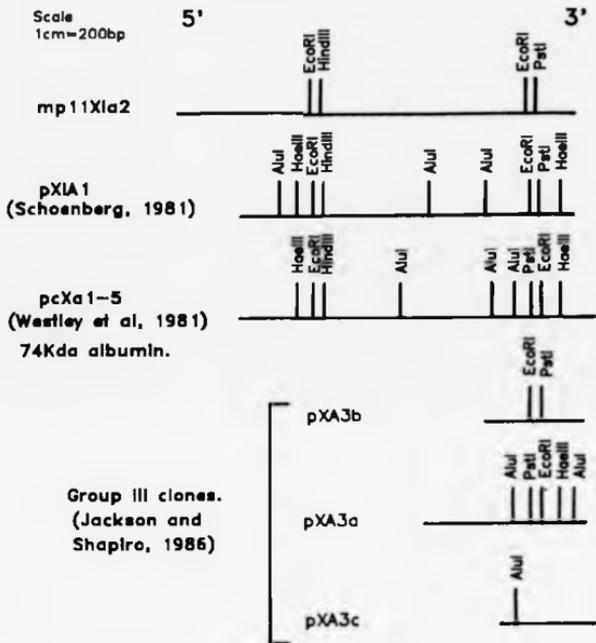
Figure 19.

The restriction enzyme map of mp11Xla2 and alignment with the maps of other albumin cDNA clones.

The X.laevis albumin cDNA clone mp11Xla2 was mapped with the restriction enzymes EcoRI, HindIII, PstI, KpnI, XbaI, XhoI, and BamHI to generate the map shown. Comparison with the published restriction maps of other X.laevis albumin cDNA clones indicated that the clones could be aligned as shown. The albumin clone pcXal-5 is known to encode the 74Kda form of serum albumin (Westley et al, 1981). The restriction map of mp11Xla2 does not resemble that of the 68Kda albumin clone isolated by Westley et al (1981). The orientation of the restriction maps with respect to the 5' end of the mRNA is based upon the known orientation of pcXal-5.

Figure 19

Restriction enzyme map of mp11Xla2 and alignment with the maps of other *Xenopus* albumin cDNA clones.



Results and Discussion

19). In the first type (mp11X1a2, pX1A1, pXA3b), the EcoRI site is on the 5' side of the PstI site, while in the second type (pcXa1-5, pXA3a) the order of these sites is reversed. The positions of the AluI sites is also consistent with the existence of two classes of 74Kda albumin cDNA clones. This would support the proposal that there are two 74Kda albumin genes in X.laevis (May et al., 1983).

Conclusion.

I have isolated an albumin cDNA clone which is very nearly full length. The restriction enzyme map of this clone indicates that it is probably a 74Kda albumin cDNA. Comparison with published restriction maps of other albumin cDNA clones indicates that mp11X1a2 can be grouped with a number of other 74Kda albumin cDNA's as shown in figure 19.

The restriction map of the albumin cDNA clone pX1A14, which was used as the probe to isolate mp11X1a2 bears no resemblance to the published restriction maps of any other albumin cDNA clones.

As well as the group III clones whose maps appear to match that of mp11X1a2 (see figure 19), Jackson and Shapiro (1986) also isolated two other classes of albumin cDNA clone (groups I and II). These groupings were made based upon both cross-hybridisation studies between the different clones and on restriction enzyme mapping. The

Results and Discussion

restriction maps of these two groups of clones (groups I and II) also do not resemble (even partially) any of the other published restriction maps. There is little doubt that the various clones isolated by the different groups are all albumin cDNA clones. I am therefore forced to conclude that there is considerable restriction enzyme site polymorphism within the coding regions of X.laevis albumin genes from different frogs.

Results and Discussion

CHAPTER 7

Transcription Of Albumin Gene Constructs Microinjected Into Xenopus Oocytes.

Introduction.

Xenopus oocytes are a useful system for studying transcription of cloned genes. Many of the genes that have been microinjected into Xenopus oocytes were found to be faithfully transcribed. However, those tissue-specific genes that have been tested in oocytes have often been found to be poorly or inaccurately transcribed. This is perhaps what would be expected given that one would not expect the oocyte to possess the trans-acting factors that are known to be required for the transcription of many tissue-specific genes. We and others have shown that the endogenous albumin genes are not transcribed in oocytes (see figure 15). Presumably this is because the oocyte is deficient in the trans-acting factors present in hepatocytes that are required for transcription of these highly liver-specific genes. However, this is perhaps a very simplistic view. Another possibility which is not mutually exclusive of the first, is that the albumin genes in the oocyte are repressed in an inactive chromatin structure.

We set out to develop the oocyte as an assay system for two complementary lines of investigation. Firstly we hoped to use biochemical complementation to identify the trans-

Results and Discussion

acting factors required for liver-specific transcription of the 68Kda albumin gene. In this assay liver mRNA is injected into the cytoplasm of the oocyte, where it will be translated. This should provide all the liver-specific factors required for efficient transcription of the albumin gene, thereby complementing deficiencies in the oocyte. When the albumin promoter is subsequently injected into the nucleus it should be transcribed more efficiently than without pre-injection of liver mRNA.

Secondly, and in conjunction with this approach, we planned to carry out an analysis of the promoter by microinjection of altered promoter constructs into oocytes that had been pre-injected with liver mRNA. This obviously depends upon transcription of the albumin gene being dependent on pre-injection of liver mRNA.

7.1 Construction of an albumin promoter:histone fusion gene.

Introduction.

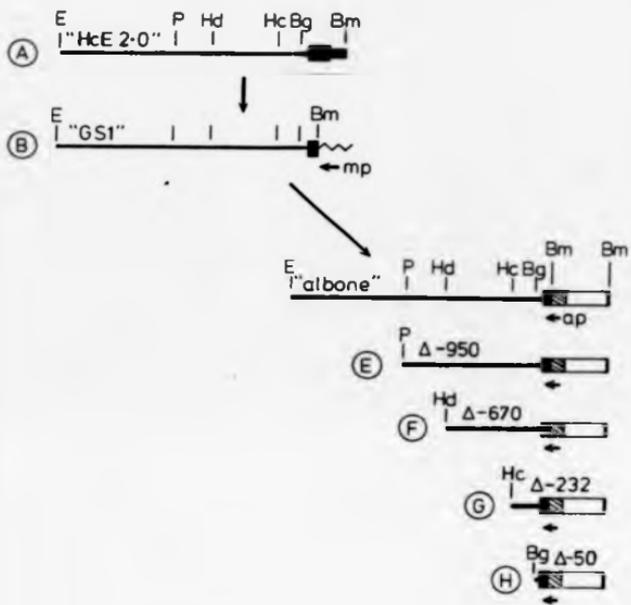
In order to do these experiments we had to be able to distinguish transcripts initiating at the injected promoter from those of the endogenous genes (even though the endogenous genes are not normally expressed). This was achieved by fusing the 68Kda albumin gene, within the first exon, to the 3' end of a histone H3 gene.

Figure 20.

Construction of an albumin promoter:histone fusion gene and a series of 5' deletion derivatives.

A 2Kb EcoRI/HincII DNA fragment from the Xenopus 68Kda albumin gene consisting of 1.7Kb of 5' flanking DNA (thin line), exon 1 (filled box) and the first 150bp of intron 1 (thick line) was cloned into M13mp19 RF DNA (zig-zag line) that had been cleaved with EcoRI and SmaI to give clone HcE2.0 (A). Site directed mutagenesis was used to create a BamHI site within exon 1 and the DNA between this site and the BamHI site in the M13 polylinker was deleted to give GS1 (B). A 370bp DNA fragment containing the sequence encoding the 3' end of the Xenopus histone H3 RNA (hatched box) and also some 3' flanking DNA from the histone H3 gene (Old et al., 1985) (clear box) was inserted into the BamHI site of GS1 to give albone (D). Clones Δ -950 (E), Δ -670 (F), Δ -232 (G), Δ -50 (H) were made by deleting albumin DNA upstream of the PstI, HindIII, HincII, and BglII sites of albone, respectively. Abbreviations used: E, EcoRI; P, PstI; Hd, HindIII; Hc, HincII; Bg, BglII; Bm, BamHI. The arrows indicate sites complementary to the M13 sequencing primer (mp) and the albone primer (ap), CGCCTCGGATCCACTTC

Figure 20



Results and Discussion

Results.

The clone called albone was constructed by G. Sweeney and R. W. Old as outlined in the legend to figure 20. A 17 mer oligonucleotide complementary to nucleotides spanning the junction between the albumin and histone sequences was synthesized. This oligonucleotide, designated the albone primer, was used to detect transcripts initiating at the albumin promoter within the albone construct in a primer extension assay. Because the primer spans the junction of the albumin and histone sequences it is specific to transcripts initiating on this construct.

7.2 Construction of a series of albumin promoter deletions for analysis in oocytes and embryos.

Introduction.

As mentioned at the beginning of this chapter, one of our main aims was to carry out a functional analysis of the albumin promoter. I therefore made a set of derivatives of albone in which various amounts of albumin 5' flanking DNA had been deleted.

Results.

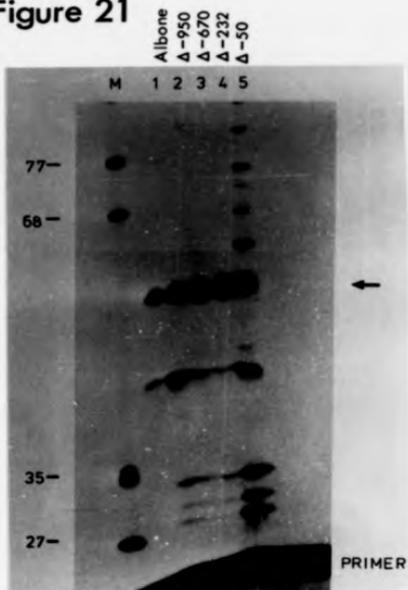
Increasing amounts of albumin 5' flanking DNA was deleted from the albone construct by digestion with PstI, HindIII, HincII and BglII. The resulting clones were designated Δ -950, Δ -670, Δ -232, and Δ -50 respectively

Figure 21.

Analysis of transcripts from oocytes injected with albone and its derivatives.

End-labelled albone primer was annealed to 20 μ g samples of RNA from oocytes each injected with 10ng of RF DNA from albone, Δ -950, Δ -670, Δ -232, and Δ -50 as indicated above each lane. Extension products were fractionated through an 8% sequencing gel and detected by autoradiography. The arrow indicates the bands (a doublet) corresponding to correctly initiated transcripts. Markers were end-labelled fragments from a digest of pBR322 DNA with HpaII.

Figure 21



Results and Discussion

(figure 20 e-h). The numbers here refer to the amount albumin DNA upstream of the transcription start site.

7.3 The albumin promoter directs accurate and efficient transcription when microinjected into oocytes.

Results.

Xenopus oocytes were given nuclear injections with 20nl aliquots of 0.5mg/ml solutions of albome RF DNA or its deletion derivatives. After incubating for 24 hours RNA was prepared from the oocytes and analysed by primer extension with end-labelled albome primer. The result of an analysis of this type is shown in figure 21. In each track 20µg of RNA (equivalent to the content of 4 oocytes) prepared from a batch of 20 oocytes injected with the RF DNA indicated was analysed. All five RNA samples gave rise to a major primer extended product of 57 nucleotides which represents correctly initiated transcripts. Each lane also has fainter bands at 58 and 45 nucleotides which represent transcripts initiating 1 nucleotide upstream and 12 nucleotides downstream of the correct start site respectively. Oocytes injected with Δ -50 gave rise to a number of larger primer extension products that represent transcripts initiating further upstream of the correct start site. However, the majority of transcripts from Δ -50 were initiated at the correct start site. On a longer exposure, some of the higher molecular weight bands seen in track 5 could also be seen in lanes 1 to 4, but they

Results and Discussion

were very much weaker than in lane 5. From this it is quite clear that all 5 clones were being accurately transcribed in oocytes even without pre-injection of liver mRNA. In order to quantitate the level of transcription from these clones the insert of Δ -50 was subcloned into pBluescribe and this was used to prepare synthetic RNA. The amount of synthetic RNA produced was calculated by incorporating [3 H] rUTP into the RNA and counting aliquots in a scintillation counter. Known amounts of this synthetic RNA were analysed by primer extension using the same albone primer used to perform the analysis shown in figure 21, and the products run on a separate sequencing gel. The slices of gel giving rise to the resulting bands together with the slices of gel giving rise to the 57 nucleotide band in lanes 1 to 5 of figure 21 were excised and counted in scintillation fluid. From these results it was calculated that clones Δ -950, Δ -670, Δ -232 and Δ -50 each gave rise to about 2.5×10^{-3} pmoles of correctly initiated transcript per oocyte. This is equivalent to about 1.5 transcripts per gene copy per day. Albone itself gave rise to about two thirds this number of transcripts, but this difference was not observed in repeat experiments. The efficiency of transcription of albone and its derivatives is greater than that of the *Xenopus* beta globin gene, which was estimated to produce between 0.005 and 0.1 correctly initiated transcripts per gene copy per 20 hours when injected into oocytes (Bendig and Williams,

Results and Discussion

1984). In addition, large amounts of incorrectly initiated transcripts were produced from the beta-globin gene.

When GS1 RF DNA (see figure 20) was injected into oocytes and transcription analysed by primer extension with the M13 universal sequencing primer, it was found that only 0.018 correctly initiated transcripts were produced per copy of GS1 per day (data not shown). GS1 is identical to albone except for the fusion of a DNA fragment encoding the 3' end of the histone H3 RNA onto the 3' end of the albumin sequence. Thus, it is probable that the albone transcript is substantially stabilised by the histone sequences, as might be expected since histone RNA is very stable in Xenopus oocytes. The possibility that a previously unidentified transcriptional enhancer was present in the histone gene fragment was eliminated by placing this fragment upstream of the albumin 5' flanking DNA in GS1. This construct gave similar levels of transcripts to that produced by GS1 when microinjected into oocytes (data not shown).

Conclusions.

Despite the fact that the endogenous 68Kda albumin gene is inactive in oocytes, the cloned promoter is able to direct accurate and relatively efficient transcription when injected into oocytes. These results are in contrast to those reported for other homologous cell-type-specific genes such as the Xenopus cardiac actin genes (Mohun et

Results and Discussion

al, 1986) and the Xenopus alpha and beta globin genes (Bendig and Williams, 1984), where transcription was found to be very inaccurate, with only a few percent of the transcripts initiating at the correct start site. However it should be noted that a recent report (Walmsey and Patient, 1987) contradicts the previous data on the Xenopus β -globin gene, suggesting that it functions efficiently and accurately when injected into oocytes.

As mentioned previously, we had hoped to use oocytes as a system in which to assay factors that stimulate expression of the albumin gene. This has not been possible because of the high constitutive activity of the albumin promoter in oocytes. Presumably, the injected albumin promoter does not require additional factors for activity in oocytes. It is not clear why the injected, but not the endogenous albumin promoter is active, but it may reflect the fact that a large number of copies (about 10^9) of the promoter are injected into each oocyte nucleus. This is therefore a very artificial situation. It is possible that the endogenous gene is under negative control by a negative transcription factor. In this case it could be that the negative element with which this factor interacts lies upstream or downstream of the albumin sequences in our constructs. Alternatively, the large number of injected genes could be in excess over the number of repressor molecules in the oocyte nucleus, leading to transcription of the unexpressed genes.

Results and Discussion

Deletion of increasing amounts of the albumin upstream sequence within albino clearly has very little effect upon either the accuracy or the efficiency of transcription in oocytes. Correct tissue-specific expression of genes, including albumin genes from other systems (Heard et al., 1985, Gorski et al., 1986) and the Xenopus 68Kda albumin gene (Schorpp et al., 1988b) requires upstream sequence, so it seems highly likely that the activity of the albumin promoter in oocytes requires only a subset of the sequences required for full activity in liver. The albumin construct Δ -50 contains only 50bp of upstream sequence and the only notable feature within this region is the TATA box. So it would seem that the TATA box alone is sufficient to direct accurate and efficient transcription in oocytes.

Similar results regarding the activity of the 68Kda albumin gene in oocytes have been reported by Schorpp et al. (1988a).

Results and Discussion

CHAPTER 8.

Analysis Of The 68Kda Albumin Gene Promoter By Microinjection Into Xenopus Embryos.

Introduction.

The microinjection of cloned genes into Xenopus embryos is a potentially powerful system for the study of gene regulation. It has a great advantage over in vitro studies in that the introduced gene is expressed inside a living organism and should therefore be subject to the normal control mechanisms. A number of cloned genes from both Xenopus and other organisms have been analysed by injection into Xenopus embryos. Busby and Reeder (1983) were able to demonstrate correct temporal activation of injected Xenopus rRNA genes. Brown and Schlissel (1985) showed competition between oocyte and somatic 5S rRNA genes injected into fertilised eggs. They found that there was considerably more transcription of the somatic 5S genes than the oocyte 5S genes, thus mimicking what is seen with the chromosomal genes.

A number of genes that are transcribed by RNA polymerase II have also been tested in Xenopus embryos. Bendig (1981) reported that when a sea urchin histone gene cluster was microinjected into Xenopus embryos, 4 out of the 5 histone genes were faithfully transcribed at the blastula stage. Curiously no H4 transcripts with the correct 5' end were detected.

Rusconi and Schaffner (1981) reported that when the

Results and Discussion

rabbit β -globin gene was injected into embryos, transcripts with the correct 5' end and splicing of at least one intron could be found in both gastrulae and swimming tadpoles (stage 35). However, the injected genes were transcribed very inefficiently (given the large number of copies of the injected gene that were present). The tissue-specificity of transcription was not tested, but the fact that transcripts could be detected at the blastula stage suggests that the injected genes were not being correctly regulated.

When the Xenopus adult alpha and beta globin genes were injected into embryos (Bendig and Williams, 1983) it was found that they were transcribed at low levels, but from the correct promoters during early development. This is in contrast to the chromosomal genes which are not expressed until metamorphosis.

Andres et al (1984) failed to find correct developmental regulation of Xenopus vitellogenin gene constructs that had been injected into fertilised Xenopus eggs.

However there have been some encouraging reports of the correct regulation of polymerase II genes injected into embryos. Krieg and Melton (1985) found that the Xenopus gene GS17 showed correct temporal regulation, whilst Wilson et al (1986) demonstrated, for the first time, the correct temporal and spatial regulation of a tissue-specific gene injected into Xenopus embryos. They showed that transcription of a X.borealis cardiac actin gene was

Results and Discussion

localized to the myotomes of tailbud stage embryos. Although the injected gene was transcribed at a low level at the mid-blastula transition (MBT), transcription increased sharply at the late gastrula stage when endogenous actin transcripts first appear.

Most if not all of the genes that have been tested in embryos appear to be activated to some degree at the MBT. Zygotic transcription is first activated at the MBT (Newport and Kirschner, 1982), so transcription of injected genes at this time may be a consequence of this general gene activation. Generally, between 100pg and 2ng of DNA has been injected into each embryo. Quantities greater than 2ng have been found to be toxic.

There have been numerous reports of, and investigations into the replication and persistence of genes injected into Xenopus embryos. Bendig (1981) injected both linear and circular plasmids containing histone genes and found that both replicated during early development. Maximum replication (about 10 fold) was attained by the late blastula stage after which the exogenous DNA was rapidly degraded. Linear DNA persisted longer, with small amounts still detectable by stage 40. The physical form of the DNA altered following injection; linear DNA was ligated to form large concatemers soon after injection. Supercoiled DNA was initially partly converted to relaxed circles before being converted back to supercoiled circles by the blastula stage. The rabbit β -globin gene was also

Results and Discussion

replicated in Xenopus embryos (Rusconi and Schaffner, 1981). The extent of replication varied between different batches of embryos but seemed to depend upon the amount of DNA injected. Replication did not depend upon whether the DNA contained a pBR322 replicon or an SV40 replicon. Small amounts of the injected plasmid were still detectable in the tissues of 6 month old frogs, but in an unintegrated form as head to tail multimers.

These observations have been extended to other DNA's by Bendig and Williams (1983) who found that the X.laevis adult alpha and beta globin genes (in the vectors Charon 4A and pAT153 respectively) were replicated 50 to 100 fold by the gastrula stage.

Etkin and Pearman and their co-workers (Etkin et al. 1984; Etkin and Pearman, 1987; Etkin et al., 1987) have carried out several investigations into the replication, persistence and germ line transmission of exogenous DNAs injected into fertilised Xenopus eggs. They tested a total of ten DNAs, most of which were recombinant pBR322 plasmids, and found that they replicated to different extents ranging from 2 fold to 300 fold. The extent of replication did not correlate with the size of the plasmid, and they proposed that replication could depend upon specific sequences (replication origins) within the plasmids. Etkin and Pearman (1984) reported that recombinant plasmids (containing a Drosophila alcohol dehydrogenase gene and a prokaryotic CAT gene) that had

Results and Discussion

been injected into fertilised eggs, persisted in the tissues of 60% of the resulting adult frogs. However they estimated that integration of the exogenous DNA into the chromosomal DNA had occurred in only 5 to 10% of those frogs in which the DNA persisted. In the majority of individuals the DNA persisted as high molecular weight concatemers. Analysis of DNA from different tissues, (Eskin and Pearman, 1984) indicated a mosaic distribution of the exogenous DNA, so integration probably occurred at some time during the first cleavage stages, but not prior to the first division. Conclusive evidence of the integration of the exogenous DNA was provided by the demonstration of the transmission of the injected DNA to the offspring. Analysis of the F₁ generation indicated that the sperm from the original transformed animal contained the injected DNA integrated at different sites within the chromosomes. This is further evidence of the extreme mosaicism of the initial transformants. Therefore, in order to generate true transgenic Xenopus (in which every cell has the transgene integrated at the same site), it may be essential to utilize the F₁ or even F₂ generations.

In conclusion, Krieg and Melton (1985) and Wilson et al (1986) have demonstrated correct expression of injected genes, but only at a relatively early stage of development (tailbud or earlier). At these early stages the majority of the injected DNA is extrachromosomal and it is likely that the transcription they detect originates from these

Results and Discussion

extrachromosomal copies. As development proceeds the majority (if not all) of this extrachromosomal DNA is degraded, with only a small percentage of the resulting animals retaining some DNA integrated in the chromosomes. These transformed frogs are highly mosaic.

Despite the problems outlined above it was decided that I should try to investigate the regulation of the Xenopus 68Kda albumin gene using this approach. This decision was made before the finding that the albumin genes are not expressed prior to metamorphosis. A set of albumin constructs (albone and its deletion derivatives) were already available so I decided to inject these into embryos. They were perhaps not the ideal constructs to use since we are unsure of the stability of transcripts with the histone 3' end in embryos. The transcript from albone is very stable in oocytes but may well be less stable in embryos.

8.1 Persistence of albumin constructs in Xenopus embryos.

Results.

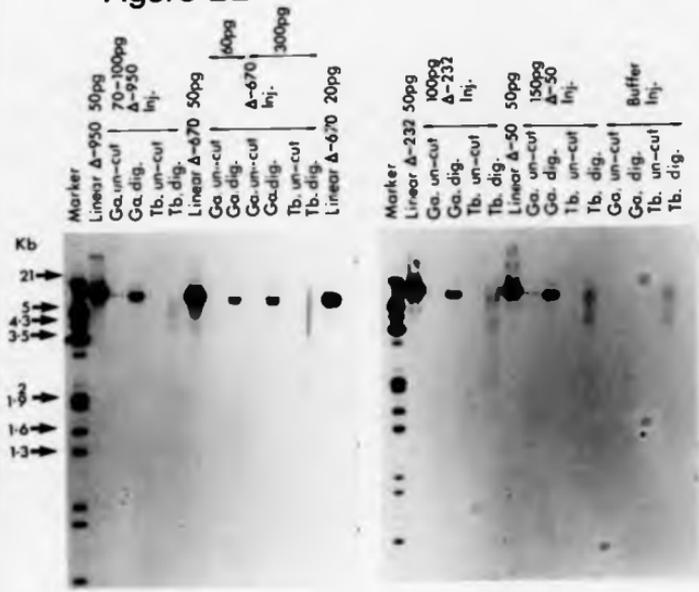
There is general agreement in the literature that linear DNA persists longer in Xenopus embryos. Therefore albone, Δ -950, Δ -670, Δ -232 and Δ -50 were linearised at the unique EcoRI site that lies in the M13 polylinker at the 3' end of the histone gene fragment. In initial experiments about 1ng of DNA was injected into each embryo, but this

Figure 22.

The persistence of albumin promoter constructs microinjected into fertilised Xenopus eggs.

X.laevis eggs were fertilised in vitro and injected at the 2 cell stage with RF DNA from either Δ -950, Δ -670, Δ -232, or Δ -50 that had been linearised by digestion with EcoRI. The amounts of DNA injected into each embryo are indicated in the figure. The injected embryos were allowed to develop and total nucleic acids prepared from pools of 10 to 15 embryos at the gastrula stage (G.A.: stage 10 to 12), and the hatching tadpole stage (T.B.: stage 42-43). One embryo worth of total nucleic acids were fractionated on agarose gels along with known quantities of EcoRI linearised RF DNA from the four albino 5' deletion constructs. The nucleic acids were transferred to nitrocellulose using the method of Southern (1975) and hybridised with a radiolabelled DNA probe prepared from non-recombinant M13mp18. The filters were washed in 2*SSC at 60°C and then exposed to X-ray film for 40 hours. Markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII.

Figure 22



Results and Discussion

appeared to be toxic, with few of the embryos gastrulating successfully. The linearised DNAs were therefore diluted and between 70 and 500pg injected into each embryo. Total nucleic acids were prepared from pools of 10 to 15 embryos at the gastrula stage and at the hatching tadpole stage (stage 43). Some injected embryos were also sacrificed when they had reached stage 49/50.

One embryos worth of DNA was digested with EcoRI and electrophoresed on a 1% agarose gel along with one embryos worth of undigested DNA. The gels were then Southern blotted and the filters hybridised to a prime cut probe prepared from non-recombinant M13. After hybridisation the filters were washed in 2*SSC at 60°C and the results are presented in figure 22. It is clear from these Southern blots that none of the DNAs injected had replicated by the gastrula stage. In fact, the opposite is true; by the gastrula stage less DNA remained than was injected. The amount of DNA remaining does not appear to depend upon the amount of DNA injected since embryos injected with 60pg of Δ -670 contained similar amounts of the DNA at the gastrula stage as those injected with 300pg of Δ -670.

In all cases the injected DNA was no longer in a linear form at the gastrula stage. In the tracks containing undigested DNA very little injected DNA was detectable. Upon digestion with EcoRI (the enzyme used to linearise all the plasmids prior to injection), a hybridising DNA fragment which co-migrates with the linear form of the

Results and Discussion

injected DNA was generated. This indicates that by the gastrula the injected DNA had been ligated to form high molecular weight concatemers, which migrated with the high molecular weight Xenopus DNA, and therefore failed to transfer efficiently to the filter. When these concatemers were digested with EcoRI a single linear species was generated, indicating that the concatenated DNA had not been grossly rearranged. A small percentage of the DNA appeared to persist as linear molecules in gastrulae. By the time the embryos had developed to stage 42 very little, if any, of the injected DNA remained.

Conclusions.

None of the plasmids injected appeared to replicate, in contrast to what has been found for other DNAs. However, the injected, linear DNA was ligated to form large concatemers as has previously been observed by other workers. There are no reports in the literature of the injection of M13 constructs into Xenopus embryos, and it could be that these albumin constructs do not contain sequences necessary for replication. Very little if any exogenous DNA remained by stage 42, although the analysis shown in figure 22 would not be sensitive enough to detect a low copy number (less than 10 copies per cell). Given that initial transformants have been reported to be highly mosaic, this system is unsuitable for a rapid analysis of the regulation of the albumin gene. One would need to

Results and Discussion

inject a large number of fertilised eggs, breed from the resulting frogs, and screen the offspring for the injected gene.

8.2 Analysis of transcription from the injected albumin constructs in early embryos and tadpoles.

Results.

The nucleic acid samples analysed in figure 22 and RNA prepared from a number of stage 49/50 tadpoles that had been injected with albone or one of its derivatives at the two cell stage, was analysed for transcripts from the injected constructs by primer extension with the albone primer.

No transcripts initiating at the correct start site were detectable in any of the samples analysed (data not shown). In some of the RNA samples prepared from gastrula and stage 42/43 embryos small amounts of a transcript initiating about 14 nucleotides downstream of the correct start site was detected. So it would appear that some inaccurate transcription from the injected albumin promoter occurred at a very low level in early stage embryos, long before the endogenous albumin genes become active.

Results and Discussion

CHAPTER 9

Neither The Rat Albumin Promoter Or The Rat β -Fibrinogen Promoter Can Be Trans-Activated In *Xenopus* Oocytes By Pre-injection With Liver Message.

Introduction.

Following our discovery that the *Xenopus* 68Kda albumin promoter has high constitutive activity in *Xenopus* oocytes (Old et al., 1988) we decided to test some other liver-specific genes in this system. Two rat β -fibrinogen constructs in which the promoter is fused to the reporter gene CAT (Courtois et al., 1987), were tested. These two clones are designated B-5400 and B-120, where the numbers refer to the amount of β -fibrinogen 5' flanking DNA upstream of the transcription start site. B-5400 had been reported to show correct liver-specific transcription whilst B-120 gave only a low level of expression when transfected into a liver cell line (Courtois et al., 1987).

We also tested a rat albumin promoter construct. This plasmid, designated pUMS Δ E1 contains the first 151bp of the rat albumin promoter fused to the reporter gene CAT (Heard et al., 1987). This plasmid also contains the so-called upstream mouse sequence (UMS) that is a potent transcription terminator in eukaryotic cells (McGeady et al., 1986), inserted immediately upstream of the rat albumin promoter. This was shown (Heard et al., 1987) to reduce readthrough transcription from the vector. pUMS Δ E1 was found to contain all the albumin promoter sequences

Figure 23.

Activity of the rat β -fibrinogen and rat albumin promoters microinjected into Xenopus oocytes.

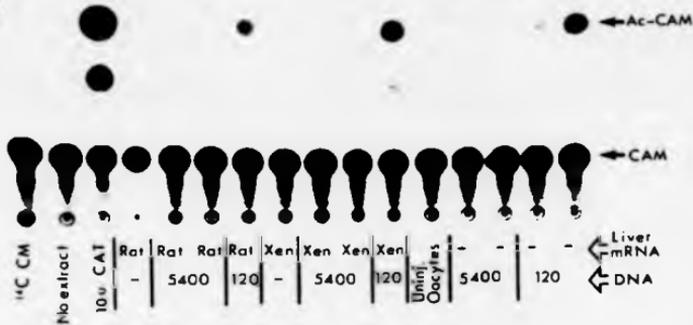
Pools of Xenopus oocytes were given cytoplasmic injections of about 20ng of either adult male X. laevis liver polyA⁺ RNA or rat liver polyA⁺ RNA. These were incubated for 2 hours in 1*BX at 22°C, along with some un-injected oocytes, after which various pools of oocytes were given nuclear injections of 4 to 10ng of the appropriate supercoiled plasmid DNA. After incubating for a further 18 hours in 1*BX at 22°C, "CAT extracts" were prepared from pools of 5 to 10 oocytes. Where sufficient numbers of oocytes had been injected, they were split into two pools which were homogenised and analysed separately in order to control for variation. One oocytes worth of extract was assayed for CAT activity as described in chapter 3.21. Acetylated chloramphenicol was detected by exposing the thin layer chromatography plate to X-ray film for 7 days. Abbreviations used: CAM and CM, chloramphenicol; Ac-CAM, acetylated chloramphenicol; CAT, chloramphenicol acetyltransferase; Xen, Xenopus: 5400, B5400; 120, B120.

Figure 23

A



B



Results and Discussion

required for correct liver-specific expression as assessed by transient transfection into various cell lines (Hearld et al., 1987).

All three of these plasmids could easily be tested in the oocyte system by assaying CAT activity.

Results.

Xenopus oocytes were injected as described in the legend to figure 23, and "CAT extracts" were prepared as described in Materials and Methods. One oocytes worth (50 μ l) of extract was then assayed for CAT activity exactly as described in Materials and Methods, and the results are presented in figure 23.

The rat albumin promoter (first 151bp upstream of the transcription start site) was completely inactive when injected alone into Xenopus oocytes (figure 23a). Pre-injection of liver mRNA failed to activate the promoter.

The β -fibrinogen construct B-120, but not B-5400, showed a low constitutive level of expression in oocytes (figure 23b) This may be the result of readthrough transcription from the vector in B-120. In B-5400 this readthrough may be prevented by the large segment (5.4Kb) of B-fibrinogen upstream DNA that lies between the CAT gene and the vector. Alternatively, sequences upstream of -120 may be required to prevent promiscuous activity of the β -fibrinogen promoter in oocytes. Neither B-120 or B-5400 were activated by pre-injection of liver mRNA.

Results and Discussion

Conclusions.

Both the rat albumin promoter and the rat β -fibrinogen promoters had very low or undetectable activity when injected into the nucleus of Xenopus oocytes. This is in complete contrast to the high constitutive activity of the Xenopus 68Kd albumin gene in oocytes. Maybe the difference in the behaviour of the rat and Xenopus albumin genes in oocytes is a reflection of the heterogeneous nature of the rat promoters.

The inability to activate the rat albumin and β -fibrinogen promoters in oocytes by pre-injection of liver mRNA would seem to suggest that this assay system is not generally applicable.

Results and Discussion

CHAPTER 10

Xenopus laevis Has a c-erb-A Gene.

Introduction.

The cellular homologues of the avian erythroblastosis (AEV) v-erb-A gene are known to encode high affinity receptors for thyroid hormone (Weinberger *et al.*, 1986, Sap *et al.*, 1986). Cellular erb-A (c-erb-A) cDNA clones have been isolated from human, rat, mouse and chicken (see chapter 1) by homology to the v-erb-A gene or other c-erb-A genes. As a probe, we employed the 500bp PstI fragment of the AEV v-erb-A gene. This fragment contains sequences homologous to the putative hormone binding domain (as defined by homology to the glucocorticoid receptor) and therefore should not cross-hybridise to other members of the steroid hormone receptor superfamily (within which the DNA binding domain is conserved).

Results.

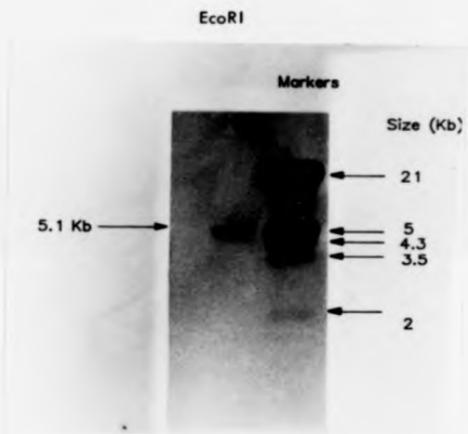
10µg of X.laevis genomic DNA was digested with EcoRI. Southern blotted, and then hybridised to the v-erb-A PstI fragment, and figure 24 shows the resulting autoradiograph. The probe hybridised to a single EcoRI fragment of 5.1Kb. This indicates that X.laevis does have a c-erb-A gene. The fact that only a single fragment hybridised to the probe indicates that X.laevis has one or at most 2 c-erb-A genes.

Figure 24.

Xenopus laevis has a c-erb-A gene.

10µg of X.laevis genomic DNA prepared from the blood of a single individual, was digested exhaustively with EcoRI and then fractionated on a 0.7% agarose gel. The DNA was then transferred to nitrocellulose by the method of Southern (1975), and hybridised to 100ng of the 500bp PstI fragment from the v-erb-A gene of avian erythroblastosis virus, that had been radiolabelled by nick translation. Prehybridisation and hybridisation was in 50% formamide, 6*SSC, 5* Denhardt's, 100µg/ml E.coli tRNA, 4mM sodium phosphate (pH6.5), 0.1% SDS at 37°C. After hybridisation the filter was washed in 1*SSC at 42°C and exposed to X-ray film for 12 days. Markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII.

Figure 24



Results and Discussion

CHAPTER 11

Isolation Of c-erb-A cDNA Clones From A *X.laevis* - Mature Oocyte cDNA Library.

Introduction.

A *X.laevis* mature oocyte cDNA library (in the bacteriophage vector lambda ZAP) was ^{kindly provided by J. Shuttleworth and A. Colman and} screened with the v-erb-A probe. This library had been prepared from polyA⁺ RNA that had been extracted from *X.laevis* oocytes that were matured in vitro with progesterone. The layer of follicle cells which surrounds each oocyte had not been removed. The cDNA had been synthesised, and the library constructed, almost exactly as described in section 3.30. After packaging, the bacteriophage had been plated and amplified in BB4. The library contains 4×10^5 independent clones (insert size 1 to 2.5Kb, 0.25% blue plaques), and we received an aliquot of the amplified library with a titre of 0.6 to 1×10^{10} pfu/ml.

Results.

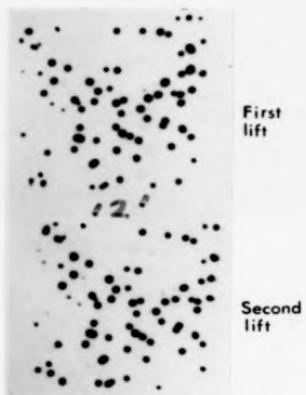
A total of 2×10^6 bacteriophage were screened with the nick translated PstI fragment of v-erb-A exactly as described in Materials and Methods (see the legend to figure 25 for hybridisation and washing conditions). After only a very short exposure (16 hours) a total of 13 positives were identified. All 13 positives were picked and re-screened with the v-erb-A probe. 11 out of the 13

Figure 25.

An example of the fourth round screen of one positive cDNA clone from the X.laevis oocyte cDNA library with the v-erb-A probe.

Bacteriophage eluted from a single plaque that hybridised strongly to the 500bp PstI fragment from the v-erb-A gene on the 3rd round screen, were plated on BB4 at low density. Phage were transferred in replica to nitrocellulose filters as described in chapter 3.34. The filters were then prehybridised in 50% formamide, 6*SSC, 5* Denhardt's, 100µg/ml E.coli tRNA, 4mM sodium phosphate (pH6.5), 0.1% SDS at 37°C. Hybridisation was in the same buffer containing approximately 10ng/ml of the 500bp PstI fragment from the v-erb-A gene that had been radiolabelled by nick translation. After hybridisation, the filters were washed in 1*SSC at 42°C and exposed to X-ray film for 16 hours. In the resulting autoradiograph the two filters are above one another in the same orientation with respect to the plate from which the lifts were made.

Figure 25



Results and Discussion

positives turned out to be true positives. This re-screening procedure was repeated twice more after which virtually every plaque hybridised to the probe. An example of this final 4th round screen is shown in figure 25.

Results and Discussion

CHAPTER 12

Automatic Excision Of The Eleven Prospective c-erb-A Clones And Initial Restriction Enzyme Analysis.

Results.

The 11 prospective c-erb-A clones were converted to plasmid form using the automatic excision protocol described in Materials and Methods. For each λ ZAP clone, 4 single colonies were picked and used to prepare miniprep DNA which was then restricted with EcoRI and analysed on agarose gels. There is some evidence that gross rearrangements of the insert DNA can sometimes occur during the excision process (Dr. J. Shuttleworth, pers. comm.), and this is why 4 clones were picked from each excision. However, in all cases the 4 clones picked gave identically sized fragments upon digestion with EcoRI, indicating that rearrangement was not a problem with these particular clones. Ten of the eleven positives all gave rise to two EcoRI insert fragments of 1.7Kb and 1.4Kb. These ten clones were assumed to be identical isolates of a single independent clone that had been over represented when the library was amplified. This clone was designated overb1. The remaining positive generated a single EcoRI fragment of 2.3Kb and this clone was designated overb12.

Results and Discussion

CHAPTER 13

Detailed Restriction Enzyme Analysis Of Overb1 And Overb12.

Introduction.

I had decided to sequence these clones by sub-cloning restriction enzyme fragments into M13 vectors. Before I began this I needed comprehensive restriction maps of both clones.

13.1 Restriction enzyme mapping of overb1 by partial digestion of end-labelled DNA.

Results.

Initial restriction enzyme digests of overb1 indicated that the insert contained one EcoRI site, 3 HindIII sites, 2 PstI sites, 4 HincII sites and no sites for BamHI, BglIII, SacI, SmaI, XbaI, XhoI, and NotI. I decided to map the sites for EcoRI, HindIII, HincII and PstI within overb1 by doing partial digests of the insert DNA labelled at one end. I therefore needed to excise the whole insert using two polylinker enzymes such that one end could be preferentially labelled. Four candidate enzymes for this were XhoI, XbaI, BamHI and NotI. In the initial digests sites for these enzymes which lay close to the polylinker site would not have been detected. To be certain that there really were no sites for these enzymes within the insert, overb1 was digested with these enzymes and the

Figure 26.

Restriction enzyme mapping of overb1 by partial digestion.

Overb1 DNA was digested with XhoI and then with BamHI and the products separated on a LMP agarose gel. The 3.4Kb fragment, that contains the complete cDNA insert, was isolated and end-labelled with the klenow fragment of DNA polymerase I in the presence of α [³²P] dGTP and no cold nucleotides. Unincorporated label was removed on a sephadex G50 column, and the end-labelled DNA recovered by ethanol precipitation. Aliquots of approximately 1200cpm of this end-labelled insert DNA were mixed with 0.8 μ g of lambda ci857 DNA and digested with the appropriate number of units (u) of various restriction enzymes, as indicated above each lane. After 15 minutes at 37°C the reactions were terminated by adding EDTA (pH8) to a final concentration of 20mM, and then fractionated on a 1.7% agarose gel. Labelled DNA fragments were detected by drying the gel and exposing it to X-ray film for 18 hours. Markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII, or pBR322 with HpaII (lane 14).

Figure 26



Results and Discussion

ends labelled by end-filling with the Klenow fragment of DNA polymerase I in the presence of α [³²P] dGTP and the appropriate cold nucleotides. After precipitating with ethanol to remove the majority of the unincorporated label, the products were separated on a 1% agarose gel which was subsequently dried down and autoradiographed. In each case a labelled vector (+insert) band was visible, but no other bands were present indicating that these enzymes really do not cut in the insert.

For partial digest mapping, overb1 DNA was digested with XhoI and then with BamHI, and the insert fragment was isolated and end-labelled with the klenow fragment of DNA polymerase I. Under the conditions used only the BamHI end was labelled. The number of units of each restriction enzyme required to get partial digestion was determined using 0.8 μ g of un-labelled lambda CI857 DNA. Partial digests of the end-labelled insert of overb1 were then carried out as detailed in the legend to figure 26. Figure 26 shows the results of two such partial digest mapping experiments. This data, together with further double and triple restriction digests, was used to construct the restriction map of overb1 shown in figure 27. It should be noted that the partial digest mapping of overb1 indicated the presence of six HincII sites. However, subsequent restriction mapping showed that there was only a single HincII site in the region between the 5' end of the insert and the 5' most HindIII site.

Figure 27.

Restriction enzyme map of overb1 showing the M13 sub-clones and extent of sequencing.

The data from the partial restriction enzyme digests of overb1 shown in figure 26, together with double and triple digests of un-labelled overb1 DNA was used to construct the restriction map shown in this figure. It should be noted that the partial digests indicated the presence of six HincII sites. However, subsequent restriction enzyme mapping with un-labelled DNA (data not shown) showed that there could only be a single HincII site in the region between the 5' end of the insert and the 5' most HindIII site. Also shown below the restriction map are the locations and identities of the 7 M13mp18 sub-clones (ov1.1 to ov1.8) derived from overb1. Each clone was constructed by isolating the appropriate restriction enzyme fragment from a LMP agarose gel and then ligating it into the appropriate M13mp18 vector. The arrows above each sub-clone indicate the direction of sequencing from the universal M13 primer. The numbers above each arrow refer to the total length of sequence (in nucleotides) obtained from that particular sub-clone. Where it is not clear exactly where a sub-clone begins or terminates the restriction site has been indicated. Abbreviations: Hc, HincII; Hd, HindIII; Pt, PstI; Kp, KpnI.

Results and Discussion

13.2 Restriction enzyme mapping of overb12 indicates that it is closely related to overb1.

Results.

Overb12 was mapped by single and double digests, i.e. not by partial digestion. This gave rise to the map shown in figure 28, in which the maps of overb12 and overb1 have been aligned according to common restriction enzyme sites. Quite clearly the two clones are related. The restriction maps are identical (within acceptable margins of error) within a region spanning the right-most PstI site to the left-most HincII site of overb12. To the left hand side of this HincII site in overb12, overb1 extends for an extra 1.8Kb not present in overb12. There are very few sites for the common restriction enzymes in this region of overb1.

13.3 Localisation of the ligand binding domain within overb1 and overb12.

Results.

Overb12 and overb1 were digested with various restriction enzymes, as indicated in figure 29, and the products separated on agarose gels. The DNA fragments were transferred to nitrocellulose using the procedure of Southern (1975). After baking, the filters were probed with the nick translated PstI fragment of v-erb-A, which by analogy to the glucocorticoid receptor, corresponds to the N-terminal 131 amino acids of the ligand-binding domain and the C-terminal 34 amino acids of the spacer

Figure 28.

Alignment of the restriction maps of overb1 and overb12 and a summary of the v-erb-A hybridisation data.

The restriction enzyme map of overb12 was elucidated by single, double, and triple digests of unlabelled DNA (data not shown). Comparison with the restriction map of overb1 indicated that the the two clones had a number of sites in common, and could be aligned as shown in this figure. Below each restriction map is a summary of the v-erb-A hybridisation data from figure 29. For each restriction enzyme digest the DNA fragments produced have been represented by double headed arrows. Those fragments which hybridised strongly to the PstI fragment from the v-erb-A gene are represented by the thick lines with large arrow heads. Fragments which hybridised only weakly to this probe are represented by the thin lines with small arrow heads, whilst fragments which did not hybridise to this probe are represented by the dashed lines with small arrow heads.

Figure 28 Alignment of the restriction maps of overb 1 and overb 12 and a summary of the v-erb-A hybridisation data.

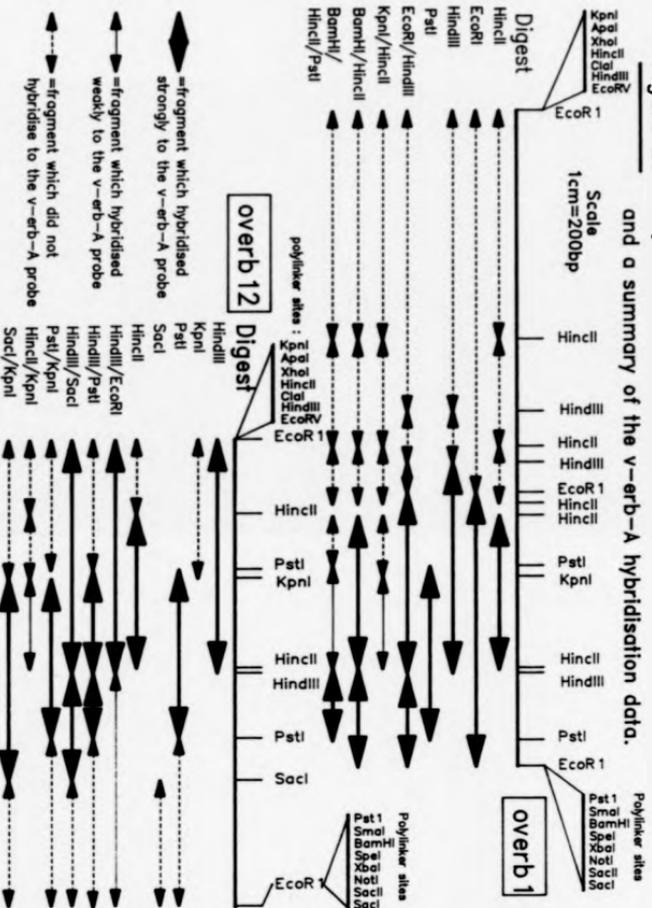
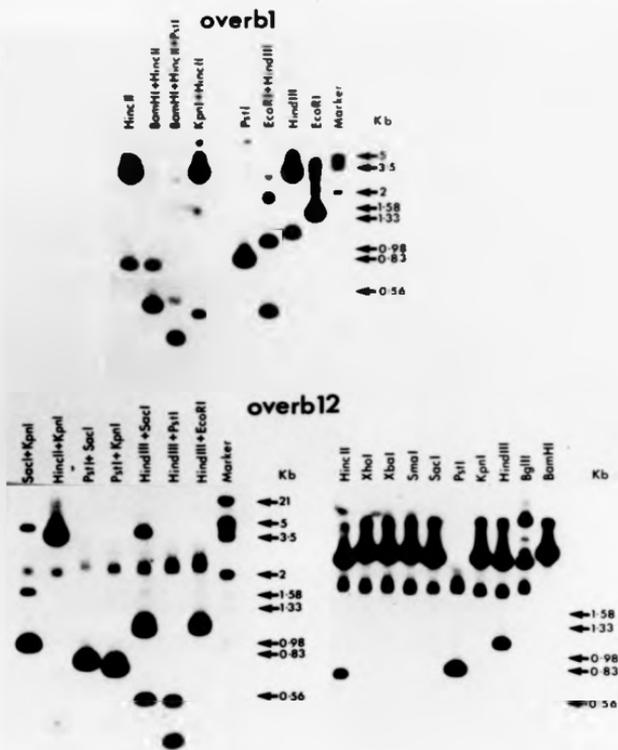


Figure 29.

Southern blot analysis of overb1 and overb12 with the 500bp PstI fragment from the v-erb-A gene.

Overb1 or overb12 DNA was digested to completion with various restriction enzymes as indicated above each lane. The products were fractionated on agarose gels and transferred to nitrocellulose using the procedure of Southern (1975). After baking, the filters were hybridised to the PstI fragment from the v-erb-A gene that had been radiolabelled by nick translation. Hybridisation and washing conditions were as described in the legend to figure 25, and five hour exposures of the filters are presented. Markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII. The digest of overb12 DNA with HincII and KpnI gave rise to a partial product of about 0.7Kb that also hybridised weakly to the v-erb-A probe.

Figure 29



Results and Discussion

region (which separates the DNA and ligand-binding domains). Final wash conditions were 2*SSC at 42°C, and the results are presented in figure 29. Clearly both clones hybridised strongly to the probe. The data has been summarised in figure 28, which shows those fragments which hybridised to the probe and those which did not. This helped to confirm that the two clones are closely related and can be aligned as shown in figure 28. From this data it can be concluded that the ligand binding domain is located to the right of the unique KpnI site in overb1 and overb12 as the maps are drawn in figures 27 and 28.

Results and Discussion

CHAPTER 14

Partial Sequencing Of Overb1 Indicates That This Clone Does Not Contain The Complete Coding Region, But Overb12 probably Does.

Results.

Various restriction enzyme fragments from overb1 were isolated from LMP agarose gels and sub-cloned in the appropriate M13mp18 vectors. A total of 7 sub-clones, designated ov1.1 to ov1.8, were constructed and these are shown in figure 27. All seven clones were sequenced by standard M13 dideoxy sequencing, and the amount of sequence data obtained is indicated in figure 27. This sequence was translated in all three reading frames and in both orientations (using the "Microgenis" sequence analysis program), and compared to the predicted protein sequence of the chicken thyroid hormone receptor (Sap et al., 1986). From this it soon became clear that the predicted protein sequence of overb1 showed considerable homology to the chicken thyroid hormone receptor. By aligning the chicken sequence with the partial sequence of overb1 I was able to identify the probable translation start site within overb1 (data not shown). The translation start site appeared to be about 80 nucleotides downstream (to the right) of the HincII site that lies at one end of the sub-clone ov1.3 (see figure 27). By comparison to the chicken thyroid hormone receptor I was able to determine the orientation of the clone with respect to the 5' and 3' end of the mRNA

Results and Discussion

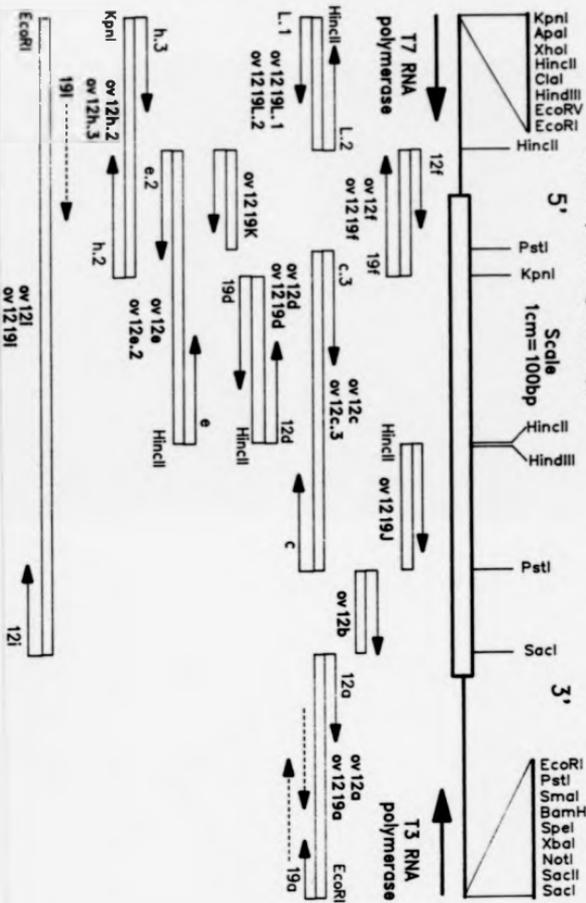
and this is also indicated in figure 27. From the sequence of clone ovl.1 it was predicted (by comparison to the chicken thyroid hormone receptor) that overb1 did not contain all the coding region of the Xenopus ovary c-erb-A mRNA. The clone terminates some 40 to 50 amino acids short of the predicted 3' end of the coding region. If it is assumed that the Xenopus ovary thyroid hormone receptor is not significantly longer than the chicken receptor at its carboxy terminus then the other clone, overb12, should contain the complete coding region.

Figure 30.

The restriction enzyme map of overb12 showing the M13 sub-clones and the sequencing strategy.

The restriction enzyme map shown in this figure was derived directly from the complete nucleotide sequence of the overb12 cDNA. The open box indicates the position of the long open reading frame. Also shown by the arrows are the directions of RNA synthesis from the T7 and T3 RNA polymerase promoters that lie on either side of the pBluescript polylinker. Shown beneath the restriction map are the 19 M13 sub-clones that were derived from overb12 by cloning gel isolated restriction fragments into the appropriate M13 RF vector. In cases where it is unclear where a particular sub-clone begins or ends, the restriction site has been shown at the end of the clone. Arrows above and below each sub-clone indicate the direction and extent of sequencing from the M13 universal primer. Where there are two arrows, the particular restriction fragment was either cloned into both M13mp18 and M13mp19, or into M13mp18 in both orientations, thereby allowing sequencing from both ends of the restriction fragment. Where the name of the sub-clone contains "19" it indicates that the vector is M13mp19, otherwise it is M13mp18. Some extended M13 dideoxy sequencing was carried out on the sub-clones ovi2191, ovi2a, and ovi219a. The extent of this extended sequencing is indicated by the dotted arrows.

Figure 30 Restriction enzyme map of overb12 showing the M13 sub-clones and the sequencing strategy.



Results and Discussion

CHAPTER 15

Complete Sequencing Of Overb12 Shows That This cDNA
Clone Contains A Complete Long Open Reading Frame
Encoding A Thyroid Hormone Receptor.

Results.

Various restriction enzyme fragments of overb12 were isolated from LMP agarose gels and subcloned into the appropriate M13 vector (mp18 or mp19). A total of 19 sub-clones (11 different restriction fragments, some in both orientations) were generated and these are shown in figure 30. The identities of all sub-clones was checked by restriction digests of miniprep DNA. These sub-clones were sequenced using M13 dideoxy sequencing as indicated in figure 30. In order to obtain the complete sequence of the 3' untranslated region, and to confirm that there was only a single HincII site in the 5' non-coding region (overb1 has two) some extended M13 dideoxy sequencing was undertaken. This is indicated by the dashed arrows in figure 30. All restriction sites except for the central HincII site and the SacI site, were sequenced across to check that there were no extra sites that may have been missed in the restriction mapping. For approximately 50% of overb12, only one strand was sequenced because of the limited time available.

Where sequence data from different sub-clones overlapped, the sequences were compared using the "Microgenie" DNA sequence analysis package and any

Figure 31.

The complete nucleotide sequence of overb12 and the predicted amino acid sequence of the encoded protein (XenTR α).

Sequence data from the various M13 sub-clones shown in figure 30 was combined to generate the complete nucleotide sequence of overb12. Examination of this sequence showed that it contained a long open reading frame encoding a protein of 418 amino acids. In addition to the ATG codon at nucleotide position 460 which is taken to be the translation start site, there are four other ATG codons upstream. The predicted protein sequence has been compared to that of the chicken thyroid hormone receptor (Sap et al., 1986) and the amino acids which differ have been underlined. The Xenopus protein has ten amino acids in the amino terminal domain that are absent from the chicken thyroid hormone receptor, and these have been boxed. Also indicated on the sequence are the locations of the DNA-binding and ligand-binding domains which were determined by homology to other steroid and thyroid hormone receptors. The ligand-binding domain extends from the arrow to the c-terminus of the protein.

Results and Discussion

differences noted. The original sequencing gels were then re-examined and alterations made as appropriate, or the sub-clones(s) re-sequenced if necessary. The DNA sequences of the different sub-clones were then merged using the "Microgenie" to generate the complete DNA sequence of overb12 which is given in figure 31. This cDNA clone contains a long open reading frame which encodes a protein of 418 amino acids with high sequence identity to thyroid hormone receptors isolated from rat, human and chicken. The predicted protein sequence of this open reading frame is also shown below the DNA sequence in figure 31. A more detailed discussion of the primary sequence of this protein in respect to thyroid hormone receptors from other systems can be found in chapter 17.

Results and Discussion

CHAPTER 16

Overb1 Contains Two Un-related cDNA Inserts, One Of Which Is Probably A Shorter Version Of Overb12.

Introduction.

In the rat, the primary transcripts from both thyroid hormone receptor genes are known to undergo differential splicing to generate a total of at least 6 mature mRNAs (see chapter 1.4.10, figure 8, and references therein). These transcripts encode six related thyroid hormone receptors which differ in either their amino or carboxyl termini. All these isoforms contain a DNA binding domain but some are unable to bind hormone. I have isolated two related thyroid hormone receptor cDNAs so this raised the possibility that they could be the products of differential splicing.

Discussion.

The partial sequence of overb1 was compared to the complete sequence of overb12 using the "Microgenie" DNA sequence analysis program. Downstream (to the right) of the HincII site which marks the start of sub-clone ov1.3 (see figure 27), the available sequence of overb1 is identical to the corresponding region of overb12 except for a few isolated differences which are almost certainly due to sequencing errors. The sequence of ov1.4 from a position 2 nucleotides downstream of the EcoRI site to the end of the sequence matched exactly the corresponding

Results and Discussion

sequence of overb12. However, the remaining 97 nucleotides of ovl.4 upstream of and including this EcoRI site did not resemble the corresponding region of overb12. Thus the sequences of overb12 and overb1 diverge precisely at the internal EcoRI site in overb1. This site lies some 200bp upstream of the translation start site in overb12 and overb1. The sequence of overb1 upstream of this EcoRI site did not match any of the sequences in the Genbank data base. These findings are consistent with the proposal that overb1 contains a double insert. During the construction of the library two EcoRI linker cDNA molecules must have annealed together and ligated into the same λ ZAP vector molecule. One of these cDNAs was a thyroid hormone receptor cDNA whilst the other was completely un-related.

Results and Discussion

CHAPTER 17

A Comparison Between The Sequence Of The Thyroid Hormone Receptor Encoded By Overb12 And Other Cloned Thyroid Hormone Receptors.

The long open reading frame (ORF) within overb12 encodes a protein of 418 amino acids. Within this open reading frame the DNA sequence shows 80% amino acid similarity to the protein coding region of the chicken thyroid hormone receptor (TR) cDNA isolated by Sap et al (1986). Note that in this comparison the region encoding the block of "extra" ten amino acids that are absent from the chicken TR (see below) were not included. At the protein level the homology is 92% (also excluding the block of ten amino acids not present in the chicken TR). Clearly overb12 does encode a thyroid hormone receptor and from now on it may be referred to as the Xenopus thyroid hormone receptor. A comparison between the predicted protein sequences of the Xenopus and chicken thyroid hormone receptors is shown in figure 31. It is interesting to note that that the Xenopus protein has an extra 10 amino acids not present in the chicken receptor (these amino acids have been boxed in figure 31). Also shown in figure 31 are the putative locations of the DNA binding domain and the hormone binding domain. These have been located by homology to the glucocorticoid receptor and other thyroid hormone receptors. The DNA binding domain is most well conserved

Figure 32.

The Xenopus oocyte thyroid hormone receptor most closely resembles the rat thyroid hormone receptor type alpha 1.

Comparisons between the predicted amino acid sequences of the Xenopus oocyte thyroid hormone receptor (XenTR α) and the rat thyroid hormone receptors (see figure 8) indicated that XenTR α most closely resembled the rat TR α 1 protein (Thompson et al, 1987). A schematic representation of these two proteins is shown. The open boxes represent the amino terminal (A/B) domains, the dot filled boxes represent the DNA binding domains, the black boxes represent the "spacer" regions, and the hatched boxes (both sizes of hatching) represent the hormone binding domains. The black boxes in the amino terminal domains indicate the locations of amino acids that are present in one protein but absent from the other. Also shown are the percentage amino acid homologies for each domain of the receptors.

Figure 32 The *Xenopus* Oocyte Thyroid Hormone Receptor Most Closely Resembles The Rat TR $\alpha 1$ Type.

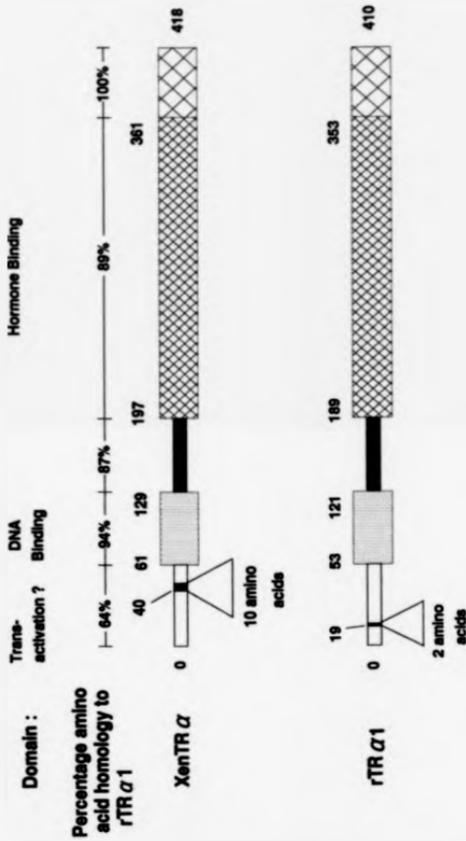


Figure 33.

The predicted amino acid sequence of XenTR α and comparison to rat TR α 1.

The predicted amino acid sequence of XenTR α was compared to that of the rat thyroid hormone receptor type alpha 1 (Thompson et al., 1987). Amino acids which differ from the rat TR alpha 1 protein are shown in red. Also shown boxed in yellow is a block of 10 amino acids that are absent from the rat TR alpha 1 protein. The DNA-binding domain has been boxed in blue and the ligand-binding domain in red. The single letter amino acid code has been used; A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

Figure 33 Predicted Amino Acid Sequence Of XenTR α
And Comparison To Rat TR α 1

M D Q N L S G L D C L S E P D E K R W P D G G K R K R K N S Q
C M G K S G M S G D S L V S L P S A G Y I P S H L D K D E P
C V V C S D K A T G Y H Y R C I T C E G C K G F F R R T I Q
K N L H P S Y S C K Y D G C C I D K I T R N Q C Q L C R F
K K C I A V G M A M D L V L D D S K R V A K R K L I E E N R
Q R R R K E E M I K T L Q Q R P E P S E E W E L I R I V T
E A H R S T N A Q G S H W K Q R R K F L P E D I G Q S P M A
S M P D G D K V D L E A F S E F T K I I T P A I T R V V D F
A K K L P M F S E L T C E D Q I I L L K G C C M E I M S L R
A A V R Y D P D S E T L T L S G E M A V K R E Q L K N G G L
G V V S D A I F D L G R S L A A F N L D D T E V A L L Q A V
L L M S S D R T G L I C T D K I E K C Q E T Y L L A F E H Y
I N H R K H N I P H F T P K L L M K V T D L R M I G A C H A
S R F L H M K V E C P T E L F P P L F L E V F E D Q E V

Amino acids shown in red differ from predicted sequence of the rat TR alpha 1.

=Extra ten amino acids not present in the rat TR alpha 1 protein.

=DNA binding domain.

=Ligand binding domain

Results and Discussion

between the two receptors, whilst the lowest level of homology lies in the amino terminal domain, which has been implicated in trans-activation of transcription.

The sequence of the Xenopus thyroid hormone receptor is also highly homologous to that of the human thyroid hormone receptor (data not shown).

As discussed in the introduction, the rat has at least six thyroid hormone receptor isoforms that are produced from two genes by alternate splicing (see figure 8). Only three of these isoforms actually bind hormone. When the predicted protein sequence of the Xenopus thyroid hormone receptor is compared to these rat isoforms it is clear that it most closely resembles the alpha 1 type, which is the only alpha type capable of binding hormone. All the other alpha types are considerably longer than the Xenopus receptor at the carboxy terminus. The beta types on the other hand are considerably longer than the Xenopus receptor at the amino terminus. Figure 32 shows schematic representations of the Xenopus receptor and the rat thyroid hormone receptor type alpha 1 (rTR α 1), and a direct amino acid sequence comparison is shown in figure 33. Clearly the two proteins are very closely related and I have therefore designated the Xenopus ovary thyroid hormone receptor an alpha type (XenTR α). The DNA binding domains of rat TR α 1 and XenTR α are 94% identical at the amino acid level. The amino terminal domains show only 64% identity, including a block of 10 "extra" amino acids in

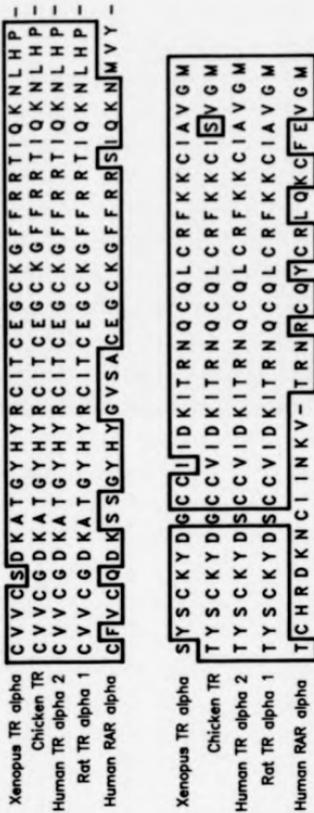
Figure 34.

Sequence comparison between the DNA-binding domains of the Xenopus thyroid hormone receptor and other members of the thyroid hormone/retinoic acid receptor family.

As discussed in chapter one, the thyroid hormone and retinoic acid receptors are closely related, and indeed can recognise the same response elements in DNA. It is therefore not surprising that their DNA-binding domains show significant sequence similarities. The predicted amino acid sequences of XenTR alpha, the chicken TR (Sap et al, 1986), the human TR alpha 2 (Nakai et al, 1988), the rat TR alpha 1 (Thompson et al, 1987) and the human retinoic acid receptor alpha (see Benbrook and Pfahl, 1988) have been aligned in this figure. Common amino acid residues have been boxed. The single letter amino acid code is used; A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

Figure 34

Sequence comparison between the DNA binding domains of the *Xenopus* thyroid hormone receptor and other members of the thyroid hormone/retinoic acid receptor family.



References to sequence data:

- Chicken thyroid hormone receptor: Sap et al (1986).
- Human thyroid hormone receptor type alpha 2: Nakai et al (1988)
- Rat thyroid hormone receptor type alpha 1: Thompson et al (1987).
- Human retinoic acid receptor type alpha: see Benbrook and Pfahl (1988).

Results and Discussion

the Xenopus protein that are absent from the rat TR α 1 protein. These are the same 10 amino acids that are "missing" from the chicken thyroid hormone receptor (see figure 31). In addition, the rat protein has 2 amino acids that are absent from the amino terminal domain of the Xenopus protein. Together this accounts for the fact that the Xenopus protein is 8 amino acids longer than rTR α 1. The hormone binding domain of XenTR α can be split into two sections on the basis of homology to rTR α 1. The first 164 amino acids are 89% identical in the two proteins, whilst the C-terminal 57 amino acids are 100% identical (figure 33). The spacer region that separates the DNA and hormone binding domains also shows a high degree of amino acid identity (87%).

On the basis of overall sequence homology the steroid hormone receptor superfamily has been divided into sub-groups (see Green and Chambon, 1988 and figure 3). The estrogen receptor, vitamin D3 receptor, retinoic acid receptors and thyroid hormone receptors form one of these sub-families. Figure 34 shows a comparison between the amino acid sequences of the DNA binding domains of some members of this sub-family. The DNA binding domain has been very highly conserved among all the members of the thyroid hormone receptor family with two large blocks of amino acids being almost absolutely conserved. In fact the DNA binding region of the Xenopus thyroid hormone receptor is the least conserved among the thyroid hormone receptors

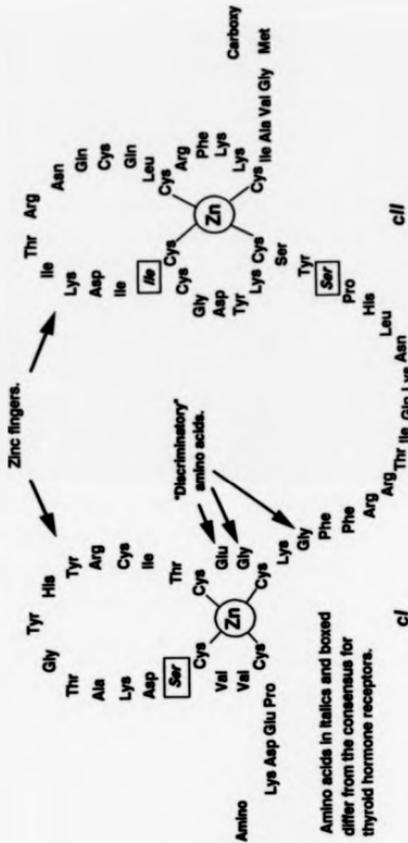
Figure 35.

The hypothetical structure of the Xenopus thyroid hormone receptor's DNA-binding region.

As discussed in chapter 1.4.4, the DNA-binding domains of steroid and thyroid hormone receptors are believed to form two finger-like structures, each coordinated by a zinc ion. The hypothetical structure of the DNA-binding domain of XentR α has been drawn by analogy with the zinc fingers of other steroid hormone receptors. Amino acids which differ from the consensus for thyroid hormone receptors (see figure 34) are drawn in italics and boxed. Also shown are the locations of the "discriminatory" amino acids whose identities (by analogy with the ER and GR) are probably important for determining target gene specificity (see chapter 1.4.13).

Figure 35

Hypothetical structure of the *Xenopus* thyroid hormone receptor's DNA binding region.



Results and Discussion

shown here. The retinoic acid receptor is less well conserved but still shows significant homology.

It has been proposed that the DNA binding domain forms a zinc finger like structure (Evans, 1988; Evans and Hollenberg, 1988; Freedman et al., 1988). Two zinc fingers would be formed by the tetrahedral coordination of two zinc molecules. The hypothetical zinc finger structure of the DNA binding domain of XenTR^α is shown schematically in figure 35. Both fingers are formed by the coordination of a zinc atom by four cysteine residues. It has been proposed that the fingers lie between the major groove of the DNA double helix (Miller et al., 1985). Specific amino acids then make contact with specific bases in the core of the helix thus providing the sequence specificity of the interaction. As expected, the three "discriminatory" amino acids identified as being important for determining target gene specificity (Mader et al., 1989; Danielsen et al., 1989; Umesono and Evans, 1989) are the same in the Xenopus thyroid hormone receptor and thyroid hormone receptors isolated from other species.

Results and Discussion

CHAPTER 18

The Xenopus Thyroid Hormone Receptor cDNA Translates Very Inefficiently In Vitro.

Results.

To confirm the presence of a long open reading frame within the XenTR α cDNA it was decided to attempt to translate the cDNA in vitro. To do this the complete cDNA was transcribed in vitro from either the T7 or T3 RNA polymerase promoters that flank the polylinker in pBluescript SK⁻ (see the legend to figure 36 for details). Aliquots of the RNA were then translated in wheat germ extract in the presence of [³⁵S] methionine. The products were electrophoresed on a denaturing SDS polyacrylamide gel which was subsequently dried down and autoradiographed. Figure 36 shows a 10 day exposure of one such gel. Clearly the sense transcript but not the antisense transcript has given rise to a number of proteins, the largest of which is about 49Kd in size. The sequence of XenTR α predicted that this cDNA encoded a protein of 418 amino acids. Given that the average molecular weight of an amino acid residue is 120, then this protein would be expected to have a molecular weight of 50Kd. This corresponds well to the size of the largest protein produced by translation of sense overb12 RNA in vitro. The smaller proteins, below the largest band, must

Figure 36.

Translation of synthetic RNA prepared from overb12 in wheat germ extract.

To synthesise sense RNA, overb12 DNA was first linearised with BamHI and then transcribed in vitro with T7 RNA polymerase (see figure 30). Antisense RNA was synthesised by transcribing XhoI linearised overb12 DNA in vitro with T3 RNA polymerase. After the transcription reaction the RNA was recovered by ethanol precipitation and resuspended in water. Approximately 0.5 μ g of synthetic RNA and 0.5 μ g of natural β -globin mRNA, and a mixture of the two were then translated in wheat germ extract (chapter 3.36.2). As an additional control, one translation reaction was carried out without added RNA. After translation, an equal volume of sample buffer was added to each reaction, and the samples heated at 100°C for 3 minutes. The samples were then fractionated on a 18% polyacrylamide/SDS gel which was subsequently dried down, and labelled proteins were detected by autoradiography for ten days. The markers were a commercial mixture of ³⁵S labelled proteins of known molecular weight.

Figure 36

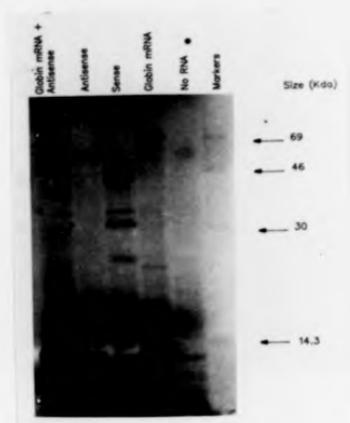


Figure 37

There is a correlation between the sizes of the truncated proteins produced by translation of XenTR alpha *in vitro* and the positions of internal methionine codons.

Positions of internal methionines.	Distance from internal methionine to the end of the XenTR alpha protein.	Predicted size (Kda) of the resultant protein (*).	Size of truncated proteins produced <i>in vitro</i> (Kda). Taken from figure 36.
(1)	(418)	(50)	(49)
32	386	46.3 } —————	46
37	381	45.7 } —————	
128	290	34.8 } —————	35
130	288	34.5 } —————	
158	260	31.2 }	32
209	209	25.1 } —————	23
212	206	24.7 } —————	
246	172	20.6 }	20
267	151	18.1 }	18
288	130	15.6 }	15
333	85	10.2 }	
377	41	4.9 }	
384	34	4.1 }	
396	22	2.6 }	

* Assuming that the average molecular weight of an amino acid is 120

Results and Discussion

be truncated versions of the full length XenTR α protein. The origin of these forms remains uncertain. They may have resulted from the action of proteases present in the wheat germ extract. Another more intriguing possibility is that they resulted from initiation by ribosomes at internal methionines within the long ORF (this is discussed in more detail later). Synthetic RNA prepared from the chicken thyroid hormone receptor cDNA gives rise to seven proteins when translated in vitro (Bigler and Eisenman, 1988). Antibody and kinetic studies suggested that these multiple proteins were produced by initiations at sites within the RNA, generating a set of nested proteins (Bigler and Eisenman, 1988). These workers also reported that a subset of the truncated proteins seen in vitro also exist in vivo, in chicken red blood cells. Their data is consistent with, but not proof of initiation at internal methionine codons in vivo.

Figure 37 demonstrates that the positions of internal methionines within XenTR α are also consistent with the idea that the truncated proteins seen in figure 36 are the result of internal initiations.

The wheat germ extract programmed with natural beta-globin mRNA has given rise to an intense band of approximately 12Kda in size, which represents beta-globin protein. By comparison to this, overb12 sense RNA has clearly been translated very inefficiently in the wheat germ extract. This basic experiment was repeated a number

Results and Discussion

of times using different extracts and new batches of synthetic RNA in an attempt to get better translation, but without success. The RNA was also translated very poorly, if at all in rabbit reticulocyte lysate (for example see figure 39). Similar results regarding the inefficient translation of synthetic RNA prepared from thyroid hormone receptor cDNAs in vitro have been reported for the chicken receptor (Sap et al, 1986), the rat TR α 1 (Murray et al, 1988) and the human ear-7 thyroid hormone receptor (Benbrook and Pfahl, 1987). In all these cases (and in fact in all the thyroid hormone receptor cDNA clones for which sequence is available) there are several ATG codons upstream of the long open reading frame. In some cases these ATG codons mark the start of short ORFs (terminated by in frame stop codons). In order to obtain efficient translation of RNA prepared from these cDNA clones in vitro, these workers removed part or all of the 5' untranslated region, thus removing some or all of these ATG codons. For a number of other thyroid hormone receptor cDNA clones (Rat TR α 1 and Rat TR α 2; Izumo and Mahdavi, 1988; Rat TR β 1; Koenig et al, 1988), the 5' untranslated region was removed before translation but it was not stated that this was done to increase translation. In contrast to these findings, Thompson et al (1987) reported that removal of two of the three upstream ORFs from the rat TR α 1 cDNA did not increase the efficiency of translation. This is in direct contrast to the results of

Figure 38

The DNA sequence of the XenTR α cDNA clone upstream of the long open reading frame showing the ATG codons and the conserved upstream open reading frame.

The nucleotide sequence of XenTR α (overb12) from nucleotide 330 to 468 is shown, and all the ATG and TGA codons have been italicized and underlined. The long open reading frame which encodes the 418 amino acid XenTR α protein begins at the ATG codon at position 460. The short upstream open reading frame that directly precedes the long open reading frame is conserved between a number of thyroid hormone receptor cDNA clones from different species as illustrated. It should be noted that the rat TR β (Murray et al. 1988), the human TR (Weinberger et al., 1986), and the rat TR β 1 (Koenig et al., 1988) do not contain this conserved upstream open reading frame. The relevant sequence data for the other four thyroid hormone receptor cDNA clones that have been isolated is not available.

Results and Discussion

Murray et al (1988) who independently isolated an apparently identical rat TR α 1 cDNA, and found that it was necessary to remove these same two upstream ORFs in order to get efficient translation in vitro.

The Xenopus thyroid hormone receptor cDNA clone also has a number of upstream ATG codons (see figure 38). There are in fact 4 in total and all of them are in frame with the long ORF. One of these upstream ATG codons is followed immediately by a stop codon, whilst the other three mark the beginning of short ORFs. Two of these ORFs are very short, containing just 4 codons. The other one would encode a protein of 23 amino acids, terminating immediately before the start of the long ORF. Thus, the 5' untranslated region of the Xenopus thyroid hormone receptor cDNA resembles those of thyroid hormone receptor cDNAs from other systems in this respect. However, the actual DNA sequence of these 5' untranslated regions show no significant homology, except for a stretch of 15 nucleotides immediately prior to the start of the long ORF (see later discussion).

Results and Discussion

CHAPTER 19.

The Xenopus Thyroid Hormone Receptor cDNA is Efficiently Translated In Vitro When All The 5' Untranslated Sequence Has Been Removed.

Results.

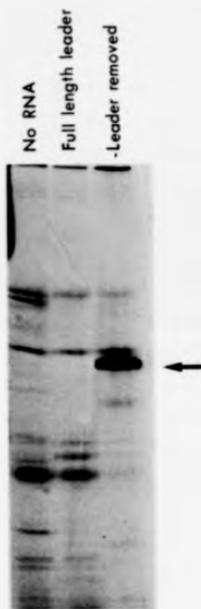
In an attempt to increase the translational efficiency of synthetic RNA prepared from the XenT_{RO} cDNA, all of the upstream ATG codons were removed. This work was carried out in collaboration with G. Sweeney. The EcoRI insert of overb12 was sub-cloned into M13mp18 and then site directed mutagenesis was used to create an EcoRI site 9 nucleotides upstream of the start of the long ORF (see figure 38). This plasmid was then digested with EcoRI to release a fragment containing the complete long ORF, all the 3' untranslated region but only 9 base pairs of the 5' untranslated region, thereby removing all the upstream ATG codons. This fragment was sub-cloned into pBluescript KS and the resulting plasmid designated pBlueXenT_{RM}HE. This plasmid was used to synthesise both sense and antisense RNA which was translated in rabbit reticulocyte lysate and the resulting labelled proteins analysed on a denaturing polyacrylamide gel. A 4 day exposure of the gel is presented in figure 39. The RNA containing the complete 5' leader did not give rise to detectable amounts of protein, whereas the RNA from which the leader had been removed gave rise to a single strong band which represents the

Figure 39.

Removal of the 5' untranslated leader significantly increases translation of XenTR α in vitro.

Synthetic RNA containing the full length 5' untranslated leader was prepared by transcribing BamHI linearised overb12 DNA in vitro with T7 RNA polymerase. To remove the 5' untranslated leader, including all four upstream ATG codons, the EcoRI insert of overb12 was cloned into M13mp18 and site directed mutagenesis was used to create an EcoRI site centred at 9 nucleotides upstream of the start of the long open reading frame (see figure 38). Digestion of the resulting RF DNA with EcoRI liberated a fragment containing the complete long open reading frame, all the 3' untranslated sequence, but only 6 nucleotides of the original 5' leader. This fragment was sub-cloned into the EcoRI site of pBlueScript KS to generate pBlueXenTR α HE. To generate sense RNA from this plasmid it was linearised with BamHI and transcribed in vitro with T3 RNA polymerase. RNA was recovered by ethanol precipitation and approximately 1 μ g of both the full length and the shortened RNA were translated in nuclease treated rabbit reticulocyte lysate. A control translation was carried out with no added RNA. Each of the samples were fractionated on an 18% polyacrylamide/SDS gel which was then dried down, and labelled proteins detected by autoradiography for 4 days. The arrow indicates the position of full length XenTR α protein.

Figure 39



Results and Discussion

full length XenTR α protein (indicated by the arrow in figure 39). This experiment shows that removal of all-but 9 nucleotides of the 5' untranslated region increases translation of the long ORF by several orders of magnitude. The ladder of smaller proteins that were produced when the RNA containing the complete 5' leader was translated in wheat germ extract (figure 36), were not observed in this experiment. This may simply reflect the fact that this is a shorter exposure than the autoradiograph shown in figure 36. However, the removal of the 5' untranslated region has clearly increased translation from what is now the first ATG codon but has not led to an equivalent increase in the synthesis of the truncated proteins.

Interestingly, removal of the most 5' of the 4 ATG codons by restriction at the HincII site did not increase translation in vitro (data not shown).

A similar stimulation of translational efficiency accompanying the deletion of the 5' untranslated region has been observed for translation of these synthetic RNAs when injected into oocytes (R. W. Old and G. Sweeney, pers. comm.). Assuming that the XenTR α cDNA is a copy of the mature mRNA molecule (and not a copy of a partially spliced RNA; see later discussion) this mRNA would appear to be a very poor template for translation, in vivo as well as in vitro.

The scanning model has been proposed to explain

Results and Discussion

translation initiation in eukaryotes. This model states that a 40s ribosomal subunit (plus associated factors) initially binds at the 5' end of the mRNA and then migrates along the mRNA until it finds the first AUG codon in a favourable context for initiating translation. The "first AUG" rule holds true for 90 to 95% of the many hundreds of mRNA sequences that have been analysed (see Kozak, 1989). Not all AUG codons at the 5' ends of mRNAs are efficient translation initiation sites. The identity of nucleotides surrounding the AUG codon has a strong influence upon the ability of the 40s subunit to initiate translation at this site (see the review by Kozak, 1989). A recent survey of 699 vertebrate mRNAs (Kozak, 1987) produced a consensus sequence for translation initiation; GCGGCC^A/GCCAUGC. The importance of the G at +4 (numbering begins with the A of the AUG codon as position +1; nucleotides 5' to that site are assigned negative numbers) and the purine (A or G) at position -3 has been confirmed by naturally occurring mutations and by site directed mutagenesis. The purine (usually A) at position -3 is the most well conserved in eukaryotic mRNAs. Alterations of this residue have a more profound effect upon translation efficiency than mutations in any other position of the consensus sequence. As long as there is a purine at position -3 deviations from the rest of the consensus sequence will only marginally impair initiation. However, in the absence of a purine at position -3, a G at +4 is

Results and Discussion

essential for efficient translation.

There are a number of viral and cellular mRNAs in which translation initiation is not limited to the first AUG codon. Kozak (1989) has concluded that initiation at downstream AUG codons (as appears to occur with synthetic RNA prepared from some thyroid hormone receptor cDNA clones in vitro, and possibly in vivo (Bigler and Eisenman, 1988) can occur only under three specific conditions. The first condition is when the first AUG codon is less than 10 nucleotides from the 5' end of the mRNA, which does not apply to any of the thyroid hormone receptor cDNA clones. The second condition is when the upstream AUG codon(s) lie in an unfavourable context in which case "leaky scanning" will enable some ribosomes to reach the downstream AUG codon and initiate translation. Obviously, with more than one upstream AUG codon the number of ribosomes reaching the "authentic" AUG codon is reduced. In the case of XenTR α , the first AUG codon is in a favourable context for initiation, having a G at -3 and a G at +4 (see figure 38). The second upstream AUG codon is in a very unfavourable context having a T at -3 and a T at +4. Similarly, the third upstream AUG codon is in a very unfavourable context and would not be expected to be an efficient translation initiation site. The fourth upstream AUG codon, however is in a favourable context for initiation. The actual AUG codon that starts the long ORF is also in a favourable context, having the required G at

Results and Discussion

+4, although it does not have a purine at -3. The third situation which allows initiation at downstream AUG codons is when the upstream AUG codons are followed by in frame stop codons. Even when the AUG codons are in a favourable context, precluding leaky scanning, ribosomes are able to translate these invariably short ORFs and then apparently resume scanning and re-initiate farther downstream. Because re-initiation is usually inefficient the presence of short upstream ORFs will usually reduce translation. This is probably how ribosomes negotiate the first, favourable upstream AUG codon in XentTM. They would then have to pass two further AUG codons in unfavourable contexts, partly by leaky scanning and partly by translating the two short ORFs (1 and 23 amino acids) and then re-initiating at the downstream ("authentic") AUG. The fourth (favourable) upstream AUG codon lies within the 23 amino acid short ORF.

Kozak (1989) notes that there are a number of cases in which upstream AUG codons identified in cDNA clones actually reside in an intron. The cDNA corresponds to a partially processed transcript in which this 5' intron had not been removed. This would not be present in the mature mRNA and would therefore not effect translation. There are in fact examples of Drosophila transcripts in which the excision of a 5' intron is developmentally regulated. This intron contains AUG codons which prevent efficient translation. Following the splicing out of this intron

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translation is activated (Bingham et al., 1988).

The DNA sequence of the 5' untranslated region is available for seven of the eleven thyroid hormone receptor cDNA clones that have been isolated so far. A short ORF of 4 amino acids immediately upstream of the long ORF is conserved in four out of these seven cDNA clones, including the Xenopus cDNA clone (figure 38). The first three amino acids of this ORF are absolutely conserved, with only one nucleotide difference in the Xenopus sequence occurring at the third (redundant) position of the codon for leucine. The fourth amino acid is less well conserved among the 4 cDNA clones. In the Xenopus and chicken receptors it is arginine with one nucleotide difference at the third position of the codon. The human receptor and the rat TR α 1 clones have a lysine codon at this position. In all four cDNA clones this short upstream open reading frame terminates with a TGA stop codon immediately prior to the ATG codon which marks the start of the long open reading frame which is presumed to encode the functional receptor protein. The strong amino acid conservation of this short open reading frame suggests that it is important. One possibility is that these four amino acids form part of the receptor protein itself. This could be accomplished by the removal of the TGA stop codon that terminates this short ORF in an RNA processing event. Comparison of 5' and 3' splice sites has shown that introns generally begin with the sequence GT and end with

Results and Discussion

the sequence AG. Indeed, all four cDNA clones have the sequence GT (shown boxed in figure 38) just 5' of the TGA stop codon. The 3' splice site would be somewhere within the long ORF, presumably quite close to the 5' end. Translation would thus begin at the most 3' of the four upstream ATG codons within the XentR α cDNA clone, and this codon is in a favourable context for initiation.

Kozak (1989) concludes that upstream AUG codons in eukaryotic mRNAs are not as common as the literature might suggest, and that where they do genuinely occur they are a clue to expect some sort of regulation such as promoter switching or regulated splicing. This certainly holds true for the rat and human thyroid hormone receptors.

Results and Discussion

CHAPTER 20

Xenopus laevis Has A Small Number Of Genes Related To

The DNA Binding Domain Of XenTR α .

Introduction.

I had already shown that X. laevis has one or possibly two genes homologous to the v-erb-A gene of avian erythroblastosis virus (chapter 10). Having isolated a X. laevis thyroid hormone receptor cDNA I wanted to use this clone to probe a Southern blot of X. laevis genomic DNA partly to confirm the result with the v-erb-A probe. As mentioned in the introduction, the DNA binding domains of the various thyroid hormone receptors show homology to the DNA binding domains of other members of the steroid hormone receptor superfamily. The degree of homology is highest with the retinoic acid receptors. It would therefore be interesting to use a fragment from the DNA binding domain of XenTR α as a probe at low stringency to see if the X. laevis genome contains such related genes.

Results.

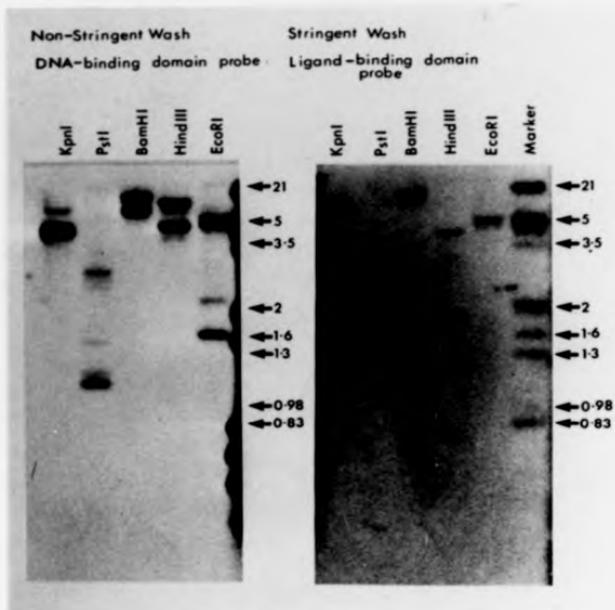
10 μ g aliquots of X. laevis genomic DNA (prepared from a single individual) that had been digested with various restriction enzymes as indicated in figure 40, were fractionated on an agarose gel and Southern blotted. After blotting, the filter was cut in half to generate two identical filters, one of which was hybridised with a probe specific for the ligand binding domain of XenTR α

Figure 40.

Southern blots of X.laevis genomic DNA hybridised with probes from both the DNA-binding and ligand-binding domains of Xentrex.

20µg aliquots of X.laevis genomic DNA prepared from the blood of a single individual was digested exhaustively with the 5 restriction enzymes indicated. After digestion, the DNA was recovered by ethanol precipitation and split into two 10µg aliquots, each of which was fractionated on a 1% agarose gel and then transferred to nitrocellulose according to Southern (1975). The filter was cut into two identical halves, baked, and prehybridised in 6*SSC, 50% formamide, 5* Denhardt's, 100µg/ml E.coli tRNA, 50µg/ml denatured, sonicated herring testis DNA, 0.1% SDS at 42°C. Hybridisation was in the same buffer containing one of two radiolabelled single stranded DNA probes prepared using the prime cut protocol. One filter was hybridised to a probe prepared from the M13 sub-clone of overb12 called ov1219d (see figure 30) and is referred to as the DNA-binding domain (DBD) probe. The second filter was hybridised to a probe prepared from the M13 sub-clone of overb12 called ov1219J (see figure 30), and is referred to as the ligand-binding domain (LBD) probe. The filter hybridised with the DBD probe was washed in 2*SSC at 55°C (non-stringent wash). The filter hybridised with the LBD probe was washed in 0.1*SSC at 65°C (stringent wash), and both filters were exposed to X-ray film for 3 weeks. Markers were end-labelled fragments from a digest of lambda DNA with EcoRI and HindIII.

Figure 40



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(LBD probe). The other, identical, filter was hybridised with a probe that contained almost all of the DNA binding domain, all of the "spacer" region and part of the ligand binding domain. The ligand binding domain-specific (LBD) probe was prepared from the M13 sub-clone of overb12 designated ov1219J (see figure 30) using the prime cut protocol. The DNA binding domain (DBD) probe was prepared using the same method from the M13 sub-clone of overb12 designated ov1219d. The filter that had been hybridised with the LBD probe was washed at high stringency, whilst the filter that had been hybridised with the DBD was washed at moderate stringency, in order to detect related sequences. The resulting autoradiographs are presented in figure 40.

In the EcoRI digest the LBD probe hybridised to a single fragment of about 5Kb, which is the same size as the EcoRI fragment detected by the v-erb-A probe (see figure 24). The DBD probe hybridised to four EcoRI fragments (the band at approximately 5Kb is a doublet) one of which is the 5Kb fragment detected by the LBD probe.

The LBD probe hybridised to two HindIII fragments of 4.3Kb and 1.8Kb. The DBD probe hybridised weakly to the 4.3Kb fragment, but did not hybridise to the 1.8Kb fragment. In addition, the DBD probe detected a HindIII fragment of 4.9kb and one of about 10-15Kb.

In the BamHI digests a single fragment of about 14-18Kb hybridised to the LBD probe. This same fragment also

Results and Discussion

hybridised with the DBD probe, but an additional fragment of 8-10Kb was also detected by this probe.

In the PstI digests the LBD probe detected 3 fragments and these 3 fragments were also detected by the DBD probe (the 1.7Kb fragment hybridised only weakly to the DBD probe, and is barely visible on the photograph). The DBD probe hybridised to an additional 4 PstI fragments. Of these 4 fragments specific for the DBD probe, one showed strong hybridisation and the other 3 only weak hybridisation, consistent with hybridisation to fragments from distinct but related genes.

The LBD probe hybridised to a single, large KpnI fragment of about 10-15Kb. The DBD probe hybridised weakly to this same fragment but very strongly to a second KpnI fragment of 4.3Kb. From examination of a shorter exposure this strong band was probably not a doublet.

The LBD probe can be assumed to detect fragments only from the XenTR α gene under these stringent conditions. If the DBD probe hybridised only to fragments from the XenTR α gene one might expect to have seen hybridisation to one or two fragments not detected by the LBD probe. The fact that in the PstI digest 4 fragments not detected by the LBD probe were detected by the DBD probe suggests (but does not prove) that X.laevis has another gene or genes related to the DNA binding domain of XenTR α . This is supported by the EcoRI digest where 3 fragments were detected specifically by the DBD probe.

Results and Discussion

This data is rather difficult to interpret without a restriction map of the *XenTR α* gene (as distinct from the cDNA which lacks introns). It would have been more informative to probe two identical genomic Southern blots with the DBD probe and then wash one at high stringency (detects only fragments from the *XenTR α* gene) and the other at low stringency. Any hybridising fragments appearing on the filter washed at low stringency but absent from the filter washed at high stringency would clearly indicate the presence of distinct but related genes. However, it is clear from this experiment that the *X.laevis* genome contains only a small number of genes (maybe just one) with sequences related to the DNA binding domain of *XenTR α* . It is likely that one of these genes is the retinoic acid receptor since the DNA binding domains of retinoic acid receptors from other organisms most closely resemble the DNA binding domains of thyroid hormone receptors.

Results and Discussion

CHAPTER 21

Northern Blot Analysis Of Thyroid Hormone Receptor Expression In *Xenopus laevis*.

Introduction.

I had constructed a *X.laevis* male liver cDNA library containing approximately one million recombinants which appeared to be of good quality on the basis of several tests (data not shown). I was unable to isolate any positive clones from this library using the v-erb-A PstI fragment as a hybridisation probe, suggesting that thyroid hormone receptor transcripts may be extremely rare in adult liver. However, I had been able to isolate a thyroid hormone receptor cDNA clone from a mature oocyte cDNA library. I therefore wanted to see if the XenTR α transcript was present only in oocytes or whether it could also be detected in adult liver. Adult male liver polyA⁺ RNA was analysed by northern blotting, using the M13 sub-clone of overb12 designated ov1219f as a hybridisation probe. No transcripts were detected, even when 15 μ g of liver polyA⁺ RNA was analysed (data not shown). Probing of an aliquot of the same RNA for albumin mRNA showed that the RNA was not degraded. It would therefore appear that XenTR α is not expressed at detectable levels in adult male liver.

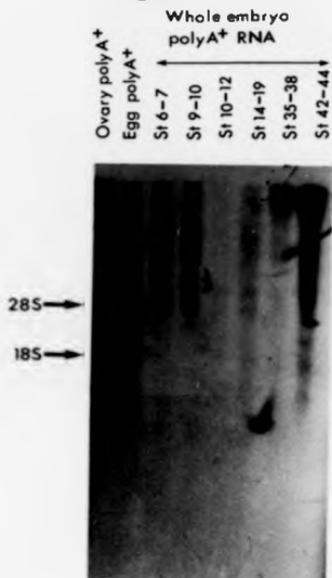
The mRNA which gave rise to the XenTR α cDNA clone could be located in the oocyte itself or in the follicle cells,

Figure 41.

Northern blot analysis of thyroid hormone receptor transcripts in early Xenopus development.

15µg aliquots of polyA⁺ RNA prepared from ovary, egg and X.laevis embryos at various stages of development, as indicated above each lane, were fractionated on a formaldehyde agarose gel. The RNA was transferred to nitrocellulose and hybridised to a single stranded DNA probe prepared from ov1219f (see figure 30) using the prime cut protocol. Prehybridisation and hybridisation was in 50% formamide, 5*SSC, 10mM sodium phosphate (pH6.5), 100µg/ml denatured herring testis DNA, 50µg/ml E.coli tRNA at 42°C. After hybridisation, the filter was washed in 0.1*SSC at 65°C and exposed to X-ray film for 12 days. The arrows indicate the positions of the 28S and 18S rRNA bands as detected by ethidium bromide staining of the gel.

Figure 41



Results and Discussion

or both. An easy but ambiguous way of addressing this question is to look for the RNA in oocytes and unfertilised eggs. Xenopus eggs have lost the layer of follicle cells, so if the RNA is absent from eggs (but present in oocytes) one could tentatively conclude that the RNA was in the follicle cells and not in the oocyte.

Results.

Total nucleic acids were isolated from pools of 100 to 200 X. laevis embryos at six stages of development, with the latest stage being stage 42-44. For the more advanced stages (stages 35-38 and 42-44) the majority of the DNA was removed by differential precipitation from 3M lithium chloride. PolyA⁺ RNA was then selected by one passage down an oligo d(T) cellulose column. 15µg aliquots of ovary polyA⁺ RNA, egg polyA⁺ RNA, and each of the six embryo polyA⁺ RNA samples were electrophoresed on a formaldehyde agarose gel and transferred to nitrocellulose. The filters were then hybridised with a single stranded DNA probe prepared from ovi219f (see figure 30), and the resulting autoradiograph is presented in figure 41. The probe hybridised to a rare RNA species of high molecular weight in ovary polyA⁺ RNA. I am convinced that this is hybridisation to a specific RNA rather than high molecular weight DNA because this band is absent from the egg RNA sample and the embryo samples. These samples contained similar amounts of high molecular weight DNA (from

Results and Discussion

examining the gel under U.V. light). In addition, three diffuse bands of lower molecular weight are visible in the ovary polyA⁺ RNA track. The high molecular weight species is not present in egg polyA⁺ RNA. The other three lower molecular weight species are much less abundant in egg polyA⁺ RNA than in the ovary polyA⁺ RNA, but a trace of them is visible. This would suggest that the majority of XenTR α transcripts are present in the follicle cells surrounding the oocyte, not in the oocyte itself.

In the stage 6-7 sample a prominent band of about 4Kb is visible. This is a surprising finding because general zygotic transcription does not begin until the mid-blastula transition (stage 7/8). The embryos from which this RNA was prepared were carefully selected to be at a stage prior to mid-blastula transition (MBT). However, the absence of transcription prior to MBT is not an absolute rule. It has been reported (Nakakura *et al*, 1987) that some new transcription does occur prior to MBT. The other surprising aspect of this result is that no transcripts of this size are detected in the next sample (stage 9-10). One might expect to see a remnant of this transcript in latter stages even if it is being rapidly degraded as development proceeds. There would seem to be little logic in the embryo producing a very short burst of transcription at this early stage of development. For these reasons I was highly suspicious that this band could be due to contamination with a small amount of XenTR α

Results and Discussion

plasmid DNA. However, a subsequent northern blot showed that this band was resistant to DNaseI (data not shown). In addition to this major band, another two bands (one of which is rather diffuse) are present in the stage 6-7 sample. These same transcripts are present in RNA prepared from stage 9-10 embryos. The stage 10-12 sample has given no signal at all, and probably this RNA is degraded. A hint of the same two transcripts can be seen in both the stage 14-19 RNA and the stage 35-38 RNA (not visible on the photograph but visible on the autoradiograph). These same two bands can be seen in the RNA prepared from stage 42-44 larvae. The stage 42-44 RNA also contains a transcript of about 5Kb (migrating just above the 28S rRNA band) which is not seen in any of the other samples. There are also three fainter, lower molecular weight bands in the stage 42-44 track, two of which are the same size as two of the lower molecular weight bands in the ovary polyA⁺ RNA.

In total 7 differently sized transcripts which hybridised to the XenT_{RM} probe are visible on the autoradiograph. Two of these transcripts are present in all of the stages of development analysed (ignoring stage 10-12). It should be noted that the abundance of these transcripts (except for the prominent band in stage 6-7) is low, and this experiment is on the limit of detection.

The existence of several different but related c-erb-A transcripts whose abundance varies in a developmental

Results and Discussion

manner is intriguing. An obvious explanation for the origin of the different transcripts is that they arise by differential splicing which is developmentally regulated. This would not be unprecedented given that the rat thyroid hormone receptor alpha primary transcript has been shown to undergo differential splicing to generate at least 4 slightly different mature mRNAs which show distinct tissue distributions.

Thyroid hormone exerts its major effect during metamorphosis in Xenopus. The thyroid hormone binding capacity of whole larvae increases markedly at stage 35 to 40 (Tata, 1970). This corresponds to the acquisition by the larvae of the ability to respond to thyroid hormone (Tata, 1970 and Tata, 1968). Clearly, c-erb-A transcripts are present in embryos at stages before this time. As will be shown in later chapters, the XenTR α cDNA encodes a biologically active thyroid hormone receptor able to bind hormone and activate transcription in a hormone dependent manner. However I have been unable positively to identify the actual transcript that gave rise to the XenTR α cDNA because more than one c-erb-A RNA species is detectable in ovary mRNA. It is possible that the transcripts detected in the early developmental stages (before stage 30) encode receptor-like proteins that do not bind hormone.

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CHAPTER 22

Expression Of The Cloned Xenopus Thyroid Hormone Receptor In COS Cells And Demonstration Of Hormone Binding.

Introduction.

The DNA sequence of the XenTR α cDNA clone indicated that it encoded a protein with a high degree of homology to other thyroid hormone receptors. To show that XenTR α is a functional thyroid hormone receptor it is essential to demonstrate that it binds thyroid hormone with high affinity. This is especially important given that a number of thyroid hormone receptor cDNAs isolated from the rat encode proteins that are defective in hormone binding. I therefore decided to express XenTR α in COS cells and carry out hormone binding studies on extracts from these cells. This same expression system could then be used to assess whether the receptor could trans-activate transcription from the Xenopus albumin gene promoter or a synthetic test gene containing a TRE. These experiments required that the XenTR α cDNA be placed in an appropriate expression vector.

22.1 Construction Of pSVLXenTR α ; A Plasmid For Expressing XenTR α In COS Cells.

Results.

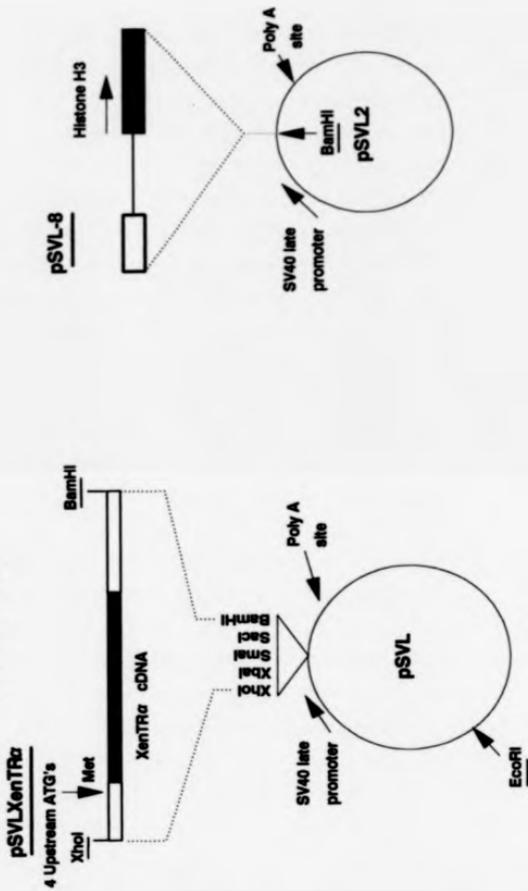
The SV40 late expression vector pSVL was designed for high level transient expression in eukaryotic cells, particularly COS cells. It has the SV40 origin of

Figure 42.

Construction of pSVLXenTR α and the structure of the histone H3 construct pSVL-8.

Overb12 DNA was digested with XhoI and BamHI and the 2.3Kb insert fragment isolated from a LMP gel. This fragment was inserted into the SV40 expression plasmid pSVL, that had been digested with XhoI and BamHI, to generate pSVLXenTR α . Note that transcription from the SV40 promoter gives rise to a transcript that contains all four of the upstream ATG codons. pSVL8 consists of a X.laevis genomic fragment from a histone gene cluster containing an intact histone H3 gene, inserted into the BamHI site of the SV40 vector pSVL2. In this plasmid, transcription of the histone H3 gene is driven by its own promoter rather than the SV40 promoter.

Figure 42 Construction of pSVLXenTRa and the structure of the histone H3 construct pSVL8



Results and Discussion

replication which allows it to replicate efficiently in COS cells, and a short polylinker in front of the powerful SV40 late promoter. The polylinker is flanked by the SV40 late polyadenylation signals so that transcripts of the inserted gene are polyadenylated. Genes inserted into the polylinker are translated from their own ATG codon.

The entire cDNA insert from overb12 was inserted into the polylinker of pSVL as described in the legend to figure 42, such that transcription of sense XenTR α RNA would be driven by the SV40 late promoter. This plasmid was designated pSVLXenTR α (figure 42). Figure 42 also contains a diagram of the plasmid pSVL-8 which was used as a control plasmid in the hormone binding experiments. This plasmid consists of a fragment from a Xenopus histone gene cluster, containing an intact histone H3 gene, inserted into the SV40 expression vector pSVL2. The histone H3 gene is expressed from its own promoter in this plasmid.

22.2 Demonstration Of Hormone Binding In COS Cells

Transiently Transfected With pSVLXenTR α .

Results.

Four flasks of COS cells were each transfected with 10 μ g of pSVLXenTR α exactly as described in Materials and Methods. Another 4 flasks were transfected with 10 μ g of pSVL-8. After 48 hours incubation in media containing serum the cells were harvested and whole cell extracts prepared, keeping each flask as a separate sample. After

Figure 43.

Triiodothyronine binding by XenTR α expressed in COS cells.

Flasks of COS cells were each transfected with 10 μ g of either pSVLXenTR α (XenTR) or pSVL8 (COS cell background) and whole cell extracts were prepared as described in chapter 3.16.

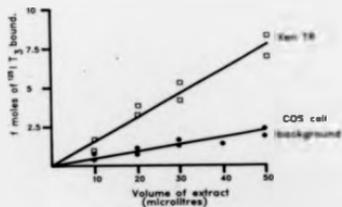
a) Various amounts of extract were incubated with 0.2nM [¹²⁵I] T3 in T3 binding buffer, and the amount of [¹²⁵I] T3 bound was determined by filtration through nitrocellulose filters and gamma counting (see chapter 3.17). The assay was carried out in duplicate with extracts prepared from different flasks of COS cells, and the results are presented as a graph.

b) For the Scatchard analysis, 20 μ l of both extracts was incubated with a range of [¹²⁵I] T3 concentrations (0 to 0.8nM) in T3 binding buffer, and the number of counts bound was determined by filtration through nitrocellulose and gamma counting. After subtracting the background (no extract control), the ratio of bound to free hormone was calculated by dividing the counts bound by the un-bound counts (input counts minus counts bound). The concentration of [¹²⁵I] T3 bound was calculated, taking 2.3*10⁵ cpm to be equivalent to 0.1nM [¹²⁵I] T3. COS cells contain significant levels of endogenous T3 binding activity (see figure 43a), and Scatchard analysis showed that this binding was of a low affinity type (data not shown). Therefore, in order to obtain the true values for binding of [¹²⁵I] T3 by XenTR α it was necessary to subtract this background, and the data are presented as a graph in figure 50b. From the negative inverse slope of this graph the Kd was calculated as 0.12nm.

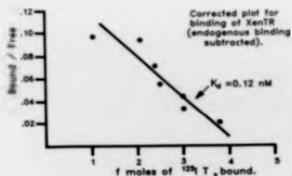
c) For competition experiments, T3 binding assays were assembled containing 40 μ l of extract from COS cells transfected with pSVLXenTR α , 0.5nM [¹²⁵I] T3 and various concentrations of the appropriate cold competitor in a final volume of 250 μ l of T3 binding buffer. After 6 hours at 4 $^{\circ}$ C the counts bound were determined as before. 100% binding was taken to be the number of counts bound in the absence of competitor. Using this figure the percentage of [¹²⁵I] bound in the presence of the various concentrations of competitor was calculated.

Figure 43

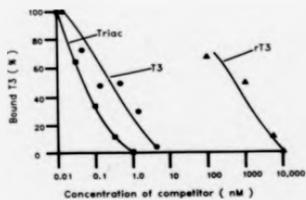
a



b Scatchard



c Competition



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determining the O.D.₂₈₀ of each extract, triiodothyronine (T3) binding assays were assembled containing 4 different quantities of each extract, and no extract. Equal O.D.₂₈₀ amounts of the pSVL-8 and the pSVLXenTR α extracts were used. An aliquot of diluted [¹²⁵I] T3 was applied directly to a nitrocellulose disc and counted in the gamma counter in order to determine the input radioactivity. The data from two duplicate experiments is presented as a graph in figure 43a. Clearly, COS cells do possess an endogenous T3 binding activity. Scatchard analysis and competition experiments showed that this was of a low affinity, non-saturable type (data not shown). These characteristics are the complete opposite of the nuclear thyroid hormone receptor which directly effects gene expression. COS cells transfected with pSVLXenTR α have significantly more T3 binding activity than those transfected with pSVL-8, indicating that XenTR α does bind thyroid hormone.

22.3 Scatchard Analysis Of Triiodothyronine Binding To XenTR α .

Results.

A Scatchard analysis involves incubating a constant amount of the receptor preparation with increasing concentrations of [¹²⁵I] T3, and then measuring the amount of [¹²⁵I] T3 bound. The data were processed as described in the legend to figure 43b, and the results are presented as a graph in figure 43b. From the negative

Results and Discussion

inverse slope, the dissociation constant (K_d), can be calculated. From the corrected plot for XenTR α shown in figure 43b (endogenous binding subtracted), the dissociation constant was calculated to be 0.12nM. This value is similar to the dissociation constants reported for other cloned thyroid hormone receptors. For example, rTR α 1; 0.4nM (Murray et al., 1988), rTR β 1; 0.49nM (Murray et al., 1988), rTR β 2; 0.46nM (Hodin et al., 1989), chicken TR; 0.3nM (Sap et al., 1986), ear-7; 0.38nM (Benbrook and Pfahl, 1987), human TR; 2.3nM (Nakai et al., 1988).

These experiments confirm that XenTR α is a high affinity thyroid hormone receptor.

22.4 Analogues Of Triiodothyronine Compete With [125 I] T3 For Binding To XenTR α .

Introduction.

Specific thyroid hormone analogues have characteristic competition patterns for T3 binding to native and cloned thyroid hormone receptors. Looking at the ability of such analogues to compete for [125 I] T3 binding to XenTR α is a way of further characterising the receptor. Generally it has been found that 3' triiodoacetic acid (TRIAc) is the most efficient competitor while 3,3',5' triiodothyronine (reverse T3) competes less well than T3 itself.

Results and Discussion

Results.

Whole cell extracts were prepared from COS cells that had been transfected with either pSVL-8 or pSVLXenTR α as previously described, except that the extracts prepared from individual flasks were pooled. Binding assays were carried out as detailed in the legend to figure 43c, and the results are presented as a graph in figure 43c.

With T3, 50% competition should in theory occur at a concentration of cold T3 equal to the concentration of [125 I] T3 in the assay, which was 0.5nM. The competition curve is similar to those that have been published for other thyroid hormone receptors. (for example Sap et al., 1986; Weinberger et al., 1986; Thompson et al., 1987; Murray et al., 1988)

Reverse T3 is a much less effective competitor than T3. Here, 50% competition occurs at about 1mM reverse T3 indicating that the Xenopus receptor binds reverse T3 with about a 1000 fold lower affinity than T3. This is typical of what has been reported for other thyroid hormone receptors (see references given above).

TRIAc competes for binding of [125 I] T3 to the Xenopus receptor more efficiently than T3 itself. 50% competition occurs at just under 0.1nM TRIAC indicating about a five fold greater affinity than for T3. This is similar to what has been reported for other cloned thyroid hormone receptors (see references given above).

The relative affinities of these analogues for the

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cloned Xenopus thyroid hormone receptor are consistent with their biological potencies.

Results and Discussion

CHAPTER 23

Trans-Activation Of Transcription From An Artificial Test Promoter By Thyroid Hormone, Mediated By The Cloned Xenopus Thyroid Hormone Receptor.

Introduction.

Having shown that XenTR α binds thyroid hormone I wanted to test its ability to function as a ligand dependent transcription factor. In particular I wanted to find out if transcription from the Xenopus 68Kda albumin promoter could be up-regulated by thyroid hormone in the presence of XenTR α .

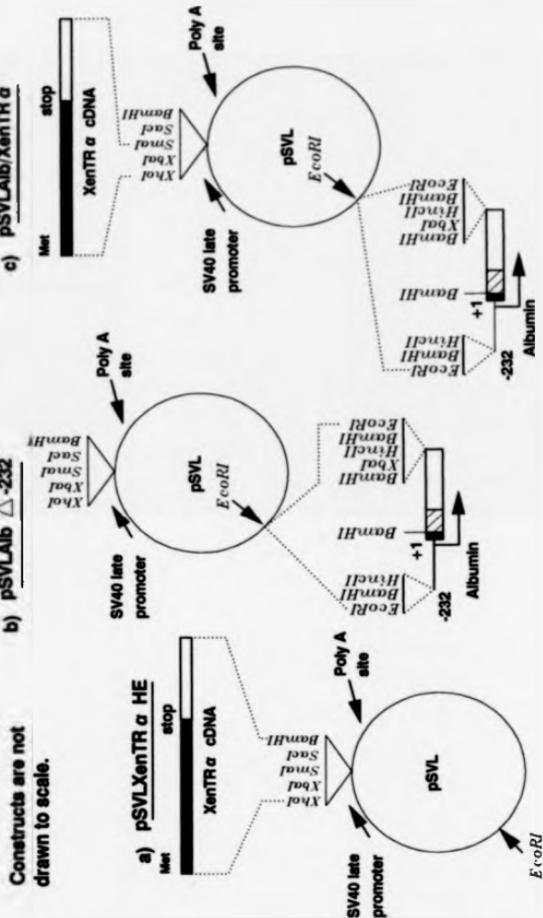
Moore and his co-workers (Brent et al., 1989a) have defined a region between -190 and -167 of the rat growth hormone promoter as the minimal thyroid hormone response element (TRE). Some mutations within this sequence were found to actually increase the response to thyroid hormone and this has lead to the synthesis of artificial TREs. When such artificial TREs were placed upstream of the minimal thymidine kinase promoter they were able to confer inducibility by thyroid hormone in a transient transfection assay (Brent et al., 1989a). These artificial TREs confer greater transcriptional activation by thyroid hormone upon a linked promoter than any natural TRE. In COS cells, which are functionally deficient in thyroid hormone receptor, this inducibility was shown to depend upon co-transfection with a plasmid expressing a functional thyroid hormone receptor (Koenig et al., 1988).

Figure 44.

SV40 constructs for expressing XenTR α in COS cells and testing for trans-activation of the albumin promoter.

pBlueXenTR α CHE, which lacks all the upstream ATG codons (see legend to figure 39), was digested with BamHI and XhoI and the 1.8Kb insert fragment isolated from a LMP agarose gel. This DNA fragment was inserted into the plasmid pSVL that had been digested with BamHI and XhoI to generate pSVLXenTR α CHE (a). To construct pSVLAlb Δ -232, Alb Δ -232 DNA (see figure 20) was digested with HincII, and the 700bp insert fragment that contains 232bp of albumin 5' flanking DNA (thin line) and part of the first exon (black box) fused to the 3' end of the histone H3 coding region (hatched box), and some 3' flanking DNA from the H3 gene (open box), was isolated from a LMP agarose gel. This fragment was inserted into the HincII site of M13mp11 and subsequently excised with EcoRI to generate essentially the same fragment but with EcoRI sticky ends, as shown in the figure. This was inserted into the EcoRI site of pSVL to generate pSVLAlb Δ -232 (b). The orientation of the albumin fragment in this clone was not determined. To construct pSVLAlb/XenTR α , pBlueXenTR α CHE DNA was digested with XhoI and SmaI, which releases the insert intact. This insert fragment was gel isolated and cloned into pSVLAlb Δ -232 that had been digested with XhoI and SmaI to generate pSVLAlb/XenTR α (c).

Figure 44 SV40 constructs for expressing XenTR α in COS cells and testing for activation of the albumin promoter by thyroid hormones.



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I therefore decided to test the response of one such artificial thyroid hormone-responsive promoter to thyroid hormone in COS cells co-transfected with the Xenopus receptor.

The plasmid designated pTK28mult (Brent et al., 1989b) contains an artificial thyroid hormone responsive promoter. It consists of the minimal TK promoter fused to the reporter gene CAT with an artificial TRE placed immediately upstream of the TK promoter. This artificial TRE is based upon the rat growth hormone gene promoter TRE. It is a 16 nucleotide sequence containing a perfect palindrome (see figure 5) and is able to confer more than a 20 fold induction by thyroid hormone on the TK promoter (Brent et al., 1989b). When two copies of this sequence (in a direct repeat organisation) were placed in front of the TK promoter in the plasmid designated pTK28mult (see figure 5), the induction by thyroid hormone was reported to be 125 fold (Brent et al., 1989b).

Results.

Removal of all the upstream AUG codons from the XenTR α cDNA gave rise to a plasmid called pBlueXenTR α HE. This plasmid was digested with XhoI and BamHI and the 2Kb insert fragment which contains the whole XenTR α cDNA, but without the upstream AUG codons, was inserted into XhoI/BamHI digested pSVL to generate the plasmid designated pSVLXenTR α HE (see figure 44). This plasmid is

Figure 45.

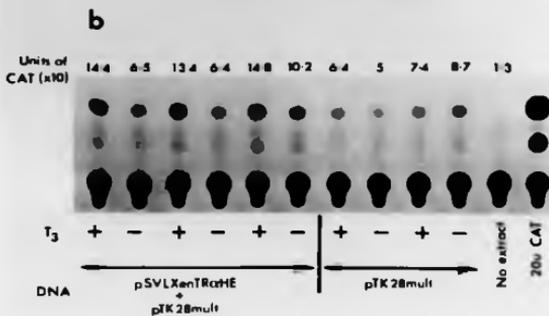
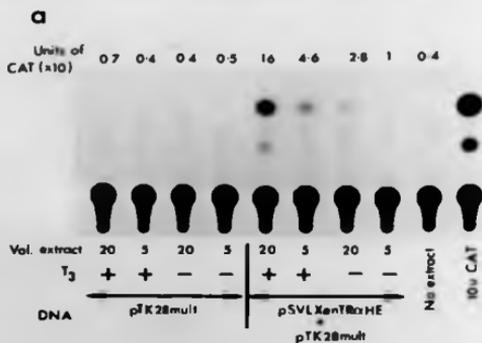
The Xenopus thyroid hormone receptor mediates up-regulation of CAT expression from the plasmid pTK28mult in response to thyroid hormone.

a) Two flasks of COS cells were each co-transfected with 2 μ g of pTK28mult and 10 μ g of pSVLXenTR_hhE. Another two flasks were each transfected with 2 μ g of pTK28mult alone. After transfection, the cells were cultured in media containing serum for 24 hours, after which they were washed twice with serum-free media. For the second of these washes the cells were incubated in serum-free media for 30 minutes. One of each pair of flasks was then incubated in serum-free medium and the other in serum-free medium supplemented with T3 at a final concentration of 10⁻⁸M. After 24 hours "CAT extracts" were prepared from each flask of COS cells, and 5 and 20 μ l of each extract assayed for CAT activity. The TLC plate was exposed to X-ray film for 2 weeks after which the area of the plate that contained the acetylated form of chloramphenicol (the most mobile radioactive spot) was carefully scraped off and counted in scintillation fluid. The number of counts present were then converted to units of CAT and are shown above each track.

b) Flasks of COS cells were transfected and cultured exactly as described for figure 45a except that each co-transfection was done in triplicate (a total of 6 flasks), and the single transfection was done in duplicate (a total of 4 flasks). "CAT" extracts were prepared and 20 μ l of each assayed for CAT activity. A 2 week exposure of the TLC plate is presented. The area of the TLC plate containing the acetylated form of chloramphenicol was scraped off into scintillation fluid and counted. The results were converted to units of CAT and are given above each track.

Abbreviations used are: CAT, chloramphenicol acetyl transferase; T3, triiodothyronine; u, units; vol., volume.

Figure 45



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identical to pSVLXenTR α except for the removal of the 5' untranslated region.

Two flasks of COS cells were each co-transfected with 2 μ g of pTK28mult and 10 μ g of pSVLXenTR α HE. This high ratio of the receptor plasmid to the test plasmid was used to try to ensure that most of the cells which were transfected with the test plasmid (pTK28mult) would also receive the receptor expression plasmid. Another two flasks of COS cells were each transfected with 2 μ g of pTK28mult alone. The cells were then cultured as detailed in the legend to figure 45, after which whole cell extracts were prepared and assayed for CAT activity. Each sample was assayed in duplicate, with 20 μ l and 5 μ l of extract, and the resulting autoradiograph is presented in figure 45a. The area of the TLC plate that gave rise to the upper acetylated form of chloramphenicol was carefully scraped off and counted in scintillation fluid. The results of this counting were converted to units of CAT enzyme and these values are also shown in figure 45a.

In the absence of the co-transfected receptor, pTK28mult produced no CAT activity above the background seen in the no extract control, either in the absence or presence of T3. It is therefore possible to argue that an induction of pTK28mult by T3 did occur in the absence of the co-transfected receptor, but the assay was not sensitive enough to detect it.

In the presence of the co-transfected receptor, CAT

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activity was increased 6 fold by treatment with thyroid hormone. In the absence of added thyroid hormone -CAT activity was slightly above background. This may reflect our inability to remove all the "endogenous" thyroid hormone from the cells. This experiment clearly demonstrates that the thyroid hormone responsive promoter in pTK28mult is activated by thyroid hormone only in the presence of the co-transfected Xenopus receptor. Given that other thyroid hormone receptors have been shown to directly effect gene expression by binding to DNA and activating transcription, it seems likely that the receptor I have cloned functions in a similar manner.

One of the problems with these types of transfection experiment is that one is comparing different flasks of cells. It can therefore be argued that differences in CAT activity are the result of different transfection efficiencies or other un-defined variations between the flasks of cells. I therefore repeated the experiment described above in triplicate, and the results are presented in figure 45b. This time the CAT activity in the absence of the co-transfected receptor was higher, significantly above the background of the no extract control. The level of T3 induction was less impressive than in the first experiment. It is difficult to explain this higher background activity given that the experimental conditions were the same as before. Treatment with T3 however, had no significant effect upon CAT

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activity in the absence of the receptor.

In the presence of the receptor there is a consistent induction of CAT activity by thyroid hormone. On average the induction is only 2 fold compared to the 6 fold induction seen in the previous experiment. Not only was the background CAT activity higher in this experiment but the CAT activity induced by T3 was also lower. Despite this the experiment does confirm that XentR κ can activate expression from a promoter containing a TRE in a ligand dependent manner.

Brent et al (1989b) reported that pTK28mult was induced 125 fold when co-transfected into GH4C1 cells (a rat pituitary tumour cell line) with a plasmid expressing a mouse thyroid hormone receptor. One explanation for the low level of induction that I have observed is that I have not optimised the experimental conditions. The level of induction could probably be increased by altering the culture conditions or perhaps using a different cell line. It is also possible that the Xenopus thyroid hormone receptor has different sequence requirements, with respect to its response element, from the mouse thyroid hormone receptor used by Brent et al (1989b).

Results and Discussion

CHAPTER 24

XenTRC Mediates Transcriptional Activation Of The Xenopus 68Kda Albumin Gene Promoter By Thyroid Hormone In A Transient Transfection Assay.

Introduction,

As discussed in the Introduction (section 1.6) there is some circumstantial evidence to suggest that expression of the Xenopus albumin genes may be regulated by thyroid hormone. Thyroid hormone response elements (TREs) appear to consist of a rather poorly conserved "half site" (see section 1.4.6) with the consensus sequence $AGGT(C/A)_A$ (Brent et al., 1989b). At least two copies of this half site in close proximity are required for a functional TRE, and these can be in either direct or inverted repeat orientations (see figure 6).

A careful examination of the sequence of the 5' flanking region of the Xenopus 68Kda albumin gene (Sweeney et al., 1987 and figure 14) revealed one perfect copy of the half site at position -140 (5' TGACCT 3'). In addition, there are two imperfect copies of the half site in close proximity to the one at -140, as shown in figure 46. These two imperfect copies match the half site consensus at four out of six positions. This putative TRE is not dissimilar to the TREs that have been identified in the rat growth hormone gene, the rat alpha myosin heavy chain gene and the rat alpha-subunit gene (see figure 6). These TREs have one half site that matches the consensus in at least five

Figure 46.

The Xenopus 68Kda albumin gene promoter contains a putative thyroid hormone response element.

The sequence of the Xenopus 68Kda albumin gene promoter spanning nucleotides -149 to -120 relative to the transcription start site is shown (Sweeney et al., 1987). Note that in the published sequence there are 2 guanines at what is now nucleotide -146, but re-examination of the sequencing gel showed that there is in fact only a single guanine at this position. The six bases which match the TRE consensus sequence defined by Brent et al. (1989b) have been boxed. On both sides of this are imperfect copies of the consensus half site (marked 2 and 3). Below the sequence these three half sites have been aligned with the consensus TRE half site. Also shown is the corresponding sequence from the promoter of the X.laevis 74Kda serum albumin gene (Schorpp et al., 1987). Nucleotides which differ from the sequence of the 68Kda albumin gene are marked with a #.

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out of six positions, with the second half site matching the consensus at only four out of six positions. The sequence of the putative TRE I have identified in the 68Kda albumin gene promoter is conserved in the corresponding region of the 74Kda albumin gene promoter (Schorpp et al, 1988a) (figure 46). However, the whole of the 5' flanking sequence extending to about 250bp upstream of the CAP site is well conserved between the the 68Kda and 74Kda genes. Given the poor conservation of thyroid hormone response elements it is essential to confirm the existence of this putative TRE in the albumin promoter in a functional assay. Having isolated a functional Xenopus thyroid hormone receptor I was in a position to carry out such an assay. I decided to do this by transient expression in COS cells. The albone construct would be suitable for this experiment because transcription from this construct can be conveniently analysed by primer extension. Co-transfection cannot ensure that both the receptor expression plasmid and the test plasmid (albone) are taken up by the majority of the transfected cells. I therefore decided to put the albone construct and the thyroid hormone receptor on the same plasmid. As an internal control I proposed to do northern blots on RNA prepared from the transfected cells using a probe for the XenTRO α RNA. The level of RNA produced by transcription from the SV40 promoter should not be affected by thyroid hormone.

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24.1 Construction of suitable plasmids for testing transcriptional activation of the 68KDa albumin promoter in COS cells.

Results.

I decided to use the albone construct that contains 232bp of albumin upstream sequence. This contains the putative TRE we had identified at -140 in the albumin promoter. It also contains all the upstream sequence required for high level transcription in liver cells (Schorpp *et al.*, 1988b).

For the control experiments I would need to transfect COS cells with the albone construct alone. I therefore decided to insert albone Δ -232 into the unique EcoRI site of pSVL, which places the albumin promoter well away from the SV40 promoter. The details of this construction are given in the legend to figure 44, and the resulting plasmid was designated pSVLAlb Δ -232 (figure 44b).

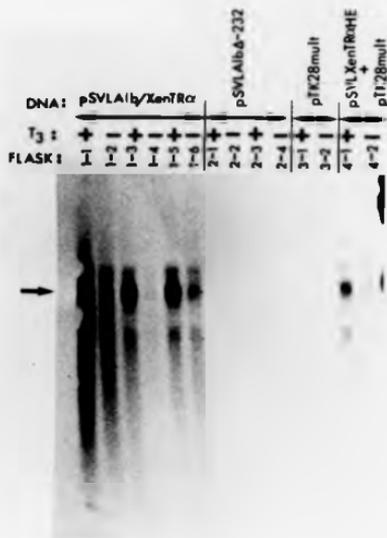
To make a combined receptor/albone construct the cDNA insert from pBlueXenTR^{OX}HE was sub-cloned into the polylinker of pSVLAlb Δ -232 (see legend to figure 44 for details). The resulting plasmid contains the XenTR^{OX} cDNA inserted in the correct orientation downstream of the SV40 late promoter, and was designated pSVLAlb/XenTR^{OX} (figure 44c).

Figure 47.

Northern blot analysis of XenTR α transcripts in transfected COS cells.

Six flasks of COS cells were each transfected with 10 μ g of pSVLAlb/XenTR α , and another 4 flasks were transfected with 10 μ g of pSVLAlb Δ -232. A further 2 flasks were transfected with 2 μ g of pTK28mult, whilst 2 more were co-transfected with 2 μ g of pTK28mult and 10 μ g of pSVLXenTR α HE. After transfection the cells were cultured in media containing serum for 24 hours, and then washed twice in serum-free medium. For the second of these washes the cells were incubated in serum-free medium for 30 minutes. The medium was then replaced with either serum-free medium or serum-free medium supplemented with T3 at a final concentration of 10⁻⁸M, as indicated. After a further 24 hour incubation RNA was prepared from the cells as described in chapter 3.5.4. 10 μ g aliquots of each RNA sample was then fractionated on a formaldehyde agarose gel, transferred to nitrocellulose and hybridised with a radiolabelled, single stranded DNA probe prepared from ov1219f (see figure 30) using the prime cut protocol. Prehybridisation and hybridisation conditions were the same as those described in the legend to figure 41. After hybridisation the filter was washed in 0.1*SSC at 60°C and then exposed to X-ray film for 2 weeks. The arrow indicates the probable position of intact XenTR α RNA.

Figure 47



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24.2 Testing for transcriptional activation of the albumin promoter in COS cells.

Results.

Six flasks of COS cells were each transfected with 10 μ g of pSVLAlb/XenTR α , and another 4 flasks were each transfected with 10 μ g of pSVLAlb Δ -232. A further two flasks were transfected with 2 μ g of pTK28mult, and another 2 were co-transfected with 2 μ g of pTK28mult and 10 μ g of pSVLXenTR α HE. After transfection the cells were incubated as described in the legend to figure 47, after which RNA was prepared from the cells as described in Materials and Methods.

10 μ g of each RNA sample was electrophoresed on a formaldehyde gel, northern blotted, and then hybridised with a single stranded DNA probe prepared from the M13 sub-clone of overb12 designated ovl219f (see figure 30). After hybridisation the filter was washed in 0.1*SSC at 60°C, and exposed to X-ray film, and the resulting autoradiograph is shown in figure 47.

Assuming that the thyroid hormone receptor transcript produced from pSVLAlb/XenTR α is terminated at the SV40 polyadenylation signal, this RNA should be about 2Kb in size. The probable position of this RNA is indicated by the arrow in figure 47. Clearly, all the hybridisation was specific to those RNA samples that were prepared from COS cells transfected with pSVLAlb/XenTR α or pSVLXenTR α . RNA samples prepared from COS cells transfected with

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pSVL1alb Δ -232 or pTK28mult showed no hybridisation to the probe. I am unsure of why there was such a smear of hybridising material on this northern, but possibly it was due to some degradation of the thyroid hormone receptor transcript (clearly the smear is specific to cells which received the receptor). I assume that the major band indicated by the arrow represents the full length receptor transcript.

In all three pairs of flasks transfected with pSVL1alb/XenTR α , and the pair of flasks co-transfected with pSVLXenTR α HE and pTK28mult, the level of this transcript was significantly greater when the cells were treated with T3. This was surprising since the SV40 late promoter is not known to be thyroid hormone responsive. It is possible that thyroid hormone has some general effect on the cells such as stimulating growth or cell survival that lead to greater levels of transcript from the transfected plasmid. Alternatively, this phenomenon could be the result of thyroid hormone-dependent stabilisation of the XenTR α transcript. Hodin et al (1989) have recently shown that the level of rat thyroid hormone receptor type β 2 mRNA in a pituitary tumour cell line is markedly reduced by treatment with thyroid hormone. It is not known whether this is a transcriptional or post-transcriptional effect. It would perhaps not be surprising to find that thyroid hormone post-transcriptionally regulates the expression of its own receptor, and this phenomenon merits further

Figure 48.

Primer extension analysis for albone transcripts in transfected COS cells.

10 μ g aliquots of the same COS cell RNA samples analysed on the northern shown in figure 47 (see the legend to figure 47 for the details of the transfections), were annealed with end-labelled albone primer. The primer was extended with reverse transcriptase and the products fractionated through an 8% sequencing gel. Extension products were detected by autoradiography for 3 days. The area containing the major primer extended product is shown enlarged at the top of the figure. Markers were end-labelled fragments from a digest of pBR322 with HpaII.

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investigation. I had intended to use the level of thyroid hormone receptor RNA in transfected COS cells as an internal control for the albumin activation experiment (see below). The finding that thyroid hormone consistently increased the level of this RNA made this internal control inappropriate. I therefore decided to simply analyse equal amounts of RNA from each flask for transcripts from the transfected albumin promoter, relying upon repetition to control for variation between flasks.

The same RNA samples as analysed on the northern blot shown in figure 47 were analysed by primer extension with the albone primer, and the result is presented in figure 48.

All the RNA samples prepared from COS cells transfected with a plasmid containing the Alb Δ -232 construct gave rise to a major primer extended product (indicated by the arrow) of between 55 and 60 nucleotides. A primer extended product of 57 nucleotides represents transcripts initiating at the correct start site. Therefore, in COS cells the major initiation site is at or close to the start site used in Xenopus liver. Further experiments would be needed to show that transcription was initiating at precisely the correct start site. This major primer extended product was not generated by primer extension analysis of RNA from COS cells transfected with pTK28mult proving that it is not an endogenous transcript.

In all three pairs of flasks transfected with

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pSVLAlb/XenTRO α , treatment with thyroid hormone resulted in an increase in the level of transcripts from the albumin promoter. The level of induction by thyroid hormone was approximately 3 to 4 fold. Of the two pairs of flasks transfected with pSVLAlb Δ -232 (which lacks the thyroid hormone receptor), one pair (2.3 and 2.4) showed no up-regulation by thyroid hormone. However, in the other pair of flasks (2.1 and 2.2), the cells not treated with thyroid hormone showed a lower level of albumin transcripts, but the level of induction by thyroid hormone in this pair of flasks was less than that seen in the presence of the receptor.

An important point to note here is that the level of albumin transcripts synthesized in the presence of the receptor but the absence of hormone was lower than was produced in the absence of the receptor. Treatment with hormone increased the level of transcripts to that produced in the absence of the receptor. So transcription was not simply increased from a basal level by thyroid hormone treatment. Rather, in the absence of hormone, the receptor appears to have actively repressed transcription. Similar results regarding the effect of the rat thyroid hormone receptor type β 1 upon transcription of a synthetic thyroid hormone responsive promoter have recently been published (Damm *et al.*, 1989; Graupner *et al.*, 1989). They concluded that in the absence of its ligand, the thyroid hormone receptor suppressed the activity of

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the promoter. Addition of thyroid hormone resulted in the stimulation of expression. If this is the case then contrary to the simple model for the action of steroid hormones, the thyroid hormone receptor must be able to bind to the response element in the absence of ligand (this was discussed in some detail in the Introduction, section 1.4.14). One could imagine that in the absence of thyroid hormone, the receptor is bound to the TRE of thyroid hormone responsive genes but makes un-productive contacts with other components of the transcriptional machinery, thereby preventing transcription. Binding of thyroid hormone could induce a conformational change in the receptor which allows it to make productive contacts with other proteins thereby activating transcription.

From the experiment reported here I tentatively conclude that the Xenopus 68Kda albumin promoter is regulated by thyroid hormone at the transcriptional level. Currently, work is under way in the laboratory to confirm these results. Site directed mutagenesis will be used to alter the DNA sequence of the putative TRE within the albumin promoter to see if this element is responsible for the apparent induction by thyroid hormone.

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CHAPTER 25

General Conclusions And Discussion.

I have isolated a full length cDNA clone encoding a thyroid hormone receptor from a mature oocyte cDNA library. This receptor binds thyroid hormone with high affinity when expressed in COS cells. Expression from the thymidine kinase promoter containing an artificial thyroid hormone response element can be induced by thyroid hormone in the presence (but not in the absence) of this receptor. Thyroid hormone receptors isolated from other organisms are known to act directly upon gene expression at the level of transcription by binding to regulatory sequences within thyroid hormone responsive genes. My results with the Xenopus thyroid hormone receptor are consistent with this mode of action.

There appear to be several rare transcripts in Xenopus ovary which hybridised to the thyroid hormone receptor cDNA clone. I have been unable to positively identify the mRNA which gave rise to the cDNA clone, but it is likely that these transcripts detected in ovary RNA are located in the follicle cells rather than the oocyte itself.

Several rare transcripts which hybridised to the cDNA clone are also present in early embryonic stages. These differently sized, but clearly related, transcripts are suggestive of differential splicing.

Synthetic RNA prepared from the entire cDNA translates

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very poorly in vitro and this is almost certainly due to the presence of several AUG codons upstream of the long open reading frame. These upstream AUG codons and the resulting poor translation are characteristics of virtually all the thyroid hormone receptors that have been cloned so far. Such upstream AUG codons are often indicative of differential splicing and indeed it has been shown that in the rat, a primary transcript is alternately spliced to generate at least four mature c-erb-A mRNAs. Presumably the mRNA which gave rise to the Xenopus thyroid hormone receptor that I have isolated is translated poorly in vivo as well as in vitro.

Thyroid hormone is known to play a vital role in inducing the many biochemical and physiological changes that occur during amphibian metamorphosis. It is almost certain that some of these changes are the result of the activation and repression of specific genes at a transcriptional level. It will be of great interest to look for thyroid hormone receptor transcript(s) during metamorphosis. One might expect to see a dramatic increase in the levels of transcripts encoding the receptor at the onset of metamorphosis. The adult 63Kd keratin genes are an example of genes whose expression is known to be induced by thyroid hormone during Xenopus metamorphosis (Mathisen and Miller, 1989). It is not known whether this induction is caused by the binding of a thyroid hormone/receptor complex to cis-acting regulatory sequences within the

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promoter, but this seems a likely possibility. However, unlike the rat growth hormone gene, the induction by thyroid hormone is not reversible (Mathisen and Miller, 1989), suggesting that the thyroid hormone receptor complex is only required for the initial induction of expression and not for the maintenance of gene expression.

I have demonstrated that transcription of the Xenopus albumin genes is activated at the onset of metamorphosis, so these are also candidates for genes that are regulated by thyroid hormone. From my experiments I tentatively conclude that thyroid hormone does increase expression of the 68Kda albumin gene, at the level of the messenger RNA, and that this increase requires the presence of the (Xenopus) thyroid hormone receptor. The liver-specific expression of albumin genes from Xenopus and other organisms is known to be controlled at the level of transcription by liver-specific trans-acting factors which interact with regulatory sequences upstream of these genes. In vivo, in the developing liver, transcription of the Xenopus albumin genes might require the presence of both tissue-specific trans-acting factors and the thyroid hormone receptor complex. I was unable to detect thyroid hormone receptor transcripts in adult Xenopus liver, suggesting that, as has been reported for the Xenopus keratin genes, thyroid hormone might only be required to activate albumin gene transcription and not to maintain it later in development.

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