The stability, movement and expression of natural and synthetic mRNAs injected into Xenopus oocytes

by

Douglas R. Drummond

B.Sc.(Hons.) (Edinburgh)

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Department of Biological Sciences

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Declaration

Except where it is acknowledged in the text, the work which I describe in this thesis is my own, and has not been accepted in any previous application for a degree. All published sources of information are acknowledged by reference.

Douglas R. Drummond

Publications

Parts of this thesis have already been published.

Most of chapters 3-5 as

Drummond, D.R., McCrae, M. and Colman, A. (1985) J. Cell Biol. 100, 1148-1156. Stability and movement of mRNAs and their encoded proteins in *Xenopus* oocytes.

Most of chapters 6-9 as

Drummond, D.R., Armstrong, J. and Colman, A. (1985) Nucleic Acids Res. (in press). The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in *Xenopus* oocytes.

Summary

The stability and movement of several natural and synthetic mRNAs in Xenopus oocytes was examined. Although the movement of injected mRNAs has important implications for experiments in oocytes, this aspect of mRNA behaviour has never been examined before.

At least 50% of the injected natural poly(A)$^+$ mRNAs (9S rabbit globin, chicken ovalbumin and lysozyme mRNAs) remained stable over a 48h period, irrespective of the amount injected. 50% of the natural poly(A)$^-$ reovirus mRNA was degraded within 24h of injection, irrespective of the amount injected, and no further degradation occurred over the next 24h.

Synthetic mRNAs coding for chicken lysozyme, calf preprochymosin and Xenopus β globin protein were transcribed in vitro using Sp6 RNA polymerase. Capping and polyadenylation increased the stability of the synthetic mRNAs with at least 42% of capped, poly(A)$^+$ transcripts remaining 48h after injection into oocytes.

Capping and polyadenylation also increased the translational efficiency of most of the synthetic mRNAs. The exception was one Xenopus β globin transcript with an unusual 3' end of 20 As and 30 Cs, where further polyadenylation decreased translational efficiency.

The movement of all the natural poly(A)$^+$ mRNAs injected into oocytes was very slow. Little movement of RNA from the animal to the vegetal half was observed, even 48h after injection. In contrast, similar amounts of mRNA were present in both halves 48h after vegetal pole injection. Similar results were obtained with poly(A)$^-$ reovirus mRNAs.

The capped poly(A)$^-$ synthetic mRNAs moved more rapidly in oocytes than either capped poly(A)$^+$ synthetic mRNAs or naturally occurring mRNAs. However equilibration of the injected RNA still did not occur even 24h after injection.

The movement of the proteins encoded by the natural poly(A)$^+$ mRNA was examined in the 6h period after injection, when little mRNA movement had occurred. The sequestered secretory proteins ovalbumin and lysozyme moved much more slowly than the cytosolic protein globin in the same oocytes.

Abbreviations

A	adenine
APS	ammonium persulphate
bisacrylamide	N,N'-methylene bisacrylamide
C	cytosine
cAMP	cyclic adenosine 5'-monophosphate
cDNA	copy DNA
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
d(N)TP	deoxyribonucleoside 5'-triphosphate (N = A,G,C or T)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
G	guanine
g	gram
h	hour
m	metre
min	minute
M_r	relative molecular mass
MOPs	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
poly(A)	polyadenylic acid
$poly(A)^+$	RNA with (3') poly(A) tail
$poly(A)^-$	RNA without (3') poly(A) tail
PPO	2,5 diphenyloxazole
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein

r(N)TP or (N)TP	ribonucleoside 5'-triphosphate (N = A,G,C, or U)
rRNA	ribosomal RNA
S	svedberg unit
s	second
SDS	sodium dodecyl sulphate
SSC	1 x SSC is 0.15M sodium chloride, 0.015M sodium citrate, pH 7.0
T	thymine
$t_{\frac{1}{2}}$	half life
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	uracil
v, vol	volume
wt	weight

Glossary

Oocytes

animal half	pigmented (brown) half of the oocyte which contains the nucleus
animal pole	the animal end of the animal-vegetal axis
equator	circle round the oocyte marked by the edge of the animal half pigment
g.v., germinal vesicle	the oocyte nucleus
vegetal half	non-pigmented half of the oocyte which contains most of the yolk protein
vegetal pole	the vegetal end of the animal-vegetal axis

synthetic RNA

anti-sense or '-' transcript	transcript with sequence complementary to the corresponding mRNA
$Chym^+$	synthetic mRNA coding for calf preprochymosin protein
dimethyl cap	cap with structure $m^7G(5')ppp(5')Gm$
Globin-Pst, Globin-Hinf	synthetic mRNAs coding for *Xenopus* β globin protein

Lys^+	synthetic mRNA coding for chicken lysozyme protein
monomethyl cap	cap with structure $m^7G(5')ppp(5')G$
natural mRNA	mRNA transcribed *in vivo*
Ov^+	synthetic mRNA coding for chicken ovalbumin protein
Ov^-	synthetic mRNA with sequence complementary to that of Ov^+
sense or '+' transcript	transcript with the same sequence as the corresponding natural mRNA
synthetic RNA	RNA transcribed *in vitro*
synthetic mRNA	mRNA transcribed *in vitro*

Lys^+	synthetic mRNA coding for chicken lysozyme protein
monomethyl cap	cap with structure $m^7G(5')ppp(5')G$
natural mRNA	mRNA transcribed *in vivo*
Ov^+	synthetic mRNA coding for chicken ovalbumin protein
Ov^-	synthetic mRNA with sequence complementary to that of Ov^+
sense or '+' transcript	transcript with the same sequence as the corresponding natural mRNA
synthetic RNA	RNA transcribed *in vitro*
synthetic mRNA	mRNA transcribed *in vitro*

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Xenopus laevis

The frog Xenopus laevis is native to the temperate zones of south and west Africa. It was first described by Daudin in 1803 who gave it the name Bufo laevis, meaning smooth-skinned toad-like creature. This has lead to the common name (or misnomer) of South African clawed toad. However it is most closely related to the genus of frogs called Pipa and belongs to the family Pipidae (a full discussion of the anatomy and classification is given by Deuchar, 1975).

Although several species belonging to the genus Xenopus have been described (Tinsley, 1975), it is Xenopus laevis (Daudin) (also called Xenopus laevis laevis), that is most widely used in laboratories. In the wild Xenopus is entirely aquatic, living in stagnant ponds and feeding on both live and dead animal material (Deuchar, 1975), which makes Xenopus convenient to both house and feed in the laboratory.

Among the many uses that have been made of Xenopus one of the most common is to remove the oocytes (the germ cells of the female), and culture them in vitro as a model cell system. The large size of the oocyte (1.2mm diameter) makes it much easier to introduce materials by microinjection than it would with normal-sized somatic cells. The widespread use of Xenopus oocytes as an experimental system, which I will describe later, makes it tempting to regard them almost as a 'neutral' cell type. However they are highly specialised and differ from somatic cells in many respects, therefore I will first describe the normal biology and formation of oocytes.

1.2 Formation and development of Oocytes

The morphological aspects of oocyte development in Xenopus laevis

have been described by Balinsky and Devis (1963), Dumont (1972) and Coggins (1973).

In the ovary of the female the germ cells or oogonia divide mitotically. After their final mitotic division they enter meiotic prophase as the primary oocyte. The oocytes remain arrested at the diplotene stage of meiotic prophase throughout their growth, and are therefore tetraploid rather than diploid like somatic cells. Meiosis only resumes with maturation of the oocyte (Masui and Clarke, 1979).

At the start of its growth the oocyte is small (25μm diameter) and transparent. It remains transparent until the vitellogenic stages of growth, which start when it has reached about 300μm diameter. Two major changes take place at this point. First the oocyte starts to accumulate yolk taken up from the small capillaries present in the single layer of folicle cells which surround the oocyte. Second, pigment granules form in the cortex of the oocyte. Initially these cover the entire surface of the oocyte but are gradually confined to one half as the oocyte increases in size. The pigmented half of the oocyte (which contains the nucleus or germinal vesicle) is called the animal half since later in development the cells of this half form the more animal like features of the embryo, such as the nervous system. The yellow, nonpigmented half, which takes its colour from the yolk granules, forms the gut or other 'lower' functions of the embryo (see Balinsky, 1975). During the vitellogenic stages of growth the diameter of the oocyte increases to about 1200μm (stage VI, Dumont, 1972). The entire growth phase can last from about three months to over a year (Dumont, 1972).

1.3 Oocyte maturation

Full-grown oocytes may remain several months in the ovary before undergoing maturation. A gonadotropin produced in the pituitary gland induces the follicle cells to produce progesterone which in turn induces

the full grown oocytes to mature (reviewed by Masui and Clarke, 1979). This process is open to experimental manipulation since the follicle cells will respond to other gonadotropins, for example human chorionic gonadotropin (HCG) which is readily available (Thibier-Fouchet *et al.*, 1976). Injection of such hormones into frogs causes the maturation response and the frogs lay eggs. Likewise oocytes can be induced to mature *in vitro* by adding progesterone to the culture medium (Wasserman *et al.*, 1984).

Maturation causes many changes in the oocyte including an increase in cytoplasmic calcium (Wasserman *et al.*, 1980), drop in cAMP level (Maller *et al.*, 1979), increase in intracellular pH (Houle and Wasserman, 1983), increase in phosphorylation of ribosomal (Wasserman and Houle, 1984) and other proteins (Maller *et al.*. 1977), and an increase in protein synthesis (Wasserman *et al.*, 1982).

On maturation the oocyte nucleus moves to the animal pole, displacing the pigment granules causing the formation of a white disc. The nuclear membrane breaks down and meiotic prophase arrest stops. The oocyte undergoes the first meiotic division and the polar body is excreted from the white disc (Brachet *et al.*, 1970).

If the oocyte has matured in the frog, the vitelline membrane, which surrounds the cell membrane, forms together with the jelly layers of the egg. If the egg is fertilised the second meiotic division takes place, leaving the haploid female pronucleus to fuse with the male pronucleus.

The morphology of the fertilised *Xenopus* egg has been described by Balinsky (1966) and the subsequent development of the embryo by Niewkoop and Faber (1967).

1.4 Accumulation of materials during oogenesis

A variety of substances are synthesised and stored in the oocyte

during oogenesis for use later in development. These include lipid stored in small protein coated inclusions (Holtfreter, 1946), glycogen (Barth and Barth, 1954), histones (Woodland and Adamson, 1977) which are stored in the nucleus, all three RNA polymerases (Roeder, 1974) and DNA polymerase (Benbow et al., 1975). Some substances such as tubulin, although they are present in a large amount, are not more concentrated than normal because of the large size of the oocyte (Pestell, 1975). However of the substances which accumulate it is the yolk protein that is the most abundant.

1.5 Accumulation of yolk and origin of the oocytes polarity

Yolk protein forms 45% of the dry weight of full grown oocytes (Barth and Barth, 1954). The yolk originates in the liver of the frog as vitellogenin (M_r 210,000) and moves to the oocyte in the blood. It is taken up into membrane bound vesicles by endocytosis. In the oocyte the vitellogenin is cleaved to form lipovitellin (M_r 120,000) and phosvitin (M_r 34,000) which form a crystalline membrane-bound structure: the yolk platelet (Wallace and Bergink, 1974). The yolk platelets may fuse with each other (Wallace and Bergink, 1974) leading to a range of sizes which are not evenly distributed within the oocyte (Gerhart et al., 1983). In the full-grown oocyte the animal half contains little yolk which is present as small platelets, whilst the vegetal half has more yolk in larger platelets.

The origin of this polarity in the distribution of yolk is uncertain. Gerhart et al. (1983) have suggested that it has its origins in the structure of the previtellogenic ooctye, where the nucleus, a concentration of mitochondria and then of Golgi apparatus, lie virtually in a line (Tourte et al., 1981; Coggins, 1973; Billet and Adam, 1976). Since the Golgi apparatus and mitochondria are involved in the protein uptake processes (Farquhar and Palade, 1981) it is proposed by Gerhart

et al. (1983) that most of the yolk platelets will form where the concentration of Golgi and mitochondria is highest. Also as the largest yolk platelets form by addition of smaller platelets (Witteck, 1952; Wallace and Bergink, 1974), they will tend to form where the concentration of yolk is highest. This means most of the yolk and the largest yolk platelets are in the opposite half of the oocyte from the nucleus.

Gerhart et al. (1983) also speculate that constant addition of new membrane into the oocytes cell membrane from the high concentration of Golgi forces the old membrane with the underlying pigment granules into the opposite (nuclear or animal) half of the oocyte.

The polarised distribution of the yolk affects the distribution of other substances in the oocyte. Thus in the oocytes of Rana pipiens. which have a similar structure to those of Xenopus. the free water accumulates in the yolk free animal half (Tluczek et al., 1984). Likewise the K^+ ion concentraton is higher in the animal half, while the Na^+ ion (which is associated with the yolk) is more concentrated in the vegetal half.

1.6 Accumulation of RNA in oocytes

In previtellogenic oocytes most transcription is of 5S RNA and tRNA (4S RNA) (Thomas, 1970; Mairy and Dennis, 1971). These RNAs combine with proteins to form ribonucleo-protein (RNP) storage particles (Johnson et al.. 1984). With the onset of vitellogenesis, mainly 18S and 28S rRNAs are transcribed (Mairy and Dennis, 1971; Scheer et al.. 1976) in the 1500 nucleoli present in the oocyte (Perkowska et al., 1968). Although the oocyte contains numerous mitochondria they only contribute 0.5% of the total rRNA (Chase and David, 1972). The full-grown oocyte contains 4µg of RNA (Brown and Littna, 1964). Most of this total is rRNA present in some 10^{12} ribosomes (Mairy and Dennis, 1971) of

which only 2% are active in protein synthesis at any given instant (Woodland, 1974).

Most of the RNA is concentrated in the yolk-free animal half of the oocyte (Niewkoop and Faber, 1967).

1.7 Poly(A) RNA in oocytes

Estimates of the poly(A)$^+$ RNA content of Xenopus oocytes vary from about 40ng (Rosbash and Ford, 1974) to 90ng (Dolecki and Smith, 1979; Sagata et al., 1980). All of the poly(A)$^+$ RNA is already present in small previtellogenic (Dumont stage 2) oocytes (Golden et al., 1980). In full-grown (Dumont stage 6) oocytes only about 4ng of the poly(A)$^+$ RNA is present in polysomes (Woodland, 1974; Taylor and Smith, 1985). About 15% is mitochondrial (Anderson et al., 1982), whilst the majority (68%) is in an unusual form that contains interspersed repetitive sequences covalently linked to unique sequence (Anderson et al., 1982). The same unique sequences are present in both the normal (mRNA like) and unusual forms of poly(A)$^+$ RNA (Thomas et al., 1981). The interspersed repetitive sequence containing poly(A)$^+$ RNA is not translatable (Richter et al., 1984), which (assuming a total of 90ng) leaves some 16ng of potentially translatable but nonpolysomal mRNA.

Smith et al. (1984) and Taylor and Smith (1985) have suggested that this nonpolysomal mRNA is stabilised and its translation suppressed by interaction with oocyte specific poly(A)$^+$ mRNA binding proteins (Dixon and Ford, 1982; Richter and Smith, 1983). In support of this they have shown that forming RNP particles with these proteins and globin mRNA inhibits its translation in vivo and in vitro (Richter and Smith, 1984). They also propose that these proteins mainly repress translation at early oocyte stages and gradually decrease up to stage 6 oocytes, to account for the increasing amount of mRNA being translated. However in full grown (stage 6) oocytes it is not the availability of mRNA but some

component of the oocytes translational machinery that limits translation (Laskey et al., 1977).

Like the ribosomal RNA, poly(A)$^+$ RNA is more concentrated in the animal than the vegetal half of the oocyte. Using in situ hybridisation to oocyte sections with a poly(U) probe Capco and Jeffery (1982) have found that poly(A) is on average 1.5 times more concentrated in the animal than the vegetal half of the full-grown oocyte. However there is also a subcortical concentration of poly(A) in the vegetal half. Other transient concentrations of poly(A) form throughout oogenesis. That these localisations reflect differences in numbers of poly(A)$^+$ molecules rather than lengths of poly(A) tail was confirmed by in situ translations of the oocyte sections, where incorporation of radioactivity into protein corresponded to concentration of poly(A) (Capco and Jackle, 1982).

Only the distribution of one specific mRNA (Histone H4) has been examined in oocytes, using in situ hybridisation, however no localisation was found (Jamrich et al., 1984). Nevertheless some of the mRNAs which are present in oocytes (ie the maternal mRNAs) are localised in eggs (Rebagliati et al., in press).

1.8 Experimental use of Xenopus oocytes

Although amphibian eggs have been used in injection experiments since 1910 (see Gurdon and Melton, 1981), continuing with nuclear transplantation experiments in eggs of Rana pipiens (Briggs and King, 1952) and Xenopus laevis (Gurdon, 1960), it is only more recently that amphibian oocytes have been used.

Initial experiments involved injection of nuclei into oocytes (Gurdon, 1968). Purified DNA was first injected to examine its replication (Gurdon et al., 1969). Specific transcription of injected DNA was demonstrated by Colman (1975) and this technique has been

refined by the development of vectors which give efficient transcription (and subsequent translation) of a cloned DNA (Krieg et al., 1984). Experiments involving injection of DNA, either to examine RNA processing or expression of proteins have been extensively reviewed (for example Gurdon and Melton, 1981; Lane, 1983; Colman, 1984a).

Although it is possible to use virtually any cultured cell for injection of DNA (or indeed any substance) (Graessman et al., 1980), amphibian oocytes have the considerable advantage of an unusually large size (1.2mm diameter). This means the injected substance can be accurately deposited in any position within the oocyte, which is impossible with other smaller cells (cf Graessmann et al., 1980; Colman, 1984). Also an individual oocyte can provide a large amount of material for subsequent biochemical analysis.

1.9 Injection of mRNA into Xenopus oocytes

Oocytes are also used for experiments involving injection of mRNA. Accurate translation of a mRNA injected into oocytes of Xenopus laevis was first demonstrated using rabbit globin mRNA (Gurdon et al., 1971; Lane et al., 1971). This technique has become so popular that over 200 types of mRNA have now been translated (Lane, 1983). These mRNAs cover virtually every group of eukaryotic animals, viruses and plants (for additional reviews see Marbaix and Huez, 1980; Colman, 1984b). The only types of mRNA not translated have been synthetic polynucleotides (Woodland and Ayers, 1974), bacteriophage mRNAs (Gurdon et al., 1971), and some mitochondrial mRNAs (Moorman et al., 1977).

Although oocytes provide a more efficient expression system, particularly for longer mRNAs, than most in vitro translation systems (Gurdon et al., 1971), most interest has centred on the oocyte's ability to correctly process the encoded protein post-translationally. Thus oocytes will accumulate nuclear proteins in their nucleus (Bonner,

1978), or process and secrete secretory proteins (Colman and Morser, 1979; Lane et al., 1980). The oocyte will also assemble complex biologically active molecules following injection of the subunit mRNAs, as demonstrated by the assembly of functional acetylcholine receptors (Barnard et al., 1982).

However despite the widespread and successful use of oocytes there are many aspects of the behaviour of the injected mRNA that have not been well characterised. In particular a knowledge of the spatial distribution of the injected mRNA is important for the correct design or interpretation of many experiments, yet to quote Lane (1983) "there is no compelling evidence to support the widely accepted view that the mRNA distributes itself evenly throughout the oocyte". Indeed given the polarity in the oocyte's structure and asymmetric distribution of most of the oocyte's endogenous substances, such an even distribution of injected molecules would seem unlikely. I have therefore investigated this and other aspects of the behaviour of injected mRNAs and their encoded proteins in Xenopus oocytes.

1.10 Stability of endogenous oocyte RNA

Before examining the movement of injected RNA it is necessary to examine its stability, to ensure that the distribution of the RNA within the oocyte reflects movement rather than differential degradation. To place the stability of injected RNAs in their correct context I will first describe the stability of the endogenous oocyte RNA.

Estimates of rRNA stability range from $t_{\frac{1}{2}}$ of 9-31 days (Leonard and La Marca, 1975) to $t_{\frac{1}{2}}$ of 1 or 2 years, equivalent to the lifetime of the oocytes (Ford et al., 1977; Anderson and Smith, 1977).

As with most animals there is rapid ($t_{\frac{1}{2}}$ of a few hours) turnover of heterogeneous RNA in the oocyte nucleus (Anderson and Smith, 1977). However some 5% of the heterogeneous nuclear transcripts enter the

cytoplasm and are not rapidly turned over. This net inflow of poly(A)$^+$ RNA into the cytoplasm, together with the constant mass of poly(A)$^+$ RNA in the oocyte throughout oogenesis (Golden et al., 1980), means that at least some poly(A)$^+$ RNA is being degraded. However no turnover has been detected using pulse-labelling methods (Ford et al., 1977; Anderson and Smith, 1978; Dolecki and Smith, 1979).

Anderson et al. (1982) have calculated that for a net inflow of 1.44ng of RNA per day an average half life of 35 days would give a steady state level of 80ng of poly(A)$^+$ RNA. Alternatively only a fraction of the poly(A)$^+$ RNA may turnover, however in this case at least 10-20% of the total must turn over to ensure a half life of at least 3 days. Anderson et al. (1982) suggest that these half lives would be too long to be detected by the short pulse labelling period used by Anderson and Smith (1978), or Dolecki and Smith (1979). Ford et al. (1977) labelled stage 2 oocytes in tadpoles. The tadpoles were allowed to grow into young frogs whose ovaries contained at least some full-sized oocytes. Since they could still recover nearly all of the labelled ovarian poly(A)$^+$ RNA, Ford et al. (1977) concluded that most of the poly(A)$^+$ RNA present in stage 2 oocytes must remain stable throughout oogenesis. However Anderson et al. (1982) have criticised this experiment since whole ovaries from the young frogs were analysed, in which most of the labelled oocytes would still be at stage 2, and any turnover of RNA in the oocytes which had grown would remain undetected.

Thus at present it is unclear whether all or only a fraction of oocyte poly(A)$^+$ RNA turns over, so there is no set 'expectation' for the 'normal' degradation of RNA in Xenopus oocytes.

1.11 Stability of RNA injected into Xenopus oocytes

Reports of the stability of RNA injected into oocytes are in many ways as uncertain as reports of the stability of endogenous RNA. Thus

although Gurdon et al. (1973) found rabbit globin mRNA to be functionally stable in Xenopus oocytes for up to 2 weeks, a result supported by Marbaix et al. (1975) who could detect no degradation after 56h using cDNA hybridisation, Richter and Smith (1981) have found rabbit globin mRNA to be very unstable. Less than 5% of the globin mRNA remained 10h after injection of more than 10ng per oocyte. Richter and Smith (1981) also found that the proportion of the RNA that remained stable decreased with injection of increasing amounts of RNA.

Similarly estimates of the half-life of radioactive reovirus mRNA injected into oocytes vary from $t_{\frac{1}{2}}$ of 18h (Furuichi et al., 1977) to a $t_{\frac{1}{2}}$ of 72h (McCrae and Woodland, 1981).

Individual reports exist for other RNAs, for example rRNA and Dictyostelium mRNA (Allende et al., 1974) and the functional stability of histone mRNA (Woodland and Witt, 1980). However it is clear that since different workers obtain different results using the same RNA (such as globin mRNA, or reovirus mRNA), that comparison of rates of degradation for different RNAs in different experiments is virtually meaningless. Obviously, even when using an mRNA whose stability has previously been determined, its stability must be determined again; under the individual experimental conditions used.

1.12 Movement and distribution of molecules injected into amphibian oocytes

The most detailed and quantitative investigation of movement of molecules injected into amphibian oocytes is the series of experiments by Horowitz and collaborators using oocytes of Rana pipiens. They measured diffusion coefficients by examining the distribution of radioactive molecules following autoradiography of oocyte sections. The diffusion coefficients of sucrose (M_r 342) (Horowitz, 1972), insulin (M_r 5,500) (Horowitz and Moore, 1976) and a series of dextrans (maximum M_r

20,000) (Paine et al., 1975), varied from 0.4 to 0.1 of their value in water (Paine et al., 1975). Thus, not surprisingly, the rate of movement in cytoplasm is relatively slower than in pure water. However, at least for sucrose, diffusional equilibrium is still achieved within 3h in the oocyte cytoplasm (Horowitz, 1972).

Bonner (1975) found, using autoradiography of sections of Xenopus oocytes injected with a variety of I^{125} labelled proteins [chicken lysozyme (M_r 14,500), whale myoglobin (M_r 17,816), chicken ovalbumin (M_r 44,000) and bovine serum albumin (M_r 67,000)], that by 24h following injection, they were evenly distributed throughout the oocyte cytoplasm with no localisations.

The even distribution of small molecules and proteins following injection into oocytes contrasts with the behaviour of RNAs injected into Xenopus oocytes. Within 24h of injection small nuclear RNAs accumulate in the nucleus, 5S RNA although mainly present in the cytoplasm also accumulates in the nucleoli, while 7S RNA and tRNA remain in the cytoplasm concentrated in the yolk free area round the nucleus (De Robertis et al., 1982).

Thus small molecules, proteins and some RNAs equilibrate rapidly (within 24h), though in the case of RNAs not evenly, in amphibian oocytes. However given the decrease in diffusion coefficients with increasing molecular size this is not necessarily true for mRNAs.

1.13 Movement and distribution of mRNA injected into Xenopus eggs

Although the movement of mRNA injected into oocytes has not been examined, some experiments have been carried out using eggs.

If endogenous poly(A)$^+$ RNAs are extracted from the animal or vegetal pole of unfertilised Xenopus eggs, then reinjected into fertilised eggs they will rapidly (within 6h) form specific gradients irrespective of the injection site used (Capco and Jeffery, 1981).

Likewise rabbit globin mRNA injected into fertilised eggs rapidly moves to become concentrated in the animal half cells of the embryo by the gastrula stage (Froehlich et al., 1971). However Woodland et al. (1974) found no specific difference in translation of rabbit globin mRNA in the dorsal or ventral tissues of embryos following injection at the one or two cell stage. Thus specific gradients form in the animal-vegetal axis with both endogenous and exogenous RNA, while no specific gradients appear in the dorsal-ventral axis of the embryo.

The results of Capco and Jeffery (1981) and Froehlich et al. (1971) suggest that mRNA may move rapidly within eggs, and Woodland et al. (1974) do not exclude rapid movement. However in none of these experiments was the distribution of the mRNA examined immediately after injection, so the actual extent of the movement remains unclear. Also, when ovalbumin mRNA is injected into fertilised eggs at the one cell stage, ovalbumin protein is only found in those regions of the gastrula corresponding to the injection site (E. Jones, H. Woodland and A. Colman, personal communication) which suggests a slow movement. However it is not clear if this result is specific to mRNAs coding for secreted proteins.

Thus in eggs rapid movement and specific localisations of the injected RNAs are possible, at least in some instances. However there are many differences between eggs and oocytes; in particular a redistribution of yolk and cytoplasm takes place on maturation (Lau et al., 1984) and fertilisation (Phillips, 1985), which means that the behaviour of injected mRNAs is not necessarily the same in oocytes and eggs.

1.14 Significance of the movement and distribution of mRNAs injected into Xenopus oocytes

There has been no direct examination of the movement or

distribution of mRNA injected into Xenopus oocytes. This is surprising since the distribution of the injected mRNA has potentially serious implications for the interpretation of many experiments, particularly competition studies, carried out using oocytes. Moreover, given the asymmetric distribution of endogenous oocyte components, and the behaviour of mRNAs injected into eggs, there is no reason to expect that injected mRNA should be evenly distributed.

Some of the interpretation difficulties that can arise are described below.

Moar et al. (1971) initially thought that full-grown oocytes had some spare translational capacity, since following injection of globin mRNA translation of globin protein increased with no corresponding decrease in translation of endogenous mRNAs. However this result was an artifact caused by using radioactive histidine to label the oocytes. Histidine is more abundant in globin than in endogenous oocyte protein; therefore even when a large amount of globin protein was produced, there would only appear to be a small decrease in the amount of endogenous protein that was labelled. When Laskey et al. (1977) repeated this experiment, but labelled the oocytes using a completely radioactive mixture of all amino acids, translation of the injected mRNA caused a decrease in translation of endogenous oocyte mRNAs. Thus the full-grown oocyte has no spare translational capacity, although the immature stages may have some (Taylor and Smith, 1985). Therefore any injected mRNA must compete with the endogenous oocyte mRNA to be translated, which makes every injection experiment essentially a competition study.

One feature of such experiments is that injection of increasing amounts of mRNA does not always give increased translation, rather a saturation point is reached. At this point up to 50% of the endogenous oocyte mRNA is still being translated (Laskey et al., 1977; Asselbergs et al. 1979; Richter and Smith, 1981). Obviously there are several

possible explanations including instability of the injected mRNA (Richter and Smith, 1981), however one simple explanation that has not been excluded is that the injected mRNA does not completely overlap spatially with the endogenous mRNA and is therefore unable to compete with it.

This possibility is consistent with the observations that after injection of mRNAs there is a lag of several hours (called the recruitment period) before the maximum rate of translation is reached (Berridge and Lane, 1976; Asselbergs et al., 1978; Asselbergs et al., 1979; Larkins et al., 1979). Berridge and Lane (1976) also found that the recruitment periods for globin and vitellogenin mRNAs were different. Indeed these authors suggested that this might be explained by different rates of movement of the two mRNAs in the oocyte, although this was not established.

The other common type of competition study is between different injected mRNAs. The mRNAs are either coinjected (Asselbergs et al., 1979; Richter and Smith, 1981), or sequentially injected with a gap of several hours between injections (Asselbergs et al., 1979).

Coinjected mRNAs coding for cytoplasmic proteins will compete with each other (Asselbergs et al., 1979; Richter and Smith, 1981; Richter et al., 1983). However if there is a delay before injecting the second mRNA the first mRNA becomes more resistant to competition. In fact the first mRNA starts to behave more like an endogenous mRNA (Asselbergs et al., 1979). An obvious explanation of this would be reduced spatial overlap of the two mRNAs when they are sequentially rather than coinjected.

A study of the movement of injected mRNAs also provides a means of examining the constraints that exist within cells for movement of endogenous mRNAs. This is of increasing interest given the potential of mRNAs as cytoplasmic determinants controlling development (see Davidson, 1976). Indeed some localised maternal mRNAs have been identified in

Xenopus eggs (Rebagliati et al., in press).

1.15 Initial aims of thesis

In view of their widespread experimental use, it is perhaps surprising that no previous study of mRNA movement in oocytes has been undertaken; especially since mRNA movement in Xenopus eggs, and the movement of proteins, smaller RNAs and other molecules in oocytes have been examined. The large size of amphibian eggs and oocytes make them ideal for such studies. Also the clear animal-vegetal polarity of the cell, marked by the brown pigment of the animal half, means the substance can be reproducibly introduced into a particular part of the cytoplasm.

The main aim of the work described in this thesis was to remedy the complete lack of information on the movement of mRNA in oocytes. A series of experiments which attempt this, by following the movement of natural mRNAs injected into oocytes, is described. However during the course of this work it became clear that by using mRNAs that were transcribed in vitro. and could therefore be made radioactive, it would be possible to improve the quantitative aspects of the study. Use of such in vitro transcripts (or synthetic RNAs) would also simplify manipulation of the RNA's structure; and allow the effect of such structural modifications to be investigated. The background to these experiments is described below.

1.16 Synthetic RNAs

It is now possible, using the RNA polymerase from either Salmonella typhimurium phage Sp6 (Melton et al., 1984) or Escherichia coli phage T7 (Davantos et al., 1984), to transcribe large quantities of discrete-sized RNAs in vitro from any DNA fragment introduced into an appropriate vector. Such transcripts have many uses: high specific activity

synthetic RNAs have been used as probes for *in situ* hybridisation (Cox *et al*., 1984), filter hybridisation (Zinn *et al*., 1983; Melton *et al*., 1984; Church and Gilbert, 1984), mapping the 5' and 3' ends of transcripts (Wood *et al*., 1984) and mapping intron-exon boundaries in genomic DNA (Melton *et al*., 1984).

Injection of anti-sense RNA transcripts has been used to block translation of the complementary mRNA in *Drosophila* embryos (Rosenberg *et al*., 1985) and *Xenopus* oocytes (Melton, 1985).

Synthetic RNAs have also been used as mRNA analogues for translation *in vitro* (Persson *et al*., 1984; Krieg and Melton, 1984a; Darveau *et al*., 1985; Mead *et al*., 1985; Pelletier and Sonenberg, 1985). Also for translation (Krieg and Melton, 1984a; Mishina *et al*., 1985) or RNA processing studies (Green *et al*., 1983; Krainer *et al*., 1984; Krieg and Melton, 1984b) after microinjection into *Xenopus* oocytes.

The use of synthetic RNAs has expanded the usefulness of *Xenopus* oocytes as an experimental *in vivo* system, utilising some of the unique aspects of *Xenopus* oocytes. Thus Green *et al*. (1983), Krainer *et al*. (1984) and Krieg and Melton (1984b) have injected radioactive mRNA precursors into the oocyte's giant nucleus and tested for processing of these precursors (eg removal of introns, correct 3' terminal processing, etc.) into mature mRNAs.

However perhaps the most ambitious experiment has been that of Mishina *et al*. (1984). They made synthetic mRNAs coding for each of the 4 subunits of a *Torpedo* acetylcholine receptor. Mutant RNAs for one subunit were made by *in vitro* mutagenesis of the corresponding cloned DNA. These mutant RNAs were coinjected with the three wild type mRNAs and, since the oocyte can assemble a functional acetylcholine receptor following injection of the mRNAs, the effect of the mutations in the coding region of subunit on the receptor's function could be tested directly.

The ability to synthesise RNA *in vitro* allows easier manipulation of the RNA's structure than is possible by altering an existing natural mRNA. Highly radioactive synthetic RNAs can be made by incorporating radioactive nucleotides during transcription, which allows easier detection and quantitation of the mRNAs for stability and movement studies than is possible with probe hybridisation methods. I have therefore exploited this to test these properties with synthetic mRNAs in oocytes.

Other manipulations of the synthetic mRNA's structure are possible, such as altering the cap structure or the poly(A) tail. This is of interest not only from the purely technical aspect of producing a synthetic mRNA that will function in *Xenopus* oocytes, but also allows an assessment of the natural function of such structures as discussed below.

1.17 Role of the mRNA cap structure

All eukaryotic mRNAs have a cap structure at their 5' end consisting of a 7-methylguanosine base whose 5' end is joined by a triphosphate bridge to the 5' end of the RNA (Shatkin, 1976; Banerjee, 1980). In addition the first and sometimes the second nucleotides of the RNA may be methylated (Shatkin, 1976; Banerjee, 1980).

The role of the cap structure has been extensively investigated. Removal of the cap structure from natural mRNAs decreases the physical stability of reovirus mRNA (Furuichi *et al*., 1977; McCrae and Woodland, 1981) and the functional stability of globin mRNA (Lockard and Lane, 1978) in *Xenopus* oocytes.

The cap structure also enhances translation of the mRNA (Shatkin, 1975; Both *et al*., 1975; Furuichi *et al*., 1977; Lockard and Lane, 1978). Although a guanosine base as the capping nucleotide will increase stability, 7-methylguanosine is required to give the increase in

translation (Furuichi et al., 1977).

I have retested these results using synthetic mRNAs, in particular to find which pattern of methylation gives the most efficient translation.

1.18 Role of the mRNA poly(A) tail

Most eukaryotic mRNAs have a poly(A) tail at their 3' end (Littauer and Soreq, 1982). However the precise physiological function of this remains unclear.

One view is that the poly(A) tail increases the stability of the mRNA; certainly polyadenylic acid itself appears resistant to nuclease attack (Allende et al., 1974). Most in vivo experiments have been conducted in Xenopus oocytes, and removal of the poly(A) tail decreases both the functional (Huez et al., 1974; Huez et al., 1975; Marbaix et al., 1975) and physical (Marbaix et al., 1975) stability of rabbit globin mRNA. Likewise polyadenylation of the normally poly(A)$^-$ HeLa cell histone mRNA increases its functional stability (Huez et al., 1978). However removal of the poly(A) tail does not affect the functional stability of either human β interferon mRNA (Sehgal et al., 1978; Soreq et al., 1981) or rat α-2u-globulin mRNA (Desphande et al., 1979).

The other view is that the poly(A) tail increases the translational efficiency of the mRNA. Early in vitro translation experiments gave variable results with some reporting decreased translation from poly(A)$^-$ mRNA (Soreq et al., 1974; Williamson et al., 1974; Sippel et al., 1974), while others found no difference (Huez et al., 1974; Bard et al., 1974; Gielen et al., 1974). However the failure to find any difference has been attributed to the low efficiency of the translation system used (generally Krebs ascites cells) (Doel and Carey, 1976). When an efficient system such as rabbit reticulocyte lysate is used, poly(A)$^+$

mRNA is more efficiently translated than poly(A)$^-$ (Doel and Williamson, 1974; Doel and Carey, 1976), and a role for poly(A) is supported by polyadenylic acid being a competitive inhibitor of translation *in vitro* (Jacobson and Favreau, 1983).

In vivo no difference has been found (at least in the initial rates) in translation of poly(A)$^+$ or poly(A)$^-$ rabbit globin mRNA (Huez *et al*., 1974; Marbaix *et al*., 1975; Nudel *et al*., 1976), HeLa cell histone mRNA (Huez *et al*., 1978) or human fibroblast β_1 and β_2 interferon (Soreq *et al*., 1981) following injection in *Xenopus* oocytes. However, Desphande *et al*. (1979) found that rat α-2u-globulin mRNA with short poly(A) tails took longer to reach their maximum rate of translation compared with those mRNAs with long poly(A) tails.

There is also some evidence for polyadenylation of mRNAs affecting translation during development. Following fertilisation in the clam *Spisula* there is a change in the pattern of protein synthesis. Some of the mRNAs that become translationally active are polyadenylated, while one that becomes translationally inactive is deadenylated; however this correlation did not hold for all mRNAs (Rosenthal *et al*., 1983). Likewise the start of development in the fungus *Dictyostelium discoideum* is marked by a decrease in translation of pre-existing mRNAs and increase in translation of new mRNAs, which correlates with a deadenylation of the pre-existing mRNAs (Palatnik *et al*., 1984). Also in sea urchin embryos poly(A)$^+$ mRNAs are more fully loaded with ribosomes than poly(A)$^-$ mRNAs (Nemer *et al*., 1975).

I have investigated the effect of polyadenylation on the stability and translation of several synthetic mRNAs. The use of high-specific activity RNAs has allowed their physical stability to be easily measured, which has made it possible to separate the stability and translation effects. In addition the role of the poly(A) tail in determining the rate of movement of mRNA was examined.

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Chemicals were of 'Analar' grade purchased from BDH Chemicals (Poole, UK).

Tris, spermidine, DTT, deoxynucleotide triphosphates, dextran sulphate, polyvinylpyrrolidone, phenylmethylsulfonylfluoride, salmon sperm DNA, polyadenylic acid, cyclohexamide, progesterone and MS 222 were purchased from Sigma (Poole, UK).

Ribonucleotide triphosphates and yeast tRNA were purchased from Boehringer (Lewes, UK).

2.2 Radiochemicals

[^{32}P-α] dGTP (3000 Ci/mmole), [^{32}P-α] dCTP (3000 Ci/mmole), [^{32}P-γ] rATP (5000 Ci/mmole), [^{32}P-α] rGTP (410 Ci/mmole), [^{35}S] methionine (>800 Ci/mmole), [^{3}H] leucine (120 Ci/mmole), [^{3}H] fucose (40-70 Ci/mmole), and [^{14}C] protein molecular weight markers were obtained from Amersham International (Amersham, UK).

2.3 Antibodies

Rabbit anti-chicken egg white antibodies were a gift from D. Cutler (EMBL, Heidelberg, West Germany). Rabbit anti-chicken ovalbumin antibodies were purchased from Miles Laboratories Inc. (Elkhart, USA). Rabbit anti-prochymosin antibodies were a gift from P. Lowe (Celltech, UK).

2.4 Chicken oviduct poly(A)$^+$ RNA

Hen oviduct RNA was prepared by a method based on Palacios *et al*. (1972) and Palmiter (1973).

The oviduct was removed from a freshly killed laying Rhode Island Red hen. The magnum portion of the oviduct was divided into 4; and each part was then homogenised in 35ml of buffer, using a motorised teflon-glass homogeniser. Homogenisation buffer is 1% (v/v) Triton X100, 1% (wt/v) sodium deoxycholate, 25mM NaCl, 5mM $MgCl_2$, 25mM Tris-Cl pH 7.6, 500µg/ml Heparin (an RNase inhibitor). The homogenate was spun at 15,000rpm for 5 min at 4°C, in a 8 x 50 fixed angle rotor in an MSE HS18 centrifuge (27,000g maximum). 1/10 vol of 1M $MgCl_2$ was added to the supernatant which was then left in ice for 60 min. The supernatant was layered over 10mls of 25mM NaCl, 5mM $MgCl_2$, 25mM Tris-Cl pH 7.6, 500µg/ml Heparin, 17% sucrose and spun for 10 min at 15,000 rpm at 4°C, in an 8 x 50 fixed angle rotor in an MSE HS18 centrifuge. The pellet was resuspended in 7.5ml of 20mM HEPES pH 7.5 and 7.5mls of 0.2M sodium acetate pH 5.0 and 0.8mls of 10% SDS added. This was then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and once with an equal volume of chloroform:isoamyl alcohol (24:1).

1/20 vol of 4M NaCl and 2.5 vols of ethanol were added to the aqueous phase, which was then precipitated overnight at -20°C.

The poly(A)$^+$ RNA was purified from the total RNA by two cycles of oligo-dT cellulose chromotography.

2.5 Rabbit globin(9S) RNA

Rabbit reticulocyte total RNA was a gift from G. Valle and S. Bahmra (University of Warwick, UK). This had been prepared by phenol/chloroform extracting reticulocyte lysate (see Jackson and Hunt, 1983).

Poly(A)$^+$ reticulocyte RNA was prepared by oligo-dT chromatography of total reticulocyte RNA.

The 9S fraction was separated by centrifuging the poly(A)$^+$ RNA

through a sucrose gradient (Berridge and Lane, 1976).

250µg of poly(A)$^+$ RNA was ethanol precipitated, then resuspended in 500µl of 10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% SDS.

Gradients were prepared in 14ml centrifuge tubes by layering 5.5ml of 5% sucrose, 10mM Tris-Cl pH 7.5, 1mM EDTA over 5.5ml of 25% sucrose, 10mM Tris-Cl pH 7.5, 1mM EDTA. The tubes were incubated at 37^{o}C for 60 min whilst lying on their sides, then cooled to 4^{o}C. The RNA sample was heated at 65^{o}C for 5 min, then cooled on ice and loaded immediately. The gradients were spun in a 6 x 14 swing-out rotor in a Beckman L8 centrifuge at 40,000rpm for 16h (284,000g) at 4^{o}C.

The gradients were fractionated using an Isco fraction collector with a UV monitor (Fisons). The 9S peak was identified from its position relative to the 5S and 18S ribosomal RNA peaks which are still present in detectable amounts.

1/10 vol of 4M LiCl and 2.5 vol ethanol was added to the 9S fraction; which was then stored at -20^{o}C.

2.6 Oligo-dT purification of RNA

Poly(A)$^+$ RNA was prepared by oligo-dT cellulose chromatography (Aviv and Leder, 1972).

6mg of total RNA was dissolved in 3.75mls of H_2O and 5mls of 20mM Tris-Cl pH 7.5, 2mM EDTA, 0.2% SDS added. The RNA was then heated at 60^{o}C for 3 min, cooled on ice and 1.25mls of 4M LiCl added.

The RNA was then loaded on a 2ml column of oligo-dT cellulose (Pharmacia, Milton Keynes, UK) that had been equilibrated with binding buffer (1M LiCl, 10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% SDS). The column was washed through with binding buffer until a constant, low OD_{260} of the eluate was obtained. The column was then washed through with 10mls of 0.3M LiCl, 10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% SDS; then 10mls of 0.12M LiCl, 10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% SDS.

Finally the column was heated to a temperature of 35°C by circulating water (at 55°C) through a jacket of plastic tubing wrapped round the column. The poly(A)$^+$ RNA was eluted with 10mls of prewarmed 10mM Tris-Cl pH 7.5, 1mM EDTA, 0.1% SDS. Fractions were collected and the amount of poly(A)$^+$ RNA estimated from the OD_{260} ($1OD_{260}$ = 40µg RNA).

The poly(A)$^+$ fraction was precipitated by adding 1/10 vol 4M LiCl, 2.5 vol EtOH and leaving at -20°C overnight.

2.7 Reovirus mRNA

Radioactive type 3 (Dearing) reovirus mRNA was a gift from M. McCrae (University of Warwick, UK).

This had been prepared as described in Drummond et al. (1985) and was labelled with ^{32}P to a specific activity of 10^4 dpm/µg.

2.8 DNA templates for in vitro transcription

pspLys$^+$ pspLys$^+$ (a gift from D. Jackson, University of Warwick, UK) is the chicken lysozyme cDNA, isolated as a Hind III fragment from pTK_2Lys^+ (Krieg et al., 1984), inserted into the Hind III site of psp64 (Melton et al., 1984), a vector which contains the sp6 RNA polymerase promoter. For transcription pspLys$^+$ was linearised using Eco RI. The resulting 541 base Lys$^+$ transcript contains (in addition to the coding region); 14 out of 31 bases from the 5', and 17 out of 117 bases from the 3' untranslated regions of lysozyme mRNA (Jung et al., 1980; Land et al., 1981).

psp82$^+$ psp82$^+$ (a gift from R. Strachan, University of Warwick, UK) is the Hind III fragment of pTK_282^+ (Krieg et al., 1981), which contains calf preprochymosin cDNA sequences, inserted into the Hind III site of psp64 (Metton et al., 1984). psp82$^+$ was linearised with Xba I. The resulting Chym$^+$ transcript (206 bases) contains in addition to the

coding region, 14 out of 25 bases from the 5', and 6 out of 136 bases from the 3' untranslated region of calf preprochymosin mRNA (Harris et al., 1982).

The first (5') 14 bases and last (3') 52 bases of the Lys^+ transcript; and the first (5') 19 bases and last (3') 24 bases of the $Chym^+$ transcript are psp64 and Hind III linker sequences (see Melton et al., 1984; Krieg et al., 1984).

pspOv$^+$ and pspOv$^-$. pspOv$^+$ and pspOv$^-$ (gifts from D. Jackson, University of Warwick, UK) contain the 1318 base Hind III fragment from pTK_2Ov^+ (Krieg et al., 1984) inserted in the Hind III site of psp64 (Metton et al., 1984) in opposite orientations. Both plasmids were linearised by Eco RI for transcription. Transcription from linearised pspOv$^+$ gave three sizes of transcript 1374 (full length), 900 nucleotides and 320 nucleotides. 72% of the radioactivity was in the 900 nucleotide transcript. This premature termination is probably caused by a prokaryotic-like termination sequence present in the ovalbumin coding region (L. Tabe, personal communication). No premature termination was found with pspOv$^-$ which gave only the 1374 base antisense transcript.

psp64-Xβm. psp64-Xβm (a gift from P. Krieg, Harvard, USA) contains the entire cDNA sequence (including untranslated regions) of Xenopus β globin mRNA inserted in psp64 (Krieg and Melton, 1984a). When linearised with Hinf I and transcribed the resulting Globin-Hinf transcript contains the entire 5' untranslated, coding and 28 bases of the 3' untranslated region of Xenopus β globin mRNA. Linearising with Pst I gives a transcript (Globin-Pst) which contains the entire mRNA sequence plus a 'tail' of 20 As and 30 Cs (Krieg and Melton, 1984a).

2.9 In vitro transcription

Uncapped transcripts Transcriptions were carried out as described by Melton et al. (1984). A reaction containing 40mM Tris pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM DTT, 2 units/µl RNasin (Promega, Madison, USA), 100µg/ml BSA (nuclease free, BRL, Paisley, UK). 500µM rUTP, rCTP and rATP, 50µM rGTP, 200µCi/ml [^{32}P-α] rGTP, 50µg/ml linearised DNA template and 300 units/ml Sp6 RNA polymerase (NEN, Southampton, UK) was incubated at 40°C for 1h.

The amount of RNA transcribed was calculated from the percentage incorporation of [^{32}P-α] GTP into RNA (measured by DE81 counting). The specific activity of the transcripts was about 10^6 dpm/µg.

Capped transcripts To produce capped transcripts the reaction mix (above) was made 500µM m^{7}G(5')ppp(5')G (monomethyl cap) or 500µM m7G(5')ppp(5')Gm (dimethyl cap) (PL-Pharmacia, Milton Keynes, UK) (Konarska et al., 1984; Pelletier and Sonenberg, 1985).

2.10 Processing of transcripts

After transcription the DNA template was digested by adding 20µg/ml DNase I (RNase free) and incubating at 37°C for 10 min. Yeast tRNA was added as carrier to 200µg/ml and the reaction mix made 10mM EDTA. The reaction mix was then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1) before adding ammonium acetate to 0.7M; 2.5 volumes of ethanol and precipitating at -20°C. The precipitation was then repeated, recovery of transcript checked by DE81 counting, and the transcripts resuspended at 100ng/µl in H_2O for oocyte injection.

2.11 DE81 counting of RNA

Incorporation of [^{32}P-α] GTP into RNA was measured using DE81 count (Maniatis et al., 1982). RNA samples were spotted onto DE81 paper. The

DE81 paper was then washed five times (2 min each) in 0.5M Na_2HPO_4, once in H_2O, once in ethanol; then air dried before scintillation counting using Beckman Ready-solv EP (Beckman, Palo Alto, USA), an aqueous sample scintillation fluid.

2.12 Preparation of RNase-free DNase I

DNase I (supplied as 'RNase free', Worthington, Irvine, UK) was made RNase free by passage over a column of agarose coupled 5'-(p-aminophenylphosphoryl) Uridine 2'(3') phosphate (Miles-Yeda Ltd, Rehovot, Israel) (Maxwell et al., 1977).

A 5ml column of the resin was equilibrated with 0.02M sodium acetate pH 5.0. 20mg of DNase I in 1ml of 0.02M sodium acetate pH 5.0 was loaded onto the column, then eluted using 0.02M sodium acetate pH 5.0. RNase activity remains bound to the column. Fractions of the eluate were collected and OD_{280} measured ($1OD_{280}$ = 1mg of protein) and fractions containing DNase I were stored at -20°C.

Purity of the DNase I was assayed using uncapped radioactive transcripts. No degradation was detectable under experimental conditions. (Ribosomal RNA was found to be too insensitive for this assay).

2.13 Polyadenylation of transcripts

Transcripts were polyadenylated using poly(A) polymerase from E. coli (Sippel, 1973). The purified transcript (up to 10µg/ml) was polyadenylated in a reaction of 50mM Tris pH 8.0, 100µM rATP, 10mM $MgCl_2$, 250mM NaCl, 1mM $MnCl_2$, 50µg/ml BSA (nuclease-free, BRL, Paisley, UK), 35 units/ml poly(A) polymerase (BRL) at 37°C for periods up to 2h. The reaction was stopped by adding EDTA to 20mM; then phenol:chloroform extraction and ethanol precipitation was carried out as described in section 2.10.

The carrier tRNA used in the preparation of transcripts for polyadenylation (as well as the corresponding non-polyadenylated control) was first treated with periodate to prevent the tRNA acting as substrate for the polyadenylation reaction.

2.14 Periodate treatment of tRNA

To remove the 3'-OH groups from the carrier tRNA it was treated with periodate (Hunt, 1968).

125μg/ml of tRNA in 10mM potassium acetate pH 5.0, 1mM potassium periodate was incubated in darkness at room temperature for 10 min; then for 40 min on ice. 0.5 volumes of 20% glucose was added to quench the reaction and the RNA precipitated by adding 1/10 vol of 3M sodium acetate pH 5.5, 2 vol ethanol at -20°C. The tRNA was then ethanol precipitated twice before use.

2.15 Gel analysis of polyadenylated transcript

Polyadenylation of transcripts was monitored using acrylamide urea sequencing type gels (Sanger *et al.*, 1977) and Ov^+ transcripts as size markers.

The gels were 6% (wt/v) acrylamide, 0.3% (wt/v) bis acrylamide, 8M urea, 1 x TBE and were polymerised by adding 0.1% (wt/v) APS and 0.08% (v/v) TEMED. Running buffer was 1 x TBE pH 8.3 (0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA).

Transcripts were dissolved in 80% (v/v) deionised formamide, 0.3% (wt/v) Xylene cyanol FF, 0.3% (wt/v) bromophenol blue, 20mM EDTA (pH 7.5); heated at 80°C for 2 min, then loaded on the gel which had already been electrophoresed for 30 min.

The poly(A) tails were also sized on agarose formaldehyde gels using ^{32}P-DNA markers.

2.16 ^{32}P-DNA size markers

A Hind III, Eco RI digest of λC_{1857} DNA (Amersham International, Amersham, UK) was endlabelled by kinasing (Maxam and Gilbert, 1980). The final restriction digest of 12µg of DNA had 1/10 vol of 1M Tris-Cl pH 8.0, 1/50 vol of 10% SDS and 5u of calf intestinal phosphatase (Boehringer, Lewes, UK) added. It was incubated for 2h at 37°C then phenol:chloroform:isoamyl alcohol (25:24:1) extracted and ethanol precipitated. 12µg of DNA was kinased in a 10µl reaction containing 30µCi [^{32}P-γ] rATP, 50mM Tris-Cl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 3u of T4 DNA kinase (Boehringer, Lewes, UK) at 37°C for 30 min before phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation.

2.17 Frogs

Xenopus laevis frogs were purchased from South African Snake Farm (Fishoek, South Africa). The frogs were kept in tanks of water at 18°C; and fed twice a week on a diet of live blow-fly maggots and minced beef.

2.18 Microinjection of oocytes

Female frogs were anaesthetised in a solution of 0.1% MS222 (ethyl m-aminobenzoate methanesulphonic acid salt) (Sigma, Poole, UK). Part of the ovary was then surgically removed as described by Colman (1984); and the frogs allowed to recover in a 0.5% solution of NaCl.

Individual oocytes were removed from the ovary manually using watch-makers forceps. This left the oocytes with an intact layer of folicle cells. The oocytes were cultured at 18°C in modified Barth X solution (88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4$, 0.33mM $Ca(NO_3)_2$, 0.41mM $CaCl_2$, 7.5mM Tris-Cl pH 7.6, 10mg l^{-1} Benzyl penicillin, 10mg l^{-1} streptomycin sulphate) (Gurdon, 1972).

Microinjection of the oocytes, using a fine glass needle supported

in a micro-manipulator was as described by Colman (1984). Typically 40-50nl of solution was injected.

For protein labelling studies the Barth X solution was supplemented with either [^{35}S] methionine or [^{3}H] leucine at 1mCi/ml.

2.19 Maturation of oocytes

Oocytes were matured by incubating them overnight in Barth X solution containing 5µg/ml progesterone (Smith and Ecker, 1969). On maturation a white disk forms at the animal pole of the oocytes.

2.20 Unfertilised eggs

Female Xenopus laevis were induced to lay eggs by injecting 500 i.u. of human chorionic gonadotropin (Chorulon, Intervet Laboratories Ltd, Cambridge, UK) into the dorsal lymph sac (Thibier-Fouchet et al., 1976).

The eggs were dejellyed by incubation in a solution of 1% cystein pH 8.0 for 10 min. The eggs were rinsed in Barth X, then for injection and subsequent incubation were transferred to 5% Ficoll in ½ strength Barth X.

2.21 Enucleation of oocytes

Enucleation of oocytes was carried out as described by Colman (1984). The animal pole of the oocyte was punctured using a needle; and the nucleus squeezed out of the hole by compressing the oocyte. In this way intact nucleii and their corresponding cytoplasm were recovered.

2.22 Dissection of oocytes

After incubation the oocytes were frozen at -70°C. Oocytes for the 'Oh' timepoint were rinsed in Barth X before freezing. The oocytes were orientated on a piece of masking tape stuck to a thin strip of

aluminium. Excess Barth X was removed from round the oocyte, then the strip of aluminium was placed on dry-ice. Freezing occurred within a few seconds. The frozen ooctyes were then bisected along the animal-vegetal equator using a scalpel blade that had been cooled to <0°C. The scalpel was held 'free-hand' and the operation was followed using a 'dissecting' stereomicroscope.

Oocytes could be stored frozen at -70°C for several weeks with no detectable degradation of their RNA.

2.23 Extraction of RNA from oocytes or oocyte halves

Extraction of RNA was by the method of Kressmann *et al*. (1978). Groups of 15-20 oocytes or oocyte halves were homogenised at 4°C in 500µl of 10mM NaCl, 1.5mM $MgCl_2$, 1% SDS, 10mM Tris-Cl pH 7.5, 1mg/ml Proteinase K (Boehringer, Lewes, UK). Before homogenisation the buffer was preincubated at 37°C for 20 min to remove ribonuclease activity.

After homogenisation the homogenate was incubated at room temperature for 30 min. The homogenate was then made 0.3M NaCl, 10mM EDTA; and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and once with an equal volume of chloroform: isoamyl alcohol (24:1). The aqueous phase was precipitated overnight with two volumes of ethanol at -20°C, and the RNA pelleted by spinning for 10 min in an eppendorf microfuge. The RNA pellet was washed in 80% ethanol, vacuum dried and resuspended in sterile distilled water.

2.24 RNA gel electrophoresis

RNA from oocytes was separated on agarose formaldehyde gels (Goldberg, 1980).

RNA equivalent to 2.5 complete or 5 half oocytes (10µg RNA) was denatured by heating for 5 min at 60°C in MOPs buffer containing 50%

(v/v) deionised formamide, 6 % (w/v) formaldehyde. MOPs buffer is 20mM 3-(N-morpholino) propanesulfonic acid, 5mM sodium acetate, 1mM EDTA pH 7.0. After the RNA samples were quenched on ice, 0.1 vol of 50% (v/v) glycerol, 0.01% (wt/v) bromophenol blue was added. The samples were then immediately loaded onto a 1.5% agarose gel containing MOPs buffer with 6 % (w/v) formaldehyde. After electrophoresis, submerged in MOPs buffer, at 100V for 5h, the gels were soaked in 10% glycine for 30 min, stained with ethidium bromide (10µg/ml) and destained in distilled water.

After staining, gels containing radioactive RNAs were dried down onto Whatman 3MM paper (Whatman Inc., New Jersey, USA), then autoradiographed at -70^{o}C using Fuiji RX film with an intensifying screen.

2.25 Northern Blotting

Northern blots (Thomas, 1980) were made of gels containing non-radioactive mRNAs. After ethidium staining the gels were soaked for 20 min in 20 x SSC (20 x SSC is 3M NaCl, 0.3M trisodium citrate pH 7.0), then blotted overnight onto a 0.45 m pore nitrocellulose filter (Schleicher and Schuell, New Hampshire, USA) using 20 x SSC as solvent. After blotting the nitrocellulose filter was air dried, then baked at 80^{o}C for 2h in a vacuum oven.

The filter was prehybridised overnight at 42^{o}C in a sealed plastic bag of prehybridisation buffer (50% deionised formamide, 5 x SSC, 50mM sodium phosphate pH 6.5, 200µg/ml sonicated salmon sperm DNA, 0.02% (wt/v) bovine serum albumin, 0.02% (wt/v) Ficoll (mw 400,000), 0.02% (wt/v) polyvinylpyrolidone (mw 360,000)), using 50µl of buffer per cm^{2} of filter.

The prehybridisation buffer was removed and hybridisation buffer (2 parts prehybridisation buffer: 1 part 50% (wt/v) Dextran sulphate)

containing up to 15ng ml^{-1} of denatured nick-translated DNA probe added, using 30µl of buffer per cm^2 of filter. (This assumes that 20µl of prehybridisation buffer is retained per cm^2 of filter).

After hybridisation overnight at 42°C, the filter was washed 4 times (5 min each) in 2 x SSC, 0.1% SDS at room temperature, then 2 times (15 min each) in 0.1 x SSC, 0.1% SDS at 50°C. The damp blot was wrapped in plastic 'cling' film and autoradiographed at -70°C using Fuiji RX film with an intensifying screen.

2.26 Preparation of DNA for probes

Recombinant plasmid DNA was prepared by standard caesium chloride centrifugation (Clewell and Helinski, 1969).

Hind III digestion of the plasmid pTK_2Ov^+ (Krieg et al., 1984) gave a 1,318 base pair fragment containing the chicken ovalbumin coding sequence.

Hind III digestion of pTK_2Lys^+ (Krieg et al., 1984) gave a 485 base pair fragment containing chicken lysozyme coding sequences.

Hae III digestion of pβG1 (Jeffreys and Flavell, 1977) gave a 333 base pair fragment containing part of the rabbit β-globin coding sequence.

Restriction enzymes were purchased from BRL (Paisley, UK) and used according to the manufacturers' instructions.

DNA fragments were purified by agarose gel electrophoresis. DNA from the restriction digest was separated by electrophoresis in an agarose gel containing TEA buffer (TEA is 0.04M Tris-acetate, 0.002M EDTA pH 8.0). After ethidium staining, a 'sandwich' of Whatman 3MM paper and dialysis membrane was inserted into the gel, in front of the required DNA band. The DNA band was run into the 'sandwich'. DNA was eluted by centrifuging the 'sandwich' in a 500µl eppendorf tube with a hole pierced in its base, inside a 1500µl eppendorf tube, for 20 sec in

an eppendorf microfuge. The DNA was then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1) before adding 1/10 vol of 3M sodium acetate pH 5.5, 2 vol ethanol and precipitating overnight at -20°C.

2.27 Nick-translation of DNA

DNA for probes was labelled by nick-translation (Rigby et al., 1977). 100ng of the DNA fragment was incubated at 15°C for 2h in a 20µl reaction containing 2.4µl of (0.3M $MgCl_2$, 0.3M Tris-Cl pH 7.6, 0.3mg/ml BSA, 60mM β-mercaptoethanol), 1µl of 0.25 mM dATP, 1µl of 0.25mM dCTP, 1µl of TTP, 20µCi of [^{32}P-α] dGTP, 1µl of 10ng/ml DNase I (Sigma, Poole, UK), 10 units of DNA polymerase I (Kornberg, whole enzyme) (Boehringer, Lewes, UK). The reaction was stopped by adding 80µl of 5mM EDTA, 0.1M NaCl and extracting with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The labelled DNA was separated from unincorporated nucleotide on a sephadex G50 (medium) column (Pharmacia, Milton Keynes, UK). Typically a specific activity of 10^8dpm/µg was obtained. The probe was denatured by boiling for 5 min, then quenching on ice immediately before use.

2.28 Acid insoluble counts of RNA

Radioactive RNA samples were spotted onto Whatman No. 1 paper (Whatman, New Jersey, USA). The paper was then washed in two changes of ice cold 10% TCA for 15 min each, rinsed with ethanol, then acetone, and air dried. The filters were then scintillation counted using Beckman Ready-solv EP (Beckman, Palo Alto, USA), an aqueous sample scintillation fluid.

2.29 Oocyte fixation and sectioning

Oocytes were fixed in Bouins fixative (71% picric acid, 24%

formaldehyde, 5% glacial acetic acid) for 15 min at 4°C (Capco and Jeffery, 1982). The oocytes were then dehydrated through an ethanol series of 30%, 50%, 70%, 80%, 90%, 95% and absolute ethanol at 4°C for 15 min each; transferred to fresh ethanol and warmed to room temperature (20 min); 1:1 ethanol:toluene for 20 min, toluene (twice) 20 min each, paraplast saturated toluene for 40 min at 60°C, 3 changes of paraplast at 60°C for 1h each before embedding in paraplast. Paraplast is a paraffin wax mp. 58°C (BDH, Poole, UK).

Serial sections were made of the oocytes at a nominal 8µm thickness using a microtome. The sections were floated on H_2O on 'subbed' slides, and then dried on a hot-plate at 40°C for two days.

Slides were subbed (Gall and Pardue, 1970) by dipping clean slides in 0.5% gelatin (300 Bloom, Sigma, Poole, UK), 0.05% chrome alum (chromic potassium sulphate) and letting them air dry. For the subbing solution to be effective the 0.5% gelatin solution must be cool before the chrome alum is added (Pouradier and Burness, 1966). After air drying the slides were baked at 80°C for 8h, which is essential for efficient retention of the sections on the slides during subsequent processing (M. Vlad, personal communication).

Sections were rehydrated by two changes of toluene (15 min each), two of absolute ethanol (5 min each) and 2 min each in 95%, 90%, 80%, 70%, 50%, 30% ethanol, and 2 changes (2 min each) of sterile distilled H_2O.

2.30 Acridine orange staining of oocyte sections

Endogenous oocyte RNA was stained using Acridine orange, which specifically stains single stranded RNA orange (Kasten, 1967).

The procedure of Hafen *et al*. (1983) was used. Slides were rinsed for 5 min in 0.2M glycine pH 2.0. Then *in darkness* slides were incubated for 30 min in 0.5mg/ml acridine orange, 0.2M glycine pH 2.0;

and washed with two changes of 15 min each in 0.2M glycine pH 2.0.

Coverslips were mounted using hydromount (National Diagnostics, New Jersey, USA); and slides examined using a Zeiss epifluorescence microscope (on FITC setting).

As a control for the specificity of the staining, some sections were predigested with RNase A (made DNAse free, by boiling) (Sigma, Poole, UK) at 50µg/ml in 10mM Tris-Cl pH 7.6, 10mM KCl, 1mM $MgCl_2$ for 4h at 37°C before staining (Capco and Jeffery, 1982).

2.31 Autoradiographs of oocyte sections

After rehydration the sections were immersed in 5% TCA for 15 min at 4°C, rinsed in H_2O, air dried and dipped in NTB-2 emulsion (diluted 1:1 with H_2O) (Kodak, Rochester, New York, USA). The autoradiographs were exposed at 4°C in lightproof boxes containing "molecular sieve 4A" (BDH, Poole, UK) as dessicant. The autoradiographs were developed in Kodak D19 developer for 5 min, rinsed in water, then fixed in fresh 'Kodafix' for 5 min and washed in running H_2O for 30 min.

After dehydration the autoradiographs were rinsed in toluene; and the coverslips mounted using DPX (BDH, Poole, UK).

Slides were examined under 'darkfield' illumination: Only the silver grains and the pigment layer surrounding the oocyte appear as bright spots of light.

2.32 Extraction of oocyte proteins

Labelled oocytes were homogenised at 4°C in 40µl per oocyte of 100mM NaCl, 20mM Tris-Cl pH 7.4, 1% Triton X100, 1mM PMSF. The homogenate was spun for 4 min in an eppendorf microfuge to pellet the yolk. The clear middle phase was taken for analysis.

2.33 Acid insoluble counts of protein

Protein samples were spotted onto Whatman No. 1 paper. The filters were immersed in ice cold 10% TCA for 5 min. Then 10 min in boiling 5% TCA containing 3% casamino acids (Difco Laboratories, Detroit, USA), and 5 min in ice cold TCA. The filters were then rinsed with ethanol and acetone before air drying; and scintillation counting using Beckman Ready-solv EP (Beckman, Palo Alto, USA) aqueous sample scintillation fluid.

2.34 In vitro translation

Wheatgerm translation system (Roberts and Paterson, 1973) purchased from Amersham International (Amersham, UK) was used according to the manufacturers' instructions. The final concentration of potassium ion in the translation assay was 140mM.

Message dependent nuclease-treated reticulocyte lysate was a gift from Tim Hunt (Cambridge University, Cambridge, UK). This was used as described by Jackson and Hunt (1983). 100ng of synthetic mRNA was translated in an 11µl reaction containing 8µl of message dependent lysate and 2µl of master-mix. Master mix is 1 vol (2M KCl, 10mM $MgCl_2$), 1 vol 200mM creatine phosphate, 1 vol amino-acid mix (2mM of each amino acid except methionine), 1 vol of 15µCi of [^{35}S] methionine.

The reaction was incubated at 30°C for 90 min.

After the initial incubation the *in vitro* translation reactions were made 100µg/ml RNase A (Sigma, Lewes, UK) and incubated at 37°C for 10 min. This degraded the radioactive synthetic mRNAs, which would otherwise cause a high background on protein gels.

2.35 Immunoprecipitation of oocyte proteins

Proteins from oocytes or their incubation medium were either run directly on gels, or they were first immunoprecipitated (Valle *et al*..

1983). The homogenate from half an oocyte or incubation medium from two oocytes was made up to 500µl final volume of detergent buffer (100mM Tris-Cl pH 8.2, 1% (v/v) Triton X100, 1% SDS, 10mM $MgCl_2$, 1% (wt/v) deoxycholate, 10mM methionine, 1mM PMSF). An excess of the appropriate antibody was added, and left for 30 min at 4°C. An excess of fixed staphlococcus aureus membranes was added, and the mixture left shaking overnight at 4°C.

The immunoprecipitated protein was spun down for 20 sec in an eppendorf microfuge, and then washed and resuspended twice with detergent buffer.

2.36 Protein gels

Immunoprecipitated proteins were resuspended in 30µl of sample buffer. Non-immunoprecipitated total protein homogenate equivalent to 1/8 oocyte (5µl), or half of an in vitro translation (5µl), were added directly to 25µl of sample buffer. Sample buffer is 200mM Tris-Cl pH 8.8, 1M sucrose, 5mM EDTA, 0.01% (wt/v) bromophenol blue, 3% (wt/v) SDS.

1µl of 0.5M DTT was added to each sample, which was then boiled for 3 min and cooled to room temperature. The protein was alkylated (Krieg et al., 1984) by adding 5µl of 0.5M iodoacetamide and incubating at room temperature for 15 min. After spinning for 1 min in an eppendorf centrifuge the samples were loaded on an SDS-polyacrylamide gel (Laemmli, 1970). The stacking gel was 5% acrylamide, 0.138% bis-acrylamide, 125mM Tris-Cl pH 6.8, 0.1% SDS, which was polymerised by adding 0.08% ammonium persulphate, 0.06% TEMED. The main separating gel was 15% acrylamide, 0.412% bis-acrylamide, 375mM Tris-Cl pH 8.8, 0.1% SDS; and was polymerised by adding 0.04% ammonium persulfate, 0.06% TEMED.

Running buffer was 28.8g l^{-1} glycine, 6g l^{-1} Tris base, 1g l^{-1} SDS.

2.37 Gel fluorography.

Gels were fixed for 30 min in 45% methanol, 10% acetic acid, then fluorographed (Bonner and Laskey, 1974). The gels were soaked in 3 changes (30 min each) of DMSO, then 3h in 20% (wt/v) PPO in DMSO. The gel was rinsed in running water for 40 min before drying down onto Whatman 3MM paper under vacuum. The dried gels were autoradiographed at -70°C using Fuji RX film.

RESULTS

CHAPTER 3

RESULTS: STABILITY OF NATURAL mRNAs

3.1 Stability of natural mRNAs

The primary aim of this thesis was to examine the movement of mRNA injected into oocytes. However to ensure that the distribution of mRNA within the oocyte was a true reflection of movement, rather than the result of differential degradation, it was essential to first establish the stability of the injected mRNA. As described in the introduction, even though the stability of all the mRNAs used had been investigated before, the variation in the results obtained was such that their stability had to be reinvestigated using the same oocytes as in the movement experiments.

3.2 Stability of poly(A) mRNA

To measure mRNA stability oocytes were injected with either chicken oviduct poly(A)$^+$ mRNA (which contains ovalbumin, lysozyme, ovomucoid and conalbumin mRNAs) or rabbit globin (9S) mRNA (a mixture of α and β globin mRNAs). In each case about 50ng of the total RNA (in a volume of 50nl of H_2O) was injected into each oocyte. None of the mRNA preparations used was a pure single species of mRNA; in addition the oviduct mRNA preparation also contained ribosomal RNA. In contrast the rabbit globin 9S mRNA, which was taken through an additional stage of purification, contained no contaminating ribosomal RNA detectable by ethidium staining of a gel containing 1μg of the RNA (not shown).

At various times up to 48h after injection total RNA was extracted from the oocytes, using at least 15 oocytes for each time point, and run on denaturing formaldehyde agarose gels. The gels were ethidium stained as a control for gross RNA degradation and equivalent RNA recovery for each time point (fig. 1). The extraction method used gave on average an

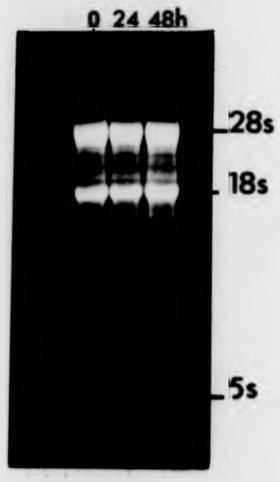
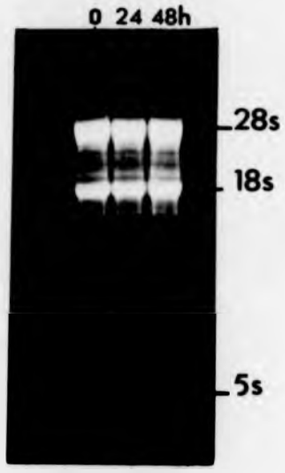


Figure 1. Stability of RNA in oocytes - gel of total RNA

RNA was extracted from whole oocytes at 0, 24 and 48 hours (h) after injection of 50ng of oviduct poly(A)$^+$ RNA per oocyte, and run on a denaturing agarose/formaldehyde gel. After electrophoresis, the gel was stained with ethidium to visualise the total RNA. A northern blot was then made of this gel and probed with nick-translated ^{32}P-labelled ovalbumin DNA (see fig. 2b).

The position of the 28S, 18S and 5S ribosomal RNAs are indicated.



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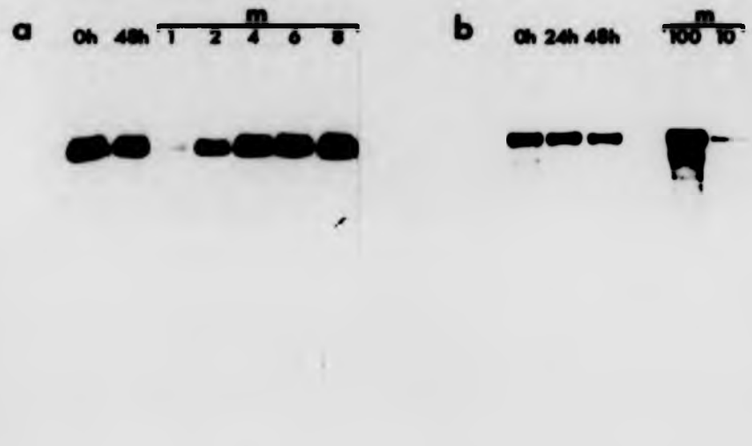
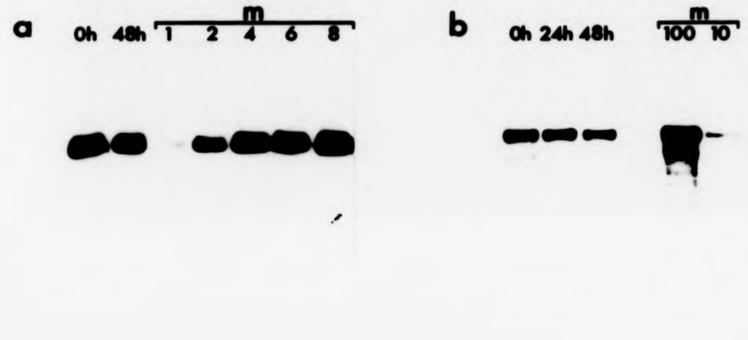


Figure 2. Stability of globin and ovalbumin mRNAs injected into oocytes

Oocytes were injected with 50ng each of either globin mRNA (a) or oviduct poly(A)$^+$ RNA (b). RNA was extracted from the whole oocytes at 0, 24 and 48 hours (h) after injection and run on denaturing agarose formaldehyde gels. The gels were stained with ethidium (see fig. 1) then blotted onto nitrocellulose filters which were probed with nick-translated ^{32}P-labelled β globin (a) or ovalbumin (b) DNAs. Marker tracks (m) contain uninjected RNA (1, 1ng; 2, 2ng; 4, 4ng; 6, 6ng; 8, 8ng; 10, 10ng; 100, 100ng).



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80% recovery of the sample RNA (data not shown). After ethidium staining the RNA from the gels was blotted onto nitrocellulose filters (northern blots). Ethidium staining did not affect the efficiency of blotting of the RNA (data not shown). The nitrocellulose filters were baked, probed with the appropriate nicktranslated DNA and autoradiographed (fig. 2). The best signal to noise ratio was obtained by using only the specific fragment of cDNA rather than the complete plasmid (data not shown).

Figure 2 shows that both the injected ovalbumin (fig. 2b) and globin mRNA (fig. 2a) remain stable up to 48h after injection. However accurate quantitation of the relative amounts of mRNA at each time point is difficult when using northern blots. This has two causes. First the response of the film used for autoradiography is linear only over a small range. Therefore the relative signal in each mRNA band is best quantified by cutting out the regions of the filter corresponding to the mRNA bands and scintillation counting them. Second the signal (amount of probe hybridised) is also only linear over a small range of mRNA concentrations, therefore a large number of calibration tracks containing different amounts of the non-injected mRNA must be used. Using this method, 70% of the β globin mRNA remained intact 48h after injection. Ovalbumin mRNA appears to be of similar stability.

3.3 Stability of poly(A)$^-$ mRNA

The stability of reovirus mRNA, which is naturally poly(A)$^-$, was also measured. This mRNA was made radioactive allowing more direct and easier quantitation than was possible with the poly(A)$^+$ mRNAs.

Oocytes were injected with 60ng each of ^{32}P-reovirus mRNA. After homogenisation of the oocytes from each time point, samples were removed and total and acid insoluble counts measured (Table 1). The remainder of the sample was purified as normal and run on a denaturing

Stability of Reo mRNA

RNA	Amount of RNA		
	0h	24h	48h
Total	100 (100)	55 (74)	48 (68)
25s	100	31	51
18s	100	37	39
12s	100	47	51

Table 1. Stability of reovirus mRNA

The experiment in Fig. 3a was quantified in two ways.

Total RNA: Aliquots of oocyte homogenate from each time point (0, 24 and 48 hours (h)) were precipitated with trichloroacetic acid. Unbracketed figures show the acid-insoluble counts for each time point as a percentage of the 0h value. Bracketed figures are the acid-insoluble counts for each time point expressed as a percentage of the total counts in the same aliquot, and corrected to give a 0h value of 100.

25S, 18S and 12S RNA: The 25S, 18S and 12S RNA bands were cut from the gel in fig. 3a and counted in a scintillation counter. The count for each time point is expressed as a percentage of the 0h value.

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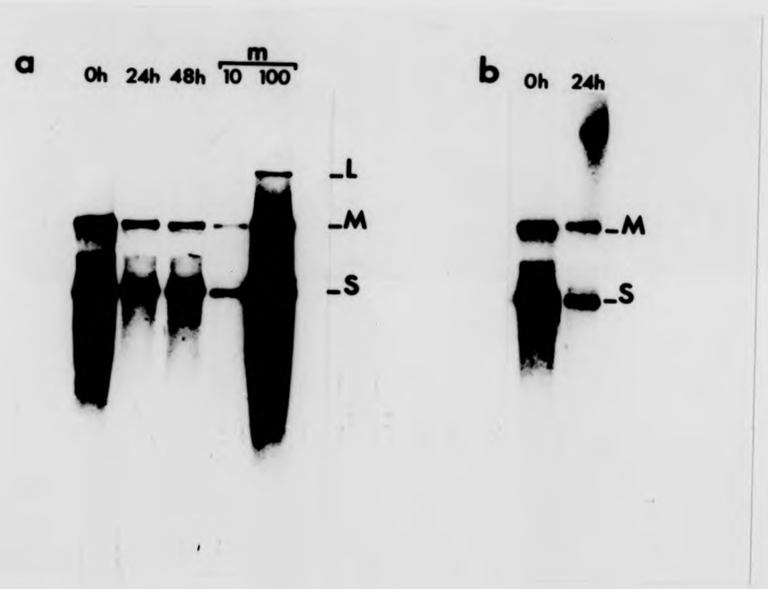
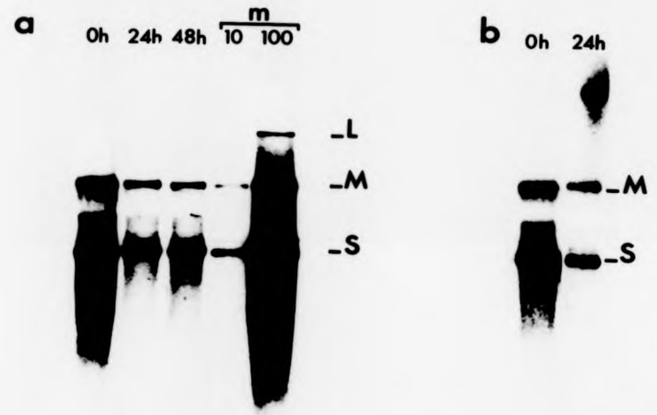


Figure 3. Stability of reovirus mRNA injected into oocytes

Oocytes were injected with either 60ng/oocyte (a) or 12ng/oocyte (b) of ^{32}P-labelled reovirus mRNA. RNA was extracted from whole oocytes at 0, 24 and 48 hours (h) after injection and run on denaturing agarose/formaldehyde gels. After electrophoresis the gels were ethidium stained, then dried down and autoradiographed directly. The marker tracks (m) contain uninjected RNA (10, 10ng; 100, 100ng). L, M and S are the large (25S), medium (18S), and small (12S) size classes of reovirus mRNA.

On the gels used the 25S size class of reovirus mRNA becomes very diffuse as it comigrates with the oocyte 28S ribosomal RNA.



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On the gels used the 25S size class of reovirus mRNA becomes very diffuse as it comigrates with the oocyte 28S ribosomal RNA.

formaldehyde agarose gel. The gel was ethidium stained, then dried down and autoradiographed directly (fig. 3a). Reovirus mRNA consists of 10 transcripts (Cashdollar et al., 1984) which run on the gels used in three size classes, Large (L) 25S, Medium (M) 18S, and small (S) 12S. The individual bands corresponding to the three size classes were cut from the gel and the amount of RNA quantified by scintillation counting (Table 1). The variation in the individual values is probably due to the smear of intermediate sizes of reovirus transcript which would be included in varying amounts in the region cut from the gel.

To test if the amount of RNA injected affected stability, oocytes were injected with 12ng each of ^{32}P-reovirus mRNA and analysed as described above (fig. 3b). After 24h, with both the high and low amounts of reovirus mRNA, 50% of the injected mRNA remains acid insoluble. Thus over a 5 fold range there is no difference in stability of the mRNA.

3.4 Stability of mRNA injected into different regions of the oocyte

In the experiments described in sections 3.2 and 3.3 the oocytes were injected at random sites. As I will describe later injected mRNA moves relatively slowly, therefore the injection site might conceivably affect stability of the mRNA. Therefore oocytes were injected with oviduct poly(A)$^+$ mRNA (50ng per oocyte) in either the animal, vegetal or equatorial region. The oocytes were incubated for 24h before extraction of RNA, northern blotting and probing with ovalbumin probe (fig. 4). No significant differences were found following injection in different regions of the oocyte.

3.5 Conclusions

All of the mRNAs tested were reasonably stable in oocytes. 70% of rabbit β globin mRNA remained stable 48h after injection into oocytes.

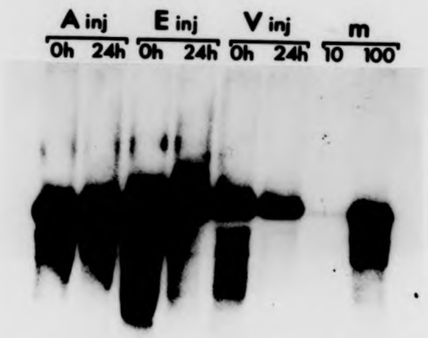
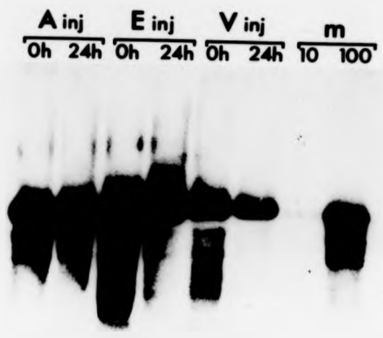


Figure 4. Stability of ovalbumin mRNA injected into the animal, equatorial and vegetal regions of the oocyte

Oocytes were injected in either the animal (A inj), equatorial (E inj), or vegetal (V inj) region with 50ng/oocyte of oviduct poly(A)$^+$ RNA. RNA was extracted from whole oocytes at 0 and 24 hours (h) after injection and run on a denaturing agarose formaldehyde gel. The gel was stained with ethidium, then blotted onto nitrocellulose and probed with nick-translated ^{32}P-labelled ovalbumin DNA. The marker track (m) contains uninjected RNA (10, 10ng; 100, 100ng).



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The amount of chicken ovalbumin mRNA which remained stable was not as accurately quantified as the β globin mRNA, however at least 50% remained stable 48h after injection. The poly(A)$^-$ reovirus mRNA had similar stability to the poly(A)$^+$ mRNAs, and 50% remained stable 48h after injection. The stability of the mRNA was independent of the amount injected, or where it was injected in the oocyte.

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CHAPTER 4

RESULTS: MOVEMENT OF NATURAL mRNAs

4.1 Movement of natural mRNAs

The movement of mRNA was followed by examining the distribution of mRNA between the animal and vegetal halves of the oocyte at various times after injection. Since, as described in chapter 3, at least 50% of the injected mRNAs remained stable, and this stability was the same in all parts of the oocyte, the distribution of mRNA at any point would be the result of movement rather than differential degradation. Equally important are Oh controls which give the initial distribution of the mRNA immediately after injection, and control for injection artifacts.

4.2 Movement of mRNA injected into oocytes

Having established that at least 50% of the injected mRNA remained stable, it was possible to examine its movement. RNA was injected at either the animal or vegetal pole of the oocyte. After incubation the oocytes were frozen on dry-ice, then sectioned along their equator to give a (brown) pigmented animal half and (yellow) non-pigmented vegetal half. The animal 'half' corresponds to 45% of the oocyte's total volume (see appendix I). At least 15 halves were pooled for each time point, the RNA extracted and run on denaturing agarose gels. Again the gels were ethidium stained as a control for RNA degradation, and the accuracy of the sectioning (fig. 5). The gels were blotted onto nitrocellulose filters and the filters probed with the appropriate nick-translated DNA. In figure 6 the oocytes were injected with oviduct poly(A)$^+$ RNA and incubated for up to 24h. The autoradiographs show little movement of ovalbumin mRNA into the non-injected half of the oocyte within the first 24h.

In case this slow movement was specific to mRNA coding for secreted

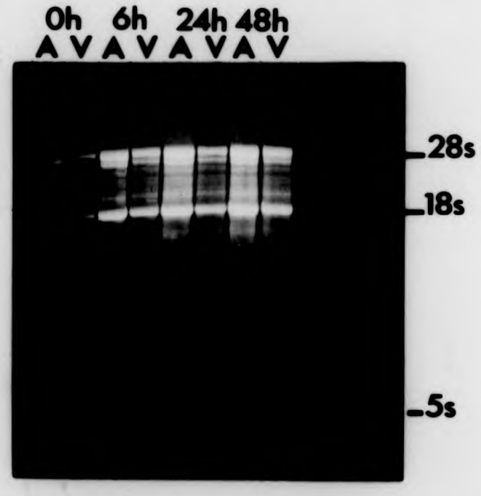

Figure 5. Movement of RNA in oocytes - gel of total RNA

Oocytes were injected in the animal pole with 10ng/oocyte of globin mRNA. At 0, 6, 24 and 48 hours (h) after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on a denaturing agarose/formaldehyde gel which was ethidium stained.

The position of the 28S, 18S and 5S ribosomal RNAs are marked.

After staining this gel was blotted onto nitrocellulose and probed with nick-translated ^{32}P-labelled globin DNA (see fig. 10a).

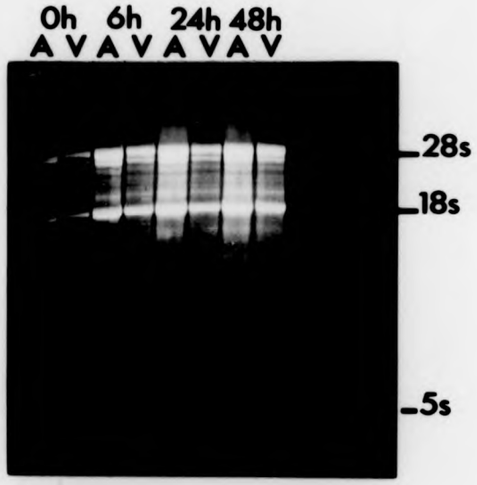

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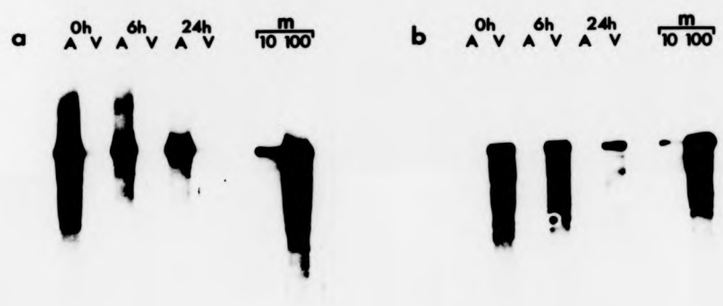
Figure 6. Movement of ovalbumin mRNA in oocytes

Oocytes were injected with 50ng/oocyte of oviduct poly(A)$^+$ RNA in either the animal (a) or vegetal pole (b). At 0, 6 and 24 hours (h) after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the oocyte halves and run on denaturing agarose/formaldehyde gels. After ethidium staining the gels were blotted onto nitrocellulose, and the nitrocellulose filters probed with nick-translated ^{32}P-labelled ovalbumin DNA. The marker tracks (m) contain uninjected RNA (10, 10ng; 100, 100ng). A, animal half. V, vegetal half.



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protein, oviduct poly(A)$^+$ RNA was coinjected with globin mRNA and movement followed up to 48h after injection (fig. 7). In this experiment some movement is apparent by 24h and very obvious by 48h, especially following injection into the vegetal pole. All the mRNAs, lysozyme, ovalbumin and globin, have a distinct asymmetry in their rate of movement: moving faster from the vegetal to the animal half than in the opposite direction. Due to quantitation difficulties with the blots (see section 3.2) it is impossible to comment precisely on the relative rates of movement of the different mRNAs. However they all appear similarly slow since in no case is equilibrium achieved within 48h of injection.

4.3 Injected mRNAs are not sequestered in the oocyte nucleus

The pigmented animal half of the oocyte contains the large germinal vesicle or nucleus, which forms 10% of the accessible volume of the oocyte (Bonner, 1975). To eliminate this as a cause of the asymmetric rates of movement, for example by injected mRNAs being deposited into the nucleus or entering it following injection, the following experiments were carried out.

Oviduct poly(A)$^+$ RNA was injected into the animal pole of oocytes and the oocytes incubated for 3h. The oocytes were enucleated by puncturing the top of the oocyte and squeezing out the nucleus. RNA was extracted from the nucleus and cytoplasm of the same oocyte, run on gels, blotted as before and probed with lysozyme or ovalbumin nicktranslated DNA. Figures 8a and 8b show that no lysozyme or ovalbumin mRNA was detectable in the nucleus.

As a second control globin mRNA was injected into the vegetal pole of oocytes. After 48h, when globin mRNA was present in both halves of the oocyte, none was detectable within the nucleus (fig. 8c).

These results imply that injected mRNA has an exclusively

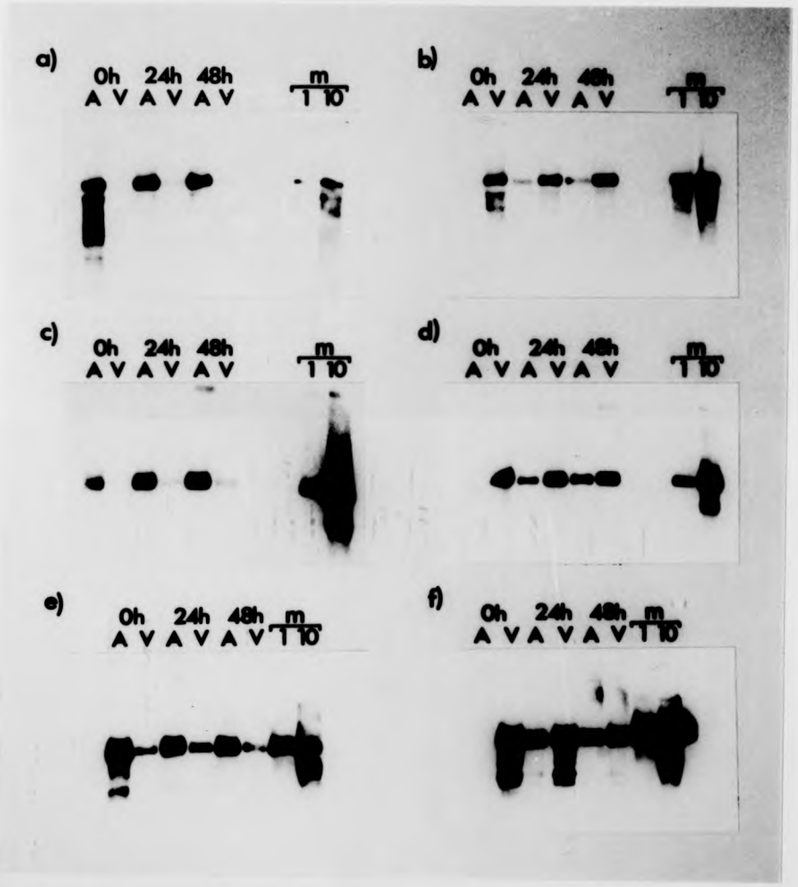

Figure 7. Movement of ovalbumin, lysozyme and globin mRNA in oocytes

Oocytes were coinjected with oviduct poly(A)$^+$ RNA (25ng/oocyte) and globin mRNA (25ng/oocyte) in either the animal (a, c and e) or vegetal (b, d and f) poles. At 0, 24 and 48 hours (h) after injection the oocytes were frozen, and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the oocyte halves, and aliquots from each sample were run on three denaturing agarose/formaldehyde gels. After ethidium staining the gels were blotted onto nitrocellulose, and the filters probed with ^{32}P-labelled nick-translated ovalbumin (a and b), lysozyme (c and d), and β globin (e and f) DNAs. a, c and e are from animal pole injected, and b, d and f from vegetal pole injected oocytes. A, animal half; V, vegetal half. Marker tracks (m) contain the appropriate uninjected RNA (1, 10ng; 10, 100ng).

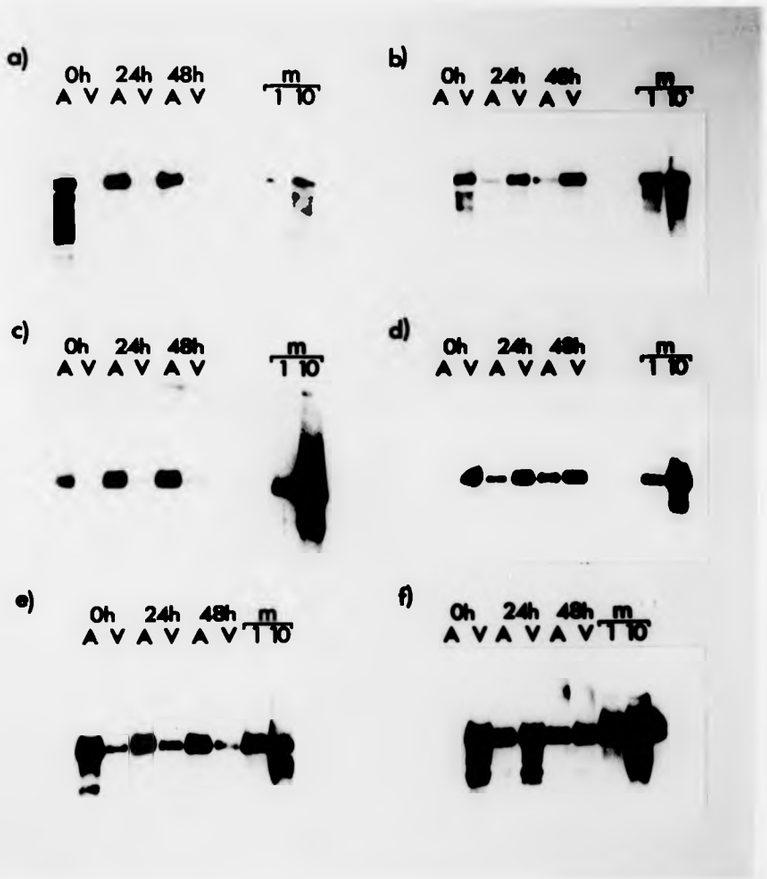

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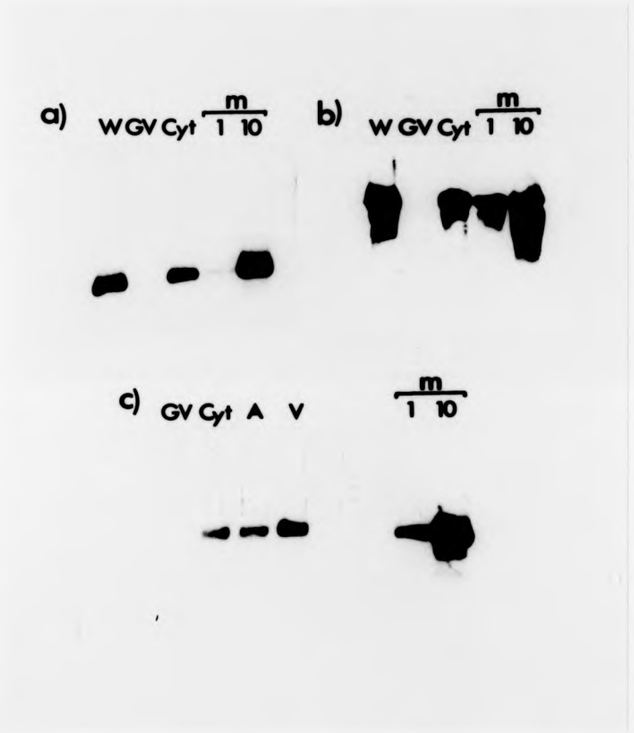
Figure 8. Partition of injected RNA between the oocyte nucleus and cytoplasm

(a and b) Oviduct poly(A)$^+$ RNA (50ng/oocyte) was injected into the animal pole of oocytes. The oocytes were manually enucleated 3 hours later and RNA extracted from the oocyte germinal vesicle (GV), and the corresponding enucleated cytoplasms (Cyt). RNA was also extracted from whole oocytes (W). Equivalent aliquots of each sample were run on denaturing agarose formaldehyde gels, blotted onto nitrocellulose filters and the filters probed with ^{32}P-labelled nick-translated lysozyme (a) or ovalbumin (b) DNAs.

(c) Globin mRNA (50ng/oocyte) was injected into the vegetal pole of oocytes. After incubation for 48 hours, RNA was extracted from the pooled animal (A) or vegetal (V) halves, or from germinal vesicles (GV) and their corresponding enucleated cytoplasm (Cyt). Aliquots were electrophoresed on a denaturing agarose/formaldehyde gel which was blotted onto nitrocellulose, and the filter was probed with ^{32}P-labelled nick-translated β globin DNA. Marker tracks (m) contain the appropriate uninjected RNA (1, 10ng; 10, 100ng).

All of the germinal vesicle (GV) samples had an equivalent number of whole non-injected oocytes added to provide carrier RNA before extraction and electrophoresis of the RNA.



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(a and b) Oviduct poly(A)$^+$ RNA (50ng/oocyte) was injected into the animal pole of oocytes. The oocytes were manually enucleated 3 hours later and RNA extracted from the oocyte germinal vesicle (GV), and the corresponding enucleated cytoplasms (Cyt). RNA was also extracted from whole oocytes (W). Equivalent aliquots of each sample were run on denaturing agarose formaldehyde gels, blotted onto nitrocellulose filters and the filters probed with ^{32}P-labelled nick-translated lysozyme (a) or ovalbumin (b) DNAs.

(c) Globin mRNA (50ng/oocyte) was injected into the vegetal pole of oocytes. After incubation for 48 hours, RNA was extracted from the pooled animal (A) or vegetal (V) halves, or from germinal vesicles (GV) and their corresponding enucleated cytoplasm (Cyt). Aliquots were electrophoresed on a denaturing agarose/formaldehyde gel which was blotted onto nitrocellulose, and the filter was probed with ^{32}P-labelled nick-translated β globin DNA. Marker tracks (m) contain the appropriate uninjected RNA (1, 10ng; 10, 100ng).

All of the germinal vesicle (GV) samples had an equivalent number of whole non-injected oocytes added to provide carrier RNA before extraction and electrophoresis of the RNA.

cytoplasmic location.

4.4 Asymmetric distribution of endogenous oocyte RNA

The animal half of the oocyte contains more RNA than the vegetal half (see fig. 5). The amount of total oocyte RNA extracted from each half was quantified by measuring its OD_{260}. On average the animal half contains 60% of the total RNA. An oocyte section stained with acridine orange (which stains RNA orange) shows that most of the RNA is concentrated in the region round the nucleus (fig. 9).

Thus the injected mRNA, which is moving towards an equilibrium distribution with most of the mRNA concentrated in the animal half, is tending towards the same distribution as the endogenous oocyte RNA.

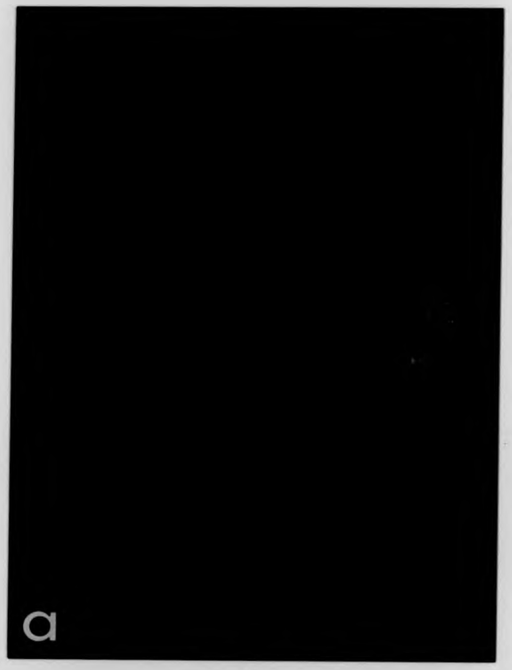
4.5 Effect of amount of mRNA injected on its rate of movement

To compare rate of movement after injection of different amounts of RNA, oocytes were injected in the animal or vegetal pole with either 50ng or 10ng of globin mRNA. The oocytes were incubated for up to 48h and analysed as before (fig. 10). There is no major difference in the rate of movement following injection of the different amounts of mRNA.

This was repeated using the poly(A)$^-$ ^{32}P-reovirus mRNA at either 60ng or 12ng per oocyte. Again there is little difference in the rates of movement of the different amounts of reovirus mRNA (fig. 11). Also the poly(A)$^-$ reovirus mRNA displays the same asymmetry in its rate of movement (faster from vegetal to animal half than in the opposite direction) as the poly(A)$^+$ mRNAs.

4.6 Movement of mRNA injected into matured oocytes and unfertilised eggs

As the injected mRNA was not entering the nucleus (see section 4.3) it was possible the nucleus might pose a large physical barrier to the



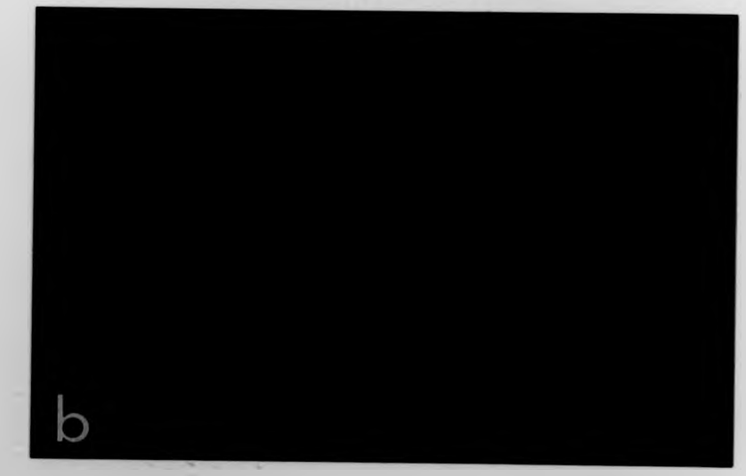


Figure 9. Distribution of endogenous oocyte RNA

Non-injected oocytes were fixed in bouins fixative, dehydrated and embedded in paraffin wax. Serial sections were made of the oocytes and, after mounting on slides, the sections were rehydrated and stained with Acridine orange (stains RNA orange). Before staining the section in (b) was predigested with RNase A, to remove RNA. The section in (a) received no pretreatment. Sections were viewed using a Zeiss epifluorescent microscope.

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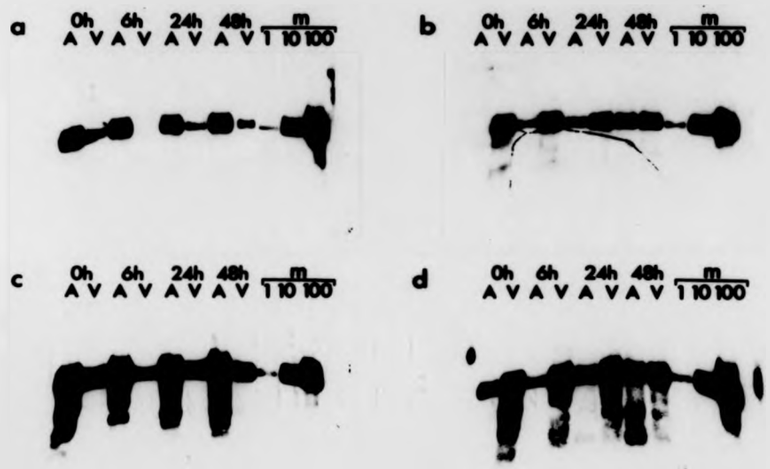


Figure 10. Effect of concentration on movement of globin mRNA

Globin mRNA at either 50ng/oocyte (c and d) or 10ng/oocyte (a and b) was injected into the animal (a and c) or vegetal (b and d) poles of oocytes. At 0, 6, 24 and 48 hours (h) after injection oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on denaturing agarose/formaldehyde gels which, after ethidium staining (fig. 5), were blotted onto nitrocellulose. The nitrocellulose filters were probed with nick-translated ^{32}P-labelled β globin DNA. Marker tracks contain uninjected RNA (1, 1ng; 10, 10ng; 100, 100ng). A, animal half; V, vegetal half.

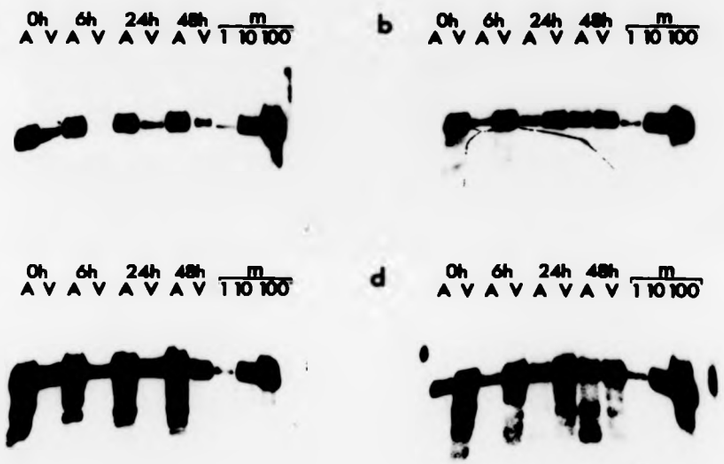


Figure 10. Effect of concentration on movement of globin mRNA

Globin mRNA at either 50ng/oocyte (c and d) or 10ng/oocyte (a and b) was injected into the animal (a and c) or vegetal (b and d) poles of oocytes. At 0, 6, 24 and 48 hours (h) after injection oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on denaturing agarose/formaldehyde gels which, after ethidium staining (fig. 5), were blotted onto nitrocellulose. The nitrocellulose filters were probed with nick-translated ^{32}P-labelled β globin DNA. Marker tracks contain uninjected RNA (1, 1ng; 10, 10ng; 100, 100ng). A, animal half; V, vegetal half.

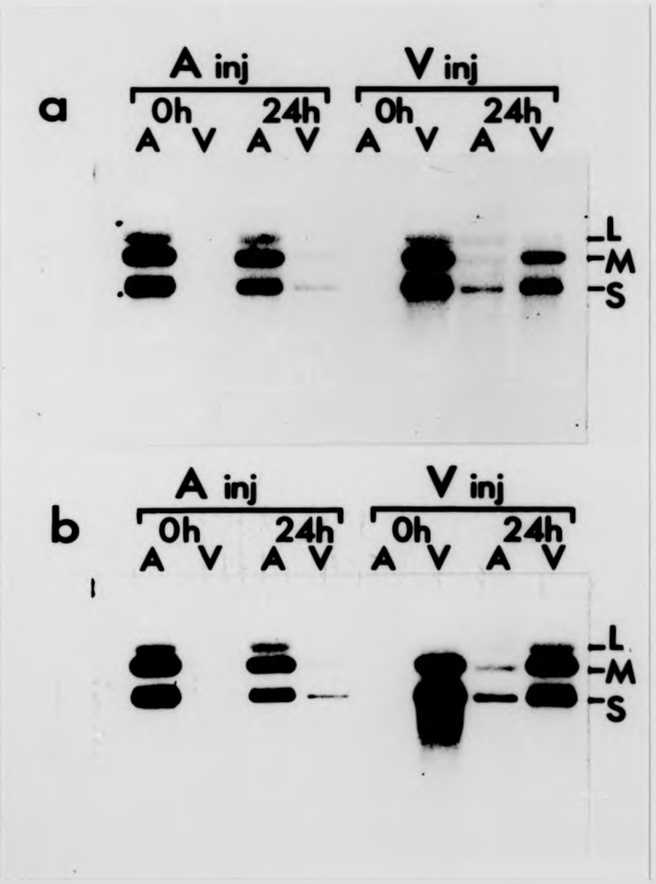


Figure 11. Effect of concentration on movement of reovirus mRNA

^{32}P-labelled reovirus RNA, at either 12ng/oocyte (a) or 60ng/oocyte (b), was injected into the animal (A inj) or vegetal (V inj) poles of oocytes. At 0 and 24 hours (h) after injection oocytes were sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on denaturing agarose/formaldehyde gels. After ethidium staining the gels were dried down and autoradiographed directly. L, M and S mark the position of the Large (25S), medium (18S) and small (12S) size classes of RNA. The large (L) size class runs as a diffuse band as it comigrates with the oocyte 28S rRNA band on these gels.

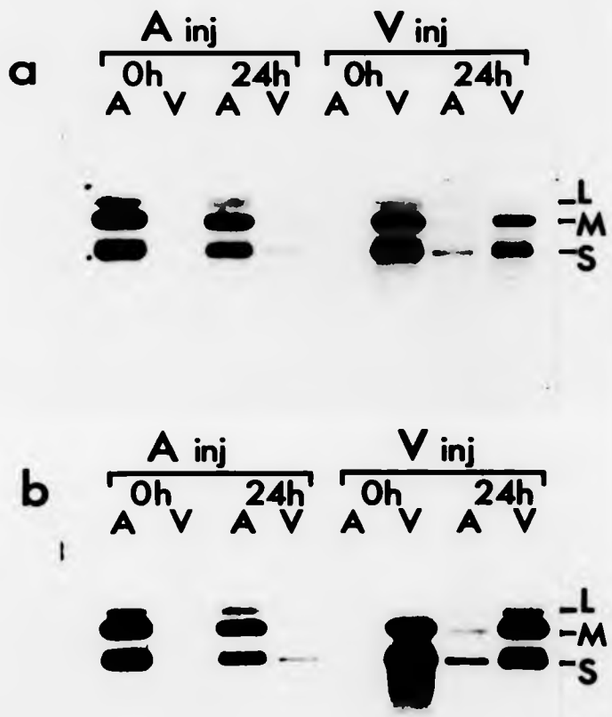


Figure 11. Effect of concentration on movement of reovirus mRNA

^{32}P-labelled reovirus RNA, at either 12ng/oocyte (a) or 60ng/oocyte (b), was injected into the animal (A inj) or vegetal (V inj) poles of oocytes. At 0 and 24 hours (h) after injection oocytes were sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on denaturing agarose/formaldehyde gels. After ethidium staining the gels were dried down and autoradiographed directly. L, M and S mark the position of the Large (25S), medium (18S) and small (12S) size classes of RNA. The large (L) size class runs as a diffuse band as it comigrates with the oocyte 28S rRNA band on these gels.

mRNA and slow its movement. Therefore mRNA movement was compared in oocytes and progesterone matured oocytes (fig. 12a and b). On treatment with progesterone the oocyte nucleus breaks down and should no longer act as a barrier to mRNA movement. In the 6h period of the experiment there is no detectable difference in the movement of lysozyme from the animal to vegetal half of the normal or matured oocytes.

However immediately after injection in the vegetal pole (< 10 min) of matured oocytes lysozyme mRNA was present in both halves of the oocyte (fig. 12b). This result was consistently obtained with different batches of matured oocytes, but not the corresponding normal oocyte controls where movement was as usual slow. A similar effect was found with unfertilised eggs (fig. 12c), except that the rapid (almost instantaneous) movement of the injected mRNA occurred following both animal and vegetal pole injections.

4.7 Effect of translation on rate of mRNA movement

At least some of the injected mRNA is being translated. To test if this influenced the rate of mRNA movement, oviduct poly(A)$^+$ RNA was injected into oocytes whose translation had been arrested prior to injection by cycloheximide (fig. 14). No increase in the rate of movement from the animal to the vegetal half was found (fig. 13), indeed even in the grossly overexposed autoradiograph shown very little movement was detectable. Also arrest of translation does not appear to cause a significant increase in degradation of the mRNA.

4.8 Effect of slow rate of mRNA movement on translation

Since the distribution of mRNA in the oocyte depended on where it had been injected, the injection site used might also influence translation of the mRNA. To test this, oocytes were injected in either the animal or vegetal pole with oviduct poly(A)$^+$ RNA, and labelled for

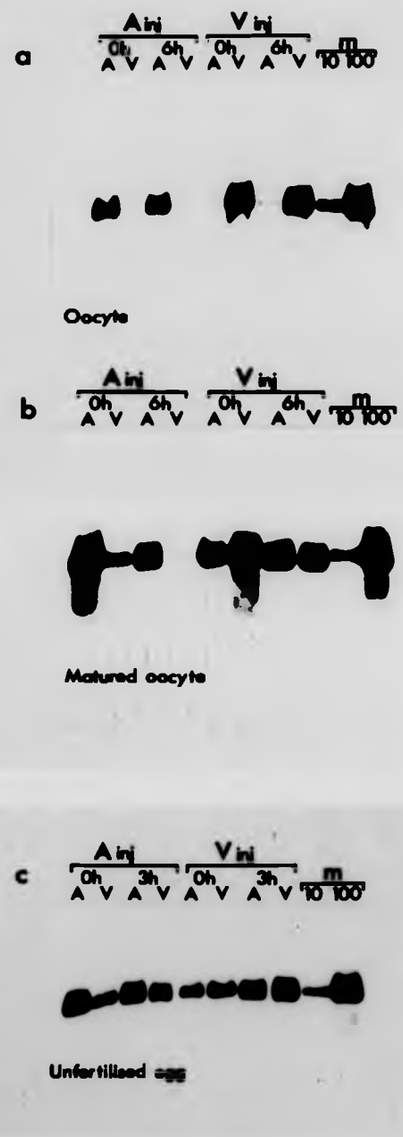
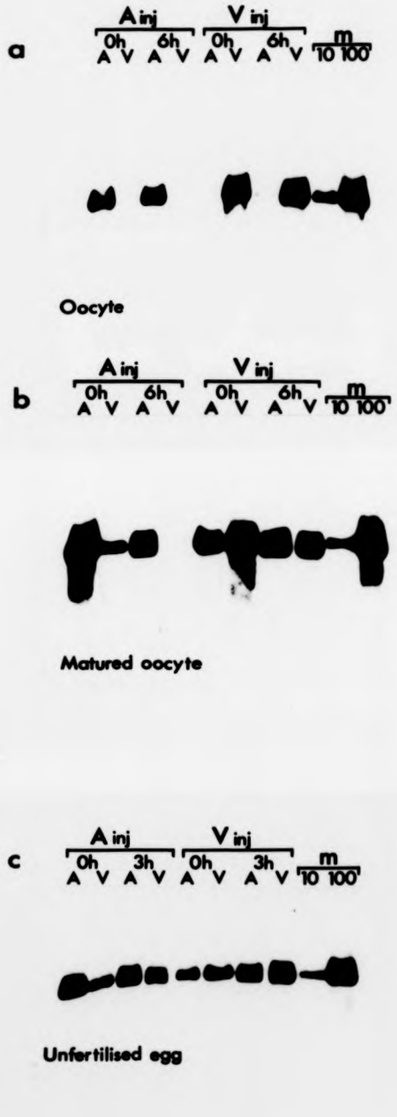


Figure 12. Movement of lysozyme in mRNA in oocytes, matured oocytes and unfertilised eggs

Oviduct poly(A)$^+$ RNA (50ng/oocyte) was injected at the animal (A inj) or vegetal (V inj) pole of control (a) or progesterone-matured oocytes (b), or unfertilised eggs (c). At 0 and 6 hours (h) (for oocytes and matured oocytes), or 0 and 3 hours (h) (for unfertilised eggs) they were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the halves and run on denaturing agarose/formaldehyde gels; which, after ethidium staining, were blotted onto nitrocellulose. The nitrocellulose filters were probed with ^{32}P-labelled nick-translated lysozyme DNA. The marker tracks (m) contain uninjected RNA (10, 10ng; 100, 100ng).



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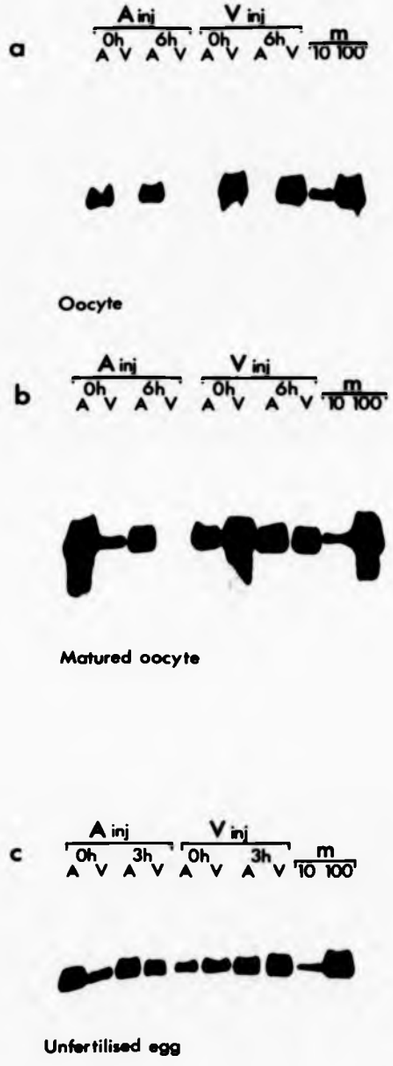

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Figure 13. Effect of cycloheximide on movement of ovalbumin mRNA

Before and after injection oocytes were incubated in medium containing 100μg/ml cycloheximide. Oviduct poly(A)$^+$ RNA (at 1mg/ml in a solution of 100μg/ml cycloheximide) was injected into the animal pole of the oocytes. At 0 and 24 hours (h) after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA was extracted from the oocyte halves and run on a denaturing agarose/formaldehyde gel. After ethidium staining the gel was blotted onto nitrocellulose and probed with ^{32}P-labelled nick-translated DNA.

As a control for the cycloheximide having prevented translation of the ovalbumin mRNA, some of the oocytes were labelled with ^{35}S-methionine over the 24h period of the experiment and analysed for protein synthesis (see Fig. 14 track C). No detectable synthesis of ovalbumin protein was found.

Figure 13. Effect of cycloheximide on movement of ovalbumin mRNA

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the 24h period immediately following injection. However, despite the different distribution of the mRNA over this period from the two injection sites (fig. 7), there was no significant difference in the amount of ovalbumin protein made (fig. 14).

4.9 Conclusions

The movement of both the poly(A)$^+$ and poly(A)$^-$ mRNAs is slow. In no case did the mRNA equilibrate within the oocyte, even by 48h after injection. This slow movement was independent of the amount of mRNA injected, and was similarly slow in oocytes whose translation was inhibited.

The injection site did affect the rate of mRNA movement, and movement was more rapid from the vegetal to animal half of the oocyte, than in the opposite direction. This asymmetric rate of movement was not caused by the nucleus which is in the animal half of the oocyte, since injected mRNA did not enter the nucleus. Also movement of mRNA was equally slow from the animal to vegetal half of matured oocytes, in which the nucleus has broken down.

CHAPTER 5

RESULTS: MOVEMENT OF PROTEINS ENCODED BY INJECTED mRNAs

5.1 Movement of ovalbumin protein in oocytes

Having established that all the mRNAs were of similar stability and were all slow moving, it was possible to examine the movement of the proteins translated from the mRNAs. Oocytes were injected with oviduct poly(A)$^+$ RNA and labelled for the 6h period immediately following injection. Injected but unlabelled control oocytes were taken for RNA analysis to confirm slow movement of the injected mRNA. During the 6h period of the experiment no movement of the mRNA into the noninjected half of the oocyte was detectable (not shown, see fig. 6). After labelling, the oocytes were sectioned into their animal and vegetal halves as for RNA analysis. Then following antibody precipitation (using anti-ovalbumin) the proteins from each half were run on an SDS-polyacrylamide gel which was fluorographed then autoradiographed (fig. 14). The ovalbumin protein moved faster than the corresponding RNA, with a large amount present in the noninjected half of the oocyte. Like mRNA the protein shows an asymmetric rate of movement: faster from vegetal to animal half than in the opposite direction.

The ovalbumin protein is present in three forms in the oocyte. The main (middle and upper) bands are glycosylated forms present in the endoplasmic reticulum, whilst the lower band is an unglycosylated form that is miscompartmentalised in the cytosol (Colman *et al.*, 1981). The cytosolic form is present in variable amounts in different batches of oocytes.

The same movement experiment was repeated in a different batch of oocytes, and the individual ovalbumin bands were cut from the fluorographed gel and quantified by scintillation counting. The cytosolic (unglycosylated) form of ovalbumin moved twice as fast as the

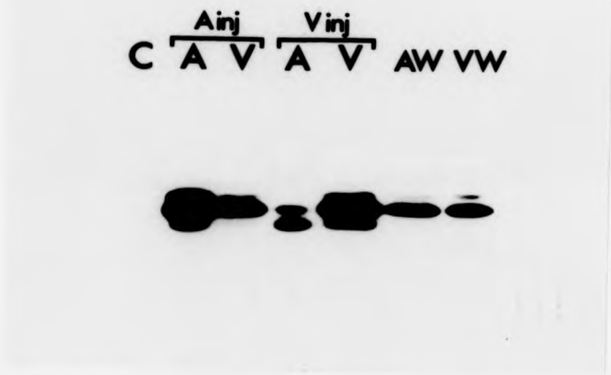


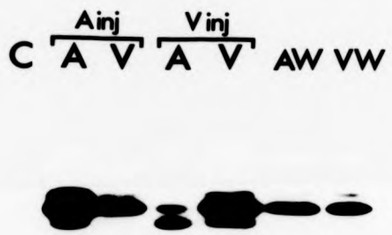
Figure 14. Ovalbumin protein synthesis and movement in oocytes

Oviduct poly(A)$^+$ RNA (50ng/oocyte) was injected at the animal (AW) or vegetal (VW) pole of oocytes, and the oocytes were cultured for 24 hours either in media containing 1mCi/ml ^{35}S-methionine (for protein analysis), or in unlabelled media (for RNA analysis). Whole ^{35}S-methionine labelled oocytes were then homogenised and immunoprecipitated with anti-ovalbumin antibody before electrophoresis on an SDS-polyacrylamide gel. RNA was extracted from unlabelled oocytes and analysed as described in fig. 2.

Track (C) is a control for the experiment described in fig. 13. Oocytes injected with 50ng/oocyte oviduct poly(A)$^+$ RNA were incubated in media containing cycloheximide (100 g/ml) and ^{35}S-methionine (1mCi/ml) for 24 hours, then analysed as described above.

A different batch of oocytes was injected at the animal (A inj) or vegetal (V inj) pole with 50ng/oocyte of oviduct poly(A)$^+$ RNA. Oocytes were then incubated in medium containing ^{35}S-methionine (1mCi/ml) for 6 hours. The oocytes were then frozen and sectioned into their animal (A) and vegetal (V) halves. The halves were then analysed for ovalbumin protein as described above.

In a parallel experiment (not shown, but see fig. 6) oocytes were incubated for 6 hours in unlabelled medium, then frozen, sectioned and analysed for movement of ovalbumin mRNA.



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In a parallel experiment (not shown, but see fig. 6) oocytes were incubated for 6 hours in unlabelled medium, then frozen, sectioned and analysed for movement of ovalbumin mRNA.

membrane bound (glycosylated) ovalbumin from the animal to vegetal half of the oocyte. In the opposite direction the cytosolic form moved 1.5 times as fast as the membrane bound form.

Both the cytosolic and membrane bound forms have an asymmetric rate of movement: faster from the vegetal to the animal half than in the opposite direction. The cytosolic ovalbumin was 2.2 times as fast moving from the vegetal to the animal half as in the opposite direction, whilst the membrane bound form was 3 times as fast.

5.2 Movement of ovalbumin, lysozyme and globin proteins in oocytes

To compare movement of different proteins, oocytes were coinjected with oviduct poly(A)$^+$ RNA (50ng/oocyte) and globin mRNA (25ng/oocyte). The oocytes were labelled with ^{35}S-methionine for the 6h period immediately following injection, then sectioned into their animal and vegetal halves. Again unlabelled control oocytes were analysed for mRNA movement (not shown). Aliquots from the pooled animal or vegetal half homogenates of the labelled oocytes were either precipitated using anti-chicken egg white antibody (precipitates both ovalbumin and lysozyme) (fig. 15a) or run directly on protein gels (fig. 15b). The appropriate bands were cut from the protein gel and scintillation counted. The distribution of each of the proteins is presented in figure 16.

Movement of all three proteins was faster from the vegetal to the animal half than in the opposite direction. Lysozyme (M_r 14,500) although the smallest protein is the slowest moving, followed by ovalbumin (M_r 44,000). Globin (M_r 16,000) is fastest, having reached an equilibrium distribution of 85:15 (animal:vegetal half) in 6h irrespective of the site of injection of the RNA.

This rapid movement and accumulation in the animal half of globin protein was also found in other batches of oocytes, although equilibrium was not always achieved within 6h.

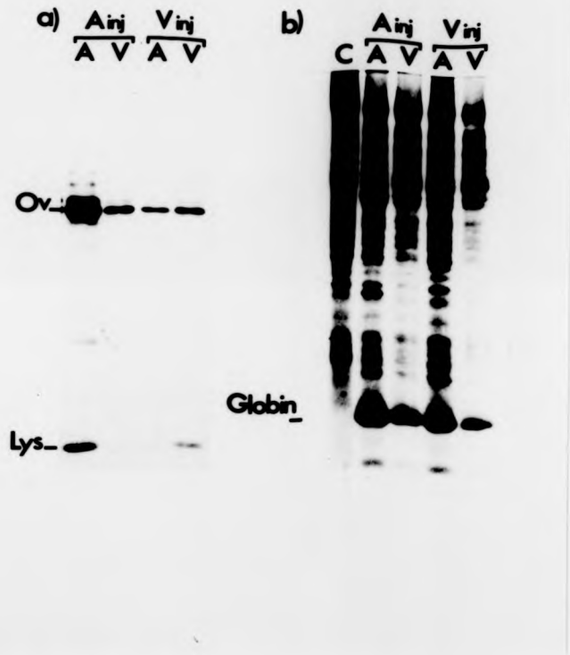
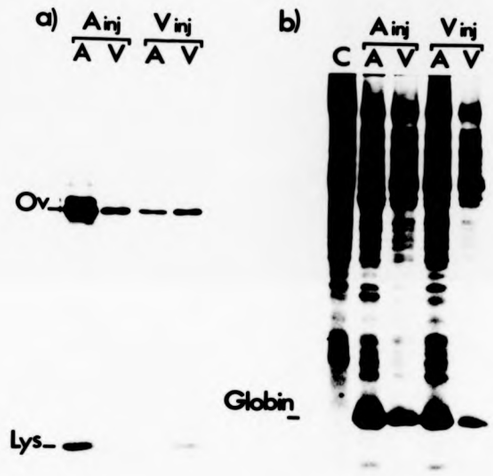


Figure 15. Distribution of ovalbumin, lysozyme and globin proteins in oocytes

Oviduct poly(A)$^+$ RNA (50ng/oocyte) and globin mRNA (25ng/oocyte) were coinjected into either the animal (A inj) or vegetal (V inj) pole of oocytes. The oocytes were incubated in media containing 0.5mCi/ml ^{35}S-methionine for the 6 hour period immediately following injection. The oocytes were then frozen and sectioned into their animal (A) and vegetal (V) halves, and homogenised. Aliquots from the homogenates were either first immunoprecipitated with anti chick egg white antibody (precipitates ovalbumin and lysozyme) (a) or run directly on an SDS-polyacrylamide gel (b). Lane C in b is from a non-injected oocyte. The position of ovalbumin (Ov), lysozyme (Lys) and globin are shown.

A parallel control experiment with non-labelled oocytes monitored RNA movement (not shown, see fig. 6, fig. 7 and fig. 10).



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A parallel control experiment with non-labelled oocytes monitored RNA movement (not shown, see fig. 6, fig. 7 and fig. 10).

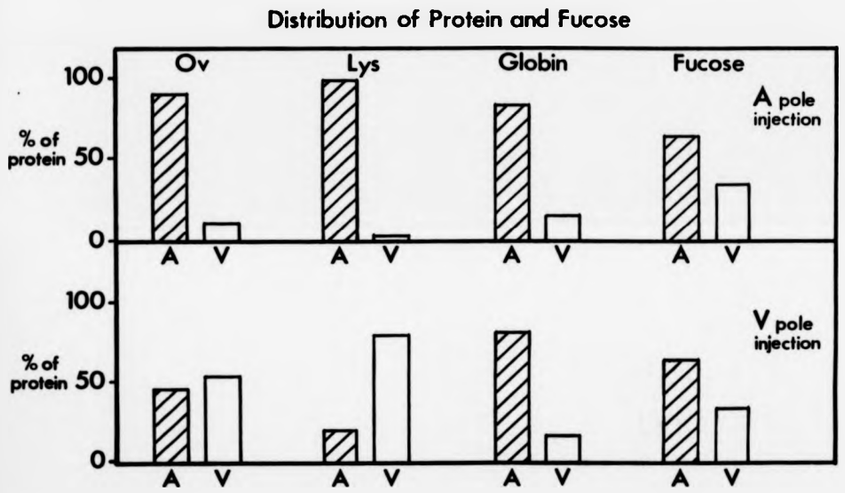
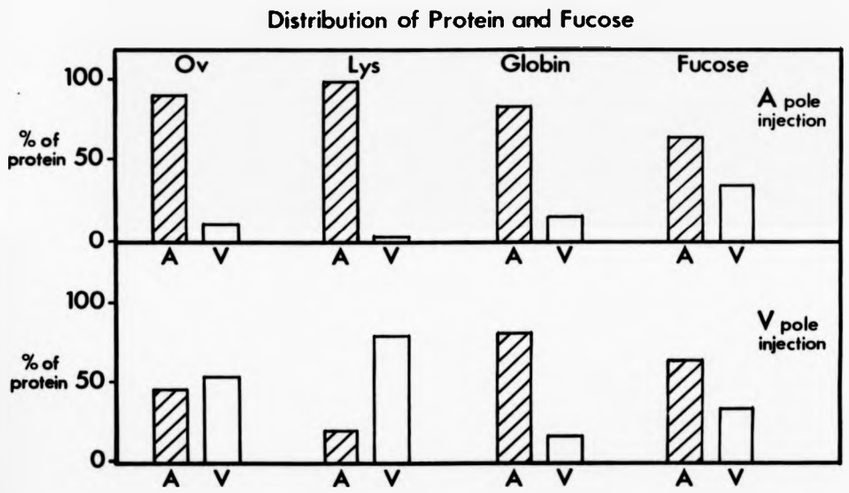


Figure 16. Distribution of proteins and fucose in oocytes

The bands corresponding to ovalbumin (Ov), lysozyme (Lys) and globin protein in the gels shown in fig. 15, were excised together with similar regions from control tracks of non-injected oocytes, and their radioactive content measured by scintillation counting. In the figure the amount of each protein in either the animal (A) or vegetal (V) half of the oocyte, is expressed as a percentage of the total amount of the protein present.

Also shown is the distribution of the sugar fucose.

Oocytes were injected in the animal or vegetal pole with 50nl of [^{3}H] fucose (1mCi/ml). After incubation for 3 hours the oocytes were frozen and sectioned into their animal and vegetal halves. The oocyte halves were homogenised in 100mM NaCl, 10mM Tris-Cl pH 7.6, 5mM $MgCl_2$ and aliquots removed and scintillation counted.



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5.3 Accessible volumes of the oocyte halves

To measure the accessible volume of cytoplasm in their animal and vegetal halves, oocytes were injected with ^{3}H-fucose. Fucose is a small sugar that is not readily metabolised in oocytes. The oocytes were incubated for 3h, frozen, sectioned and the amount of fucose in each half measured by scintillation counting. Irrespective of the injection site a distribution of 65:35 (animal:vegetal half) was found (fig. 16).

5.4 Conclusions

Proteins moved faster in oocytes than their corresponding mRNAs. Also, unlike mRNAs, there were significant differences in rate of movement of the different proteins. Globin protein was fastest, and equilibrated within the oocyte in the 6h period of the experiment with a distribution of 85:15 (animal:vegetal half). Both ovalbumin and lysozyme, which are sequestered in a membrane bound fraction of the oocyte moved more slowly. All of the proteins displayed asymmetry in their rate of movement, and were faster moving from the vegetal to animal half of the oocyte, than in the opposite direction.

CHAPTER 6

RESULTS: STABILITY OF SYNTHETIC RNAs

6.1 Synthetic RNAs

As described in section 3.2, there were difficulties in quantifying the amount of natural mRNAs using northern blots. These difficulties could be overcome by using radioactively labelled mRNAs, which allow a more direct quantitation of mRNA stability and movement. However it was only practical to make radioactively labelled reovirus mRNA; and even this was not entirely satisfactory as reovirus produces a mixture of 10 different sizes of mRNA.

While the work described in chapters 3-5 was in progress, a system was developed using Sp6 RNA polymerase which simplified transcription of RNA *in vitro*. This allowed production of specific single species of radioactively labelled transcripts; and essentially similar stability and movement studies were carried out using these synthetic RNAs, as have been described for natural mRNAs. The synthetic RNAs also extended the type of experiment possible, since *in vitro* transcription simplifies the manipulation of the RNA's structure; and the effect of capping and polyadenylation of the mRNA could be examined.

However, as I will describe below, such synthetic mRNAs are not exact copies of the natural mRNAs. Therefore results obtained with these synthetic mRNAs should be treated with caution.

6.2 The synthetic mRNAs

The behaviour of four types of transcript was examined.

Lys^+: a 541 base transcript encoding chicken lysozyme. It contains (in addition to the coding region), 14 out of 31 bases from the 5', and 17 out or 117 bases from the 3' untranslated regions of lysozyme mRNA (Jung *et al*., 1980; Land *et al*., 1981).

$Chym^+$: a 1206 base transcript encoding calf preprochymosin, which contains 14 out of 25 bases from the 5' and 6 out of 136 bases from the 3' untranslated regions of calf preprochymosin mRNA (Harris et al., 1982).

Ov^-: a 1318 base transcript that gives the chicken ovalbumin anti-sense strand transcript.

Lys^+, $Chym^+$ and Ov^- also have short vector and Hind III linker sequences at their 5' and 3' ends (Melton et al., 1984; Krieg et al., 1984).

Globin transcripts: Two transcripts which both encode Xenopus globin were used. Globin-Hinf contains the entire 5' untranslated, coding and 28 bases of the the 3' untranslated region of the Xenopus globin mRNA.

Globin-Pst contains the entire mRNA sequence plus 20 As and 20 Cs at its 3' end (Krieg and Melton, 1984a) (a fuller description of the transcripts is given in Materials and Methods, see section 2.8).

These synthetic mRNAs were transcribed in vitro, using Sp6 RNA polymerase, as run-off transcripts from linearised vectors (see Methods). The basic transcript contains neither a 'cap' structure nor the 3' poly(A) tail which are common features of most eukaryotic mRNAs.

6.3 Effect of capping on RNA stability

Most eukaryotic mRNAs have a cap structure at their 5' end consisting of a 7 methylguanosine base joined 5'-5' to the first nucleotide of the RNA by a triphosphate bridge. In addition usually the first and sometimes the second nucleotide of the RNA is methylated (Banerjee, 1980). The synthetic RNAs were capped by including a 10-fold excess of 'capping dinucleotide' in the transcription reaction (Konarska et al., 1984; Pelletier and Sonenberg, 1985). Either 7mG(5)ppp(5)G forming a monomethyl cap, or 7mG(5)ppp(5)Gm forming a dimethyl cap

structure was used.

To compare their relative stabilities; uncapped, monomethyl or dimethyl capped ^{32}P-Lys^{+} transcripts were injected into oocytes (about 5ng per oocyte). At various times up to 24h after injection RNA was extracted from the oocytes and run on denaturing agarose gels which were then dried down and autoradiographed (fig. 17a and b). The bands corresponding to the RNAs were cut from the gels and quantified by scintillation counting (Table 2). Only 10% of the uncapped Lys^{+} RNA remained after 24h. Capping increased the stability of the RNA with 45% and 41% of the monomethyl and dimethyl capped transcripts respectively remaining intact.

6.4 Stability of anti-sense transcripts

To compare the stability of an anti-sense transcript with a coding strand transcript ^{32}P-Ov^{-} (2.5ng per oocyte) was coinjected with ^{32}P-Lys^{+} (2.5ng per oocyte). Both transcripts were either uncapped (fig. 18a) or monomethyl capped (fig. 18b). The oocytes were incubated for up to 24h before extraction and analysis of RNA as described. No significant difference was found in the stability of the sense and anti-sense transcript in either the uncapped or capped form (Table 3).

6.5 Polyadenylation of synthetic RNAs

After transcription the synthetic RNAs were polyadenylated using E. coli poly(A) polymerase (Sippel, 1973). The length of poly(A) tail added was assessed by running the transcripts on an acrylamide sequencing type gel (fig. 19). Although in the preparation illustrated the transcripts have a reasonably uniform length of tail, in other experiments with different preparations of enzyme the polyadenylated transcript tended to form a continuous smear from the normal non-polyadenylated length of transcript upwards. In these experiments

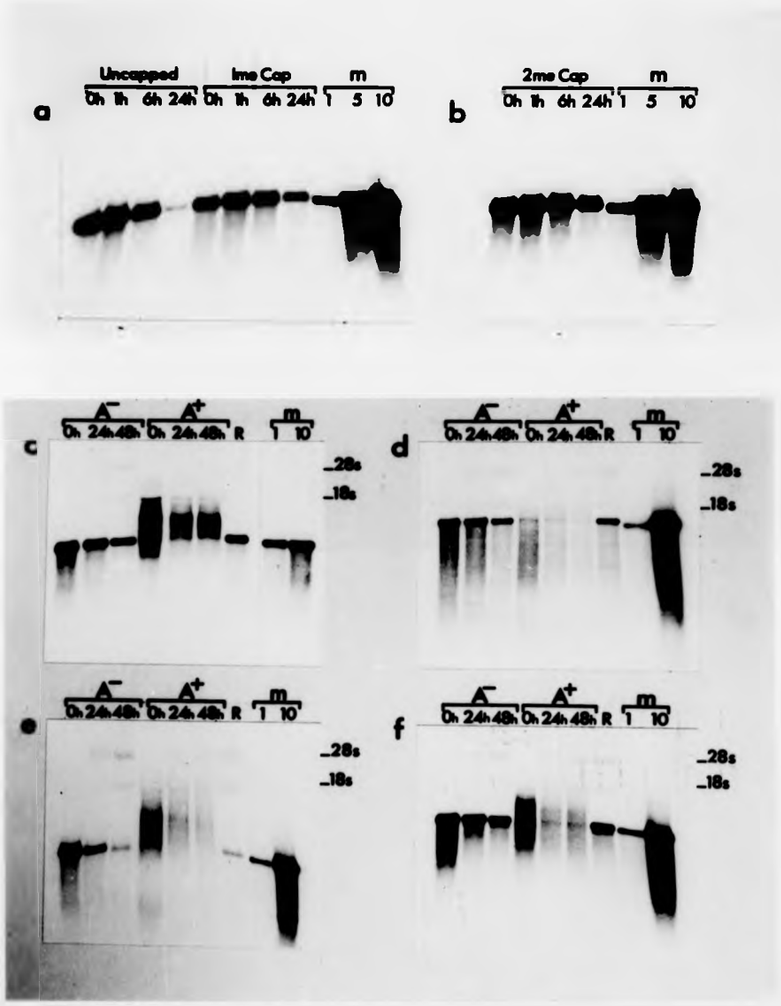
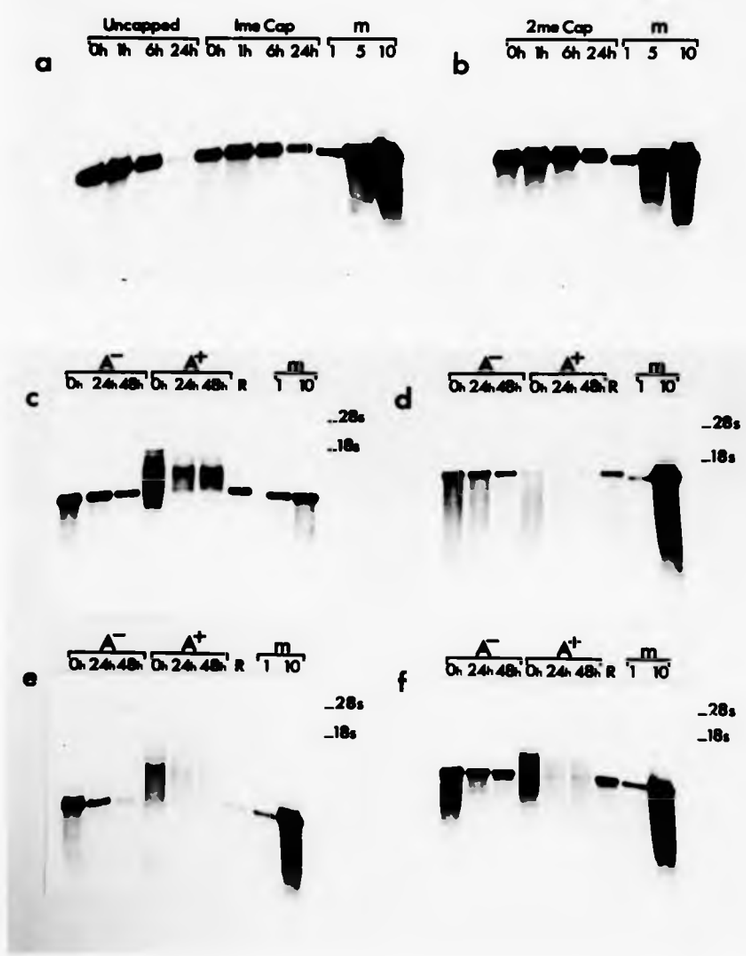


Figure 17. Stability of synthetic RNAs in oocytes

Oocytes were injected with the following ^{32}P-labelled in vitro transcripts (5ng/oocyte) (a) Lys$^+$ uncapped, Lys$^+$ monomethyl cap (1me cap); (b) Lys$^+$ dimethyl cap (2me cap); (c) Lys$^+$ monomethyl cap poly(A)$^-$ and poly(A)$^+$; (d) Chym$^+$ monomethyl cap poly(A)$^-$ and poly(A)$^+$; (e) Globin-Hinf monomethyl cap poly(A)$^-$ and poly(A)$^+$; (f) Globin-Pst monomethyl cap poly(A)$^-$ and poly(A)$^+$. At either 0, 1, 6 and 24 hours (h) after injection (a and b) or 0, 24 and 48 hours (h) after injection (c, d, e and f), RNA was extracted from whole oocytes and run on denaturing agarose/formaldehyde gels. After ethidium staining, the gels were dried down and autoradiographed directly.

(A)$^-$ nonpolyadenylated, and (A)$^+$ polyadenylated transcripts. The marker tracks (m) contain uninjected poly(A)$^-$ transcript (1, 1ng; 5, 5ng; 10, 10ng). Track R is a repeat of the poly(A)$^-$ 48h track. 28s and 18s are the oocyte 28S and 18S ribosomal RNA bands. These bands become prominent due to reincorporation of labelled nucleotides from degraded transcript.



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Stability of Capped RNA

Transcript	Amount of RNA		
	0h	6h	24h
Lys	100	61	10
Lys 1me Cap	100	90	49
Lys 2me Cap	100	82	45

Table 2. Stability of capped RNA

The experiment shown in fig. 17a and b was quantified by excising the RNA bands from the gels and scintillation counting them. The radioactive content of each band is expressed as a percentage of the 0h value. Lys, Lys^+ uncapped; Lys 1me cap, Lys^+ monomethyl cap; Lys 2me cap, Lys^+ dimethyl cap.

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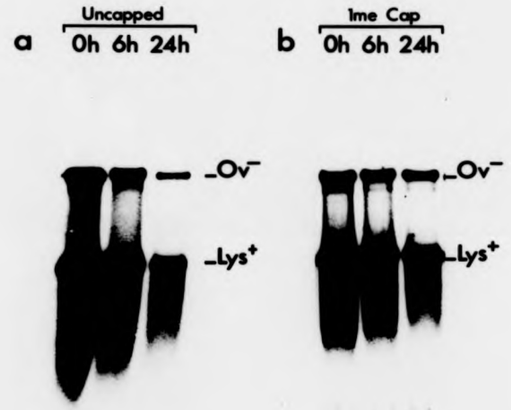

Figure 18. Stability of Lys^+ and Ov^- RNA in oocytes

^{32}P-labelled Ov^- (2.5ng/oocyte) and Lys^+ (2.5ng/oocyte) either uncapped (a) or monomethyl capped (1me cap) (b) were coninjected into oocytes. At 0, 6 and 24 hours (h) after injection RNA was extracted from whole oocytes and run on denaturing agarose/formaldehyde gels. The gels were stained with ethidium, then dried down and autoradiographed directly. The position of the Lys^+ and Ov^- transcripts are indicated.

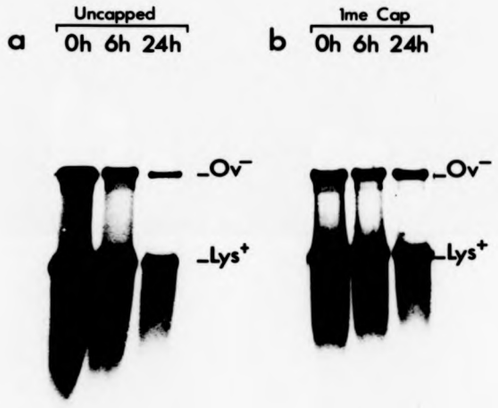

Figure 18. Stability of Lys^+ and Ov^- RNA in oocytes

^{32}P-labelled Ov^- (2.5ng/oocyte) and Lys^+ (2.5ng/oocyte) either uncapped (a) or monomethyl capped (1me cap) (b) were coninjected into oocytes. At 0, 6 and 24 hours (h) after injection RNA was extracted from whole oocytes and run on denaturing agarose/formaldehyde gels. The gels were stained with ethidium, then dried down and autoradiographed directly. The position of the Lys^+ and Ov^- transcripts are indicated.

Stability of Lys^+ and Ov^- RNA

Transcript	Amount of RNA		
	0h	6h	24h
Lys^+	100	62	24
Ov^-	100	48	19
Lys^+ 1me Cap	100	78	51
Ov^- 1me Cap	100	72	60

Table 3. Stability of Lys^+ and Ov^- RNA

The experiment shown in fig. 18 was quantified by excising the RNA bands from the gels, and scintillation counting them. The amount of radioactivity in each band is expressed as a percentage of the 0h value. Lys^+, Lys^+ uncapped; Ov^-, Ov^- uncapped; Lys^+ 1me cap, Lys^+ monomethyl cap; Ov^- 1me cap, Ov^- monomethyl cap.

Stability of Lys^+ and Ov^- RNA

Transcript	Amount of RNA		
	0h	6h	24h
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Ov^-	100	48	19
Lys^+ 1me Cap	100	78	51
Ov^- 1me Cap	100	72	60

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because of the longer length of the poly(A) tails the size of the tail was estimated using denaturing agarose gels and ^{32}P labelled λC_{I857} Hind III/Eco RI digest DNA markers (gels not shown). These gels were dried down and autoradiographed. As the poly(A) tails had an asymmetric distribution of sizes the median and maximum length of tail is given (minimum length about 0) see Table 4). The median length was estimated from densitometer tracings of the gel autoradiographs.

6.6 Effect of polyadenylation on RNA stability

Oocytes were injected with either poly(A)$^-$ or poly(A)$^+$ ^{32}P labelled transcripts (at 5ng per oocyte) using Lys$^+$ monomethyl cap, Lys$^+$ dimethyl cap, Chym$^+$ monomethyl cap, Globin-Hinf monomethyl cap and Globin-Pst monomethyl cap. The oocytes were incubated for up to 48h before extracting and running the RNA on denaturing agarose gels which were dried down and autoradiographed (fig. 17c,d,e and f). The amount of transcript at each time point was quantified by cutting the appropriate regions from the gel and scintillation counting them (Table 4). For the poly(A)$^+$ transcripts the smear corresponding to the RNA was used.

Polyadenylation had only a slight effect on stability over the first 24h. However after 48h the polyadenylated transcripts were all clearly more stable. The Xenopus 28S and 18S ribosomal RNA bands become prominent due to reincorporation of labelled nucleotides from degraded injected transcripts. The ribosomal bands are more strongly labelled in the oocytes injected with the less stable poly(A)$^-$ transcripts.

In a different experiment using Lys$^+$ monomethyl cap RNA with two different lengths of poly(A) tail (one of median length 120 and maximum length 280, the other of median length 210 and maximum length 550) both had similar stabilities in oocytes.

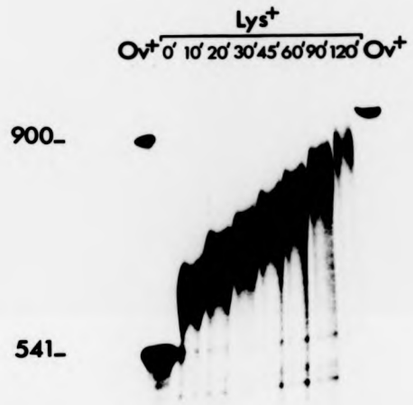
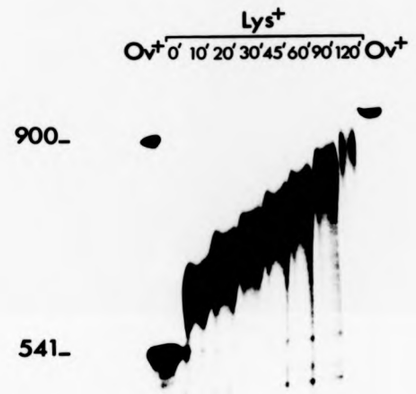


Figure 19. Polyadenylation of synthetic RNA

^{32}P-labelled Lys^{+} monomethyl cap transcript (540 nucleotides) was polyadenylated for 0, 10, 20, 30, 45, 60, 90 and 120 minutes (') as described in materials and methods section 2.13. Samples of the polyadenylated transcripts were run on a 6% acrylamide/urea sequencing type gel. Ov^{+} transcripts were run as size markers, only the 900 nucleotide is shown. After electrophoresis the gel was fixed, then dried down and autoradiographed directly.



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Stability of PolyA$^+$ RNA

Transcript		Length of poly A		Amount of RNA		
		Median	Maximum	0h	24h	48h
Lys 1me Cap	A^-	—	—	100	62	33
	A^+	250	1400	100	47	54
Lys 2me Cap	A^-	—	—	100	65	14
	A^+	250	1400	100	79	54
Chym 1me Cap	A^-	—	—	100	70	38
	A^+	410	2100	100	90	71
Globin Hinf 1me Cap	A^-	—	—	100	30	13
	A^+	280	2900	100	58	42
Globin Pst 1me Cap	A^-	—	—	100	48	33
	A^+	250	2700	100	53	52

Table 4. Stability of polyadenylated synthetic RNA

The experiment shown in fig. 17c, d, e and f, plus a similar experiment using Lys$^+$ dimethyl cap RNA (gel not shown), was quantified by cutting the appropriate bands or regions (for polyadenylated transcripts) from the gel and scintillation counting. The amount of RNA at each time point is expressed as a percentage of the 0h value.

The median and maximum length of the poly(A) tail was estimated by densitometric scanning of autoradiographs of denaturing agarose/formaldehyde gels loaded with the various RNA transcripts and ^{32}P-labelled DNA size markers (not shown). The minimum length of poly(A) tail was about 0. Lys 1me cap, Lys$^+$ monomethyl cap; Lys 2me cap, Lys$^+$ dimethyl cap; Chym 1me cap, Chym$^+$ monomethyl cap; Globin Hinf 1me cap, Globin-Hinf monomethyl cap; Globin Pst 1me cap, Globin-Pst monomethyl cap. A^-, nonpolyadenylated; A^+ polyadenylated transcript.

Stability of PolyA^+ RNA

Transcript		Length of poly A		Amount of RNA		
		Median	Maximum	0h	24h	48h
Lys 1me Cap	A^-	—	—	100	62	33
	A^+	250	1400	100	47	54
Lys 2me Cap	A^-	—	—	100	65	14
	A^+	250	1400	100	79	54
Chym 1me Cap	A^-	—	—	100	70	38
	A^+	410	2100	100	90	71
Globin Hinf 1me Cap	A^-	—	—	100	30	13
	A^+	280	2900	100	58	42
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6.7 Conclusions

Capping increased the stability of all the transcripts, and at least 45% of the monomethyl or dimethyl capped transcript remained intact, 24h after injection. Anti-sense strand transcripts were as stable as the sense strand transcripts.

Polyadenylation also increased the stability of all the transcripts, and at least 40% of capped and polyadenylated transcript remained stable up to 48h after injection into oocytes.

CHAPTER 7

RESULTS: EXPRESSION OF SYNTHETIC mRNAs IN OOCYTES

7.1 Expression of synthetic mRNAs

During the course of the stability experiments described in chapter 6, some of the oocytes were labelled using radioactive amino acids to compare translation of the synthetic mRNAs. In this way it was possible to differentiate between the effects of capping and polyadenylation on stability and on translation of the same mRNA during the same experiment.

7.2 Incorporation of ^{35}S-methionine in oocytes

Translational efficiency was used as a measure of the expression of the different synthetic mRNAs: that is the amount of protein produced per stable transcript. For this to be a valid comparison the incorporation of label into protein must be linear throughout the duration of the experiment.

To test this under the experimental conditions groups of 5 non-injected oocytes were labelled using 30μCi of ^{35}S-methionine in 30μl of Barth X. At 2h intervals over a 24h period a batch of 5 oocytes was removed and the percentage incorporation of ^{35}S-methionine into hot acid insoluble counts measured (this eliminates measurement of ^{35}S-methionine-tRNA molecules). The results are plotted in figure 20. The data is consistent with linear incorporation of label throughout the 24h period (probability > 99.9% using student t test).

7.3 Effect of capping on translation of synthetic mRNAs

Oocytes injected with uncapped, monomethyl or dimethyl capped Lys$^+$ RNAs were labelled with ^{35}S-methionine for the 6h period immediately following injection. The oocytes or the corresponding incubation medium

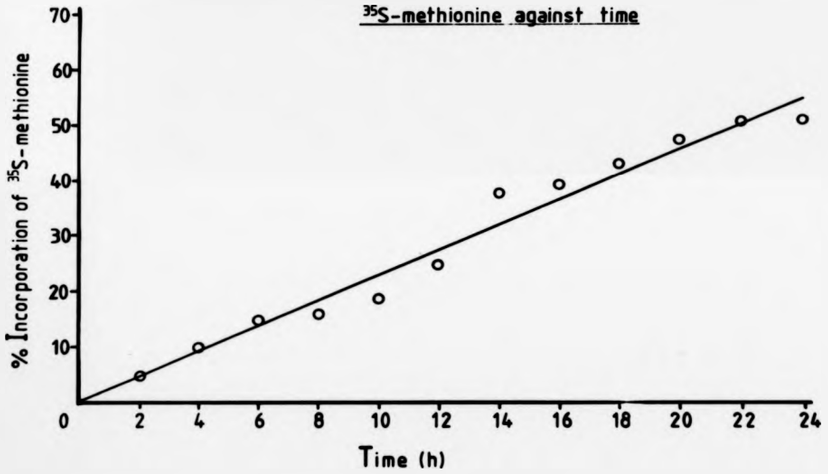


Figure 20. Percentage incorporation of ^{35}S-methionine into oocyte protein against time

Batches of 5 non-injected oocytes were incubated in 30µl of Barth X containing 30µCi of ^{35}S-methionine. At the times shown, a batch of oocytes was homogenised in protein extraction buffer and aliquots taken to measure total (including counts still in incubation medium), and hot trichloroacetic acid insoluble counts by scintillation counting. The acid insoluble counts are expressed as a percentage of the total counts for each time point.

The line of best fit was found by least-squares analysis, and the goodness of fit checked using student t test. The data fit a straight line with a probability of > 99.9% (data not shown).

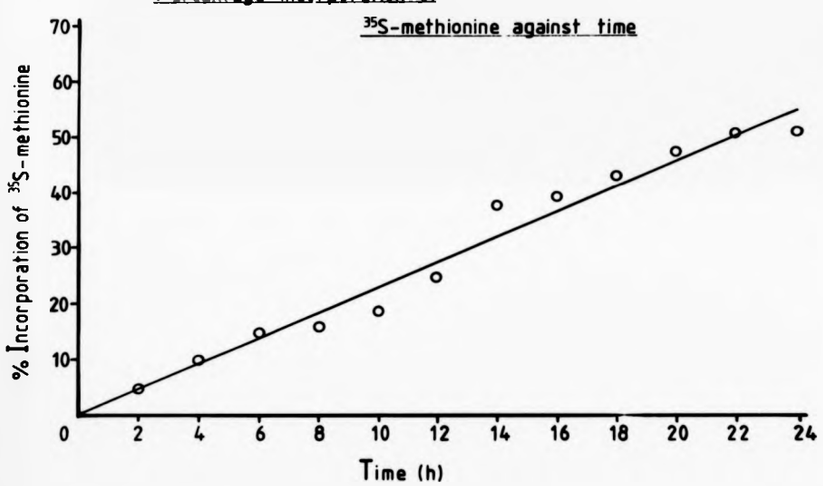


Figure 20. Percentage incorporation of ^{35}S-methionine into oocyte protein against time

Batches of 5 non-injected oocytes were incubated in 30µl of Barth X containing 30µCi of ^{35}S-methionine. At the times shown, a batch of oocytes was homogenised in protein extraction buffer and aliquots taken to measure total (including counts still in incubation medium), and hot trichloroacetic acid insoluble counts by scintillation counting. The acid insoluble counts are expressed as a percentage of the total counts for each time point.

The line of best fit was found by least-squares analysis, and the goodness of fit checked using student t test. The data fit a straight line with a probability of > 99.9% (data not shown).

was precipitated using anti-chick egg white antibody and run on SDS polyacrylamide gels which were fluorographed then autoradiographed (fig. 21). The stability of the RNA in this batch of oocytes has been described (Table 2) and 61%, 90% and 82% of uncapped, monomethyl capped and dimethyl capped Lys^+ RNA respectively remained intact after 6h.

More lysozyme protein was obtained from the capped than the uncapped transcripts, although there is a detectable amount of protein from the uncapped transcript. The monomethyl capped transcript clearly gave more protein than the dimethyl capped transcript; a result which was consistently obtained with several transcript preparations in different batches of oocytes.

The amount of protein made was quantified by cutting out the corresponding areas of the gel and scintillation counting them. Since the relative stabilities of the three transcripts were known it was possible to calculate their relative translational efficiencies (the amount of protein made per stable transcript). Relative to the monomethyl capped RNA (=1) the translational efficiencies of the uncapped and dimethyl capped RNAs were 0.04 and 0.20 respectively.

7.4 Effect of polyadenylation on the translation of synthetic mRNAs

The relative stabilities of the $poly(A)^+$ and $poly(A)^-$ synthetic mRNAs were described in Table 4. Over the first 24h the $poly(A)^+$ RNAs were at most twice as stable as the $poly(A)^-$. Some of the same batch of oocytes used for the stability study were labelled with ^{35}S-methionine over the same 24h period. Then the oocytes or their surrounding incubation medium was antibody precipitated using anti-chicken egg white or anti-preprochymosin and run on SDS polyacrylamide gels. The gels were fluorographed and autoradiographed (fig. 22a). The amount of protein was quantified by cutting the bands from the gels and scintillation counting them.

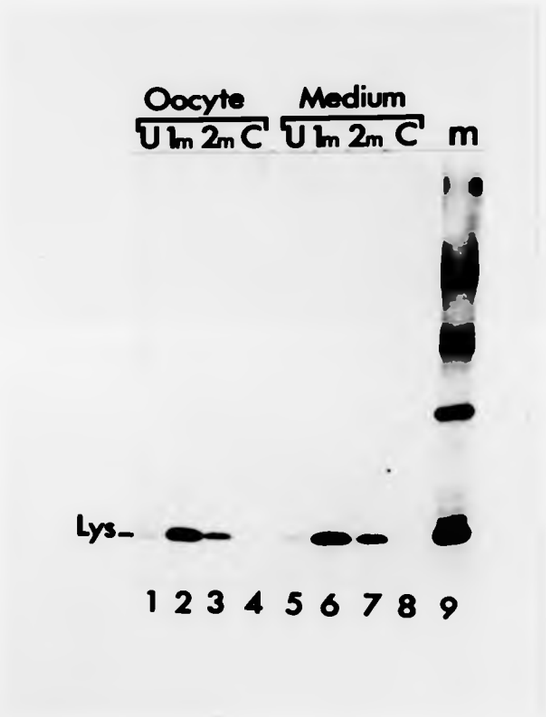
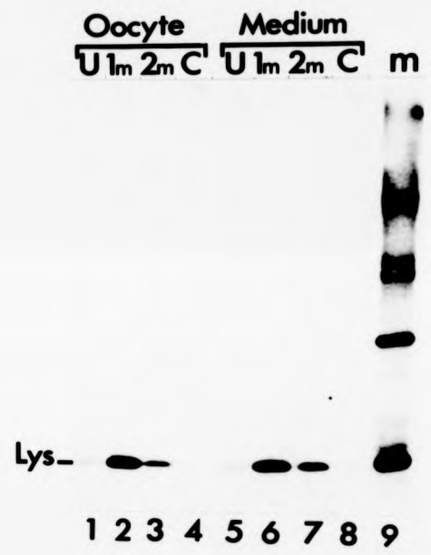


Figure 21. Translation of capped synthetic RNAs in oocytes

Oocytes injected with 5ng/oocyte of Lys^+ uncapped transcript ((u) tracks 1,5); Lys^+ monomethyl cap ((1m) tracks 2,6); Lys^+ dimethyl cap ((2m) tracks 3,7); also non-injected control oocytes ((c) tracks 4,8); were labelled with ^{35}S-methionine for the 6 hour period immediately following injection. The proteins from the oocytes (tracks 1-4) and the surrounding medium (tracks 6-8) were immunoprecipitated with anti-chick egg white antibody, and separated on an SDS-polyacrylamide gel which was fluorographed and autoradiographed. Track 9 (m) contains ^{14}C-labelled marker proteins. Lys marks the position of lysozyme protein.

The stability of the RNAs during this experiment is described in fig. 17 and Table 2.



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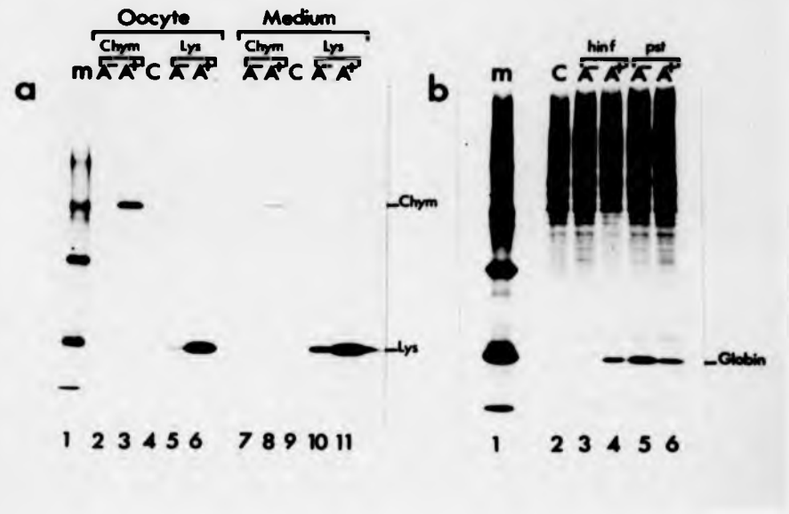


Figure 22. Translation of polyadenylated synthetic mRNAs in oocytes

(a) Oocytes injected with 5ng/oocyte of either Chym$^+$ monomethyl cap transcript (chym), poly(A)$^-$ (A$^-$, tracks 2,7) or poly(A)$^+$ (A$^+$ tracks 3,8); or Lys$^+$ monomethyl cap transcript (Lys), poly(A)$^-$ (A$^-$, tracks 5,10) or poly(A)$^+$ (A$^+$, tracks 6,11); or noninjected controls (c, tracks 4,9); were labelled using ^{35}S-methionine for the 24 hour post-injection period. The proteins from the oocytes (tracks 2-6), or the surrounding medium (tracks 7-11) were immunoprecipitated with either anti-prochymosin or anti-chicken egg white antibody, and separated on an SDS-polyacrylamide gel which was fluorographed and autoradiographed. Chym and Lys at the right-hand side of the gel mark the position of the chymosin and lysozyme proteins. Track 1 (m) contains ^{14}C-labelled protein molecular weight markers.

(b) Oocytes injected with 5ng/oocyte of Globin-Hinf monomethyl cap transcript (hinf), poly(A)$^-$ (A$^-$, track 3) or poly(A)$^+$ (A$^+$, track 4); or Globin-Pst monomethyl cap transcript (pst), poly(A)$^-$ (A$^-$, track 5), or poly(A)$^+$ (A$^+$, track 6); or noninjected control oocytes (c, track 2) were labelled for the 24 hour period following injection with [^{3}H]-leucine. Samples of total oocyte protein were run on an SDS-polyacrylamide gel, which was fluorographed and then autoradiographed. Track 1 contains ^{14}C protein molecular weight markers. Globin marks the position of rabbit globin protein

The stability of the RNAs during this experiment is described in fig. 17 and Table 4.

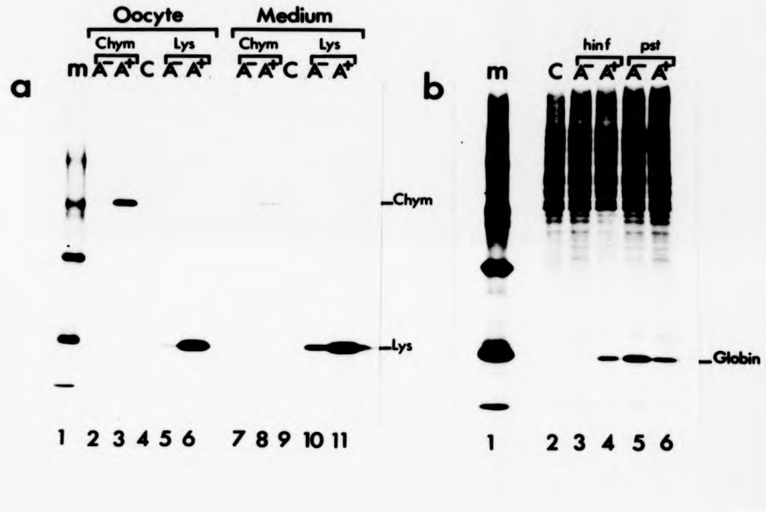


Figure 22. Translation of polyadenylated synthetic mRNAs in oocytes

(a) Oocytes injected with 5ng/oocyte of either Chym$^+$ monomethyl cap transcript (chym), poly(A)$^-$ (A$^-$, tracks 2,7) or poly(A)$^+$ (A$^+$ tracks 3,8); or Lys$^+$ monomethyl cap transcript (Lys), poly(A)$^-$ (A$^-$, tracks 5,10) or poly(A)$^+$ (A$^+$, tracks 6,11); or noninjected controls (c, tracks 4,9); were labelled using ^{35}S-methionine for the 24 hour post-injection period. The proteins from the oocytes (tracks 2-6), or the surrounding medium (tracks 7-11) were immunoprecipitated with either anti-prochymosin or anti-chicken egg white antibody, and separated on an SDS-polyacrylamide gel which was fluorographed and autoradiographed. Chym and Lys at the right-hand side of the gel mark the position of the chymosin and lysozyme proteins. Track 1 (m) contains ^{14}C-labelled protein molecular weight markers.

(b) Oocytes injected with 5ng/oocyte of Globin-Hinf monomethyl cap transcript (hinf), poly(A)$^-$ (A$^-$, track 3) or poly(A)$^+$ (A$^+$, track 4); or Globin-Pst monomethyl cap transcript (pst), poly(A)$^-$ (A$^-$, track 5), or poly(A)$^+$ (A$^+$, track 6); or noninjected control oocytes (c, track 2) were labelled for the 24 hour period following injection with [^{3}H]-leucine. Samples of total oocyte protein were run on an SDS-polyacrylamide gel, which was fluorographed and then autoradiographed. Track 1 contains ^{14}C protein molecular weight markers. Globin marks the position of rabbit globin protein

The stability of the RNAs during this experiment is described in fig. 17 and Table 4.

The translation of the Lys^+ monomethyl cap $poly(A)^+$ was 20 times that of the $poly(A)^-$ transcript (median length of poly(A) tail 250 As). The translation of the $Chym^+$ monomethyl cap $poly(A)^+$ was 10 times that of the $poly(A)^-$ (median length of poly(A) tail 250 A residues). Using the relative stabilities of the transcripts from Table 4 the translational efficiency of the Lys^+ $poly(A)^+$ was 26 times that of Lys^+ $poly(A)^-$, while $Chym^+$ $poly(A)^+$ was 8 times that of $Chym^+$ $poly(A)^-$.

The Lys^+ dimethyl capped transcript also gave the same stimulation of translation on polyadenylation as the Lys^+ monomethyl capped transcript, although a lower absolute level of protein was made.

The extent of the stimulation of translation by polyadenylation did vary in different batches of oocytes, however in no case was it less than a five-fold increase.

In a direct comparison in the same batch of oocytes two Lys^+ monomethyl cap $poly(A)^+$ RNAs with different lengths of poly(A) tail (either median length 120, maximum 280 or median length 210, maximum 550), showed no difference in their stimulation of translation over the $poly(A)^-$ RNA.

Polyadenylation of the Globin-Hinf monomethyl capped RNA also increased its translation (fig. 22b). However even better translation was obtained with the Globin-Pst monomethyl capped but non-polyadenylated RNA. This RNA, although the only transcript used which had the complete sequence of the corresponding mRNA, also had an unusual 3' end of 20 As and 30 Cs. Surprisingly polyadenylation of this transcript led to a decrease in its translation (fig. 22b).

7.5 Competition with exogenous polyadenylic acid

If the poly(A) tail is having a direct effect on translation it may be possible to compete out the factor responsible by coinjecting an excess of polyadenylic acid. To test this oocytes were injected with

either Lys^+ monomethyl cap $poly(A)^-$ or $poly(A)^+$ RNA (median length of tail 250 As, maximum 1400 As) at 2.5ng per oocyte, both with and without polyadenylic acid at 25ng per oocyte (fig. 23). The polyadenylic acid used was a commercial preparation which, from the manufacturers' information, was at least 200 A residues in length. Assuming a length of 200 As this would mean the polyadenylic acid molecules were in 5-fold molar excess over the Lys^+ transcripts.

This batch of oocytes gave a 10-fold stimulation in translation with the Lys^+ $poly(A)^+$ over the Lys^+ $poly(A)^-$. The Lys^+ $poly(A)^+$ coinjected with polyadenylic acid gave half (0.5 times) the lysozyme protein compared with Lys^+ $poly(A)^+$ alone. The translation of the Lys^+ $poly(A)^-$ RNA increased on coinjection with polyadenylic acid (1.8 times) compared with Lys^+ $poly(A)^-$ alone. However it must be emphasised that these are the results from a single experiment.

7.6 Comparison of translation of natural and synthetic mRNAs

To find the amount of lysozyme mRNA present in the chick oviduct $poly(A)^+$ RNA preparation a northern blot was made using 1ng of synthetic Lys^+ transcript together with calibration tracks of different amounts of oviduct $poly(A)^+$ RNA. The blot was probed with a nicktranslated fragment of DNA that had sequence common to both the natural and synthetic RNAs and then autoradiographed (fig. 24a). The relevant regions of the blot were cut out and quantified by scintillation counting. From this it was found that 2.2% of the mass of oviduct $poly(A)^+$ RNA was lysozyme mRNA.

To compare translation of the two mRNAs oocytes were injected with 5ng per oocyte of either oviduct $poly(A)^+$ RNA (equivalent to 0.11ng of lysozyme mRNA), or Lys^+ monomethyl cap $poly(A)^+$ RNA (fig. 24b). The relative amounts of protein made were quantified by scintillation counting the relevant parts of the gel. After correcting for the

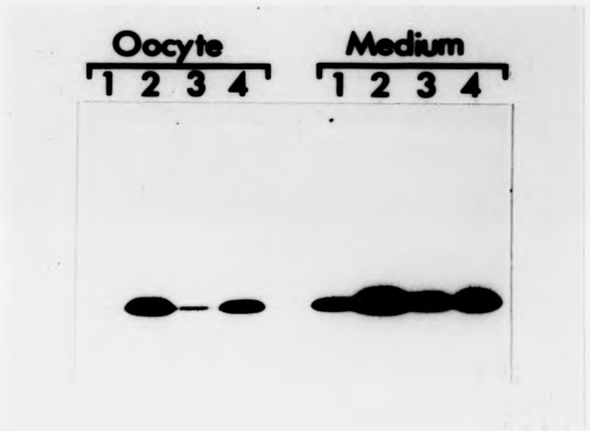


Figure 23. Effect of coinjected polyadenylic acid on translation of synthetic mRNAs in oocytes

Oocytes injected with Lys^+ monomethyl cap $poly(A)^-$ RNA (2.5ng/oocyte), (track 1); Lys^+ monomethyl cap $poly(A)^+$ RNA (2.5ng/oocyte), (track 2); or coinjected with Lys^+ monomethyl cap $poly(A)^-$ RNA (2.5ng/oocyte) and polyadenylic acid (25ng/oocyte), (track 3); or coinjected with Lys^+ monomethyl cap poly(A) RNA (2.5ng/oocyte) and polyadenylic acid (25ng/oocyte), (track 4); were labelled using ^{35}S-methionine for the 24 hour period following injection. The proteins from the oocytes and their corresponding incubation medium were immunoprecipitated using anti-chicken egg white, and run on an SDS-polyacrylamide gel which was fluorographed and autoradiographed.

Figure 23. Effect of coinjected polyadenylic acid on translation of synthetic mRNAs in oocytes

Oocytes injected with Lys^+ monomethyl cap poly(A)$^-$ RNA (2.5ng/oocyte), (track 1); Lys^+ monomethyl cap poly(A)$^+$ RNA (2.5ng/oocyte), (track 2); or coinjected with Lys^+ monomethyl cap poly(A)$^-$ RNA (2.5ng/oocyte) and polyadenylic acid (25ng/oocyte), (track 3); or coinjected with Lys^+ monomethyl cap poly(A) RNA (2.5ng/oocyte) and polyadenylic acid (25ng/oocyte), (track 4); were labelled using ^{35}S-methionine for the 24 hour period following injection. The proteins from the oocytes and their corresponding incubation medium were immunoprecipitated using anti-chicken egg white, and run on an SDS-polyacrylamide gel which was fluorographed and autoradiographed.

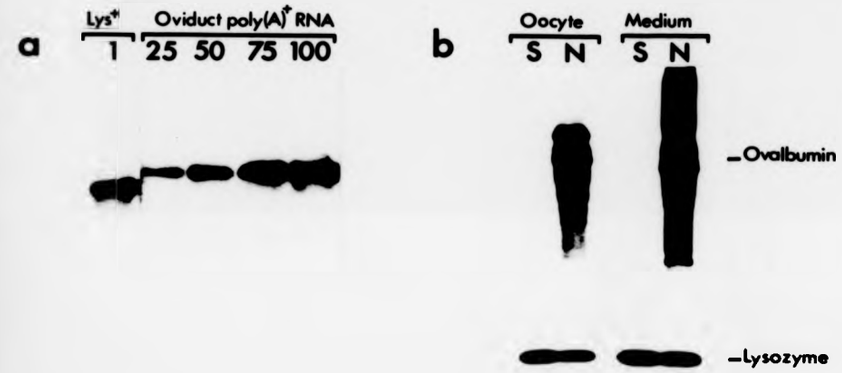
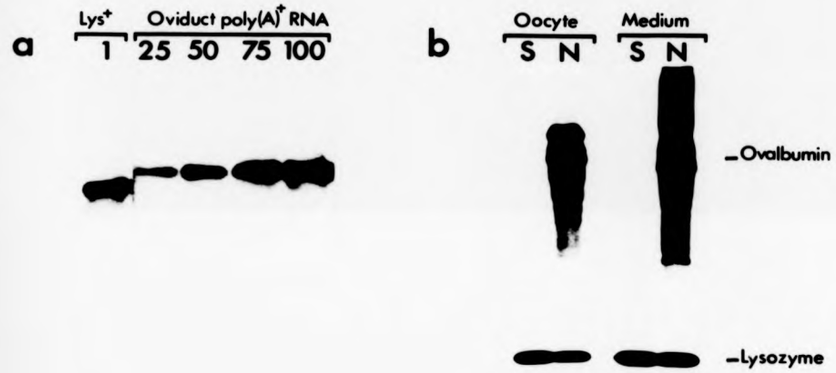


Figure 24. Efficiency of translation of natural and synthetic lysozyme mRNAs in oocytes

(a) A lng (1) sample of Lys^+ monomethyl capped $poly(A)^-$ synthetic RNA (Lys^+) was run on a denaturing agarose/formaldehyde gel together with 25, 50, 75 and 100ng (25, 50, 75, 100) samples of natural oviduct $poly(A)^+$ RNA. The gel was blotted onto nitrocellulose and the blot was probed using a nick-translated ^{32}P-labelled fragment of lysozyme DNA which was common to both the natural and synthetic RNAs. After autoradiography the appropriate bands were cut from the nitrocellulose filter and the radioactivity quantified by scintillation counting (not shown).

(b) Oocytes injected with 5ng/oocyte of either Lys^+ monomethyl cap $poly(A)^+$ RNA (S), or 5ng/oocyte of oviduct $poly(A)^+$ RNA (equivalent to 0.11ng/oocyte of lysozyme mRNA) (N), were labelled with ^{35}S-methionine for the 24 hour period immediately following injection. The proteins from the oocytes and surrounding medium were precipitated with anti-chick egg white antibody, and separated on an SDS-polyacrylamide gel which was fluorographed and autoradiographed. The position of ovalbumin and lysozyme proteins are indicated. The amount of lysozyme protein made was quantified by excising the appropriate bands from the gel and scintillation counting them.



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relative amounts of lysozyme RNA present (and assuming similar stabilities) the lysozyme mRNA is translated 25 times more efficiently than the synthetic Lys^+ $poly(A)^+$ mRNA.

7.7 Conclusions

Capping increased the translational efficiency of the transcripts, and monomethyl capped Lys^+ transcript was translated 25 times as efficiently as uncapped Lys^+. Monomethyl capped transcript was translated 5 times as efficiently as dimethyl capped transcript.

Polyadenylation also increased the translational efficiency of most of the transcripts, the translational efficiency of monomethyl capped Lys^+ transcript increased up to 26 times on polyadenylation. The single exception to this was a Globin-Pst transcript, that had an unusual 3' end of 20 As and 30 Cs, where polyadenylation decreased translation.

However even the translation of Lys^+ monomethyl capped $poly(A)^+$ transcript was less efficient than the corresponding natural lysozyme mRNA.

CHAPTER 8

RESULTS: TRANSLATION OF SYNTHETIC mRNAs IN VITRO

Translation of the synthetic mRNAs in Xenopus oocytes was compared with translation in vitro using the wheatgerm and reticulocyte lysate cell free translation systems.

8.1 Effect of capping on translation in vitro

Translation of Lys^+ uncapped, monomethyl and dimethyl capped RNAs (the same RNA preparations used in oocytes) was compared with the wheatgerm system (fig. 25). As in oocytes capping stimulates translation; although, in contrast to oocytes, it is the dimethyl capped transcript which gives the best translation.

8.2 Effect of polyadenylation on translation in vitro

All of the $poly(A)^-$ mRNAs were translated in both the wheatgerm and reticulocyte lysate systems (fig. 26). The $Chym^+$ RNA was less well translated in the wheatgerm relative to the other shorter RNAs. This was not unexpected as wheatgerm is usually regarded as less efficient for long mRNAs (compare the amounts of ovalbumin and lysozyme protein in the oviduct $poly(A)^+$ RNA control tracks).

In contrast to oocytes, where the Globin-Pst transcript gave more translation than Globin-Hinf, in both wheatgerm and reticulocyte lysate systems the Globin-Hinf gave the best translation.

Polyadenylation caused a decrease in translation of all RNAs in both systems (fig. 26). These were the same $poly(A)^+$ RNA preparations which had stimulated translation in the oocyte. Translation of Lys^+ was decreased by 70% in both systems on polyadenylation, and translation of all the other transcripts ($Chym^+$, Globin-Hinf and Globin-Pst) decreased by at least 90% in both translation systems upon polyadenylation.

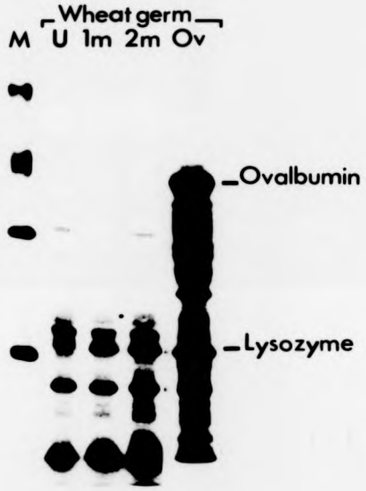
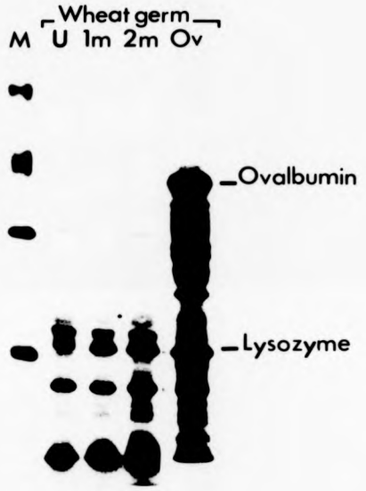


Figure 25. Effect of capping on translation of synthetic mRNAs in vitro

50ng aliquots of Lys^+ uncapped RNA (U), Lys^+ monomethyl capped RNA (1m), Lys^+ dimethyl capped RNA (2m), and 100ng of natural oviduct $poly(A)^+$ RNA (Ov), were translated in vitro. in the wheatgerm cell free system. Track (M) contains ^{14}C-labelled protein molecular weight markers. The position of ovalbumin and lysozyme proteins are indicated.

After translation the reactions were incubated for a further period with RNase A to remove background caused by the ^{32}P-labelled synthetic RNAs, and then run directly on an SDS-polyacrylamide gel which was fluorographed and autoradiographed.



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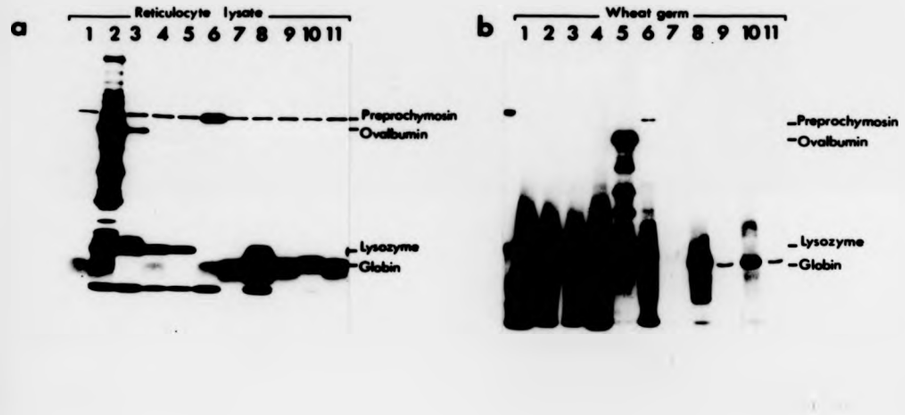

Figure 26. Effect of polyadenylation on translation of synthetic mRNAs in vitro

100ng aliquots of synthetic RNAs were translated in vitro in the rabbit reticulocyte (panel a) or wheatgerm (panel b) cell-free systems. After translation the ^{32}P-labelled synthetic RNAs were digested with RNase A, and then the reaction mixes were run on SDS-polyacrylamide gels which were fluorographed and autoradiographed.

Panel (a) track 1, no RNA; track 2, natural oviduct poly(A)$^+$ RNA; track 3, Lys$^+$ poly(A)$^-$ RNA; track 4, Lys$^+$ poly(A)$^+$ RNA; track 5, Lys$^+$ poly(A)$^+$ RNA, different preparation; track 6, Chym$^+$ poly(A)$^-$ RNA; track 7, Chym$^+$ poly(A)$^+$ RNA; track 8, Globin-Hinf poly(A)$^-$ RNA; track 9, Globin-Hinf poly(A)$^+$ RNA; track 10, Globin-Pst poly(A)$^-$ RNA; track 11, Globin-Pst poly(A)$^+$ RNA.

Panel (b) track 1, Lys$^+$ poly(A)$^-$ RNA; track 2, Lys$^+$ poly(A)$^+$ RNA; track 3, Lys$^+$ poly(A)$^+$ RNA, different preparation; track 4, Lys$^+$ poly(A)$^-$ RNA that has been through a mock polyadenylation reaction at 4^{o}C; track 5, natural oviduct poly(A)$^+$ RNA; track 6, Chym$^+$ poly(A)$^-$ RNA; track 7, Chym$^+$ poly(A)$^+$ RNA; track 8, Globin-Hinf poly(A)$^-$ RNA; track 9, Globin-Hinf poly(A)$^+$ RNA; track 10, Globin-Pst poly(A)$^-$ RNA; track 11, Globin-Pst poly(A)$^+$ RNA.

All of the synthetic RNAs were monomethyl capped. The position of preprochymosin, ovalbumin, lysozyme and globin protein are indicated. Note the endogenous rabbit reticulocyte band which comigrates with preprochymosin on the 15% acrylamide gels which were used.

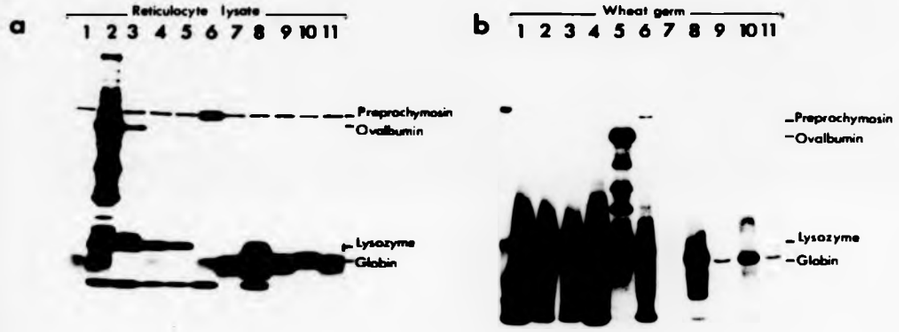

Figure 26. Effect of polyadenylation on translation of synthetic mRNAs in vitro

100ng aliquots of synthetic RNAs were translated in vitro in the rabbit reticulocyte (panel a) or wheatgerm (panel b) cell-free systems. After translation the ^{32}P-labelled synthetic RNAs were digested with RNase A, and then the reaction mixes were run on SDS-polyacrylamide gels which were fluorographed and autoradiographed.

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Panel (b) track 1, Lys$^+$ poly(A)$^-$ RNA; track 2, Lys$^+$ poly(A)$^+$ RNA; track 3, Lys$^+$ poly(A)$^+$ RNA, different preparation; track 4, Lys$^+$ poly(A)$^-$ RNA that has been through a mock polyadenylation reaction at 4^{o}C; track 5, natural oviduct poly(A)$^+$ RNA; track 6, Chym$^+$ poly(A)$^-$ RNA; track 7, Chym$^+$ poly(A)$^+$ RNA; track 8, Globin-Hinf poly(A)$^-$ RNA; track 9, Globin-Hinf poly(A)$^+$ RNA; track 10, Globin-Pst poly(A)$^-$ RNA; track 11, Globin-Pst poly(A)$^+$ RNA.

All of the synthetic RNAs were monomethyl capped. The position of preprochymosin, ovalbumin, lysozyme and globin protein are indicated. Note the endogenous rabbit reticulocyte band which comigrates with preprochymosin on the 15% acrylamide gels which were used.

As a control for a toxic factor from the polyadenylation reaction copurifying with the poly(A)$^+$ RNA causing the inhibition, a Lys$^+$ transcript was put through a mock polyadenylation reaction on ice (4^{o}C) before being purified as normal. This transcript caused no inhibition of translation (fig. 27).

However despite the apparent absence of a toxic factor the poly(A)$^+$ RNAs exhibited a distinct trans effect, and inhibited translation of poly(A)$^-$ transcripts in the same reaction (fig. 27). Translation of Lys$^+$ poly(A)$^-$ RNA mixed with Chym$^+$ poly(A)$^+$ RNA was reduced by 40% compared with Lys$^+$ poly(A)$^-$ mixed with Chym$^+$ poly(A)$^-$. Likewise mixing 50ng of Lys$^+$ poly(A)$^-$ with 50ng of Lys$^+$ poly(A)$^+$ reduced translation by 70% compared with translation of 50ng of Lys$^+$ poly(A)$^-$ alone. (The level of lysozyme protein produced was the same with 50ng of Lys$^+$ poly(A)$^-$ alone, or 50ng Lys$^+$ poly(A)$^-$ mixed with 50ng Chym$^+$ poly(A)$^-$).

This experiment suggested a generally inhibitory effect of poly(A), possibly related to the large amounts of poly(A) present. This was confirmed by adding a 10-fold excess (by weight) of polyadenylic acid to the translation reactions, which caused a general inhibition of translation (not shown).

8.3 Conclusions

Capping increased translation in the wheatgerm cell-free translation system, and the dimethyl capped transcripts gave the best translation.

In contrast to oocytes, polyadenylation decreased translation of transcripts in both the wheatgerm and reticulocyte lysate systems. This appeared to be a specific effect of the poly(A) tail, rather than for example a non-specific toxic factor copurifying with the poly(A)$^+$ transcript.

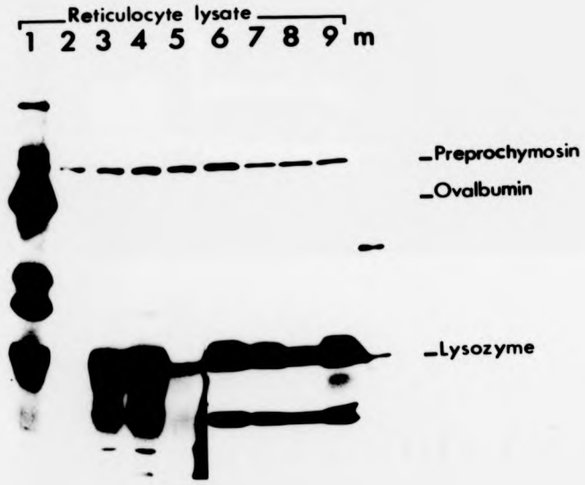
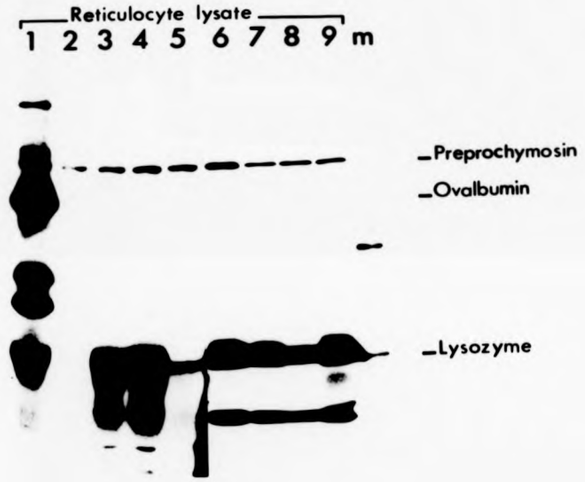


Figure 27. Inhibition of translation in vitro by the poly(A) tail of synthetic RNAs

The following RNAs were translated in vitro in the rabbit reticulocyte system to characterise the nature of the inhibitory effect of the poly(A) tail. Track 1, 100ng natural oviduct poly(A)$^+$ RNA; track 2, no RNA; track 3, Lys$^+$ poly(A)$^-$ RNA; track 4, Lys$^+$ poly(A)$^-$ RNA which has been through a mock polyadenylation reaction at 4^{o}C; track 5, Lys$^+$ poly(A)$^+$ RNA.

Track 6, 50ng Lys$^+$ poly(A)$^-$ RNA and 50ng Chym$^+$ poly(A)$^-$ RNA; track 7, 50ng Lys$^+$ poly(A)$^-$ RNA and 50ng Chym$^+$ poly(A)$^+$ RNA; track 8, 50ng Lys$^+$ poly(A)$^-$ RNA and 50ng Lys$^+$ poly(A)$^+$ RNA; track 9, 50ng Lys$^+$ poly(A)$^-$ RNA. Track (m) contains ^{14}C-labelled protein molecular weight markers.

After the translation reaction, the reaction mixes were incubated with RNase A to remove the ^{32}P-labelled synthetic RNAs, and then were run directly on an SDS-polyacrylamide gel which was fluorographed and autoradiographed. The position of preprochymosin, ovalbumin and lysozyme proteins are indicated. Note the endogenous reticulocyte lysate band which comigrates with preprochymosin.



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CHAPTER 9

RESULTS: MOVEMENT OF SYNTHETIC RNAs IN OOCYTES

9.1 Movement of synthetic mRNAs

Use of radioactively labelled synthetic mRNAs allowed a more quantitative analysis of their movement than was possible with natural mRNAs. They also extended the type of experiment that was possible, and movement of the radioactive mRNA could be followed *in situ* by autoradiography of oocyte sections. It was also possible to examine the effect of polyadenylation on the synthetic mRNAs rate of movement.

9.2 Movement of poly(A) RNA

Use of ^{32}P labelled synthetic mRNAs made it possible to follow their movement in oocytes by autoradiography. Oocytes were injected in either the animal or vegetal pole with ^{32}P-Lys^{+} dimethyl cap RNA. At various times, up to 24h after injection, the oocytes were fixed, embedded in wax and serial sections made. The distribution of the ^{32}P-Lys^{+} RNA in the sections was followed *in situ* using autoradiography (fig. 28). Immediately (< 10 min) after injection the RNA is localised in the injected region (fig. 28c and d). Although the injected RNA occupies a large volume in the oocyte, it does not appear to have caused any major disruption of the cytoplasm as judged by the pattern of yolk platelets in the section (fig. 28a and b). Since serial sections were made of the injected oocytes it was possible to estimate the shape of the region occupied by the injected RNA. In most cases it appeared ellipsoid or ovoid rather than spherical.

After 6h some movement of the RNA had taken place, although most remained localised round the injection site (fig. 28e and f). By 24h the injected RNA had reached all parts of the oocyte. However, although the vegetal pole injected oocyte had an almost even distribution of

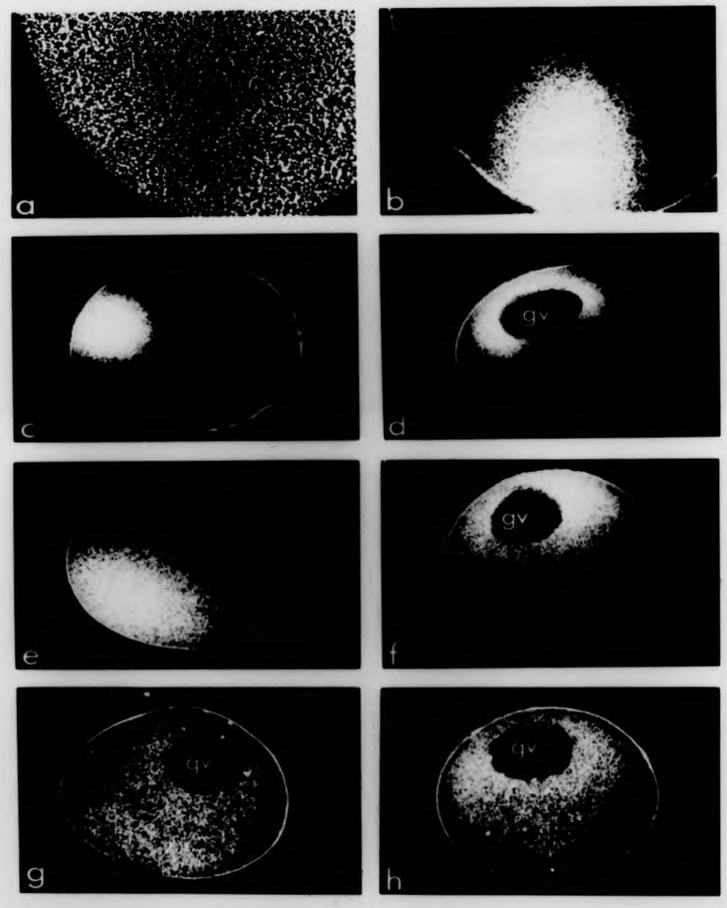
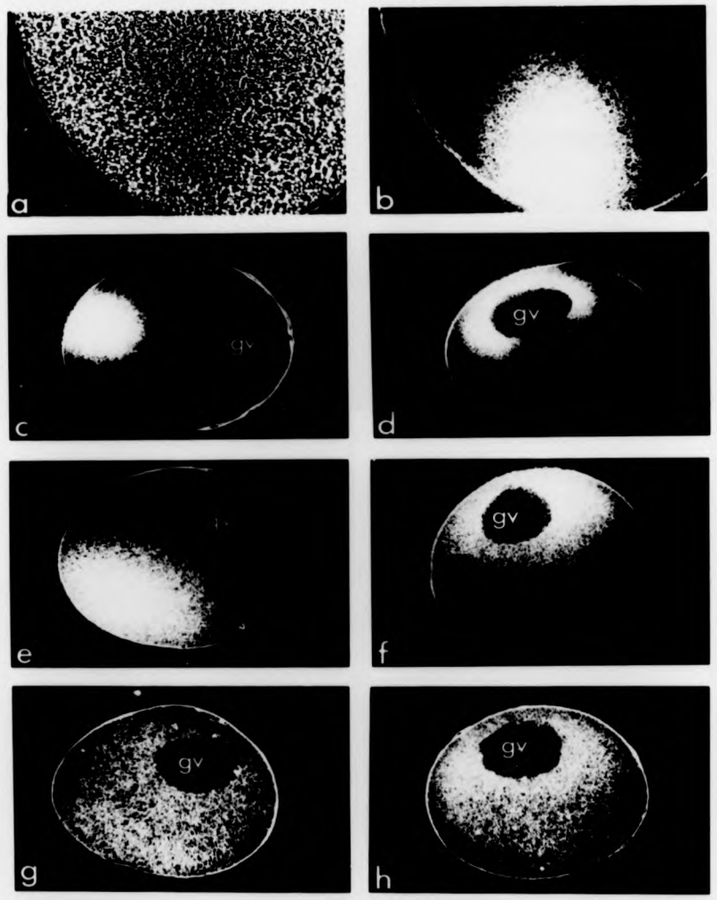


Figure 28. Autoradiographs of injected oocytes

^{32}P-labelled Lys^{+} dimethyl cap RNA was injected into oocytes at the vegetal (a, b, c, e and g) or the animal pole (d, f and h), and the oocytes were fixed in bouins fixative within 10 min (a, b, c, d), 6 hours (e, f) and 24 hours (g, h) of injection. After fixation the oocytes were dehydrated and embedded in paraffin wax. Serial sections were made of the oocytes and mounted on slides. After removal of the wax the sections were autoradiographed by coating the slides with a liquid photographic emulsion. After development of the autoradiographs, the sections were visualised by phase-contrast (a), or dark-field (b-h) illumination. Panels (a) and (b) show the same section under phase-contrast and dark-field illumination.

Under dark-field illumination (b-h) the silver grains in the autoradiographs appear as bright sources of light. The bright rim round the oocytes in panels b-h, is due to light scattering by the dense pigment granules (not silver grains) around the periphery of the oocytes. The nucleus of the oocyte is indicated (g.v., germinal vesicle). The concentrations of grains in the g.v. of the oocytes in panels (g) and (h) lie over nucleoli. The position of the nucleoli in these sections was found using phase-contrast illumination (data not shown).



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grains (fig. 28g), the animal pole injected oocyte still had a distinct concentration round the germinal vesicle (fig. 28h). This correlates with the concentration of endogenous oocyte RNA in this region (see fig. 9). One major limitation of this method of analysis is that the distribution of grains is caused both by intact injected RNA, partially degraded RNA, single labelled nucleotides and nucleotides that have been reincorporated into endogenous RNA. In particular detectable amounts of ribosomal RNA form (see fig. 17), which accounts for the localisation of grains over the nucleoli found in the autoradiographs of some of the sections. However from the measurement of RNA stability (Table 2) at least 45% of the grains are formed by injected RNA at 24h. Also to confirm that the pattern of grains in the autoradiographs is a genuine reflection of the RNAs distribution, movement was analysed as before. Frozen oocytes were sectioned and the RNA from the animal and vegetal halves was run on gels, which were dried down and autoradiographed (fig. 29). The distribution of RNA was quantified by cutting up and scintillation counting the relevant parts of the gels (Table 5). The distribution of RNA on the gels at 24h agrees with the distribution of grains in the *in situ* autoradiographs. There is a concentration of RNA in the animal half and movement from the vegetal to the animal half of the oocyte is 1.4 times faster than in the opposite direction. In a separate experiment using a different batch of oocytes movement from the vegetal to animal half was 1.7 times faster than in the opposite direction.

9.3 Comparison of movement of natural and synthetic mRNAs

To compare rate of movement of synthetic RNAs with natural mRNAs, oocytes were coinjected with Lys^+ monomethyl cap RNA (5ng per oocyte) and ^{32}P-reovirus mRNA (60ng per oocyte) (fig. 30a). Because of the difference in the specific activity of the two types of RNA two

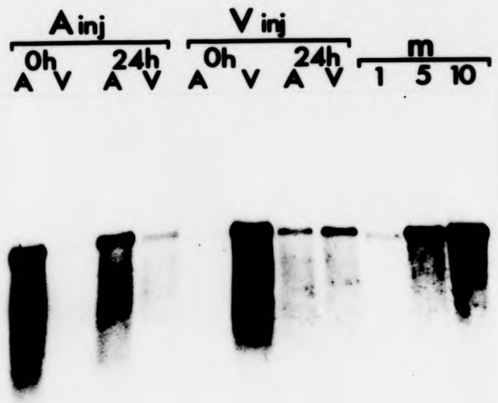

Figure 29. Movement of Lys^+ RNA in oocytes

^{32}P-labelled Lys^+ monomethyl cap RNA was injected (5ng/oocyte) into the animal (A inj) or vegetal pole (V inj) of oocytes. At 0 and 24 hours (h) after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA extracted from the oocyte halves was run on a denaturing agarose/formaldehyde gel, which, after ethidium staining, was dried down and autoradiographed directly. Tracks (m) contain non-injected RNA (1, 1ng; 5, 5ng; 10, 10ng).

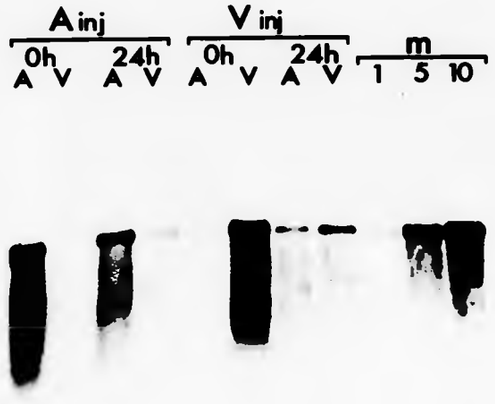

Figure 29. Movement of Lys^+ RNA in oocytes

^{32}P-labelled Lys^+ monomethyl cap RNA was injected (5ng/oocyte) into the animal (A inj) or vegetal pole (V inj) of oocytes. At 0 and 24 hours (h) after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA extracted from the oocyte halves was run on a denaturing agarose/formaldehyde gel, which, after ethidium staining, was dried down and autoradiographed directly. Tracks (m) contain non-injected RNA (1, 1ng; 5, 5ng; 10, 10ng).

Movement of Lys$^+$ RNA

Time	Distribution of RNA			
	A inj		V inj	
	A	V	A	V
0h	92	8	2	98
24h	76	24	32	68

Table 5. Movement of Lys$^+$ RNA

The experiment shown in fig. 29 was quantified by cutting the appropriate bands from the gel and scintillation counting them. The amount of RNA in each half of the oocyte is expressed as a percentage of the total amount of RNA in the oocyte at that time. A, animal half; V, vegetal half. A inj, RNA was injected into the animal pole; V inj, RNA was injected into the vegetal pole.

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different exposures of the same gel are shown. Again both RNAs display asymmetry in their rate of movement.

Scintillation counting of the relevant parts of the gel showed that the Lys^+ RNA was moving 1.7 times faster than the 12S size class of reovirus mRNA. This does not appear to be simply the result of the difference in size of the reovirus mRNA and Lys^+ RNA; since when Lys^+ RNA was coinjected with $Chym^+$ RNA (fig. 30b), the Lys^+ moved only 1.3 times faster than the $Chym^+$ RNA. The $Chym^+$ RNA is as large (1206 bases) as the smallest sizes of the reovirus 12S mRNA (1198, 1200, 1329 and 1416 bases; Cashdollar et al., 1982; Richardson and Furiuchi, 1983; Cashdollar et al., 1984).

9.4 Effect of polyadenylation on rate of movement of RNA

To find if polyadenylation affected movement of the synthetic mRNAs, the movement of Lys^+ monomethyl cap $poly(A)^-$ RNA was compared with various Lys^+ monomethyl cap $poly(A)^+$ RNAs. The RNA was injected into either the animal or vegetal pole and, 24h later, movement analysed by freezing and sectioning the oocytes, extracting RNA from the two halves and running it on denaturing agarose gels. The gels were dried down and autoradiographed (not shown) and the appropriate regions cut from the gels and scintillation counted. The distribution of RNA at 24h after injections of different RNA preparations in different frogs is shown in Table 6. Polyadenylation causes a retardation in the rate of movement, and the $poly(A)^-$ RNAs move at up to twice (2x) the rate of $poly(A)^+$ RNAs. However the magnitude of this effect varies between experiments.

That the retardation in movement is specific to the poly(A) tail rather than simply the increase in length of the $poly(A)^+$ RNAs is confirmed by the difference in rates being greater than was found between Lys^+ $poly(A)^-$ and $Chym^+$ $poly(A)^-$. The $Chym^+$ $poly(A)^-$ RNA is

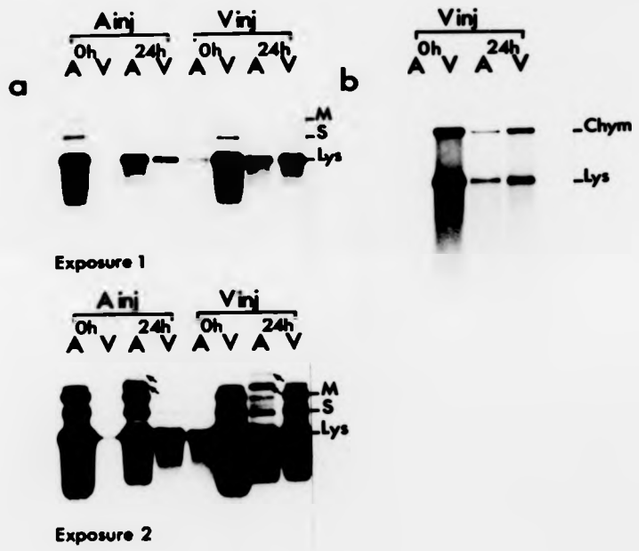
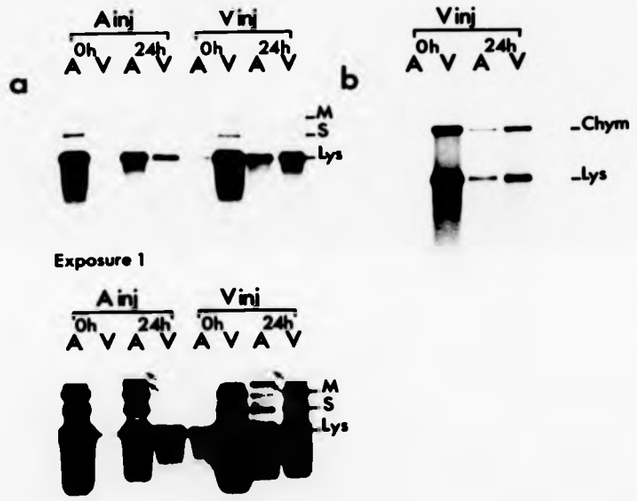


Figure 30. Comparison of movement of natural and synthetic mRNAs in oocytes

(a) ^{32}P-labelled reovirus mRNA (60ng/oocyte) and ^{32}P-labelled Lys^{+} monomethyl cap RNA (5ng/oocyte) were coinjected into either the animal (A inj) or vegetal (V inj) pole of oocytes.

(b) ^{32}P-labelled Chym^{+} monomethyl cap RNA (2.5ng/oocyte) and ^{32}P-labelled Lys^{+} monomethyl cap RNA (2.5ng/oocyte) were coinjected into the vegetal (V inj) pole of oocytes.

At 0 and 24 hours (h) after injection oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA extracted from the oocyte halves was run on denaturing agarose/formaldehyde gels. After ethidium staining, the gels were dried down and autoradiographed directly. Panel (a) contains two different exposures of the same gel. (M) and (S) are the medium (18S) and small (12S) size classes of reovirus mRNA. Lys, Lys^{+} monomethyl cap RNA; Chym, Chym^{+} monomethyl cap RNA. The arrows in exposure 2 of panel (a) mark the position of the 28S and 18S ribosomal bands, which become prominent in the animal half 24 hours after injection due to reincorporation of radioactive nucleotides.



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Movement of Poly A$^+$ RNA

Frog	Transcript		Length of poly A		Distribution of RNA at 24h			
					A pole inj		V pole inj	
			Median	Maximum	A	V	A	V
1	Lys 1me Cap	A$^-$	—	—	76	24	43	57
	Lys 1me Cap	A$^+$	120	280	80	20	35	65
	Lys 1me Cap	A$^+$	210	550	88	12	24	76
2	Lys 1me Cap	A$^-$	—	—	ND	ND	40	60
	Lys 1me Cap	A$^+$	250	1400	ND	ND	30	70

Table 6. Movement of polyadenylated synthetic RNAs

Oocytes were injected in the animal (A pole inj) and vegetal (V pole inj) pole with 5ng/oocyte of Lys$^+$ monomethyl cap (Lys 1me cap) poly(A)$^-$ (A$^-$) or poly(A)$^+$ (A$^+$) RNA. AT 0 and 24 hours after injection the oocytes were frozen and sectioned into their animal (A) and vegetal (V) halves. RNA extracted from the oocyte halves was run on denaturing agarose/formaldehyde gels which, after ethidium staining, were dried down and autoradiographed (not shown). The amount of RNA in each half of the oocyte was quantified by cutting the appropriate bands from the gels and scintillation counting, and is expressed as a percentage of the total present in the oocyte (only the 24 hour values are shown). ND, values not determined.

The median and maximum lengths of poly(A) tail were estimated from densitometer tracings of autoradiographs of denaturing agarose/formaldehyde gels loaded with the various RNA transcripts and ^{32}P-labelled DNA markers (not shown).

Movement of Poly A$^+$ RNA

Frog	Transcript		Length of poly A		Distribution of RNA at 24h			
					A pole inj		V pole inj	
			Median	Maximum	A	V	A	V
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longer than most of the Lys^+ $poly(A)^+$ RNAs that were used.

9.5 Conclusions

The $poly(A)^-$ synthetic mRNAs moved slightly faster in oocytes than naturally occurring (reovirus) $poly(A)^-$ RNAs. However movement of the synthetic mRNA was still sufficiently slow to prevent its equilibration within 24h of injection into the oocyte. Polyadenylation of the synthetic mRNAs did slow their rate of movement slightly.

The synthetic mRNAs had the same asymmetric rate of movement as naturally occuring mRNAs, and moved faster from the vegetal to animal half of the oocyte, than in the opposite direction. Autoradiography of oocyte sections confirmed that this differential rate of movement was not due to the synthetic mRNA entering the nucleus, or to any specific 'streaming' of the mRNA injected into the vegetal half.

DISCUSSION

CHAPTER 10

DISCUSSION: NATURAL mRNAs

10.1 Stability of natural mRNAs in Xenopus oocytes

Following injection of about 50ng per oocyte of rabbit globin mRNA, 70% of the β globin mRNA (which forms 40% of the total, Phillips et al., 1977) remained stable for at least 48h. This was less stable than some previous reports: Gurdon et al. (1973) found no decrease in the functional stability of globin mRNA over 14 days, and Marbaix et al. (1975) found no decrease in the physical stability of globin mRNA over a 56h period. However there are difficulties with their methods of analysis since functional stability only follows the fate of the injected mRNA that is being translated and, as discussed in the introduction, it is not clear that all of the injected mRNA is necessarily translated. This could cause discrepancies between estimates of functional and physical stabilities. The cDNA hybridisation method used by Marbaix et al. (1975) does not distinguish between intact and partially degraded mRNAs. This could lead to apparently greater stabilities than my measurements which only concerned intact mRNA.

However these explanations cannot account for the discrepancy between my results and those of Richter and Smith (1981). They found, using cDNA hybridisation, that 10h after injection of 40ng per oocyte of globin mRNA only 3.8% remained. Richter et al. (1983) also found ovalbumin mRNA equally unstable, and only 2.5% remained 12h after injection of about 30ng of ovalbumin mRNA per oocyte.

Although the oviduct poly(A)$^+$ RNA which I used was contaminated with rRNA, it is possible to estimate the proportion that was genuine mRNA. I found that 2.2% of the oviduct poly(A)$^+$ RNA preparation was lysozyme mRNA. Therefore, since lysozyme mRNA forms 3.4% of oviduct

mRNA (Schutz et al., 1977), the mRNA content of the oviduct poly(A)$^+$ RNA was about 65%, and the injection of 50ng per oocyte of the oviduct poly(A)$^+$ RNA preparation was equivalent to injecting about 32ng of pure mRNA. Although its stability was not as accurately quantified as the globin mRNA, at least 50% of the ovalbumin mRNA remained intact, a result which clearly differs from Richter et al. (1983).

The stability of reovirus mRNA in oocytes appears equally variable. Furuichi et al. (1977) found it had a half life of about 18h in oocytes while McCrae and Woodland (1981) found a half life of 120h (although it did vary between batches of oocytes to as low as 72h). I did not make sufficient measurements to assign a half-life, although 50% remains stable after 24h. This fraction remains stable over the next 24h, so 50% still remains 48h after injection, which suggests, unlike the results of Furuichi et al. (1977) or McCrae and Woodland (1981), that the reovirus mRNA is decaying with biphasic rather than linear kinetics, although there are insufficient measurements to be certain. Such biphasic degradation was also found for Dictyostelium and vesicular stomatitis virus mRNAs in oocytes (Allende et al., 1974).

I also found that 50% of the reovirus mRNA remained intact 24h after injection whether 12.5ng or 60ng was injected per oocyte. Allende et al. (1974) also found the stability of Dictyostelium mRNA to be independent of the amount injected. This again contrasts with the results of Richter and Smith (1981) who found that the percentage of globin or zein mRNA remaining intact at least halved over the same range of amounts of mRNA injected that I used.

The simplest explanation for the variation in the absolute values of mRNA stability in oocytes is variation between different batches of oocytes. This is to some extent confirmed by the large variations in half-life of reovirus mRNA found by McCrae and Woodland (1981) using different batches of oocyte. However it is not clear exactly what

property of the oocytes does vary; it may simply be differences in the level of ribonuclease activity, or in some other factor required to stabilize the mRNA.

Allende et al. (1974) originally suggested that translation of mRNAs helped stabilise them. However I did not find any reduction in stability of ovalbumin mRNA in cycloheximide treated oocytes; also Richter and Smith (1981) and Richter et al. (1983) have demonstrated that stable non-polysomal mRNAs can exist in oocytes, at least with mRNAs coding for secretory protein. In addition oocytes contain a fraction of endogenous non-translated but stable mRNA (Laskey et al., 1977; Taylor and Smith, 1985).

Another factor which may stabilise mRNAs is binding of proteins to the mRNA, since ribosomal RNA is more stable when injected as intact ribosomes rather than naked RNA (Allende et al., 1974). Specific RNA binding proteins have been demonstrated in Xenopus oocytes (Darnbrough and Ford, 1981; Richter and Smith, 1983) and, at least in vitro. naked mRNA will form a ribonucleoprotein particle with oocyte (Richter and Smith, 1984) or rabbit reticulocyte proteins (Greenberg and Carroll, 1985). Variation in the amount of such proteins present in the oocyte might affect the stability of injected mRNA; however it remains to be demonstrated that formation of such complexes takes place with injected mRNAs, and that such complexes are actually required for stability of the mRNA.

Whatever the cause, variations do exist in mRNA stability in different batches of oocytes; but what matters from a practical standpoint is that the mRNA's stability is known in any particular experiment. For movement experiments it was essential for a sufficiently large proportion of the injected mRNA to remain stable for its distribution at any time to actually reflect its movement. With at least 50% of the injected mRNAs remaining stable 48h after injection

this was fulfilled.

10.2 Slow movement of natural mRNAs in Xenopus oocytes

The movement of all the injected mRNAs, chicken ovalbumin and lysozyme, rabbit globin and reovirus mRNA, was slow. Only 48h after injection into the vegetal pole did the oocyte even have equivalent amounts of mRNA in both halves. Even this did not necessarily represent equilibrium (the point where mRNA distribution is the same, irrespective of where it was injected), since in oocytes injected in the animal pole most of mRNA was still in the animal half. This slow rate of movement contrasts with small molecules such as sugars like sucrose (Horowitz, 1972) and fucose which equilibrate within 3h. Small RNA molecules such as 5S RNA and tRNA also seem to equilibrate within 24h of injection into Xenopus oocytes (De Robertis et al., 1982).

That the rate of movement of mRNA injected into oocytes is so slow is perhaps, with hindsight, not surprising given the results obtained with other substances. Paine et al. (1975) found for a range of small molecules in amphibian oocytes, that over a 70 fold molecular weight range the diffusion coefficients were 0.4 to 0.1 of their value in water. With larger molecules, such as bovine serum albumin in fibroblast cells, the diffusion coefficient was 0.015 of its equivalent in water (Wojcieszyn et al., 1981). Indeed given the view that cellular cytoplasm resembles a hydrated protein crystal (Fulton, 1982), it is perhaps surprising that much detectable movement takes place at all. Also, because of the giant dimensions of the amphibian oocyte (1.2mm diameter), molecules will take longer to equilibrate by diffusion than in smaller somatic cells.

The decreased rate of diffusion of substances in the cytoplasm of amphibian oocytes has been attributed to an increased diffusional path length (Horowitz, 1972). However the slow movement of mRNA may not

simply be due to its intrinsic rate of diffusion. An additional factor is that the injected mRNA may form ribonucleoprotein particles with RNA binding proteins (Darnbrough and Ford, 1981; Richter and Smith, 1983). The resulting increase in molecular weight would greatly slow the rate of movement.

As well as increasing the mass of the mRNA-protein particle the RNA binding proteins might also cause specific interactions with other cellular components. The poly(A) tail has been proposed as mediating binding to the cytoskeleton (Milcarek and Penman, 1974). However since the reovirus mRNA, which is poly(A)$^-$, has a similarly slow rate of movement as the other poly(A)$^+$ RNAs it seems unlikely that a specific poly(A) effect via the poly(A) binding protein (Richter and Evers, 1984) is important in determining the rate of movement.

The mRNA cap-binding protein also appears to be associated with the cell's cytoskeleton (Zumbe et al.. 1982) and in many cells some mRNA is bound to the cytoskeleton (Lenk et al., 1977; Jeffery, 1982); however this association may only occur while the mRNA is being translated (Venrooij et al., 1981; Cervera et al., 1981; Bonneau et al., 1985). Certainly inhibition of translation did not cause an increase in the rate of movement of ovalbumin mRNA. This also suggests that the association of mRNAs that code for secretory proteins with the cells endoplasmic reticulum whilst being translated (Richter and Smith, 1981; Sabatini et al., 1982) is not an important factor in determining its rate of movement. However given the oocyte's limited translational capacity of 4ng of mRNA (Woodland, 1974; Laskey et al., 1977), and less than 4ng for mRNAs coding for secretory proteins (Richter and Smith, 1981) it is not certain what proportion of the injected mRNA was actually being translated. So the role of such factors as translation in determining rate of movement remains unclear until the exact form and translational status of the injected mRNA is determined.

If there is a factor which binds to the injected mRNA to slow its movement, it could not be saturated over the range of 10-50ng with globin mRNA or 12-60ng with reovirus mRNA. Indeed a similar rate of movement following injection of different amounts of mRNA is what would be expected for a molecule moving by simple diffusion (see Appendix II).

Intriguingly transcripts from DNA injected into the oocyte nucleus do seem to equilibrate more rapidly, with almost equivalent amounts in both halves of the oocyte 24h after injection (Drummond et al.. 1985). However it is not clear if this is really due to a faster rate of movement for endogenous RNAs, or merely reflects the less polarised position of the oocyte nucleus compared to the mRNA injection sites.

10.3 Assymmetry in rate of movement of natural mRNAs

The exact rate of movement of the injected mRNA depended on where it was injected in the oocyte. Following injection at the vegetal pole movement of all the mRNAs was more rapid than following injection at the animal pole. Although an equilibrium distribution (the same distribution irrespective of the injection site) was never reached, the results suggest that at equilibrium there would be a net accumulation of the mRNA in the animal half of the oocyte.

Many of the endogenous oocyte substances such as Na^+ ions and water (Tluczek et al., 1984), ribosomes (Niewkoop and Faber, 1967) and poly(A)$^+$ RNA (Capco and Jeffrey, 1982) are concentrated in the animal half. It is thought that this concentration is caused by displacement of cytoplasm from the vegetal half (Gerhart et al., 1983). To assess the relative volumes of the two halves I measured the equilibrium distribution of fucose, a small sugar not readily metabolised by the oocyte. Although the animal half forms only 45% of the oocyte's geometrical volume (Appendix I) it contains 65% of the volume accessible to fucose. However fucose, unlike the injected mRNAs, enters the

nucleus, which forms 12% of the accessible volume (Bonner, 1975). Correcting for this the cytoplasmic distribution of the fucose is 60:40 (animal:vegetal half). This agrees with the distribution of total endogenous oocyte RNA between the two halves, where 60% of the RNA was found in the animal half. An accumulation of injected mRNA in the animal half is therefore consistent with most of the accessible cytoplasm being in this half, as with the distribution of endogenous RNA.

If the injected mRNA is moving by simple diffusion, this assymetric distribution of accessible cytoplasm would also explain the difference in rate of movement. Following injection, the mRNA would be about $1\frac{1}{2}$ times more concentrated in the vegetal than the animal half, assuming the water which is also injected equilibrates rapidly. Since the amount of mRNA which diffuses depends on the concentration gradient of the mRNA, the amount which diffuses will be about $1\frac{1}{2}$ times greater moving from the vegetal to the animal half than in the opposite direction (Appendix II).

However since neither the relative rate of movement nor the equilibrium distribution of the injected mRNA was determined, it is not clear if the injected mRNA is behaving in a similar, though slower, way to fucose.

10.4 Movement of natural mRNAs in matured oocytes and unfertilised eggs

In matured oocytes, although the nucleus has broken down (Smith and Ecker, 1969), the injected mRNA does not move any faster from the animal to the vegetal half than in normal oocytes. Therefore, although the injected mRNA is unable to enter the nucleus in the oocyte, the nucleus does not seem to be a major obstacle to movement.

Immediately following injection into the vegetal pole of matured oocytes, or into the vegetal or animal pole of unfertilised eggs, the

mRNA was present in almost equivalent amounts in both halves of the oocytes or eggs. The cause of this is not clear. Certainly there is a shift of the oocyte's accessible cytoplasm into the animal half during maturation (Lau et al., 1984); caused by shifts in yolk and the addition of the nuclear sap on breakdown of the nucleus. However this shift would have to be considerable to account for the results. Alternatively the cytoplasm in the vegetal half of matured oocytes, and both halves of eggs, is perhaps less able to resist the initial surge of the injected material.

From my results it is impossible to tell how rapidly the lysozyme mRNA injected into the vegetal pole of matured oocytes may be moving. However, 6h after injection into the vegetal pole there does not seem to be any further shift of the lysozyme mRNA into the animal half, which suggests that following the initial injection surge the intrinsic rate of movement of the mRNA is still slow. This would agree with the observation of Colman, Woodland and Jones (personal communication) that movement of ovalbumin mRNA injected into fertilised eggs is slow. Yet Froelich et al. (1977) found that globin mRNA appeared to move rapidly in fertilised eggs to form a net accumulation in the animal half of the gastrula; although this may be specific to globin mRNA, since no corresponding concentration of injected polyadenylic acid was found. Likewise the rapid movements and formation of specific gradients by reinjected endogenous RNAs (Capco and Jeffrey, 1981) may be due to some effect specific to certain mRNAs.

10.5 Implication of the slow movement of mRNA

That the movement of mRNA injected into Xenopus oocytes is slow and equilibration not instantaneous as has often been assumed (see Lane, 1983), has many potentially serious implications for experiments in oocytes.

The translation of mRNAs injected into oocytes is usually followed by 'labelling' the protein using either single or a combination of radioactive amino acids. Labelling may be carried out by either incubating the oocyte in medium containing the amino acid or directly injecting the amino acid (Gurdon et al., 1971). With the incubation method of labelling there is a short delay in entry of the amino acid into uninjected oocytes (Gurdon et al., 1971). The mRNA injection site may therefore provide a preferential entry point for the amino acid. Since for up to 6h little movement of the injected mRNA is detectable its position close to the injection site may cause a preferential labelling of translation products from the injected mRNA. However no difference has been found in the incorporation of label into the translation products of injected or endogenous mRNAs, even when additional injection wounds are made in other parts of the oocyte or when the label is injected into the oocyte (A. Colman, personal communication). This is presumably because the amino acid can equilibrate rapidly once it is within the oocyte.

Although with short labelling periods the injected mRNA is unequally distributed within the oocyte and the exact distribution depends on where the mRNA was injected into the oocyte, this does not seem to affect translation. Thus there is no difference in the amount of protein produced irrespective of whether ovalbumin mRNA is injected in animal or vegetal pole.

Therefore despite the slow movement of the injected mRNA, the method of labelling would not appear to be a problem.

Berridge and Lane (1976) found that vitellogenin mRNA took 20-24h to reach its maximum rate of translation, while globin and albumin took only 2-4h. They attributed the delay in vitellogenin mRNA translation to its much larger size (about 10 times bigger than globin), which they suggested would reduce its rate of diffusion and equilibration

throughout the oocyte. However Asselbergs et al. (1979) found no difference in the time (both 6-7h) for lens crystalline mRNAs (14S) and globin mRNAs (9S) to reach maximum translation, and I found no differences in the actual rate of movement of different mRNAs, although these only varied in size over a 4 fold range from globin mRNA to reovirus 18S mRNA. Therefore unless very large mRNAs, such as vitellogenin mRNA, do indeed move more slowly than the smaller mRNAs which I tested, my results and those of Asselbergs et al. (1979) suggest that the recruitment delay for vitellogenin mRNA observed by Berridge and Lane (1976) has some cause, other than a slower rate of movement, specific to vitellogenin mRNA.

The slow movement of injected mRNA in oocytes is most significant in competition studies where inhomogeneous mixing of the competing mRNAs could seriously affect the results. Both Laskey et al. (1977) and Asselbergs et al. (1979) have found that when globin mRNA is injected in saturating amounts, endogenous protein synthesis is only reduced by 50%. One explanation is an inhomogenous distribution of the injected mRNA so that it was unable to compete with all of the endogenous mRNA. Asselbergs et al. (1979) also found that two mRNAs which are coinjected will compete with each other more than with endogenous mRNA; but if the two mRNAs are injected at different times, the first mRNA becomes resistant to competition by the second and behaves more like endogenous mRNA. This was thought to demonstrate some special property of endogenous mRNAs that rendered them resistant to competition. Obviously a reduced overlap of the mRNAs when injected sequentially rather than coinjected provides an alternative explanation. However Asselbergs et al. (1979) is the only report of reduced competition that might be explained by an incomplete spatial mixing of the injected mRNAs. Although Richter and Smith (1981) found no translational competition between coinjected mRNAs coding for secreted and non-secreted proteins,

they did demonstrate that two coinjected mRNAs for non-secreted proteins would compete under the same conditions. Since I found no significant difference in rate of movement of mRNAs for secreted or non-secreted proteins, the lack of competition must depend on some other property of the two types of mRNA.

10.6 Movement of proteins translated in oocytes

Having established that injected mRNAs showed little movement over the first 6 hours following injection it was possible, by labelling the oocytes over this period, to follow the movement of the proteins translated from the mRNAs. The movement of the proteins was faster than that of the encoding mRNAs. Globin was particularly fast and achieved an equilibrium distribution of 85:15 (animal to vegetal half) within 6h. As with injected mRNA, the globin protein does not enter the oocyte nucleus (A. Wilson, personal communication). Thus the nucleus does not appear to be the cause of this rapid shift of globin protein into the animal half. This accumulation contrasts with the failure of Bonner (1975) to find any localisation of a range of proteins following their injection, although it is not clear along which axis the oocytes were sectioned in this study.

The accumulation of globin protein in the animal half of the oocyte exceeds the 60:40 distribution (animal:vegetal) expected from the measurement of accessible cytoplasmic volume using fucose (see section 11.3); although it has been found, at least with smaller sizes of molecule, that increasing size decreases the volume of accessible cytoplasm for that molecule (Horowitz and Paine, 1976). This cytoplasmic exclusion seems to be caused by the cell's cytoskeleton. Therefore differences in the cytoskeletal structure between the animal and vegetal halves of the oocyte, causing differences in the extent of cytoplasmic exclusion, would account for the accumulation of globin. If

this were true other cytosolic proteins should have a similar equilibrium distribution. Alternatively since some endogenous oocyte proteins are localised along the animal-vegetal axis (Moen and Namenwirth, 1977), globin protein may have some specific property that causes its localisation in the animal half.

Not all of the proteins examined were moving in the cytosol; some were secreted proteins which are normally within other cellular compartments (see Sabatini et al., 1985 for a review of protein secretion). Ovalbumin is one secretory protein of particular interest. In oocytes, as in other cells, it is normally present only in the endoplasmic reticulum and other membrane-bound compartments of the cell. However in some oocytes a small, variable proportion can be miscompartmentalised and is present free in the cytosol (Colman et al., 1981). This allowed direct comparison of the movement of the same protein in the cytosol and endoplasmic reticulum. Ovalbumin protein moved 1½ to 2 times faster in the cytosol than the endoplasmic reticulum, which may be due to the more contorted path of the protein in the endoplasmic reticulum. It is interesting that protein within the endoplasmic reticulum is able to pass along the length of the oocyte; which suggests continuity of the endoplasmic reticulum throughout the oocyte. Also the protein within the endoplasmic reticulum displays the same polarity in rate of movement as cytosolic proteins. Indeed the rate of movement from the vegetal to the animal half compared with movement in the opposite direction, is even greater in the endoplasmic reticulum than the cytosol. Thus, if the proteins are simply diffusing, the 'volume' of endoplasmic reticulum in the animal half must be even more concentrated than the volume of accessible cytoplasm.

The other secretory protein examined was lysozyme, which despite being the smallest protein (Mr 14,500 compared to ovalbumin Mr 44,000; Bonner, 1975) had the slowest rate of movement. Lysozyme is secreted

from oocytes 12 times faster than ovalbumin (Cutler et al., 1981) so it may be mainly present in a part of the secretory apparatus that is, unlike the endoplasmic reticulum, non continuous in the oocyte. Although a fraction of the lysozyme protein is present in an unusual form that is never secreted (Cutler et al., 1981), this forms an insignificant fraction of the total during the time (6h) of the movement experiment (A. Colman, personal communication).

Colman et al. (1982) have demonstrated that MOPC 21 immunoglobulin light and heavy chain polypeptides are only secreted from the oocyte when present in equimolar amounts. Injection of only one type of mRNA into one pole of the oocyte results in the protein being trapped in the oocyte's endoplasmic reticulum. The injection, 24 hours later, of the second mRNA into the opposite pole of the oocyte allows rescue (secretion) of the trapped protein over the subsequent 24 hour period. It was not clear if this rescue was due to overlap of the mRNAs or proteins. My results indicate that over the period of the experiment overlap of both mRNA and protein may have occurred. However if the immunoglobulin proteins are as free to move within the endoplasmic reticulum as ovalbumin, no overlap of mRNA would be required for rescue and the 'rescuing' mRNA could be translated in any part of the oocyte. In more general terms assembly of complex proteins does not seem to require spatial coordination of translation of their sub-units.

CHAPTER 11

DISCUSSION: SYNTHETIC mRNAs

11.1 Effect of capping on the stability of synthetic RNAs

All eukaryotic and many viral mRNAs have a cap structure consisting of a 7-methylguanosine base joined by a triphosphate bridge to their 5' end. In addition usually the first, and sometimes the second nucleotide of the mRNA is methylated (Shatkin, 1976; Banerjee, 1980). It has been shown that enzymatically removing the cap (the 7-methylguanosine base) from natural mRNAs, decreases both the functional stability of globin mRNA (Lockard and Lane, 1978) and the physical stability of Reovirus mRNA (Furuichi et al.. 1977; McCrae and Woodland, 1981) injected into Xenopus oocytes. Capping is also essential for the physical stability of synthetic mRNAs in oocytes (Krieg and Melton, 1984a; Green et al.. 1983). I retested these results using Lys^+ transcript and two forms of cap structure. One had the 7-methylguanosine base at the cap (called the monomethyl cap); while the other had, in addition, the first nucleotide of the transcript methylated (called the dimethyl cap).

The uncapped Lys^+ transcript was more stable with 61% left 6h after injection, than has been reported for other in vitro transcripts: Krieg and Melton (1984a) could detect no uncapped transcript by 15 minutes after injection. However as described in section 11.1 such comparisons between results obtained by different workers in different batches of oocytes are so variable as to be meaningless.

Despite the already high stability of the transcript, capping the Lys^+ transcript with either the monomethyl or dimethyl caps increased stability to 5 times that of uncapped RNA 24 hours after injection. This stability was not confined to coding strand transcripts and similar stability was also found with ovalbumin antisense strand transcript (Ov^-) in both the capped and uncapped form. The Ov^- transcript is,

unlike the Lys^+ transcript, not detectably translated (A. Colman, personal communication). The similar stability of translated and untranslated transcripts suggests that translation is not an important factor in determining mRNA stability.

Likewise, although the monomethyl capped Lys^+ mRNA is translated 5 times more efficiently than the dimethyl capped transcript, it has the same stability. This contrasts with the suggestion of Huez et al. (1977) that the degradation of poly(A)$^-$ mRNA is enhanced by its translation; although since the proportion of the injected synthetic mRNA that is actually engaged on polysomes is not known, my results do not conclusively disprove a connection between translation and degradation.

11.2 Effect of polyadenylation on the stability of synthetic mRNAs

The role of polyadenylation in the stability of mRNAs injected into Xenopus oocytes remains controversial. Using cDNA hybridisation to RNA recovered from injected oocytes Marbaix et al. (1975) have shown that rabbit globin mRNA is destabilised by deadenylation. This however is the only study which has directly measured the physical stability of the mRNA. Most other studies have used functional stability; that is the relative amount of translation from the mRNA at various times following injection. Using this criterion the functional stability of globin mRNA has been shown to follow its physical stability, and decreases upon deadenylation (Huez et al., 1974; Marbaix et al., 1978). In contrast the functional stabilities of human interferon (Sehgal et al., 1978; Soreq et al., 1981) and rat α-2u globulin mRNA (Desphande et al., 1979) are unaffected by deadenylation. However the functional stability of an injected mRNA does not necessarily reflect its physical stability as not all of the injected mRNA may be translated. Also the mRNAs may only be gradually recruited onto active polysomes (Berridge and Lane, 1976;

Asselbergs et al., 1979). Therefore the amount of protein produced over the initial labelling period may underestimate the initial amount of mRNA.

Since synthetic mRNAs are easily made radioactive I have been able to follow their physical stability directly. Also separating the extracted mRNAs on gels before quantitation eliminates the possibility of including partially degraded mRNAs in stability measurements, which is a danger with solution hybridisation methods such as that used by Marbaix et al. (1977). I found that the physical stability of all the transcripts, Lys^+, $Chym^+$, Globin-Hinf and Globin-Pst, increased upon polyadenylation. The Globin-Pst $poly(A)^-$ transcript, which has an unusual 3' end of 20 As and 30 Cs, was already more stable than the Globin-Hinf $poly(A)^-$ transcript. Krieg and Melton (1984a) have also found the functional stability of Globin-Pst to be greater than that of the Globin-Hinf transcript. The 20 As of the Globin-Pst transcript are at the lower limit for a functional poly(A) tail, at least for rabbit globin mRNA in oocytes (Nudel et al., 1976). However the inclusion of 30 Cs may be fortuitous as poly(C) has been found more stable than either poly(U) or rRNA in oocytes (Allende et al., 1974).

11.3 Effect of capping on translation of synthetic mRNAs in oocytes

Removal of the cap structure from natural reovirus mRNA (Both et al., 1975; Furuichi et al., 1977) and globin mRNA (Lockard and Lane, 1978), has shown the presence of the 7-methylguanosine capping nucleotide to be essential for efficient translation of mRNA. In agreement with these observations I found the translational efficiency of Lys^+ monomethyl cap transcript to be about 25 times that of uncapped Lys^+ transcript, (translational efficiency being the amount of protein synthesised per stable transcript). Also the monomethyl capped RNAs are translated 5 times as efficiently as the dimethyl capped RNA. This is

surprising as the dimethyl cap is the more common form among natural eukaryotic mRNAs (Banerjee, 1980). Also oocytes will methylate injected capped (but unmethylated) RNAs to form both the monomethyl and dimethyl cap patterns of methylation (Furuichi et al., 1977). Thus raising the question of whether the oocyte can regulate translation by a mechanism of differential methylation.

Shatkin (1985) has speculated that the role of cap-binding proteins in translation is unwinding secondary structure in the 5'-untranslated region of the mRNA as well as promoting initiation complex formation, although the relative contribution of each function remains unclear. The Lys^+ transcripts lacked 50% of their normal 5'-untranslated region, and possessed no detectable local secondary structure. Yet the monomethyl cap RNA was translated 25 times as efficiently as the uncapped. Thus even in the apparent absence of mRNA secondary structure the cap still has a significant effect on translation.

11.4 Effect of polyadenylation on translation of synthetic mRNAs in oocytes

Two general hypotheses have been advanced for the role of poly(A) tails on mRNAs. One is that the poly(A) tail stabilises the mRNA (discussed in section 11.2). The other view is that polyadenylation enhances translation of mRNA. This is largely based on the correlation between polyadenylation and translation during development in several species (Nemer et al., 1975; Rosenthal et al., 1983; Palatnik et al.. 1984).

I have found that in oocytes the translation of most of the transcripts (Lys^+, $Chym^+$ and Globin-Hinf) increased up to 20 fold upon polyadenylation. This increase in translation was much greater than the corresponding increase in stability which was at most 2 fold. A similar increase in translation in oocytes has been found with rat α-2u globulin

mRNA with long (175 As) compared with short (40 As) lengths of poly(A) tail (Desphande et al., 1979); although in this study only the functional stability of the mRNA was examined. In contrast to this I found no difference between the translation of Lys^+ transcripts with tails of median lengths 280 As and 550 As.

The single exception to the increase in translation was the Globin-Pst transcript, translation of which decreased upon adding a poly(A) tail. Although this was the only transcript used which had the complete 5' and 3' untranslated regions of the corresponding mRNA, it also had an unusual 3' end of 20 As and 30 Cs (Krieg and Melton, 1984a). So the physiological significance, if any, of this result remains unclear.

The stimulation of translation in oocytes by polyadenylation contrasts with most previous reports which found no difference, at least in the initial rates, of translation of $poly(A)^+$ and $poly(A)^-$ rabbit globin mRNA (Huez et al., 1974; Marbaix et al., 1975; Nudel et al., 1976), HeLa cell histone mRNA (Heuz et al., 1978) or human fibroblast β_1 and β_2 interferon mRNAs (Soreq et al., 1981).

That the effect was specific to the poly(A) tail is supported by the inhibition of translation of $poly(A)^+$ Lys^+ transcript by coinjected polyadenylic acid. The slight increase in translation of the $poly(A)^-$ transcript on coinjection of polyadenylic acid is possibly caused by the polyadenylic acid inhibiting translation of endogenous $poly(A)^+$ mRNAs, which would be preferentially translated compared with the $poly(A)^-$ transcript. This result is analagous to that of Jacobson and Favreau (1983) who inhibited translation in vitro by adding excess polyadenylic acid; although Woodland and Ayers (1974) found no effect in oocytes.

There are however some reservations about the applicability of results obtained using these synthetic mRNAs to the normal situation in vivo.

First, even the Lys^+ monomethyl cap $poly(A)^+$ was not as efficiently

translated as normal Lysozyme mRNA. This may simply have been because the synthetic mRNA was used at a higher concentration than the natural mRNA, and high amounts of injected mRNA are translated less efficiently than small amounts (Gurdon et al., 1971). Alternatively the 5' or 3'-untranslated regions of the natural mRNA, which are missing from the synthetic mRNA, may contain sequences essential for efficient translation. The role of these regions is unclear (see Littauer and Soreq, 1981; Kozak, 1983) and although as I have confirmed, these regions are not essential for translation either in vitro (Kronenberg et al., 1979; Krieg and Melton, 1984a) or in vivo (Soreq et al., 1981) their effect on the efficiency of translation has not been tested. Indeed the mutation rate in some parts of the untranslated regions of mRNA is lower than expected for non-functional sequence (Miyata et al., 1980; Martin et al., 1981), and in rabbit globin mRNA the 3' untranslated region seems to interact with the poly(A) tail (Albrecht et al., 1984), which suggests some function for these regions.

Second the length of poly(A) tail added to the synthetic mRNAs (most of median length 200-300 As) exceeds the length of 40-100 As found on steady-state populations of mRNA in vivo (Greenberg and Perry, 1972; Pemberton and Baglioni, 1972; Sheiness and Darnell, 1973; Gorski et al., 1974; Rosbash and Ford, 1974; Jeffery and Brawerman, 1974; Brawerman and Diez, 1975; Greenberg, 1975; Palatnik et al., 1980), or even the length of 150-200 As of newly synthesised poly(A) tails (Kates, 1970; Nakazoto et al., 1973). However even with the shortest length of poly(A) tail tested (median length 180 As) a stimulation of translation exceeding the increase in stability of the transcript was found.

Thus efficient translation of synthetic mRNAs in Xenopus oocytes depends on the transcripts being polyadenylated. This is also true with both natural (Huez et al., 1981) and synthetic mRNAs (Drummond et al., in press) injected into cultured cells, although it is not known if the

effect in cultured cells is due to increased stability or efficiency of translation of the mRNA.

11.5 Effect of capping on translation of synthetic mRNAs in vitro

In the wheatgerm cell-free translation system the relative translation of Lys^+ transcripts was dimethyl cap (100%), monomethyl cap (80%) and uncapped (60%). As in oocytes the capped RNAs were more efficiently translated than the uncapped; though the relative efficiency of the two capped forms was different from oocytes with the dimethyl rather than the monomethyl cap giving the best translation. However such translational differences are merely the result of the potassium ion concentration in the particular wheatgerm assay used (Mead et al., 1985).

11.6 Effect of polyadenylation on translation in vitro

The inhibition of translation in the reticulocyte lysate and wheatgerm systems by polyadenylation of the mRNAs is unexpected. Previous studies on the contribution of the poly(A) tract to translation in vitro always involved the deadenylation of $poly(A)^+$ RNAs. Although some of the initial studies found no difference in the translation of $poly(A)^+$ and $poly(A)^-$ mRNAs (Huez et al., 1974; Bard et al., 1974; Gielen et al., 1974), later work using more efficient translation systems found that $poly(A)^+$ mRNA was more efficiently translated than $poly(A)^-$ (Doel and Carey, 1976).

With the reticulocyte lysate system that I used Lys^+ $poly(A)^-$ RNA was being reinitiated over 50 times, indicating the high efficiency of the system. It is therefore surprising that polyadenylation reduced translation by at least 3 fold, especially as the same RNA preparations stimulated translation in both oocytes and cultured cells. Also control experiments have established that although $poly(A)^+$ mRNA exhibited a

strong trans effect and inhibited translation of poly(A)$^-$ mRNAs in the same reaction, this was not due to a non-specific toxic factor. Since adding polyadenylic acid to an in vitro translation system reduces translation of natural poly(A)$^+$ mRNAs (Jacobson and Favreau, 1981) the inhibition I observed may be due to adsorption of crucial translational components by the long poly(A) tracts present in the transcripts. These poly(A) tracts are longer than those normally found on natural mRNAs (see section 12.4).

11.7 Movement of synthetic mRNAs in oocytes

Melton (1984) has suggested that synthetic RNAs can move rapidly in oocytes. This was based on an experiment where globin sense-strand transcript was injected into oocytes and 10h later globin anti-sense strand was injected. Over 90% of the sense-strand transcripts could then be recovered in hybrids. However the anti-sense transcript was used in such vast excess (50 fold) that even a little movement could provide sufficient overlap to ensure efficient hybridisation. I directly compared the rate of movement of radioactive Lys$^+$ and Chym$^+$ synthetic RNAs with that of radioactive reovirus mRNA, and found that the synthetic RNAs do indeed move more rapidly than the natural mRNAs; although even by 24h after injection the synthetic mRNAs have still not equilibrated within the oocytes.

Since the synthetic RNAs that I used were not as efficiently translated as their natural counterparts, the synthetic mRNAs may not be in the same structural state following injection as the natural mRNAs. They might not, for example be binding mRNA specific proteins as efficiently as natural mRNAs, and the smaller size of their RNA-protein particles (RNPs) may permit more rapid movement. However a proper analysis of the state of injected mRNA would be required to confirm this.

One specific difference was that the synthetic RNAs used lacked the poly(A) tail of the corresponding natural mRNAs. I found that polyadenylation of the synthetic mRNAs did slow their movement up to 1.5 times more than would be expected purely from their increased length. However it is not clear that the reduction in rate is sufficient to completely explain the difference in rate of natural and synthetic mRNAs. Certainly the slow movement of the naturally poly(A)$^-$ reovirus mRNA shows that a poly(A) tail is not an absolute requirement for slow movement.

An explanation of the slower movement of polyadenylated mRNAs would be the interaction of the poly(A) tail with poly(A) binding proteins (Richter and Evers, 1984) leading to an increase in the size of the mRNP. Goldenberg et al. (1980) found that the poly(A) tail (length 80 As) on duck globin mRNA had 4-5 proteins (each M_r 73,000) bound to it. This would also explain why increasing length of poly(A) tail correlated with decreased rate of movement, since the longer poly(A) tail would bind more poly(A) binding proteins than shorter poly(A) tails.

However *if* this explanation is true, it would also mean that the slowing effect of the poly(A) tail would be much more significant in the synthetic mRNAs, which had unusually long poly(A) tails (see section 10.2), than in natural mRNAs with their normal length of poly(A) tail; and this might explain why no difference was detected in the rate of movement of natural poly(A)$^+$ and poly(A)$^-$ mRNA. This would also leave unanswered the question why the natural poly(A)$^-$ and poly(A)$^+$ mRNAs moved more slowly than the synthetic poly(A)$^-$ mRNA.

Like the natural mRNAs there was also asymmetry in the rate of movement of the synthetic mRNAs. The synthetic mRNAs again moved more rapidly from the vegetal to the animal half than in the opposite direction. Autoradiography of oocyte sections confirmed that this

asymmetry was not due to the RNA either entering or being injected into the nucleus, also there was no specific streaming of the RNA injected into the vegetal pole that might explain its preferential movement.

Since the animal half of the oocyte contains 60% of the fucose accessible cytoplasm, the concentration gradient of an injected substance will be larger moving from the vegetal to the animal half than in the opposite direction. Also as the amount of a substance that diffuses is proportional to the concentrations gradient that it moves along (see Appendix II and Crank, 1975) more will move from the vegetal to the animal half than in the opposite direction. If the injected RNA distributes at equilibrium like fucose (60% in the animal half) the amount that moves from the vegetal to the animal half should be 1.5 times the amount moving in the opposite direction. Since the synthetic mRNAs were radioactive this difference in rate of movement could be measured. In different experiments Lys^+ monomethyl cap $poly(A)^-$ RNA moved 1.3 times, 1.7 times and 1.8 times faster, and Lys^+ monomethyl cap $poly(A)^+$ RNA 1.5 times and 2 times faster, from the vegetal to the animal half than in the opposite direction, which is close to the expected difference of 1.5 times.

This, together with the data from natural mRNAs that amount of mRNA does not affect rate of movement, suggests that injected mRNAs at least behave as if they move by diffusion, and will distribute within the oocyte at equilibrium according to the distribution of accessible cytoplasm. In addition the rate of mRNA movement may simply be determined by the size of the mRNP particle.

CHAPTER 12

Further experiments

In this final chapter I will describe a few of the ways in which the work described in this thesis might be improved or extended.

One of the major difficulties encountered in this work was accurate quantitation of movement. Although this was partially overcome by using radioactively labelled RNA, the basic experimental design was such that it still suffered from variation that made comparison of the movement of different RNAs difficult. The main causes of this variation were differences in the exact position at which the mRNA was injected into oocytes, and variation during dissection of the oocyte into its animal and vegetal halves. It would be difficult to eliminate either of these sources of variation.

A more satisfactory comparison of movement would be by directly measuring the diffusion coefficients of the mRNAs, as Horowitz (1972), Horowitz and Moore (1974), and Paine *et al*. (1975) did for other molecules. They injected radioactively labelled molecules, sectioned the oocytes, and made autoradiographs of oocyte sections. By measuring the distribution of grains from the injection site, the diffusion coefficient could be calculated (Horowitz, 1972). The advantage of this approach is that all measurements are made relative to the actual injection site, so variations in the precise location of the injection site within the oocyte no longer matter.

Two methods for detecting injected mRNA are available which would allow measurement of its distribution in oocyte sections, and therefore calculation of the mRNA's diffusion coefficient. One is to inject non-radioactive mRNAs, then detect their position in oocyte sections by *in situ* hybridisation of radioactive probes, as described by Capco and Jeffery (1982), Jamrich *et al*. (1984), and Phillips (1985). This method

of detection is not however very satisfactory since, at least in my hands, the methods of Capco and Jeffery (1982) and Phillips (1985) do not readily work (data not shown).

The second method of detection avoids the hybridisation step by injecting radioactively labelled RNAs. The simplest method of obtaining high-specific activity mRNAs is to use synthetic mRNAs which are transcribed *in vitro* (Melton *et al*., 1984). I carried out some experiments using such radioactively labelled RNAs; but found that the movement of the synthetic mRNAs which I used was faster than that of natural mRNAs. Obviously the structure of such synthetic mRNAs would have to be refined until they functioned like natural mRNAs, to give physiologically meaningful results.

As well as moving faster, I also found that the synthetic mRNAs were not as efficiently translated as natural mRNAs. These results may have been because the synthetic mRNAs lacked the 5' and 3' untranslated regions of the natural mRNA; although this is not certain since the function (if any) of these regions is not known (Littauer and Soreq, 1982; Kozak, 1983; also see section 11.4). What is required is a systematic study of the effect of these regions on stability, translation and movement, also to find if the 3' untranslated region, by interacting with the poly(A) tail (Albrecht *et al*., 1984), affects the stimulation of translation which I found upon polyadenylation of the synthetic mRNAs. Such studies should use a single transcript which resembles as closely as possible the natural mRNA. Cloning of the required complete cDNA to make such transcripts is possible using the methods of Okayama and Berg (1982) and Lang and Spritz (1985). Although a synthetic transcript of the complete *Xenopus* β globin mRNA is available (Krieg and Melton, 1984a) this is not ideal, since the complete transcript includes 30 Cs at the 3' end (from the cloning procedure), and the rest of its sequence does not contain suitable

restriction sites to allow easy manipulation of the transcript's structure.

As well as optimising the synthetic mRNAs themselves, a further refinement towards obtaining physiologically meaningful results from movement studies would be to inject the mRNA in its normal form, that is as an RNP particle, rather than injecting large amounts of naked mRNA. This might be important if mRNA movement is slowed by the binding of proteins, since the naked mRNA will be moving faster than it would in its normal form, leading to an overestimate of its diffusion coefficient. Such RNPs could be assembled from naked mRNA and oocyte (Richter and Smith, 1984) or reticulocyte (Greenberg and Carrol, 1985) proteins *in vitro*. before injection.

RNA transcribed within the oocyte from DNA injected into nucleus appears to equilibrate rapidly (Drummond *et al*., 1985), however this may simply be due to the position of the nucleus close to the oocyte's equatorial region. As an alternative to the bulk movement study *in situ* hybridisation (if it could be got to work!) could be used to follow the distribution of the transcripts as they moved from the nucleus; although even this would not be entirely satisfactory as the large size of the nucleus, and constant addition of new transcripts to the cytoplasm, would prevent measurement of actual diffusion coefficients.

Interest in the movement of injected mRNA is likely to increase for several reasons. It is now, for example, possible to block translation by injecting anti-sense transcripts, that have been transcribed *in vitro*. which hybridise to endogenous mRNAs *in vivo* and block their translation (Melton, 1985). Using this method Rosenberg *et al*. (1985) have been able to mimic the effect of the *Kruppel* mutation in genetically normal *Drosophila* embryos. To be effective such injected anti-sense transcripts must spatially overlap with the endogenous mRNA; therefore knowledge of the movement of the injected RNA and the

limitations on such movement are essential for correct experimental design. This is particularly true in Xenopus or other amphibian embryos where, if an even distribution of the anti-sense transcript throughout the embryo is required, movement must be rapid enough to permit this in the short time before the first cell divisions occur and prevent further movement of the transcript. This problem is more acute in Xenopus embryos where the first cell division is 90 min after fertilisation compared with 3h in Drosophila (see Slack, 1983), especially given the larger size of the Xenopus embryo.

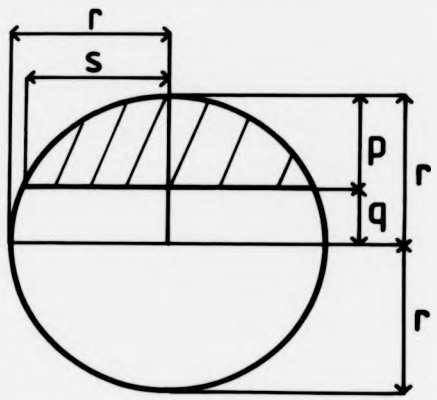
There is also increasing interest in localised mRNAs, especially those found in oocytes and embryos which are potentially important during animal development (see Davidson, 1976). Some such localised mRNAs have already been identified in Xenopus laevis (Rebagliati et al., in press). It would be interesting to examine the behaviour of such mRNAs upon reinjection; for example to test if they always return to their normal (localised) position. Obviously this would not be possible if these mRNAs suffer from the same constraints and slow movement which I have found in oocytes, and following injection into the animal half of matured oocytes. I also did not find any evidence for rapid movement, other than an initial surge throughout the egg on injection, in unfertilised eggs; although the results of Capco and Jeffery (1981) suggest that reinjected egg RNA may be able to move rapidly. Clearly movement of mRNA in the egg and early embryo require further investigation.

Movement of mRNA in the Xenopus oocyte is of considerable interest in its own right given the experimental use of oocytes, and the oocyte's normal role in development; however it would also be of interest to know if mRNA movement is as slow in other cell types as it is in Xenopus oocytes, and therefore if the oocyte might serve as a model system for movement studies. This is not an easy question to answer since, unlike

the giant Xenopus oocyte, it is not possible with present methods to introduce mRNA into only one part of a small somatic cell, and follow movement of the mRNA away from the injection site. Rather one must follow movement of molecules which are already evenly distributed in the cell following injection. Although this is more difficult it is not impossible and Wojcieszyn et al. (1981) have measured the diffusion coefficient of bovine serum albumin (BSA) in fibroblast cells. They injected fluorescein labelled BSA then, using a laser beam, photobleached a small area of the cell. By measuring the time taken for non-bleached molecules to enter the bleached zone, their diffusion coefficient could be calculated. This method could also be used with RNA. The RNA could be labelled either by incorporating biotin nucleotide analogues during its synthesis (Langer et al., 1981) or by cross-linking biotin to existing natural RNAs (Forster et al., 1985). Using this method the fluorescent label would be conjugated to an intermediate molecule such as avidin which has a high affinity for biotin, and would therefore bind the fluorescent label to the RNA. Alternatively it may be possible using the photo-activated cross-linking technique of Forster et al. (1985) to directly join the fluorescent label to the RNA, removing the need for biotin. Another approach would be to join fluorescein to the proteins (Wojcieszyn et al., 1981) of an RNP particle. Care would have to be taken that such modifications did not affect subsequent interactions of either the RNA or RNP in the cell, which would affect the rate of movement of the RNA; however if successful such a method would in many ways provide the most satisfactory measurement of mRNA movement, as the mRNA would be in its normal RNP form, at equilibrium within the cell.

Appendix I. Volumes of the animal and vegetal halves of the oocyte

Crossection of an oocyte: shaded area represents the pigmented animal half.



From measurements of oocytes, on average

radius $r = 600\mu m$ $p = 541\mu m$ $q = 59\mu m$

$s = (r^2 - q^2)^{\frac{1}{2}} = 597\mu m$.

Assuming the oocyte is a perfect sphere:

Volume of animal half (shaded area of diagram)

$= (\pi r^2 p) - (\frac{1}{3}\pi r^3) - (\frac{1}{3}\pi s^2 q)$

$= (\pi \times (600)^2 \times 541) - (\frac{1}{3}\pi(600)^3) - (\frac{1}{3}\pi \times (597)^2 \times 59)$

$= 4.078 \times 10^8 \mu m^3$

Total volume of oocyte $= \frac{4}{3}\pi r^3 = \frac{4}{3}\pi(600)^3 = 9.048 \times 10^8 \mu m^3$

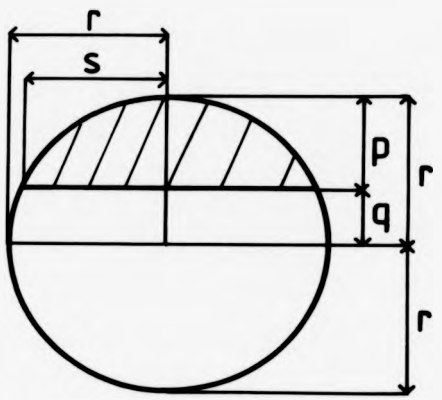
Volume of animal half/volume of oocyte $= 4.078 \times 10^8 / 9.048 \times 10^8 = 0.450$

i.e. The animal half forms 45% of the oocyte's total volume.

For full details of the equation for the volume of the animal half see Moise (1963) or any geometry text-book.

Appendix I. Volumes of the animal and vegetal halves of the oocyte

Crossection of an oocyte: shaded area represents the pigmented animal half.



From measurements of oocytes, on average

radius r = 600μm p = 541μm q = 59μm

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Volume of animal half (shaded area of diagram)

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i.e. The animal half forms 45% of the oocyte's total volume.

For full details of the equation for the volume of the animal half see Moise (1963) or any geometry text-book.

Appendix II. Fick's first law of diffusion

Fick's first law of diffusion is

$$F = DA(\frac{dc}{dx})$$

F is the flux or amount of substance moving per unit time, through an area of crossection A, along a concentration gradient $\frac{dc}{dx}$. D is the diffusion coefficient which depends on the substance moving (the solute) and the properties of the solvent.

In other words the amount of substance moving is directly proportional to the concentration gradient along which it moves.

This has several implications for movement in oocytes: Following injection of different amounts of mRNA the concentration gradient will obviously differ, however since the product of (DA) is constant the proportion of the mRNA moving will remain constant.

The accessible volume of cytoplasm is unevenly distributed between the animal and vegetal halves of the oocyte; the animal half contains 60% of the accessible volume of cytoplasm (see section 5.3). Following injection the concentration gradient of the mRNA will be 1.5 times larger moving from the vegetal to the animal half, than in the opposite direction. Therefore the flux or amount of mRNA moving from the vegetal to the animal half will be 1.5 times that moving in the opposite direction.

In conclusion

(1) Irrespective of the amount of mRNA injected, the proportion or percentage of the total which moves remains constant.

(2) The amount of mRNA moving from the vegetal to animal half will be 1.5 times that moving from the animal to vegetal half.

This however depends on several assumptions.

(i) The mRNA is moving by diffusion.

(ii) Following injection the H_2O that was injected with the mRNA equilibrates more rapidly than the mRNA itself.

(iii) It ignores any effects caused by reflection of the mRNA from the oocyte cell membrane or nuclear membrane.

(iv) It ignores any effects caused by different diffusional path lengths in the animal and vegetal halves because of the different sizes of the yolk platelets in the two halves.

A full discussion of diffusion is given by Crank (1975).

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