STUDIES ON NUCLEAR RNA POLYMERASES AND THE ROLE OF CYCLIC NUCLEOTIDES ON TRANSCRIPTION

A Thesis submitted to the University of Warwick in fulfillment of the requirements of the Degree of Doctor of Philosophy

by

AMIRA NADJAT-SHOKOURI

Department of Chemistry and Molecular Sciences, University of Warwick, Coventry, CV4 7AL, England. February, 1978.
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TO MY FAMILY

TO WHOM I AM GREATLY INDEBTED
ACKNOWLEDGMENTS

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SUMMARY

1. An assay for measuring the endogenous RNA polymerase activity in *Tetrahymena pyriformis* nuclei was developed. The reaction rate was linear for 60 minutes at 25°C. This rate of the reaction was dependent on the presence of four nucleoside triphosphates (ATP, GTP, CTP, and UTP), Mg$^{++}$ or Mn$^{++}$ ions, and was optimal at high concentrations of KCl or (NH$_4$)$_2$SO$_4$ (0.15 M).

2. α-amanitin (a specific inhibitor of RNA polymerase II) was used to differentiate between the different classes of nuclear RNA polymerases, RNA polymerase I and RNA polymerase II. 3.6 x 10$^{-6}$ M α-amanitin inhibited approximately 60% of total RNA polymerase activity in nuclei isolated from logarithmically growing cells. Thus about two-thirds of the nuclear RNA polymerase activity was due to RNA polymerase II.

3. Cyclic AMP or dibutyryl cyclic AMP stimulated nuclear RNA polymerase activity at physiological concentrations (10$^{-6}$ - 10$^{-8}$ M). Maximum stimulation was obtained using 10$^{-7}$ M cyclic AMP or 10$^{-7}$ M dibutyryl cyclic AMP. The increase in activity stimulated by cyclic AMP in isolated nuclei was dependent on salt concentration. Cyclic AMP stimulated endogenous RNA polymerase I and inhibited endogenous RNA polymerase II activity. Chromatin-bound RNA polymerase activity was also stimulated by this cyclic nucleotide.

4. Cyclic GMP or dibutyryl cyclic GMP in the presence of CaCl$_2$ stimulated nuclear RNA polymerase activity at physiological concentrations (10$^{-8}$ - 10$^{-10}$ M). Maximum stimulation of nuclear RNA...
polymerase activity by cyclic GMP in the presence of 2 mM CaCl₂ occurred at 10⁻¹⁰ M cyclic nucleotide. 10⁻⁸ M dibutyryl cyclic GMP plus 2 mM CaCl₂ produced maximum stimulation. The stimulation was CaCl₂ dependent.

5. No significant stimulation of nuclear RNA polymerase activity was observed with 5'-AMP, 5'-GMP or 3',5'-cyclic CMP.

6. Chromatin-bound cyclic GMP and cyclic AMP phosphodiesterases (degrading enzymes of cyclic GMP and cyclic AMP respectively) were demonstrated to occur in Tetrahymena pyriformis nuclei.

7. The levels of total RNA polymerase activity were determined during the course of the natural cell cycle. By using α-amanitin, approximately two-thirds of the activity during the S phase of the cell cycle was found to be due to RNA polymerase II. RNA polymerase I activity was predominant in the G₂ phase of the cell cycle.

8. An assay for endogenous protein kinase activity in Tetrahymena pyriformis nuclei was developed and validated. The cyclic AMP-dependent phosphorylation of nuclear proteins was found to be a salt-dependent process. Dibutyryl cyclic AMP stimulated the phosphorylation of the endogenous protein(s) and exogenous substrates in a partially purified nuclear fraction containing RNA polymerase activity by 5-fold.
### Abbreviations

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<td>Cyclic AMP</td>
<td>Cyclic adenosine 3’:5’-monophosphate</td>
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<td>Dibutyryl cyclic AMP</td>
<td>N6,O2’-dibutyryl cyclic adenosine 3’:5’-monophosphate</td>
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<td>Cyclic GMP</td>
<td>Cyclic guanosine 3’:5’-monophosphate</td>
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<td>Dibutyryl cyclic GMP</td>
<td>N6,O2’-dibutyryl cyclic guanosine 3’:5’-monophosphate</td>
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<td>Cyclic CMP</td>
<td>Cyclic cytidine 3’:5’-monophosphate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
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<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
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<tr>
<td>CTP</td>
<td>Cytidine 5’-triphosphate</td>
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<tr>
<td>UTP</td>
<td>Uridine 5’-triphosphate</td>
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<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
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<tr>
<td>GMP</td>
<td>Guanosine 5’-monophosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris (hydroxy methyl) aminomethane hydrochloride</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>ECTA</td>
<td>Ethyleneglycolbis (β-aminostyly-ether)-N,N’-tetra-acetic acid</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-di 2-(5-phenyloxazyl) benzene</td>
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<td>Theophylline</td>
<td>1,3-dimethylxanthine</td>
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<td>Caffeine</td>
<td>1,3,7-trimethylxanthine</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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INTRODUCTION

1.1 Tetrahymena pyriformis

1.1.a General Background (Morphology and life cycle)

* Tetrahymena pyriformis is the type species of the genus Tetrahymena*. Antony van Leeuwenhoek observed them three centuries ago. In 1923 Andrew Lwoff first discovered *Tetrahymena pyriformis* in fresh water. He was able to grow this organism in axenic culture. Since that time, many thousands of strains have been isolated from fresh water in various parts of the world.

Normally, Tetrahymena is a pyriform-shaped and uniformly ciliated cell as shown in Fig. (1), but the extreme plasticity of the pellicle allows considerable temporary distortion. The average cell size is about 30 x 50 microns. Sixteen to twenty-six ciliary rows (Primary meridians) cover most of the cell surface and alternate with secondary meridians of mucocyst pores. The oral area is located close to the anterior pole of the cell. The macronucleus in both micronucleate and amicronucleate strains is typically oval to spherical body and always centrally positioned Fig. (2). The micronucleus when present is spherical in shape and located near the macronucleus.

The Protozoan *Tetrahymena pyriformis* has typical eukaryotic organelles including a nucleus, mitochondria and food vacuoles. The mitochondria of *Tetrahymena* contain all the enzymes of the Krebs cycle and are the major sites for energy production under aerobic conditions. The mitochondria change in number, shape and distribution during the growth cycle. In early exponential phase mitochondria are minimal in number, oval in shape and peripherally located near the basal bodies of
Fig. 1

A Chatton-Lwoff silver stain of a cell from a logarithmic culture. The oral area (OA) is in the anterior quarter of the cell. The dark granules of the primary meridians (PM) represent sites of cilia. Alternating with them are secondary meridians (SM) composed of mucocyst pores. x 480.

Fig. 2

A longitudinal section through a stationary-phase cell (strain GL). The oral area (OA) is in the anterior of the cell. The macronucleus (MA) is centrally located. Peripherally, the mucocysts (MU) and mitochondria (M) are indicated. x 1500.

[Reproduced from reference (2)]
the cilia, but become numerous, uniformly spherical and randomly distributed throughout the cytoplasm in the stationary phase. Some mitochondria were observed to be present within large autophagic vacuoles at this stage as well. Such variation in the mitochondrial distribution may be co-ordinated with the function of these organelles.

Mucocysts, small granules lying beneath the pellicle along the meridians have been observed in several strains of Tetrahymena. The function of mucocysts in Tetrahymena is unknown. They could coat the cell with amucoid substances that might protect it against osmotic shock. *Tetrahymena pyriformis* contains lipid droplets distributed throughout the cytoplasm. They increase in number as the culture matures (2). The particulate peroxisomes are also found in Tetrahymena. These organelles contain the key enzymes of glyoxylate cycle. This cycle plays a role in the carbohydrates metabolism of this protozoan by converting lipids to glycogen by shunting acetate. Lysosomes according to deDuve and Wattiaux (3) are subcellular organelles enveloped by a unit membrane which contain acid hydrolases which have been identified in this protozoan. More than one type of lysosome appears to exist. These are primary lysosomes, secondary lysosomes (food vacuoles) and tertiary ones, which contain other subcellular organelles (2). Two basic types of endoplasmic reticulum have been described in this protozoan (4). One, devoid of ribosomes and including the Golgi apparatus, is the smooth (agranular) endoplasmic reticulum. The other is the ribosome containing rough (granular) endoplasmic reticulum.

Most strains of *Tetrahymena pyriformis* which contain micronuclei are sexually active. They reproduce by conjugation when cells of different mating types within the same syngen are mixed under appropriate conditions (5).
By this process, shortly after cell pairs are formed, the micronuclei undergo meiosis, resulting in four presumably haploid nuclei. Three of the four meiotic products degenerate, while the fourth divides mitotically once more. One of the two nuclei produced by this division migrates into the other cell and fuses with the nucleus which has remained behind. The derived diploid nucleus divides twice mitotically; and the two anterior products enlarge and begin to differentiate into macronuclei. Meanwhile, the old macronucleus becomes pycnotic and begins to disappear. One of the posterior nuclei becomes the new micronucleus, but the other disintegrates after separation of the cells. The micronucleus divides mitotically for the first cell division, in which each daughter cell receives one of the new macronuclei. This process is illustrated in Fig. (3). Vegetative divisions occur until

Fig. (3) Nuclear events in conjugation of Tetrahymena pyriformis.
[Reproduced from reference (9) Chapter 7]
the next conjugation (6). The micronucleate cells may lose their micronuclei with time in the laboratory; the cells then become amiconucleate. Amiconucleated cells are sexually inactive and they always reproduce by vegetative growth in which the polyploid macronuclei divide amitotically. The macronucleus elongates and pulls apart as the cell itself constricts to form two daughters (7).

The evolutionary development of Tetrahymena places it close to the divergence between plant and animal forms (8). The biochemistry of this ciliate is also similar in certain respects to that of mammals, plants and bacteria; it bears a striking resemblance to mammals in certain aspects of its metabolism and nutritional requirements. It has the ability to manufacture a vitamin B 12-like compound (9). Therefore it would be a promising model system in which to study the metabolic control systems of a simple unicellular eukaryote and, from an evolutionary point of view, the controls may be transitional between those of very primitive cells and those from highly organised ones. Tetrahymena pyriformis grows rapidly under laboratory conditions and has a generation time of about three hours. Such growth can be achieved in an axenic culture. In addition, many strains can be easily induced to divide synchronously by physical or chemical means (10). The manipulation of amiconucleate strains of Tetrahymena pyriformis in the laboratory is simpler than the manipulation of the micronucleate ones because the former reproduce the vegetative growth only. An amiconucleate, Tetrahymena pyriformis, strain w has been used throughout the present work.

1.2 Nuclei

1.2.a Macronucleus

Tetrahymena macronucleus is bounded with a porous envelope, consisting
of two distinct membranes with a space between them. The outer membrane of the envelope is continuous with the endoplasmic reticulum, thus providing a channel from the space in the nuclear envelope to the cisternae of the endoplasmic reticulum. Feulgen-positive small granules, chromatin bodies, as observed in the light microscope fill most of the macronucleus. These are 45 - 90 granules in number and it is evident from electron-microscopic-cytochemistry that these bodies contain the bulk of the macronuclear DNA (2). Chromatin bodies change in structure during the cell cycle and they also respond to environmental conditions (11). Nucleoli, 500 - 1000, dense small bodies containing ribosomal RNA and DNA, are found in a peripheral layer of the macronucleus. They are somewhat irregular in size and shape, but consistently larger than the chromatin bodies. Under certain conditions these bodies take on a specific pattern suggesting a type of maturation of the ribosomes. During the cell cycle they also change in structure (12). Fine fibres and microtubules have been resolved by quantitative, high resolution polyacrylamide gel electrophoresis of isolated nuclei (13). The function of the chromatin granules in nucleoli is associated with RNA synthesis. The isolation of Tetrahymena macronuclei has not proved particularly difficult and isolated nuclei have been used to study transcriptional activities. Successful methods for their isolation have been developed in a number of laboratories (14,15,16,17,18,19).

1.2.b. Micronucleus

Although I have worked with the amicronucleate strain of Tetrahymena pyriformis, strain w, throughout my research, I will describe the micronucleus very briefly.
A diploid micronucleus is a characteristic of most sexually active strains of Tetrahymena pyriformis. This micronucleus lies in an indentation of the macronucleus. It is about one-fifth the diameter of the macronucleus. It divides mitotically and shows five pairs of chromosomes during mitosis. The specific activity of micronuclear DNA as a template for RNA synthesis is at least 100 times lower than that of the macronuclear DNA as shown quantitatively by light microscopic autoradiographic studies (20). Unlike the macronucleus, histones H1 and H3 are absent from the micronucleus (21,22,23).

1.3 The Cell Cycle.

Mitchison has defined the cell cycle as "the period between the formation of the cell by the division of its mother cell and the time when the cell itself divides to form two daughters" (24). One complete cell cycle consists of several stages in which many biochemical events and morphological changes occur. These stages are divided into the following periods: G1, is the period from the end of cell division to the start of the S period; S, is the macronuclear DNA synthesis period; G2, is the period from the end of the S period to the start of division; D, is the division period. Synchronised cultures (when nearly all of the cells are in the same stage of the cell cycle) provide an excellent system in which to study the different biochemical events and their relationship to each other. There are many methods cited in the literature concerning the production of synchronous cultures, but these fall into two main categories - Induction Synchrony and Selection Synchrony (25).
1.3.a Induction Synchrony

For induction synchrony a normally growing exponential culture is subjected to a procedure which results in all the cells accumulating at a certain stage of the cell cycle. Several methods which produce such synchrony have been applied to *Tetrahymena pyriformis*.

The use of repetitive heat shocks is one of the most important and commonly used methods. In this method, which was first developed by Zeuthen and Scharbaum in 1954 (26), cells from exponentially growing culture are transferred from optimal to higher temperature for up to seven cycles of 30 minutes at 34°C, at 30 minutes intervals (28°C). This treatment causes the cells to stop dividing and, about 80 - 100 minutes after the end of the heat shocks, a synchronous burst of division occurs. As subsequent divisions occur, the cells become gradually less synchronous. However, although division is stopped during the heat shock treatment, growth and macronuclear DNA and RNA synthesis continues. This results in larger than average cells with more DNA per cell than normal. Approximately 70% of the cells synthesise DNA in synchrony between the first two synchronised divisions after the heat shocks (27). The rest of the cells do not undergo synchronous DNA synthesis during this cell cycle (28). The technique has been improved recently by giving seven cycles of 30 minutes heat shocks per generation time (2j - 3 hours) (29). Under these conditions DNA synthesis and cell division remain coupled.

In an oxygen-deficient environment *Tetrahymena pyriformis* cells stop dividing and the cells accumulate in G2 (30). This may be due to energy deprivation. Recently in our laboratory a single hypoxic shock has been used to generate a synchronous population of *Tetrahymena*.
pyriformis (31). In earlier papers repeated cycles of hypoxia (32,33) were used to obtain cell synchrony. Anderson and co-workers have reviewed other methods of induction synchrony (29). The general methods of induction synchrony are outlined in Table (1).

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<tr>
<th>Methods</th>
<th>Principle</th>
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<td>Changes in temperature are used as the treatment for inducing cell synchrony.</td>
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<tr>
<td>2) Nutritional Methods</td>
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<td></td>
<td>a) Inhibition of DNA synthesis: By the use of thymidine or by addition of methotrexate and uridine (which prevents the synthesis of thymidine).</td>
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Table (1) General Methods of Induction Synchrony
In this technique, cells which are in the same stage of the cell cycle are separated or selected from an exponential culture and grown up as a separate synchronous culture. Several techniques of selection synchrony have been developed by many workers in order to study the natural cell division cycle. One of the most widely used techniques is that of differential sedimentation through a linear sucrose gradient (42). This method has not been used with success to synchronise Tetrahymena pyriformis, because of its great motility and the effects of environmental changes on the shape and size of the cell (43). Lloyd et al. have developed a continuous-flow size selection technique to allow large-scale preparations of synchronous cultures of micro-organisms (44). This technique can be used for many types of eukaryotic cells as long as they do not aggregate. More recently Lloyd and co-workers (45) have shown that a continuous-flow cell cycle fractionation in a Sharples Supernatrizuge has all the advantages of the continuous-flow size selection technique in a conventional centrifuge. The authors have stated that the former method is technically simpler and more rapid than the latter method. It also avoids the prior harvesting of organisms and their exposure to density gradients.

Cells undergoing division lose their ability to ingest particulate matter (46) due to the lack of the oral apparatus at this stage of the cell cycle. On the basis of this observation, Wolfe (46) has developed a technique for selecting synchronous population of Tetrahymena pyriformis by addition of tantalum particles to a culture of logarithmically growing cells (asynchronous culture). Centrifugation through a Ficoll step-gradient was used by Wolfe to separate the non-feeding
population from the heavy feeding, interphase population.

Hildebrandt and Duspiva (47) have developed a technique for selecting synchronised cells by addition of iron particles to exponentially growing *Tetrahymena pyriformis*. Cells which have ingested iron particles are retained by a magnetic field. Cells at the division stage do not ingest iron particles and can pass through this magnetic field. These cells can be collected as a synchronous culture. Very recently a uniform electromagnetic field has been used in our laboratory to select synchronous *Tetrahymena pyriformis* cells which have been exposed to iron particles (48). This technique was found to be the best at producing a synchronous culture with a high synchrony index. The summary of general methods of selection synchrony is outlined in Table (2).

<table>
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<tr>
<th>Methods</th>
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b) Continuous-flow cell cycle fractionation in a Sharpless Supercentrifuge; this technique is simple and rapid (10L culture/15 mins). | 44, 45     |
| 3) Differential Density Labelling | a) Selective separation of late division and post-division cells from a tantalum-containing growing culture by centrifugation through a Ficoll step-gradient.  
b) Selective separation of late division cells from an iron-containing growing culture by passage through a magnetic field. | 46         |

Table (2) General Methods of Selection Synchrony
1.4 Control of Transcription in Bacteria

Up to this time most work has been done with bacteria to elucidate the fundamental aspects of transcriptional control because of their simpler transcriptional unit than that of eukaryotes. Jacob and Monod in 1961 (49) described the lac operon and they showed that the related genes in bacteria exist in control structural and functional units. Each unit is termed an operon which consists of a group of cistrons that function co-ordinately under the combined control of an operator and a repressor. An operator is defined as the DNA sequence which the repressor molecule recognises and to which it binds. In addition, there is a promotor region on the DNA with which the DNA dependent RNA polymerase interacts. The discovery of specific repressor proteins that interact with specific operators in the cell genome and prevent the transcription of the particular region of the chromosome controlled by these operators was of particular significance (50,51). The binding of various allosteric effectors to these repressors changes their protein conformation resulting in a loss of repressor affinity for DNA. Thus, the transcription of the derepressed genome region can occur. Another protein that interacts with the bacterial genome is the cyclic-AMP binding protein (CRP or CAP) which was discovered by two groups of workers (52,53). This protein was purified by Anderson et. al in 1971 (54) and found to be involved in the control of transcription of the Lac and Gal operons in E. coli (55,56). This protein combines with cyclic AMP and then binds to promoters on the bacterial chromosome. Such binding induces a conformational change in promotor DNA which allows binding of RNA polymerase (55).
Proteins which specifically interact with RNA polymerase and are involved in control of bacterial transcription have been isolated by some workers. These proteins have effects on initiation of RNA synthesis, elongation of RNA chains, and on termination of RNA synthesis. Some of these proteins are the sigma factor (σ) (57), the p factor (p) (58), the M- factor (59), the V factor (V) (60) and the H- factor (61).

Bacterial cells can also regulate transcription by the mechanism of "stringent" control (62). In such cells, two nucleotides, guanosine tetraphosphate and guanosine pentaphosphate, can accumulate and these nucleotides interact directly with RNA polymerase and inhibit transcription (63).

1.5 Control of Transcription in Eukaryotic cells:

The eukaryotic chromosomes are very complex compared to the bacterial genome (64). The question of the existence of an operon system in eukaryotes remains open. The model for the structure of the chromosomes of higher organisms put forward by Crick (65) suggests that chromosomal DNA falls into two classes: globular DNA containing unpaired regions for control and a much smaller fraction consisting of fibrous DNA which alone codes for proteins. Eukaryotic DNA is complexed with a wide variety of non-histone proteins and histones in contrast to the predominantly naked bacterial chromosome. Therefore, there is a major structural difference between eukaryotic and prokaryotic genetic material that results in major differences in modes of transcriptional control. However, several protein factors which are analogous in a way to the repressors of bacteria have also
been found in eukaryotic cells (66, 67, 68, 69). Some of these proteins seem to have their effects on RNA chain elongation (70) although the factor of Stein and Hausen may stimulate chain initiation (66). Another, which has been reported by Crippa (67) inhibits ribosomal DNA transcription by binding specifically to ribosomal DNA and inhibiting transcription. There is also a process directly analogous to bacterial stringent control exhibited by a multicellular eukaryotes. Such systems have been demonstrated in ascites cells and also in yeast (71,72).

1.5. a. Histones and Transcription Control

Histone-DNA interactions determine the structure of the eukaryotic chromosome and may ultimately prove to be an important part of the control of the genetic activity in the chromatin itself (73). In recent years the interaction between DNA and histones and the packing of eukaryotic DNA has attracted much attention. It was found that histones act as non-specific repressors of transcription by binding to DNA, hence inhibiting the ability of DNA to serve as a template for RNA synthesis (74,75). The process by which histones block transcription has not yet been elucidated (73). However, extensive research has shown that histone structure is conserved from the evolutionary point of view. Since there are only five different types of histone it is unlikely that histones are wholly responsible for the regulation of eukaryotic transcription (76,77,78,79,80).

1.5.b. Non-histone Proteins and Transcription Control

Non-histone proteins are a heterogenous, ill-characterised group of proteins associated with eukaryotic chromosomes (81,82,83). It has been shown that these proteins show both tissue and species specificity (84,85,86). Several lines of evidence strongly suggest
that the non-histone proteins are specific positive effectors of transcription in eukaryotes. Unlike histones, the non-histone proteins are found to be in higher concentrations in the chromatin of metabolically active cells than in chromatin from inert cell types (87). Moreover, it has also been observed that these proteins are preferentially localised in those regions of the chromatin that are most active in RNA synthesis (88,89). It has been reported that non-histone proteins can stimulate transcription in cell-free systems (90). The non-histone proteins are also synthesised in cells at times of gene activation (91).

Paul and Gilmour in 1968 were the first to report that non-histone proteins could interact with DNA in a tissue-specific fashion and thereby modify transcription (92). Later it was shown that some of these proteins were able to bind specifically to homologous DNA (79,84). Tsanev and Sendov in 1971 postulate that the non-histone proteins act by interacting in a specific manner with histone complexes, thus activating transcription (93). This hypothesis provides a mechanism for positive control and it also postulates repressors, analogous to those reported in prokaryotes, as negative controls. Another hypothesis illustrates another possible role of non-histone proteins in the transcriptional unit of chromatin (94). This is shown in the model depicted in Fig. (4).
Figure (4)

(A) Diagrammatic representation of part of a chromatid, showing alternating 'fibrous' and 'globular' DNA. One globular locus has formed a transcription loop.

(B) Some details are enlarged. Globular regions consist of DNA and histones (nucleohistone), forming an irregularly supercoiled structure which contains transcribable sequences. Fibrous regions contain address sites to which non-histone proteins are attached. A configurational change may be induced e.g. by an effector such as a steroid-acceptor protein, and this permits an RNA polymerase molecule to bind to DNA. If repressor binding sites are unoccupied, the polymerase may start to transcribe at the initiation site and unwind the globular DNA as it progresses along the transcriptional unit, generating polymers (RNA and informofer proteins). Among non-histone proteins may, therefore, by RNA polymerase subunits, informofer proteins, repressors, acceptors and effectors. Address and repressor binding sites should contain repetitive sequences whereas structural genes in the globular regions should contain unique sequences.
Fig. (4) A speculative model for the transcriptional unit of chromatin showing possible roles for non-histone proteins. Reproduced from reference (96).
1.3c RNA Polymerases

DNA-dependent RNA polymerase (nucleosidetriphosphateRNA nucleotidyltransferase, E.C. 2.7.7.6) is the key enzyme implicated in the first step of genetic information flow from DNA to RNA. It catalyses the sequential assembly of the four ribonucleoside triphosphates into RNA molecules. The polymerisation reaction can be summarised as follows:

\[
\text{DNA} + \text{Mg}^{++} \rightarrow \text{pppPu} + \text{npppX} \rightarrow \text{ppp} \left( \text{pX} \right)_n + \text{npp}_{ii}
\]

[pppX = ribonucleoside triphosphate, X = adenosine, guanosine, cytosine or uridine, Pu = Purine ribonucleoside, pp$_i$ = inorganic pyrophosphate]

The RNA product, pppPu(pX)$_n$, is formed in the presence of Mg$^{++}$ and DNA as template, with the concomitant release of inorganic pyrophosphate. The total reaction is irreversible, since the pyrophosphorolysis of RNA does not take place with the enzyme (95, 96). The steps involved in the synthesis of RNA by RNA polymerase in vitro occur in the following sequence: The binding of the enzyme to DNA at discrete sites, initiation of RNA chains involving strand selection and the exclusive selection of a purine ribonucleoside triphosphate at the 5' end, elongation of the RNA chains from 5' → 3' and with concomitant elimination of inorganic pyrophosphate until a termination site is reached, at which newly synthesised single stranded RNA chains are released from the DNA-enzyme complex (97).

DNA-dependent RNA polymerase activity was first demonstrated by Weiss in rat liver nuclei (98). The first solubilisation and demonstration of several forms of eukaryotic RNA polymerase was
accomplished by Roeder and Rutter (99). It is the activity of these enzymes that is ultimately modified by any transcription control mechanism as they are bound within the chromatin matrix during transcription. Nuclear DNA-dependent RNA polymerases, although not generally considered as such, are another group of non-histone proteins. Since the discovery of multiple forms of RNA polymerase by Roeder and Rutter, it has been found that these multiple forms have different subcellular localisation, different functions, and distinct subunit structures.

The three forms of eukaryotic RNA polymerases have been isolated from a wide variety of tissues and organisms (100,101,102,103,104,105). In bacteria a single RNA polymerase enzyme is responsible for the synthesis of all types of RNA. In higher eukaryotes class I or A polymerases are localized in the nucleolus (99,102) and are responsible for the synthesis of ribosomal RNA (106). Class I polymerases are resistant to the mushroom toxin α-amanitin at concentrations up to 1 mM (1 mg/ml) (70). Enzymes of class II or B are nucleoplasmic (99,100) and are responsible for the synthesis of heterogeneous nuclear RNA, the presumed precursor of messenger RNA (107). Class II enzymes are distinguished from Class I enzymes by their extreme sensitivity to α-amanitin. Nanomolar concentrations of this toxin can inhibit class II enzymes (70). Class III or C enzymes are also nucleoplasmic and possibly cytoplasmic enzymes (99,100,108). Type C enzymes have not been isolated from all eukaryotes so far examined. Class III enzymes are responsible for the synthesis of transfer RNA and 5S ribosomal RNA (109) and exhibit an intermediate sensitivity to α-amanitin (0.1 - 0.01 mM) (70). An additional function
for class II and III enzymes is the synthesis of viral messenger RNA precursors and viral 5.5S RNA in cells infected with type 2 adenovirus (110). Class II enzymes were also found to be responsible for the transcription of viral DNA in cells infected by an RNA tumour virus (111) and for the transcription of polyoma virus DNA in infected cells (112).

The proportions of the three enzymes as well as their cellular levels can vary considerably among different cell types in the same organism and even in the same cell under different physiological conditions (113,114). During the cell cycle the activities of the different forms of RNA polymerase have been found to be independently regulated (115).

In bacteria, DNA-dependent RNA polymerase is resistant to the toxin α-amanitin but it shows extreme sensitivity towards the drug rifampicin (116,117). The drug blocks RNA chain initiation by binding tightly to the σ subunit of the enzyme and probably prevents the binding of the initial nucleoside triphosphate to the enzyme (118). The bacterial RNA polymerases are large molecules (m.w. between 400,000 and 500,000) and have complex subunit structures as judged by polyacrylamide gel electrophoresis in 6 M urea and 0.1% SDS (119). These subunits have been designated by Burgess et al. as β−, β, σ, α and ω, in order of decreasing molecular weight. There are many reports which indicate that β−, β, σ and α are functional subunits in bacteria (120, 121). Sigma factor is an important subunit for initiation of transcription by the holoenzyme. It can be dissociated from the whole enzyme as shown in the figure depicted below.
Fig. (5) **Summary of the subunit structure of E. coli: RNA polymerase**
(Burgess and Travers, 1970) (122)

In eukaryotes, the three forms of DNA-dependent RNA polymerase also possess distinct subunit structures as determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS and 8 M urea. All of the enzymes examined so far consist of two large molecular weight subunits and from four to eight subunits of decreasing molecular weight that differ slightly depending on the species (70,105). In yeast, an exceptional case, it has been found that the enzyme contains 10 - 11 subunits, two large subunits in equimolar amounts and several smaller components (123,124,125). In Tetrahymena pyriformis analysis of subunit structure has not yet been determined.

A variety of studies have shown conclusively that the three classes of eukaryotic RNA polymerase are not interconvertible, although a common pool of small molecular weight subunits has not been ruled out (70,101,105). In addition, each of the three classes of polymerase exhibits chromatographic and electrophoretic heterogeneity when carefully analysed (70). It seems that the basic structure of the mammalian nuclear enzymes is similar to that of the bacterial enzyme since they both consist of two high molecular subunits accompanied by several smaller subunits. Such studies suggest that no drastic change has occurred in the structure of these RNA polymerases during evolution.
1.6 Control of Transcription by phosphorylation of nuclear proteins and RNA polymerases

Extensive research in recent years has established that the major regulatory process affecting nuclear proteins is the phosphorylation and dephosphorylation of these proteins \((86,126,127)\). Langan has found that the administration of glucagon to rats causes a marked increase in the phosphorylation of a specific serine residue in the lysine-rich \((F_1)\) histone fraction of liver during a one-hour period following the administration of the hormone \((128)\). Such phosphorylation of the histone fraction was found at times of gene activation and cell division as well \((79,80)\). It has been found that acetylation of nuclear proteins can also play a role in the regulatory process of these proteins. Allfrey et al. have found that acetylated histones did not inhibit RNA synthesis when added to isolated calf thymus nuclei \((129)\). However, more recently, Marushige \((130)\) reported that the ability of calf thymus chromatin to support DNA-dependent RNA synthesis is markedly increased by modification of the histones with acetic anhydride. He suggested that the acetylation of histone side chains stimulates the rate of chain elongation during transcription of chromatin. It is thought that these covalent modifications of histones result in increased genetic activity throughout the chromatin, due primarily to an increase in the negative charges of the proteins caused by the presence of the extra phosphate or acetyl groups.

Allfrey and co-workers \((79)\) suggested that this increase in negative charges causes the histones to dissociate from the DNA and permit general transcription of most of the chromatin. Although the possibility still exists, there is no direct evidence that histones have any role in the control of gene activity in vivo \((80)\).
Correlations between the phosphorylation of non-histone chromosomal proteins and gene activity observed in a number of systems suggested the involvement of these acidic proteins in gene activation (131,132,133). These proteins, as mentioned earlier, are both heterogenous and of numerous different types (84,134). Therefore if the phosphorylated non-histone proteins are involved in specific gene regulation, then one would expect this protein fraction to be heterogenous and exhibit specific differences in tissues and cell types where differences in gene expression occur. Allfrey and co-workers have reported that these non-histone phosphoproteins can bind specifically to homologous DNA (79) and can stimulate RNA synthesis in cell-free systems. Shea and Kleinsmith have also found that the addition of rat liver phosphoprotein in a system employing rat DNA as a template with purified rat liver RNA polymerase, can cause more than a doubling in the synthesis of RNA (135). Interestingly, it was found by the same workers that this effect is blocked by pre-incubating the phosphoprotein with E. coli alkaline phosphatase.

It has been reported by many research workers that the phosphorylation of different species of non-histone proteins is mediated through a number of nuclear protein kinases (ATP: Proteinphosphotransferase, E.C. 2.7.1.37)(136,137,138) and this phosphorylation can alter the effect of non-histone proteins on transcription. For example, non-histone proteins phosphorylated by pre-incubation with a purified nuclear protein kinase and ATP were able to stimulate the levels of RNA synthesis seen in chromatin reconstituted with these proteins as opposed to non-phosphorylated proteins (139). The nuclear protein kinase alone had no effect on transcription. These results suggest that
phosphorylation of non-histone chromosomal proteins by nuclear kinases is related to transcriptional control and gene activation. A model summarising the relationships between the phosphorylation and dephosphorylation reactions of non-histone phosphoproteins is depicted in Fig. (6) taken from the report of Kleinsmith (140).

In this model, Serine (and threonine) residues in the proteins are phosphorylated via the terminal phosphate of various nucleoside triphosphates and deoxynucleoside triphosphates in a typical protein kinase reaction. In a separate phosphatase reaction the phosphoserine (and phosphothreonine) bonds are broken, releasing inorganic phosphate.

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Fig. (6) Model summarising the relationships between the phosphorylation and dephosphorylation reactions of non-histone phosphoproteins.

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Although phosphorylation and dephosphorylation control the activity
of non-histone proteins, it is not known what regulates the activity of the nuclear protein kinases responsible for phosphorylating them. Johnson and Allfrey (141) have demonstrated that cyclicAMP stimulated the phosphorylation of non-histone proteins in rat liver, and administration of dibutylryl cyclicAMP to rats increased the phosphorylation of these proteins within fifteen minutes, but few investigators have isolated protein kinases from nuclei responsive to cyclicAMP (136,137,142,143). There is, however, a report by Kish and Kleinsmith (138) which reveals the presence of twelve nuclear protein kinases from bovine liver, some of which were stimulated by cyclicAMP, others were unaffected by this cyclic nucleotide, and some were inhibited by cyclicAMP. In many cases, the cyclicAMP dependence was seen only when the enzymes were phosphorylating their endogenous substrate (8).

This report shows clearly how complex the regulation of nuclear protein kinases might be. The picture is complicated still further by several reports that some of the effects of cyclicAMP in nuclei may be mediated by the translocation of cyclicAMP-dependent protein kinase catalytic subunits from the cytoplasm into the nucleus (114,145). Experiments by O'Malley and Means (146) have shown that a cytoplasmic protein kinase can be taken up by nuclei in the presence of a cyclic AMP binding protein and the complex can phosphorylate several non-histone proteins. These observations suggest that the cytoplasmic cyclicAMP-dependent protein kinases may be involved in the phosphorylation of non-histone proteins and thus in the control of transcription. However, the general role of cyclicAMP in the phosphorylation of non-histone proteins with subsequent transcription effects is still unclear.

When any regulation of transcription by protein phosphorylation is postulated, the role of dephosphorylation of these proteins by phospho-
protein phosphatases must be taken into consideration (126,127,134). Dephosphorylation of non-histone chromosomal proteins in vitro abolished their ability to stimulate RNA synthesis (84,135) and dephosphorylation may play an integral role in transcription control in vivo. The importance of this process is not clear at present and has not yet been intensively investigated.

Histones, in addition to their possible role as gene repressors, may also play a role in the control of non-histone protein phosphorylation. Addition of histones to partially purified non-histone proteins stimulated their phosphorylation (147) and it was observed that exogenous histones readily enter the nucleus and bind throughout the nucleoplasm and to the nuclear membrane (148,149). Furthermore, specific histones can stimulate the phosphorylation of some non-histone proteins and inhibit the phosphorylation of others (86). One of the non-histone proteins is a histone deacetylase (150). This may act to regulate histone function and suggests that there may be an interaction of control mechanisms for nuclear protein function. It has been postulated that the binding of histones to non-histone proteins results in an increase in the number of sites susceptible to phosphorylation in the non-histone proteins (147). More recently Kleinsmith has suggested a mechanism for the control of transcription involving the interaction of phosphorylated histones and non-histone proteins and has defined the role of nuclear protein phosphorylation in the derepression of the DNA template that occurs during gene activation. This mechanism is shown in a model presented in Fig. (7).
Histone I 

Histone I

Acidic phosphoprotein (synthesis or activation)

Specific binding

Phosphorylation

Displacement

RNA synthesis

Fig. (7) A model shows some of the properties of non-histone phosphoproteins in gene activation. This model is based on the specific binding of non-histone proteins to DNA, their subsequent phosphorylation and finally histone displacement. The resulting naked DNA is then capable of synthesizing RNA. [Kleinsmith (1975) (140)]

Briefly, this model is based on the specific binding of non-histone proteins to DNA and their subsequent phosphorylation. The negatively charged phosphate groups of the phosphoprotein might interact with the positively charged histones, thereby displacing the inhibitory histones from the DNA-histone complex and thus allowing the DNA to become active as a template for RNA synthesis.
DNA-dependent RNA polymerases, if considered as non-histone proteins, are probably subject to the same phosphorylation that governs the activity of the other nuclear proteins. Since phosphorylation can modify the activity of a variety of enzymes (134), the possibility that the activity of protein kinases could modify transcription has been investigated. Martelo et al. (151) were the first to report a marked stimulation of E. coli RNA polymerase activity by cyclic AMP-dependent protein kinases from rabbit skeletal muscle and rabbit reticulocytes. As expected, cyclicAMP augmented this stimulation. These workers also showed that E. coli RNA polymerase was phosphorylated by the two protein kinases and that this phosphorylation was stimulated by cyclicAMP and ATP. A subsequent report by Martelo and co-workers (152) demonstrated that the primary site of phosphorylation by the protein kinases was the sigma (σ) subunit of RNA polymerase and that serine residues were phosphorylated. In addition, the beta (β) subunit of the core polymerase was phosphorylated when greater amounts of the protein kinases were used. It was clearly demonstrated by these workers that phosphorylation was responsible for the enzyme activation and such activation occurs in the presence of cyclicAMP and ATP.

Although a cyclicAMP-dependent protein kinase has been reported in E. coli (153), Martelo and co-workers were unable to demonstrate its presence (152). Rhamendorf et al. (154) have reported that bacteriophage T7 induced a protein kinase in E. coli upon infection.

In Eukaryotes, the effects of protein kinases on transcription have also appeared in recent years. Martelo in 1973 and Martelo and Hirsch in 1974 (155,142) have reported that two partially purified nuclear protein kinases from rat liver were able to stimulate primarily RNA polymerase I activity on homologous native DNA and to phosphorylate...
partially purified polymerases I and II. It was also reported by Rutter and co-workers (156) that RNA polymerase II could be phosphorylated by cyclicAMP-dependent protein kinases from rabbit skeletal muscle and rat liver. However, no effect of this phosphorylation on transcription was observed in this case. Jungmann et al. (157) have reported that calf ovary cytosol protein kinases stimulated and possibly phosphorylated partially purified RNA polymerases Ia, Ib and II from this tissue. They found that the cyclicAMP-dependent phosphorylation of the RNA polymerase preparations was associated with an increase of the RNA-synthesising activity of all three enzyme preparations. On the basis of this evidence, they suggested that cyclicAMP and protein kinase may play an important role in the hormonal control of ovarian nuclear RNA synthesis by means of phosphorylation of the ovarian nuclear RNA polymerases.

Johnson and co-workers (158) have extracted cAMP-dependent protein kinase and proteins which specifically bind cyclicAMP from calf thymus nuclei and have analysed for their abilities to bind to DNA. They showed that cyclicAMP increases the capacity of the calf thymus cyclic AMP-dependent protein kinase preparation to bind labeled calf thymus DNA. They suggested that the binding of protein kinases to DNA may be part of a mechanism for localising cyclic nucleotide stimulated protein phosphorylation at specific sites on the chromatin. However, Kleinsmith (140) has reported that phosphorylation of a protein associated with one of the RNA polymerases in rat liver was inhibited by cyclicAMP and this inhibition was responsible for an enhancement of RNA synthesis in vitro. This author also reported that cyclicAMP alone could stimulate RNA synthesis in vitro provided histone was present. Desjardins, et al. (137) have reported that cyclicAMP alone could
stimulate partially purified RNA polymerases I and II from rat mammary gland. The purified protein factor isolated from Novikoff ascites tumour cells was found to stimulate the purified RNA polymerase II by 5 - 7 fold. It was shown that this factor had extensive protein kinase activity and catalysed the incorporation of $\gamma^32P$ from ATP into protein under normal RNA polymerase assay conditions (137). More recently, Kuroiwa and co-workers (159) have purified a stimulatory factor for RNA polymerase II from Ehrlich ascites tumour cells. However, these authors observed that this factor could not be co-purified with protein kinase and that protein kinase activity was not essential for the stimulation of RNA polymerase II.

Early reports of cyclicAMP stimulation of RNA polymerase I have appeared, but the role of polymerase phosphorylation in these instances was not established (160,161,162). Later, Hirsch and Martelo (163) showed that phosphorylation of rat liver RNA polymerase I occurred when intact rat liver nuclei were incubated with $[\gamma ^{32}P]$ ATP and dibutyl cyclicAMP. In addition they also demonstrated that partially purified RNA polymerase I could be phosphorylated in vitro by an endogenous protein kinase. By extensive purification of the enzyme they showed that $^32P$ remained bound to the enzyme throughout purification. Radioautography revealed that the $^32P$ was located primarily on enzyme subunits SA1, SA3 and SA6. Bell et al. (164), in a short communication, have reported the isolation of phosphorylated RNA polymerase I from yeast cells. They identified phosphorylation of the enzyme subunits in vivo. They also observed the same pattern of phosphorylation obtained when highly purified RNA polymerase I was incubated with a yeast protein kinase preparation. The presence of
phosphorylated subunits in yeast RNA polymerases I and II was also reported (165). Quite recently, Bell et al., 1977 (166) have demonstrated the phosphorylation of the three enzymes I, II and III in vivo when yeast cells were grown continuously in $^{32}$P$_i$ and the RNA polymerases were isolated simultaneously by a new purification procedure. These observations reveal that such phosphorylation of RNA polymerases subunits from mammalian cells to lower eukaryotes could be subjected to the same type of regulatory mechanisms.

Although cyclicGMP has been implicated as an active signal that may induce cell division (167), the role of this cyclic nucleotide on transcription has not been studied in detail. However, some reports appeared in the literature concerning the effect of cyclicGMP on RNA synthesis since the synthesis of RNA appears as one of the earliest events following initiation of cell division process. A preliminary study with isolated lymphocyte nuclei indicated that low concentrations of cyclicGMP and Ca$^{++}$ increased RNA synthesis (168). A subsequent study by Johnson and Hadden (169) demonstrated that cyclicGMP in the presence of Ca$^{++}$ stimulated RNA polymerase I activity in lymphocyte nuclei isolated from both non-stimulated and phytohemagglutinin stimulated lymphocytes. Subunit phosphorylation of the enzyme (S) by cyclicGMP-dependent phosphorylation has not yet been demonstrated. However, the report of Johnson and Hadden (170) showed that cyclicGMP and cholinergic agents stimulate incorporation of phosphate into specific nuclear acidic proteins of horse peripheral blood lymphocytes.
1.7 Control of Transcription in Tetrahymena pyriformis

For the reasons mentioned in Section (1.1) a micronucleate Tetrahymena pyriformis, strain W was the unicellular organism used throughout the present work. Although these ciliates are classified as animals in the current taxonomy, it has been shown that they possess a cytochrome c of a bacterial type (171). Furthermore, it was found that their ribosomal RNA has a very low G-C content (172), shows low thermal stability (173) and has sedimentation properties intermediate between those shown by ribosomal RNA isolated from higher eukaryotes and bacteria (174). On the other hand, it has been shown that the isolated macronuclei of these ciliates contain enzymes that are similar in many respects to the mammalian DNA-dependent RNA polymerases (175, 176, 177). The multiple forms of DNA-dependent RNA polymerase were isolated from sonicated nuclei and purified by column chromatography on DEAE-Sephadex (176, 177, 178, 179).

The role of nuclear proteins on the RNA synthetic capacity has not yet been studied in detail in Tetrahymena pyriformis. However, Cameron and co-workers (180) have reported the changes in the nuclear acidic proteins and chromatin structure in starved and refed Tetrahymena. They showed that in Tetrahymena as in other eukaryotic cell systems, there was a significant increase in the quantity of acidic proteins early in G1. This increase occurs at the same time as the synthesis of whole cell protein is increased but prior to the sequential transcription of the messenger RNAs necessary for DNA synthesis (-S-phase) and cell division.

It is now widely recognised that cyclic nucleotides are of fundamental importance in regulating the many biochemical processes in
prokaryotic and eukaryotic cells. It has been suggested that cyclic AMP and cyclic GMP may operate in a co-ordinate, yet opposing manner to influence the metabolism of a cell (167). In *Tetrahymena pyriformis* the existence of cyclic GMP (181) and cyclic AMP (182) has been detected and their intracellular concentrations at different stages of the cell cycle and cell growth have been measured. Wolfe (183) has showed that dibutyryl cyclic AMP inhibits ciliary regeneration in these ciliates. Dickens *et al.*, (184) have reported that the induction of thymidylate synthetase activity was stimulated by an exogenous addition of cyclic GMP or its dibutyryl derivative. They also showed that caffeine mimicked the cyclic GMP response. They suggested that the regulation of the rate of formation of this enzyme which is required for DNA synthesis may be effective in the regulation of cellular division. Cyclic GMP phosphodiesterase and cyclic GMP-dependent protein kinases have also been reported in *Tetrahymena pyriformis* (181, 185). It has been proposed that calcium may play a central role in the mediation of the effect of cyclic nucleotides (186, 187, 188). It was found that *Tetrahymena pyriformis* accumulates calcium in storage granules under certain conditions (189) and cyclic nucleotides may be involved in the mobilisation of this store.

*Tetrahymena pyriformis* possesses all the necessary components of a fully functioning cyclic AMP regulatory system, cyclic AMP (182), adenylate cyclase (190), cyclic AMP phosphodiesterase (191) and cyclic AMP-dependent protein kinase (185, 192). The intracellular localisation of these different components has not been fully characterised because most of the work has been done with whole cell homogenates. However,
Majumder and co-workers have reported a cytoplasmic cyclic AMP-dependent protein kinase. Only a small amount of the enzyme activity was associated with nuclei. Cyclic AMP-dependent protein kinase has also been reported in cilia of *Tetrahymena pyriformis* (185). A cyclic AMP-dependent protein kinase activity was also demonstrated in the axonemes of *Tetrahymena* cilia and the enzyme was found to catalyse the phosphorylation of ciliary tubulins in vitro (193).

1.8. **Aims of this Project:**

The primary aim of this project was to study the effects of cyclic nucleotides on transcription of nuclear DNA. Nuclei from the unicellular protozoan, *Tetrahymena pyriformis* were isolated for the study of the mechanisms which control the transcription of nuclear DNA by RNA polymerases. The secondary aim of the project was to test if any of the biological effects of cyclic nucleotides in the nucleus are mediated through nuclear cyclic nucleotide-dependent protein kinases.
CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Biochemicals

The following materials were purchased from Sigma Chemical Company Ltd.; cyclicAMP, Dibutyryl cyclicAMP, cyclicGMP, Dibutyryl cyclicGMP, calf thymus DNA (Type I; sodium salt "highly polymerised"), Theophylline, Dithiothreitol and all nucleoside triphosphates. EDTA was obtained from Hopkins and Williams. α-Amanitin was kindly donated by Professor Th. Wieland, Chemical Department, Max-Plank Institute of Medical Research, Heidelberg, Federal Republic of Germany. Nonidet-P40 was kindly offered by Shell Chemical Company Ltd., Industrial Chemicals Division, Dawntree Building, Shell Centre (U.K.). [4-14C]UTP (60Ci/mole) and [γ-32P] ATP (2.7 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (U.K.). All other chemicals used were of the highest purity commercially available. Proteose Peptone ("Difco") was purchased from Difco Laboratories, Detroit, Michigan, (U.S.A.). Powdered yeast extract was purchased from London Analytical and Bacteriological Media Ltd., London (U.K.). Tetrahymena pyriformis, strain L1630/1W, was obtained from the Culture Centre of Algae and Protozoa, Cambridge, U.K.).

2.2 Buffers

A. 0.25M Sucrose buffer: 0.25M sucrose, 10mM MgCl₂ (pH 7.2).

B. TCMED buffer: 0.05M Tris-HCl (pH 7.9), 30% (v/v) glycerol, 3mM Magnesium acetate, 0.1mM EDTA, 5mM DTT.

C. NaCl buffer: 0.14M NaCl, 10mM Tris-HCl (pH 7.9), 5mM DTT, 0.1mM EDTA, 5mM MgCl₂.
D. **1.0M Sucrose buffer**: 10mM Tris-HCl (pH 7.9), 1.0M Sucrose
5mM MgCl₂, 5mM DTT.

E. **Tris-Cl buffer**: 40mM Tris-HCl (pH 7.5), 2mM MgSO₄.

2.3 **Organism and Growth Conditions**:

*Tetrahymena pyriformis*, strain w, was grown in a medium containing 2% Proteose-peptone (Difco), 0.1% yeast extract and 5 μg/ml crystalline Ferric chloride. The medium was made up with tap water. 7.0 - 7.2 was the pH of the medium. Glucose supplemented medium (+0.5% glucose) was used only in certain experiments stated in the text. The medium was sterilised by autoclaving at 15 lb/in² for 20 minutes. 400 ml cultures were grown in 2L Erlenmeyer flasks to give high surface/volume ratio. The cultures were shaken at 140 - 150 oscillations/min in an orbital shaker. The temperature was maintained at 28 ± 1°C. The cultures were generally harvested between $3 \times 10^5 - 4 \times 10^5$ cells/ml. The mean doubling time of log phase cultures was 160 - 180 minutes. If the cell density was allowed to reach $1.5 \times 10^6 - 2 \times 10^6$ cells/ml the cells were in stationary phase and immediately after that a rapid decline in cell number followed by lysis of the cells was observed.

Stock cultures were kept statically in 100ml medium in 250ml conical flasks and were grown at room temperature. The cells were transferred twice a week to fresh medium. To inoculate a new medium, 1ml of 3 - 4 day old culture at a density of about $3 \times 10^5 - 4 \times 10^5$ cells/ml was transferred to 100ml of fresh medium under aseptic conditions. Under these conditions a stationary phase cell density of about $5 \times 10^5$ cells/ml was obtained after 3 - 4 days and cells
remained viable for a further 7 - 8 days, at least.

2.4 Fixing and Counting Tetrahymena pyriformis cells:

Cells were examined under the microscope to assess the viability of the culture before counting. Cells were fixed by taking an aliquot of culture and adding it to an equal volume of 20% formaldehyde neutralised with 0.01M phosphate buffer (pH 7.2). Fixed cells were then counted in a Neubauer haemocytometer.

2.5 Isolation of whole nuclei:

Tetrahymena pyriformis nuclei were isolated by the Nonidet - P40 procedure as described by Mita et al. (194) and Higashinakagawa et al. (176). Cells were harvested at either the logarithmic phase or the early stationary phase. 2 - 3L of the cultures were grown each time, at least, to give the total number of about $10^9$ cells. Cells were harvested in 250ml batches and sedimented at 5000 r.p.m. (4080 xg) for 10 minutes in a Sorval RC2 - B (GSA head) at 0°C. All the following procedures were performed at 0 - 4°C. The sedimented cells were washed twice in a small volume of ice-cold sucrose buffer (A). The packed cells were then resuspended in nine volumes of the sucrose buffer. One-fifth volume of 1% Nonidet-P40 in sucrose buffer (A) was added to the cell suspension to give 0.16% final concentration of Nonidet-P40. This concentration was enough to disrupt the cell membrane and release cell nuclei within 1 - 5 minutes with gentle stirring at 0°C. The degree of cell lysis was checked by the light microscope and the integrity of the whole nuclei was checked by phase contrast microscope. The cell lysate was made 2.1M with respect to sucrose by adding solid sucrose. This was done in the cold room with stirring. The final
viscous cell suspension was centrifuged at 26000 r.p.m. (59000 xg) for one hour at 0°C in an Ultracentrifuge, spinco L50 using the 30 rotor. The nuclei were separated from the cell debris in the 2.1M sucrose and were centrifuged to the bottom of the tubes. The nuclei were gently resuspended in TGMED buffer (B) and washed twice at low speed centrifugation 3000 r.p.m. (1085 xg) in a Sorval RC2 - B (SS 34 head). The resulting clean nuclei were resuspended in the TGMED buffer (B) to give $2 \times 10^8$ nuclei/ml. The nuclear suspension was divided in 1 ml portions in small plastic tubes. The tubes were quick-frozen in liquid nitrogen and kept in this liquid at -20°C. This procedure for the isolation of Tetrahymena nuclei gave approximately 80 – 90% recovery of the nuclei relative to DNA determination. The nuclei prepared by Nonidet - P40 procedure lose their outer membrane as shown in Fig. (A) taken from reference 179.
Electron micrographs of isolated meronts of Leptocoma californica CL.

(a) Stained with Anex C. (b) A higher magnification of (a), stained with Anex C.

Fig. 8. Electron micrographs of isolated meronts of Leptocoma californica CL. The nuclear pellet was fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4). After washing with the buffer, the specimens were postfixed with 1% OsO₄ in phosphate buffer. The specimens were then dehydrated with a series of ethanol solution and QE-1. After dehydration, the specimens were embedded in Epon 812 and cut with a Servoll MT II-9 Ultra-microtome. They were double-stained with 2% uranyl acetate and lead solution. Final samples were observed with JEM 10 electron microscope with an acceleration voltage of 60 kV. Note the nuclear membrane is partly impaired or completely lost in most of the nuclei. Cross section of contaminating cilia is also seen. NM: nuclear membrane, CB: chromatin body, NO: nucleolus, CL: cillum. a) x3100, b) x3900, c) x5900, d) x8700.
Photomicrographs of isolated macronuclei of *Tetrahymena pyriformis* GL. (a) Stained with Azur C. (b) A higher magnification of (a). Stained with Azur C.

Fig. 9. Electron micrographs of isolated macronuclei of *Tetrahymena pyriformis* GL. The nuclear pellet was fixed with 3% glutaraldehyde in phosphate buffer (pH 7.4). After washing with the buffer, the specimens were postfixed with 1% OsO₄ in phosphate buffer. The specimens were then dehydrated with a series of ethanol solution and QX-1. After dehydration, the specimens were embedded in Epon 812 and cut with a Sorvall MT II-B Ultra-microtome. They were double-stained with 2% uranyl acetate and lead solution. Final samples were observed with JEM T7 electron microscope with an acceleration voltage of 60 kV. Note the nuclear membrane is partly impaired or completely lost in most of the nuclei. Cross section of contaminating cilia is also seen. NM: nuclear membrane, CB: chromatin body, NO: nucleolus, CL: cilium. a) x3100, b) x3900, c) x5900, d) x8700.
2.6 Preparation of nuclear subfractions:

Whole intact *Tetrahymena pyriformis* nuclei were subjected to the sub-fractionation procedures outlined below in Fig. (9).

Whole Intact Nuclei (2 ml, 2.46 x 10^6 nuclei/ml)

- Whole nuclei
  - 1.7 ml resuspended in 3 ml of buffer (C) 0.14M NaCl containing 10 mM Tris-HCl (pH 7.9), 5 mM DTT, 0.1 mM EDTA, 5 mM MgCl₂.
  - Homogenised by hand for 5 minutes in a teflon-glass Potter-Elvehjem homogeniser.
  - Centrifuged at 3000 xg for 10 minutes (0 - 4°C).

- Pellet
  - Supernatant A¹
    - Resuspended in buffer (C), centrifuged at 3000 xg for 10 minutes (0 - 4°C).
    - Supernatant A¹' and A¹'' combined to give a total 6 ml of material, N₂.

- Pellet
  - Supernatant A²
    - Resuspended in buffer (C), centrifuged at 3000 xg for 10 minutes (0 - 4°C).

- Pellet
  - Supernatant A³
    - Resuspended to 6 ml with 2M NaCl buffer (2 M NaCl containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM DTT and 30% (v/v) glycerol) stirred at 0°C for 2 hours. Centrifuged for 1 hour at 20000 g.
    - Supernatant N₃ (5.5 ml).

Fig. (9) Preparation of Nuclear Subfractions
$N_2$ represents the soluble nucleoplasmic proteins. Fraction $N_3$ is the 2 M NaCl-soluble portion of chromatin and contains essentially, histones, non-histone chromosomal proteins and RNA polymerase. Fraction $N_4$ is the "chromatin residue" which contained mainly the DNA of the nucleus and less than 1X of chromosomal proteins (195, 196).

2.7 Preparation of Chromatin:

The crude *Tetrahymena pyriformis* chromatin was isolated by a slight modification of the method of Panyim *et al.* (78).

The frozen nuclei were thawed and resuspended in 40 - 50 volumes of buffer (C) and homogenised by hand for 1 - 2 minutes in a Potter-Elvehjem homogeniser using a fairly tight-fitting Teflon pestle. The nuclear suspension was centrifuged at 5500 r.p.m. (3640 g) for 10 minutes in a Sorvall RC2 - B (SS 34 head). The supernatant was discarded. The precipitate was washed in the same buffer. Washing with 0.14 M buffered NaCl (C) removes the soluble nucleoplasmic proteins which are not associated with the chromatin (78). After centrifugation the sticky crude chromatin pellets were homogenised gently by hand for 2 minutes in 40 - 50 volumes of double distilled water to remove the salt and centrifuged at 10000 r.p.m. (12100 g) for 10 minutes. The washing procedure was repeated once more. At this stage all the nuclei were broken and released the chromatin as observed by light and phase contrast microscope. The gelatinous crude chromatin preparation was finally resuspended in buffer (B). The presence of high salt concentrations and of divalent cations was avoided since they are known to cause the loss of chromatin proteins (197). Ingredients known to stabilise labile enzymes, such as DTT and glycerol,
were included in the buffer of chromatin preparation. However, the RNA polymerase activity in the chromatin prepared in this way was unstable, even when stored in liquid nitrogen at -20°C and therefore chromatin was freshly prepared for each experiment. All the operations described above were performed at 0-4°C.

2.8 Extraction of RNA Polymerase:

Total RNA polymerase activity was solubilised from the nuclei either by sonication of the nuclei in a medium of high ionic strength (2.8.a.) or by homogenisation of the nuclei in high salt buffer (2.8.b.).

2.8.a By Sonication Procedure:

This procedure was performed exactly as described by Higashinakagawa et al. (176).

The isolated nuclei were thawed and resuspended in a few ml of buffer (D). An equal volume of the same buffer containing 0.6 M ammonium sulphate was added to the suspension. The viscous mixture was sonicated in a MSE (150 W) sonicator at maximum power and amplitude for 60 seconds (15 second intervals at 0°C). The resulting solution was mixed with 2 volume of buffer (B) (TGMED buffer) and centrifuged at 37000 r.p.m. (100000 g) for 1 hour in an ultracentrifuge, spino-L50 using rotor type 40. Solid ammonium sulphate 0.42 g/ml (65% saturation) was added to the supernatant and dissolved by stirring at 0°C. The white precipitate which formed was collected by centrifugation at 37000 r.p.m. (100000 g) for 1 hour, dissolved in a few ml of TGMED.
buffer (B) and dialysed against 0.01 M ammonium sulphate in buffer (B). This was done at 0°C in the cold room for at least 6 hours. The dialysate was centrifuged at 160000 g for 1 hour to remove particulate material, which contained little enzyme activity. The clear final supernatant was used as a source of RNA polymerase activity. This was either used freshly immediately after preparation or quick-frozen in liquid nitrogen. When kept in liquid nitrogen at -20°C there was no appreciable loss of activity in a period of 3 - 4 weeks.

2.8.b. By Salt Extraction

Extraction of total RNA polymerase activity by salt method was carried out according to the scheme outlined in section (2.6). The supernatant N3, which contains histones, non-histone chromosomal protein and DNA-dependent RNA polymerase activity was dialysed for 8 hours against TGMED buffer (B) to remove the salt. The dialysate was centrifuged for 1 hour at 37000 r.p.m. (100000 g) to get rid of any remaining contaminant matter. The supernatant was used as the source of RNA polymerase activity.

2.9 Selection of a synchronous population of Tetrahymena pyriformis

The procedure used to select a synchronous culture of Tetrahymena pyriformis was essentially the same as that described by Hildebrandt and Duspiva (47). The procedure is based on selecting the dividing cells from an exponential culture, since these cells are unable to ingest the particulate matter. Cells were grown in a proteose-peptone medium, as described in Section (2.3.), with shaking at 28°C. Rapidly growing cells at the early logarithmic phase (10^5 - 2 x 10^5 cells/ml) were incubated with 10 g/ml iron particles for 12 minutes. This was
done with rapid shaking by hand at 28°C in a water bath. More than 90% of the cells, at this stage, would ingest the iron. A slight positive pressure was used to drive the culture onto a 35 x 4.5 cm column of dry acid-washed sand (30 - 90 mesh). The column stood vertically, and was surrounded by four barium Ferrite magnets. The magnets were arranged around the column as shown in Figure (10) with their poles horizontal; the maximum magnetic field strength at the axis of the column was approximately 100 Gauss (190, 199). The two upper magnets were placed with their poles 5.5 cm from the axis of the column, the lower two slightly nearer at 4 cm. Preliminary experiments had revealed a small proportion of the very smallest iron particles was not retained by the four magnets arranged round the column, so a sector-shaped magnet was placed against the column outflow pipe to catch these very fine particles. The synchronous cells were collected at growth temperature with shaking. When sufficient volume of cells had been collected for an experiment, the synchronous culture was divided into separate portions, which were incubated as usual, and taken one at a time for cell cycle analysis. Separation of the synchronous cells into several aliquots reduces the risk of contamination which might result from frequent sampling from one culture.
Fig. 10. The apparatus for the magnetic method of selection of a synchronous population of Tetrahymena pyriformis
2.10 Assay of RNA polymerase:

2.10.1 Endogenous RNA polymerase assay:

RNA polymerase activity was assayed by measuring the incorporation of \( [\text{4} - ^{14}\text{C}] \) UMP into acid-(TCA) precipitable material. Nuclei were assayed for total endogenous DNA-dependent RNA polymerase activity using the endogenous DNA as a template. The standard assay system comprised: 50 mM Tris-HCl (pH 7.9), 1 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.4 mM UTP, 0.05 \( \mu \)g \( [\text{4} - ^{14}\text{C}] \) UTP (60 c\( _{i} \)/mole), 0.15 M KCl, 2.5 mM DTT, 1.5 mM MnCl\(_{2}\) and 4 mM MgCl\(_{2}\) in a total volume of 0.15 ml. The reaction was started by the addition of nuclei to the cocktail mixture with rapid mixing in a whirli-mixer and was terminated after 10 minutes. (The incubation was performed in a water bath at 25°C.) 100 \( \mu \)l aliquots were then immediately charged onto glass fibre discs (Whatman, GF/C, 2.4 cm diameter) and the discs were then immersed in ice-cold TCA solution containing 5\% (w/v) TCA, 1\% (w/v) sodium pyrophosphate (pH 1.5) and then these discs were washed three times in ice-cold TCA solution (30 minutes for the first wash and 10 minutes for the second and third washes). The discs were then washed twice in absolute ethanol and then twice in diethyl ether. Discs were finally dried under an infra-red lamp and then assayed for radioactivity in a liquid scintillation spectrometer using a Packard 2425 tri-carb spectrometer. 4 ml of toluene scintillator which comprised 5 gm PPO and 0.2 gm POPOP per litre of toluene was sufficient to cover the dry discs. Zero time incubation blanks contained less than 0.2\% of the total radioactivity. Assays were performed in duplicate and the mean and standard deviations were evaluated.
2.10. b. Assay of extracted RNA polymerase:

The total extracted DNA-dependent RNA polymerase from whole nuclei was assayed using exogenous calf thymus DNA as a template. The reaction mixture was the same as described in the preceding section (2.10.a.) except that 0.15 M KCl was omitted and 20 µg heat-denatured DNA was substituted. The heat-denatured DNA was prepared as follows: Native DNA was denatured by heating the DNA solution in boiling water in a stoppered tube for 10 minutes. After heating, the sample was cooled immediately in an ice bath for about 20 minutes (198). Assays were performed in duplicate.

2.11 Phosphodiesterase assay:

CyclicAMP and cyclicGMP phosphodiesterase activities were measured in whole nuclei and subnuclear fractions as described by Dickinson (199). The assay is based on the methods of Butcher and Sutherland (200). The assay consisted of measuring the release of inorganic phosphate with the use of an excess of 5'—nucleotidase that is present in the venom of Ophiophagus hannah. CyclicAMP phosphodiesterase reaction mixture contained 0.36 µmole cyclicAMP in 0.8 ml of buffer (E) (40 mM Tris-HCl (pH 7.5), 2 mM MgSO₄). The assay was initiated by addition of 0.1 ml of the sample tested, diluted as appropriate with buffer (E). Incubation was at 30°C for 30 minutes in a water bath. After the first 20 minutes of the incubation, 0.1 ml of a solution of Ophiophagus hannah venom in buffer (E) was added, containing 0.1 mg of venom. The reaction was terminated by the addition of 0.1 ml of ice-cold 55% TCA. After addition of TCA, the precipitate was removed by centrifugation, and aliquots of the super-
natum analysed for inorganic phosphate in a 1 ml assay, according to the method of Fiske and Subba Row (201). Conditions of the cyclic AMP phosphodiesterase assay were organised such that no more than 10% of the substrate was utilised during the course of the incubation. The quantity of snake venom used was capable of hydrolysing all of the 5'-AMP produced in the reaction. Under the conditions described the cyclicAMP phosphodiesterase reaction was linear for at least 30 minutes (199). Assays were performed in duplicate.

CyclicGMP phosphodiesterase assay was performed in an identical fashion to the assay of cyclicAMP phosphodiesterase, except that 1 mM cyclic GMP was used as substrate instead of cyclicAMP.

2.12 Protein Kinase Assays

An assay for endogenous protein kinase activity from Tetrahymena pyriformis nuclei was developed and validated. The protein kinase activity was determined by measuring the incorporation of $^{32}$P from $[\gamma-32P]$-ATP into acid precipitable protein. The standard assay mixture contained (final concentration): 50 mM Tris-HCl (pH 7.5), 0.1 mM ATP, 1.8 mM $[\gamma-32P]$-ATP (specific activity 2.7 c/min/µmol), 10 mM MgCl$_2$, 10 mM NaF, 100 mM NaCl and 0.2 mM EDTA.

100 µg F$_1$-lysine rich histone (Sigma, type I), 100 µg phosvitin or 300 µg casein were used as exogenous substrates in different experiments. The total assay volume was 0.15 ml, of which 40 µl contained the reaction cocktail (except for the labelled ATP), 50 µl contained the nuclei or a protein kinase fraction and the volume was made up with 50 µl of exogenous substrates, distilled water or cyclic nucleotides to be tested. The assay was initiated by the addition of
10 μl of labelled \( \gamma ^{32}P \)-ATP. The reaction mixture was incubated at 25°C for 10 minutes after which time the reaction was terminated by charging 100 μl aliquots onto glass fibre discs and immersing immediately in ice-cold TCA sodium pyrophosphate solution as described previously in Section (2.10.a.). The discs were washed and dried under an infrared lamp and then assayed for radioactivity in a similar way to that described for the RNA polymerase assay (Section 2.10.a.). The blank value was obtained by omitting MgCl₂ from the reaction mixture. Similar blank values were obtained if kinase containing samples were boiled for three minutes or if labelled ATP was added after the addition of the TCA solution to the discs to stop the reaction. Assays were performed in duplicate.

Greenaway has observed that an acid insoluble product can be formed between ATP, Mg²⁺ and NaF which can give rise to an artifact in the protein kinase assay. Precautions have to be taken to prevent this artifact which does not occur in the presence of 0.1 M NaCl and 1 mM EDTA (202).

2.13 DNA Determination

Nuclear DNA was determined according to the method of Burton (203, 204).

For cell cycle work measurement of cellular DNA enables identification of the cell cycle phase at a particular point in the experiment. Cells at different points of the cell cycle were harvested by spinning at 2000 g for five minutes at growth temperature. Growth media was carefully removed and the pellet of cells resuspended in a small volume of ice-cold distilled water. The suspension of cells was transferred to a sonication vial and an equal volume of ice-cold 20X
TCA was added. Sonication was performed at 0°C, using a MSE 150 W sonicator at maximum power and amplitude for one minute (15 second intervals) by which time cell disruption was judged to be complete. The TCA precipitated material was centrifuged and the supernatant discarded. The pellet was washed by resuspending in 5 ml ice-cold 5% (w/v) TCA and centrifuged again. The supernatant from cells treated with TCA which contained free deoxynucleotides were removed and discarded. DNA was extracted from the pellet by heating to 70°C for 15 minutes in the presence of 0.5 M perchloric acid. After centrifugation the process was repeated. The supernatants from both perchloric acid steps were combined and the DNA assayed. Calf thymus DNA was used to construct a standard calibration curve.

The DNA assay described by Burton (203, 204) is based on the reaction between the deoxyribose of the DNA and diphenylamine; this method is probably the most frequently used colour reaction for the determination of DNA. Diphenylamine was purified by recrystallising from hexane and dissolved in 1% glacial acetic acid and 2.75 ml concentrated H$_2$SO$_4$ was added. The standard DNA curve was linear between 0 - 60 µg DNA.

2.14 Protein Determination

Protein was estimated by the method of Lowry et al. (205). Crystallised bovine serum albumin was used to construct a standard curve.
CHAPTER THREE

GENERAL ENZYMATIC PROPERTIES OF NUCLEAR DNA-DEPENDENT RNA POLYMERASE

Studies on the synthesis of RNA by DNA-dependent RNA polymerase in vitro have contributed considerably to the understanding of the control of gene activity. In this Chapter, some properties of DNA-dependent RNA polymerase from isolated *Tetrahymena pyriformis* nuclei were studied in order to establish conditions that approach in vivo RNA synthesis.

3.1. The dependence of Nuclear RNA Polymerase Activity on the Concentration of Ribonucleoside Triphosphates.

In the literature, different concentrations of labelled UTP have been used to measure the incorporation of this nucleoside triphosphate into an acid-precipitable RNA product (206, 207). It was therefore decided to determine the apparent $K_m$ value for UTP in *Tetrahymena pyriformis* nuclei. A plot of the $[4\text{--}^{14}C]$ UMP incorporated into an acid-precipitable RNA as a function of the concentration of UTP is shown in Figure (11.A.). The Line weaver-Burke Plot (Figure 11.B.) was non-linear. This was not unexpected since nuclei contain two different RNA polymerases (as will be seen later in this Chapter) with different $K_m$'s. The apparent $K_m$ value for UTP for the high $K_m$ RNA polymerase was 357 $\mu$M. This figure is 1.23-fold higher than the $K_m$ value for UTP of calf thymus RNA polymerase II and 22-fold higher than that obtained with RNA polymerase I from calf thymus (208). The inhibition by a-amanitin (which specifically inhibits RNA Polymerase II (50 $\mu$g/assay)) remained constant at about 58 - 62% throughout the UTP concentration range indicating that high UTP concentrations did not preferentially activate one of the
Nuclei were isolated as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity. Conditions for assay were as described in Section (2.10.a) except that UTP concentration was varied as indicated in Fig. (11.A). Fig. (11.B) is the Line weaver-Burke plot.

Nuclear DNA was determined as described in Section (2.13).

\[ (n = 4), \text{n = number of experiments performed.} \]
Graph A shows the incorporation of [4-14C]-dUMP into DNA as a function of UTP concentration. The graph illustrates a linear relationship with an increase in incorporation as UTP concentration increases. The data points are indicated with error bars, suggesting variability in the measurements.

Graph B presents the Lineweaver-Burk plot, which is a double reciprocal plot. The x-axis represents the reciprocal of UTP concentration (UTP^-1), and the y-axis represents the reciprocal of the reaction rate (1/Vo) times 10^2. The graph intersects the y-axis at a value of 2, and the x-axis at a value of 4, indicating the Michaelis-Menten constant (Km) for the reaction. The Km value is given as 357 μM.
RNA polymerase forms over the other in isolated nuclei.

All of the three ribonucleoside triphosphates, GTP, ATP and CTP are required for incorporation of labelled UTP into acid-precipitable material. As shown in Table (3) omission of any one or more of the nucleoside triphosphates results in an 85 - 89% loss of the activity. This result indicates that the nuclei did not contain a high pool of endogenous nucleoside triphosphates.

To check whether the acid-precipitable product in the RNA polymerase assay mixture is really RNA; pancreatic RNase (a ribonucleic acid degrading enzyme) was applied directly to the glass fibre discs after the "complete" assay reaction procedure had been completed. It can be seen from Table (3) that 10 μg of RNase gave the blank rate (about 0.2% of the total radioactivity incorporated in the complete assay). Thus the polymerised reaction product is RNA.

3.2. The Time Course of Nuclear RNA Polymerase Assay:

Figure (12) shows the time course of a typical DNA-dependent RNA polymerase assay. The synthesis of RNA was linear in the intact isolated nuclei up to 60 minutes, after which time the nuclei began to lyse spontaneously as observed by phase contrast microscopy. This observation is in contrast to the result obtained by Lee and Byfield (175). In their assay system, the initial rate of RNA synthesis in isolated Tetrahymena pyriformis nuclei was almost linear for only the first two minutes of the incubation and the rate of radioactivity incorporated into RNA declined after ten minutes. Omission of cold UTP added in the assay mixture (0.4 mM) gave a similar pattern to that observed by these authors. Thus to achieve linearity of the assay sufficiently high concentrations...
Table (3) The Dependence of Nuclear RNA Polymerase Activity on the Concentration of Ribonucleoside Triphosphates

<table>
<thead>
<tr>
<th>Assay System</th>
<th>Pmoles UMP incorporated per 10 min per μg DNA</th>
<th>% Activity of complete assay system</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Complete&quot;</td>
<td>19.34 ± 1.00</td>
<td>100</td>
</tr>
<tr>
<td>- ATP</td>
<td>2.11 ± 0.06</td>
<td>11</td>
</tr>
<tr>
<td>- GTP</td>
<td>2.66 ± 0.09</td>
<td>14</td>
</tr>
<tr>
<td>- CTP</td>
<td>2.78 ± 0.09</td>
<td>14</td>
</tr>
<tr>
<td>- ATP, GTP</td>
<td>2.63 ± 0.06</td>
<td>14</td>
</tr>
<tr>
<td>- CTP, GTP</td>
<td>2.36 ± 0.07</td>
<td>12</td>
</tr>
<tr>
<td>- ATP, GTP, CTP</td>
<td>2.68 ± 0.07</td>
<td>14</td>
</tr>
<tr>
<td>5 μg RNase</td>
<td>2.77 ± 0.06</td>
<td>14</td>
</tr>
<tr>
<td>10 μg RNase</td>
<td>Blank Rate</td>
<td>0</td>
</tr>
</tbody>
</table>

Nuclei were isolated as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity as described in Section (2.10.a.) "Complete". Ribonucleoside triphosphates were partly or completely omitted from the reaction cocktail.

In experiments with RNase, samples were treated as usual but before counting, 5 μg/100 μl or 10 μg/100 μl of pancreatic ribonuclease in distilled water was applied directly to the filter paper discs. After incubation for one hour at 25°C, the discs were again washed with 5% TCA - 1% sodium pyrophosphate, dried and counted. Controls not treated with RNase did not lose radioactivity during the second wash.

\[ n = 2, \text{ } n = \text{ number of experiments performed.} \]
Fig. (12) The Time Course of Nuclear RNA polymerase Assay

Nuclei were isolated as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity as described in Section (2.10.a). DNA was determined as described in Section (2.13). Nuclei containing a total of 45 µg DNA were used per assay. [n = 2, n = number of experiments performed.]
of the substrate are required to prevent a substantial decrease in substrate concentration during the assay period.

3.3. **The Effect of Temperature on Nuclear RNA Polymerase Activity:**

As has been observed for isolated nuclei from mouse myeloma cells (209) and yeast cells (210), the temperature of the incubation mixture has a pronounced effect on the kinetics of RNA synthesis by isolated *Tetrahymena pyriformis* nuclei. As shown in Figure (13), at low temperatures (25°C) the reaction continues at a nearly linear rate for 60 minutes. At 37°C the reaction rate reaches a plateau after 30 minutes. At 30°C the plateau was reached at 40 minutes.

3.4. **The Effect of Salt Concentration on Nuclear RNA Polymerase Activity:**

Endogenous DNA-dependent RNA Polymerase activity in isolated *Tetrahymena pyriformis* nuclei was dependent on the ionic strength of the reaction mixture. A rise in the KCl or (NH₄)₂SO₄ concentration from zero to 0.2 M was accompanied by an increase in enzyme activity. The reaction rate reached a plateau at approximately 0.15 M KCl as shown in Figure (14.A.) and remained at a constant level until 0.3 M when it began to fall again. However, it was consistently observed that a high ionic strength of (NH₄)₂SO₄ (0.6 ionic strength at 0.2 M) produces immediate disruption of macronuclei and chromatin precipitation. Pogo et al. (211) have also observed that 0.4 M (NH₄)₂SO₄ produced extensive changes in the organisation of rat liver chromosomes. Such a complete disruption of nuclear structure by high ammonium sulphate concentrations may not only lead to the changes in template efficiency through the
Nuclei were isolated from exponentially growing culture 
(4 x 10^5 cells/ml) as described in Section (2.5) and assayed for 
endogenous DNA-dependent RNA polymerase activity as described in 
Section (2.10.a). In these experiments different incubation 
temperatures were used to determine the enzyme activity. DNA 
was determined as described in Section (2.13).

\[ n = 2, \text{ } n = \text{number of experiments performed.} \]
Fig. (14) The Effect of Salt concentration on nuclear RNA polymerase Activity

Nuclei were isolated from exponentially growing cells (4.0 x 10^5 cells/ml) as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity as described in Section (2.10.a) except that KCl and (NH_4)_2SO_4 concentrations were varied as indicated in the Figure.

(--o--) plot A - represents the activity with KCl

(--o--) plot B - represents the activity with (NH_4)_2SO_4

DNA was determined as described in Section (2.13)

\[ n = 2, n = \text{number of experiments performed} \]

Curve C - represents the activity with KCl and (NH_4)_2SO_4 against the ionic strength.
Pmoles (4 - $^{14}$C)UMP incorporated/10 min/μgDNA

Ionic strength

(MgSO$_4$) (KCl)
removal of histones as suggested by Widnell and Tate (212), but also
dissociates other chromosomal components. In *Tetrahymena pyriformis* the
optimum salt concentration required for the optimum enzyme activity in
whole nuclei differs from that required for each of the isolated RNA
polymerase activities. Higashinakagawa et al., (176) have observed
that all the three enzyme forms are progressively inhibited by increasing
\((\text{NH}_4)_2\text{SO}_4\) concentrations. At 0.2 M \((\text{NH}_4)_2\text{SO}_4\), the activities of the
three RNA polymerase forms were almost 100% inhibited. It has been
established that high salt concentrations inhibit initiation, but not
elongation in the overall transcription process (213). The RNA
polymerase activity measured in nuclei at high salt concentrations is
therefore probably preinitiated enzyme as suggested by some research
workers (209, 214, 215).

Addition of 2 mg/ml of lysine-rich (F₁) histone to the assay mixture
at 0.15 M KCl caused about 35% inhibition of the incorporated radio-
activity, while addition of 2 mg/ml of either casein or phosvitin to the
above assay mixture did not cause any inhibitory effects. These
observations stress the inhibitory effect of basic proteins (histones),
but not the acidic proteins (phosvitin and casein) on the control of DNA
transcription. The removal of histones by increasing salt concentration
from the chromatin allows the RNA polymerase to elongate or possibly
re-initiate RNA chains along the DNA template. These preliminary
observations are shown in Table (4).

Addition of 20 μg of native or denatured calf thymus DNA to the assay
mixture (calf thymus DNA is known to be a good template for the isolated
*Tetrahymena* RNA polymerases) did not increase the activity of the
endogenous RNA polymerase in the absence or presence of 0.15 M KCl. Under
Table (4) The Effect of Exogenous Nuclear Proteins and DNA on the RNA Polymerase Activity

<table>
<thead>
<tr>
<th>Assay System</th>
<th>Pmoles $[^{14}C]UmP$ incorporated per 10 min. per µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Complete&quot;</td>
<td>21.95 ± 1.40</td>
</tr>
<tr>
<td>+ Histone (F1)</td>
<td>14.31 ± 0.82</td>
</tr>
<tr>
<td>+ Casein</td>
<td>21.37 ± 1.33</td>
</tr>
<tr>
<td>+ Phosvitin</td>
<td>21.88 ± 1.25</td>
</tr>
<tr>
<td>+ Native DNA</td>
<td>21.43 ± 1.36</td>
</tr>
<tr>
<td>+ Denatured DNA</td>
<td>21.99 ± 0.89</td>
</tr>
<tr>
<td>&quot;Complete&quot; (-KCl)</td>
<td>8.80 ± 0.40</td>
</tr>
<tr>
<td>+ Native DNA (-KCl)</td>
<td>8.83 ± 0.38</td>
</tr>
<tr>
<td>+ Denatured DNA (-KCl)</td>
<td>8.65 ± 0.44</td>
</tr>
</tbody>
</table>

Nuclei were isolated as described in Section (2.5) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a.) "Complete".

2 mg/ml of (F1)-histone, casein or phosvitin were added to the "Complete" assay system. 20 µg of native or denatured calf thymus DNA was added to the "Complete" assay system. DNA was determined as described in Section (2.13.).

($n = 2, n =$ number of experiments performed.)

* It has been reported that 95% of histone F1 can enter the nuclei freely (148), possibly it is the case for casein and phosvitin.
these conditions RNA polymerases bound to the endogenous chromatin at the time of nuclear isolation are active in the assay (engaged RNA polymerase).

Recently, the presence of free RNA polymerase(s) in isolated Tetrahymena pyriformis nuclei has been demonstrated in our laboratory, but this enzyme does not re-initiate transcription with endogenous chromatin in isolated nuclei at high salt concentrations under the assay conditions described above (216).

5.5. The Effect of Divalent Metal Ions on Nuclear RNA Polymerase Activity:

RNA synthesis in isolated Tetrahymena pyriformis nuclei shows an absolute requirement for a divalent cation. Figure (15) shows the effect of increasing concentrations of Mg$^{++}$ and Mn$^{++}$ on the DNA-dependent RNA polymerase activity under standard assay conditions. The optimum Mn$^{++}$ concentration as shown in Figure (15.A.) was 1.5 mM. Higher concentrations of Mn$^{++}$ were inhibitory. It has been reported by Stein (217) that addition of high concentrations of Mn$^{++}$ in the assay mixture for RNA synthesis by isolated giant chromosomes from Drosophila hydei caused gross alterations in chromosomal morphology and hence alteration in engaged enzyme activity. This might also be true in the case of Tetrahymena pyriformis. In Figure (15.B.) addition of Mg$^{++}$ across a broad concentration range between 4 mM and 20 mM gave maximal activity under the standard assay conditions. However, when Mn$^{++}$ was replaced by Mg$^{++}$ the rate of radioactivity incorporated into RNA was reduced by 25%. In the routine standard assay cocktail 1.5 mM MnCl$_2$ together with 4 mM MgCl$_2$ were included for optimal RNA polymerase activity.

The optimal concentrations of Mn$^{++}$ and Mg$^{++}$ ions for endogenous RNA polymerase activity correlate with the optimal concentrations of Mn$^{++}$ and
Nuclei were isolated from exponentially growing cells (3.8 x 10^5 cells/ml) as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity as described in Section (2.10.a) except that the concentrations of MgCl₂ and MnCl₂ were varied as indicated in the Figure.

(---) plot A - represents activity of the enzyme with MnCl₂
(---) plot B - represents activity of the enzyme with MgCl₂
DNA was determined as described in Section (2.13).
Mg$^{++}$ found for the isolated enzymes by Higashinakagawa et al. (176).

The values of the optimal MnCl$_2$ concentration (1.5 mM - 2 mM) is close to the level of the combined nucleotides, in the assay mixture probably indicating that metal ion-ribonucleotide complexes may be the true substrates for the enzyme. However, it is also possible that these metal ions may have a direct stimulatory effect on the enzyme.

3.6. The Dependence of RNA Polymerase Activity on Nuclei Concentration:

The net rate of incorporation of labelled UTP into an acid-precipitable product in isolated nuclei was found to be dependent on the concentration of nuclei. Figure (16) shows a plot of radioactivity incorporated as a function of increasing amount of nuclear DNA per assay. It can be seen clearly from the Figure that the enzyme activity was proportional to the amount of nuclei used. In these experiments DNA is used as a measure of nuclear concentration. 10$^6$ cells in exponential phase contain about 10 µg DNA.

3.7. The Effect of α-amanitin on Nuclear RNA Polymerase Activity:

α-amanitin, a toxic Octapeptide isolated from the toadstool Amanita phalloides, has been found to inhibit RNA polymerase II from mammalian tissues and also from lower eukaryotes (70, 176). The first observations directly supporting the hypothesis of multiple RNA polymerases in animal nuclei were obtained in experiments performed by Wieland (218) who measured the effects of α-amanitin on RNA synthesis in vitro using isolated mouse liver nuclei. Stirpe and Fiume (219) have observed that α-amanitin preferentially inhibited the high salt Mn$^{++}$ stimulated RNA polymerase activity. This early observation suggested that nuclei contained two RNA polymerases; one sensitive and the other resistant.
Fig. (16) **Variation of RNA polymerase activity with nuclei concentration**

Nuclei from exponentially growing culture (3.6 x 10^5 cells/ml) were isolated as described in Section (2.5) and assayed for endogenous DNA-dependent RNA polymerase activity as described in Section (2.10.a) except that the amount of nuclear DNA was varied/assay as indicated in the Figure. DNA was determined as described in Section (2.13).

**I Curve (A)** - represents the activity as nmoles [4 - ^14C]UMP incorporated/10 min as a function of nuclear µg DNA/assay.

**II Curve (B)** - represents the specific activity as pmoles [4 - ^14C] UMP incorporated/10 min/µg DNA as a function of nuclear µg DNA/assay.
Graph (A) shows the relationship between \( \mu g\) DNA/assay and the number of moles of \((4-1^4C)\)UMP incorporated/10 min. The data points are plotted with error bars indicating variability.

Graph (B) depicts the relationship between \( \mu g\) DNA and the moles of \((4-1^4C)\)UMP incorporated/10 min. The line is relatively flat with error bars showing similar variability across different DNA concentrations.
With *Tetrahymena pyriformis* nuclei, the effect of α-amanitin on total nuclear RNA polymerase activity was investigated. Figure (17) shows the effect of increasing the amount of this drug on the rate of 

\[ 4 - ^{14}C \text{UMP} \] 

incorporated into an acid-precipitable product. As can be seen from the Figure, the maximum inhibition of radioactivity incorporated into RNA was obtained approximately at 3.6 \( \times 10^{-6} \) M of α-amanitin (50 μg/assay). Under standard assay conditions (Section 2.10.a.), the maximum inhibition of nuclear RNA polymerase activity ranged from 58 - 62% of the total activity using nuclei from exponential phase cells. This result is consistent with the presence of two major RNA polymerase activities in the nucleus, one α-amanitin sensitive and the other α-amanitin insensitive. Thus, 50 μg/assay of α-amanitin was routinely used to differentiate between the two major nuclear RNA polymerase activities.

RNA polymerase I is responsible for the majority of the activity at low salt concentrations and RNA polymerase II at higher concentrations. In whole nuclear preparations, the inhibition of enzyme activity by α-amanitin when KCl was omitted from the reaction mixture was studied. Table (5) shows that the percentage of inhibition by α-amanitin was much less than when 0.15 M KCl was present. Approximately 14% of the total enzyme activity was inhibited by 50 μg of α-amanitin present in the assay mixture. This indicates that the major species detected under these assay conditions is the α-amanitin insensitive enzyme.

The presence of either Mg" or Mn" in the assay mixture did not alter the maximal amount of inhibition by α-amanitin in the absence of KCl. These observations suggested that the different metal ions (Mn" or Mg") cannot be used to differentiate RNA polymerase I and II activity from whole nuclei.
Fig. (17) **Inhibition of Nuclear RNA polymerase Activity by α-amanitin**

Nuclei were isolated from exponentially growing culture (3.5 x 10⁵ cells/ml) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a). α-amanitin was added to the incubation mixture at the concentrations indicated in the Figure. The reaction was started by the addition of nuclei containing 30 μg nuclear DNA. DNA was determined as described in Section (2.13).

Plot (A) - represents the activity of RNA polymerase in the presence of α-amanitin.

Plot (B) - represents the percentage inhibition by α-amanitin.

[n = 3, n = number of experiments performed]
Pioles (4 - C)UMP incorporated/10 min/μgDNA

(A)

μg α-amanitin/assay

3.6 x 10^{-4}M α-amanitin
Inhibition of Nuclear RNA polymerase activity by α-amanitin

μg α-amanitin/assay
Table (5) The Effect of KCl and Metal Ions on the Nuclear RNA Polymerase inhibition by α-amanitin

<table>
<thead>
<tr>
<th>Addition of α-amanitin µg/assay</th>
<th>Pmoles ([4 - ^{14}C\text{]}\text{UMP}) incorporated/10 min /µg DNA</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (− KCl)</td>
<td>6.33 ± 0.20</td>
<td>−</td>
</tr>
<tr>
<td>8 &quot;</td>
<td>5.82 ± 0.00</td>
<td>8</td>
</tr>
<tr>
<td>25 &quot;</td>
<td>5.60 ± 0.11</td>
<td>12</td>
</tr>
<tr>
<td>50 &quot;</td>
<td>5.41 ± 0.15</td>
<td>14</td>
</tr>
<tr>
<td>50 (−KCl, −MgCl₂)</td>
<td>5.48 ± 0.16</td>
<td>13</td>
</tr>
<tr>
<td>50 (−KCl, −MnCl₂)</td>
<td>5.52 ± 0.10</td>
<td>13</td>
</tr>
</tbody>
</table>

Nuclei were isolated from exponentially growing culture (3.8 x 10⁵ cells/ml) as described in Section (2.5) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a.) except that KCl was omitted from the assay cocktail in the presence or absence of MnCl₂ or MgCl₂ as indicated in the Table.

Nuclear DNA was determined as described in Section (2.13).

\[ n = 2, \text{n = number of experiments performed.} \]
Higashinakagawa et al. (176), using DEAE-Sephadex chromatography to separate and purify *Tetrahymena pyriformis* nuclear RNA polymerases, suggested that *Tetrahymena* nuclei possess only two major RNA polymerase activities, namely T1b and T11 which according to their behaviour towards α-amanitin correspond to RNA polymerase I and II of rat liver nuclear enzymes respectively. However, higher concentrations of α-amanitin were needed to bring about 100% inhibition of *Tetrahymena pyriformis* polymerase II when compared to mammalian polymerase II. Approximately 3.2 x 10^{-8} M of α-amanitin gave 100% inhibition of mammalian RNA polymerase II (70), while about a 100-fold higher concentration of α-amanitin was required to inhibit the yeast enzyme (approximately 1 x 10^{-6} M) (220). In *Tetrahymena pyriformis*, 3 x 10^{-5} M α-amanitin did not give 100% inhibition of RNA polymerase T11 of Higashinakagawa et al. (176). However, these authors suggested that these results might be due to the T11 enzyme being contaminated by T1b, the enzyme which is α-amanitin insensitive. With whole nuclear work, approximately 3.6 x 10^{-4} M of α-amanitin was needed to give the highest inhibition of total nuclear RNA polymerase activity. This concentration is about 10-fold higher than that used by Higashinakagawa et al. in their work on the T11 enzyme.
CHAPTER FOUR

THE EFFECT OF CYCLIC NUCLEOTIDES ON THE ACTIVITY OF NUCLEAR RNA POLYMERASE

Several recent experiments support the hypothesis that the cyclic nucleotides, cyclic AMP and cyclic GMP, exert opposing influences on the induction of cell proliferation in eukaryotic cells. These experiments proved that cyclic AMP acts as a growth inhibitor. It inhibits the rate of cell proliferation in a wide variety of cell types, while cyclic GMP and some mitogenic agents which elevate the intracellular level of cyclic GMP act as a signal to accelerate the rate of cell proliferation.

In this Chapter, it was of interest to study the effect of various cyclic nucleotides on nuclear DNA-dependent RNA polymerase activity, since RNA synthesis appears as one of the earliest events following initiation of cellular proliferation.

4.1. The Effect of Cyclic AMP on the Activity of Nuclear RNA Polymerase

The effect of cyclic AMP and its dibutyryl analogue (dibutyryl cyclic AMP) on the nuclear RNA polymerase activity was studied. In these experiments whole intact Tetrahymena pyriformis nuclei were used as a model to represent an in vivo situation. Figure (18) shows the effects of decreasing concentrations of cyclic AMP on the nuclear RNA polymerase activity. As can be seen from the Figure, cyclic AMP stimulates the enzyme activity at physiological concentrations (10^{-6} M - 10^{-8} M). Cyclic AMP at 10^{-7} M produced maximum stimulation and the rate of incorporation of UTP into acid-insoluble RNA was approximately doubled. Dibutyryl cyclic AMP gave a similar pattern of stimulation of nuclear RNA polymerase activity in the same concentration range as cyclic AMP. This indicates that nuclei
Fig. (18) The Effect of cyclic AMP on Nuclear RNA polymerase Activity

Nuclei were isolated from exponentially growing culture (3.8 x 10^5 cells/ml) as described in Section (2.5) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a). DNA was determined as described in Section (2.13). Cyclic AMP or dibutyryl cyclic AMP at the concentrations indicated in the Figure were added to the reaction mixture. The reaction was started by the addition of nuclei containing 40μg DNA.
prepared by the Nonidet-P₄₀ procedure, which is known to remove the outer membrane of the nuclei, are freely permeable to cyclic AMP. Since dibutyryl cyclic AMP is known to pass through membranes more freely than cyclic AMP itself, it would appear that the Nonidet prepared nuclei have no membrane barrier to cyclic AMP. Furthermore, dibutyryl cyclic AMP is less susceptible to enzymatic degradation by a cyclic AMP phosphodiesterase, thus indicating that if a cyclic AMP phosphodiesterase is present in these nuclear preparations, it is not rapidly degrading added cyclic AMP.

Stimulation of UTP incorporation into RNA by cyclic AMP was independent of the amount of nuclei used. Figure (19) shows the rate of stimulation of nuclear RNA polymerase activity by 10⁻⁷ M cyclic AMP as a function of nuclear DNA concentrations used under the standard assay conditions.

The effect of cyclic AMP on the different forms of nuclear RNA polymerase activity was investigated. A specific assay for RNA polymerase I can be done in the presence of 3.6 x 10⁻⁸ M α-amanitin which gives maximum inhibition of nuclear RNA polymerase II activity. The stimulation of nuclear RNA polymerase activity by 10⁻⁷ M cyclic AMP did not alter in the presence of α-amanitin. This indicates that stimulation of nuclear RNA polymerase activity by cyclic AMP is primarily due to the activation of RNA polymerase I (α-amanitin insensitive enzyme).

The data of such an experiment is shown in Table (6). When the data are analysed as shown in Table (6.B.), it appears that RNA polymerase I is stimulated 3.4-fold while RNA polymerase II is actually inhibited by cyclic AMP.

It was found that cyclic AMP stimulation of transcription was very dependent on the ionic strength of the assay mixture. As mentioned in the preceding Chapter, in the absence of added KCl and in the presence of
The experimental conditions used were as described in Fig. (18) except that the amount of nuclear DNA used per assay was varied.

- Curve (A) represents the RNA polymerase activity in the presence of $10^{-7}$M cyclic AMP.
- Curve (B) represents the activity of the enzymes in the absence of $10^{-7}$M cyclic AMP.
- Curve (C) represents the effect of varying the amount of nuclei on the specific activity of RNA polymerase in the presence and absence of cyclic AMP.

Fig. (19) The Effect of varying the amount of nuclei on RNA polymerase activity stimulated by cyclic AMP.
### Table (6.A.) The Effect of α-amanitin on the Nuclear RNA Polymerase Activities stimulated by Cyclic AMP

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>nmoles [4 - 14C] UMP incorporated per 10 min.</th>
<th>% Activity of the complete assay system</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Control&quot;</td>
<td>0.78 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>+ 10^{-7} M cyclic AMP</td>
<td>1.38 ± 0.09</td>
<td>177</td>
</tr>
<tr>
<td>+ α-amanitin (50 μg)</td>
<td>0.34 ± 0.03</td>
<td>44</td>
</tr>
<tr>
<td>[ + α-amanitin ]</td>
<td>1.15 ± 0.05</td>
<td>147</td>
</tr>
</tbody>
</table>

The experimental conditions used were as described in Figure (18).

\[ n = 2, \ n = \text{number of experiments performed.} \]

### Table (6.B.) Analysis of Data in Table (6.A.)

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>RNA Polymerase I*</th>
<th>RNA Polymerase II*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Control&quot;</td>
<td>0.34</td>
<td>0.44</td>
</tr>
<tr>
<td>+ 10^{-7} M cyclic AMP</td>
<td>1.15</td>
<td>0.23</td>
</tr>
</tbody>
</table>

This table is only a hypothetical analysis of the data RNA polymerase. It may not be affected by the α-amanitin.

* Units of activity are as in Table (6.A.)

nmoles UMP incorporated/10 min.
either Mn\(^{++}\) or Mg\(^{++}\) ions in the assay mixture, RNA polymerase I is the major enzyme species which is measured in a nuclear preparation. However, since there is some residual RNA polymerase activity inhibited by \(\alpha\)-amanitin, a small amount of the total activity measured under these conditions must be attributed to RNA polymerase II. The stimulation of nuclear RNA polymerase I by cyclic AMP was investigated in the absence of added KCl but with either Mn\(^{++}\) or Mg\(^{++}\) ions present. No stimulation was observed at any cyclic AMP concentrations between \(10^{-3}\) - \(10^{-10}\) M.

Using nuclear DNA concentrations between 10 \(\mu\)g - 60 \(\mu\)g in the presence of \(10^{-7}\) M cyclic AMP, the values of radioactivity incorporated into acid-precipitable product were comparable to the control values when no cyclic AMP was present. Time-dependent stimulation was also investigated between 10 - 20 minutes. Again no stimulation by cyclic AMP was observed. Addition of 50 mM KCl to the reaction mixture (the conventional concentration of KCl which is used for preferential RNA polymerase I assay in mammalian nuclei) in the presence of Mg\(^{++}\) ion and \(\alpha\)-amanitin gave no or very slight stimulation by \(10^{-7}\) M cyclic AMP. 6 - 10% stimulation was observed in one set of experiments, but consistent results were not obtained when the experiment was repeated and the small amount of stimulation might be considered to be within the range of experimental errors between duplicates.

Highest stimulation of nuclear RNA polymerase activity by \(10^{-7}\) M cyclic AMP was observed when 0.15 M KCl was included in the assay mixture. Such stimulation occurred even in the presence of \(\alpha\)-amanitin. From the above observations one may conclude that the rate of stimulation of DNA-dependent RNA polymerase activity in whole intact nuclei by cyclic AMP must be due to a salt-dependent stimulation process. Further experimental work is needed to elucidate such observations. However, it
is known that high salt concentrations cause structural changes within the nucleus. These may in some way facilitate the cyclic AMP dependent stimulation.

The effect of cyclic AMP on RNA polymerase activity associated with a chromatin preparation was also studied. A fresh crude chromatin preparation had less transcriptional activity than the whole nuclei. 35 - 50% of the nuclear transcriptional activity was observed in different chromatin preparations. This variation may be due to the different amounts of chromatin shearing from one experiment to another or may be due to the loss of some of the RNA polymerase proteins during the washing procedure in 0.14 M buffered NaCl which removes the soluble nucleoplasmic proteins. However, the stimulation of the residual RNA polymerase activity in such chromatin preparations by cyclic AMP was consistently obtained in repeated experiments. Under standard assay conditions (see Section 2.10.a.) maximum stimulation by $10^{-7}$ M cyclic AMP was at best 25%. Table (7) shows such stimulation of the RNA polymerase activity associated with the chromatin. This result demonstrates that the stimulation of nuclear RNA polymerase activity by cyclic AMP is associated with the chromatin. Probably a protein kinase present in the chromatin structure mediates the action of this cyclic nucleotide.

4.2. The Effect of Cyclic GMP on Nuclear RNA Polymerase Activity:

The effect of cyclic GMP and its dibutyryl analogue was studied on nuclear RNA polymerase activity. Whole intact Tetrahymena pyriformis nuclei prepared by the Nonidet procedure were used for such studies. Figure (20) shows the effects of decreasing concentrations of cyclic GMP on the nuclear RNA polymerase activity. As can be seen from the Figure,
Table (7). Stimulation by Cyclic AMP of RNA Polymerase Activity associated with Chromatin Preparation

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>Pmoles $[^{14}C]$ UMP incorporated/10 min/µg chromatin DNA</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Complete&quot;</td>
<td>9.22 ± 0.80</td>
<td>-</td>
</tr>
<tr>
<td>+ 10^{-6} M cyclic AMP</td>
<td>9.01 ± 0.25</td>
<td>-</td>
</tr>
<tr>
<td>+ 10^{-7} M cyclic AMP</td>
<td>12.35 ± 0.66</td>
<td>25</td>
</tr>
<tr>
<td>+ 10^{-8} M cyclic AMP</td>
<td>9.07 ± 0.98</td>
<td>-</td>
</tr>
</tbody>
</table>

Fresh chromatin fraction was prepared as described in Section (2.7.).

Endogenous RNA polymerase activity was assayed as described in Section (2.10.a.) "complete". Chromatin DNA was determined as described in Section (2.13). 37 µg chromatin DNA was used per assay.

$[n = 2, n = number of experiments performed.]$
Fig. (20). The Effect of Cyclic GMP and Ca$^{++}$ on Nuclear RNA polymerase Activity

Nuclei were isolated from exponentially growing culture (4.0 x 10$^5$ cells/ml) as described in Section (2.5) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a). DNA was determined as described in Section (2.13). 2 mM CaCl$_2$ plus cyclic GMP or dibutyryl cyclic GMP at the concentrations indicated in the Figure were added to the reaction mixture. The reaction was started by the addition of nuclei containing 38 µg DNA.
The additional interpretation is that EGTA is chelating another metal ion, e.g. Zn$^{2+}$ which is required for the activity of the enzyme.
cyclic GMP in the presence of 2 mM CaCl$_2$ stimulates the enzyme activity at concentrations as low as $10^{-9} - 10^{-11}$ M. The cyclic nucleotide in the presence of Ca$^{++}$ (2 mM) produced maximum stimulation at $10^{-10}$M. The rate of radioactivity incorporated into an acid-insoluble product was approximately double that of the control value when no cyclic GMP and CaCl$_2$ were present. Interestingly, when dibutyl cyclic GMP was used for such a study, the maximum stimulation was at $10^{-8}$ M in the presence of 2 mM CaCl$_2$.

Addition of 2 mM CaCl$_2$ alone to the reaction mixture had no effect on the rate of radioactivity incorporated into RNA. But, in some experiments, addition of cyclic GMP at $10^{-10}$M alone gave some stimulation of radioactivity incorporated into RNA. This stimulation ranged between 15 - 36% in different repeated experiments. Addition of 0.2 mM EGTA (a specific chelating agent for Ca$^{++}$) to the reaction mixture abolished such stimulation. This indicates that in some nuclear preparations, the nuclei contained a pool of endogenous Ca$^{++}$. Only cyclic GMP plus CaCl$_2$ produced maximum stimulation of nuclear RNA polymerase activity. The result correlates with the proposed hypothesis put forward by Berridge (186) on the role of calcium and cyclic nucleotides in the control of cell division. Cyclic GMP may function together with Ca$^{++}$ to initiate the cell division programme at the transcription level.

The effect of cyclic GMP and CaCl$_2$ on the RNA polymerase activity associated with a chromatin preparation was also studied. In this case, no stimulation of the RNA polymerase activity was observed in repeated experiments. The chromatin prepared by the procedure described in Section (2.7) might have lost some important component(s) which mediated the action of cyclic GMP and Ca$^{++}$ on transcriptional activity. If this proves to be the case, it will be of interest to isolate these components.
and use them to restore the cyclic GMP mediated transcriptional activity of the chromatin preparation.

So far, it has not been established whether cyclic GMP and Ca\(^{++}\) stimulates either RNA polymerase I or II or both of these enzymes in *Tetrahymena pyriformis* nuclei. This could be done by designing experiments in which α-amanitin is used to distinguish between the different RNA polymerases. This has not been done so far because of the high cost of α-amanitin.

4.3. The Effect of 5'-GMP, 5'-AMP and cyclic-3', 5'-CMP on the Activity of Nuclear RNA Polymerase

GMP, AMP and cyclic CMP had no effect on the activity of nuclear RNA polymerase. No significant stimulation of nuclear RNA polymerase by any of these compounds was observed at concentrations between 10\(^{-3}\) - 10\(^{-1}\)M.

The experimental data are shown in Figure (21).

4.4. The Effect of Cyclic Nucleotides on Extracted RNA Polymerase Activity:

Since a crude preparation of soluble RNA polymerase from *Tetrahymena pyriformis* nuclei also contained an endogenous cyclic AMP-dependent protein kinase, the effect of cyclic AMP on extracted RNA polymerase activity was investigated.

Table (8) shows a partial purification of RNA polymerase extracted from nuclei by the sonication method described in Section (2.8.a.). The specific activity of the enzyme after ammonium sulphate fractionation was 43.7 ± 2.3 nmoles of [\(^{14}\)C]UMP incorporated/10 min./mg protein. This value is higher than that reported by other authors in the literature, i.e. 44- and 22-fold higher than the corresponding fractions reported for *Tetrahymena pyriformis* and rat liver respectively (176, 108). In the
Fig. (21) The Effect of GMP, AMP and cyclic CMP on Nuclear RNA polymerase activity

Nuclei were isolated from exponentially growing culture (3.8 x 10^5 cells/ml) as described in Section (2.5) and assayed for endogenous RNA polymerase activity as described in Section (2.10.a). DNA was determined as described in Section (2.13). GMP, AMP and cyclic CMP at the concentrations indicated in the Figure were added to the reaction mixture. The reaction was started by the addition of nuclei containing 35 μg DNA.

(Δ-Δ) represents the activity with AMP

(○-○) represents the activity with GMP

(■-■) represents the activity with cyclic CMP
### Table (8) Extraction of Nuclear RNA Polymerase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume (ml)</th>
<th>Total Protein (mg)</th>
<th>RNA Polymerase Activity</th>
<th>RNA Polymerase specific Activity</th>
<th>Total Activity</th>
<th>% of Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant of sonicated nuclei</td>
<td>22</td>
<td>18.7</td>
<td>0.90 ± 0.04</td>
<td>21.2 ± 1.0</td>
<td>396</td>
<td>100</td>
</tr>
<tr>
<td>0 - 65% ((NH_4)_2SO_4) fraction (after dialysis)</td>
<td>2</td>
<td>5.2</td>
<td>1.31 ± 0.07</td>
<td>43.7 ± 2.3</td>
<td>227</td>
<td>57</td>
</tr>
</tbody>
</table>

Nuclei were isolated from 4 litres exponentially growing culture \((3.8 \times 10^5\) cells/ml) as described in Section (2.5). The packed nuclei were resuspended in 4 ml of buffer (D) (Section 2.2.) and subjected to the sonication procedure as described in Section (2.8.a.). RNA polymerase activity was assayed as described in Section (2.10.b.) using 20 \(\mu\)g/assay of heat-denatured calf thymus DNA as a template. Protein was determined as described in Section (2.14).

RNA polymerase activity was expressed as nmoles of \([4-^14C]\)UMP incorporated/10 min.

RNA Polymerase specific activity was expressed as nmoles of \([4-^14C]\)UMP incorporated/10 min./mg protein.

Total RNA polymerase activity was expressed as nmoles of \([4-^14C]\)UMP incorporated/10 min./total protein.
present study high concentrations of unlabelled UTP (0.4 mM) have been
used in the assay mixture and this may account for the differences in
the specific activity.

When using calf thymus DNA as a template in the assay mixture for
extracted RNA polymerase, addition of $10^{-6}$ or $10^{-7}$ M cyclic AMP did not
increase the enzyme activity in either the sonicate supernatant or the
ammonium sulphate fractions. It has been reported that cyclic AMP will
stimulate the activity of a solubilized RNA polymerase fraction in an
assay system in which the DNA template is prepared from the same species
(135, 157). It has also been reported that the binding of cyclic AMP-
dependent protein kinase to specific sites on the DNA is species specific
(158). It will therefore be of interest to determine whether cyclic AMP
can stimulate the activity of a solubilized RNA polymerase(s) fraction
from *Tetrahymena pyriformis* nuclei when isolated DNA from this organism
is used as the template.

Although it has been reported that cyclic GMP stimulates the activity
of extracted RNA polymerase from rat mammary gland nuclei (221, 222) in a
system employing calf thymus DNA as a template, the effect of this cyclic
nucleotide on the extracted RNA polymerase activity from *Tetrahymena
pyriformis* nuclei has so far not been investigated.

Apart from the sonication method, RNA polymerase can be extracted
from nuclei using high salt concentrations. The RNA polymerase containing
fraction isolated from *Tetrahymena pyriformis* nuclei by the 2 M NaCl
method (Section 2.8.b.) showed very little activity even when large
numbers of nuclei (approximately $4 \times 10^9$ nuclei/4 ml) were used. A
specific activity of only $4.32 \pm 0.28$ nmoles $[4 - ^{14}C]$ UMP incorporated
/10 min/mg protein was detected after dialysis compared to the specific
activity of $21.2 \pm 1.0$ nmoles $[4 - ^{14}C]$ UMP incorporated/10 min/mg protein
of the enzyme fraction prepared by sonicating nuclei (Section 2.8.a.) (Table (8)). In subsequent experiments, the sonication method was used to extract the nuclear RNA polymerase. However, the 2 M NaCl extraction method was the procedure used for the measurement of other important nuclear enzymes, namely cyclic AMP and cyclic GMP phosphodiesterases. The 2 M NaCl extraction procedure was found to be a useful method for obtaining high activities of these enzymes in whole nuclei and subnuclear fractions as will be seen from the following Section (4.5).

4.5. Detection of Cyclic AMP and Cyclic GMP Phosphodiesterases in Whole Nuclei and Subnuclear Fractions:

Early in the present work, the lack of stimulation of extracted RNA polymerase by cyclic AMP and cyclic GMP was thought to be possibly due to the presence of cyclic nucleotide phosphodiesterases. Thus it was decided to look for the presence of cyclic nucleotide phosphodiesterases in the extracted RNA polymerase fraction. Interestingly, cyclic AMP and cyclic GMP phosphodiesterase activities were detected in whole intact nuclei and subnuclear fractions. Measurement of enzyme activities were kindly performed by J. R. Dickinson.

Table (9) shows the distribution of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase in subnuclear fractions. The subnuclear fractions were prepared as described in Section (2.6.), Figure (9). As can be seen from the Table, the specific activity of cyclic AMP phosphodiesterase in the 2 M NaCl chromatin residue (Fraction N₄) was almost double that found in whole nuclei (Fraction N₁). Interestingly, cyclic GMP phosphodiesterase specific activity in the 2 M NaCl chromatin residue was almost equal to that activity found in whole nuclei. These observations suggest that cyclic AMP phosphodiesterase is more tightly bound to the DNA than cyclic GMP phosphodiesterase because the enzyme
Table (9) Subnuclear Distribution of Cyclic AMP Phosphodiesterase and Cyclic GMP Phosphodiesterase

### Cyclic AMP Phosphodiesterase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cyclic AMP Phosphodiesterase Activity</th>
<th>Protein (mg)</th>
<th>Specific Activity</th>
<th>% of Total Activity in Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei (Ni)</td>
<td>1.62</td>
<td>1.02</td>
<td>1.58</td>
<td>100.00</td>
</tr>
<tr>
<td>0.14 M NaCl soluble extract (N2)</td>
<td>0.85</td>
<td>21.18</td>
<td>0.04</td>
<td>7.5</td>
</tr>
<tr>
<td>2 M NaCl soluble extract (N3)</td>
<td>1.75</td>
<td>4.01</td>
<td>0.44</td>
<td>15.4</td>
</tr>
<tr>
<td>2 M NaCl chromatin residue (N4)</td>
<td>4.70</td>
<td>1.50</td>
<td>3.13</td>
<td>41.4</td>
</tr>
</tbody>
</table>

### Cyclic GMP Phosphodiesterase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cyclic GMP Phosphodiesterase Activity</th>
<th>Protein (mg)</th>
<th>Specific Activity</th>
<th>% of Total Activity in Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei (Ni)</td>
<td>2.17</td>
<td>1.02</td>
<td>2.12</td>
<td>100.00</td>
</tr>
<tr>
<td>0.14 M NaCl soluble extract (N2)</td>
<td>0.21</td>
<td>21.18</td>
<td>0.01</td>
<td>1.4</td>
</tr>
<tr>
<td>2 M NaCl soluble extract (N3)</td>
<td>1.45</td>
<td>4.01</td>
<td>0.36</td>
<td>9.5</td>
</tr>
<tr>
<td>2 M NaCl chromatin residue (N4)</td>
<td>3.77</td>
<td>1.50</td>
<td>2.51</td>
<td>24.8</td>
</tr>
</tbody>
</table>

Cyclic AMP and cyclic GMP phosphodiesterase activities were expressed as μmole cyclic AMP and cyclic GMP hydrolysed/30 min.

The enzymes' specific activities were expressed as μmole cyclic AMP and cyclic GMP hydrolysed/30 min/mg protein.

The subnuclear fractions were prepared as described in Section (2.6). Protein was determined as described in Section (2.14). Cyclic AMP and cyclic GMP phosphodiesterases were assayed as described in Section (2.11). The activity of the enzymes was measured by J. R. Dickinson.
was not released from the chromatin by the high salt extraction method. Dickinson (199) has found that the total activity of cyclic AMP and cyclic GMP phosphodiesterase was highest in the 50000 g supernatant of cell homogenate prepared by the sonication of whole cells. He suggested that the high activity found in the 50000 g supernatant could be explained by the fact that sonication of whole cells might disrupt the chromatin structure thereby solubilising the enzymes.
CHAPTER FIVE

THE DETERMINATION OF TOTAL RNA POLYMERASE LEVELS DURING THE CELL CYCLE

The investigation of the activity of the different forms of RNA polymerase during the cell cycle offers an approach to study the regulation of these enzymes and their relationship with other biochemical events.

Many reports in the literature are concerned with the control of the synthesis of cellular RNA in intact cells. In such studies, the rate of labelled uridine incorporated into cellular RNA is often monitored during the cell cycle. In Hela cells, Terasima and Tolmach (223) showed that a three-fold increase in the rate of incorporation of $^3$H-uridine into RNA occurred during the period from early G\textsubscript{1} to late G\textsubscript{2}. Pfeiffer and Tolmach (224) have reported that $^{14}$C-uridine incorporated into Hela cell culture increased about 2.5-fold between mid G\textsubscript{1} and late G\textsubscript{2}. However, Kim and Perez (225), using Hela cells claimed a ten-fold increase in the rate of $^3$H-uridine incorporated between early G\textsubscript{1} and the middle of S phase. In Chinese hamster cells, Crippa (226) has studied the relationship between the rate of $^3$H-uridine incorporation and DNA content during the cell cycle. He observed that the rate of both chromosomal and nucleolar RNA synthesis was lowest in G\textsubscript{1}, increased continuously through S concomitantly with the progressive increase of the DNA content per cell. However, all the above reports showed that the increase in the rate of labelled uridine incorporated into a cell culture coincides with the rate of DNA synthesis during the S phase of the cell cycle.
In the unicellular eukaryote, Physarum polycephalum, it has been reported that the solubilised activities of RNA polymerase I and II from plasmodial homogenates showed equal levels throughout the synchronous mitotic cycle (227). However, Fouquet and Braun (228) have shown by RNA:DNA hybridisation techniques that more DNA sequences were transcribed in the S phase than in the G2 phase in this organism. On the basis of this finding, the authors have claimed that there is a differential transcription of DNA during the cell cycle. In work with isolated nuclei and nucleoli from this organism, Grant (229) has shown that there is independent regulation of nuclear RNA polymerases I and II during the cell cycle. The major peak of α-amanitin-sensitive enzyme (RNA polymerase II) activity coincided with the S phase of the cell cycle while the α-amanitin-insensitive enzyme activity peaked during the G2 phase of the cell cycle. More recently, Davies and Walker (230) have been able to obtain similar results with this organism.

For yeast cells, there are reports by two different groups of research workers (115, 231) in which it has been shown that there is independent regulation of the RNA polymerase I and II activities during the cell cycle.

In this Chapter, the levels of total endogenous RNA polymerase activity during the natural Tetrahymena pyriformis cell cycle were studied. α-amanitin was used to distinguish between RNA polymerases I and II.

Figure (22.A.) shows the analysis of DNA/cell throughout the cell cycle in selection synchronised Tetrahymena pyriformis. As can be seen from the Figure, DNA synthesis is synchronised relative to cell division. Knowing the timing of cell division and DNA synthesis,
Fig. (22.A) Analysis of cellular DNA in selection-synchronised *Tetrahymena pyriformis*

Cells were grown in a protease-peptone medium supplemented with 0.5% (w/v) glucose as described in Section (2.3). The presence of glucose in the medium resulted in better cell synchrony (199). The cells were synchronised by the magnetic method as described in Section (2.9). Cellular DNA was determined as described in Section (2.13).
Endogenous RNA polymerase activity in cell homogenates from the different phases of the cell cycle was assayed by the standard assay procedure described in Section (2.10.a).

RNA polymerase activity was assayed either in the presence (---) and or the absence (—) of 50 µg α-amanitin per assay.

\[ n = 3, \quad n = \text{number of experiments performed} \]
one can therefore assign the various phases of the cell cycle. The amount of DNA is doubled on completion of the S phase.

Figure 22B shows the rate of $[\text{4-}^{14}\text{C}]\text{UMP}$ incorporated into acid-precipitable RNA as a function of the cell cycle. As can be seen from the Figure, the total endogenous RNA polymerase activity from the sonicated cells is highest at the end of S phase. A specific activity of $1.5 \pm 0.07$ nmoles of $[\text{4-}^{14}\text{C}]\text{UMP}$ incorporated/10 min/10$^6$ cells was observed at S phase and declined during the G$_2$ phase of the cell cycle. A specific activity of only $0.1$ nmoles $[\text{4-}^{14}\text{C}]\text{UMP}$ incorporated/10 min./10$^6$ cells was observed in G$_2$ phase. Virtually no significant RNA polymerase activity could be detected during the cell division.

Addition of 50 $\mu$g of a-amanitin (which gives maximum inhibition of nuclear RNA polymerase II) to the assay mixture inhibited about 58% of the rate of radioactivity incorporated into RNA during S phase. No inhibition by a-amanitin was observed during the remainder of the cell cycle. This result suggests that RNA polymerase II activity might account for almost two-thirds of the total RNA polymerase activity observed in S phase and that RNA polymerase I activity is the predominant one during the G$_2$ phase of the cell cycle. However, these are only preliminary studies and the reasons why RNA polymerase I and II activities fluctuate during the cell cycle are as yet unknown. Many factors might account for such fluctuations:

1. Availability of the template might be a regulatory factor during the cell cycle.
2. Variation during the cell cycle in the number of enzyme molecules which are responsible for transcription of particular genes on the DNA genome.
3. Alterations in the number of available promoter sites on the DNA throughout the cell cycle.

4. Finally, alterations in the activity of the enzymes might have already occurred at the time of cell disruption because of some change for example, in cellular cyclic AMP, cyclic GMP, Ca^{++} or other allosteric or covalent modifier of activity.

It is clear that the results reported here can provide the basis for further work on the control systems which operate on transcription.
CHAPTER SIX

THE RELATIONSHIP BETWEEN NUCLEAR PROTEIN KINASE AND DNA-DEPENDENT RNA-POLYMERASE

In *E. coli*, it has been shown that cyclic AMP regulates transcription at the Lac and Gal operons (55, 56) through a mechanism that involves binding of the cyclic nucleotide to a cyclic AMP receptor protein with a variable affinity for DNA (56, 54, 53). In the presence of cyclic AMP, the receptor protein binds to the DNA at the operator-promoter region and promotes the initiation of transcription by RNA polymerase (55).

In eukaryotic systems, the effects of cyclic AMP upon transcription may be mediated through binding of the cyclic nucleotide to receptor proteins which act in the cell nucleus. The most extensively characterised receptor proteins for cyclic AMP in mammalian cells are cyclic AMP-dependent protein kinases (128, 158) and it has been proposed that in higher organisms many, if not all, the biological effects of cyclic AMP are mediated through the activation of this class of enzymes (232). Cyclic AMP-dependent protein kinases are composed of regulatory (R) and catalytic (C) subunits. Cyclic AMP binds to the R subunit, which splits from the kinase holoenzyme, leaving an activated C subunit (233).

\[
\text{RC} + \text{cyclic AMP} \rightleftharpoons \text{C} + \text{R} - \text{cyclic AMP}
\]

The present chapter contains a report of the discovery of the existence of protein kinase activity in isolated *Tetrahymena pyriformis* nuclei. The effects of the cyclic nucleotides, cyclic AMP and cyclic GMP, on the
activity of nuclear protein kinases were studied; because it had already been established that cyclic nucleotides stimulated endogenous nuclear RNA polymerase activity (Chapter 4). Before detailed studies could be started, however, it was necessary to optimise the assay conditions for nuclear protein kinase.

6.1. The Effect of MgCl\textsubscript{2} on the Protein Kinase activity in Intact Nuclei.

Endogenous protein phosphorylation in intact Tetrahymena pyriformis nuclei was found to be Mg\textsuperscript{++}-dependent. Figure (23) shows the effect of increasing MgCl\textsubscript{2} concentrations on the rate of $[^{32}\text{P}]$ incorporation from $[\gamma-^{32}\text{P}]$ATP into an acid precipitable protein under standard assay conditions (Section 2.12). As can be seen from the Figure, addition of MgCl\textsubscript{2} to a final assay concentration of between 10 - 25 mM gave maximal activity. 40 mM MgCl\textsubscript{2} was found to inhibit the activity of the enzyme. In the routine standard assay cocktail 10 mM MgCl\textsubscript{2} was used to obtain optimal protein kinase activity.

6.2. The Time Course of $[^{32}\text{P}]$ incorporation in Intact Nuclei.

Figure (24) shows the time course of $[^{32}\text{P}]$ incorporation in the presence or absence of 10 mM NaF under standard assay conditions (Section 2.12.). The assay was linear for 10 minutes in the presence or absence of NaF after which time the rate of $[^{32}\text{P}]$ incorporation into acid-precipitable material declined rapidly in the absence of 10 mM NaF but less rapidly in its presence. Since it is known that NaF is an inhibitor of phosphoprotein phosphatase, these results indicate that NaF inhibits the dephosphorylation of phosphoproteins. In a routine assay for protein kinase 10 mM NaF was added to assay mixture to inhibit dephosphorylation of the product.
Fig. (23) The Effect of MgCl₂ on the nuclear protein kinase activity

Nuclei were prepared as described in Section (2.5) from an exponentially growing culture (3.9 x 10⁵ cells/ml). 50 μl nuclei containing 100 μg protein were assayed for endogenous protein kinase activity as described in Section (2.12) except MgCl₂ concentration was varied as indicated in the Figure. Protein was determined as described in Section (2.14). [n = 3, n = number of experiments performed.]
Fig. (24) The time course of $^{32}$P incorporation in protein assays on isolated nuclei in the presence and absence of NaF.

Nuclei were isolated as described in Section (2.5).

50 μl nuclei containing 100 μg protein were assayed for endogenous protein kinase activity as described in Section (2.12), in the presence or absence of 10 mM NaF. Protein was determined as described in Section (2.14).

$[n = 3, n = \text{number of experiments performed}]$
From the above result, one can deduce that phosphorylation and dephosphorylation of nuclear proteins are regulated by nuclear protein kinase (S) and phosphoprotein phosphatase (S) respectively. The presence of protein kinase and phosphatase activities in isolated nuclei is of interest because of the hypothesis put forward by Kleinsmith (140) on the importance of the phosphorylation and dephosphorylation of chromatin proteins in the control of transcription.

6.3. The Effect of cyclic AMP on Nuclear Protein Kinase Activity:

Bearing in mind the observations of many research workers that cyclic AMP in eukaryotic cells acts by means of the activation of protein kinases and since it was also observed that this cyclic nucleotide increased the nuclear RNA polymerase activity it was of interest to investigate the effect of cyclic AMP on endogenous nuclear protein phosphorylation.

Stimulation of endogenous protein phosphorylation by cyclic AMP was only observed in the presence of salt added to the assay mixture. It has been shown that NaCl stimulates some nuclear protein kinase activities while inhibiting others when nuclear protein kinases are separated into several peaks by phosphocellulose chromatography (234). $5 \times 10^{-7}$ M cyclic AMP stimulated endogenous protein kinase activity when 0.1 M NaCl was added to the assay mixture. The effect was very dependent on concentration since $5 \times 10^{-5}$ or $5 \times 10^{-6}$ M cyclic AMP had no effect. An experiment of this type is shown in Figure (25). Figure (26) shows the time course of $[^{32}P]$ incorporation into whole nuclei in the presence and absence of $5 \times 10^{-7}$ M cyclic AMP. As can be seen from the Figure, cyclic AMP stimulated the rate of $[^{32}P]$ incorporation into an acid-precipitable protein by 23% after 10 minutes incubation. The rate of stimulation by
Fig. (25) The effect of cyclic AMP on nuclear protein kinase activity at different NaCl concentrations

Nuclei were isolated as described in Section (2.5) from exponentially growing cells (3.4 x 10⁵ cells/ml). 50 µl nuclei containing 110 µg protein were assayed for endogenous protein kinase activity as described in Section (2.12) except that NaCl concentration was varied as indicated in the Figure. The assay was performed in the absence or presence of 5 x 10⁻⁷M cyclic AMP.

[n = 2, n = number of experiments performed.]
Fig. (26) The time course of $^{32}$P incorporation in a protein kinase assay on nuclei in the presence and absence of added cyclic AMP.

Nuclei from an exponentially growing culture ($3.8 \times 10^5$ cells/ml) were isolated as described in Section (2.5). 50 µl nuclei containing 105 µg protein were assayed for endogenous protein kinase in the absence and presence of $5 \times 10^{-7}$M cyclic AMP as described in Section (2.12). Protein was determined as described in Section (2.14).

[n = 4, n = number of experiments performed.]
cyclic AMP reaches a plateau between 10 - 20 minutes of incubation.

The stimulation of nuclear protein kinase by cyclic AMP (like the stimulation of endogenous nuclear RNA polymerase activity) is a salt-dependent stimulation process. This stimulation may have been due to activation of cyclic AMP-dependent protein kinase(s) in nuclei or to inhibition of phosphatase activity by cyclic AMP or to both.

6.4. The Effect of cyclic GMP on Nuclear Protein Kinase Activity:

All concentrations of cyclic GMP tried (between $10^{-6}$ M - $10^{-12}$M) whether in the presence or the absence of 2 mM CaCl$_2$ failed to stimulate nuclear protein kinase activity. No stimulation could be detected in the absence or presence of 0.05 - 0.2 M NaCl. It is therefore not established at this time if there is a specific cyclic GMP dependent protein in the nucleus. These preliminary results would seem to exclude its presence, but if this was the case it would be difficult to explain the mechanism of stimulation of nuclear RNA polymerase by cyclic GMP and Ca$^{++}$.

6.5. The Effect of Theophylline on Nuclear Protein Kinase Activity:

At an early stage in my work on endogenous nuclear protein phosphorylation, the lack of stimulation of nuclear protein kinase by cyclic AMP was observed. This was thought to be probably due to the presence of a cyclic AMP phosphodiesterase activity in the nuclei which degrades the cyclic nucleotide. For this reason experiments were designed in which theophylline was added to the assay mixture to inhibit any phosphodiesterase. Unexpectedly, addition of theophylline to the protein kinase reaction mixture was found to inhibit the rate of $\left[^{32}\text{P}\right]$ incorporated into acid-precipitable protein. Under standard assay conditions (Section 2.12.)
the inhibition of nuclear protein kinase activity by theophylline was found either in the presence or in the absence of added cyclic AMP. Table (10) shows the results of such experiments. As can be seen from the Table, highest inhibition of $[^{32}\text{P}]$ incorporated into acid-precipitable protein was 66% when 20 mM theophylline was added to the assay mixture. The extent of inhibition was the same in the absence or presence of $5 \times 10^{-7}$ M cyclic AMP.

Methylxanthines, caffeine and theophylline, are very well known as inhibitors of cyclic nucleotide phosphodiesterases. More recently, Ehmann et al. (235) have shown that these compounds inhibit postreplication repair of DNA in mammalian cells by binding to DNA. Interestingly, it has also been shown that human cell cultures treated with caffeine show chromosomal abnormalities, including dicentrics, breaks and gaps in the chromosome structure in proportion to the dose of caffeine used (236).

6.6. Phosphorylation of Extracted RNA Polymerase Fraction:

A preparation of partially purified RNA polymerase was found to contain an endogenous protein kinase activity. Figure (27) shows the rate of $[^{32}\text{P}]$ incorporation into acid-precipitable protein as a function of enzyme concentration. The rate of the reaction was not linear with enzyme concentration. The plateau seen at higher concentrations of protein may be due to the presence of ATPase, protein phosphatase, or various inhibitors of the phosphorylation reaction. However, the specific activity of the endogenous phosphorylation of RNA polymerase fraction near the plateau was approximately 1.04 Pmoles of $[^{32}\text{P}]$ incorporated/10 min/μg protein. This value is half of the value for the endogenous phosphorylation of the proteins in whole intact nuclei. It can therefore be concluded that about 50% of the protein kinase activity
Table (10) The Effect of Theophylline on Nuclear Protein Kinase Activity

<table>
<thead>
<tr>
<th>Addition of Theophylline</th>
<th>Specific Activity*</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ 5 x 10^-7 M cyclic AMP</td>
<td>- cyclic AMP</td>
</tr>
<tr>
<td>0 mM</td>
<td>2.62 ± 0.10</td>
<td>2.01 ± 0.07</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.75 ± 0.09</td>
<td>1.34 ± 0.05</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.30 ± 0.06</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>20 mM</td>
<td>0.88 ± 0.04</td>
<td>0.67 ± 0.03</td>
</tr>
</tbody>
</table>

* Specific activity was expressed as Pmoles[^32P] incorporated per 10 min per μg of nuclear protein.

Nuclei were isolated as described in Section (2.5.) and assayed for endogenous protein kinase activity as described in Section (2.12). 50 μl nuclei containing 95 μg protein was added to the assay mixture. Protein was determined as described in Section (2.14.).

[n = 3, n = number of experiments performed]
Fig. (27) The endogenous phosphorylation of partially purified nuclear RNA polymerase

The preparation of a nuclear RNA polymerase fraction was performed by the sonication procedure described in Section (2.8.a). The fraction was assayed for RNA polymerase activity (Table 8). The fraction after dialysis was also used for an assay of endogenous phosphorylation activity as described in Section (2.12). Protein was determined as described in Section (2.14).
Table (11) The Effect of Cyclic Nucleotides on the Phosphorylation of extracted RNA Polymerase Fraction in the absence and presence of Exogenous Substrates

<table>
<thead>
<tr>
<th>Substrate added to the assay system</th>
<th>Pmoles$[^32P]$ incorporated/10 min/µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- cyclic AMP</td>
</tr>
<tr>
<td>None (Endogenous)</td>
<td>1.04</td>
</tr>
<tr>
<td>Histone</td>
<td>1.04</td>
</tr>
<tr>
<td>Casein</td>
<td>2.40</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>1.35</td>
</tr>
</tbody>
</table>

The preparation of a soluble nuclear RNA polymerase fraction was accomplished by the sonication procedure described in Section (2.8.a.). This fraction was assayed for RNA polymerase activity (Table 8). The fraction after dialysis at a concentration of 65 µg protein/25µl was also used as a source of protein kinase activity. Protein kinase was assayed as described in Section (2.12) in either the absence or the presence of exogenous substrates as indicated in the Table. Protein was determined as described in Section (2.14.).

$[n = 2, n = number of experiments performed.]$
originally present in the nuclei was recovered in the partially extracted RNA polymerase fraction.

Direct phosphorylation of RNA polymerase might play a role in regulating the enzyme activity and thereby the rate of gene transcription. However, one would need to have isolated completely pure phosphorylated RNA polymerase to prove this hypothesis. Cyclic AMP was found to enhance the rate of endogenous phosphorylation in the crude RNA polymerase fraction by approximately five-fold but the proteins undergoing phosphorylation have so far not been identified. Table (11) shows the effect of cyclic AMP, cyclic GMP and various exogenous substrates on the rate of protein phosphorylation in the crude RNA polymerase fraction. As can be seen from the Figure, casein was a better substrate for enzymic phosphorylation than histone or phosvitin. 10^{-6} M dibutyryl cyclic AMP stimulated the phosphorylation of all the exogenous substrates added to the assay system, as well as endogenous phosphorylation. 10^{-8} M dibutyryl cyclic GMP did not give any stimulation of protein phosphorylation in this fraction.
CHAPTER SEVEN

DISCUSSION

For a better understanding of the regulatory mechanisms which control transcription in an organism, detailed investigations are required of all the factors involved in transcription including nuclear kinases and the different RNA polymerases. However, it is difficult to build up a picture of the interaction between all the components by studying the purified enzymes in isolation. For a study of the control of RNA synthesis a suitable in vitro system in which to study such interactions is a preparation of purified nuclei. However, even studies on the operation of control systems in nuclei are not necessarily representative of the operation of the same controls in the cell because of the interactions between nuclei and cytoplasm. In the present work, transcriptionally active Tetrahymena pyriformis nuclei were isolated by the Nonidet-P₄₀ procedure and an assay for measuring the total endogenous activity of RNA polymerase by these nuclei was developed.

A number of properties of in vitro RNA synthesis by isolated nuclei were examined. The temperature of the reaction has a pronounced effect on the duration of RNA synthesis. The reaction continued at a near-linear rate for a longer time and the total amount of labelled UTP incorporated into acid-precipitable RNA was greater at low temperatures (25°C) than at high temperatures (30°C or 37°C). Similar results have been reported for the isolated nuclei from mouse myeloma cells (209), Hela cells (236), and Brewers' yeast (210, 237). Marzluff et al. (209), Dakloet and Belts (233) have reported that more RNA was made with a
higher molecular weight when the nuclei were incubated at a low temperature than at a higher temperature.

This may be the case for *Tetrahymena pyriformis* nuclei as well. However, only analysis of the RNA transcripts will directly confirm this idea. Several factors may be responsible for the decrease in the activity of nuclear RNA polymerase with time at high temperatures. For HeLa cell nuclei, Busiello and DiGirolamo (238) have observed that the decreased rate of RNA synthesis with time was due to both the destruction of the ribonucleoside triphosphates in the nuclei and an inactivation of the nuclear polymerising activities. Preliminary studies by DeKloet and Beltz (237) suggest that the RNA polymerising activities in yeast nuclei, as well, are inactivated with time. This would seem to be the case for *Tetrahymena pyriformis* nuclei as well. However, the presence of ribonuclease (a ribonucleic acid degrading enzyme) which is active at high temperatures (30°C - 37°C) in *Tetrahymena pyriformis* nuclei might account for an apparent decrease in nuclear RNA polymerase activity due to destruction of the transcript.

Ionic strength influences the relative activities of the endogenous RNA polymerases in isolated *Tetrahymena pyriformis* nuclei. At high ionic strength (0.15 M KCl) much of the RNA synthesis is probably transcribed by RNA polymerase II (which is responsible for synthesis of heterogeneous messenger RNA). Approximately 60% of the total RNA polymerase activity in nuclei was inhibited by $3.4 \times 10^{-4}$ M α-amanitin (a specific inhibitor of RNA polymerase II). At low ionic strength, the majority of RNA synthesis is probably transcribed by RNA polymerase I (which is responsible for synthesis of ribosomal RNA). In this case only 13% of radioactivity incorporated into RNA was inhibited by $3.4 \times 10^{-4}$ M α-amanitin. Similar results were obtained with isolated hen oviduct nuclei (239) using similar RNA polymerase assay conditions.
However, in the latter case, approximately 100-fold less α-amanitin (2.7 x 10^{-6} M) was needed to bring about 60% inhibition of nuclear RNA polymerase activity. The existence of multiple classes of eukaryotic RNA polymerases with distinct transcriptional functions suggests that the relative levels of the different RNA polymerases may in part determine the patterns of cellular gene expression.

Cyclic nucleotides, cyclic AMP and cyclic GMP, have been implicated in the control of cell division. Generally it is now well established that cyclic AMP acts as inhibitor of cell division and cyclic GMP acts as promoter of cell division. Berridge has postulated that calcium is the primary division signal in all cells. An increase in the intracellular calcium level is viewed as being the primary stimulus to divide and cyclic AMP exerts either positive or negative feedback control on the intracellular calcium level. Figure (28) shows the proposed role of calcium and cyclic nucleotides in the control of cell division put forward by Berridge (186). As can be seen from the Figure, the major intracellular signal regulating division is calcium which usually enters the cytoplasm from the outside, but it can also be released from intracellular reservoirs such as mitochondria. Cyclic AMP may modulate this signal. In Figure (28.A.), cyclic AMP augments the calcium signal by stimulating the release of internal calcium. In Figure (28.B.) cyclic AMP opposes the calcium signal by stimulating its removal from the cytoplasm or by inhibiting the uptake of external calcium. In both cases, a high level of calcium may stimulate guanyl cyclase (GC) to increase the level of cyclic GMP which may function together with calcium to initiate the cell division programme.

Hadden and co-workers (167) were the first to implicate cyclic GMP in cellular growth control. They found that stimulation of lymphocytes
Figure (28) The Proposed Role of Calcium and Cyclic Nucleotides in the Control of Cell Division

\[\text{Berridge (186)}\]
by the mitogenic agents, concanavalin A and phytohemagglutinin produced up to a 50-fold increase in intracellular cyclic GMP within 20 minutes of exposure to the mitogens. They therefore proposed the view that cyclic GMP is the active signal which induces cell proliferation. The site(s) of action of cyclic GMP which promote cell proliferation are as yet unknown. However, Pogo et al. (240) have observed some changes in the lymphocyte nuclei within minutes after stimulation of whole lymphocytes by phytohemagglutinin. These changes include an increase in histone acetylation, phosphorylation of nuclear proteins and increased nuclear RNA synthesis. Cyclic GMP in the presence of Ca++ was found in the present work to enhance nuclear DNA transcription by means of an increase in endogenous RNA polymerase activity. Maximum stimulation was found at $10^{-10}$M cyclic GMP in the presence of 2 mM CaCl₂. Cyclic GMP alone had no effect on nuclear RNA polymerase activity. This observation seems to point to the importance of Ca++ in the control of transcription. However, 2 mM CaCl₂ alone had no effect on RNA polymerase activity. It appears that cyclic GMP must function together with Ca++ to control transcription and possibly to initiate the cell division programme at the transcriptional level. It is known that the transcription of DNA into RNA by RNA polymerases is one of the earliest events following the stimulation of cellular proliferation by mitogenic signals.

Johnson and Hadden (169) showed that cyclic GMP in the presence of Ca++ stimulated RNA polymerase I activity in lymphocyte nuclei isolated from both non-stimulated and phytohemagglutinin stimulated lymphocytes. Such stimulation of ribosomal RNA polymerase activity may be required for rapid production ribosomal RNA which is necessary for initiation of cell division.
Cyclic AMP and dibutyryl cyclic AMP have also been observed to stimulate nuclear RNA polymerase activity at physiological levels ($10^{-6}$ M - $10^{-7}$ M). Maximum stimulation was induced by $10^{-7}$ M cyclic AMP or $10^{-7}$ M dibutyryl cyclic AMP. The rate of nucleotide incorporation into acid-precipitable RNA was doubled. Further studies, however, showed that only the activity of RNA polymerase I was stimulated by this cyclic nucleotide. This was achieved by using α-amanitin to differentiate between enzyme I and II activities in a nuclear preparation. Analysis of the data showed that RNA polymerase II actually was inhibited by cyclic nucleotide. This observation may be consistent with the inhibitory effect of cyclic AMP on cell division, since the synthesis of messenger RNA (which is synthesised by RNA polymerase II) is inhibited.

Roeder (241) showed that cyclic AMP was able to stimulate RNA synthesis in rat liver nucleoli, the cellular location of RNA polymerase I. Hirsch and Martelo (163) also showed that cyclic AMP was able to stimulate RNA polymerase I in whole rat liver nuclei incubated under conditions which favoured the measurement of RNA polymerase I. However, these authors showed that cyclic AMP had no effect on RNA polymerase II in whole rat liver nuclear preparation. The selective stimulation of ribosomal RNA synthesis by cyclic AMP in intact nuclei can also be detected by analysis of the RNA transcripts. Roeder and Roeder (106) have shown that 45S, 28S and 18S ribosomal RNA's were the products of transcription by RNA polymerase I.

Cyclic AMP was also able to stimulate chromatin-bound RNA polymerase activity suggesting the site of action of this cyclic nucleotide probably is directly on the chromatin.

It appears likely, on the basis of several recent studies, that certain of the effects of cyclic AMP upon growth and differentiation of
Eukaryotic cells are due to the ability of this cyclic nucleotide to influence transcription. In lymphoblasts, MacManus and Whitfield (243) have reported that $10^{-8}$ M - $10^{-6}$ M cyclic AMP or dibutyryl cyclic AMP were able to stimulate proliferation of thymic lymphocytes in culture. However, concentrations of cyclic AMP above $10^{-6}$ M were found by these authors to inhibit cell proliferation. It was also found that agents which produced an elevation of intracellular cyclic AMP such as epinephrine or prostaglandin E, caused initiation of DNA synthesis and cell division (243, 244, 245). It seems likely that cyclic AMP in this case promotes DNA synthesis and mitosis in a subpopulation of already activated lymphoblasts (246), since DNA synthesis as measured by $[^3H]$-thymidine incorporation occurred within an hour after cyclic AMP treatment and cell division began within three to four hours. It has also been suggested by Willingham et al. (247) that an increase in intracellular cyclic AMP in late G1 of the cell cycle might be required to initiate DNA synthesis.

The increase in nuclear RNA polymerase activity by cyclic AMP and cyclic GMP and Ca++ may be due to an increase in initiation sites for RNA synthesis along the DNA in the chromatin. Another possibility for the site of action of the cyclic nucleotides might be the stabilisation of RNA chains which have already been initiated in vivo. Thus the cyclic nucleotides might inhibit the rapid turnover of RNA chains in the nucleus. There are as yet no definitive experiments to distinguish between these hypotheses.

The finding that a cyclic AMP-dependent protein kinase is associated with the chromatin (158) seems to suggest a possible mechanism by which cyclic nucleotides can regulate the DNA transcription i.e. by the phosphorylation of nuclear proteins at specific sites in the chromatin,
possibly even the direct phosphorylation of RNA polymerase.

A cyclic AMP-dependent protein kinase, but not a cyclic GMP-dependent protein kinase, has been detected in *Tetrahymena pyriformis* nuclei. Addition of cyclic AMP at a physiological concentration (5 x 10^{-7} M) to nuclei caused a stimulation of endogenous protein phosphorylation (the rate of $[^{32}P]$ incorporation into acid-precipitable proteins) by approximately 23%. This stimulation only occurred when 0.1 M NaCl was included in the reaction mixture. Recent studies employing labelled mammalian DNA indicate that high concentrations of NaCl decreased the binding of certain non-histone chromatin proteins to DNA while enhancing the binding of a few other specific proteins to DNA (248). Possibly, the binding of cyclic AMP to the cyclic AMP-dependent protein kinase associated with the chromatin requires under in vitro conditions the changes in chromatin structure induced by high salt concentration before protein phosphorylation can occur. Cyclic AMP usually binds to the regulatory sites of cyclic AMP-dependent protein kinases leaving the activated catalytic sites free to phosphorylate other chromatin-associated proteins. By this mechanism the direct phosphorylation of chromatin bound RNA polymerase molecules might also occur.

The cyclic AMP induced stimulation of phosphorylation of nuclear proteins in intact rat liver nuclei has been reported by Johnson and Allfrey (141) and Castagna et al. (144).

The only previous work on cyclic AMP protein kinases in *Tetrahymena pyriformis* was done by Majumder et al. (192). However, in their studies they concentrated on the properties of the cytoplasmic enzymes and not the nuclear chromatin associated protein kinases.

It should be pointed out that a net increase of phosphorylation of
nuclear proteins in whole nuclei could either have been due to activation of cyclic AMP-dependent protein kinase(s) in nuclei or to inhibition of phosphatase activity by this cyclic nucleotide or to both.

Interestingly, addition of theophylline to the protein kinase reaction mixture was found to inhibit the rate of $^{32}P$ incorporated into Tetrahymena pyriformis nuclei. This compound had no effect on the rate of RNA synthesis by isolated nuclei. Methylxanthines, caffeine and theophylline, besides being very well known inhibitors of cyclic nucleotide phosphodiesterases, have recently been shown to inhibit postreplication repair of DNA in mammalian cells by direct binding to DNA (235). Another group of research workers have also shown that human cell cultures, treated with caffeine, show chromosomal abnormalities, including dicentrics and breaks and gaps in the chromosome structure (236). It is possible that these compound by binding to the DNA may inhibit or inactivate the protein kinase(s) bound to the chromatin. The reason why they do not also inhibit chromatin bound RNA polymerase is not clear (5 - 10 mM theophylline had no effect on nuclear transcription) (data not presented in the thesis).

It has recently been recommended that caffeine present in soft drinks, tea, coffee and some drug preparations should be reduced to minimise a possible hazard to the general population.

The detection of cyclic AMP-dependent protein kinase in a partially purified RNA polymerase fraction is of particular interest. Cyclic AMP stimulated the phosphorylation of endogenous and exogenous substrates in the partially purified RNA polymerase fraction. Casein was found to be a better substrate than phosvitin and histone (F1) for phosphorylation. However, the protein kinase associated with this fraction showed a wide substrate specificity, phosphorylating not only the acidic proteins...
casein and phosvitin, but also the basic protein histone (F₁). This is in agreement with the observations reported by Rubin and Rosen (134) about the non-specificity of almost all protein kinases in vitro and sheds no light on how specificity of phosphorylation is regulated in the cell nucleus. However, Kish and Kleinsmith (138) report the presence of twelve nuclear protein kinases from bovine liver. Some of these kinases were stimulated by cyclic AMP, others were unaffected by this cyclic nucleotide, and some were even inhibited by cyclic AMP. These observations show how complex the regulation of nuclear protein kinases must be. The picture is still more complicated by the finding that a cytoplasmic protein kinase can be taken up by nuclei in the presence of cyclic AMP binding protein and the complex can phosphorylate several non-histone proteins. The phosphorylation of non-histone proteins is a major mechanism for the regulation of gene activity (146).

Although the direct phosphorylation of RNA polymerase subunits was not investigated in the present work, phosphorylation of the RNA polymerase subunits has been reported by other research workers (163, 164, 165, 166). Phosphorylation of rat liver RNA polymerase I subunits has been shown to occur both in vivo and in vitro experiments (163). The presence of phosphorylated subunits in yeast RNA polymerase I, II and III has also been reported from in vivo experiments (164, 165, 166). Rutter and co-workers (156) have reported the phosphorylation of purified rat liver RNA polymerase II by a cyclic AMP-dependent protein kinase from both rat liver and rabbit muscle. Jungmann et al. (157) have reported the stimulation of ovarian RNA polymerase II by a partially purified cyclic AMP-dependent protein kinase from calf ovary cytosol. The activity of polymerase II was increased nine-fold whereas the activity of polymerase Ia and Ib was stimulated about three-fold. The phosphoryl-
ation of purified E. coli RNA polymerase with beef skeletal protein kinase has also been reported by Martelo et al. (151, 152). In vivo phosphorylation of E. coli RNA polymerase has also been demonstrated in bacteriophage T7 infected cells (249). The β subunit and to a lesser extent the β subunit of E. coli RNA polymerase are phosphorylated by a phage coded protein kinase. The fact that mutants that lack early transcriptional control were also deficient in protein kinase suggests the phosphorylation of RNA polymerase may be the mechanism by which early transcription is regulated.

From the above review, it seems likely that the activity of RNA polymerase molecules may in part be regulated by a protein kinase.

A demonstration of enzyme phosphorylation, however, is not sufficient evidence to establish a causal relationship between protein phosphorylation and the stimulation of polymerase activity. To establish such a relationship would also require a demonstration that the selective inhibition of protein kinase inhibits polymerase stimulation and that the extent of stimulation is dependent on the extent of enzyme phosphorylation. A careful analysis of the extent of the in vivo phosphorylation of RNA polymerase molecules is also required for a detailed theory of control to be formulated.

If RNA polymerase is subject to control by phosphorylation, it must also be dephosphorylated by a phosphatase under certain conditions. By modulating the charge density at a specific site in a polypeptide such as RNA polymerase subunits, phosphorylation and dephosphorylation could control the extent of interaction between enzyme subunits or between the whole enzyme and other molecules. In this way, phosphorylation of RNA polymerase could have a direct effect on transcription by altering the specific activity of the enzyme. It could also have an indirect effect on
activity by modulating the association of polymerase with regulatory molecules or the chromatin. Alternatively, phosphorylation may alter the turnover of the enzyme.

Detection of other important enzymes, cyclic GMP phosphodiesterase and cyclic AMP phosphodiesterase associated with *Tetrahymena pyriformis* chromatin is reported in this thesis. These cyclic nucleotides degrading enzymes in the *Tetrahymena pyriformis* cell nucleus may play an integral role in regulating the effects of cyclic nucleotides on the DNA transcription. It is now clear that both cyclic AMP and cyclic GMP and Ca**+** have a positive effect on total transcription. Specific cyclic nucleotide dependent protein kinases could possibly bring about the differential phosphorylation of nuclear proteins and thereby control transcription. If cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase are integral components of the chromatin in *Tetrahymena pyriformis* nuclei, modulation of cyclic nucleotide concentrations in the environment of the chromatin could also be regulated by these enzymes.

Fluctuation of RNA polymerase I and II activities during the natural cell cycle of *Tetrahymena pyriformis* was observed. Total endogenous RNA polymerase activity was maximum at the end of S phase. The activity of the enzyme declined rapidly during the G2 phase of the cell cycle. Two-thirds of the total RNA polymerase activity observed in S phase was due to RNA polymerase II. This was detected by means of the inhibition of this enzyme by α-amanitin, the specific inhibitor of RNA polymerase II. In the G2 phase of the cell cycle the RNA polymerase I activity was the predominant one. Independent regulation of these enzymes during the cell cycle might account for the differential
synthesis of different classes of RNAs necessary for the cell division processes.

A ten-fold variation of the total endogenous RNA polymerase activity during the cell cycle does not, however, correlate well with the RNA synthesis in a cell during the cell cycle because RNA synthesis is expected to be double at the initiation of the cell division programme (224). The experiments performed in the present work were only of a preliminary type utilising the endogenous RNA polymerase and DNA for measuring the RNA polymerase activity. Many factors might account for the observed variation in enzyme activity during the cell cycle. These factors are enumerated below:

1. Using the endogenous DNA template, the levels of activity measured presumably reflect the number of RNA polymerase molecules actually engaged in RNA synthesis when the cells were disrupted. A change in the ability of the chromatin DNA to function as a template for the RNA polymerase during the cell cycle might be a regulatory factor in transcription. This could be due to the changes in the interaction between DNA and chromatin proteins (histones are known as repressors of DNA transcription which mask the DNA and prevent its transcription by RNA polymerase(s) thereby altering in the number of available promoter sites on the DNA throughout the cell cycle.

2. Control of transcription can also occur through variation during the cell cycle in the number of enzymes molecules which are responsible for transcription of particular genes on the DNA genome.

3. Alteration in the activity of the enzymes might have already occurred at the time of cell disruption because of some changes, for example, in cellular cyclic AMP, cyclic GMP, Ca++ or other allosteric or covalent modifier of activity.
The above factors may all or in part function together to generate fluctuations in enzyme activity. Further questions which would help to resolve the precise mechanism of control of transcription during the cell cycle are:

1) what are the levels of extractable RNA polymerase(s) during the cell cycle?
2) how do these relate to the actual rate of RNA synthesis in intact cells?
3) how does the activity compare with the kind of activity measured in the present work? (RNA polymerase activity with endogenous DNA was measured in crude homogenates throughout the cell cycle.)

It is clear from the literature and the present work, how complex the control of transcriptional activity in a cell nucleus can be. Many factors appear to work together to control the activity of the DNA transcription. However, it has been established that cyclic nucleotides, cyclic AMP and cyclic GMP and the enzymes which integrate with their function (cyclic nucleotides dependent protein kinases and cyclic nucleotides phosphodiesterases) appear to play a major role in regulating the gene activity in a cell nucleus.
REFERENCES


