

A Thesis Submitted for the Degree of PhD at the University of Warwick

Permanent WRAP URL:

<http://wrap.warwick.ac.uk/137488>

Copyright and reuse:

This thesis is made available online and is protected by original copyright.

Please scroll down to view the document itself.

Please refer to the repository record for this item for information to help you to cite it.

Our policy information is available from the repository home page.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk

THE CONTROL OF RNA SYNTHESIS IN VITRO

by

JULIAN M. CRAMPTON

Submitted for the degree of Doctor of Philosophy

University of Warwick
Department of Biological Sciences
November, 1978

SUMMARY

Conditions for the isolation and incubation of Xenopus cultured cell nuclei were optimised for maximal synthesis of RNA. The Xenopus nuclei showed all three RNA polymerase activities and appeared to process rRNA to 18S and 28S species, and possibly to initiate new RNA chains.

The rate of RNA synthesis in isolated XTC-2 nuclei was changed when they were incubated with Xenopus oocyte and egg cell extracts in an optimised assay system (which included a preincubation step), the oocyte being stimulatory, and the egg inhibitory. Cell extracts from other developmental stages assayed in the same way indicated that extracts of early cleavage stages were also inhibitory, whilst stages 7½ to 13 became increasingly stimulatory. Some possible trivial causes of these effects were eliminated.

Four main stimulatory factors were isolated from mature oocytes and were purified to varying degrees by gel filtration and ion exchange chromatography. Three were found to be relatively non-specific in their action; the fourth specifically stimulated RNA polymerase I activity and the synthesis of rRNA. The activity of this factor was found to vary during oogenesis and early development (though possibly because of poor recovery), correlating reasonably with the known rates of rRNA synthesis in vivo. The factors were not species specific.

A fifth minor stimulatory factor was found mainly to affect RNA polymerase II activity. The factor's activity changed little between extracts from different developmental stages. However, this was the only purified factor which also stimulated RNA synthesis in isolated Xenopus blood nuclei.

The factors stimulated elongation and possibly initiation also. All four triphosphates were required for factor stimulation to occur. Activity of the rRNA-specific factor was dependent on continued polymerase II activity.

The factors stimulated RNA synthesis when assayed with Xenopus chromatin and added homologous polymerases. They were ineffective, however, when assayed with homologous purified DNA and RNA polymerases.

Experiments involving the factors in vivo were inconclusive; their relevance and that of the nuclear assay system is discussed.

CONTENTS

	<u>page</u>
Summary	i
Contents	ii
Index of Figures	v
Index of Tables	viii
Index of Plates	x
Acknowledgements	xi
Declaration	xi
Abbreviations	xii
<u>Chapter 1</u>	
<u>Introduction</u>	1
(a) RNA synthesis during <u>Xenopus</u> early development	2
(b) Evidence for the cytoplasmic control of RNA synthesis in <u>Xenopus</u>	5
(c) Methods of analysing cell cytoplasms in terms of their control capabilities	7
(d) <u>In vitro</u> transcription systems	8
(e) Experimental system	10
(f) Experimental approach	11
<u>Chapter 2</u>	
<u>Materials and Methods</u>	
(a) Materials	12
(b) Buffers	13
(c) Cell culture	14
(d) Preparation of <u>Xenopus</u> oocytes, eggs and embryos	15
(e) Isolation of nuclei, nucleoli and chromatin	16
(f) Preparation of cell extracts	17
(g) Extraction of nucleic acids	17
(h) Scintillation counting	18
(i) RNA polymerase preparation	19
(j) Enzyme assays	19
(k) Fractionation methods	20
(l) DNA, RNA and protein estimation	22
(m) DNA-RNA hybridization on nitrocellulose filters	23
(n) DNAase I digestion of nuclear DNA	23

Chapter 3Characterisation of conditions for isolating and incubating nuclei

(a) Introduction	24
(b) Methods of isolating nuclei	24
(c) Optimisation of RNA synthesised by isolated XTC-2 nuclei	28
(d) Characterisation of the RNA synthesising activity	31
(e) Characterisation of the RNA product	31
(f) Processing of the <u>in vitro</u> product	34
(g) Initiation of RNA synthesis in isolated XTC-2 nuclei	35
(h) Discussion	36

Chapter 4Addition of Xenopus cell extracts to isolated XTC-2 nuclei

(a) Introduction	38
(b) Preparation of cell extracts	38
(c) RNA synthesis by nuclei incubated with cell extracts	39
(d) Optimisation of the assay for regulatory factors	40
(e) Experiments to control against trivial effects of the cell extracts on isolated nuclei	43
(f) Identification of the activity in cell extracts	45
(g) Characterisation of the effects of cell extracts on isolated nuclei	47
(h) Variation in the stimulatory properties of extracts from embryos at different stages of development	49
(i) Discussion	50

Chapter 5Purification of factors stimulating RNA synthesis in isolated nuclei from Stage 5/6 oocytes. Their levels during oogenesis and early development

(a) Introduction	53
(b) Purification of stimulatory factors from Stage 5/6 oocytes	53
(c) The activity of the factors during oogenesis	58
(d) The activity of the factors during <u>Xenopus</u> ' early development	59
(e) Discussion	61

	<u>page</u>
<u>Chapter 6</u>	
<u>Specificity of the purified factors</u>	
(a) Introduction	62
(b) The activities of the different RNA polymerases in factor treated nuclei	63
(c) Electrophoretic analysis of RNA synthesised <u>in vitro</u> by factor stimulated nuclei	64
(d) Discussion	65
<u>Chapter 7</u>	
<u>Mode of action of the factors</u>	
(a) Introduction	67
(b) Possible identity of the factors	67
(c) Mode of action of the factors	69
(d) Discussion	77
<u>Chapter 8</u>	
<u>Micro-injected factors and their fate in the nuclear assay</u>	
(a) Introduction	81
(b) Recovery of injected labelled factors	81
(c) What happens to the factors in the nuclear assay?	84
(d) Discussion	85
<u>Chapter 9</u>	
<u>The effect of <u>Xenopus</u> cell extracts on RNA synthesis in isolated BHK and <u>Xenopus</u> blood nuclei</u>	
(a) Introduction	87
(b) Results with BHK nuclei	88
(c) Results with <u>Xenopus</u> blood nuclei	89
(d) Discussion	91
<u>Chapter 10</u>	
<u>Sephadex Peak 3</u>	
(a) Discussion	93
<u>Chapter 11</u>	
<u>Conclusions and Prospects</u>	
	95
References	99

Index of Figures

	<u>following page</u>
<u>Chapter 3</u>	
3.1 Salt optimisation of XTC-2 nuclei	28
3.2 Optimisation of XTC-2 nuclei for temperature, tri-phosphate concentration and pH	30
3.3 Time course of incorporation of [³ H] UTP by XTC-2 nuclei in the presence and absence of α -amanitin	31
3.4 The inhibition of [³ H] UTP incorporation by XTC-2 nuclei in the presence of varying levels of α -amanitin	31
3.5 Time course of incorporation by XTC-2 nuclei under conditions selecting for RNA polymerases I, II + III and III	31
3.6 Assay for the presence of RNAase activity in XTC-2 nuclear incubations	32
3.7 Gel fractionation of RNA products synthesised by XTC-2 nuclei <u>in vitro</u>	33
3.8 Hybridization of labelled 18S and 28S and 6-18S RNA synthesised by XTC-2 nuclei <u>in vitro</u> to total <u>Xenopus</u> DNA fractionated on a CsCl gradient	33
3.9 Migration of RNA from XTC-2 nuclei incubated <u>in vitro</u>	34
3.10 P ³² I -cellulose thin layer chromatography of hydrolysed [γ - ³² P]GTP or [γ - ³² P]ATP labelled RNA from XTC-2 nuclei	35
3.11 Time course of incorporation of [³ H] UTP by XTC-2 nuclei in the presence and absence of AF/013	36
<u>Chapter 4</u>	
4.1 Time course of incorporation of [³ H] UTP by XTC-2 nuclei in the presence of oocyte and egg extracts	39
4.2 Optimisation of preincubation time using oocyte and egg extracts	41
4.3 Optimisation of the preincubation conditions using oocyte and egg cell extracts	42
4.4 Time course of [³ H] UTP incorporation by XTC-2 nuclei after preincubation in the presence of oocyte and unfertilised egg cell extracts	42
4.5 Concentration curve of protein present in the preincubation of XTC-2 nuclei using oocyte and egg cell extracts and BSA	44
4.6 Assay of RNA synthesis in isolated XTC-2 nuclei preincubated with varying proportions of oocyte and unfertilised egg cell extracts	45

Index of Figures (continued)

	<u>following page</u>
4.7 Gel fractionation of RNA products synthesised by XTC-2 nuclei using oocyte and egg cell extracts	47
4.8 Time course of incorporation of oocyte and egg preincubated nuclei in the presence and absence of AF/013	49
4.9 RNA synthesis by XTC-2 nuclei preincubated with cell extracts from different stages of <u>Xenopus</u> early development	49
 <u>Chapter 5</u>	
5.1 Purification of factors affecting RNA synthesis in XTC-2 nuclei from mature oocytes	53
5.2 DEAE-cellulose chromatography of whole oocyte cell extract and Peaks 1 and 2 purified from oocyte cell extracts on G-100 Sephadex	54
5.3 Fraction mixing experiment on DEAE-ion exchange chromatographed, Sephadex Peak 2 material	55
5.4 Concentration curves of oocyte stimulatory factors	57
5.5 Molecular weight determination of stimulatory activities by Sephadex gel filtration and SDS gel electrophoresis	58
5.6 Nuclear assays of Stage 1 and Stage 2/3 ovary cell extracts fractionated on G-100 Sephadex	59
5.7 DEAE-cellulose chromatography of Sephadex-purified Peak 2 from Stage 1 and Stage 2/3 ovary cell extracts	59
5.8 Nuclear assays of oocyte, egg and blastula cell extracts fractionated on G-100 Sephadex	59
5.9 Nuclear assay of tailbud cell extract fractionated on G-100 Sephadex	60
5.10 DEAE-cellulose chromatography of Sephadex purified Peaks 1 and 2 from unfertilised egg and blastula cell extracts	60
 <u>Chapter 6</u>	
6.1 The α -amanitin sensitivity of RNA synthesis by nuclei preincubated with Sephadex G-100 fractions of Stage 5/6 oocyte extract	62
6.2 The α -amanitin sensitivity of RNA synthesis by nuclei preincubated with DEAE fractions of Stage 5/6 oocyte Peaks 1 and 2	63

Index of Figures (continued)

	<u>following page</u>
6.3 Gel fractionation of RNA products synthesised by XTC-2 nuclei <u>in vitro</u> after preincubation with factors IA, IB, IIA and IIB	64
6.4 Gel electrophoresis of RNA products synthesised in isolated XTC-2 nuclei <u>in vitro</u> after preincubation with and without factor IIA	64
6.5 Hybridization of labelled RNA synthesised by XTC-2 nuclei <u>in vitro</u> after preincubation with factor IIA	65
 <u>Chapter 7</u>	
7.1 G-100 fractionated oocyte cell extract assayed for RNA polymerase and protein kinase activity	68
7.2 DEAE-cellulose fractions of oocyte Sephadex Peak 1 assayed for RNA polymerase activity	68
7.3 Nuclear assays using an enucleated extract fractionated on G-100 Sephadex	68
7.4 The effect of factor IIA on transcription by isolated XTC-2 chromatin <u>in vitro</u>	70
7.5 DNAase I sensitivity of XTC-2 nuclei preincubated with cell extracts	72
7.6 The effect of Sephadex fractionated oocyte extract on transcription in isolated XTC-2 nucleoli	76
 <u>Chapter 8</u>	
8.1 Recovery of factor IIA activity added during preparation of unfertilised egg extract	81
8.2 Recovery of labelled Sephadex Peak 1 injected into oocytes, eggs and embryos	83
8.3 Recovery of labelled Sephadex Peak 2 injected into oocytes, eggs and embryos	83
8.4 Migration of labelled oocyte proteins into XTC-2 nuclei <u>in vitro</u>	85
8.5 Densitometer scans of labelled proteins present in isolated nuclei and their incubation medium after preincubation with labelled oocyte proteins	85
 <u>Chapter 9</u>	
9.1 Time course of RNA synthesis in <u>Xenopus</u> blood nuclei incubated with and without cell extracts	89
9.2 RNA synthesis in <u>Xenopus</u> blood nuclei incubated with Sephadex column fractions of oocyte extract	90

Index of Tables

	<u>following page</u>
<u>Chapter 3</u>	
3.1 Characterisation of [^3H] UMP incorporation by isolated XTC-2 nuclei	28
<u>Chapter 4</u>	
4.1 The effect of dialysis on the properties of cell extracts in the nuclear assay	44
4.2 Addition of <u>E. coli</u> and <u>Xenopus</u> RNA polymerases to extract treated nuclei	45
4.3 The effect of trypsin digestion on the stimulatory activity of an oocyte extract	46
4.4 Intracellular location of stimulatory factors and RNA polymerase activity in Stage 6 oocytes	46
4.5 Initiation assays on extract-treated nuclei	48
<u>Chapter 5</u>	
5.1 Purification of factors stimulating RNA synthesis in isolated nuclei from Stage 5/6 oocytes	56
5.2 The activity of stimulatory factors in cell extracts from different stages during <u>Xenopus</u> oogenesis and embryogenesis	59
<u>Chapter 6</u>	
6.1 The activity of each RNA polymerase form in factor treated nuclei	63
6.2 Absolute fold stimulation of individual RNA polymerase forms in Stage 5/6 oocyte factor treated nuclei	63
6.3 Absolute fold stimulation of individual RNA polymerase forms in Stage 5/6 factor treated nuclei, normalised with respect to polymerase III activity	63
<u>Chapter 7</u>	
7.1 Hybridization of [^3H] rRNA to DNAase I digested DNA, derived from factor treated nuclei	73
7.2 Free and template-engaged RNA polymerases in factor treated nuclei	73
7.3 The effect of omitting CTP during the preincubation step on the stimulatory activity of the purified factors	75
7.4 The effect of α -amanitin on factor IIA stimulation	75
7.5 Total RNA and 18S and 28S rRNA synthesis per hour by isolated XTC-2 nuclei	79

Index of tables (continued)following pageChapter 8

- 8.1 The fate of oocyte factors during their pre-
incubation with isolated nuclei 84

Chapter 9

- 9.1 The effects of cell extracts and oocyte factors on
RNA synthesis in isolated XTC-2 and BHK nuclei 89
- 9.2 The effect of cell extracts on the RNA polymerase
activities of isolated Xenopus blood nuclei 90

Index of Plates

	<u>following page</u>
<u>Chapter 3</u>	
3.1 Isolated XTC-2 nuclei	27
<u>Chapter 5</u>	
5.1 Polyacrylamide gel electrophoresis of oocyte Peak 2	57
5.2 Polyacrylamide gel electrophoresis of oocyte factor IIA	57
5.3 Polyacrylamide gel electrophoresis of oocyte factor IB	57

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. H.R. Woodland, for his guidance and interest during the course of this research.

I would also like to thank all members of the Developmental Biology Research group for their support, in particular:

Ms Elizabeth Ballantine for general support and for ensuring a smooth-running laboratory.

Dr. Alan Colman for doing the endonuclease assays (Chapter 7).

Ms. Anna Mayall for help in preparing this thesis.

Dr. H.R. Woodland for performing the microinjections described in Chapter 8 (b) and the DNA binding assays (Chapter 7).

Ms. A. Wyllie for help in some of the nuclear assays.

I am grateful to Dr. R. Avery for supplying Influenza RNA, and would also like to thank Christine Woodland for sustenance and Jacynth McKeand for her quick and accurate typing of this thesis.

Finally, I thank the Science Research Council for financial support throughout this project.

DECLARATION

I declare that this thesis has been composed by myself, has not been accepted in any previous application for a degree and that the work is my own except where specifically acknowledged.

ABBREVIATIONSNucleic acids

DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
ssDNA	single stranded DNA
rDNA	DNA containing genes for rRNA
RNA	ribonucleic acid
dRNA	RNA having a base composition similar to DNA
hnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
poly(A)	polyadenylic acid

Others

A ₂₆₀	absorbance at 260 nm
A ₂₈₀	absorbance at 280 nm
ATP	adenosine 5'-triphosphate
BHK	babyhamster kidney (cells)
BSA	bovine serum albumin
cAMP	cyclic 3':5'-adenosine monophosphate
Cl	Curie (3.7×10^{10} disintegrations per second)
CMP	5'-cytidine monophosphate
cpm	counts per minute
CTP	cytidine 5'-triphosphate
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNAase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
g	gravitational field
GMEM	Glasgow modified Eagle's minimal essential medium
GTP	guanosine 5'-triphosphate
GV	germinal vesicle

Abbreviations (continued)

LL-BSA	lysolethicin-BSA method (Gurdon, 1976)
MW	molecular weight
NMR	nuclear magnetic resonance
PAS	para-aminosalicylic acid
PBS	phosphate buffered saline
POPOP	1,4-bis-(5-phenyloxazol-2-yl) benzene
PPO	2,5-diphenyloxazole
RM	resuspension medium
RNAase	ribonuclease
rpm	revolutions per minute
S	Svedberg unit
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TCA	trichloroacetic acid
TNS	tri-iso-propylnaphthalenesulphonic acid
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	octyl phenoxy polyethoxy ethanol
UTP	uridine 5'-triphosphate
XTC	<u>Xenopus</u> tissue culture cells

Chapter 1

Introduction

One of the most stimulating problems in biology concerns the way in which the processes of development and differentiation in higher organisms are regulated. This must involve the control of gene expression at some level, and it is thought that control of transcription of the genome may be principally involved (for example see Gurdon, 1974).

Much of the recent speculation about the control of gene transcription in eukaryotes has been influenced by the elegant studies with bacteria, especially the pioneering work on the lac operon by Jacob and Monod (1961). The application of conclusions based on prokaryotes to eukaryote systems is, however, only possible with reservations. Jacob and Monod were the first to stress that conditions for RNA synthesis in bacteria are very different from those in a eukaryotic cell, although the principles may be the same. These differences and the increased complexity of control systems in eukaryotes place demands on the eukaryote system which are not present in the prokaryote.

The main stumbling blocks in studying transcriptional control in eukaryotes are the complexity of the problem and (in the absence of control mutants) finding a system amenable to investigation. Perhaps the best situation in which to study the control of any natural process is circumstances in which this process changes as abruptly as possible. As far as transcription is concerned, two main types of system seem to fulfil this requirement.

One is the transition of progenitor cells to a particular pathway of terminal differentiation. Examples include erythropoiesis (Attardi et al., 1976), myogenesis (Paterson et al., 1974) and the differentiation of the chick oviduct (O'Malley et al., 1977). These systems have the advantages that the agent inducing the transition is often known (for example, it may be a hormone), the pathway of differentiation can be well defined, and the synthesis of only one or a few proteins may be studied.

Advantages such as these have enabled a thorough investigation of some of the control processes and specific functions involved. Particularly interesting results have been obtained concerning the process of hormone

induction in the chick oviduct (e.g. Harris et al., 1975) and the sequences of specific regulable genes, their primary transcripts and mRNA's, (e.g. Jeffreys and Flavell, 1977; Tilghman et al., 1977).

The other type of system which has been widely investigated, where abrupt changes in transcription occur, is the early stages of animal development. Again, a defined sequence of events can be studied, but such a system has the disadvantage that the transcription of large populations of genes are involved in these changes. Investigation of transcription during the early development of amphibians, especially Xenopus laevis, overcomes this disadvantage to some extent. In this case, dramatic quantitative changes occur in the synthesis of all classes of RNA. It is useful to describe these changes in more detail.

(a) RNA synthesis during Xenopus early development

Several different classes of RNA are synthesised during oogenesis and embryonic development, and these are briefly discussed.

18S and 28S ribosomal RNA

Ribosomal RNA (which is synthesised by RNA polymerase I) is the major species of RNA synthesised during oogenesis (over 90% of the total RNA synthesised in Stages 3-6, (Brown and Littna, 1964; Colman, 1974). This provides the mature oocyte with the $\sim 10^{12}$ ribosomes it requires for the early stages of development. To enable this requirement to be fulfilled in a reasonable time, amplification of the rDNA occurs at about metamorphosis (at pachytene in oocyte meiosis) (Bird and Birnstiel, 1971). It results in a 4,000-fold increase over the haploid rDNA content of 450 genes, the amplified DNA being extrachromosomal and dispersed in about 1500 nucleoli. Although nucleoli are present before amplification, it is only by the beginning of vitellogenesis that the full complement of extrachromosomal nucleoli is reached (MacGregor, 1972). This suggests that there is a gradual recruitment and transcription of the amplified rDNA and this is supported by the work of Ford (1972). He found a 10-fold increase in the rate of rRNA synthesis (from an accumulation rate of 0.7 ng/oocyte/hour to 6.7 ng/oocyte/hour) at about the onset of vitellogenesis.

More recent work by Lamarca et al. (1973) has provided estimates of the absolute rate of rRNA synthesis after gene amplification. The results

indicate that Stage 4 oocytes synthesise RNA at 0.73-1.39 ng/oocyte/hour and the Stage 6 oocytes at 1.1-1.69 ng/oocyte/hour. These rates suggest that fully grown (Stage 6) oocytes continue to synthesise rRNA at an appreciable rate.

Synthesis of rRNA is greatly reduced during the conversion of the oocyte to an egg, which takes place by hormonally induced maturation, and is undetectable during the early stages after fertilisation (reviewed by Gurdon, 1968). The appearance of nucleoli, usually correlated with the onset of rRNA synthesis, occurs in the late blastula stage and rRNA may first be recognised unequivocally at the early gastrula stage (Brown and Littna, 1964). However, there is some controversy as to whether this represents a switch from inactive to active rRNA synthesis or merely a problem of detecting small amounts of rRNA synthesis occurring during cleavage stages. Recently, Nakahashi and Yamana (1976), by estimating the amount of rRNA synthesised per nucleus at different stages of development, have argued that the rRNA synthesis observed at the late blastula stages represents a true activation of ribosomal gene transcription. However, the interpretation of their results may be open to criticism.

Messenger RNA

It seems clear that protein synthesis during the early stages of Xenopus development is largely dependent on mRNA which is synthesised (by RNA polymerase II) and stored during oogenesis (Ford, 1972). The majority of this stored mRNA is synthesised during the early diplotene stages of oogenesis when the lampbrush chromosomes are fully developed (Davidson, 1976). Synthesis appears to continue, however, after this stage of maximum genome activity.

Study of the synthesis of mRNA during the early stages of development is complicated by the very large store of maternal mRNA. However, it seems that synthesis of mRNA continues during ovulation (Brown and Littna, 1964). The total mRNA content (defined loosely as dRNA) increases during cleavage relative to the DNA content of the embryo and then remains as a constant proportion of the DNA content between gastrula and early swimming stages (Brown and Littna, 1966a).

5S RNA

5S, 18S and 28SrRNA are present in equimolar amounts in ribosomes and work by Brown and Littna (1966b) suggested that they may be coordinately synthesised during oogenesis and the early development of Xenopus. This, despite the fact that the 18S/28S genes and the 5S genes are present in very different numbers (450 compared to 24,000 genes/haploid genome respectively). In fact it is now clear that the 5S genes are transcribed by a separate RNA polymerase (polymerase III) and that synthesis of 5S RNA is not coordinated with 18S and 28S rRNA synthesis during early oogenesis, 5S being synthesised in great excess (Ford, 1971). About half of the Stage 6 complement of 5S RNA is accumulated by previtellogenic oocytes.

5S RNA synthesis is first detected again after fertilization at the blastula stage (Miller, 1974) which is before the synthesis of 18S/28S RNA becomes clearly detectable and again indicates non-coordinate synthesis. An interesting point about 5S RNA is that the 5S genes expressed in oocytes are inactive in somatic cells. (Ford and Southern, 1973). The mechanism controlling this differential expression of the 5S genes is unknown.

Transfer RNA

Investigation of the accumulation of tRNA during oogenesis shows that it parallels 5S RNA synthesis (Brown and Littna, 1966b). This may reflect the fact that both species of RNA are synthesised by RNA polymerase III (Weinmann and Roeder, 1974). About half the tRNA required by the Stage 6 oocyte is synthesised in the previtellogenic period and at this time represents about 45% of the total oocyte RNA (Brown and Littna, 1966b).

After fertilisation, tRNA synthesis is first detected during late cleavage stages. Its rate of synthesis then increases until the late neurula stage, after which time there is a reduction to a constant rate of synthesis by the hatching stage (Brown and Littna, 1966b).

This brief review of RNA synthesis during Xenopus early development indicates that the major species of RNA are synthesised at independent rates. The implication is that the synthesis of each class is under some form of independent control. A similar conclusion may be arrived at by the study of other animal species including the sea urchin (Emerson and Humpreys, 1971) and mice (Woodland and Graham, 1964; Knowland and Graham, 1972). However, the stages of development at which particular RNA species are synthesised does appear to vary considerably between species.

What is the nature of the mechanisms controlling the synthesis of RNA in Xenopus early development?

(b) Evidence for the cytoplasmic control of RNA synthesis in Xenopus

The changes in gene activity outlined above, occurring in cell types derived from each other in a period of a few hours, presents a favourable situation for studying the overall regulation of transcription. An obvious mechanism for producing such variation in the transcription of the different species of RNA was that there were changes in the amounts of the various RNA polymerases activities present at different developmental stages. This possibility has been ruled out by measuring the different polymerase activities at stages during oogenesis and embryonic development (Roeder, 1974).

An initial analysis of the regulatory processes has been made by Gurdon and his associates, using nuclear transplantation (Gurdon, 1974). Early experiments involved the injection of adult frog brain nuclei (which synthesise small amounts of RNA, and DNA occasionally) into oocytes and unfertilised eggs (Gurdon, 1968). These two stages are in many ways similar, but differ fundamentally in their rates of nucleic acid synthesis, the oocyte synthesises RNA but no DNA, and the unfertilised egg synthesises no RNA but does synthesise DNA after activation. The brain nuclei rapidly conformed to the synthetic activity characteristic of their new cytoplasmic environment. This result indicates that the oocyte and unfertilised egg differ in terms of some cytoplasmic component which influences nuclear activity.

A later series of experiments involved the transplantation of single neurula nuclei into enucleated eggs. The nuclei before transplantation were synthesising rRNA, 4S RNA and HnRNA. After transplantation,

the pattern of RNA synthesis in the embryo was indistinguishable from that of normal embryos (Gurdon and Woodland, 1969). This confirms the presence of cytoplasmic factors influencing RNA synthesis during the early stages of Xenopus development. It is probably also true in the case of oocyte cytoplasm and the genes coding for oocyte-specific proteins, as judged by the analysis of the proteins for which they code (De Robertis et al., 1977). Some of the factors also seem to be species specific, e.g. in the situation where embryos are developing (Woodland and Gurdon, 1969), but some are not, e.g. De Robertis et al., (1977).

There have been some reports of the isolation of factors from mature oocytes, unfertilised eggs, cleavage and blastula stage embryos, which inhibit rRNA synthesis in neurula cells (Shiokawa and Yamana, 1967; Crippa, 1970). These are described in more detail in Chapter 6. One of the most recent reports (Laskey et al., 1973) has confirmed Shiokawa and Yamana's (1967) report that an acid soluble component of Xenopus blastulae (but not neurulae) will reduce rRNA synthesis relative to 4S RNA synthesis in dissociated neurula cells by a factor of 10. The nature of such regulatory molecules is, however, not yet known. There is ample evidence for the migration and accumulation ~~within~~ nuclei of proteins synthesised by maturing oocytes and early cleavage stage embryos (Merriam, 1969; Ecker and Smith, 1971). It may be that these proteins act as specific regulators of gene activity. However, there is no evidence that the entry of a specific protein into a nucleus has altered its transcriptional activity in a specific way.

Factors which influence nuclear activity have also been detected in cell fusion experiments between differentiated somatic cells. These heterokaryons show extinction of the differentiated functions of each parent (Thompson and Gelehrter, 1971). Presumably nuclear activity was involved in this extinction, so signals must pass through the cytoplasm. Similarly, there are factors which may reactivate the rRNA genes of an erythrocyte as well as some coding for particular proteins (Harris, 1970).

(c) Methods of analysing cell cytoplasm in terms of their control capabilities

It seems that alterations in nuclear activity during early amphibian development are brought about by changes in the cytoplasm. The next stage in analysing these control processes is clearly to find in what relevant way the cytoplasm changes. It might be possible to do this by injecting substances into oocytes and eggs, but we have no clue in advance as to the identity of such substances. Another approach might be to attempt the purification of the molecules controlling the transcription of specific gene sequences by their possible DNA-binding properties (in an analogous way to the lac repressor, Gilbert and Muller-Hill, 1970). Drawbacks may be high backgrounds from non-specific DNA-binding proteins and the lack of evidence that regulatory molecules bind directly to DNA.

One alternative is to construct a cell-free system in which cytoplasm may be added to an RNA transcription system in the hope of reproducing in vitro the changing events observed in vivo. An obvious advantage of this type of approach is that it is easily applicable to the purification of active agents, if any can be found. One could, for example, analyse the effects on transcription of extracts of oocytes and unfertilised eggs (which differ fundamentally in terms of their transcriptional activity and their ability to induce RNA synthesis in injected nuclei, and which may be obtained in the large quantities required for biochemical analysis).

The problem is to choose a suitable transcription system to assay these cytoplasm. Clearly, such a system should fulfil certain criteria if it is to respond to a cytoplasm as observed in vivo. Some of these criteria are listed below.

- (i) As far as possible, the system should be an homologous one (i.e. Xenopus derived in the case of Xenopus oocyte and egg extracts) because of the known species specificity of some of the control mechanisms.
- (ii) The system would be capable of prolonged synthesis of RNA in a defined environment. All three polymerase types should be active and Xenopus derived (non-homologous polymerases, such as E. coli, may initiate incorrectly, Wilson *et al.*, 1975). This would allow the control of all classes of RNA to be studied.

(iii) Initiation of new RNA chains should occur in vitro. This is clearly an essential if a particular cytoplasm is to alter gene expression qualitatively as well as quantitatively.

(iv) The system should transcribe faithfully, i.e. give strand selective transcription with correct initiation and termination.

(v) The system should be as free of contaminating cytoplasm as possible; an obvious requirement if added cytoplasm are to be analysed by this method.

(vi) Components should preferably be storable. This facilitates assays and reduces material waste.

At present there is no defined system which fulfils all the above criteria. What are the available in vitro transcription systems and how well does each fulfil the criteria outlined above ?

(d) In vitro transcription systems

(i) Purified DNA and RNA polymerases

Transcription systems consisting of a pure DNA template and RNA polymerase incubated in a defined medium have been used successfully in the analysis of bacterial transcription (Creplin et al., 1975) and in the purification of some factors directly affecting the activity of the RNA polymerases themselves (Biswas et al., 1975). Although high rates of RNA synthesis can occur, and initiation of new RNA chains must occur in this system, there is no evidence that initiation is at the correct in vivo sites. Indeed the template is usually nicked and degraded during isolation and purification. One exception may be nucleoids (protein depleted nuclei) (Colman and Cook, 1977) which contain superhelical DNA. Again there is no indication of correct initiation occurring with added polymerases, but these may be useful in reconstitution studies.

(ii) Isolated chromatin

Isolated chromatin has been used with added E. coli polymerase to analyse the involvement of chromosomal proteins in the control of gene expression (Gilmour and Paul, 1973). Work such as this has been criticised on the basis that the polymerases do not appear to use the correct initiation sites (Honjo and Reeder, 1974; Wilson et al., 1975).

Isolated chromatin without added polymerase has also been used as a transcription system. There is a controversy about the integrity of the transcription occurring in such chromatin (Marzluff and Huang, 1975; Konkel and Ingram, 1978), possibly as a result of the different preparation procedures (De Pomerai *et al.*, 1974). One drawback of using chromatin is that it has to be used for transcription immediately after preparation. An advantage may be that it allows dissection and reconstitution of the transcription system.

(III) Isolated nuclei and nucleoli

One form of crude nuclear preparation is the so-called 'nuclear monolayer'. These are prepared by lysing cells with detergent while they are still adhering to a support (Tsai and Green, 1973). They synthesise RNA efficiently but suffer from considerable cytoplasmic contamination and inconvenience in handling, and thus have not been used extensively (Bombik and Baserga, 1974).

Isolated nuclei have been used to analyse many aspects of transcription and its control, including the transcription of viral genes (Weber *et al.*, 1977) and the mechanism of hormone action inside its target cells (O'Malley and Means, 1974). They have been shown to synthesise RNA when incubated under suitable defined conditions, at close to (about 1/10th) calculated *in vivo* rates for prolonged periods, and to give faithful transcription of various genes *in vitro* (Smith and Huang, 1976; Reeder and Roeder, 1972; Orkin, 1978; Detke *et al.*, 1978). There is increasing evidence that initiation of RNA chains occurs in isolated nuclei (Bustello and Di Girolamo, 1975; Gilboa *et al.*, 1977), and that various aspects of RNA processing such as methylation (Rickwood and Klemperer, 1971), 5'-capping (Wei and Moss, 1977), polyadenylation (De Pomerai and Butterworth, 1975) and RNA transport (McNamara *et al.*, 1975) also occurs. Isolated nuclei may be stored in a simple medium for prolonged periods without apparent loss of activity, as reported by most of the above authors.

Finally, isolated nucleoli have been used very effectively to study the synthesis and especially the processing of rRNA (Grummt and Lindigkeit, 1973; Grummt and Grummt, 1976). However, they contain mainly RNA polymerase I and rRNA is the major product. Also, initiation does not seem to occur in isolated nucleoli (Ballal *et al.*, 1978) and their preparation (by sonication of isolated nuclei) degrades the rDNA to some extent (Beebee and Butterworth, 1977).

From this brief review of the systems available, it seems that isolated nuclei fulfil most of the criteria set out above, although the other systems may have their uses in examining some specific aspects of the transcriptional process. The fact that nuclei injected into oocytes and eggs respond in the same way as the endogenous nuclei also indicates their suitability for an *in vitro* assay system.

(e) Experimental system

The work described in this thesis was initially undertaken to investigate more thoroughly the cytoplasmic factors shown to be important in regulating gene activity during *Xenopus* development. It was decided to analyse these cytoplasmic factors, in terms of their effects on RNA synthesis, by coupling them with isolated nuclei in an *in vitro* transcription system. Somewhat similar systems have been used by Thomson and McCarthy (1968) and Hilder and Maclean (1974) to detect factors controlling RNA synthesis and by Benbow and Ford (1975) to test for cytoplasmic factors influencing DNA synthesis. All these have indicated the presence of regulatory factors, but they have not been analysed in any great detail.

The remaining problem was to decide on the source for isolated *Xenopus* nuclei. Sources available which give large numbers of more or less homogeneous cells were adult brain, blood, liver, embryos, oocytes and a permanent cell line derived from *Xenopus* tadpoles. The first two were eliminated because both cell types synthesise relatively small amounts of RNA. Nuclei actively synthesising RNA were required, initially at least, in order to detect factors inhibiting as well as stimulating RNA synthesis. Also erythrocyte nuclei retain only RNA polymerase II (Hentschel and Tata, 1978).

Oocytes and embryonic cells were not used because of difficulties of nuclear preparation and storage. The final choice of tissue culture cells as the primary source was because these were available in large numbers and their nuclei could be prepared and purified easily. It also represented a homogeneous population of relatively undifferentiated cells, actively synthesising all classes of RNA.

(f) Experimental approach

The experimental approach adopted may best be summarised by listing a series of aims. These are given below.

- (i) To establish suitable methods of isolating, purifying and incubating nuclei from Xenopus tissue culture cells and to investigate how well these nuclei fulfil the transcriptional requirements already described.
- (ii) To incubate isolated nuclei in the presence of cell extracts of Xenopus oocytes and eggs, to see if there is any change in the template activity of the nuclei. The criterion used for a faithful regulatory system is one in which transcription is stimulated when the cytoplasm is derived from oocytes, but inhibited when it is derived from unfertilised eggs. If such changes occur, to analyse them in terms of the polymerases involved and the RNA product. Finally, to assay cell extracts from other developmental stages in the same way, to see how well the in vitro assay mirrors the RNA synthesis occurring in vivo.
- (iii) To purify any regulatory molecules involved and to analyse these activities during oogenesis and embryonic development.
- (iv) To establish the specificity of any purified factors by the RNA products of the system and the RNA polymerases involved (using the toxin α -amanitin, which inhibits polymerase II at concentrations over 0.1 $\mu\text{g}/\text{ml}$, inhibits polymerase III at concentrations over 100 $\mu\text{g}/\text{ml}$ and has no inhibitory effect on polymerase I).
- (v) To analyse the mode of action of any purified factors if possible.
- (vi) To investigate whether the factors have an effect on RNA synthesis in vivo.
- (vii) To determine whether the factors exhibit any species specificity.

The results obtained following this program of investigation, using the system already outlined, are described in the following chapters.

Oocytes and embryonic cells were not used because of difficulties of nuclear preparation and storage. The final choice of tissue culture cells as the primary source was because these were available in large numbers and their nuclei could be prepared and purified easily. It also represented a homogeneous population of relatively undifferentiated cells, actively synthesising all classes of RNA.

(f) Experimental approach

The experimental approach adopted may best be summarised by listing a series of aims. These are given below.

- (i) To establish suitable methods of isolating, purifying and incubating nuclei from Xenopus tissue culture cells and to investigate how well these nuclei fulfil the transcriptional requirements already described.
- (ii) To incubate isolated nuclei in the presence of cell extracts of Xenopus oocytes and eggs, to see if there is any change in the template activity of the nuclei. The criterion used for a faithful regulatory system is one in which transcription is stimulated when the cytoplasm is derived from oocytes, but inhibited when it is derived from unfertilised eggs. If such changes occur, to analyse them in terms of the polymerases involved and the RNA product. Finally, to assay cell extracts from other developmental stages in the same way, to see how well the in vitro assay mirrors the RNA synthesis occurring in vivo.
- (iii) To purify any regulatory molecules involved and to analyse these activities during oogenesis and embryonic development.
- (iv) To establish the specificity of any purified factors by the RNA products of the system and the RNA polymerases involved (using the toxin α -amanitin, which inhibits polymerase II at concentrations over 0.1 $\mu\text{g/ml}$, inhibits polymerase III at concentrations over 100 $\mu\text{g/ml}$ and has no inhibitory effect on polymerase I).
- (v) To analyse the mode of action of any purified factors if possible.
- (vi) To investigate whether the factors have an effect on RNA synthesis in vivo.
- (vii) To determine whether the factors exhibit any species specificity.

The results obtained following this program of investigation, using the system already outlined, are described in the following chapters.

Chapter 2

(a) Materials

Animals

Mature Xenopus laevis toads were obtained from the South African Snake Farm, Fish Hoek, South Africa. Immature Xenopus laevis were laboratory reared as described by Gurdon and Woodland (1975).

Tissue Culture Cells

Xenopus XTC-2 cells, derived by Pudney et al. (1973) from tadpoles, were obtained from Dr. P. Cook. BHK-21/13 cells (Macpherson, 1963) were obtained from Flow Laboratories Ltd., Scotland. All tissue culture materials, medium etc. were also obtained from this source.

General Chemicals

[For non-standard abbreviations, see page xii].

Most chemicals were of 'Analar' grade (where commercially available). The following firms supplied the materials listed.

British Drug Houses Ltd., Dorset: p-amino-salicylic acid (PAS); sodium dodecyl sulphate (SDS).

Eastman Organic Chemicals, USA: acrylamide; NN'-methylenebisacrylamide; urea; tri-isopropyl-naphthalene sulphonic acid (TNS).

Sigma (London) Chemical Co. Ltd.: bovine serum albumin (BSA); Triton X-100; cyclic 3':5'-AMP; N, N, N'N'-tetramethylethylenediamine (TEMED); calf thymus histones; marker proteins (for gel electrophoresis and gel filtration).

Pharmacia (GB) Ltd.: Sephadex G-10, G-25, G-100.

Packard Instrument Company Inc. USA: Soluene-350; Insta-gel.

Nuclear Enterprises (G.B.) Ltd.: 2, 5-diphenyloxazole (PPO); 1, 4-bis-(5-phenyloxazole-2-yl) benzene (POPOP).

Whatman Ltd., Kent: GF/C (glass fibre discs): 3 MM and 54 filter paper; DE 52.

Fisons Ltd., UK: caesium chloride.

Enzymes

RNAase (bovine pancrease, type IIIA)

DNAase I

Sigma (London) Ltd.

Trypsin

DNAase (electrophoretically pure): Worthington Biochemical Corp., USA.

RNA polymerase: The Boehringer Corp. Ltd., UK.

Nucleotides etc.

Poly d(A-T) GTP, ATP, UTP and CTP: Boehringer Corp. Ltd., UK.

5'-CMP: Sigma (London) Ltd.

Inhibitors

Actinomycin D (supplied free:): Merck, Sharpe and Dohme, USA.

α -amanitin: Boehringer Corp. Ltd., UK.

Rifampicin AF/013: a gift from the Department of Chemistry and Molecular Sciences, University of Warwick.

Trypsin Inhibitor (soybean, type II-0): Sigma (London) Ltd.

Radiochemicals - from the Radiochemical Centre, Amersham.

[5-³H] Uridine (~ 20 Ci/mmol)

[5-³H Uridine 5'-triphosphate (20 Ci/mmol)

Adenosine 5'-[γ -³²P] triphosphate (15 Ci/mmol)

Guanosine 5'-[γ -³²P] triphosphate (15 Ci/mmol)

[³²P] orthophosphate in dilute HCl solution (40-70 Ci/mg of phosphorous).

Iodine-125 (~ 100 mCi/ml)

L-[³⁵S] methionine (200-300 Ci/mmol)

L-[4, 5-³H] Lysine monohydrochloride (40 Ci/mmol)

(b) Buffers

Barth X (modified from Barth and Barth, 1959).

NaCl	88 mM	Ca(NO ₃) ₂ ·4H ₂ O	0.33 mM
KCl	1 mM	CaCl ₂ ·6H ₂ O	0.41 mM
NaHCO ₃	2.4 mM	MgSO ₄	0.82 mM
Tris-HCl pH 7.5	15 mM	crystamycin	10 μ g/ml

Kirby Buffer

KCl	50 mM	TNS	1 %
Tris-HCl pH 7.6	10 mM	phenol/cresol	6 %
PAS	6 %		

Phenol/Cresol

phenol (redistilled)	500 g	H ₂ O	55 ml
m-cresol (redistilled)	70 ml	8-hydroxyquinoline	0.5 g

Homogenization medium

sucrose	0.3 M	Tris-HCl pH 8.0	10 mM
Mg(Ac) ₂	0.2 mM	Triton X-100	0.1 %
CaCl ₂	3 mM	DTT	0.5 mM

Centrifugation medium

sucrose	2 M	Tris-HCl pH 8.0	10 mM
Mg(AC) ₂	5 mM	DTT	0.5 mM

Resuspension medium

glycerol	25 %	DTT	5 mM
Mg(AC) ₂	5 mM	EDTA	0.1 mM
Tris-HCl pH 8.0	50 mM		

Incubation medium (for XTC-2 nuclei)

glycerol	12.5 %	KCl	100 mM
MnCl ₂	1 mM	GTP, ATP, CTP	0.4 mM
Tris-HCl pH 8.0	25 mM	[³ H] UTP (2Ci/mmol)	0.025 mM
EDTA	0.05 mM	5'-CMP	1 mM
DTT	2.5 mM		

Column buffer

Tris-HCl pH 8.0	10 mM	EDTA	0.05 mM
DTT	0.5 mM		

SSC (saline sodium citrate)

NaCl	0.15 M	sodium citrate	0.015 M
------	--------	----------------	---------

(c) Cell culture

XTC-2 cells were maintained at 25^o C in Glasgow modified Eagle's Minimal Essential Medium (GMEM) supplemented with non-essential amino acids, 10% foetal calf serum (FCS), glutamine and antibiotics. They were grown in glass or plastic tissue culture bottles and were subcultured every 5 days. Briefly, the cells were subcultured, when confluent, by removing the medium, washing the cells with PBS and subsequently detaching the cells with a trypsin (0.25%)/versene (0.02% in PBS) mixture (1:4). An equal volume of culture medium was added, the cells were dissociated by pipetting, and inoculated into fresh medium.

BHK-21/13 cells were grown in roller culture at 37° C in GMEM modified for BHK cells (Flow Laboratories), supplemented with 10% FCS and antibiotics. They were subcultured, as described for the XTC-2 cells, every 3 days. Both cell lines were regularly tested for the presence of mycoplasma by the method of Perez *et al.* (1972).

Cells were stored in aliquots in liquid nitrogen at 5×10^6 cells/ml in 10% DMSO in FCS. A haemocytometer was used for all cell counting.

XTC-2 or BHK total nucleic acids were labelled by incubating the cells overnight in the presence of [³H] uridine at 20 µCi/ml in normal culture medium or in the presence of [³²P] orthophosphate in phosphate-free medium supplemented with dialysed FCS.

(d) Preparation of Xenopus oocytes, eggs and embryos

Xenopus laevis were kept and oocytes, eggs and embryos prepared as described by Gurdon and Woodland (1975). Stages in development are as described by Nieuwkoop and Faber (1956).

Ovary was dissected from female toads and immediately transferred to Barth X where it was separated into small clumps of oocytes. The oocytes were maintained at 18° C in fresh Barth X until required. Large oocytes (Stages 5 and 6) were removed manually as required. Stage 1 and 3 oocytes were obtained from young laboratory reared toads which had been treated with hormone to induce sex reversal in males, as described by Gallian (1956). When necessary, follicle cells were removed manually from Stage 6 oocytes and the oocytes were further dissected into G.V's and enucleated oocytes as described by Ford and Gurdon (1977) and Woodland and Adamson (1977).

Total oocyte proteins were labelled by incubating separated full grown oocytes overnight at 18° C in lyophilised [³H] lysine or [³⁵S] methionine dissolved in Barth X at 1 mCi/ml.

Microinjection of oocytes, eggs and embryos was carried out by Dr. H.R. Woodland.

Xenopus blood was removed from freshly killed toads by cardiac puncture. The blood was removed into heparinized tubes, the buffy coat removed and the cells resuspended in PBS for further use.

(e) Isolation of nuclei, nucleoli and chromatin

Nuclei

Nuclei were prepared essentially as described by Marzluff *et al.* (1973). Cells were washed in ice-cold PBS after harvesting with trypsin, then they were resuspended in ice-cold homogenization medium at about 10^7 cells/ml. They were left on ice for 1-2 minutes, then homogenized in a tight-fitting Teflon-glass homogenizer on ice, using about 30 strokes. An equal volume of ice-cold centrifugation medium was added and 5 ml of this mixture was layered over 8 ml of centrifugation medium and spun at 4,000 g max for 1 hour at 4° C in an MSE 6L centrifuge. The supernatant was removed and the pellets were resuspended in RM at 2×10^7 nuclei/ml. The preparation was stored in aliquots at -70° C. Counting of nuclei was by haemocytometer with phase contrast microscopy.

Nucleoli

Nucleoli were prepared by the method of Grammt and Lindigkeit (1973). The method consisted of washing the previously isolated nuclei in 0.5% (w/v) Triton X-100, 5 mM $MgCl_2$, 0.25 M sucrose and then sonicating the nuclei in 0.25 M sucrose, 50 mM $MgCl_2$ for 4×10 second bursts with 30 second cooling intervals. The sonicate was layered over a 0.88 M sucrose pad and the whole centrifuged for 30 minutes at $2,000 \times g$ and 4° C. The nucleolar pellet was resuspended in 0.25 M sucrose, 50 mM $MgCl_2$ and centrifuged again through a 0.88 M sucrose pad. The nucleolar pellet was resuspended in RM at $100 \mu g$ DNA/ml (determined by the absorbance at 260 nm) and stored at -70° C in 500 ml aliquots. Nucleoli prepared in this way were found to be free of nuclei when examined by phase contrast microscopy. There was no loss of template activity on storage at -70° C.

Chromatin

Chromatin was prepared as described by De Pomerai et al. (1974) for their form VI chromatin. This consists of homogenizing previously isolated nuclei briefly in ice-cold 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5 mM DTT, 12.5% glycerol. The lysed nuclei were then centrifuged at 15,000 xg for 15 minutes. The chromatin pellet was resuspended on ice in the same homogenization buffer by gentle homogenization and the concentration adjusted to 500 μ g DNA/ml as judged by the A_{260} . Chromatin was used in assays immediately after preparation.

(f) Preparation of cell extracts

Extracts were prepared after washing the oocytes, eggs or embryos in column buffer. Unfertilised eggs and embryos were first dejellied in 1% cysteine hydrochloride, adjusted to pH 8.0 with NaOH. The cells were transferred to a homogenizer on ice, the supernatant was removed and the residuum homogenized gently. The homogenate was centrifuged at 2,500 x g max for 30 minutes at 4^o C. The clear supernatant was removed, avoiding the lipid, and recentrifuged at 30,000 x g max for 30 minutes at 4^o C. The protein concentration was adjusted to 10 mg/ml (determined by the method of Lowry (1951) using column buffer and the extract was stored in aliquots at -70^o C.

(g) Extraction of nucleic acids

The phenol detergent method of Parish and Kirby (1966) as modified by Loening (1969) was used omitting the high salt step during deproteinization. All operations were carried out at 0-4^o C. Pelleted material was homogenized in 4 ml of Kirby Buffer and then shaken vigorously with an equal volume of phenol/cresol and centrifuged at 2,500 xg for 10 minutes. Cell or nuclear suspensions or aqueous samples were made up to 2 ml with water and an equal volume of double strength Kirby Buffer was added and treated as above. After centrifugation, the phenol layer was removed and another 4 ml of phenol/cresol added to the aqueous layer and

extracted as above. The nucleic acid was precipitated from the resulting aqueous layer by the addition of 2 volumes of ethanol and storage overnight at -20°C . The precipitated nucleic acid was recovered by centrifugation at $2,500 \times g$ for 20 minutes and washed twice by resuspending in 70% (v/v) ethanol containing 50 mM NaCl. The drained pellet was dissolved in a small volume of 50 mM Tris-HCl (pH 7.6), 3 mM MgCl_2 on ice. If RNA was required, DNAase (RNAase-free) was added to 0.4 $\mu\text{g}/\text{ml}$ and incubated for 30 minutes on ice. The RNA was then re-extracted as above, carrier RNA being added if required. If DNA was the required product, the dissolved nucleic acid was incubated with RNAase (pancreatic A, 50 $\mu\text{g}/\text{ml}$, 30 minutes at 25°C), re-extracted and precipitated as above.

Purified rRNA (both unlabelled and labelled) was prepared by homogenizing cells in 0.25 M sucrose and centrifuging at $700 \times g$ for 5 minutes and at 4°C to pellet the nuclei and debris. The supernatant was recentrifuged at $10,000 \times g$ for 10 minutes to pellet the mitochondria. This supernatant was centrifuged finally at $100,000 \times g$ for 1 hour at 4°C to pellet the ribosomes. The rRNA was extracted from this pellet as described above.

(h) Scintillation counting

Total radioactivity in aqueous samples was estimated by spotting a sample on to GF/C filter discs, drying and counting in 4 ml of PPO/POPOP/toluene scintillant (0.5% PPO, 0.03% POPOP in toluene). Larger volumes of aqueous samples were counted by mixing with Insta-Gel as described by the manufacturers.

Acid-precipitable radioactivity from enzyme assays etc. was estimated as follows. Samples were spotted directly on to numbered (in pencil) 2 cm squares of Whatman 54 filter paper and plunged immediately into ice-cold TCA, 1% tetra-sodium pyrophosphate. When all the samples had been collected in the TCA mixture they were bulk washed, on ice, in 3 changes of the same TCA mixture, 2 changes of ethanol and one of diethyl ether. The filters were sorted, pinned out on aluminium foil and placed in an oven at 60°C . When dry, they were placed in vials and counted in 4 ml of PPO/POPOP/toluene scintillant.

Gel slices were solubilised in 0.1 ml of 100-volume hydrogen peroxide at 80° C for 4-8 hours in capped scintillation vials. 4 ml of Triton/toluene scintillation fluid (0.4% PPO, 0.05% POPOP in toluene-Triton X-100 (2:1, v/v)) was added to each vial, and the mixture shaken to ensure dispersion, and counted.

All radioactivity determinations were carried out in a Packard Tri-carb model 3320. These methods gave counting efficiencies of 25% for ^3H (background 20-25 cts/min) and 98% for ^{32}P (background 10-15 cts/min).

(i) RNA polymerase preparation

RNA polymerases were prepared from Xenopus ovary exactly as described by Roeder (1974) using differential $(\text{NH}_4)_2\text{SO}_4$ precipitation. The final pellet containing the RNA polymerase was taken up in TCME (0.05 M Tris-HCl (pH 7.9), 5 mM MgCl_2 , 0.1 mM EDTA, 0.5 mM DTT, 25% glycerol) to give a $(\text{NH}_4)_2\text{SO}_4$ concentration of, at most, 40 mM (measured by conductance). The activity of the polymerases in this preparation was assayed by the method of Roeder (1974) using either Xenopus purified DNA or poly d(A-T) as template at 100 $\mu\text{g}/\text{ml}$ in a 100 μl assay with 30 μl of the polymerase preparation. Incubation was for 20 minutes at 30° C. The units of enzyme activity are those used by Roeder (1974). These crude polymerase preparations contained about equal amounts of polymerase I, II and III activity as assayed using α -amanitin at 1 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$.

(j) Enzyme assays

1. Assays of RNA synthesis

Isolated nuclei

The basic assay was similar to that of Marzluff *et al.* (1973). For Xenopus nuclei the final incubation medium is given in section (b) in this chapter. Incubation was at 25° C. For BHK nuclei the same medium was used except the KCl concentration was 150 mM.

Nuclei were incubated with cytoplasm, or column fractions, as follows. 100 μl incubations contained 10^5 nuclei, 40 μl of cell extract (or column fraction), 20 mM KCl, 1 mM MnCl_2 and 0.02 mM

for each ribonucleotide. They were incubated for 1 hour at 25° C. The nuclei were centrifuged at 1,000 x g for 5 minutes at 4° C and resuspended in incubation medium as described above and incubated at 25° C.

Isolated nucleoli

These were assayed in the same way as the nuclei except that the $MnCl_2$ was replaced by 5 mM $Mg(AC)_2$. A 100 μ l assay contained ~1-1.5 μ g of DNA and incubation was at 25° C for 30 minutes. When assayed with cell extracts or column fractions, no preincubation step was used. A 100 μ l assay contained 40 μ l of extract or column fraction. Again, incubation was at 25° C for 30 minutes.

Chromatin

Chromatin from XTC-2 cells was assayed at 25° C under the same conditions as the isolated nuclei, the assays contained an equivalent amount of DNA in a 100 μ l assay (~1 μ g DNA). When assayed with cell extracts or column fractions, no preincubation step was used, 40 μ l of extract was present in a 100 μ l standard assay mixture.

DNA and purified RNA polymerase

RNA polymerases in column fractions and extracts were assayed as described by Roeder (1974) (see above). When column fractions were assayed for their effect on purified RNA polymerase activity, the same assays were used except that a 100 μ l incubate contained 15 μ l of column fraction and 15 μ l of crude *Xenopus* RNA polymerase. Incubation was at 30° C for 20 minutes.

ii. Protein kinases

Column fractions were assayed for the presence of cyclic AMP-activated protein kinase activity by the method of Maler and Krebs (1977). An 80 μ l assay contained 20 μ l of column fraction, 16 nmol [γ -³²P] ATP (~1000 cts/min/pmol), 200 μ g calf thymus histone and cyclic AMP to 10^{-4} M. Incubation was at 30° C for 10 minutes.

(k) Fractionation methods

i. Gel filtration

Cell extracts were fractionated at 4° C on G-100 Superfine Sephadex (Pharmacia) in a 2 x 30 cm Pharmacia column equilibrated with column buffer. 50 drop fractions were collected. The effect of each fraction on RNA synthesis was measured by adding 20 μ l of

column extract to isolated nuclei in a 50 μ l incubation under the standard preincubation conditions.

ii. DEAE-cellulose chromatography

DEAE-cellulose chromatography of cell extracts or Sephadex fractions was carried out at 4^o C using 1.5 x 4 cm columns of DE52 (Whatman) equilibrated with column buffer. A 40 ml 0 to 0.45 M linear gradient of NaCl in column buffer was passed through the column after washing the loaded DE52 to constant UV absorbance. 30 drop fractions were collected. The NaCl gradient was measured by conductance.

iii. Fractionation of nucleic acids by electrophoresis on 6 mm disc polyacrylamide gels

The procedure of Loening (1967) was followed, but using the E-buffer (36 mM Tris, 30 mM NaH₂PO₄, 1 mM EDTA) described by Loening (1969) as the electrophoresis buffer. SDS was added to the buffer in the electrophoresis reservoirs at a concentration of 0.2%. 9 cm gels were pre-electrophoresed for 1 hour (as described by Loening, 1967) with a 1 cm layer of half-strength E-buffer containing 8 M urea on the top of the gels. During the pre-electrophoresis, the urea diffused into the upper region of the gels. The washed nucleic acid pellets were drained to remove excess ethanol and dissolved in half-strength E-buffer containing 8 M urea. The samples were made 7% (w/v) with respect to sucrose (RNAase free) and were layered on to the top of the gels underneath the layer of 8 M urea. Split gels were prepared by overlaying 4.5 cm of 7.5% gel with 4.5 cm of 2.4% gel. Electrophoresis was carried out at 6 mA per gel for about 2 hours at room temperature. Gels were washed by soaking in distilled water for 30 minutes and scanned at 260 nm in a Gilford 2000 spectrophotometer fitted with a 2410-S linear transport scanning attachment. BHK RNA was used to provide molecular weight markers.

Scanned gels were frozen in solid carbon dioxide and sectioned transversely into 1 mm slices with a Mickle gel slicer. Radio-activity in the gel slices was determined as already described.

iv. Fractionation of RNA by sucrose gradient centrifugation

RNA was fractionated on 5-25% exponential sucrose gradients by the method of Covey (manuscript in preparation). Sucrose solutions were prepared using a standard buffer of 150 mM LiCl, 50 mM Tris-HCl (pH 8.0), 0.5% SDS and 2 mM EDTA. 8 ml gradients were formed in 10 ml centrifuge tubes and the samples loaded in 500 μ l of 3% sucrose. BHK rRNA was used as a marker in parallel gradients. The gradients were centrifuged at 15^o C for 1.5 hours at 50,000 rpm in the MSE 10 x 10 ml aluminium angle rotor. The samples were analysed on an ISCO density gradient fractionator. Total and acid insoluble radioactivity in each fraction was determined as already described. Required fractions were pooled, precipitated with ethanol and stored until required.

v. Buoyant density centrifugation of DNA on neutral CsCl gradients

Purified DNA in 0.1 x SSC was centrifuged to equilibrium on 4.5 ml, 1.715g/cc CsCl gradients at 42,000 rpm and 15^o C for 22 hours in an MSE 10 x 10 ml aluminium angle rotor. They were analysed using an ISCO density gradient fractionator.

vi. SDS-polyacrylamide slab gel electrophoresis of proteins

Protein samples from cell extracts, column fractions and nuclei were fractionated on slab gels as described by Laemmli (1970). 18% gels were cast between 20 x 25 cm glass plates. Samples were precipitated, by the addition of TCA to 20%, and pelleted by centrifugation. The pellets were washed twice with 95% acetone, dried in vacuo and taken up in a small volume of sample buffer (Laemmli, 1970). The samples were boiled for 3 minutes before being applied to the gel for electrophoresis.

Proteins were visualised by staining the gel with 0.1% Coomassie Brilliant Blue, 45% methanol, 10% acetic acid and destained in 45% methanol, 10% acetic acid.

Gels were fluorographed as described by Laskey and Mills (1975). Autoradiographs were scanned using a Joyce-Loebl densitometer.

(1) DNA, RNA and protein estimations

DNA was determined by the method of Burton (1968) using Salmon testis DNA as standard.

RNA was determined by measuring the A_{260} of the solution. A 1 mg/ml solution was taken to have an $A_{260}^{1 \text{ cm}}$ of 20.

Protein concentration was determined by the method of Lowry et al. (1951).

(m) DNA-RNA hybridization on nitrocellulose filters

DNA samples were denatured and immobilized on 13 mm HAWP Millipore filters (Millipore (UK) Ltd.) by the method of Gillespie and Spiegelman (1965) as modified by Birnstiel et al. (1972). All hybridizations were carried out in 2 x SSC, 50% formamide at 61.5°C for 17 hours. RNA concentrations are given in the individual figure legends. The filters were washed and treated with RNAase as described by Birnstiel et al. (1972). Radioactivity on dried filters was determined using PPO/POPOP/toluene scintillation fluid as already described. The levels of radioactivity obtained were corrected for radioactivity binding to filters carrying no DNA.

(n) DNAase I digestion of nuclear DNA

DNAase I sensitivity of nuclear DNA was tested by the method of Garel and Axel (1976). Nuclei were suspended in Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and DNAase I was added to 20 $\mu\text{g}/\text{ml}$. The nuclei were incubated at 37°C and the kinetics of digestion followed by measuring the A_{260} absorbing material soluble in 1 M $HClO_4$, 1 M NaCl.

Chapter 3

Characterisation of conditions for isolating and incubating nuclei

(a) Introduction

The first essential in devising an assay system for cytoplasmic factors influencing RNA synthesis is obviously to have an RNA synthesising system in the first place. As discussed in Chapter 1, this synthesising system should fulfil several criteria if it is to be capable of responding to any factors present. These criteria are also important if the results are to relate to the in vivo situation.

The starting point for this investigation was to test the various methods available for isolating nuclei from cultured cells. In the first instance, ease of extraction, yield and purity were the criteria applied. Two basic methods for the isolation of nuclei have been extensively used. Firstly, cells have been lysed by the use of detergents such as Triton X-100 and lysolecithin or secondly, by hypo-osmotic shock. These several methods were tried and the nuclei tested for purity and RNA synthesising activity. The chosen method of isolation was then used exclusively in all subsequent characterisation experiments which make up the major part of this chapter. The RNA synthesising activity, as judged by trichloroacetic acid (TCA) precipitable incorporation of radioactive UTP, was optimised for ion and salt concentrations, pH, temperature and nucleotide triphosphate concentration. Various controls have been applied to be sure the synthesis observed was actually of RNA and was being carried out in the nuclei. Also the level of activity of the different RNA polymerases involved has been assayed by different methods. The RNA product has been characterised by gel electrophoresis and the level of transport of this RNA from the nuclei to the surrounding medium has been measured. Finally, some estimate has been made of the amount of initiation of new RNA molecules that occurs in isolated XTC-2 nuclei.

(b) Methods of isolating nuclei

Three methods of nuclei isolation were tried initially as outlined below.

(i) Hypo-osmotic shock

Cells were washed in phosphate buffered saline (PBS) after removal with trypsin, then resuspended at 5×10^6 cells/ml in hypotonic buffer (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM MgCl₂) as described by Busiello and Di

Girolamo (1975). The cells were allowed to swell on ice for about 10 minutes and then broken open with 10 strokes of a tight fitting teflon/glass homogeniser. An equal volume of a 30% sucrose solution (22 mM NaHCO_3 , 13 mM KHCO_3 , 14 mM KH_2PO_4 , 5 mM MgCl_2 , 90 mM KCl , 1 mM DTT, 30% sucrose) was then mixed with the suspensions of nuclei and the whole centrifuged at 365 x g for ten minutes. The nuclear pellet was resuspended in the 30% sucrose solution (see above) at 5×10^6 nuclei/ml.

(ii) Lysolecithin rupture (LL-BSA method)

Cells were harvested and washed as above, and then the nuclei were isolated using lysolecithin as a detergent, as described by Gurdon (1976). The cells were pelleted by low speed centrifugation and resuspended (to single cell suspension) in 0.25 M sucrose, 2 mM MgCl_2 at 18-24°C and 5×10^6 cells/ml. 20 μl of a 1 mg/ml solution of lysolecithin (in water) per ml of cell suspension was added at the same temperature. The cell suspension was then swirled every 15 seconds for one minute (for XTC cells) and then 2 ml per 5×10^6 cells of 0.25 M sucrose, 2 mM MgCl_2 and 3% BSA was added at 0°C. The nuclear suspension was then centrifuged at 320 x g for 30 seconds and the nuclear pellet resuspended in a small volume of the sucrose/BSA solution at 20×10^6 /ml.

(iii) Triton lysis

Nuclei were prepared essentially by the method of Marzluff *et al.* (1973). The method is the one described in the Methods section and consists of resuspending the harvested cells in a Triton/Tris sucrose solution, then homogenising in a tight fitting homogeniser. An equal volume of buffered 2 M sucrose was mixed with the homogenate and the whole layered over a 2 M sucrose pad and spun at 4,000 g_{max} for 1 hour at 4°C. The nuclear pellet was then resuspended in buffered 25% glycerol at 20×10^6 nuclei/ml.

The nuclei prepared by these three methods were then compared for yield, purity and the presence of cytoplasmic tags by phase contrast microscopy and by staining released nuclei with 0.2% Trypan blue in PBS. The following conclusions were reached from these observations.

(1) The level of contamination of the nuclear preparation by whole cells, as assayed by staining with Trypan Blue (nuclei in unlysed cells remain

unstained while isolated nuclei stain blue), was found to vary between a high level of contamination for method (i) (10-20 cells per 100 particles), and a very low level for (ii) and (iii), which both showed less than one whole cell per 1000 particles.

(2) The yield of nuclei was in general good, method (iii) at 80% being slightly lower than (i) and (ii).

(3) Phase contrast microscopy of the different nuclear preparations showed that methods (i) and (ii) both produced nuclei with considerable cytoplasmic contamination. In contrast, method (iii) gave nuclei which were essentially free of cytoplasmic tags. In an effort to reduce cytoplasmic contamination in nuclei prepared by methods (i) and (ii), they were purified further by centrifuging through a 2 M sucrose pad as in method (iii). This resulted in a lowering of cellular contamination and a slight decrease in cytoplasmic tags on the nuclei.

Thus in terms of purity, yield and the level of cytoplasmic contamination, the Triton X-100 method of Marzluff *et al.* (1973) proved the best overall. It especially fulfilled one of the primary requirements for the basis of a cytoplasmic assay system, that of absence of substantial adhering cellular cytoplasm. This method is essentially the same as that used by Reeder and Roeder (1972), also for *Xenopus* tissue culture cells.

The three methods of nuclei preparation were further tested by assaying for their RNA synthesising capacity. A starting point for these RNA synthesis assays was to choose an appropriate incubation medium. Those used by previous workers (for example Marzluff *et al.* (1973), Busiello and Di Girolamo (1975) and Reeder and Roeder (1972)) vary little in their general composition. Therefore, that of Marzluff *et al.* was chosen since it gives good levels of RNA synthesis in myeloma nuclei. Another reason for this choice was that these workers have shown that initiation of new RNA chains occurs in nuclei prepared and incubated by their method.

Nuclei prepared by methods (i), (ii) and (iii) were resuspended in Resuspension Medium (RM) and then incubated at 25°C in the incubation medium of Marzluff *et al.* (12.5% glycerol, 25 mM Tris-HCl, 2.5 mM DTT, 0.05 mM EDTA, 1 mM MnCl₂, 5 mM Mg(Ac)₂, 150 mM KCl and 0.4 mM unlabelled triphosphates (GTP, CTP and ATP) and 0.025 mM

[³H] UTP at 2.5 Ci/mmol). RNA synthesis was assayed by taking aliquots after 0 minutes and 30 minutes incubation and estimating the TCA precipitable incorporation of [³H] UTP as outlined in the Methods section. A general conclusion was that all three preparations of nuclei actively synthesised RNA as assayed by this method. Preparation (iii) was slightly superior in terms of total incorporation per unit DNA.

It was decided from these initial observations to use the methods for preparation and incubation of nuclei outlined by Marzluff *et al.* as the starting point for this investigation. Plate 1 illustrates the nuclei prepared in a typical isolation by the method of Marzluff *et al.* Note the almost complete absence of adhering cytoplasm and the obvious nucleoli. XTC-2 nuclei prepared in this manner have a DNA content of 11 pg per nucleus (the *Xenopus laevis* diploid DNA content is 6 pg/nucleus Dawid, 1965) and a DNA:RNA:protein ratio of 1:0.2:4. This is a lower RNA content than found by most workers (*e.g.* Birnstiel, 1964), and may reflect a lower cytoplasmic contamination, for example from ribosomes, etc.

Some general points follow about the isolation and incubation of nuclei, particularly in relation to method (iii). During the purification step of centrifuging the nuclei through a sucrose pad, a most important point was the concentration of the nuclei in the suspension which overlays the pad. It was found to be essential to keep this concentration below 2.5×10^7 nuclei per ml, that is below 5×10^7 cells/ml in the original homogenising medium. If this value was exceeded, the nuclei either completely failed to penetrate the interphase during centrifugation or the yield of nuclei was much reduced.

It was noticed that in the early stages of this investigation some variability occurred between different preparations of nuclei. This was thought to correlate with the confluency of the cells and the state of the medium in terms of substrate levels, etc. The following procedure was used to obtain consistent preparations of nuclei, in terms of maximum numbers of nuclei for a given amount of medium, and good RNA synthesising activity. XTC-2 cells were grown, as in the Methods section, to near confluency; the medium was replaced on the evening

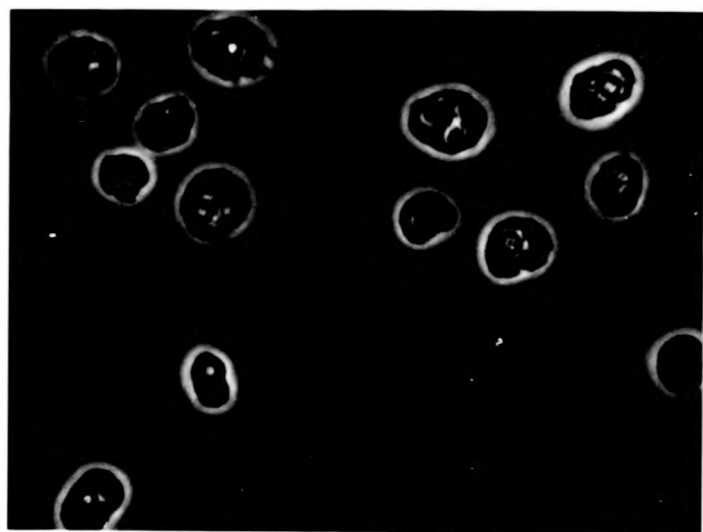


Plate 3.1 Isolated XTC-2 nuclei

XTC-2 nuclei were prepared by the method of Marzluff et al. (1973) and then visualised by phase contrast microscopy. (Magnification was 400 x).

before the nuclei were to be isolated. The cells were harvested the next morning after 16 hours in the new medium and the nuclei isolated. This method gave consistent results and also gave in general a better RNA synthetic activity per nucleus.

A final point about the assay for RNA synthesis was that nuclei prepared by the Triton method sometimes aggregated, although this could be overcome by gentle agitation. Read and Mauritzen (1970) have suggested that Triton treated nuclei may clump as a result of a change in the surface charge on the nuclear membrane. However, it was important that the nuclei did not clump during the assay for RNA synthesis to avoid local substrate starvation. For this reason all nuclei incubations were agitated every 15 minutes.

(c) Optimisation of RNA synthesis by isolated XTC-2 nuclei

Some basic controls have been performed to show that the labelled RNA precursor ($[^3\text{H}]$ UTP) was indeed incorporated into a ribopolymer by DNA-dependent RNA polymerases. The results are shown in Table 3.1. The incorporation of $[^3\text{H}]$ -UTP by a standard assay has been defined as 100%. If the UTP is incorporated into RNA, all four nucleoside triphosphates will be required. It can be seen that the omission of one resulted in a complete inhibition of label incorporation. This also suggested that the pool of nucleoside triphosphates was very small in the nuclear preparations.

The incorporation of $[^3\text{H}]$ UTP was shown to be dependent on the availability of DNA by complexing the DNA with a DNA binding agent, actinomycin D, and by digesting the DNA with DNAase. In both cases the incorporation was much reduced, by 98% and 94% respectively. 97% of the labelled product was found to be susceptible to RNAase digestion. No incorporation of $[^3\text{H}]$ UTP into TCA precipitable material was ever observed in the absence of the nuclei.

The conditions of Marzluff *et al.* (1973) were used as a starting point in "optimising" the transcription of XTC-2 nuclei by maximisation of total $[^3\text{H}]$ UTP incorporation into TCA-precipitable radioactivity. The response of RNA synthesis to changes in Mg^{2+} , Mn^{2+} and KCl concentrations is shown in Fig. 3.1. The optimal environment for XTC-2

before the nuclei were to be isolated. The cells were harvested the next morning after 16 hours in the new medium and the nuclei isolated. This method gave consistent results and also gave in general a better RNA synthetic activity per nucleus.

A final point about the assay for RNA synthesis was that nuclei prepared by the Triton method sometimes aggregated, although this could be overcome by gentle agitation. Read and Mauritzen (1970) have suggested that Triton treated nuclei may clump as a result of a change in the surface charge on the nuclear membrane. However, it was important that the nuclei did not clump during the assay for RNA synthesis to avoid local substrate starvation. For this reason all nuclei incubations were agitated every 15 minutes.

(c) Optimisation of RNA synthesis by isolated XTC-2 nuclei

Some basic controls have been performed to show that the labelled RNA precursor ($[^3\text{H}]$ UTP) was indeed incorporated into a ribopolymer by DNA-dependent RNA polymerases. The results are shown in Table 3.1. The incorporation of $[^3\text{H}]$ -UTP by a standard assay has been defined as 100%. If the UTP is incorporated into RNA, all four nucleoside triphosphates will be required. It can be seen that the omission of one resulted in a complete inhibition of label incorporation. This also suggested that the pool of nucleoside triphosphates was very small in the nuclear preparations.

The incorporation of $[^3\text{H}]$ UTP was shown to be dependent on the availability of DNA by complexing the DNA with a DNA binding agent, actinomycin D, and by digesting the DNA with DNAase. In both cases the incorporation was much reduced, by 98% and 94% respectively. 97% of the labelled product was found to be susceptible to RNAase digestion. No incorporation of $[^3\text{H}]$ UTP into TCA precipitable material was ever observed in the absence of the nuclei.

The conditions of Marzluff *et al.* (1973) were used as a starting point in "optimising" the transcription of XTC-2 nuclei by maximisation of total $[^3\text{H}]$ UTP incorporation into TCA-precipitable radioactivity. The response of RNA synthesis to changes in Mg^{2+} , Mn^{2+} and KCl concentrations is shown in Fig. 3.1. The optimal environment for XTC-2

Table 3.1

Characterisation of [^3H] UMP incorporation by isolated XTC-2 nuclei

<u>Assay</u>	<u>[^3H] UMP incorporation (% of control)</u>
Complete	100
-CTP	0
-GTP	0
-UTP	0
-ATP	2
+ Actinomycin D	3
Pre-incubated with DNAase	6
Post-incubated with RNAase	3
-Nuclei control	0

Standard assays were carried out (see Chapter 2). Incorporation was for 30 minutes at 25⁰ C. Actinomycin D was added to 10 µg/ml. DNAase treatment was pre-incubation of the nuclear mix with 100 µg/ml DNAase for 30 minutes on ice. RNAase treatment was after the standard incubation. RNAase (pretreated by heating to 80⁰ C for 10 minutes to remove DNAase) was added to 50 µg/ml and the whole incubated for 30 minutes at 25⁰ C. The minus nuclei control was addition of RM to the assay.

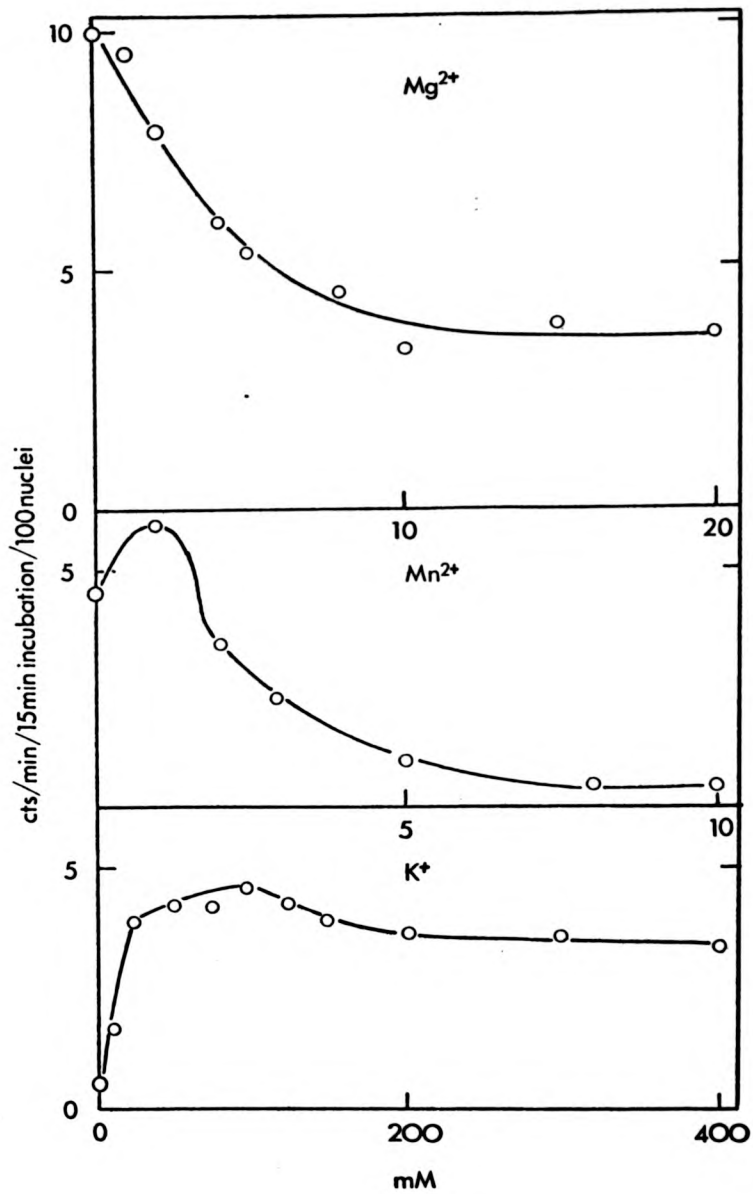


Fig. 3.1

Figure 3.1 Salt optimisation of XTC-2 nuclei

Incorporation of [^3H]UTP by XTC-2 nuclei, incubated under standard assay conditions for 15 minutes. Salt concentrations were 5 mM Mg^{2+} , 1 mM Mn^{2+} and 150 mM K^+ , except when varied as specified in the figure. Other constituents were as indicated in Chapter 2.

0
20
40
60
80

Fig 3.1

nuclei seemed to be 100 mM KCl and 1 mM MnCl_2 and these concentrations were used subsequently. This monovalent cation concentration of 100 mM is lower than that used for mammalian nuclei (150 mM for myeloma nuclei (Marzluff *et al.*, 1973) and BHK nuclei (Chapter 9, this thesis) but agrees with the optimal value for *Xenopus* nuclei found by Ramage and Barry (1975). These concentrations correlate well with the respective isotonic Ringer's monovalent cation concentration, mammalian Ringer's being 150 mM KCl and amphibian 110 mM. NH_4^+ substitutes equally for K^+ in these assays. Mg^{2+} was found to be inhibitory and was omitted from the assays. This result is in marked contrast to those of Marzluff *et al.* (1973) and Ramage and Barry (1975) who found 5 mM Mg^{2+} to be optimal and higher concentrations to be less inhibitory than found here for XTC-2 nuclei. It is possible that Mg^{2+} is carried over in the nuclei during their isolation, so any further addition of Mg^{2+} is inhibitory. The use of Mn^{2+} in transcription systems has been criticised because it is unphysiological. This point is discussed further (see below), but Mn^{2+} was used initially because this gave optimal total [^3H] UTP incorporation.

Transcription in isolated XTC-2 nuclei was further optimised for temperature, nucleotide triphosphate concentration and pH. The results are shown in Fig. 3.2. The effect of temperature (Fig. 3.2A) was perhaps as expected; at 25°C, the temperature at which XTC-2 cells are cultured, incorporation was better over a prolonged incubation than at 37°C, with a two-fold difference after 30 minutes incubation. However, this was also found to be the case for BHK nuclei (Chapter 8, this thesis) and for mammalian myeloma nuclei (Marzluff *et al.*, 1973). The 37°C incubation gives a rapid incorporation of [^3H] UTP for the first 5 minutes, then incorporation stops. Marzluff *et al.* (1973) have speculated that this could be due to a temperature sensitive regulatory molecule, not a polymerase, which is inactivated at 37°C. It seems unlikely to be merely an effect on initiation of new RNA chains as a much more gradual shut off would result, the shorter molecules being finished first and the longer, for example ribosomal RNA's, being transcribed for longer. It appears that all the polymerases stop in unison. Incubation at 30°C gives a time course similar to that at 37°C. The temperature optimum is quite marked and is fairly critical.

The NTP concentration was optimised by altering concentrations of unlabelled GTP, CTP and ATP while keeping the UTP concentration constant. The result (Fig. 3.2B) shows that incorporation levels plateau at 0.4 mM and then rise slowly at higher concentrations. 0.4 mM nucleotide triphosphate was used throughout as a compromise between better incorporation and the cost of nucleotide triphosphates.

Fig. 3.2C shows a fairly broad pH optimum between pH 7.5 and 8.5. pH 8.0 was used in all subsequent assays. Incorporation of [^3H] UTP was found to be linearly related to the number of nuclei per assay (Fig. 3.2D). A standard 50 μl assay contained 5×10^4 nuclei. The addition of an energy generating system consisting of 6 mM creatine phosphate and 50 units/ml of creatine phosphokinase showed no effect on the RNA synthesis in these nuclei. Addition of BSA to 1% to nuclear incubation had no effect on prolonged synthesis as reported by Ernest (1976). Nuclei incubated in these these optimised conditions showed a [^3H] UTP incorporation of about 10 pmoles/ μg of DNA in 30 minutes. This is about three times the incorporation observed by Marzluff et al. (1973) with myeloma nuclei. This rate of synthesis was linear for up to two hours.

An important point, essential in the following investigation, was how intact the nuclei remained during prolonged incubation. This was tested by counting the nuclei before and after a 30 minute incubation at 25 $^{\circ}$ C. There was no observable reduction in the number of nuclei present after incubation. Incubation in high salt (0.4 M and above) resulted in spontaneous lysis of the nuclei.

A property related to reproducibility and consistency in the assays and, of course, convenience was how well the nuclei could be stored. Nuclei in RM were frozen quickly and stored at -70 $^{\circ}$ C. They were subsequently thawed and reassayed. There was no loss in the transcriptional activity and no reduction in the number of nuclei present. It was subsequently found that the nuclei could be stored equally well at -20 $^{\circ}$ C and that on repeated freezing and thawing there was considerable loss in activity and lysis of nuclei. All subsequent nuclear preparations were therefore divided into 1 ml aliquots before storage to avoid waste and the use of nuclei thawed more than once.

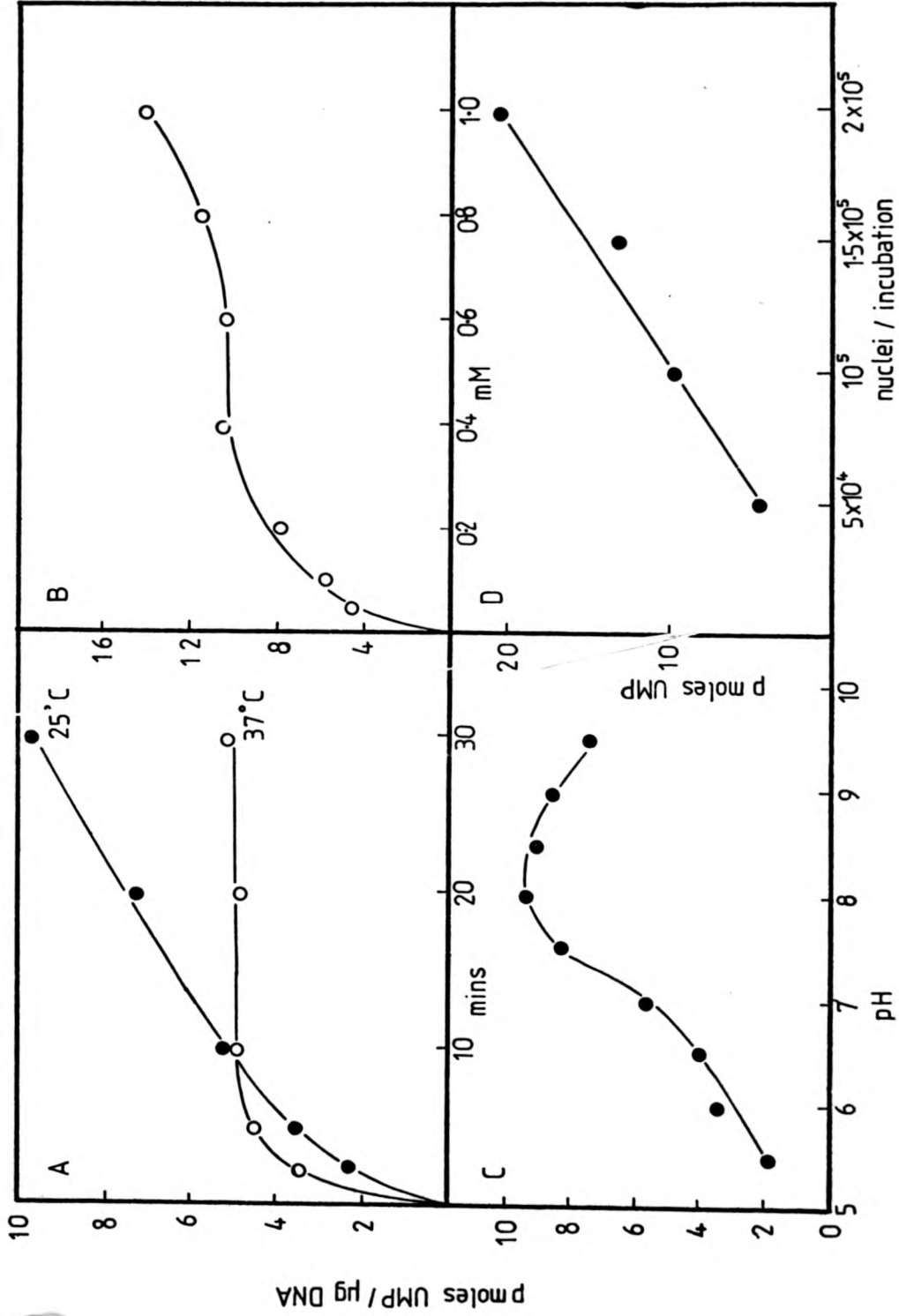


Fig. 3.2

Figure 3.2 Optimisation of XTC-2 nuclei for temperature, triphosphate concentration and pH.

Incorporation of [^3H] UTP by XTC-2 nuclei incubated for 15 minutes under standard assay conditions, except where specified.

- (A) The effect on incubating at 25 $^{\circ}$ C (●) and 37 $^{\circ}$ C (○) with time of incubation.
- (B) The effect of varying the unlabelled (GTP, CTP and ATP) triphosphate concentration between 0 and 1 mM.
- (C) The effect of varying the pH in otherwise standard assays between pH 5.5 and 9.5.
- (D) The relationship between the number of nuclei per 50 μl standard incubate and [^3H] UTP incorporation.

nuclei / incubation

pH

10.5

(d) Characterisation of the RNA synthesising activity

As described in Chapter 1, the different DNA-dependent RNA polymerase activities may be estimated by: (1) their different susceptibilities to the toxin α -amanitin and (2) their different mono- and divalent cation requirements. These two methods, separately and in combination, were used to estimate the activities of the different RNA polymerases present in isolated XTC-2 nuclei.

Fig. 3.3 shows the time course of incorporation of [^3H] UTP by control nuclei compared to nuclei incubated in the presence of 1 $\mu\text{g}/\text{ml}$ α -amanitin. This concentration inhibits only RNA polymerase II and the result suggests that 40-45% of the total incorporation is a result of RNA polymerase II activity. A better estimate of the different RNA polymerase activities involved may be gained by incubating nuclei with varying concentrations of α -amanitin. Concentrations in the range of 0-1 mg/ml were used in this way and the resulting concentration curve of incorporation by the nuclei is shown in Fig. 3.4. The curve is triphasic, showing that all three forms of RNA polymerase are present. It suggests that under standard assay conditions 50% of the polymerase activity is polymerase III, 40% polymerase II and about 10% polymerase I.

The above estimates are supported by experiments involving varying the mono- and divalent cation concentrations and using low levels of α -amanitin to inhibit polymerase II in the nuclei assays (Widnell and Tata, 1964). Fig. 3.5 shows the time course of incorporation by nuclei incubated under conditions selecting for particular RNA polymerase activities. The levels of polymerase activities from this data correlate very well with the α -amanitin concentration curve described above. The figures are also in good agreement with the results of other workers (Marzluff *et al.*, 1973; Sarma *et al.*, 1976).

(e) Characterisation of the RNA product

Two important features of a system to identify factors regulating transcription are: (1) that the synthesis of entirely new RNA chains should occur and (2) that the RNA product should resemble RNA synthesised in vivo as much as possible. The first is an obvious essential, if regulatory factors are to qualitatively influence the product in the system.

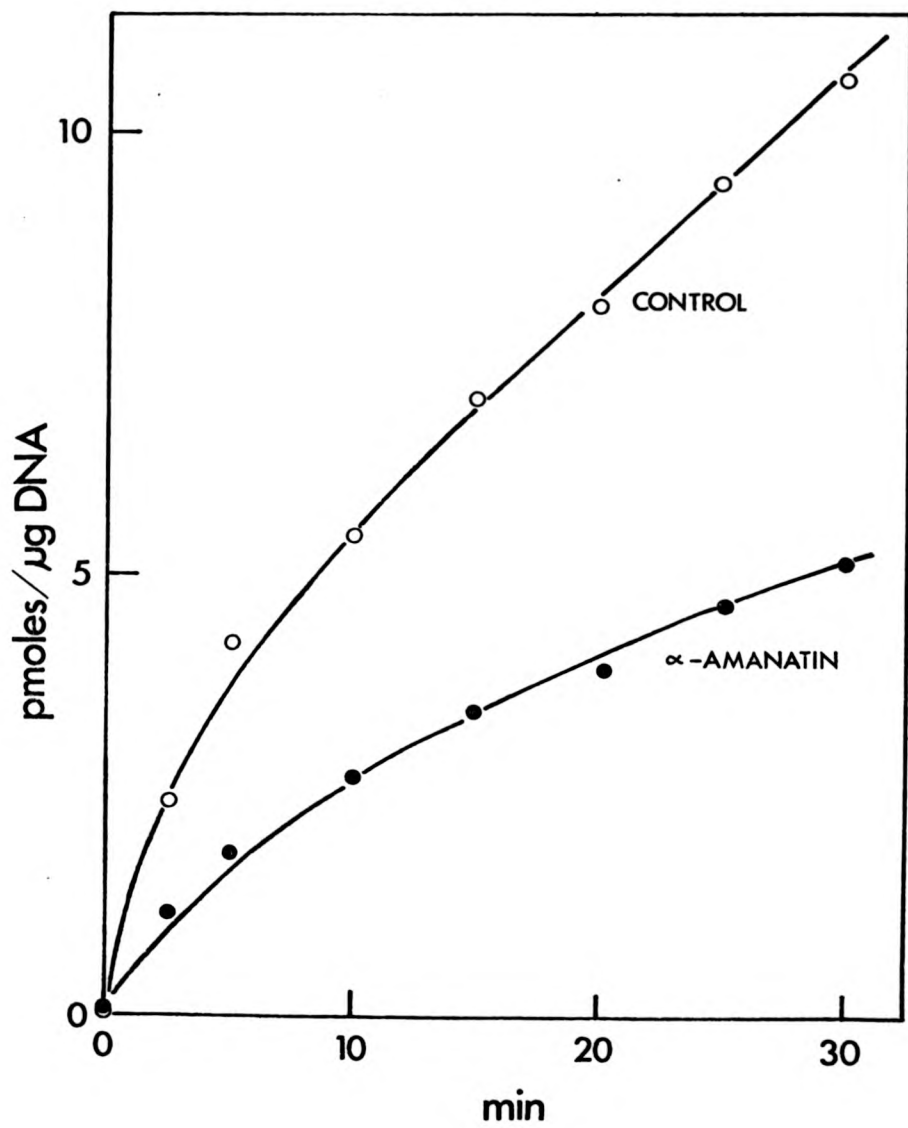


Figure 3.3 Time course of incorporation of [³H] UTP by XTC-2 nuclei in the presence and absence of α amanitin

Standard 500 μ l incubations were prepared with XTC-2 nuclei in the absence (O) and presence (●) of α -amanitin. 25 μ l duplicate samples were removed and counted at each time point. α -amanitin was present at a final concentration of 1 μ g/ml which was sufficient to inhibit RNA polymerase II, but not I or III.



30

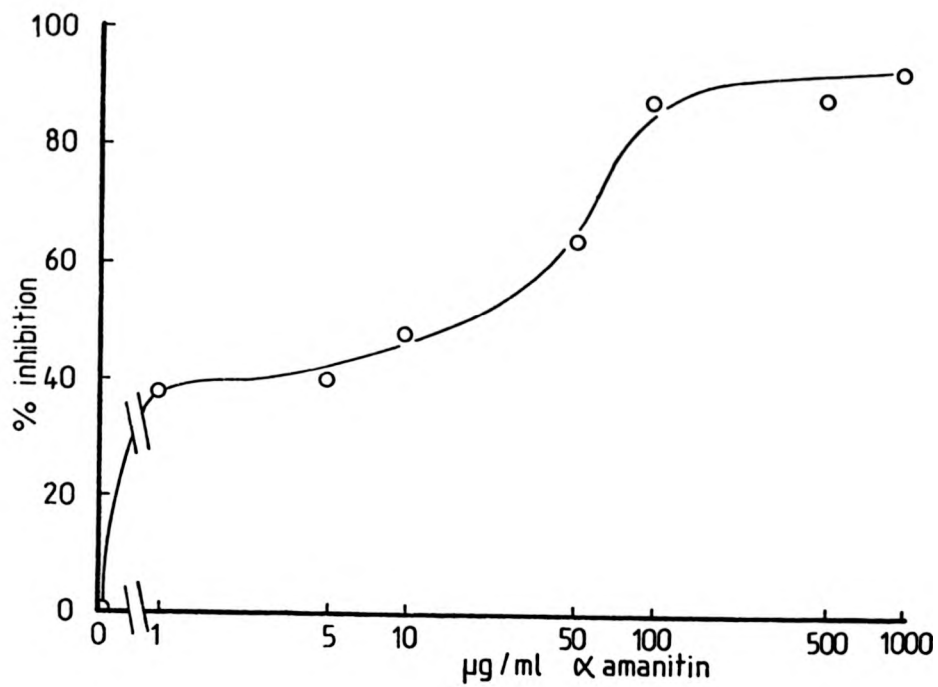


Figure 3.4 The inhibition of [³H] UTP incorporation by XTC-2 nuclei in the presence of varying levels of α -amanitin.

Standard duplicate 50 μ l incubations were prepared using XTC-2 nuclei with α -amanitin added to the indicated concentrations using a 10 mg/ml stock. Incubations were for 30 minutes at 25^o C. The percentage inhibition was calculated from the [³H] UTP incorporation of the assay without α -amanitin added.

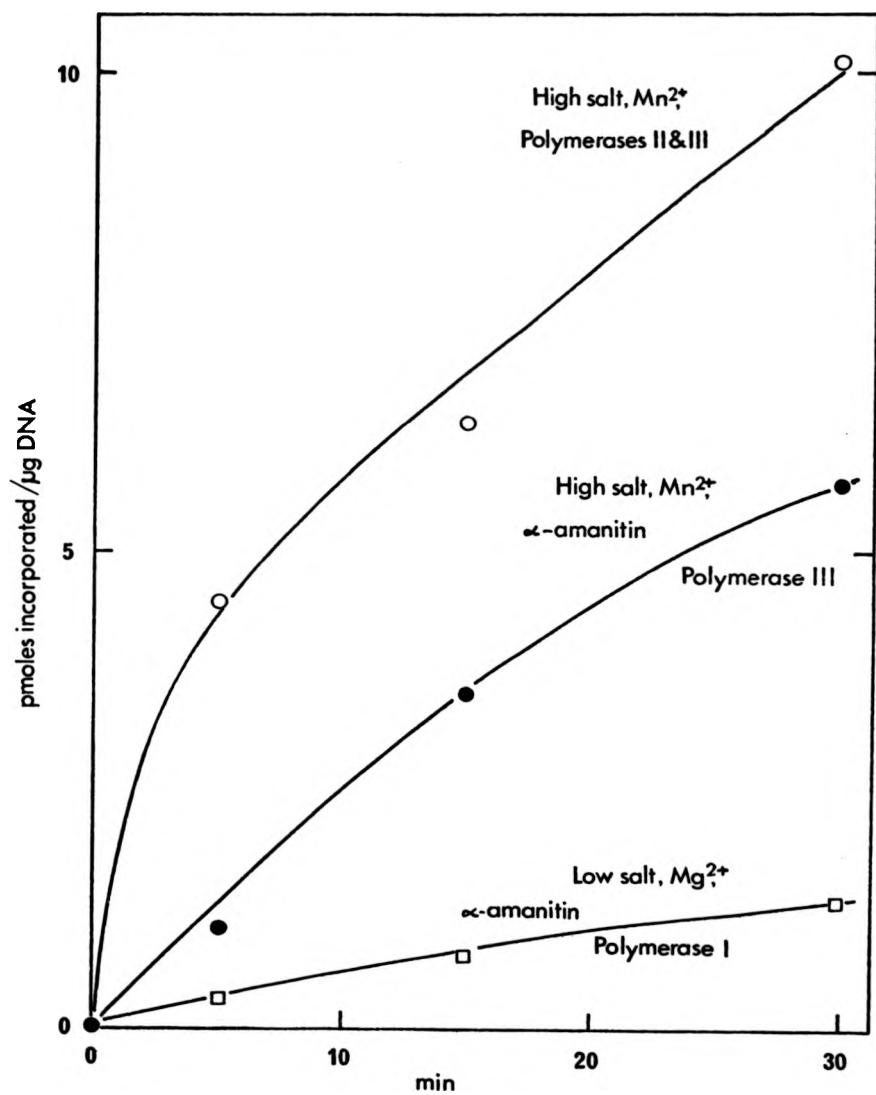


Figure 3.5 Time course of incorporation by XTC-2 nuclei under conditions selecting for RNA polymerases I, II + III and III.

XTC-2 nuclei were incubated under standard assay condition. 250 μ l incubates were prepared, selecting for RNA polymerases II + III (O, 200 mM K^+ , 1 mM Mn^{2+}), RNA polymerase III (\bullet , 200 mM K^+ , 1 mM Mn^{2+} , 1 μ g/ml α -amanitin) and RNA polymerase I (\square , 20 mM K^+ , 5 mM Mg^{2+} , 1 μ g/ml α -amanitin). Duplicate 25 μ l aliquots were removed from each at the times shown. The rationale for this experiment was discussed by Biswas (1974).



The second is desirable in that results may be compared more meaningfully to the normal situation if synthesis by isolated nuclei resembles synthesis in vivo.

This section deals with an attempt to reveal the nature of the transcription product in vitro. The main method used was that of polyacrylamide gel electrophoresis (Loening, 1969). This technique has the advantage of better resolution than, for example, sucrose density gradient analysis. By this method it was hoped to determine the molecular weights of the RNA products, and if possible to identify the transcription of discrete products, especially rRNA. These results could then be compared to the RNA synthesised by XTC-2 cells in vivo.

A starting point was to assay for RNAase activity in the nuclear preparations. This was achieved by ^{using} standard conditions for XTC-2 nuclei, incubation being allowed to proceed normally for 5 minutes. After this time, unlabelled UTP was added to a final concentration of 1 mM. The assay was then incubated for a further 25 minutes. The assay depends upon any nuclease present reducing some of the in vitro synthesised RNA to nucleotide fragments too small to be acid precipitable. Fig. 3.6 shows the result of this experiment; the amount of TCA precipitable radioactive material was estimated in 25 μ l samples which were withdrawn at the times indicated. It is clear that there is a significant reduction in the amount of acid precipitated radioactive material suggesting the presence of high levels of RNAase. This assay would not detect RNAase activity resulting in larger fragments, although this activity is still relevant if a complete profile of the RNA synthesised in vitro is to be obtained.

A satisfactory inhibitor of the RNAase activity in the nuclear preparation was essential if the RNA product of the nuclei was to be studied further. Any inhibitor should have the dual properties of not affecting the morphology of the nuclei or the activity of the RNA polymerases in any way. This limited the choice of known inhibitors. Fig. 3.6 shows the RNAase assay in the absence and presence of two inhibitors which proved useful. The addition of large amounts of yeast RNA as a bulk substrate was an obvious choice but proved less successful than the addition of 5'-CMP to a concentration of 1 mM. This nucleoside stopped

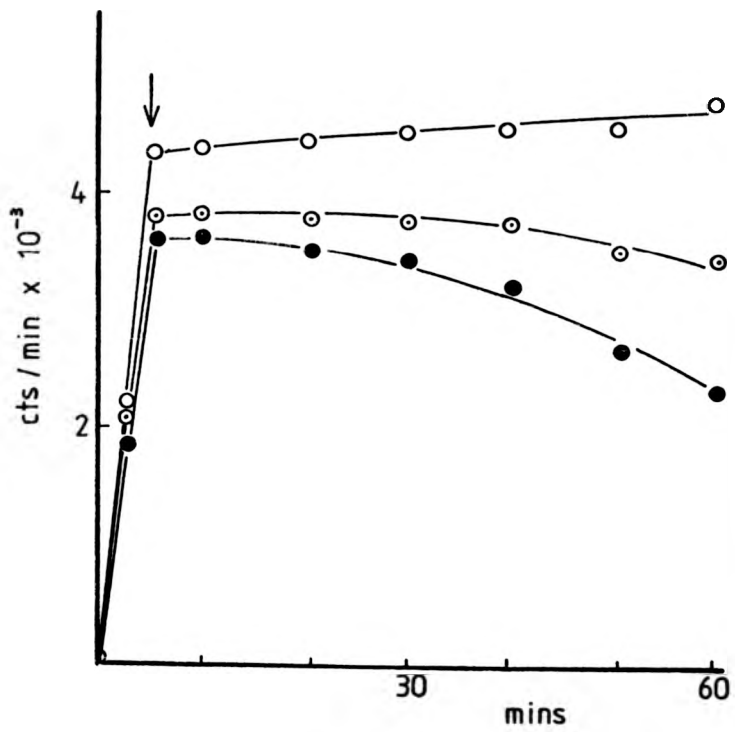


Figure 3.6 Assay for the presence of RNAase activity in XTC-2 nuclear incubations

Three sets of 250 μ l nuclear incubations were prepared. (1) The standard assay without CMP, (●). (2) The standard assay with CMP added to 1 mM, (○). (3) The standard assay with 50 μ g of yeast RNA added, (⊙). These were incubated for 5 minutes at 25^o C. After this time, unlabelled UTP was added to a final concentration of 1 mM and the assays were incubated for a further 55 minutes at 25^o C. 25 μ l samples were withdrawn and assayed for acid insoluble radioactivity at the times indicated.

all loss of acid precipitable material and, when added to the standard assay, resulted in a slight stimulation of [^3H] UTP incorporation. This is consistent with the observation that loss of TCA precipitable material was minimal in the presence of 5'-CMP. This concentration was used during all subsequent assays with XTC-2 nuclei.

RNA from nuclei incubated for 30 minutes in the standard way, with the addition of 5'-CMP, was extracted by the method of Parish and Kirby (1966) (see Chapter 2), treated with DNAase and subjected to polyacrylamide gel electrophoresis under denaturing conditions as described in Chapter 2, (under non-denaturing conditions the labelled RNA did not enter the gel and probably aggregated during extraction). Fig. 3.7 shows RNA prepared in this way compared with the RNA product in the absence of 5'-CMP and in the presence of 5'-CMP and 1 $\mu\text{g}/\text{ml}$ α -amanitin.

It is clear that in the absence of 5'-CMP the RNA product shows the characteristic profile of degraded RNA; a large accumulation of radioactivity in the low molecular weight region of the gel. In the presence of 5'-CMP the RNA made was much larger with peaks corresponding to 18S, 28S and 40S RNA. Addition of α -amanitin to 1 $\mu\text{g}/\text{ml}$, which inhibits only RNA polymerase II, resulted in a general lowering of the background around the 18S and 28S peaks. This is consistent with the known specificity of this enzyme (which is not involved in rRNA synthesis) and supports the idea that the peaks migrating with the ribosomal RNA markers are indeed rRNA. To support this view further, nuclei were incubated in the presence of 5'-CMP for 30 minutes and the extracted RNA fractionated on sucrose gradients (Chapter 2). The 18S and 28S peaks were pooled, as were the fractions between 6S and 18S. These radioactive RNAs were then hybridized on filters to fractions of *Xenopus* liver DNA which had been banded in caesium chloride density gradients. Hybridization of the labelled RNA to the heavy side of the main band DNA, where rDNA would be expected, would indicate the presence of rRNA. Hybridization to the main band would indicate the absence of rRNA. Fig. 3.8 shows that the 28S and 18S RNA contained large amounts of rRNA, whereas little, if any, was present in lower molecular weight regions. This result also suggests that little degradation of rRNA occurs in the nuclei. What has happened

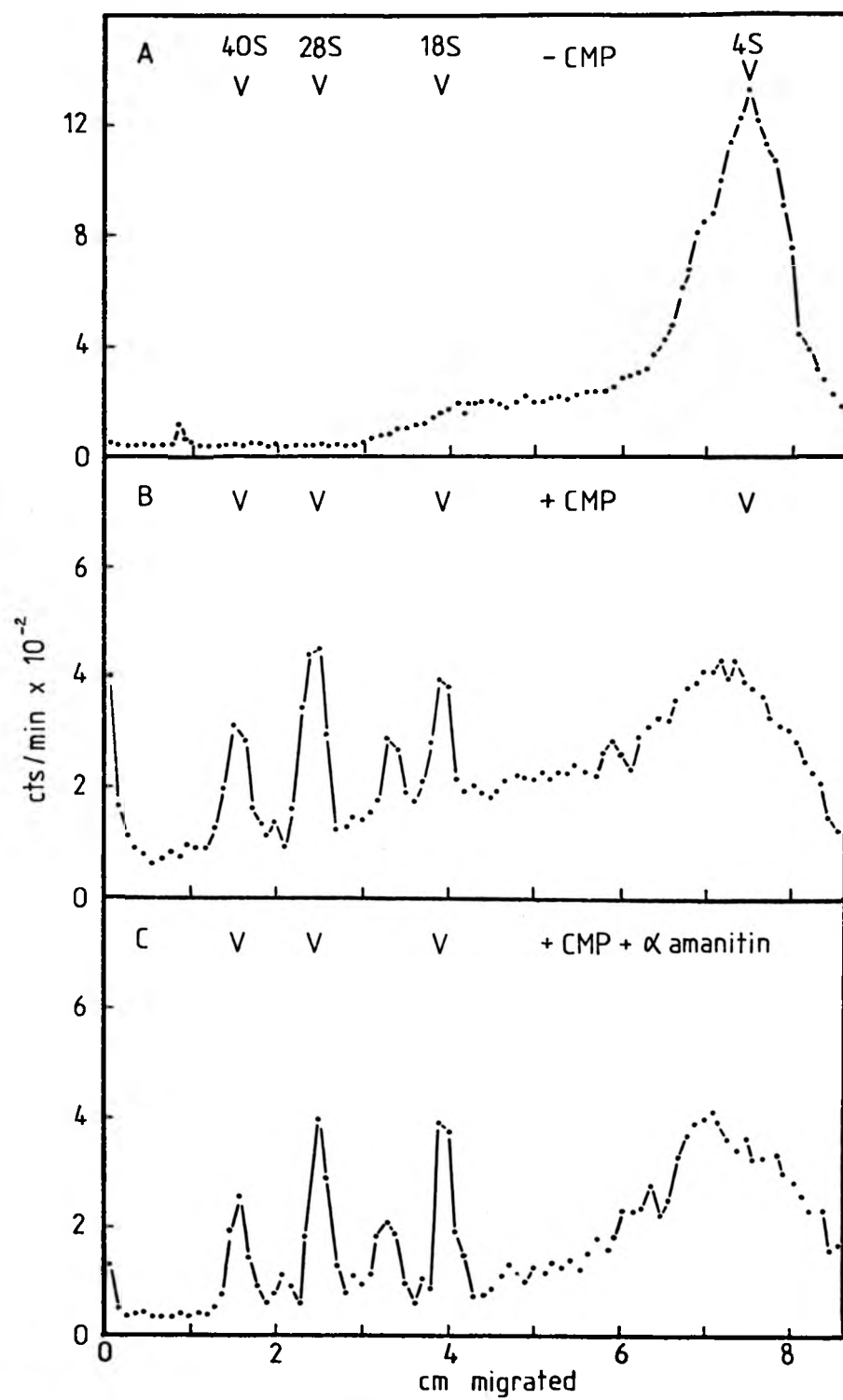


Figure 3.7 Gel fractionation of RNA products synthesised by XTC-2 nuclei in vitro.

RNA was extracted from 200 μ l incubates of XTC-2 nuclei, (A) the standard assay without CMP added, (B) the standard assay mixture with CMP added to 1 mM, (C) the standard assay mixture with CMP added to 1 mM and α -amanitin added to 1 μ g/ml. The extracted RNA was run on 2.4% RNA gels as described in Chapter 2, with BHK RNA as markers. Gels were scanned, sliced into 1 mm slices and assayed for the presence of radioactivity as already described (Chapter 2). Arrows indicate the position of BHK RNA markers.

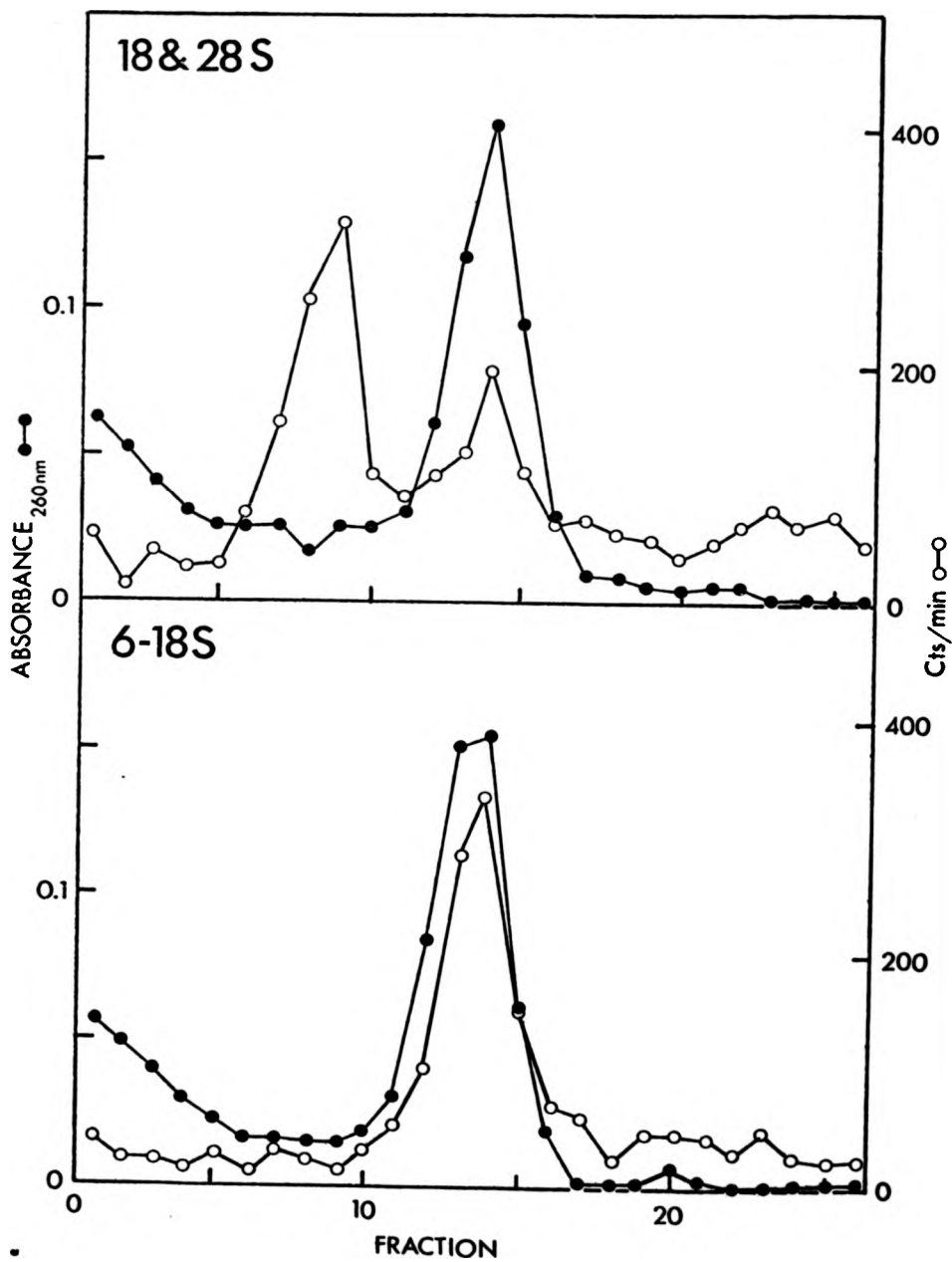
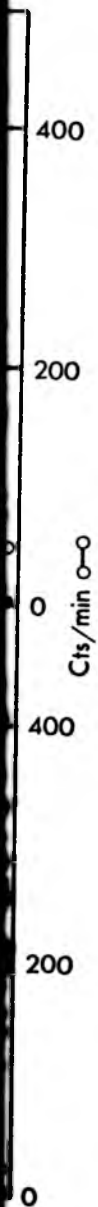


Figure 3.8 Hybridization of labelled 18S and 28S and 6-18S RNA synthesised by XTC-2 nuclei in vitro to total Xenopus DNA fractionated on a CsCl gradient.

RNA from 500 μ l incubates of XTC-2 nuclei incubated under standard conditions (+ CMP) was extracted and fractionated on sucrose gradients as described in Chapter 2. The 18S and 28S peak fractions were pooled, as were the fractions between 18S and 6S. These were then hybridized to CsCl-fractionated total Xenopus liver DNA on Millipore filters as described in Chapter 2. 50 μ g of DNA were loaded on each CsCl gradient. Input counts for the 18S + 28S hybridization were 8,500 cpm and for the 6S - 18S 6,500 cpm. (\bullet — \bullet) A_{260} ; (\circ — \circ) hybridized cpm.



to the transcribed spacer ? This may be a good system for finding out. The RNA product, as judged by gel electrophoresis, was not altered in profile by the addition of Mg^{2+} to 5 mM, indicating that there was no increase in integrity of the product in the presence of this divalent cation.

If the in vitro RNA product of XTC-2 nuclei is compared with RNA synthesised by XTC-2 cells labelled with [3H]-uridine, some similarities exist. Both show the presence of the rRNA peaks; however, the ratio of 28S to 18S in the nuclear product is not 2:1 as in the cellular product, i.e. there is more 18S than seen in vivo. The reason for this is not clear, but there may be residual levels of RNAase activity in the nuclei. Other alternatives are that incorrect initiation or processing is occurring in the nuclei or that elongation is inefficient. Essential factors may be lost during the nuclear preparation.

(f) Processing of the in vitro product

The rRNA peaks at 28S and 18S (Fig. 3.7B) suggest that some processing from the 40S rRNA precursor has taken place. This implies that the enzymes involved in the processing of RNA in the nucleus are not lost during nuclear isolation.

Another form of processing is the transport of the RNA out of the nucleus. To estimate how much transport of the in vitro transcription product from the isolated nuclei was occurring, standard assays with XTC-2 nuclei were prepared. In one the time course of [3H] UTP incorporation was followed as usual; in the other at each time point an aliquot was removed and layered over a pad of RM, and the whole spun for 2 minutes in the Beckman microfuge. This procedure efficiently separated the nuclear pellet from the incubation medium. Both the nuclear pellet and the supernatant were assayed for TCA precipitable material in the standard way. After 30 minutes incubation ^{200 μ l of} the remaining mixture was separated into pellet and supernatant as above, and the RNA extracted from both. This RNA was then fractionated on a 7-25% exponential sucrose gradient.

Fig. 3.9A shows both the total incorporation and the labelled material present in the separated nuclear pellet and supernatant at each time point. It is clear that the majority of the RNA is released from the nuclear pellet

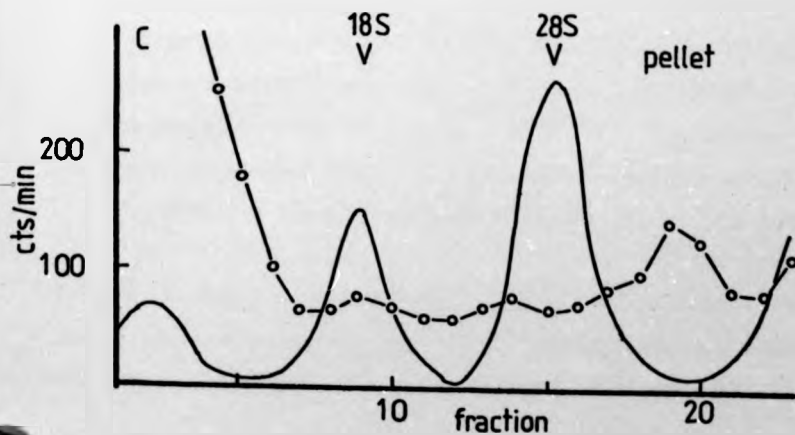
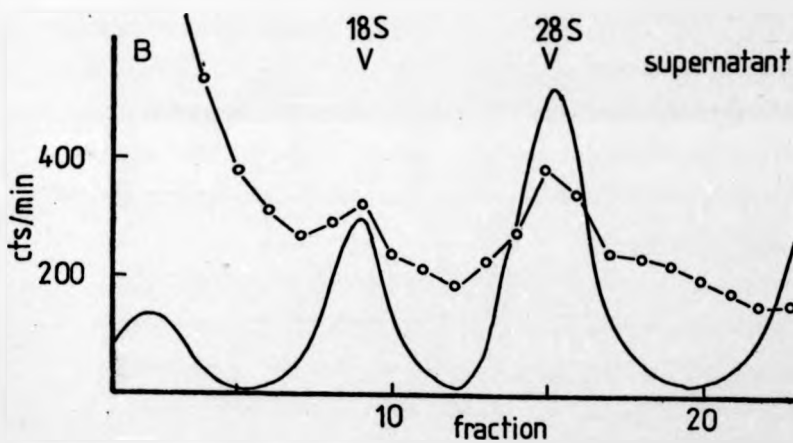
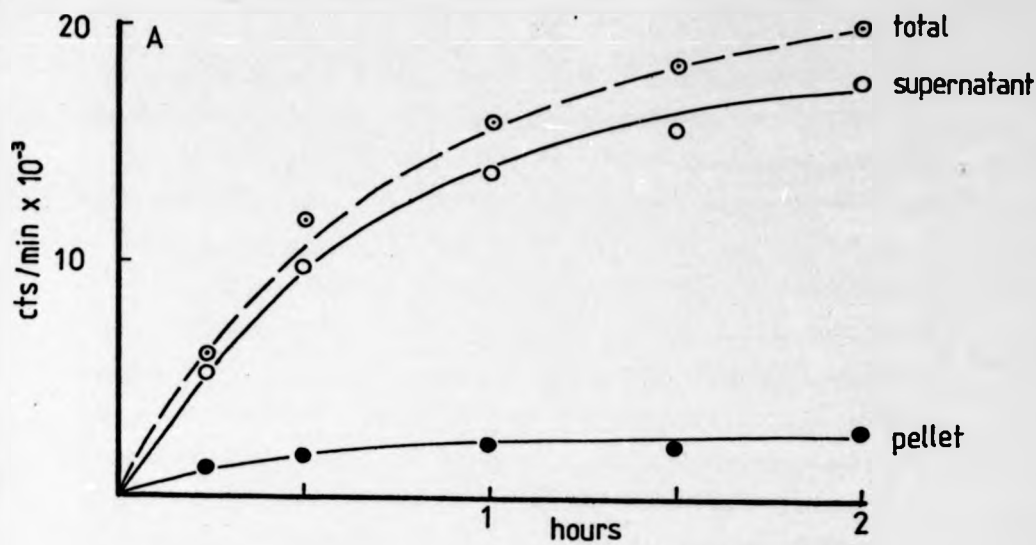
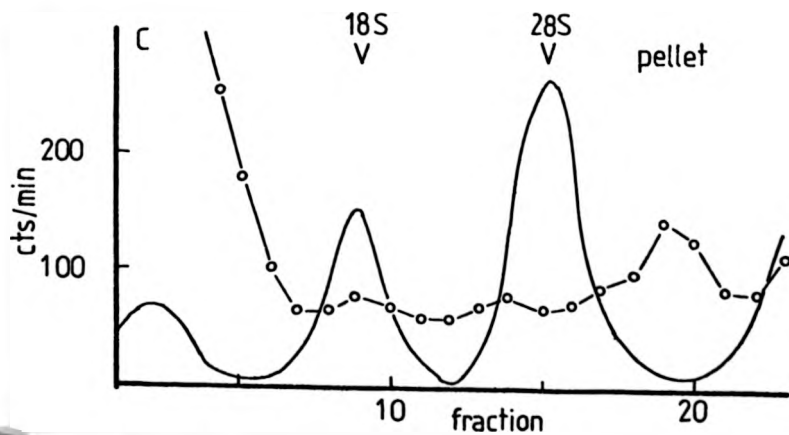
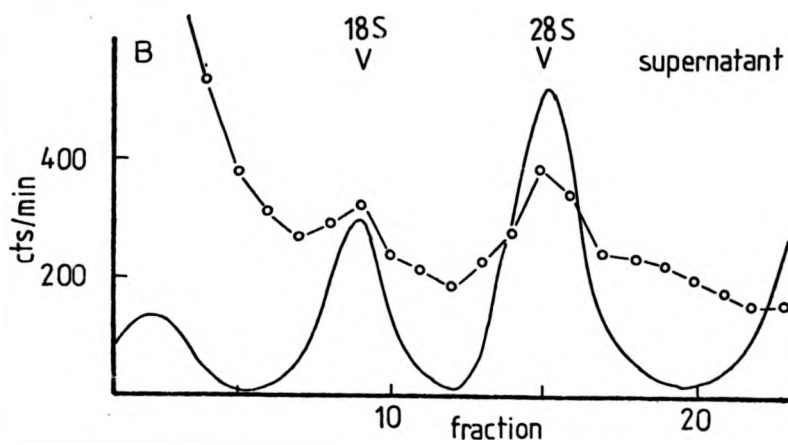
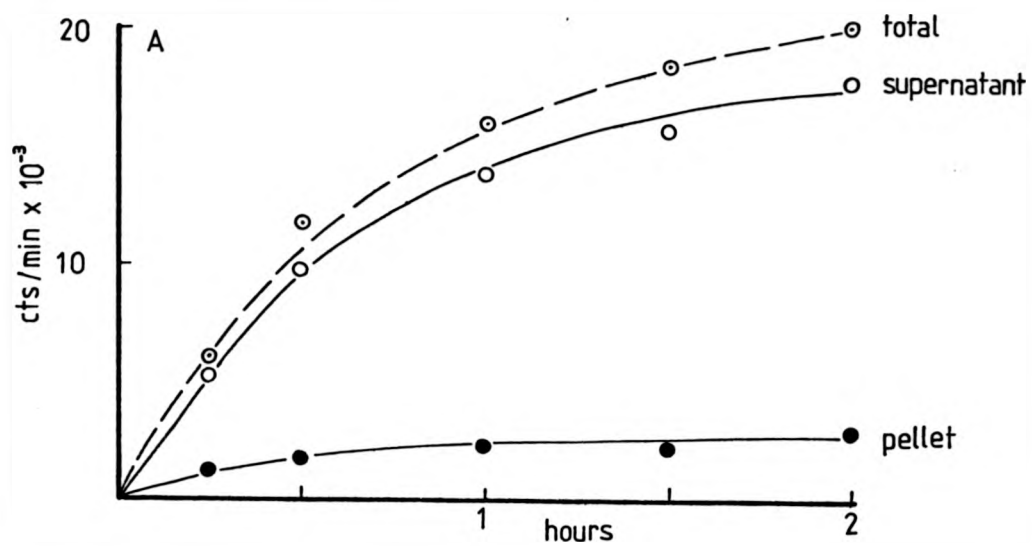


Fig. 3.9.



3.9

atant

Figure 3.9 Migration of RNA from XTC-2 nuclei incubated *in vitro*.

Standard 800 μ l XTC-2 nuclear incubations were prepared and incubated at 25^o C. 25 μ l samples were withdrawn at the times indicated in part (A). One set of samples were counted as usual, and this gave the total incorporation (\odot). The other set were layered over a 100 μ l pad of RM and centrifuged for 2 minutes in a microfuge to separate the nuclei from the incubation media. The resulting pellet and supernatants were counted separately to give an estimate of the RNA in the nuclei (\bullet) and the incubation medium (\circ).

200 μ l aliquots were withdrawn from each incubation after 30 minutes and separated into nuclear pellet and supernatant fractions as above. The RNA was extracted from these samples (100 μ g of BHK RNA was added), and the whole fractionated in 7-25% exponential sucrose gradients as described in Chapter 2. The gradients were analysed on an ISCO gradient fractionator, and samples of the fractions collected were assayed for the presence of radioactivity. (B) shows the radioactivity profile of the RNA present in the incubation medium and (C) the profile of labelled RNA present in the nuclear pellet. (—) in both cases is the A_{260} profile of the gradient resulting from the added BHK RNA.

3.9

during the incubation. It is interesting that the total amount of labelled material remaining in the nuclei remains fairly constant throughout the time course. Figs. 3.9B and 3.9C show the profile of the RNA present in the supernatant and pellet fractions respectively after a 30 minutes incubation. The gradients do not fractionate the low molecular weight species at all. It is clear that the 28S and 18S RNA species are in the main lost from the nuclei. In contrast, there is a predominance of higher molecular weight RNA ($\sim 40S$) in the nuclear fraction. This may be the rRNA precursor which has to be processed before release from the nuclei. If this is the case it is another indication that the nuclei are behaving in a physiological manner.

(g) Initiation of RNA synthesis in isolated XTC-2 nuclei

The final essential feature required for the regulatory factor assay system is that the isolated nuclei should initiate new RNA chains efficiently. This is not an easy thing to quantify; however, some estimate of whether initiation is occurring may be obtained by two methods.

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporation has been used to assay for initiation in isolated nuclei (Busiello and Di Girolamo, 1975; Chuang and Chuang, 1975). The $[\gamma\text{-}^{32}\text{P}]$ label should only be incorporated into the 5'-triphosphates of the RNA chain and thus only newly initiated chains should contain radioactivity. Initiation may best be monitored by hydrolysing the RNA under alkaline conditions and fractionating the nucleotides by PEI thin layer chromatography. The radioactivity should be in the pppGp region if initiation has occurred. More recently, Gilboa et al. (1977) have used $[\beta\text{-}^{32}\text{P}]$ labelled ribonucleoside triphosphate in the same way. This overcomes to a certain extent the problems of phosphorylase activity and removal of the ^{32}P during RNA capping (Wei and Moss, 1977). Fig. 3.10 shows the result of the initiation assay using $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in parallel incubations. Depending on the precursor used, radioactivity is present in the pppGp and the pppAp positions after 15 minutes incubation. Also present, as at zero time, is a large amount of unincorporated $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, persisting even after repeated washing of the RNA before hydrolysis. It is apparent that similar levels of initiation occur with GTP and ATP. Radioactivity did not

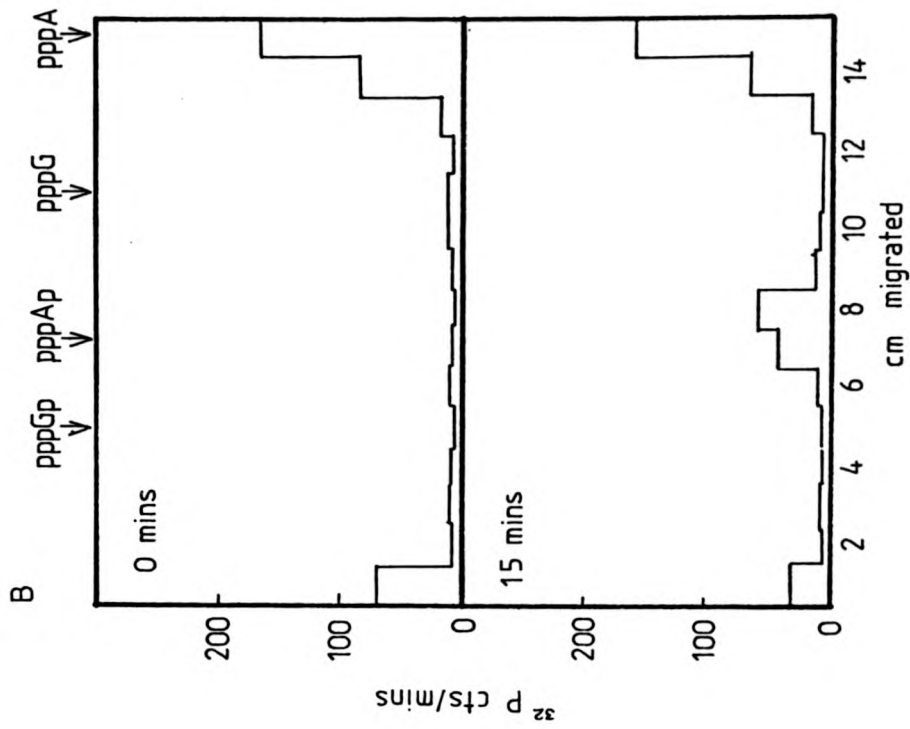
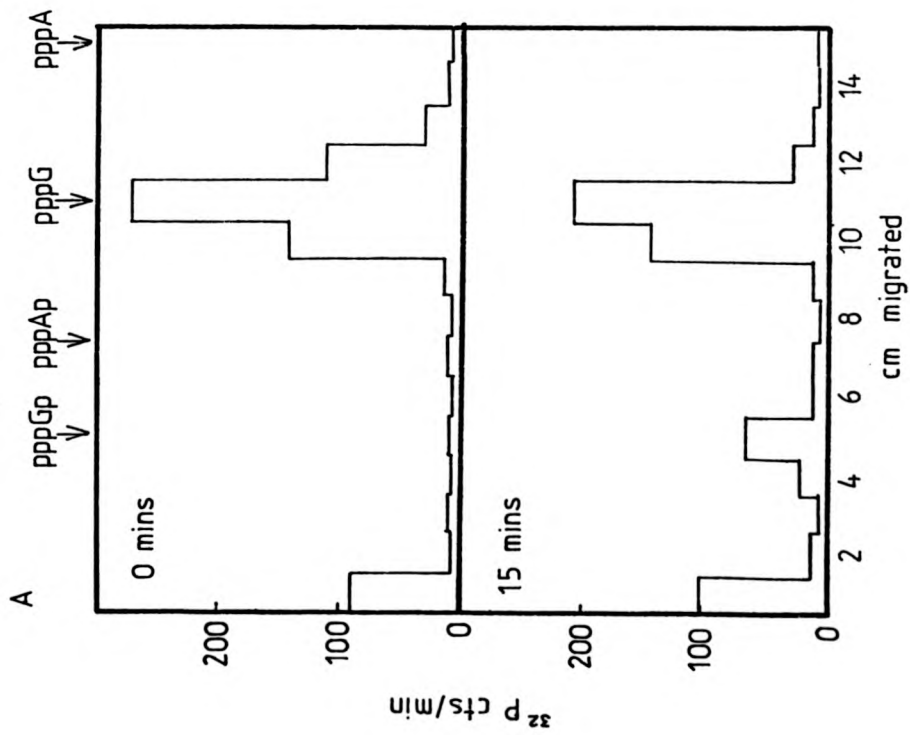


Figure 3.10 PEI-cellulose thin layer chromatography of hydrolysed
[γ - 32 P] GTP or [γ - 32 P] ATP labelled RNA from XTC-2 nuclei.

XTC-2 nuclei were incubated under standard conditions, except that the label was either [γ - 32 P] GTP or [γ - 32 P] ATP (50 Ci, in a 250 μ l incubate). 100 μ l was removed at 0 minutes and at 15 minutes to follow the course of the reaction. RNA was extracted from these aliquots and hydrolyzed at 37 $^{\circ}$ C overnight in 0.3 M KOH. The K $^{+}$ was removed by addition of 0.3 M HClO $_4$ and the supernatant analysed on PEI-cellulose using 1.6 M LiCl (Randerath and Randerath, 1967). Each track was cut into 1 cm sections and counted in PPO/POPOP/toluene scintillant supplemented with 9% soluene-350 and 1% water.

(A) shows the resulting profile at 0 and 15 minutes when the label was [γ - 32 P] GTP and (B) when the label was [γ - 32 P] ATP. Arrows indicate markers run in parallel.

cm migrated

cm migrated

appear in other tetraphosphate fractions, arguing strongly against terminal transfer of the γ - 32 P to pre-existing RNA molecules. For the reasons given above, this assay probably underestimates initiation and detects mainly initiation of small RNA molecules. It appears that initiation has occurred in the XTC-2 nuclei after 15 minutes incubation.

Another way of estimating what proportion of the RNA synthesis observed in XTC-2 nuclei is due to initiation of new RNA chains is by the use of rifampicin. Derivatives of rifampicin with highly hydrophobic side-chains, e.g. AF/013, completely inhibit initiation of calf thymus RNA polymerases at concentrations of 20-40 μ g/ml (Meilhac *et al.*, 1972). This has also been shown for *Xenopus* polymerases in isolated embryonic nuclei by Ramage and Barry (1975).

XTC-2 nuclei, incubated under standard conditions in the presence of 50 μ g/ml AF/013, were assayed for transcription in the normal way. Fig. 3.11 shows the time course of incorporation by XTC-2 nuclei in the presence and absence of the inhibitor. This shows clearly that the inhibitor almost completely eliminated incorporation after 20 minutes incubation. From this result it seems that the first 15-20 minutes of incorporation is partly the result of elongation of previously initiated RNA chains. After this time, initiation is required for continued RNA synthesis *in vitro*. That inhibition by AF/013 is not complete suggests either that continued elongation is occurring or that an RNA polymerase species is insensitive to the inhibitor.

(h) Discussion

The results described in this chapter indicate that the method of Marzluff *et al.* (1973) produces *Xenopus* nuclei which meet the criteria outlined in the introduction. In contrast Gurdon (1976) has shown that LL-BSA prepared nuclei remain morphologically healthy after injection into oocytes and Triton prepared nuclei deteriorate rapidly. LL-BSA nuclei were not used here because of their cytoplasmic contamination.

The RNA synthetic activity in the nuclei has been optimised and characterised to some extent. The optimal conditions do not vary significantly from those of other workers except for the absence of Mg^{2+} and the slightly lower KCl concentration. All three forms of DNA

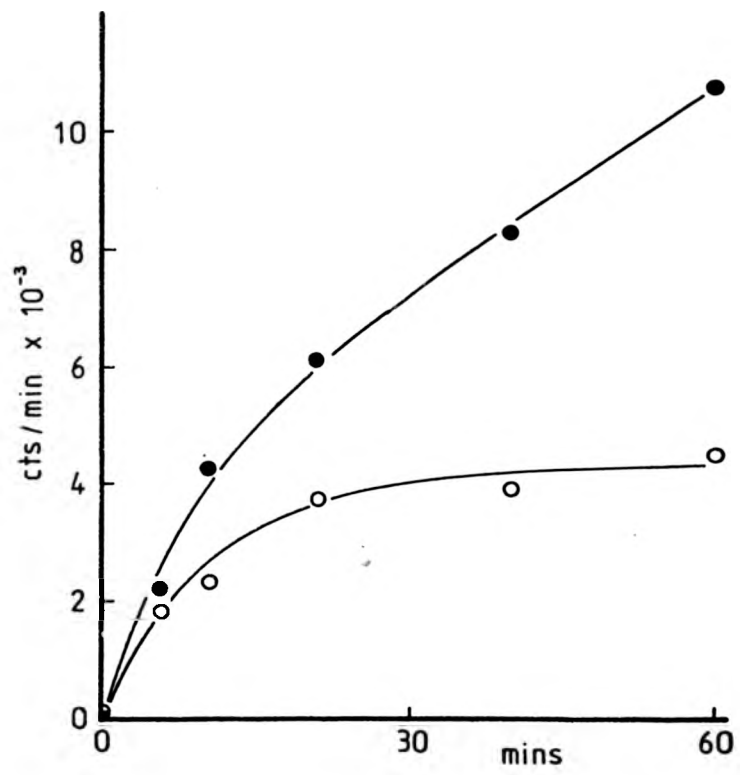


Figure 3.11 Time course of incorporation of [³H] UTP by XTC-2 nuclei in the presence and absence of AF/013

Duplicate 200 μ l nuclear incubations were prepared with XTC-2 nuclei in the absence (●) and presence (○) of 50 μ g/ml AF/013. 25 μ l samples were removed and counted at each time point in the usual way.

dependent RNA polymerases are active in the isolated nuclei. XTC-2 nuclei synthesise RNA initially at the rate of about 5 picoles of UMP incorporated/ μg of DNA_{in 10 minutes} which corresponds to a rate of 0.4 pg/nucleus/hour; after 5 minutes the rate falls to 0.084 pg/nucleus/hour.

Davidson (1976) calculated, from the data of others, that a Xenopus neurula cell makes 0.6-1.2 pg of heterogeneous RNA per hour, total RNA synthesis perhaps being double this amount. Gurdon *et al.* (1976) have calculated that HeLa cells synthesise about 2 pg of stable RNA/hour. The rates observed for the XTC-2 nuclei in vitro are thus not greatly different from these calculated rates of synthesis in vivo.

RNA synthesised by XTC-2 nuclei in vitro in the presence of 5'-CMP, an inhibitor of RNAase, was found to be heterogeneous in size with defined peaks of radioactivity comigrating with rRNA markers. These peaks were shown to hybridize to rDNA. This observation and data on the species of RNA being transported from the nuclei suggest that the ability to synthesise, process and transport RNA normally has been retained to some extent by the isolated XTC-2 nuclei. Other workers have reported the normal synthesis of specific genes by isolated nuclei (see Chapter 1, this thesis): examples include the ribosomal genes in Xenopus nuclei (Reeder and Roeder, 1972), the 5S and tRNA genes in HeLa and myeloma cells (Price and Penman, 1972; Marzluff *et al.*, 1974), and the immunoglobulin kappa light chain gene, also in myeloma nuclei (Smith and Huang, 1976). Some of these authors have shown that this synthesis is a result of initiation of new RNA molecules and not just elongation of half-formed chains initiated in vivo. The data described in this chapter strongly suggests that initiation of new RNA chains is occurring in nuclei isolated from XTC-2 cells.

Once the isolated nuclei had been characterised, as described in this chapter, the main object of the work could be approached, that is combining nuclei with cell extracts from Xenopus oocytes, eggs and early embryos. This is the subject of the next chapter.

Chapter 4

Addition of *Xenopus* cell extracts to isolated XTC-2 nuclei

(a) Introduction

The data compiled in Chapter 3 is, in general, consistent with that reported by other workers (Reeder and Roeder, 1972; Marzluff *et al.*, 1973). It suggested that the RNA synthesising system based on isolated nuclei had the characteristics required of an assay for cytoplasmic factors regulating RNA synthesis.

This chapter deals with the initial experiments coupling cell extracts to isolated XTC-2 nuclei. At first, only cell extracts from *Xenopus laevis* oocytes and unfertilised eggs were assayed for their effect on RNA synthesis. The reasoning here was that these two cells represent extremes in terms of their rates of RNA synthesis; oocytes actively synthesise RNA and eggs are relatively inactive (see Chapter 1, this thesis and Gurdon (1974) for a review). These two extracts were used to optimise the assay system such that transcription was stimulated when the cell extract was derived from oocytes but inhibited when it was derived from unfertilised eggs. To some extent the egg was used like an RNA-deficient mutant to establish conditions under which nuclei respond to additives in the same way that they do naturally, or when they are injected into unfertilised eggs.

A final assay system for regulatory factors, described in the first half of this chapter, used these optimal conditions with a preincubation step which allows the nuclei to be incubated in the normal way after being exposed to a particular cytoplasm.

The second half of this chapter is concerned with characterising the effects of the cell extracts on RNA synthesis in isolated nuclei and eliminating some trivial causes of these effects. In all the optimisation and characterisation experiments, the 'oocyte extract' used was an extract of Stage 5 and 6 oocytes.

(b) Preparation of cell extracts

The method of preparing the cell extracts, as described in Chapter 2, is essentially to obtain a 30,000 xg supernatant from whole cells. It involved gentle homogenisation of the oocytes, eggs or embryos essentially

in their own volume with minimal dilution. The homogenate was cleared by centrifugation at 2,500 x g for 30 minutes at 4°C and the clear supernatant separated from the lipid, pigment and yolk. The supernatant was subsequently recentrifuged at 30,000 x g_{max} for 30 minutes at 4°C and this supernatant was used as the cell extract in the assays. It gave about 1-2 µl of extract per oocyte, egg or embryo. Given that an oocyte is 1 µl and about 40% aqueous phase (Bonner, 1975a), this means that the extract was a 2.5-5-fold dilution of the original cytosol.

This method is more gentle than that described by Benbow and Ford (1975) in their cell free assay for factors regulating DNA synthesis in Xenopus early development. It closely resembles that used by Laskey et al. (1977) who showed that a similar extract from Xenopus eggs was capable of assembling SV40 DNA into chromatin. As shown later in this chapter, the extracts prepared by the above method were essentially RNAase free, this being an important feature of the assay.

(c) RNA synthesis by nuclei incubated with cell extracts

Initially nuclei were incubated in the standard way, except that a 100 µl nuclear incubation contained 40 µl of cell extract derived from oocytes or unfertilised eggs. This corresponded to about one oocyte or egg equivalent of cell extract per 5×10^4 nuclei (i.e. an early neurula stage). The time course of RNA synthesis by these nuclei in the presence of oocyte and egg extracts compared with a control (containing Column buffer instead of extract) was followed by tracing [³H]-UTP incorporation as usual. Fig. 4.1 shows the results of this experiment over a 2 hour period. Oocyte extract stimulates the rate of RNA synthesis and allows incorporation to continue at a constant rate for 2 hours (in fact for at least 4 hours, data not shown), whereas the control begins to plateau after an hour. Egg extract is somewhat inhibitory and the plateau effect is more pronounced. Because the control and egg incubates plateau, the stimulation produced by the oocyte extract becomes more pronounced after one hour of incubation.

Thus the cell extracts do influence the rates of RNA synthesis in isolated nuclei. The next step in developing this assay was to introduce a preincubation step. The time course in Fig. 4.1 suggested that the effect was most pronounced after one hour's incubation. Preincubation and

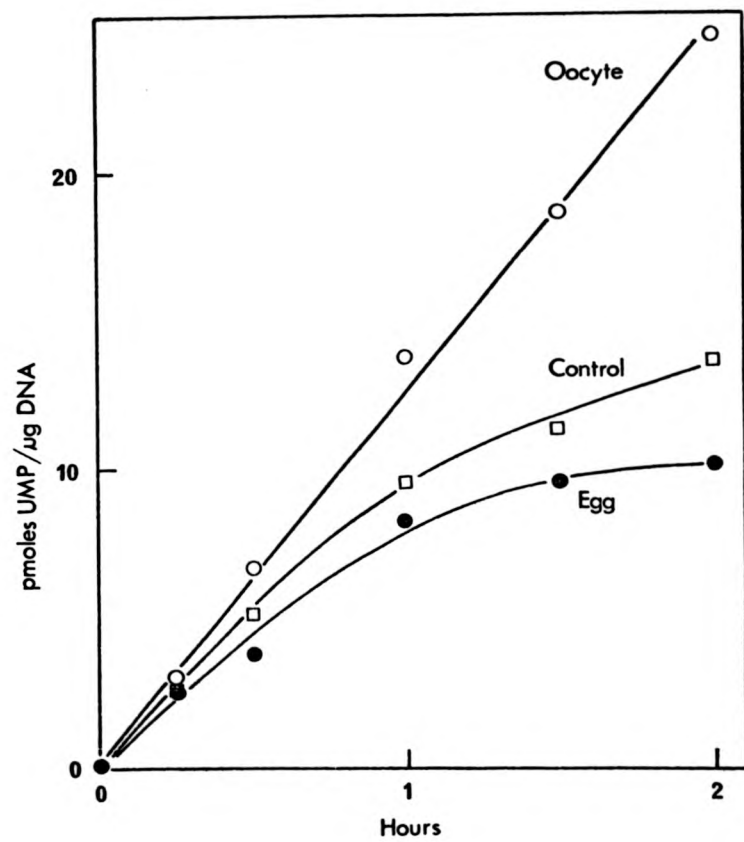


Figure 4.1 Time course of incorporation of [³H] UTP by XTC-2 nuclei in the presence of oocyte and egg extracts.

XTC-2 nuclei were incubated under standard assay conditions with oocyte (O), egg (●) and Column buffer (□) without preincubation as described in Chapter 2. 400 μl incubates were prepared and duplicate 25 μl samples were counted at the times shown.



subsequent labelling may thus give a quicker assay with as large an effect on the synthetic rate in the nuclei. This could have been achieved in two ways: (1) by incubating the nuclei with the cell extracts, adding labelled precursors after a given time and then following its incorporation and (2) by incubating the nuclei as in (1), then removing them from the cytoplasm by centrifugation and assaying for transcription in the standard way. Method(2) has the disadvantage that the effect may depend on continued presence of the extracts but the advantage that if the effect persisted, many trivial explanations of its cause could be eliminated. Both of these methods were tried with 1 hour preincubations. Very little difference in the incorporation rates between the oocyte and egg preincubated nuclei were obtained. The difference between these and the original assay was that UTP, which was added after 1 hour, was not present during preincubation. When unlabelled UTP was added to the normal level, the normal effect of the extracts was apparent in both preincubation assays. Method (2), that of preincubating the nuclei in extracts with all four ribonucleotide triphosphates and then removing the nuclei and assaying as usual, was used hereafter, unless otherwise stated.

It is interesting that the addition of all four triphosphates to the assay is necessary for the effect of the extracts to be pronounced. This will be discussed later in more detail; however, it does suggest that RNA synthesis is necessary for the effect. It would seem that the extracts themselves do not contain enough triphosphates or at least available triphosphates, to produce the effect. Preincubation and reisolation also suggests that the continuous presence of the extracts in the assay medium is not necessary. These experiments also rule out, to some extent, trivial explanations for the observed effects, including different triphosphate concentrations and the ionic composition of the extracts.

(d) Optimisation of the assay for regulatory factors

This assay system for regulatory factors was optimised for preincubation time, salt and triphosphate concentrations in the preincubation step. It was optimised on the basis of maximising the difference between oocyte and egg treated nuclei as already explained. After preincubation under varying conditions, the nuclei were removed in each case by

centrifugation and assayed for RNA synthesis under the optimal conditions for the standard assay (see Chapter 3).

The period of preincubation was investigated first, nuclei being preincubated for varying times in the presence of the different extracts and in conditions as for the standard nuclear assay. The nuclei were subsequently labelled for 30 minutes in the normal way, the final incorporation of [^3H] UTP being plotted against the time of preincubation, as shown in Fig. 4.2. It is clear that the difference between the oocyte and the egg treated nuclei increases with the preincubation time. For convenience and because a 2-fold effect showed sufficient difference to be workable, 1 hour's preincubation was routinely used. Another reason for not using prolonged incubation times was that it became difficult to exclude bacterial contamination. The control in this case was nuclei preincubated in the same conditions but with Column buffer replacing the cell extracts. Nuclei preincubated with egg extract were slightly inhibited compared with control nuclei.

Also included in Fig. 4.2 is the result of a similar experiment with oocyte extracts but preincubated in the absence of CTP. The same result was found for all four nucleotide triphosphates. This clearly shows the need for triphosphates during the preincubation, as the effect of the extract is lost in the absence of CTP.

The preincubation conditions were finally optimised for the triphosphates and salt concentrations. As before, these conditions were varied (using the standard assay conditions as a starting point) during the preincubation. The final assay conditions after preincubation were as for the standard assay, labelling being allowed to proceed for 30 minutes.

Fig. 4.3 shows the results of the optimisations. All four unlabelled triphosphates were varied as indicated during the preincubation and Fig. 4.3A shows the result of this with oocyte and egg extracts. As noted before, triphosphates are required during the preincubation for the stimulation by the oocyte extract to occur, and the difference between oocyte and egg to be apparent. There is a sharp increase in the difference between the two extracts up to 0.02 mM; little further increase in the difference at higher concentrations. Triphosphate concentrations of 0.02 mM were used in all further assays of this nature.

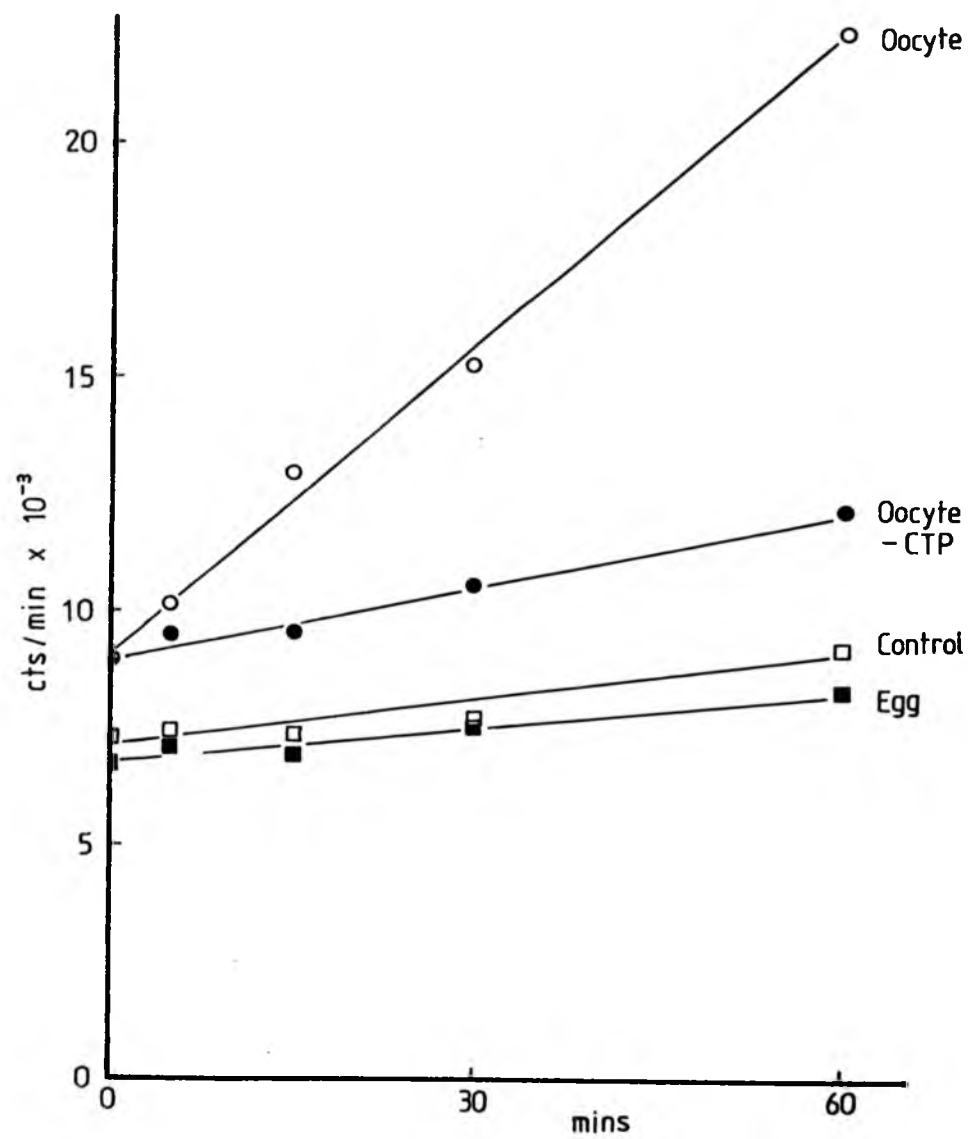


Figure 4.2 Optimisation of preincubation time using oocyte and egg extracts .

XTC-2 nuclei were incubated at 25^o C under the standard assay conditions in the presence of all four unlabelled triphosphates and oocyte extract (○), egg extract (■), Column buffer (□) and oocyte extract in the absence of unlabelled CTP (●). Samples were taken at the times indicated, the nuclei were removed by centrifugation and assayed for RNA synthesis as usual with [³H] UTP. Labelling was for 30 minutes at 25^o C.

yte

yte
ITP

ntrol

g

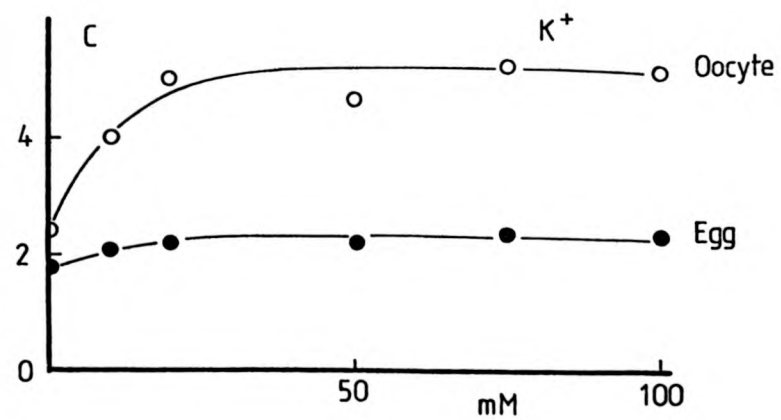
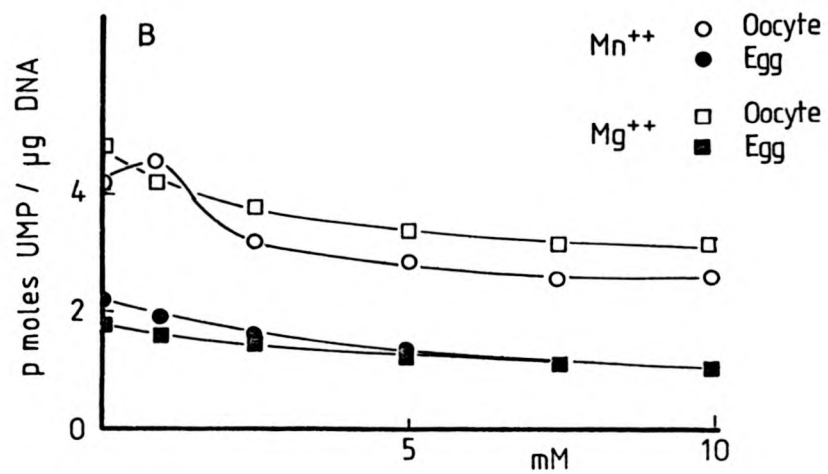
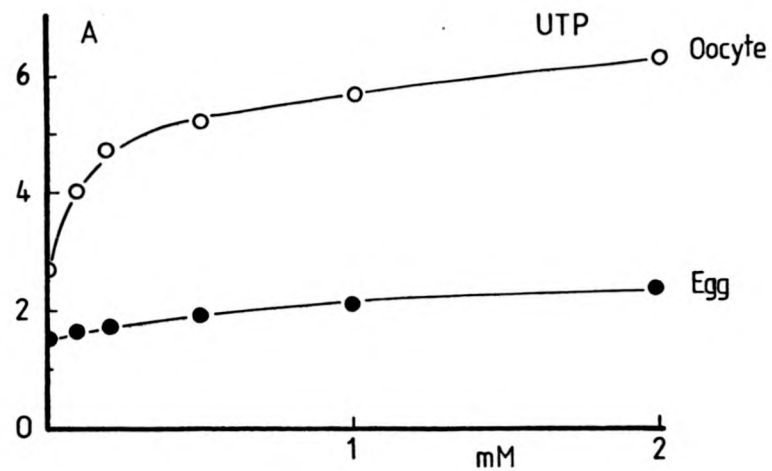


Figure 4.3 Optimisation of the preincubation condition using oocyte and egg cell extracts

XTC-2 nuclei were preincubated with oocyte and egg cell extracts in 0.4 mM UTP, GTP, CTP and ATP, 1 mM Mn^{2+} and 100 mM K^+ except when varied as specified in the figure. Other constituents were as indicated in Chapter 2. After 1 hour's preincubation, the nuclei were removed and assayed for RNA synthesis in the usual way with [3H] UTP. Labelling was for 30 minutes at 25^o C.

(A) Preincubation with oocyte (○) and egg (●) extracts and all four triphosphates at the concentration indicated. (B) Preincubation with oocyte (□) and egg (■) extracts with Mg^{2+} at the concentration indicated. Preincubation with oocyte (○) and egg (●) extracts with Mn^{2+} at the concentrations indicated. (C) Preincubation with oocyte (○) and egg (●) extracts with K^+ at the concentration indicated.

Optimisation involving Mg^{2+} (0-10 mM), Mn^{2+} (0-10 mM) and K^+ (0-100 mM) are shown in Figs. 4.3B and 4.3C. The results may be briefly summarised. Mg^{2+} had little effect on the oocyte:egg difference, and was omitted from further assays. Mn^{2+} was slightly optimal at 0-1 mM, 1 mM being used in subsequent assays. It is interesting to note here that these Mn^{2+} conditions are probably unphysiological. Colman and Gadian (1976) found that the NTP in oocytes and eggs are nearly all bound to Mg^{2+} and Ca^{2+} , there being little Mn^{2+} bound NTP (0.4% of the total). This may suggest that Mg^{2+} is more physiological and the conditions in which the nuclei are incubated are abnormal in terms of the divalent cation. Mn^{2+} was used here because it gave the largest difference in transcription induced by oocyte and egg extracts. Optimisation of the K^+ concentration was more interesting. K^+ concentrations of 20 mM or over were required for maximum difference between oocyte and egg treated nuclei. Below this concentration the difference was not apparent. This requirement for salt may be explained in many ways. Butterworth *et al.* (1971) showed in isolated chromatin that salt is required for the initiation of RNA polymerase, and this may explain the requirement in this case.

Experiments were carried out involving purifying the nuclei between the preincubation and labelling steps by layering the preincubation mix, after 1 hour at 25^o C, over a small pad of RM. The whole was then centrifuged in a Beckman microfuge, the nuclear pellet being assayed as usual. This precaution did not alter the difference between oocyte and egg preincubated nuclei. However, the purified nuclei showed slightly better overall incorporation of radioactivity in the final assay. This was probably due to the carry-over of unlabelled UTP from the preincubation in the absence of this step, which lowered the specific activity of the [³H] UTP. The difference was not great and the step was omitted from the assay.

The time course of incorporation by control, oocyte and egg preincubated nuclei using the optimised conditions (see above and outlined in Chapter 2) is shown in Fig. 4.4. The difference between the nuclei preincubated in the different extracts was immediately apparent. A

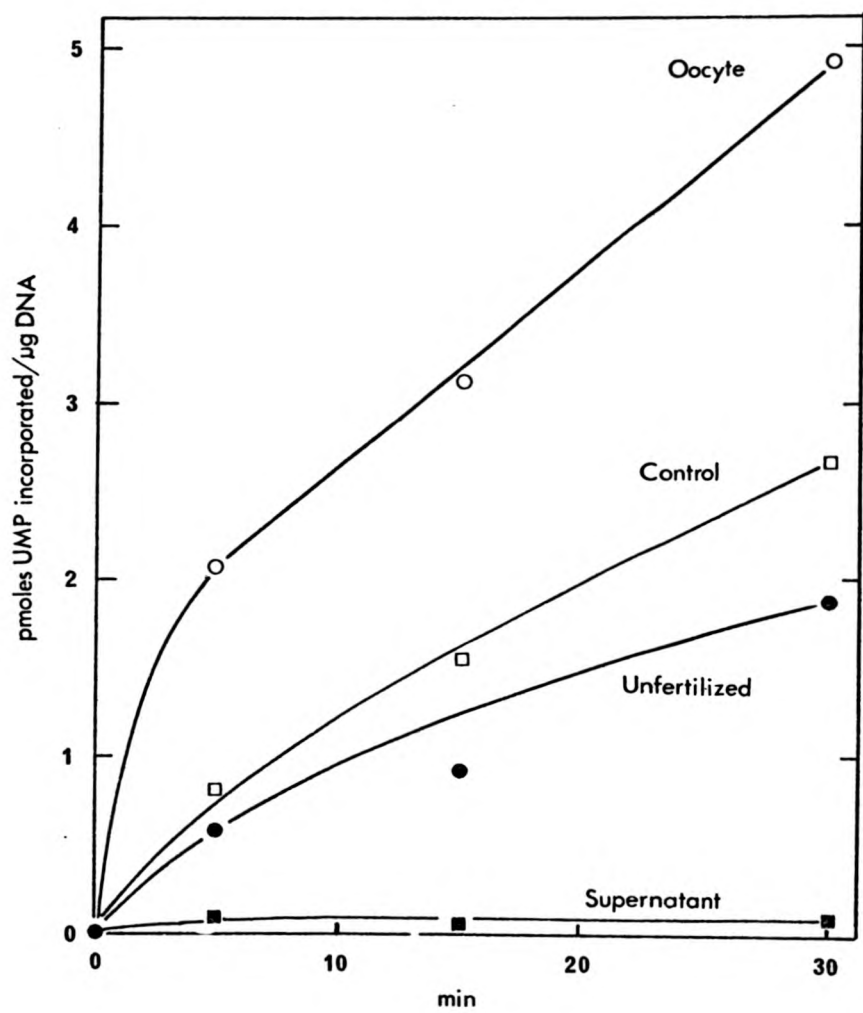


Figure 4.4 Timecourse of [³H] UTP incorporation by XTC-2 nuclei after preincubation in the presence of oocyte and unfertilised egg cell extracts.

XTC-2 nuclei were preincubated for 1 hour at 25^o C under the optimised conditions in the presence and absence of cell extracts. The nuclei were removed by low speed centrifugation and assayed for RNA synthesis as usual with [³H] UTP. (○), oocyte treated nuclei; (●), unfertilised egg treated nuclei; (□), Column buffer control. The supernatants from the preincubation step were also incubated in the presence of triphosphate and [³H] UTP and the acid insoluble counts measured (■).

comparison of Figs. 4.1 and 4.4 shows that the stimulation by the oocyte extract was the same almost immediately after preincubation as after 2 hours without preincubation. Also shown in Fig. 4.4 is the incorporation, in the presence of unlabelled CTP, GTP and ATP and [^3H] UTP, by the supernatants which were removed after preincubation. There was no significant incorporation in any of these. This rules out the possibility of incorporation of [^3H] UTP into acid precipitable material by the oocyte extract under non-preincubation conditions. More importantly, it reduces the possibility that large scale lysis of the nuclei was occurring during the prolonged incubations. Cell extracts prepared as described here contain polymerase activity (see below and Chapter 7). If the nuclei have lysed, releasing chromatin or DNA to any great extent, the polymerases may be expected to be active on these templates in the preincubation supernatants. The lack of incorporation rules this out and the conclusion is confirmed by counting the nuclei before and after incubation. Very little reduction in the number of nuclei was observed. Thus most of the incorporation of [^3H] UTP occurred in the nuclei which all remained at least superficially undamaged.

Other experiments showed that the extracts prepared for these experiments could be stored for prolonged periods at -70°C without altering their effect on isolated nuclei. In general extracts were prepared and stored in $500\mu\text{l}$ aliquots at -70°C . If part of an aliquot remained, it was discarded and not refrozen for further use.

(e) Experiments to control against trivial effects of the cell extracts on isolated nuclei

Some possible trivial effects of cell extracts on nuclei were examined. These included: (1) the addition of any protein to the nuclei may alter their activity in some way; (2) the cell extracts may contain different pools of triphosphates, in particular UTP, which may alter the specific activity of the radioactive precursors; (3) ionic constituents of the extracts may produce the effects observed; (4) the extracts may contain varying levels of available or active RNA polymerases. All these possibilities are to some extent dealt with below and in general may be eliminated by experiments described later in this thesis.

It is unlikely that the extracts from oocytes and eggs should differ sufficiently in their protein content to give the results described so far. As mentioned in Chapter 3, the addition of BSA to the assays had little effect on transcription by isolated nuclei. However, the extracts were assayed for their protein content (see Chapter 2) by the method of Lowry *et al.* (1951). Both oocyte and egg cell extracts were found to contain between 15 and 20 μg of protein per oocyte or egg equivalent. A concentration curve for the two extracts was obtained using the pre-incubation assay with a constant nuclear input in parallel with a similar range of added BSA. Fig. 4.5 shows that the stimulation by oocyte extracts is not merely due to protein content. The concentration curve of the oocyte extract shows a simple dilution effect on the activity of the nuclei. There is no evidence for a threshold effect.

The problem of pool sizes in the two extracts may be tackled in a number of ways. Woodland and Pestell (1972) have directly measured the ribonucleoside triphosphate amounts in oocytes and eggs. They found little difference between the two cell types, suggesting this is not the reason for the observed differences in RNA synthetic activity. More recently, Colman and Gadian (1975) have measured the NTP pools in oocytes and eggs by NMR spectroscopy. Although their estimates were higher (3-4.5 nmols/cell NTP compared with about 1.5-2 nmols), again little difference was observed between oocytes and eggs. These results imply that there should be little difference between oocyte and egg cell extracts in terms of their NTP pools. To eliminate the possibility of pool sizes or ions causing the difference between the extracts, the simplest control is to dialyse the extracts. Oocyte and egg extracts were thus dialysed extensively against Column buffer. The dialysed and undialysed extracts were then assayed in the nuclear assay and the results are shown in Table 4.1. Dialysis has no effect on the activity of the extracts and the difference between them in this assay. This suggests that dialysable molecules are not involved in the oocyte stimulation and that macromolecules are involved. These experiments do not, however, rule out a pool-size.

Another possible cause of the nuclear stimulation is that the oocyte extract contains more active RNA polymerase molecules than the egg

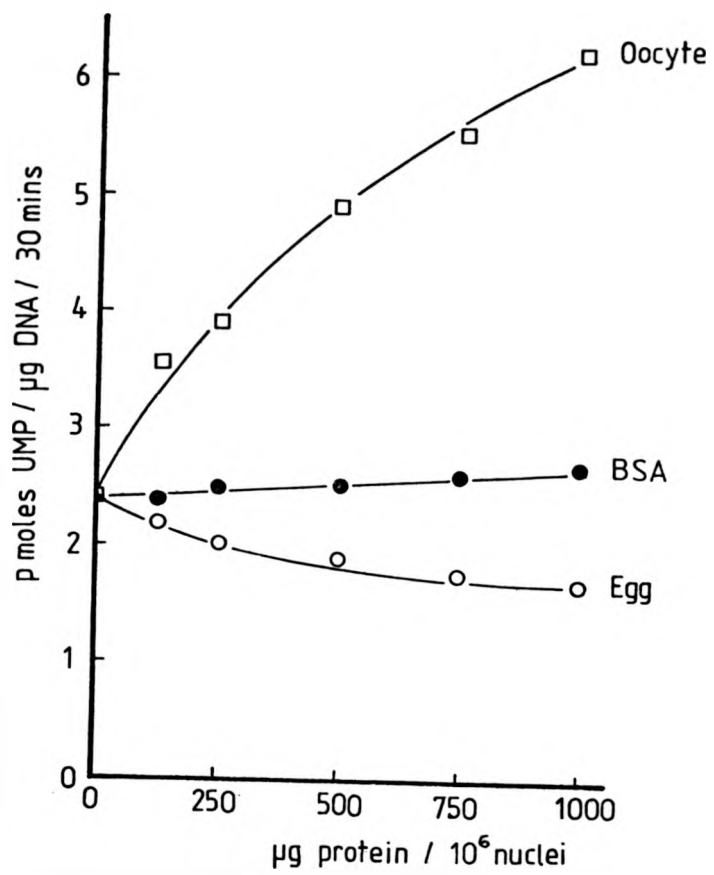


Figure 4.5 Concentration curve of protein present in the preincubation of XTC-2 nuclei using oocyte and egg cell extracts and BSA.

XTC-2 nuclei were preincubated in the standard way with oocyte and egg cell extracts and BSA present at the concentration indicated. After 1 hour's preincubation at 25⁰ C the nuclei were removed and assayed for RNA synthesis with [³H] UTP in the usual manner. (□) oocyte treated nuclei; (○) egg treated nuclei; (●) BSA treated nuclei. Protein concentrations were estimated by the method of Lowry et al. (1951).

Table 4.1

The effect of dialysis on the properties of cell extracts in the nuclear assay

	<u>Undialysed extract</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>	<u>Dialysed extract</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>
Control	2382	1	2983	1
Oocyte	5635	2.3	5715	2
Egg	2355	0.98	2237	0.75

Dialysis of the cell extracts was for 16 hours at 4° C against 2 changes of 1 litre of Column buffer . An aliquot of each extract was kept undialysed at -70° C. The dialysed and undialysed extracts were assayed in duplicate 50 µl standard nuclear assays. Column buffer replaced the extracts in the control.

extract. Roeder (1974) has shown that Stage 6 oocytes and unfertilised eggs contain very similar total and individual RNA polymerase activities. As shown later, in this chapter and in Chapter 7, there is RNA polymerase activity in the cell extracts. To partially eliminate the involvement of RNA polymerase in the oocyte stimulation, RNA polymerases were added to control and extract treated nuclei during the preincubation step. This was done initially using purified E. coli RNA polymerase as this may be obtained at a higher specific activity than amphibian polymerases. The same experiment was later repeated with partially purified RNA polymerase from Xenopus laevis oocytes. Both results are shown in Table 4.2. Clearly, the addition of RNA polymerase does not affect transcription in control nuclei and does not alter the difference between the oocyte and egg treated nuclei. The source of the polymerase did not alter the result. Addition of either polymerase to the standard nuclei assay (i.e. the RNA polymerase is present during the labelling incubation) also does not alter the incorporation. These results suggest that exogenous RNA polymerases are not involved in the extracts' effects and do not enter the nuclei. They may also imply that the nuclei remain relatively intact during the incubations. Lysed nuclei would release chromatin which would act as a ready template for added RNA polymerases.

The result that added RNA polymerase does not alter transcription in the isolated nuclei contradicts the result of Sklar and Roeder (1977) who found that nuclear RNA synthesis was stimulated by exogenous polymerases. Their result has recently been challenged by Hagopian and Ingram (1978) who suggest that the stimulation is due to the presence of free chromatin in the nuclear preparation.

(f) Identification of the activity in cell extracts

Some simple experiments may be carried out with these crude extracts to give some indication of the nature of the factors involved. It might be asked if the egg extract contains factors which inhibit the action of stimulating agents. This was investigated by assaying mixtures of oocyte and egg extracts, a sort of cell hybrid experiment in vitro. Fig. 4.6 shows that egg cytoplasm acts like a simple diluent of the oocyte

Table 4.2

Addition of E. coli and Xenopus RNA polymerases to extract treated nuclei

(a) Addition of E. coli RNA polymerase (Boehringer)

	<u>-RNA poly</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>	<u>+RNA poly</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>
Control	2686	1.0	2626	1.0
Oocyte	4818	1.8	5201	1.9
Egg	2518	0.9	2826	1.1

(b) Addition of Xenopus laevis RNA polymerases (from ovary)

	<u>-RNA poly</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>	<u>+RNA poly</u> <u>(counts/minute)</u>	<u>Stimulation</u> <u>over control</u>
Control	2519	1.0	2643	1.0
Oocyte	5029	2.0	5148	1.9
Egg	2362	0.9	2415	0.9

Either 1 unit of E. coli RNA polymerase or 0.2 units of Xenopus polymerases, were added to 50 μ l of assay mixture during the preincubation step of the assay. The nuclei were then assayed under standard conditions. Control incubation contained Column buffer instead of extract.

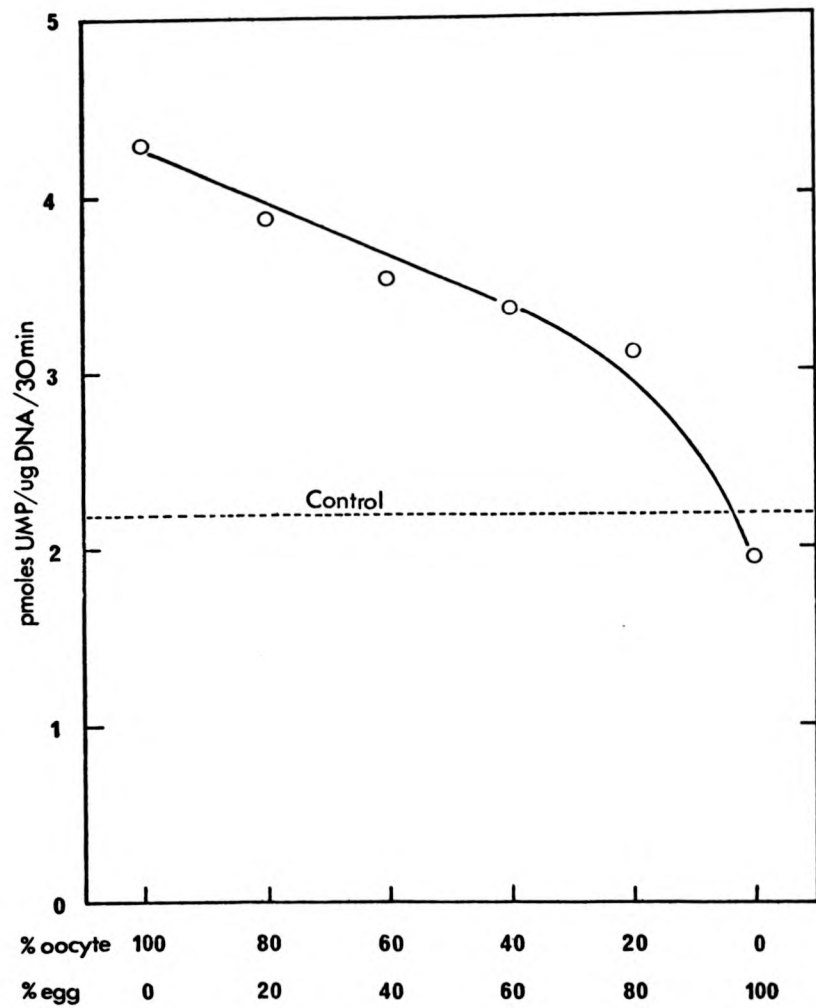


Figure 4.6 Assay of RNA synthesis in isolated XTC-2 nuclei preincubated with varying proportions of oocyte and unfertilised egg cell extracts.

XTC-2 nuclei were assayed for RNA synthesis with [³H] UTP for 30 minutes at 25° C after preincubation with varying percentages of cell extracts as indicated in the figure.

stimulatory property (as seen in Fig. 4.5). This argues that the egg extract does not contain excess amounts of factors that inhibit the stimulatory agents of oocytes, nor do oocytes contain excess amounts of factors inactivating such inhibitors in eggs. It seems most likely that the egg extract lacks stimulatory activities which are present in the oocyte. This conclusion is supported by the purification of factors from oocytes and eggs (Chapter 5).

Other experiments showed that the stimulatory activity in the oocyte extract was heat labile (the stimulation was abolished by heating to 100° C for 5 minutes) and was sensitive to treatment with trypsin. Table 4.3 shows the result of the nuclear assay in the presence of oocyte extract undigested and digested with trypsin before the extract was used in the nuclear assay. This suggests that the active agents in oocyte extracts are partly or wholly protein.

It is of interest to know the distribution of the stimulatory agents between the nucleus of the oocyte, its cytoplasm and the layer of follicle cells surrounding it. Stage 6 oocytes were dissected by hand, as described by Ford and Gurdon (1977), and extracts made from the intact follicle/oocyte complex, oocytes from which most of the follicle cells were removed, enucleated oocytes minus most follicle cells and the germinal vesicles (GV's). Assays of these fractions are shown in Table 4.4. Almost 98% of the stimulatory activity is extractable from the cytoplasm of the oocyte, and little can be located in the nucleus or the follicle cells. This is consistent with an equal distribution of factors between cytoplasm and GV (the difference in volume between the two could account for the observed distribution) or an entirely cytoplasmic location. This experiment does, however, support the idea that the stimulatory agents do not include RNA polymerases since these are located in the GV (Roeder, 1974; Hollinger and Smith, 1967). Confirmation of this point was obtained by assaying the extracts made from dissected oocytes for their RNA polymerase activity. Table 4.4 includes these results, showing a predominance of the polymerase activity in the GV extract. Some loss of total polymerase activity is apparent in the dissected compared to the total oocyte, and is probably a result of leakage during the dissection.

Table 4.3

The effect of trypsin digestion on the stimulatory activity of an oocyte extract

	<u>Counts/min</u>	<u>Stimulation over control</u>
(a) Nuclei alone	4320	1
(b) Nuclei + oocyte extract	11104	2.5
(c) Nuclei + oocyte extract + trypsin	3991	0.92
(d) Nuclei + oocyte extract + trypsin + trypsin inhibitor	10237	2.36
(e) Nuclei + oocyte extract + trypsin inhibitor	10593	2.43

Duplicate 50 μ l assays were made, the nuclei being preincubated for 1 hour with oocyte cell extract previously treated in the following ways:

- (a) No extract added.
- (b) Oocyte extract incubated for 15 min at 37^o C.
- (c) Oocyte extract incubated for 15 min at 37^o C in the presence of 20 μ g/ml trypsin followed by the addition of 200 μ g/ml soybean trypsin inhibitor (Type II-0, Sigma).
- (d) Oocyte extract incubated for 15 min at 37^o C in the presence of 20 μ g/ml trypsin and 200 μ g/ml trypsin inhibitor.
- (e) Oocyte extract incubated for 15 min at 37^o C in the presence of 200 μ g/ml trypsin inhibitor.

Nuclei were then assayed in the normal way for RNA synthesis.

Table 4.4

Intracellular location of stimulatory factors and RNA polymerase activity in Stage 6 oocytes

	Nuclear assay		RNA polymerase assay	
	Counts/minute	Stimulation over control	Counts/minute	% of Total
Control	4893	1	-	-
Nucleus	5340	1.1	9854	58%
Cytoplasm	12,101	2.5	3746	22%
Whole oocyte	11,125	2.3	16,274	96%
Oocyte + follicle cells	12,345	2.52	16,918	100%

100 oocytes were dissected as described by Ford and Gurdon (1977), then homogenised in the standard way, except that the nuclei were made up to the same volume as the other samples with Columnn buffer. Nuclear assays were for 1 hour at 25^o C with preincubation; the labelled precursor was [³H] UTP.

Polymerase assays were as described by Roeder (1974), 100 μ l assays containing 30 μ l of extract and 10 μ g of poly d(A-T) as template. Incubation was for 30 minutes at 30^o C.

(g) Characterisation of the effects of cell extracts on isolated nuclei

Characterisation of the effects of cell extracts on isolated XTC-2 nuclei may give information regarding the mode of action of the factors. There is no dramatic change in the morphology of the nuclei during incubation: they may swell slightly but not consistently. Gurdon (1976) has found that HeLa nuclei injected into oocytes swell considerably and increase their rate of RNA synthesis. However, this is over a longer time period than used here.

Initial experiments were to assay the treated nuclei for the different RNA polymerase activities using α -amanitin. This was added, at a final concentration of 1 μ g/ml (sufficient to inhibit RNA polymerase II only), to the labelling step of XTC-2 nuclei preincubated in oocyte and egg extracts. In the control about 40% of the incorporation is removed in this way. The figure is lower in nuclei treated with oocyte extract (24%), but rises to 64% in those treated with egg extract. This suggests that the oocyte extract stimulates all polymerases, but mainly polymerase I and/or III. The egg effect is not so easily explained because there is only slight inhibition of overall RNA synthesis. The simplest interpretation is that the egg extract stimulates polymerase II but inhibits one or both of the other polymerases.

Fig. 4.7 shows an electrophoretic analysis on 2.4/7.5% polyacrylamide gels of RNA synthesised by XTC-2 nuclei after preincubation in oocyte and unfertilised egg extracts. There is no discernible selective stimulation of any prevalent RNA species by oocyte extracts, merely a general increase in synthesis across the whole range of molecular weights (including the rRNA peaks). This observation supports the conclusion from the α -amanitin data above.

RNA synthesised by nuclei treated with egg extracts shows no sharp peaks (except for the artifactual accumulation of material at the junction between the high and low percentage gels). Although this might, at first sight, suggest the presence of nucleases in the egg extract, there was no great build up of low molecular weight material. RNAase activity was assayed by the method previously described. Also, purified, labelled rRNA from BHK cells was added to the cell extracts and subsequently

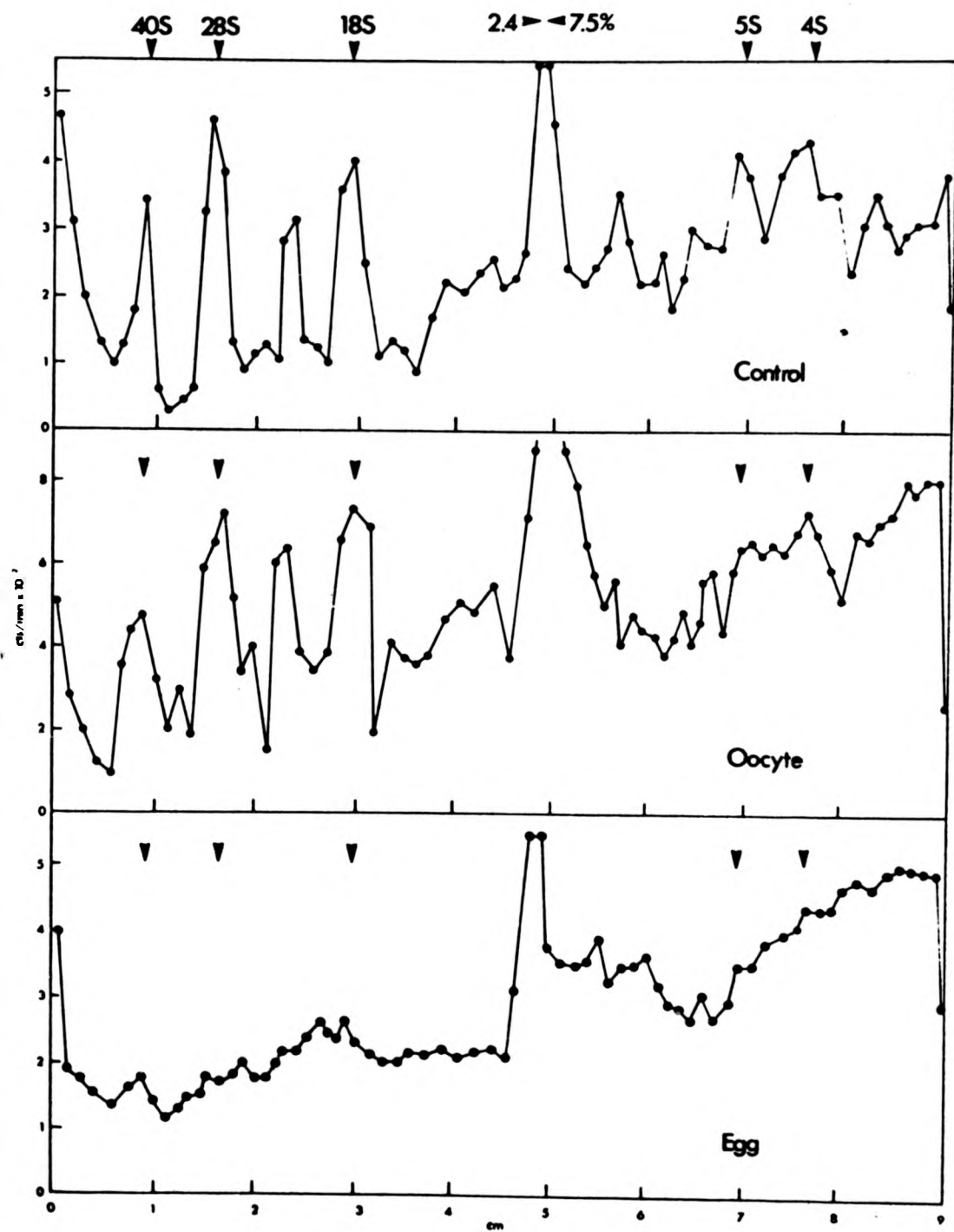


Figure 4.7 Gel fractionation of RNA products synthesised by XTC-2 nuclei *in vitro* after preincubation with and without cell extracts.

RNA was extracted from 200 μ l incubates of XTC-2 nuclei which had been preincubated with oocyte and egg cell extracts and labelled as described in Chapter 2. The extracted RNA was run on gels which were then scanned at 260 nm and sliced into 1 mm sections. The slices were hydrolysed and counted as already described (see Chapter 2). Arrows indicate the position of BHK RNA markers.



analysed by gel electrophoresis (under denaturing conditions). There was no detectable RNAase activity in either assay, the labelled rRNA remaining essentially intact after a 1 hour incubation in both extracts. A similar result was obtained with purified Influenza RNA.

The effect of cell extracts on the initiation of new RNA chains has been investigated, as described in Chapter 3, by measuring the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into pppGp and by inhibiting initiation with rifampicin. As described in Chapter 3, there are draw-backs to the $[\gamma\text{-}^{32}\text{P}]$ assay for initiation. Phosphorylating enzymes in the extracts could cause differential reduction in the $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ specific activity. Different amounts of processing of the 5'-triphosphates on the newly formed RNA chain could occur. Results described above indicate that this is a problem because processing of rRNA seems to occur in XTC-2 nuclei even in the absence of added extracts. This assay underestimates the initiation and may only be used to compare nuclei treated in different ways. Preincubation helps in this respect; all the nuclei are assayed under as near final conditions as possible. They were reisolated after preincubation with the cytoplasmic additives and assayed under the normal conditions with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[^3\text{H}]\text{UTP}$ as precursors. After 30 minutes' incubation an aliquot was TCA precipitated as usual to determine $[^3\text{H}]\text{UTP}$ incorporation. A further aliquot was phenol extracted, and the RNA precipitated with ethanol. The content of $^{32}\text{pppGp}$ was determined by alkaline hydrolysis and PEI-cellulose chromatography. Table 4.5 shows that the usual effects on $[^3\text{H}]\text{UTP}$ incorporation were produced. The $[\gamma\text{-}^{32}\text{P}]$ incorporation was stimulated to a smaller extent in the case of the oocyte extract and the egg extract was slightly inhibitory. It is reasonable to conclude that the oocyte extract produces some increase in initiation, although it may also affect elongation.

Use of the inhibitor rifampicin should make it possible to tell whether or not initiation and/or elongation are being affected by the oocyte and egg extracts. This could be achieved by preincubating the XTC-2 nuclei with oocyte and egg extracts and then following the time course of incorporation in the presence and absence of the inhibitor. The time taken for incorporation to cease should give some indication about the

Table 4.5

Initiation assays on extract-treated nuclei

	<u>[³H] UTP</u> <u>counts/minute</u>	<u>Stimulation</u> <u>over control</u>	<u>[γ-³²P] GTP</u> <u>counts/minute</u>	<u>Stimulation</u> <u>over control</u>
Control	4,192	1	224	1
Oocyte	10,911	2.5	289	1.25
Egg	4,201	1.01	211	0.95

Standard 200 μ l assays were made with a pre-incubation step. Both labelled precursors were added during the labelling step. [³H] UTP incorporation was determined by acid precipitation of duplicate 25 μ l aliquots. The [γ -³²P] GTP incorporation (50 μ Ci, 20 Ci/mmol, in a 200 μ l incubate) was determined by extracting the RNA from a 100 μ l aliquot as described in the Methods. The RNA was hydrolyzed overnight at 37^o C in 0.3 M KOH, the K⁺ was subsequently removed by addition of 0.3 M HClO₄. The resulting supernatant was analysed on PEI cellulose, using 1.6 M LiCl (Randerath and Randerath, 1967). Each track was cut into 1 cm sections and counted in a toluene based scintillant supplemented with 9% soluene 350 (Packard) and 1% water.

average elongation and termination rate, and the final plateau level should indicate the relative stimulation of initiation or its inhibition, i.e. the relative number of polymerase molecules engaged with the template. Fig. 4.8 shows the result of such an experiment. It is clear that, with oocyte treated nuclei, the incorporation was essentially complete after 10 minutes, whereas the control nearly reached its plateau after 20-30 minutes. In this experiment the egg treated nuclei did not reach a final plateau. To summarise the conclusions: (1) there is initiation occurring in all the nuclei, rifampicin causes a 55% inhibition in the oocyte treated nuclei compared to a 44% inhibition in the control and egg treated nuclei after 30 minutes, suggesting that there has been a stimulation of initiation in the oocyte treated nuclei (as seen in Fig. 3.11, there is a 43% inhibition by rifampicin after 30 minutes in untreated nuclei); (2) nuclei exposed to oocyte extracts reach their maximum incorporation faster in the presence of the inhibitor than control or egg treated nuclei, suggesting that elongation is also stimulated in oocyte treated nuclei. To quantify these conclusions thoroughly, values for the number of growing RNA molecules and the chain length distribution would be required. However, the above conclusions support the data from the [γ - 32 P] initiation assay.

(h) Variation in the stimulatory properties of extracts from embryos at different stages of development

A possibility arising from the above data was that either the oocyte or egg extracts were affecting the transcription in isolated XTC-2 nuclei in an artefactual manner. The most obvious way to test this and to investigate the general applicability of the assay system is to assay extracts made from other developmental stages of Xenopus early development. The aim was to see if the extracts changed the rates of RNA synthesis in isolated nuclei in a way analogous to the changes shown by nuclei passing through normal development.

Extracts were prepared from oocytes, eggs and embryos through the hatching stages of development and their effects on nuclei assayed by the standard preincubation assay. Fig. 4.9 summarises the results. There is apparent agreement with events occurring in vivo up to the late

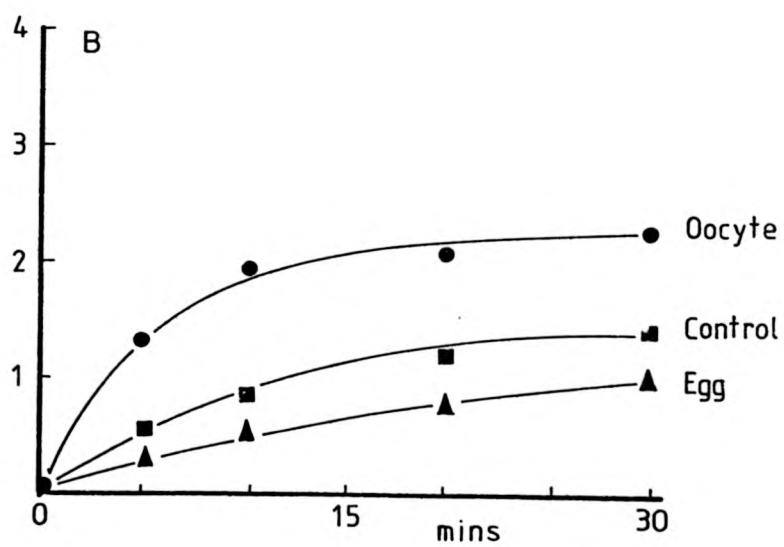
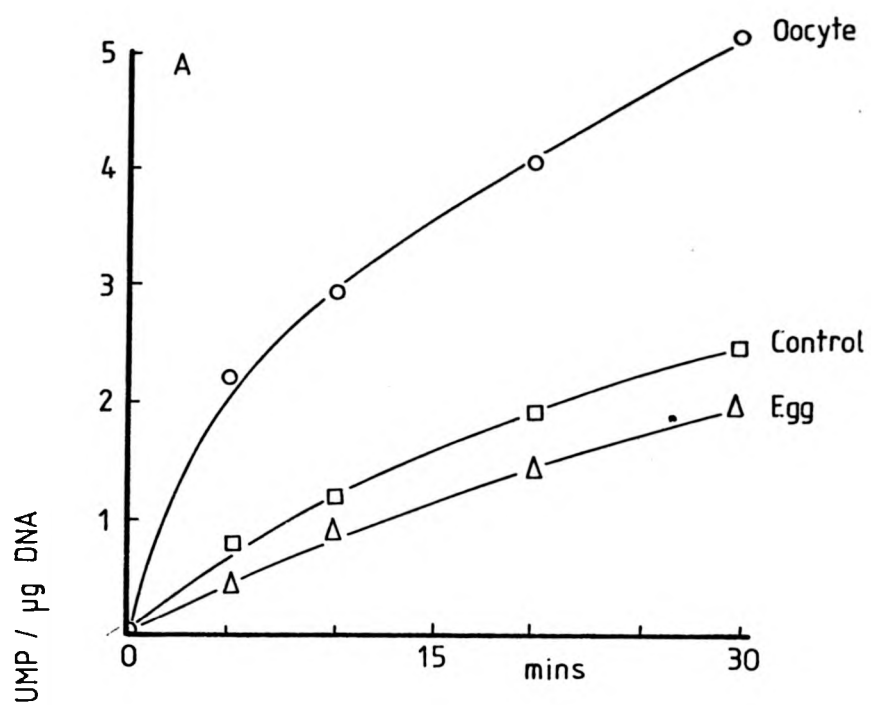
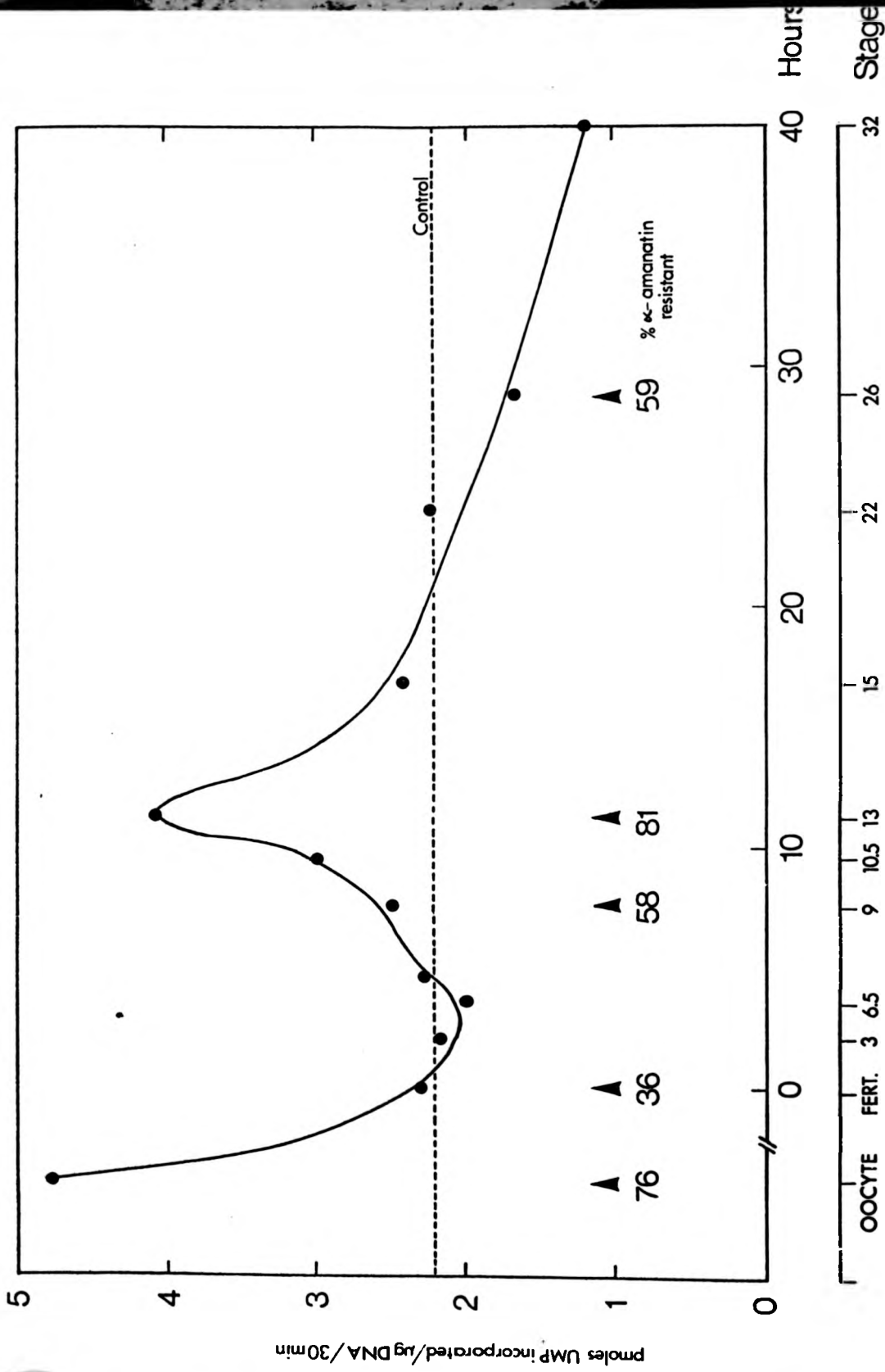


Figure 4.8 Time course of incorporation of oocyte and egg preincubated nuclei in the presence and absence of AF/013.

XTC-2 nuclei were preincubated as usual with oocyte and egg cell extracts and Column buffer as control. The nuclei were then assayed for RNA synthesis with [3 H] UTP in the presence and absence of AF/013 at 50 μ g/ml, 25 μ l aliquots being removed (from each incubation) and counted at the times indicated. (A) Time courses in the absence of AF/013 after preincubation with oocyte (\circ) and egg (Δ) extracts and control (\square). (B) Time courses in the presence of AF/013 after preincubation with oocyte (\bullet) and egg (\blacktriangle) extract and control (\blacksquare) preincubation.



Stage

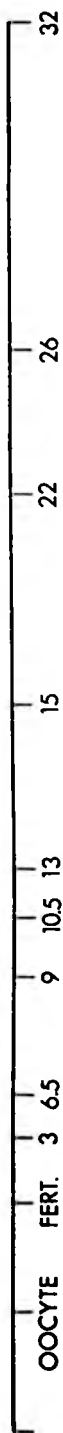


Figure 4.9 RNA synthesis by XTC-2 nuclei preincubated with cell extracts from different stages of *Xenopus* early development .

XTC-2 nuclei were preincubated for 1 hour with cell extracts from the stages indicated and assayed with [3 H] UTP for RNA synthesis as described in Chapter 2. Duplicate 100 μ l assays were carried out with α -amanitin at a final concentration of 1 μ g/ml, sufficient to inhibit RNA polymerase II. Stages of development are taken from Nieuwkoop and Faber (1956).

gastrula stage (Stages 13-14). As shown above, oocyte cytoplasm is stimulatory, perhaps reflecting the high rate of RNA synthesis occurring during oogenesis. Unfertilised egg and early cleavage extracts are all slightly inhibitory, which is consistent with the low rate of RNA synthesis occurring in normal nuclei at these stages. In development, intense nuclear RNA synthesis becomes detectable at about Stage 7½, which is when the extracted cytoplasm starts to be stimulatory again. The peak of stimulation is reached at the gastrula stage, later stage extracts becoming very inhibitory.

This inhibition, which deviates from the expected result, may be the result of loss of stimulatory factors, the presence of inhibitory factors, the unavailability of stimulatory factors or their loss during isolation. One possibility was that the factors, although present, were localised in the nuclei and subsequently lost during extract preparation. This possibility was investigated by sonicating hatching stage total homogenates before the centrifugation steps. It was hoped that this would solubilise any factors present in the embryonic nuclei and prevent their loss during the preparation. Sonication had no effect in overcoming the inhibition. This argues for the loss of stimulatory activity or the presence of inhibitors.

The effect of cell extracts on the activities of polymerase II versus polymerases I plus III was investigated by inhibiting the former with low levels of α -amanitin as shown in Fig. 4.9. The action of this inhibitor on the activity of nuclei in the presence of egg and oocyte extracts has been discussed above. The reactivated blastula and gastrula extracts re-acquire the oocyte proportion of polymerase II activity, i.e. polymerase I and III levels rise.

(i) Discussion

In this chapter an assay system for cytoplasmic regulatory factors has been devised and optimised. The assay is based on the assumption that cell extracts of Xenopus oocytes and eggs differ in the content of factors which regulate transcription, oocyte extracts stimulating and egg extracts inhibiting RNA synthesis. The validity of this assumption has been discussed in Chapter 1. The assay can only be considered relevant

if: (a) the assay follows closely the in vivo situation and (b) any factors purified by this method may be shown to have some importance in the control of transcription in vivo. The first validation has been, to some extent, carried out. Events, in terms of RNA synthesis, up to gastrulation in Xenopus embryos are mimicked by the assay. In this respect, the inhibition by later stages (i.e. stage 32) is worrying, and is dealt with later in this thesis. The other proof of the value of the assay depends on further purification of the factors involved and this occupies the next chapter.

So far, it may be said that the factor(s) involved in the stimulation of RNA synthesis in isolated XTC-2 nuclei treated with oocyte extracts is not dialysable, is heat labile and probably protein in nature. The possibility that the bulk addition of RNA polymerases is involved in the stimulation of transcription has not been supported by the experiments carried out so far. A point to note here, is that nuclei prepared using the LL-BSA method (Curdon, 1976) showed less stimulation in the presence of oocyte extracts than did Triton prepared nuclei. The reason for this is not clear but may be related to the cytoplasmic contamination in the LL-BSA nuclear preparations (see Chapter 3).

The location of the stimulatory factors in the cytoplasm of the oocyte is interesting. Stored nuclear proteins studied previously have been shown to be located in the nucleus. These include the developmental store of RNA polymerases (Hollinger and Smith, 1976) and of histones (Woodland and Adamson, 1977). There is no evidence that the final location of the factors involved is predominantly nuclear (but see Chapter 8). Factors which influence nuclear activity have also been detected in cell fusion experiments between differentiated somatic cells, as already described.

The use of low levels of α -amanitin show that the stimulation of RNA synthesis by oocyte and Stage 10-13 extracts probably involves all three polymerases. This is also in line with electrophoretic analyses of the RNA products. The synthesis of all species of RNA seems to increase roughly proportionately. The egg extract inhibits incorporation slightly, probably by inhibiting one or both of the α -amanitin insensitive RNA polymerases.

It would be interesting to know how the modulating effects of the extracts are achieved. There are two obvious questions here. First, is the effect on initiation or elongation of RNA chain formation? Secondly, is the effect directly on the RNA polymerases or on some other component of the nucleus?

Effects on initiation and elongation are not easily distinguished. Two methods have been used to assay for initiation and both have their limitations. In general they indicate that the oocyte extract may act by stimulating both initiation and elongation. It is also hard to tell if the extracts act directly on the RNA polymerases. This is partly because adding polymerases to XTC-2 nuclei has little effect on RNA synthesis, probably because the polymerases do not penetrate the nuclear membrane. Clarification of these points can only come from using a more defined system coupled with the purification of the factors involved.

Chapter 5

Purification of factors stimulating RNA synthesis in isolated nuclei from Stage 5/6 oocytes. Their levels during oogenesis and early development

(a) Introduction

Results described in the last chapter indicated that Stage 6 oocytes probably contain more than one protein factor which stimulates RNA synthesis in isolated nuclei. Further characterisation of these factors, their effects and mode of action depended on their purification. This chapter deals with this purification and the subsequent analysis of activities of the factors during oogenesis and early development of Xenopus laevis.

(b) Purification of stimulatory factors from Stage 5/6 oocytes

Simple methods were first applied to the problem of purifying the active agents from Stage 6 oocytes. The fact that the stimulatory activity was not dialysable suggested that a first step in the purification might be achieved by gel filtration on Sephadex.

Gel filtration of oocyte extracts

Crude oocyte extract was applied directly to Sephadex G-25 and eluted with Column buffer. 40 μ l samples were assayed in the standard preincubation assay. Most of the stimulatory activity eluted near the void volume. This suggested that macromolecules were involved in the stimulation of RNA synthesis. There was a small peak of activity eluting with a very low molecular weight and it was probably produced by nucleotides (see also below for G-100 analysis). As shown in Chapters 3 and 4, increased nucleotide concentrations above 0.02 mM stimulated incorporation to some extent.

Initial fractionation was achieved on G-100 Sephadex (see Chapter 2). The resulting profile of incorporation in the nuclear assay is shown in Fig. 5.1A. There were two main peaks of activity; Peak 1 eluted just after the void volume; Peak 2 gave a consistently higher stimulation and eluted with a much lower molecular weight. Several less stimulatory peaks were obtained quite consistently and are discussed to some extent later in this thesis. One in particular (labelled Peak 3), which seemed insignificant, is discussed in more detail in Chapter 10. The stimulations

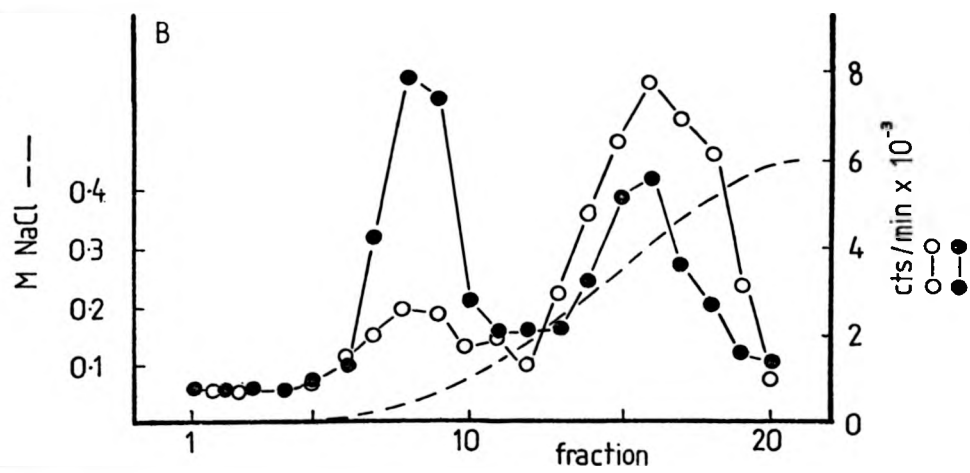
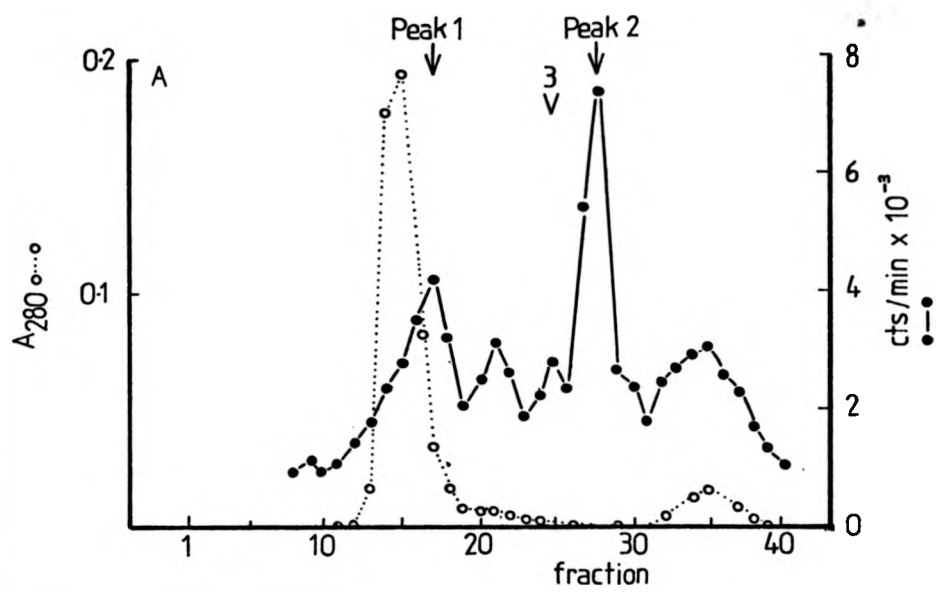


Figure 5.1 Purification of factors affecting RNA synthesis in XTC-2 nuclei from mature oocytes.

(A) Cell extracts prepared from ~ 200 oocytes (4 mg protein) were fractionated on Sephadex G-100 superfine (see Chapter 2). 20 μ l from each fraction was assayed for its effect on RNA synthesis in isolated XTC-2 nuclei under standard preincubation conditions. (O.....O), A_{280} ; (●), [^3H]UTP incorporation in the nuclear assay.

(B) The peak fractions (Peaks 1 and 2) from the Sephadex fractionation (A) were eluted from 1.5 x 4 cm DE52 columns with 40 ml linear 0-0.45 M salt gradients (see Chapter 2). The salt concentration (---) of each fraction was measured and 20 μ l assayed with XTC-2 nuclei for its effect on RNA synthesis as described in Chapter 2. (O), Peak 1 from the oocyte Sephadex; (●), Peak 2 from the oocyte Sephadex.

produced by Peaks 1 and 2 were very large. Column buffer had no effect on the incorporation of [^3H] UTP by isolated nuclei: Fig. 5.1A shows that Peak 1 stimulated RNA synthesis by about 4-fold and Peak 2 by up to 8-fold. On the other hand, the original crude extract stimulated incorporation only 2.5-fold. The partially purified factors were up to 3 times more stimulatory and were much more dilute than the original extract. Purification (which is quantified later in this chapter) therefore increased the apparent activity quite considerably.

No inhibitory fractions were found. This was slightly surprising, since the total stimulatory activity of the column eluent was more than that of the original extract. It is possible that inhibitory factors were lost or destroyed during column chromatography or that they were separated into components that were not inhibitory alone. This could have been tested by adding together all the fractions to the same concentration as the original extract and reassaying it, but proved impossible. As described later, no method was found for concentrating the eluent by the required amount which did not also destroy the activity.

It could be argued that any inhibitors function only on the stimulatory factors, and not on untreated nuclei. The column fractions were thus reassayed with nuclei to which Peak 1 or Peak 2 was always added. No inhibitory factors were found.

The finding that the total activity was greater on fractionation than the original extract is not an uncommon one. Similar findings have been reported for other cytoplasmic fractionation experiments (MacLean and Hilder, 1977) and for enzyme purifications such as RNA polymerase (Roeder, 1974).

Analysis of oocyte stimulatory factors by DEAE-cellulose chromatography

Further purification of the oocyte stimulatory factors was achieved by DEAE-cellulose chromatography. The crude extract, when eluted with a linear gradient of NaCl, gives two separable activities, both binding to the ion exchanger (Fig. 5.2A). No stimulatory activity was found to pass directly through the column. Fig. 5.2A shows that the two activities elute at very different counter ion molarities corresponding to 0.05 M NaCl and 0.2 M NaCl. The relation between the DEAE and Sephadex fractions was

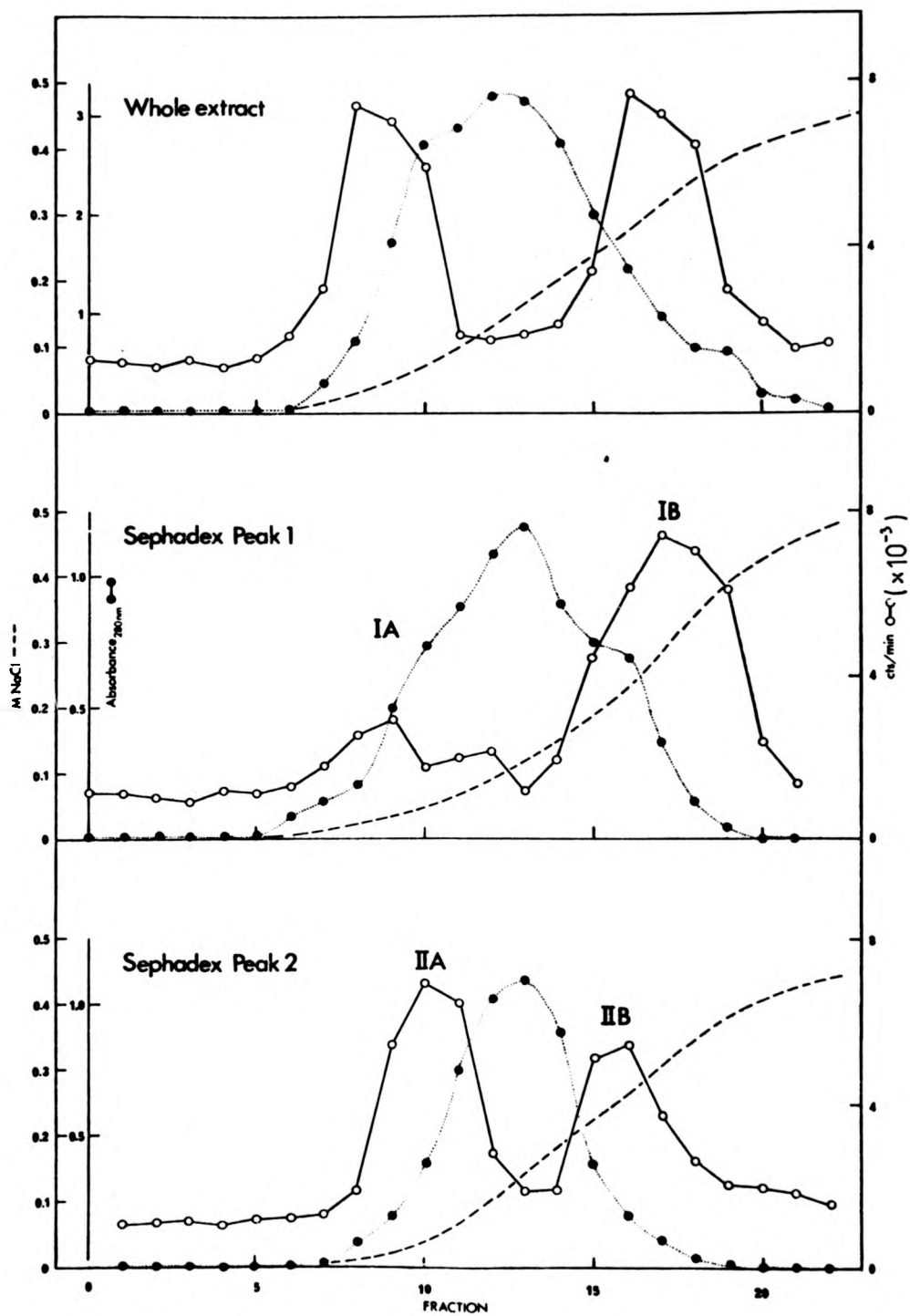


Figure 5.2 DEAE-cellulose chromatography of whole oocyte cell extract and Peaks 1 and 2 purified from oocyte cell extracts on G-100 Sephadex.

An extract from 200 oocytes and pooled fractions from Peaks 1 and 2 off G-100 Sephadex were eluted from DE52 gradients as in Figure 5.1B. The A_{280} (●—●) and salt concentration (— —) of each fraction were measured and 20 μ l was assayed with XTC-2 nuclei for its effect on RNA synthesis (○—○) as described in Chapter 2.

cts/min (x 10⁻³)

determined by running Sephadex Peaks 1 and 2 on DEAE-cellulose. Figs. 5.2B and C show that the first crude extract DEAE peak is derived mainly from Sephadex Peak 2, whereas the second is derived from both Sephadex peaks. These DEAE activities are called IA, IB, IIA and IIB as shown in Fig. 5.2. Peak IA is of variable magnitude, but is always smaller than peak IB.

Again, no inhibitory factors were found in the DEAE-cellulose fractions of total extract or of fractionated Peaks 1 and 2. The minimum between the two peaks on DEAE corresponded to the bulk protein. This may itself inhibit the assay and produce two peaks from one agent. This was shown not to be the case by fraction mixing experiments. Each column was reassayed with nuclei to which the bulk protein fractions had been added in each case. An example of the results obtained is shown in Fig. 5.3. This shows the fraction mixing assay described above applied to the Peak 2 DEAE column. There is reduction in the stimulation by peaks IIA and IIB but their effects are not eliminated. The reduction is consistent with the dilution of the factor resulting from the experimental design. Similar results were obtained for the total extract and Peak 1 DEAE column. This experiment also tests for the presence of inhibitors acting only on the factors. As for the Sephadex fractionation, no such inhibitors were found.

When the DEAE column fractions were tested in the nuclear assay, the final salt concentrations in the preincubation step varied with each fraction. The NaCl concentrations attained were thought to be in the range tested during the optimisation experiments (Chapter 3). However, to be sure that the salt concentration was not affecting the assay, the column fractions were dialysed before being reassayed as usual. There was little change from the original assay in all the cases tested, and this step was omitted.

Other methods of purification

Other methods of fractionating the extracts were tried. Amongst these were $(\text{NH}_4)_2\text{SO}_4$ differential precipitation as a first step in the purification. This method resulted in too great a loss in activity and was

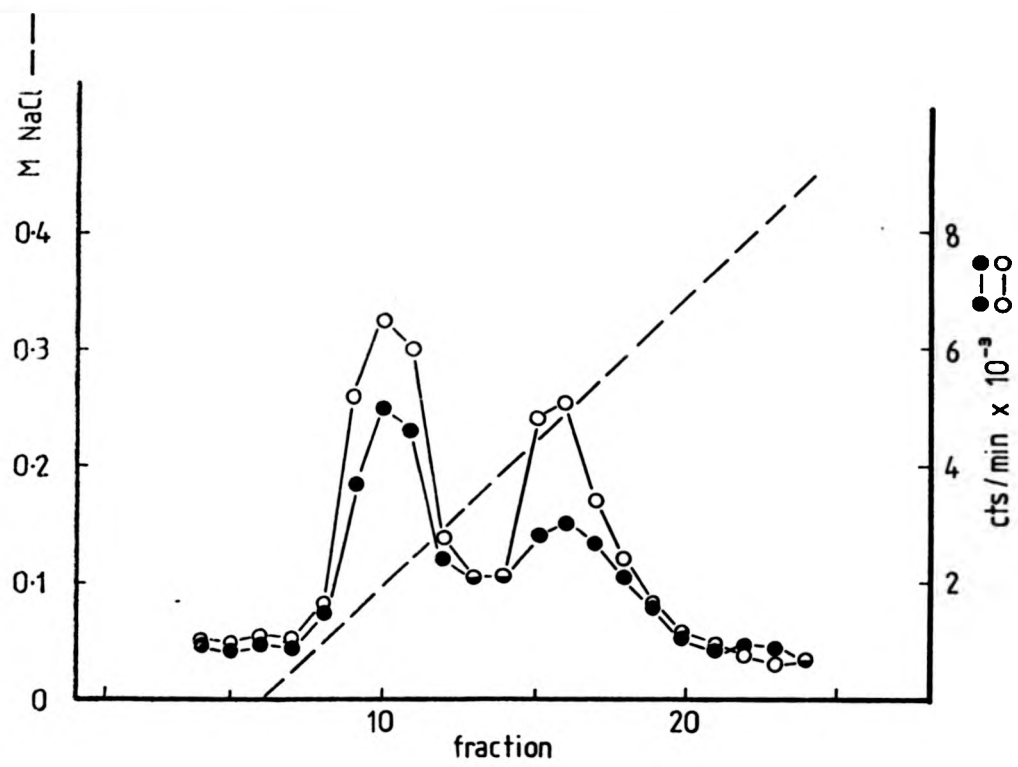


Figure 5.3 Fraction mixing experiment on DEAE-ion exchange chromatographed Sephadex Peak 2 material.

The fractions obtained after DEAE-cellulose chromatography of oocyte Sephadex Peak 2 shown in Figure 5.2 were reassayed as follows: 20 μ l of each fraction was assayed as before (Figure 5.2) with XTC-2 nuclei (O). A parallel set of assays was carried out with 10 μ l from fraction 13 added to 10 μ l of each column fraction in the standard assay (●). (—) salt concentration. In both sets of assays, labelling was for 30 minutes at 25^o C.

8
● — ●
○ — ○
6 cts/min x 10⁻³
4
2

not adopted. Other methods were applied to concentrate the factors purified so far. These included various methods of precipitation and lyophilisation. In general they resulted in varying amounts of loss of the activities and were not further used.

The final purification procedure for the factors has been already described (and summarised in Fig. 5.1A and B). A combination of gel filtration on Sephadex G-100 and subsequent fractionation by DEAE-cellulose chromatography was used, and this resulted in four stimulatory activities.

Purifications achieved

The protein content of the original extracts and the purified fractions Peaks 1 and 2 and the factors IA, IB, IIA and IIB were estimated by the method of Lowry *et al.* (1951). The purification, in terms of protein content, was calculated for each activity after G-100 fractionation and after DEAE-cellulose chromatography. Table 5.1 gives a summary of the results and the stimulatory activity of each peak or factor. It is clear that factors IIA and IIB have been purified considerably, much more so than IA and IB. The reason is that Peak 2 avoids the bulk protein peak on Sephadex completely. It was felt that the purification of these factors was sufficient to warrant further characterisation in terms of their protein make-up, their activities during development, their specificity and their mode of action.

Concentration curves of the purified factors

As described above, attempts to concentrate the factors by precipitation had failed. In order to analyse the full stimulatory effect of the factors, more concentrated preparations were required. To obtain these, a much larger G-100 column than described in the methods section was used.

Bulk fractionation of oocyte extract was achieved with a 30 cm x 10 cm column of G-100 Superfine Sephadex, prepared in the same way as the smaller column. The definition achieved by the large column was not very good. However, it enabled the purification of the factors to a slightly higher concentration. This enabled concentration curves of the factors to be constructed to see if there was a limit to the stimulation in

Table 5.1

Purification of factors stimulating RNA synthesis in isolated nuclei from Stage 5/6 oocytes

		Total protein content	% of original protein	Stimulation in the nuclear assay
Total oocyte extract		4 mg in 400 μ l	100%	2.5
	Peak 1	1 mg in 4 ml	25%	4.5
Sephadex peaks	Peak 2	40 μ g in 4 ml	1%	8.2
	Factor IA	72 μ g in 2 ml	1.8%	2.7
DEAE peaks	Factor IB	200 μ g in 2 ml	5%	6.8
	Factor IIA	3.5 μ g in 2 ml	0.09%	7.3
	Factor IIB	10.5 μ g in 2 ml	0.26%	5.0

Extracts and column fractions were prepared as described in Chapter 2 and in the text. Protein estimations were by the method of Lowry et al. (1951). Stimulation in the nuclear assay refers to the stimulation over control caused by the presence of 20 μ l of each fraction in a standard 50 μ l nuclear assay with preincubation.

transcription. The concentration curves for Peaks 1 and 2 and for factors IB, IIA and IIB are shown in Fig. 5.4. Neither Peak 1 or 2 reached a true plateau. Factor IIA did reach a plateau of incorporation at concentrations over 50 ng protein per 50 μ l nuclear assay (equivalent to 10 ng protein per μ g of DNA). The other factors were not prepared in high enough concentrations for their saturation values to be ascertained.

Gel electrophoresis of purified factors

It was hoped to identify at least some of the proteins involved in stimulating transcription by running SDS polyacrylamide gels (Laemmli, 1970) of the column fractions. Some tentative identification may be made by correlating the stimulatory activity in the fractions with the different concentrations of the proteins in the same fraction.

This was first done for Peaks 1 and 2. The number of proteins present in Peak 1, as expected, was too complex for further analysis. Plate 5.1 shows a SDS slab gel, stained for proteins, of the fractions through Peak 2, and the corresponding activity of each fraction in the nuclear assay. A major protein component with a molecular weight of about 15,000 does correlate with activity in the nuclear assay.

Factor IIA was analysed in the same way. Plate 5.2 shows the resulting stained slab gel and the transcriptional activity of the XTC-2 nuclei in the preincubation assay. Essentially only two proteins are visible on this gel and these have been called protein A1 and protein A2. The amount of these proteins in the different fractions correlates well with the level of activity in the nuclear assay, and they seem to co-purify with the stimulatory activity. These proteins were tentatively assigned to factor IIA. A final assignment may only be made after further analysis.

No stainable proteins were observed when fractions through factor IIB were subjected to SDS slab gel electrophoresis. By comparison with IIA, there was sufficient protein in the samples for visualisation after staining. Possible causes were bad recoveries during sample preparation or the presence of many proteins. The factor must then have a higher specific activity than IIA.

Fractions through Peak IB from a DEAE column were also subjected to slab gel electrophoresis. As for Peak 1, a complex pattern of proteins was obtained as shown in Plate 5.3. Some proteins do appear to be most

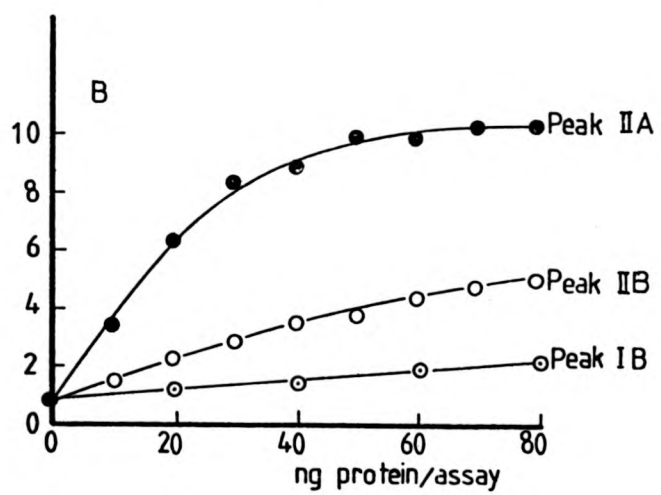
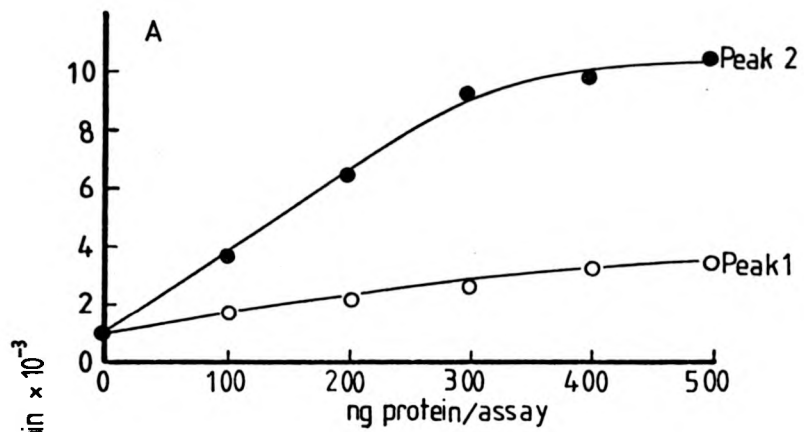


Figure 5.4 Concentration curves of oocyte stimulatory factors

XTC-2 nuclei were preincubated with varying amounts of Sephadex Peak 1 and Peak 2.

(A) Varying amounts of Sephadex Peak 1 and Peak 2 were assayed with XTC-2 nuclei for their effect on RNA synthesis with [³H] UTP in the standard way. Each 50 μ l assay contained 5×10^5 nuclei and the amounts of protein derived from Sephadex Peak 1 (O) or Peak 2 (●) are indicated in the figure. (B) Varying amounts of factors IA, IB and IIA were assayed with XTC-2 nuclei as above. Each 50 μ l assay contained 5×10^5 nuclei and the amounts of protein derived from DEAE factors IB, (⊙); IIA, (●); and IIB (O). All protein estimations were by the method of Lowry et al. (1951).

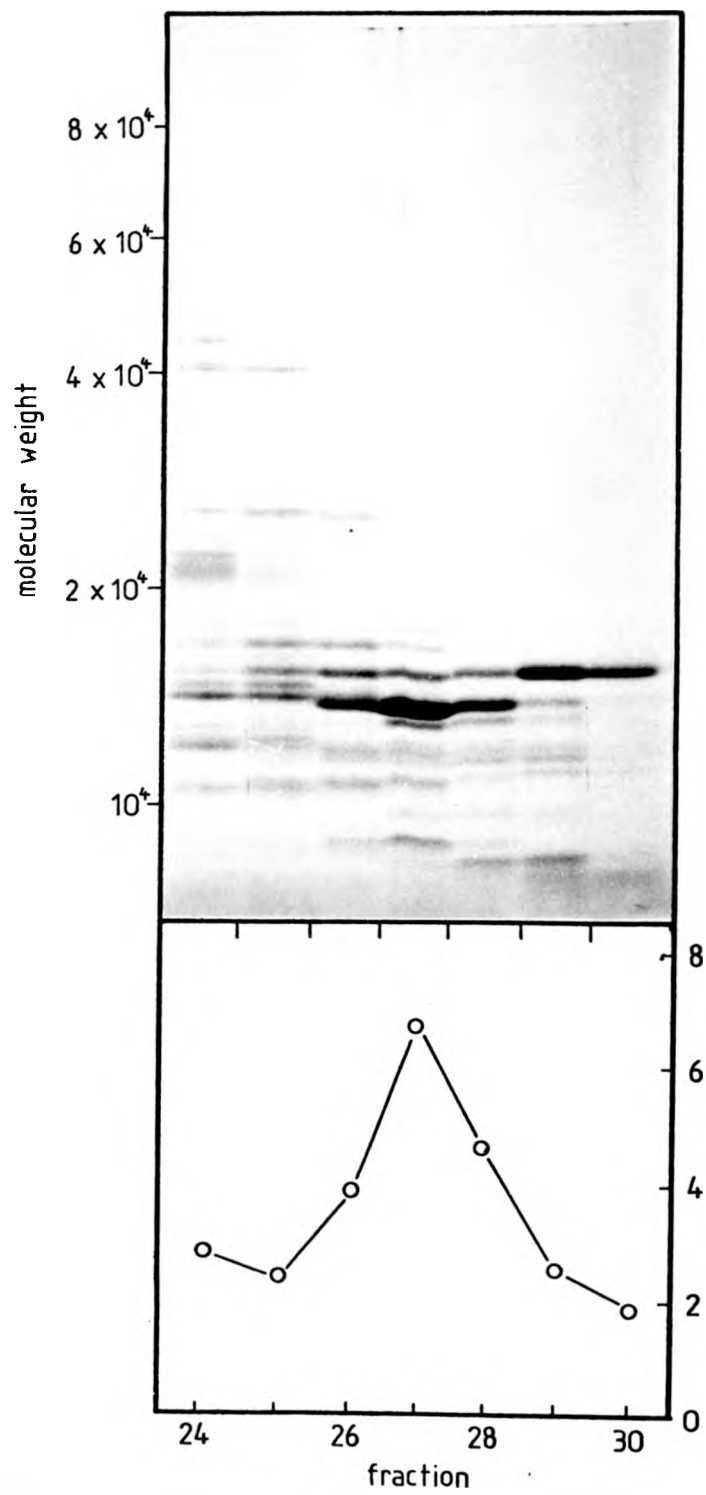


Plate 5.1 Polyacrylamide gel electrophoresis of oocyte Peak 2

Sephadex fractions through oocyte Peak 2 were TCA precipitated and subjected to gel electrophoresis on an 18% gel as described in Chapter 2. The figure shows the resulting gel stained for protein with Coomassie Brilliant Blue and the corresponding incorporation of [³H]UTP in the nuclear assay for each fraction (○—○).

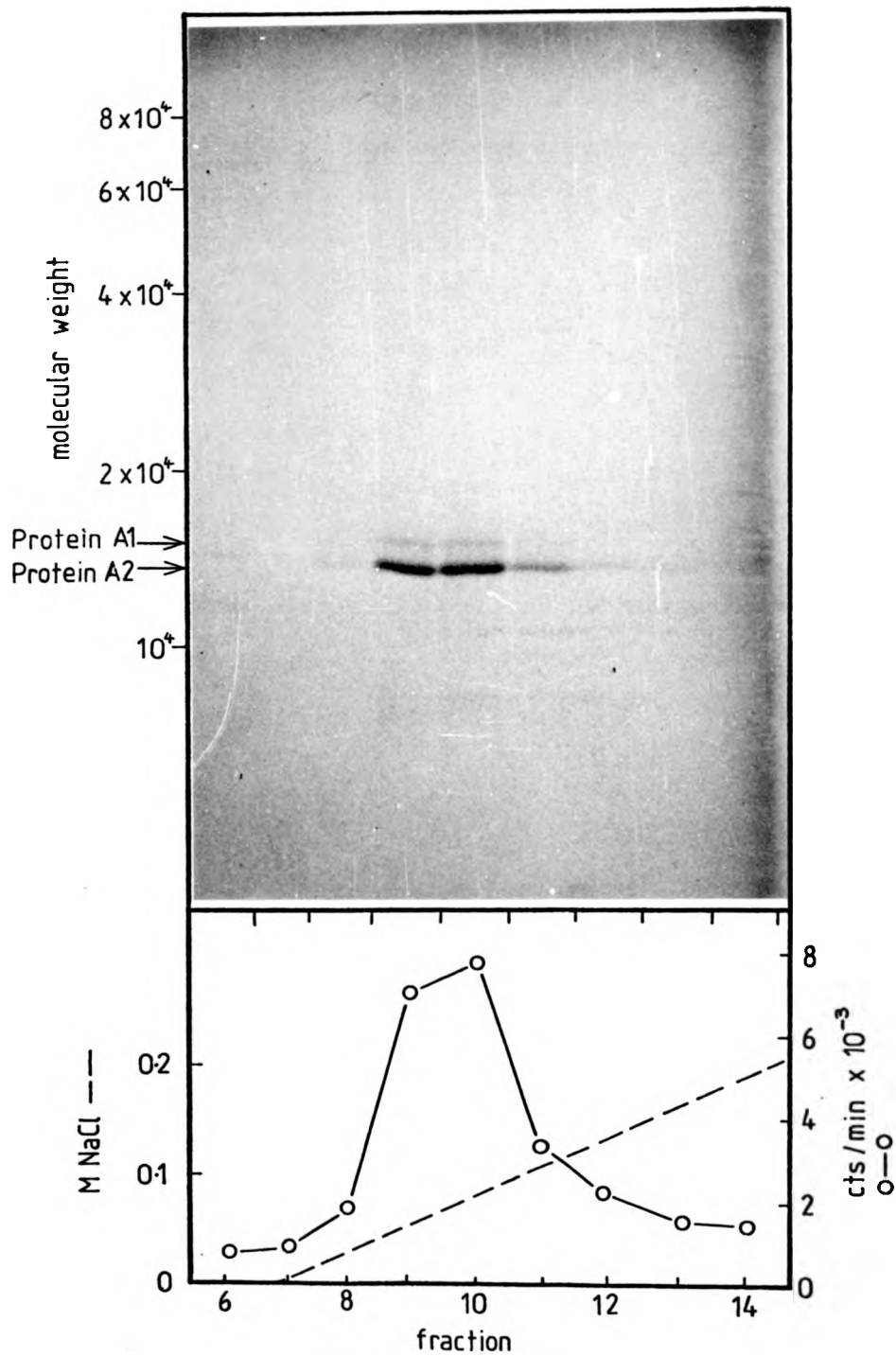


Plate 5.2 Polyacrylamide gel electrophoresis of oocyte factor IIA

DEAE column fractions through peak IIA (from oocytes) were TCA precipitated and subjected to gel electrophoresis on an 18% SDS gel as described in Chapter 2. The figure shows the resulting gel stained for protein as in Plate 5.1 and the corresponding [³H] UTP incorporation in the nuclear assay (○—○). Salt concentration (— — —).

cts/min x 10
○—○

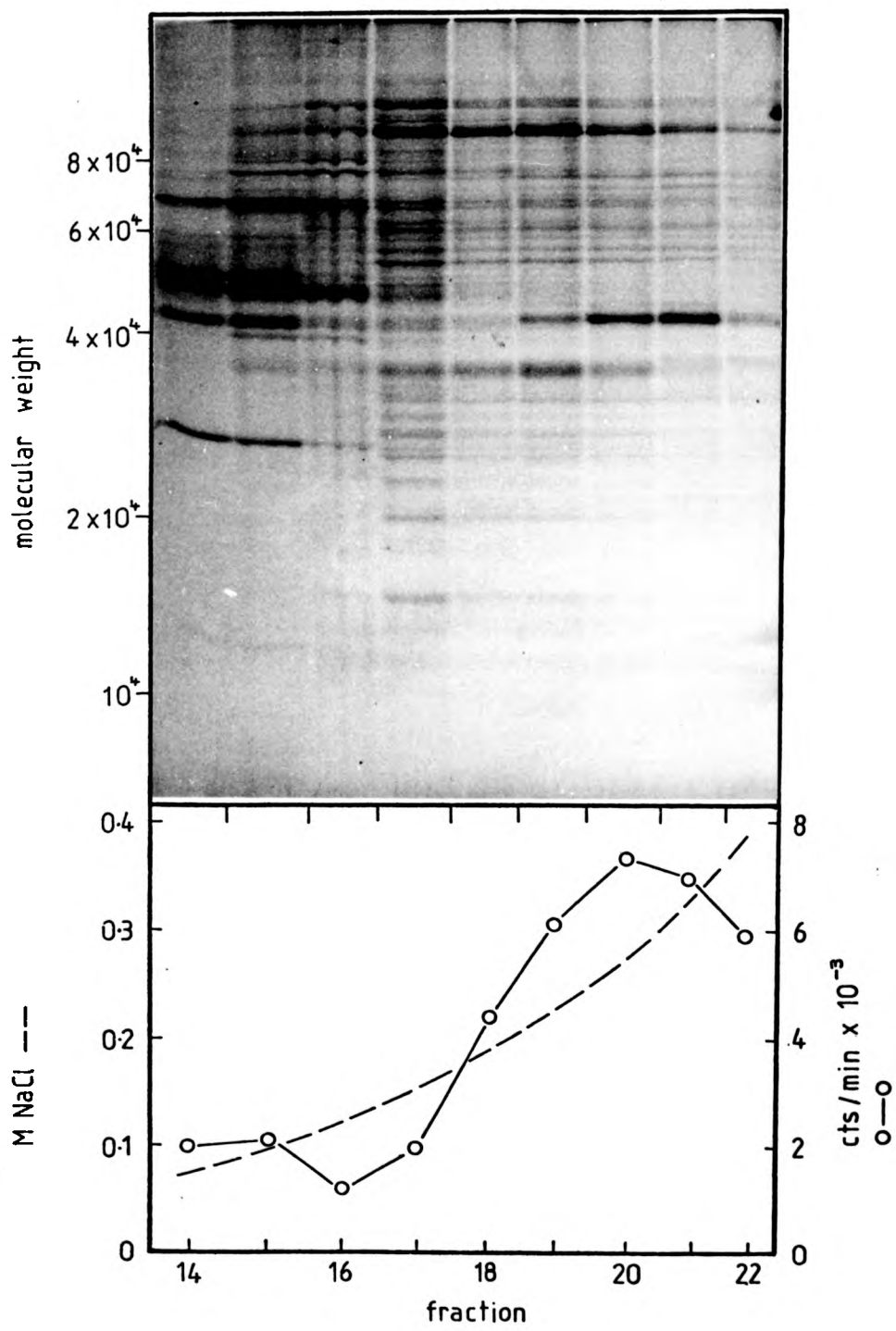


Plate 5.3 Polyacrylamide gel electrophoresis of oocyte factor IB

DEAE column fractions through peak IB (from oocytes) were TCA precipitated and subjected to gel electrophoresis on an 18% SDS slab gel as described in Chapter 2. The figure shows the gel stained for protein and the corresponding [³H] UTP incorporation in the nuclear assay (○—○). Salt concentration (— — —).

cts/min x 10⁻³
○—○

concentrated in the most active fractions (for example around 4×10^4 molecular weight), however, no conclusion may be drawn from this result. The same was true for Peak IA.

Factors IIA and IIB were investigated further by iodination of the proteins present in each fraction with ^{125}I by the chloramine T method of Greenwood *et al.* (1963). This was to test for proteins present in low concentrations. The ^{125}I -labelled proteins were then subjected to SDS slab gel electrophoresis as before. The gel was dried and subjected to autoradiography. Several proteins were present in the factor IIA fraction including the major components seen on stained gels. Factor IIB fractions also contained a number of proteins, and so further purification is necessary in both cases before assigning the activities to particular proteins.

Molecular weight determinations

The molecular weights of the interesting peaks from Sephadex and those of proteins A1 and A2 were determined by running marker proteins. The calibration graphs for the Sephadex peaks and the slab gels are shown in Fig. 5.5. Included are the marker proteins used. The positions where Peaks 1, 2 and 3 elute from Sephadex G-100 are shown (Fig. 5.5A) and indicate that Peak 1 has a molecular weight of about 65,000, Peak 2 about 23,000 and Peak 3, 30,000. Fig. 5.5B shows that proteins A1 and A2 have molecular weights of 15,750 and 17,400 respectively. These, however, elute with a molecular weight of about 23,000 from Sephadex. A possibility is that the active factor eluting with this molecular weight is made up of a number of sub-units. Given the errors in molecular weight determination on Sephadex and gels, the native protein could have one 15,750 and one 17,400 sub-unit.

(c) The activity of the factors during oogenesis

During normal oogenesis and early development, the RNA synthesised by the nuclei undergoes drastic qualitative and quantitative changes as discussed in Chapter 1. With the partial purification from Stage 5/6 oocytes of factors which stimulate RNA synthesis in isolated nuclei, it is of obvious relevance to ask if the activities of the factors changes during these periods. Stages during oogenesis were analysed by preparing cell

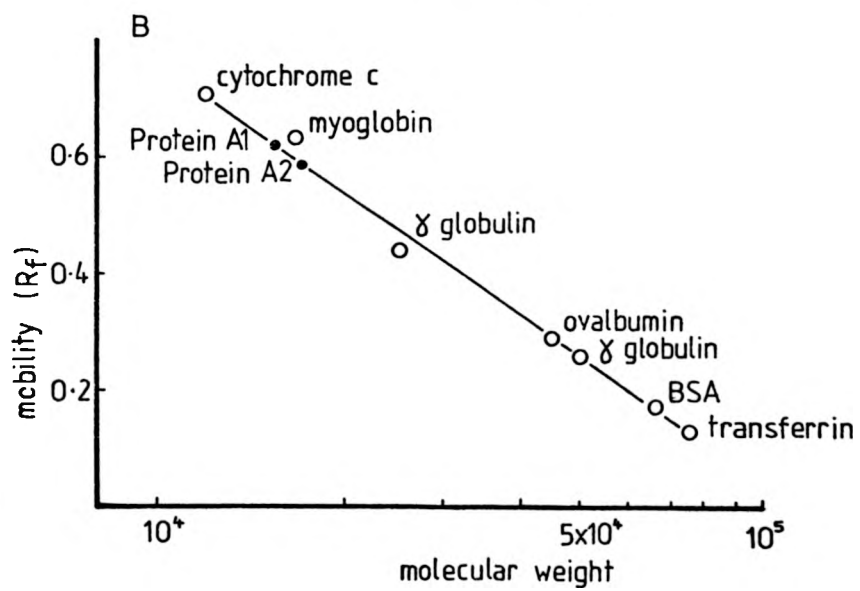
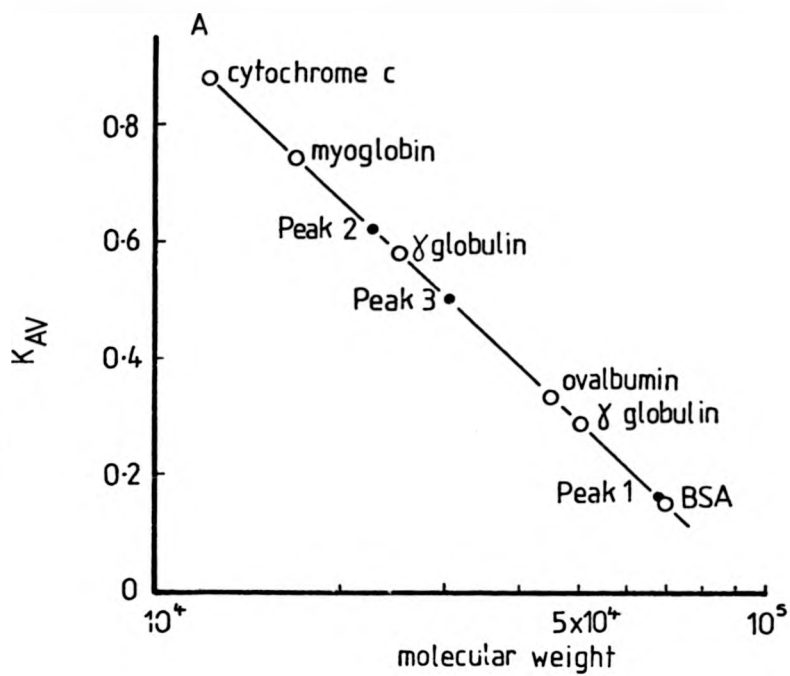


Figure 5.5 Molecular weight determination of stimulatory activities by Sephadex gel filtration and SDS gel electrophoresis.

(A) A Sephadex G-100 column used for fractionating oocyte extracts was calibrated by running the marker proteins indicated in a similar manner to the extract (see Chapter 2). Their presence was detected by measuring the A_{280} of the eluate. The graph shows the relative elution volumes of these markers (K_{AV}) against their known molecular weights. Also shown are the elution volumes of Peaks 1, 2 and 3 which have apparent molecular weights of 65,000, 23,000 and 30,000 daltons respectively.

(B) A mixture of marker proteins (shown in the figure) was applied to an 18% SDS slab gel in parallel with a sample of factor IIA, and electrophoresed as described in Chapter 2. The gel was stained for protein with Coomassie Brilliant Blue, destained, and the relative mobilities of the markers and proteins A1 and A2 measured from the origin. The graph shows these relative mobilities (R_f) plotted against the markers' known molecular weights. Proteins A1 and A2 have apparent molecular weights of 15,750 and 17,400 daltons respectively.

extracts as previously described for Stage 1, Stage 2/3 and Stage 5/6 oocytes. Extracts containing 4mg of protein were then fractionated by gel filtration on Sephadex G-100 and the fractions assayed as before. Fig. 5.6 shows the results of these fractionations and the incorporation in the nuclear assay for Stage 1 and Stage 2/3 oocytes. These profiles may be compared with that for Stage 5/6 oocytes shown in Fig. 5.8 (top). In each case the stimulation by the crude extract is also indicated.

The easiest way to summarise these results is to compare the incorporation (in the nuclear assay) of the peak fractions and express this as a stimulation over background. Table 5.2 shows the result of these calculations. Peak 1 activity increases gradually during oogenesis per unit protein. Peak 2 increases much more markedly and Peak 3 remains fairly constant or decreases slightly (per unit protein).

The Peak 2 fractions from each stage were further fractionated, as before, by DEAE-cellulose chromatography. The results for the Stage 1 and Stage 2/3 oocytes are shown in Fig. 5.7. Fig. 5.2 (bottom) shows the Stage 5/6 oocyte Peak 2 result. These results have been summarised in the same way as above and are also included in Table 5.2. Briefly, factor IIA activity is almost absent in Stage 1 oocytes and is very active in both Stage 2/3 and Stage 5/6 oocytes. There is thus an increase in the activity of factor IIB during oogenesis per unit soluble protein. It is tempting to speculate that this increase in the activity of the factors during oogenesis may be correlated with the increase in RNA synthesis in vivo.

(d) The activity of the factors during *Xenopus* early development

In normal development nuclei make little RNA in egg cytoplasm, but resume intensive RNA synthesis in the blastula. It may be asked if the activity in the Sephadex peaks and the purified factors changes during these stages in development.

Fig. 5.8 shows a Sephadex G-100 analysis of oocyte, egg and blastula (Stage 9) extracts. Peak 1 is present at all times, but Peak 2 diminishes very greatly in the egg and reappears in the blastula. It is always noticeable that a little of Peak 2 remains in the egg.

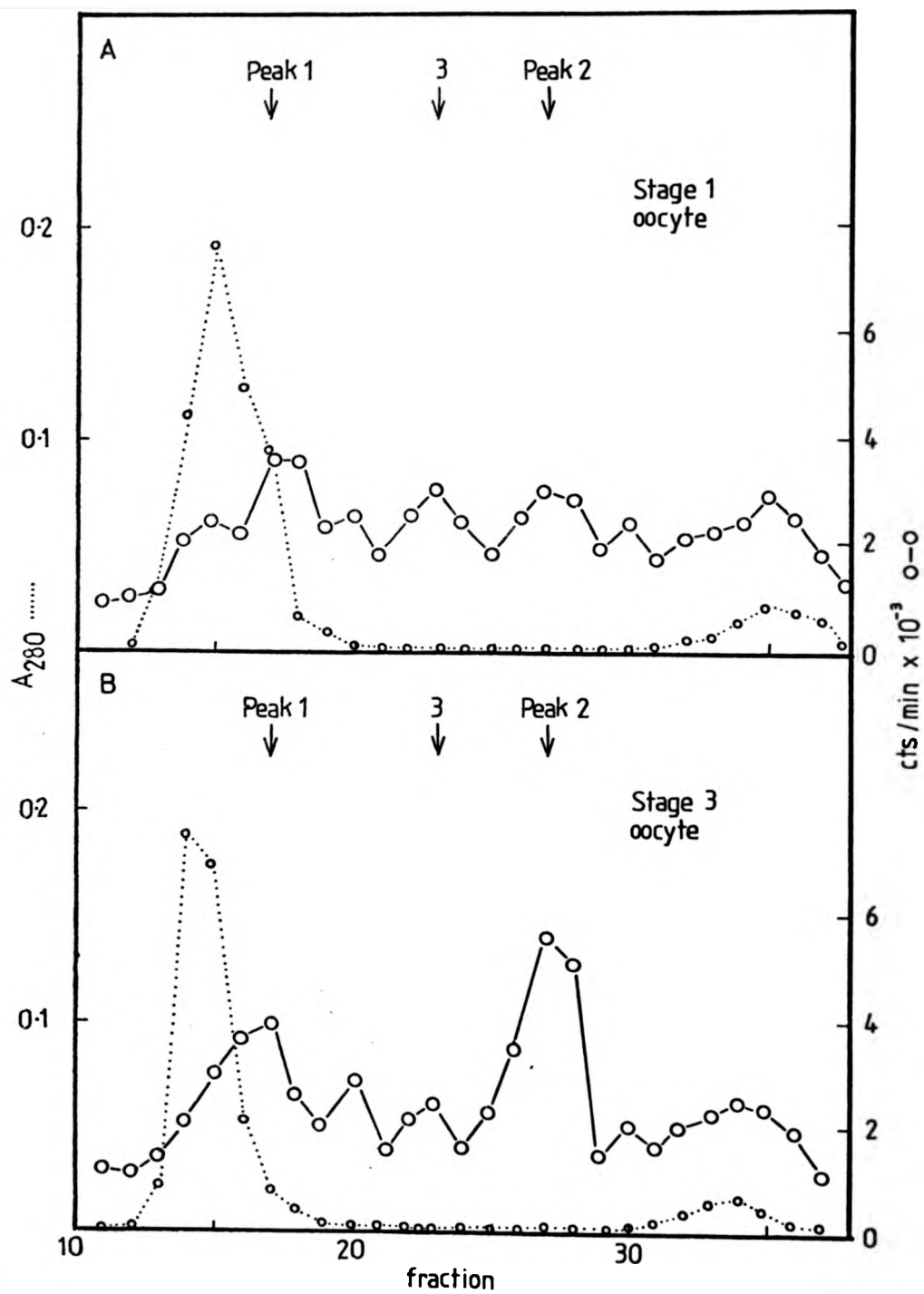


Figure 5.6 Nuclear assays of Stage 1 and Stage 2/3 ovary cell extracts fractionated on G-100 Sephadex.

Cell extracts containing 4 mg of protein prepared from Stage 1 ovary were fractionated on Sephadex G-100 superfine. 20 μ l from each fraction was assayed for its effect on RNA synthesis in isolated XTC-2 nuclei under standard preincubation conditions. (O.....O), A_{280} ; (O—O), [3 H]UTP incorporation in the nuclear assay.

The initial total extracts assayed in the same way gave incorporations of 1300 cpm (Stage 1) and 2200 (Stage 2/3). The markers indicate where Peaks 1, 2 and 3 from Stage 5/6 oocytes elute under the same conditions.

cts/min $\times 10^{-3}$ o—o

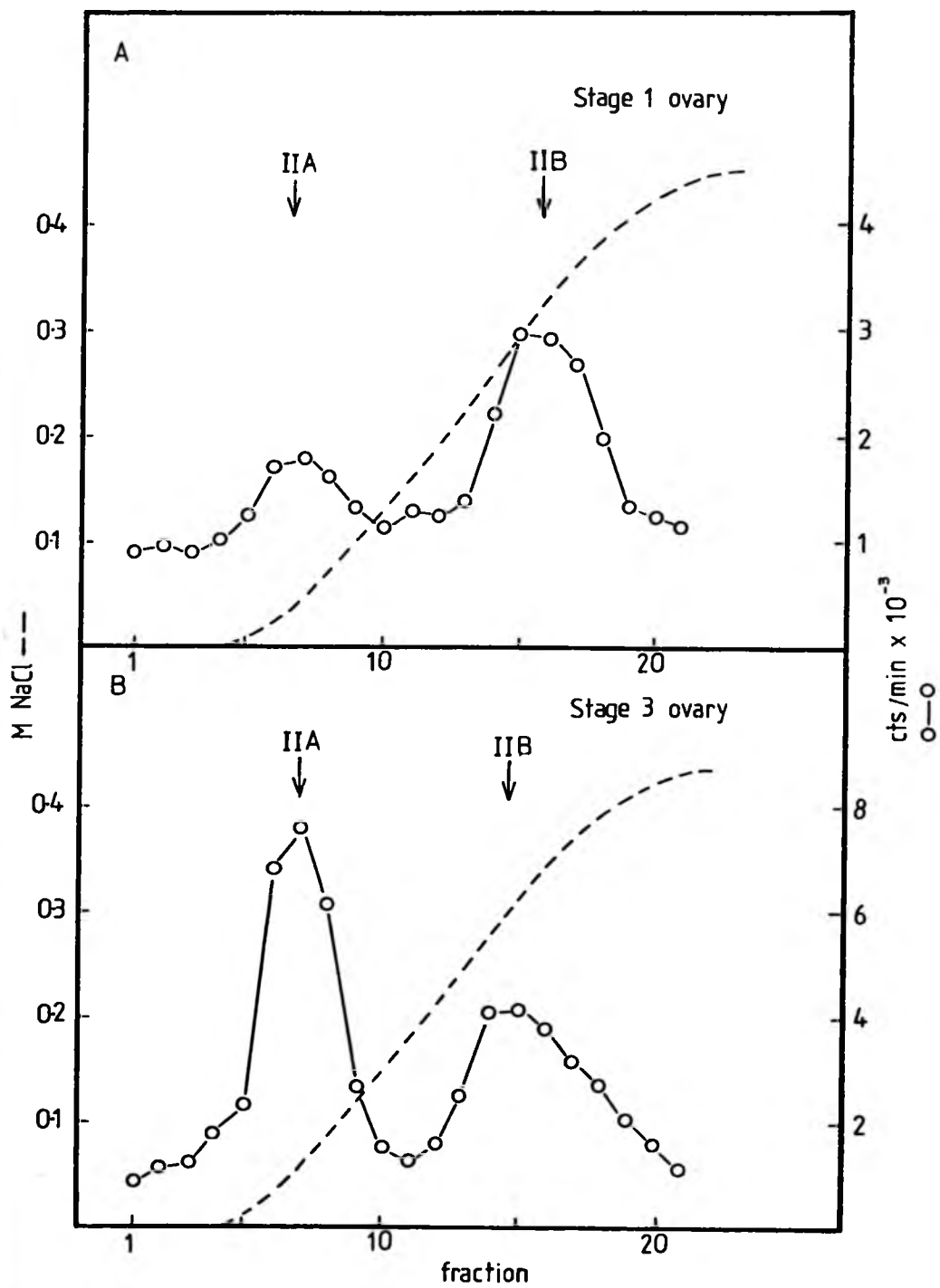


Figure 5.7 DEAE-cellulose chromatography of Sephadex purified Peak 2 from Stage 1 and Stage 2/3 ovary cell extracts.

Peak 2 pooled from Stage 1 and Stage 2/3 ovary Sephadex columns (see Figure 5.6) were run on DE52 columns and assayed as described in Figure 5.1B. (O—O), [³H] UTP incorporation in the nuclear assay; (— —), salt concentration.

cts/min x 10

O—O

Table 5.2

The activity of stimulatory factors in cell extracts from different Stages during Xenopus oogenesis and embryogenesis

	Total extract	Sephadex	Sephadex	Sephadex	Factor IA	Factor IB	Factor IIA	Factor IIB
		Peak 1	Peak 2	Peak 3				
Stage 1 oocyte	1.2	3.5	3.0	3.0	-	-	1.75	3.0
Stage 2/3 oocyte	2.0	4.0	5.5	2.5	-	-	7.5	4.0
Stage 5/6 oocyte	2.5	5.0	8.3	3.0	2.7	6.8	7.3	5.0
Egg	1.0	4.5	3.3	2.1	1.2	6.0	1.6	2.6
Blastula	2.4	3.8	8.0	3.9	2.5	6.5	5.6	4.0
Tailbud	0.4	3.3	4.3	1.6	-	-	-	-

Cell extracts were prepared from the stages indicated in the standard way; 4 mg of protein were fractionated as already described. The figures relate to the stimulation over background for each activity in the standard nuclear assay.

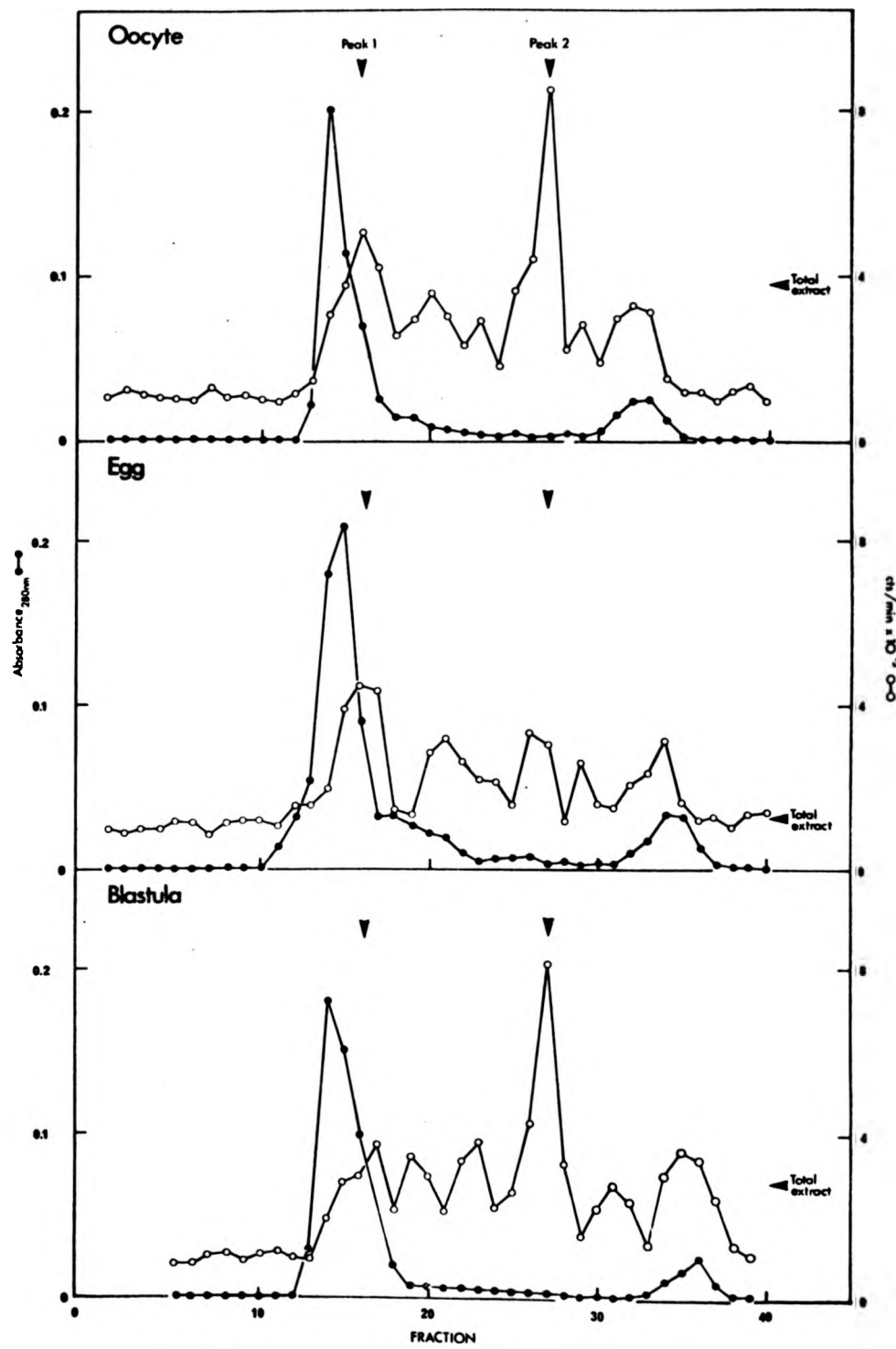
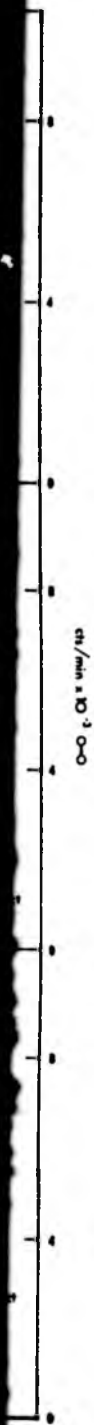


Figure 5.8 Nuclear assays of oocyte, egg and blastula cell extracts fractionated on G-100 Sephadex.

Cell extracts prepared from 200 oocytes, eggs or Stage 9 embryos, (containing ~ 4 mg of protein) were fractionated on Sephadex G-100 superfine. 20 μ l from each fraction was assayed for its effect on RNA synthesis in isolated XTC-2 nuclei under standard preincubation conditions. (\bullet — \bullet), A_{280} ; (\circ — \circ), [3 H] UTP incorporation in the nuclear assay.

The results of parallel nuclear assays using the initial total extracts are indicated. Peak 3 elutes at about fraction 23 under these conditions.



No inhibitory fractions were obtained from the egg extract. This is even more surprising than their absence from the oocyte.

Crude extracts of neurulae and later embryonic stages were shown to inhibit RNA synthesis by isolated nuclei very effectively (see Chapter 4). Since later embryonic stages make RNA very actively, it was important to know if they contained the stimulatory factors separable on Sephadex. If they were absent, these factors would be unlikely to be of general importance in controlling RNA synthesis. Fig. 5.9 shows that Peaks 1 and 2 are both present in tail-bud embryos, even though a crude extract of this stage inhibits RNA synthesis by about 40%. There are a few inhibitory fractions eluting between Peaks 1 and 2 and these might inhibit the stimulatory activities in the crude extract. A mixing experiment was performed to test this. Each fraction was reassayed with nuclei in the presence of the inhibitory fractions. The result is also shown in Fig. 5.9. There is a reduction in incorporation but this may be accounted for by dilution of the added fractions. Thus the inhibitory fractions do not seem to affect the stimulation produced by Peaks 1 and 2. The inhibitory property of the crude extract therefore remains obscure.

It is interesting to note here that Peak 3 is reduced in the egg extract but is much more prominent in the blastula in comparison with the Stage 5/6 oocyte activity. There is a reduction in Peak 3 in the tail-bud, however, as can be seen from Fig. 5.9. The inhibitory fractions affect the activity of this peak to some extent. As Peak 3 elutes close to the inhibitory fractions, its actual activity may be altered.

Peaks 1 and 2 from oocytes, eggs and blastula Sephadex columns were further analysed by DEAE-cellulose chromatography. The results of these experiments for the egg and blastula are shown in Fig. 5.10 and may be compared with the oocyte results shown in Fig. 5.7. DEAE peaks IA and IB are present in similar amounts throughout the developmental stages studied, the egg and blastula profiles looking just like those of the Stage 5/6 oocytes. Sephadex Peak 2 is much reduced in the egg and, as expected, the DEAE profiles also show lowered stimulatory activity. Peak IIA is reduced much more than IIB. The blastula profiles are very similar to those obtained for the oocyte Peak 2. All these results are

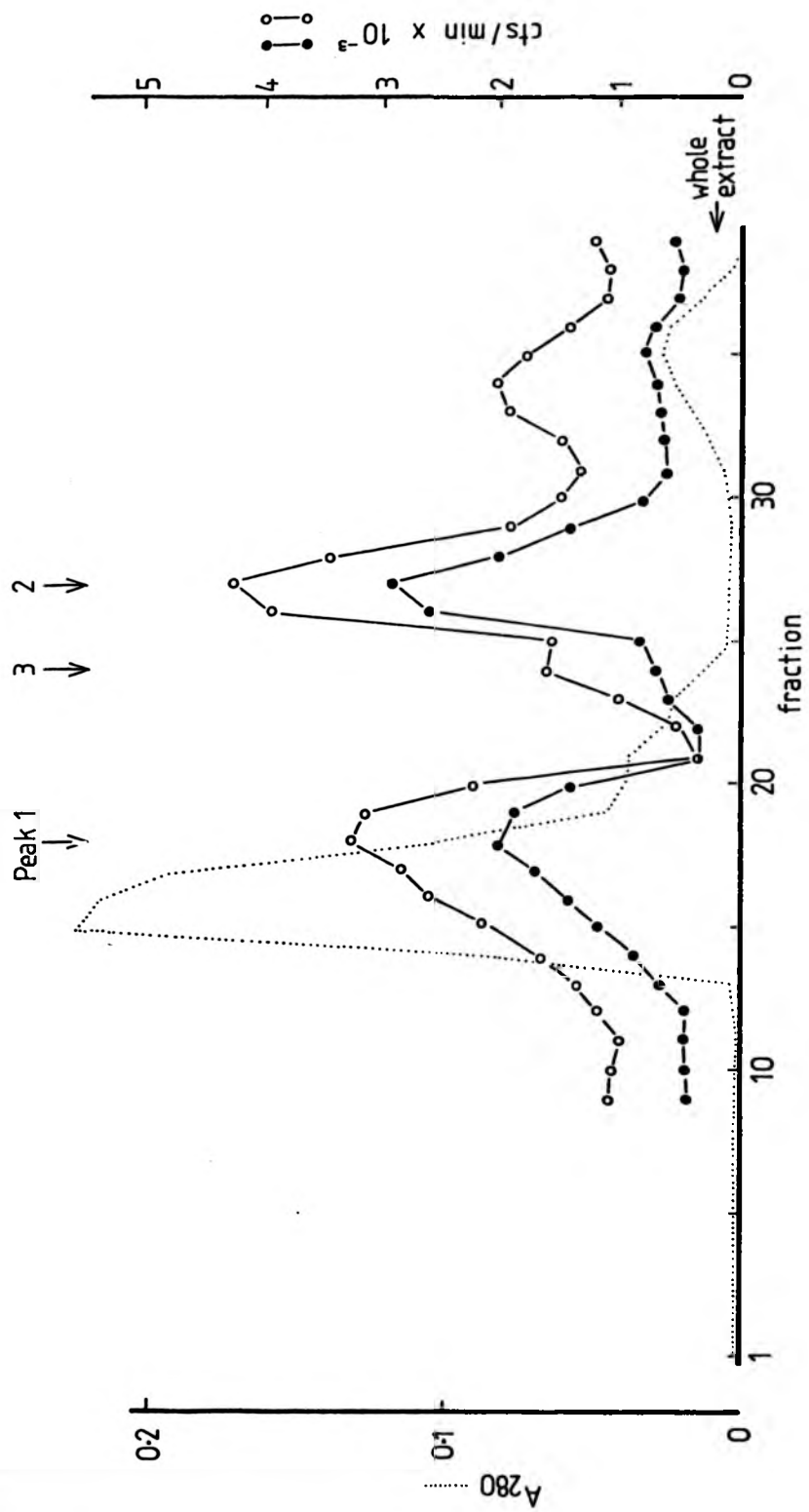


Figure 5.9 Nuclear assay of tailbud cell extracts fractionated on G-100 Sephadex.

A cell extract prepared from ~ 200 tailbud embryos (containing ~ 4 mg protein) was fractionated and assayed as described in Figure 5.1A. (.....), A_{280} ; (○—○), [^3H] UTP incorporation in the nuclear assay. The arrow indicates the incorporation of [^3H] UTP by XTC-2 nuclei preincubated with the unfractionated extract.

The column fractions were reassayed as above except that each 50 μl assay contained 10 μl of fraction 21 and 10 μl of the particular fraction to be assayed. (●—●), [^3H] UTP incorporation in the nuclear assays of the mixed fractions.

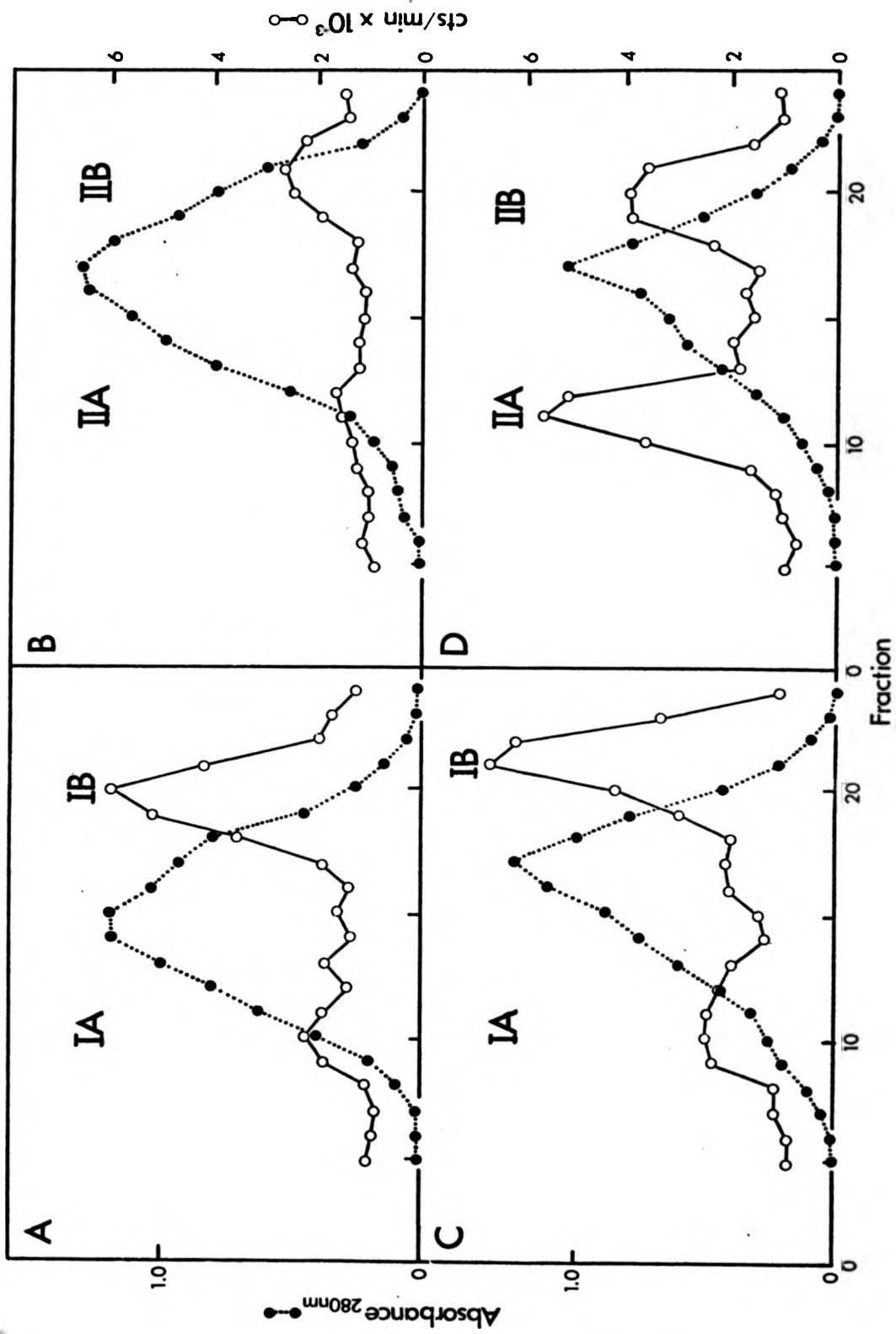


Figure 5.10 DEAE-cellulose chromatography of Sephadex purified Peaks 1 and 2 from unfertilised egg and blastula cell extracts.

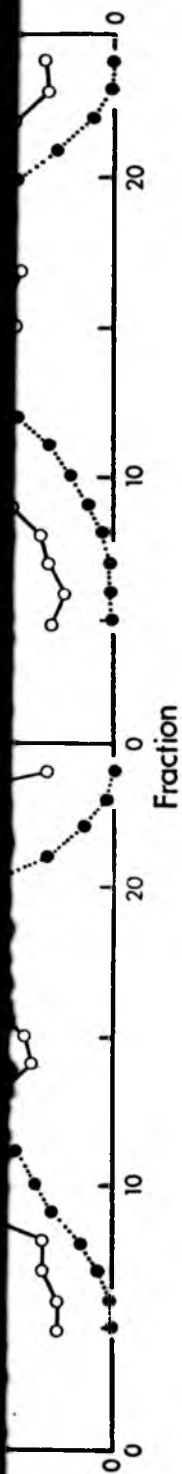
Peaks 1 and 2 pooled from egg and blastula Sephadex columns were run on DE52 and assayed as described in Figure 5.1B. (●.....●), A_{280} ; (○—○), [^3H]UTP incorporation in the nuclear assay.

(A) Peak 1 from unfertilised egg Sephadex column.

(B) Peak 2 from unfertilised egg Sephadex column.

(C) Peak 1 from blastula Sephadex column.

(D) Peak 2 from blastula Sephadex column.



summarised in Table 5.2 in the same way as the oogenesis results. Clearly the most obvious change is in the activity in Peak 2 and in factors IIA and IIB. These are present in the oocyte, are much reduced in the egg and reappear in the blastula and tail-bud stages.

(e) Discussion

This chapter has described the first steps in the purification from Stage 5/6 oocytes of the factors which stimulate RNA synthesis in isolated nuclei. The two step purification procedure, involving gel filtration on Sephadex G-100 followed by DEAE-cellulose chromatography, indicates that four components may be separated from this extract. Purity of these factors is not very good. However, one, factor IIA, which is probably the most pure, has a saturating level of 10 ng protein/ μ g DNA (i.e. 10 ng protein per 2 ng rDNA) which causes a 10-fold stimulation in RNA synthesis in isolated nuclei. Two proteins with molecular weights of 15,750 and 17,400 are present in large amounts in this active fraction (although there are other proteins present) and may be involved in causing the stimulation in transcription. The other factors are too impure for any tentative assignments to be made.

Some of these purified factors change in activity during Xenopus oogenesis and early development. Table 5.2 summarises the results. Sephadex Peaks 1 and 2 do have different activities at the different stages, Peak 2 most significantly. This effect is most obvious in factor IIA whose activity changes markedly, being least active in the Stage 1 oocyte and the unfertilised egg. The possibility that the changes in the activity of the factors is responsible for the changes observed in RNA synthesis in vivo is an attractive one. However, proof of this depends on further characterising the purified factors and their activities. The next chapter describes experiments designed to go some way towards this.

Chapter 6

Specificity of the purified factors

(a) Introduction

The previous chapter described the partial purification of four factors from Stage 5/6 oocytes. This chapter is concerned with investigating the specificity of these factors, that is, whether they affect one particular or all the RNA polymerases present in the isolated nuclei. On a finer level of specificity is the possibility that the factors may affect the synthesis of one or a few RNA species. How they may have these effects is another question, one which will be dealt with in the next chapter.

The main methods for investigating specificity of the factors were the use of α -amanitin and polyacrylamide gel electrophoresis. By using α -amanitin at different concentrations in the assay after preincubation, it was possible to measure the activities of the different RNA polymerases after stimulation had occurred. These results occupy the first half of this chapter. The second half is confined to the electrophoretic analysis of the RNA products synthesised by nuclei treated with the different factors. These experiments are devoted to factors isolated from Stage 5/6 oocytes only.

(b) The activities of the different RNA polymerases in factor-treated nuclei

Nuclei were preincubated in the normal way with fractions from a Sephadex G-100 column of Stage 5/6 oocytes. The nuclei were then assayed for transcription as before except that nuclei preincubated with each fraction were labelled in the absence of α -amanitin and also exposed to two levels of α -amanitin at 1 μ g/ml (sufficient to inhibit polymerase II) and at 100 μ g/ml (sufficient to inhibit polymerases II and III). The incorporation in these assays was measured after 30 minutes as usual. Fig. 6.1 shows the result of this experiment. Clearly there is variation in the percentage of α -amanitin insensitive incorporation between the different fractions. This suggests that the different peaks of stimulation may have different RNA polymerase specificities. Peaks 2 and 3 seem particularly interesting. Peak 2 stimulation is relatively

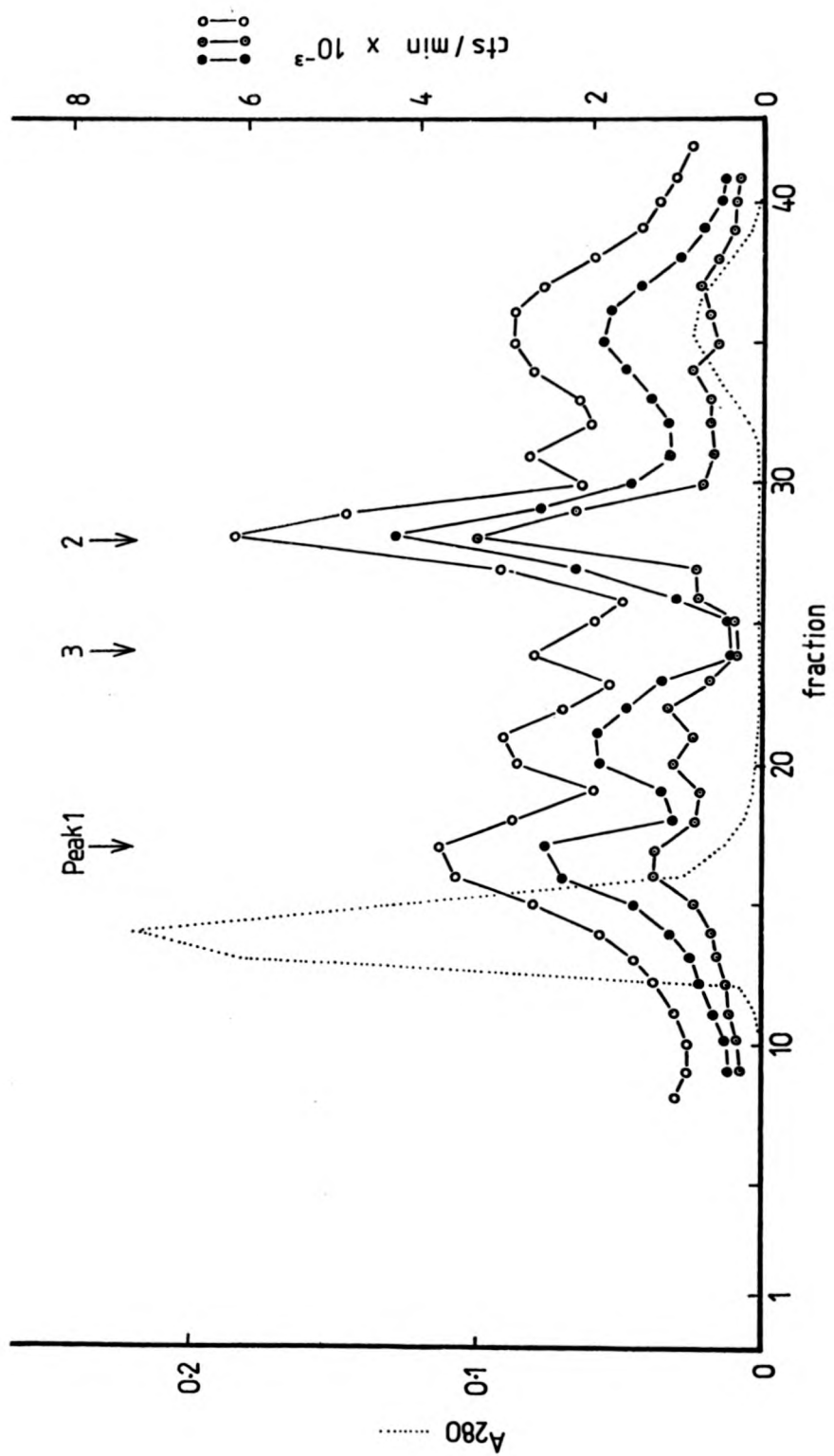


Figure 6.1 The α -amanitin sensitivity of RNA synthesis by nuclei preincubated with Sephadex G-100 fractions of Stage 5/6 oocyte extract.

A cell extract prepared from Stage 5/6 oocytes (containing ~ 4 mg protein) was fractionated on Sephadex G-100 superfine. 20 μ l samples from each fraction were preincubated in standard nuclear assays. After preincubation, the nuclei were assayed for RNA synthesis with [3 H] UTP in the standard way except that α -amanitin was added to 1 μ g/ml and 100 μ g/ml in parallel sets of assays. (.....), A_{280} ; [3 H] UTP incorporation in the nuclear assay in the absence of α -amanitin (O); in the presence of 1 μ g/ml α -amanitin (●); in the presence of 100 μ g/ml α -amanitin (⊙).

insensitive to α -amanitin, whereas Peak 3 is very sensitive to the toxin.

The same form of experiment was carried out for DEAE-cellulose column fractions of Peaks 1 and 2 from Sephadex. These results are shown in Fig. 6.2. Most of the factors appear to stimulate all the RNA polymerase species. However, the stimulation by factor IIA is almost entirely α -amanitin insensitive. This suggests that the factor preferentially affects polymerase I activity.

To clarify these results they have been quantified in the following manner. The percentage of the total incorporation in the nuclear assay resulting from the activity of each RNA polymerase form for the control, the Sephadex peaks and the purified factors has been calculated and is shown in Table 6.1. Also shown in this table is the fold stimulation by each factor. It is necessary to compare the absolute levels of each polymerase activity in the control and the factor treated nuclei. For this purpose the absolute polymerase activity of the total control assay was defined as 1. This was then used to calculate the absolute relative polymerase activity for all the factor treated nuclei for each of the RNA polymerase forms. One unit of polymerase I activity was then defined as the absolute level in the control. From this an absolute fold stimulation of polymerase I may be calculated for each of the different factors. The same calculation was carried out for polymerase II and III activities. These figures are given in Table 6.2. They indicate the fold stimulation over control of each individual polymerase. These were then normalised to the activity of one polymerase. Polymerase III was chosen as its stimulation is, in general, the least. This normalisation, shown in Table 6.3, allows for the varying amount of each factor added to the assay.

These data indicate that most of the factors stimulate all three polymerases, in general polymerase I more than the others. There are, however, exceptions to this. As already noted, Peak 2 and factor IIA both stimulate polymerase I considerably, factor IIA particularly so. It causes an almost 50-fold stimulation of polymerase I alone. Polymerases II and III are unaffected.

The other interesting result here is that Peak 3 appears to stimulate polymerase II preferentially, although polymerase I is also stimulated

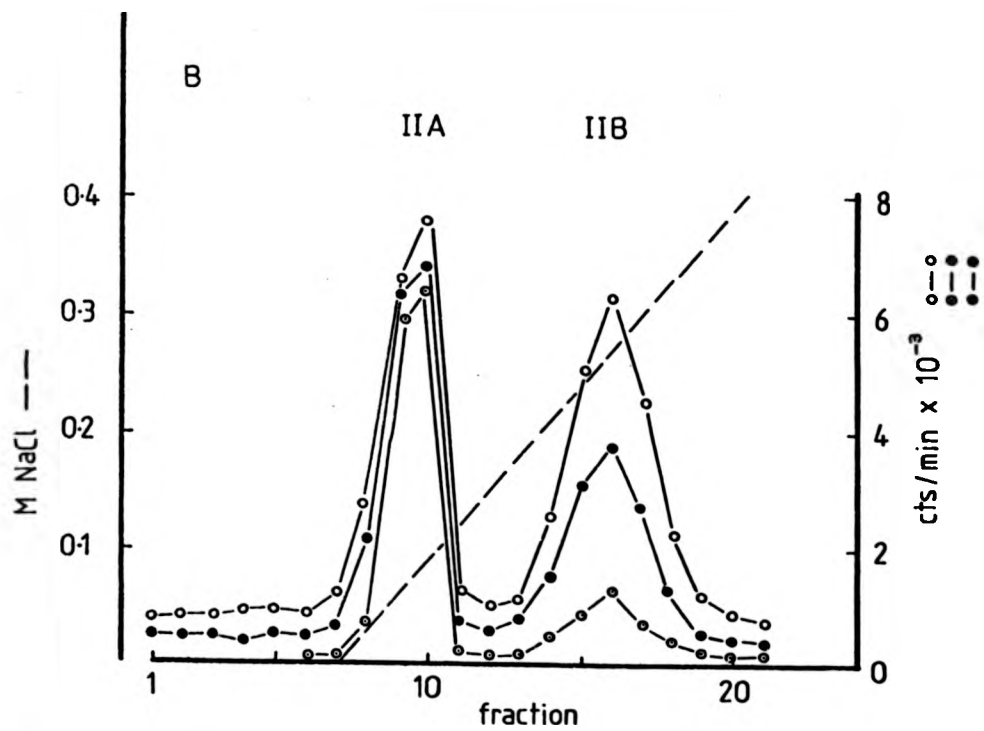
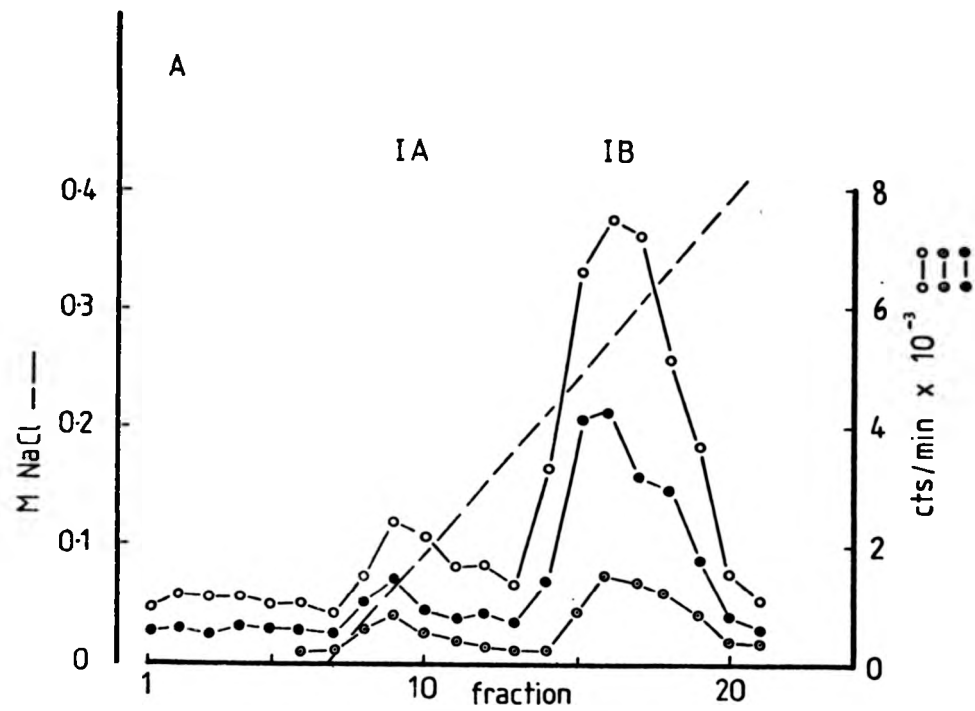


Figure 6.2 The α -amanitin sensitivity of RNA synthesis by nuclei preincubated with DEAE fractions of Stage 5/6 oocyte Peaks 1 and 2.

Peaks 1 and 2 pooled from a Stage 5/6 oocyte Sephadex column were run on DE52 as described in Figure 5.1B. 20 μ l samples from each fraction were preincubated with XTC-2 nuclei in the standard way. After preincubation, the nuclei were assayed for RNA synthesis with [3 H] UTP in the standard way with α -amanitin added to 1 μ g/ml and 100 μ g/ml in parallel sets of assays. (— —), salt concentration; [3 H] UTP incorporation in the nuclear assay in the absence of α -amanitin, (\circ); in the presence of 1 μ g/ml α -amanitin (\bullet); in the presence of 100 μ g/ml α -amanitin (\odot).

(A) Peak 1 from oocyte Sephadex column.

(B) Peak 2 from oocyte Sephadex column.

Table 6.1

The activity of each RNA polymerase form in factor treated nuclei

		Fold stimulation in the nuclear assay	% RNA polymerase I	% RNA polymerase II	% RNA polymerase III
	Control	1	13	37	50
Sephadex peaks	Peak 1	5	34	32	34
	Peak 2	8.3	52	32	16
	Peak 3	3	9	89	2
	Factor IA	2.7	30	45	25
DEAE peaks	Factor IB	6.8	26	41	33
	Factor IIA	7.3	88	5	7
	Factor IIB	5	37	38	25

Nuclei were preincubated with the indicated fractions and factors purified from Stage 5/6 oocytes in the standard way. α -amanitin was either left out or added to 1 μ g/ml or 100 μ g/ml during the labelling step. The percentages for the different polymerase activities were calculated from these assays after 30 minutes of labelling.

Table 6.2

Absolute fold stimulation of individual RNA polymerase forms in Stage 5/6 oocyte factor treated nuclei

		Fold stimulation		
		polymerase I	polymerase II	polymerase III
	Control	1	1	1
Sephadex peaks	Peak 1	13	4	3
	Peak 2	33	7	3
	Peak 3	2	7	0.1
<hr/>				
	Factor IA	6	3	1.2
DEAE peaks	Factor IB	13	7	4
	Factor IIA	49	1	1
	Factor IIB	14	5	2

These figures are derived from Table 6.1 (as described in the text) by taking the control total incorporation as one arbitrary polymerase unit. Using the fold stimulation induced by the factors and the percentage due to each polymerase activity, the above figures were calculated taking the control level of each polymerase as 1.

Table 6.3

Absolute fold stimulation of individual RNA polymerase forms in Stage 5/6 factor treated nuclei, normalised with respect to polymerase III activity

		Fold stimulation		
		polymerase I	polymerase II	polymerase III
Control		1	1	1
Sephadex peaks	1	4.3	1.3	1
	2	11	2.3	1
	3	20	70	1
<hr/>				
IA		5	2.5	1
DEAE peaks	IB	4.2	1.7	1
	IIA	49	1	1
	IIB	7	2.5	1

These figures are from Table 6.2 except they are normalised to a polymerase III activity of 1 in each case.

but to a lesser extent. This activity will be discussed in more detail in Chapter 10.

No polymerase III-specific factor is apparent in the data shown here. The other lesser peaks of stimulation from Sephadex have not been investigated fully, but there is no obvious polymerase III stimulatory factor, at least in Stage 5/6 oocytes.

The specificity of the factors from other developmental stages has been assayed using α -amanitin. They display similar specificities, in terms of the α -amanitin sensitivity of the stimulation, to the Stage 5/6 oocyte factors.

(c) Electrophoretic analysis of RNA synthesised *in vitro* by factor stimulated nuclei

The RNA synthesised *in vitro* by nuclei preincubated with the different factors was subjected to polyacrylamide gel electrophoresis under denaturing conditions (see Chapter 2). Fig. 6.3 shows the RNA product of IA, IB, IIA and IIB treated nuclei. Clearly, most of the factors do not alter the general profile of the RNA product. Again, factor IIA is an exception. There is a considerable stimulation of rRNA in nuclei treated with this factor. This effect of rRNA synthesis was further clarified by incubating the nuclei, in 1 mg/ml α -amanitin, during the labelling step. The resulting RNA product fractionated on a 2.4% polyacrylamide gel is shown in Fig. 6.4. A massive and specific stimulation of rRNA synthesis is apparent. This figure also shows the RNA product of control nuclei under these conditions and the time course of incorporation by the control and IIA treated nuclei.

The stimulation of 40S precursor synthesis (comparing the 40S ribosomal peaks in the control and the IIA RNA product) was about 20-25-fold. The stimulation of 18S and 28S RNA was lower, at 11-fold, but this may be because these have been degraded in some way or because processing is slowed down or rate limiting. This fold stimulation, even for the 40S precursor, is lower than expected from the α -amanitin data. As already suggested, this may be due to a nuclease problem. To test this possibility a hybridization experiment was carried out, as described in Chapter 3. The RNA products of factor IIA treated nuclei, synthesised

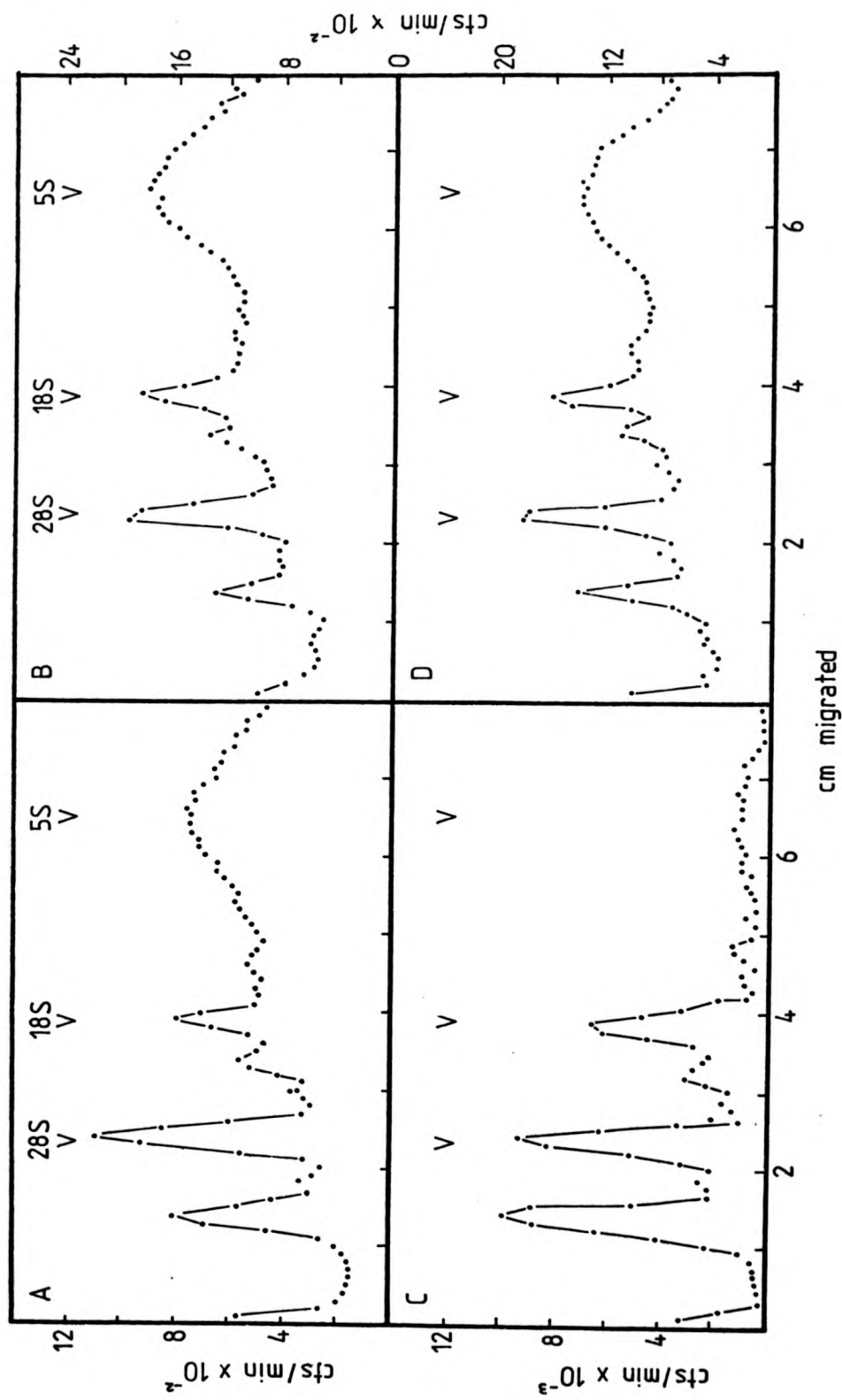


Figure 6.3 Gel fractionation of RNA products synthesised by XTC-2 nuclei *in vitro* after preincubation with factors IA, IB, IIA and IIB.

RNA was extracted from standard 200 μ l nuclear assays of factors IA, IB, IIA and IIB after 30 minutes labelling with [3 H] UTP. The extracted RNA was run on 2.4% RNA gels scanned at 260 nm and sliced into 1 mm sections. The radioactivity present in each slice was determined as described in Chapter 2.

- (A) The RNA product of factor IA treated nuclei.
- (B) The RNA product of factor IB treated nuclei.
- (C) The RNA product of factor IIA treated nuclei.
- (D) The RNA product of factor IIB treated nuclei.

The arrows indicate the position of BHK RNA markers.

6
4
2
cm migrated
6
4
2

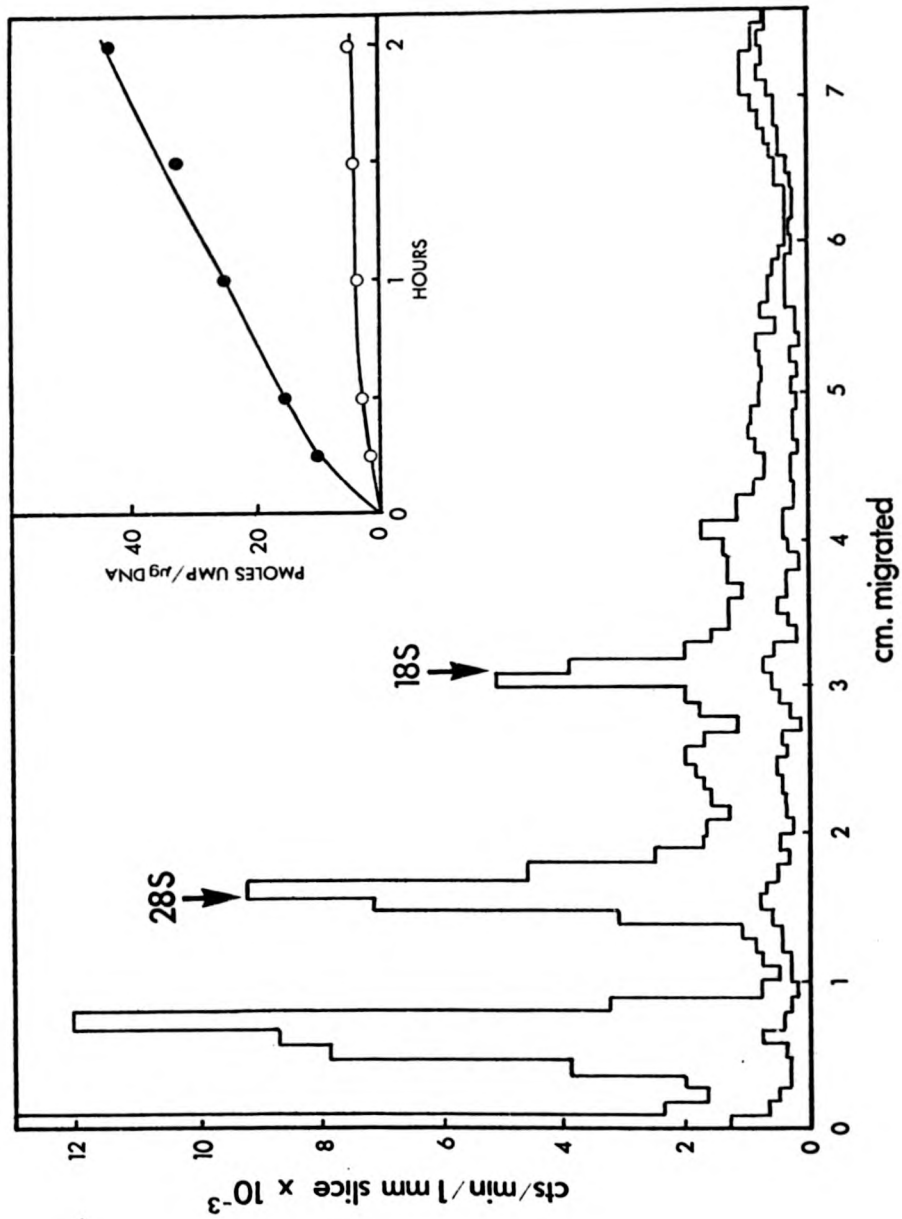
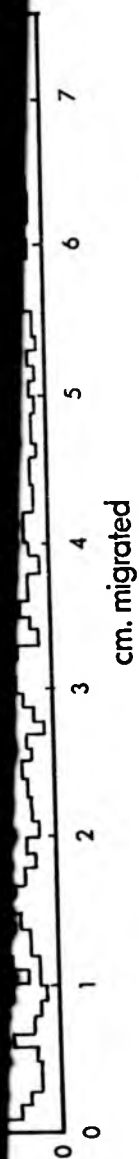


Figure 6.4 Gel electrophoresis of RNA products synthesised in isolated XTC-2 nuclei *in vitro* after preincubation with and without factor IIA.

RNA was extracted from 200 μ l incubates of XTC-2 nuclei which had been preincubated with and without factor IIA from Stage 5/6 oocytes. α -amanitin was added to 1 mg/ml during the labelling step. The purified RNA was run on 2.4% RNA gels, which were scanned, sliced and counted as already described. The lower profile is the product of control nuclei; the upper is of IIA treated nuclei. The arrows indicate the position of BHK RNA markers. The inset shows the time course of incorporation of control (\circ) and IIA treated nuclei (\bullet).



in the presence of α -amanitin (1 mg/ml) were hybridized on filters to fractions of Xenopus liver DNA which had been banded in a CsCl density gradient. This should determine whether all the RNA synthesised in these conditions is rRNA.

The result of the hybridization experiment is shown in Fig. 6.5, 81% of the RNA hybridized to the rDNA suggesting that factor IIA stimulated rRNA synthesis by almost 40-fold. It is clear that some non-ribosomal RNA, that is RNA that hybridizes to the main band DNA, is present. This may be due to residual polymerase II or III activity which is not inhibited by the α -amanitin, but with an α -amanitin concentration of 1 mg/ml this seems unlikely. Another possibility is that the RNA is a product of RNA polymerase I molecules which are incorrectly initiating on non-ribosomal genes. Incorrect initiation may be expected in isolated nuclei. Hybridization experiments of this type may indicate just how much is occurring. There is another possibility however. The RNA product hybridizing to the main band DNA may be a true product of polymerase I. Indeed, Serecks and Penman (1977) have reported such a class of RNA molecules in HeLa cells. These 6-10S non-ribosomal molecules are synthesised at α -amanitin concentrations of 150-400 μ g/ml. Experiments with factor IIA may help to elucidate the role of this RNA in the cell.

(d) Discussion

Results in this chapter indicate that most of the purified factors are non-specific, that is they stimulate all three forms of RNA polymerase. The exceptions are factor IIA, which seems to be polymerase I specific, and Sephadex Peak 3, which preferentially stimulates polymerase II.

The finding that Stage 5/6 oocytes contain factors which stimulate RNA synthesis may not perhaps be a surprising one. Over 90% of the RNA synthesised by these oocytes is ribosomal (Colman, 1974), and so the observation that one of these factors (factor IIA) is polymerase I specific may also be expected. It is clear from Chapter 5 that the activity of this factor, in general, follows the level of rRNA synthesis occurring at the different stages of oogenesis and early development. It is not present in Stage 1 oocytes and eggs, but is present in large

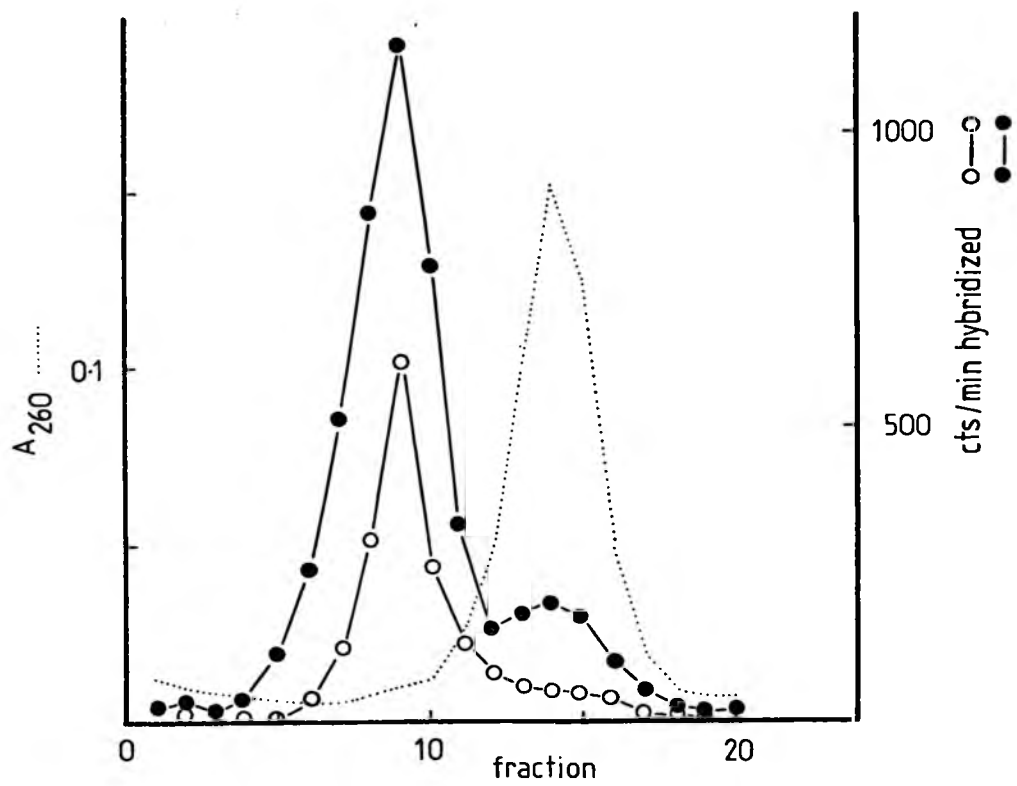


Figure 6.5 Hybridization of labelled RNA synthesis by XTC-2 nuclei *in vitro* after preincubation with factor IIA.

RNA was extracted from standard nuclear assays of factor IIA from Stage 5/6 oocytes. α -amanitin was added to 1 mg/ml during the labelling step with [3 H] UTP. 50 μ g of *Xenopus* DNA was loaded on to a CsCl gradient. After centrifugation, the gradient was fractionated and each fraction was divided into two. These were loaded separately on to 13 mm millipore filters. One set was hybridized to the purified RNA from the nuclear incubation (●). The other set was hybridized to [32 P] labelled rRNA purified from XTC-2 cells (○). (.....), A_{260} .

Input counts for the [3 H] RNA were 15,000 cpm and for the [32 P] RNA, 10,000 cpm. Details of RNA extractions, CsCl gradients and hybridizations were as described in Chapter 2.

cts/min hybridized
○—○
●—●

amounts in the Stage 9 (blastula) extract and this correlates with the observed early gastrula start of rRNA synthesis (Nakahashi and Yamana, 1976).

In contrast to the stimulatory compounds described here, most reports to date have described the search for, and analysis of factors from mature oocytes, unfertilised eggs or cleaving embryos which inhibit rRNA synthesis. Shiokawa and Yamana (1967) first reported a fairly specific inhibitor of rRNA synthesis which was released from dissociated blastula cells. The small, non-protein molecule inhibited rRNA synthesis in intact neurula cells, which synthesise rRNA rapidly. These experiments have not been exactly repeated successfully (Hill and McConkey, 1972), but Laskey *et al.* (1973) have reported that a 0.5 N perchloric acid extract of *Xenopus* eggs completely inhibits rRNA synthesis in dissociated neurula cells. Crippa (1970) has also reported the isolation of a rDNA binding molecule from *Xenopus* oocytes which remained in an ovary 3 days after hormone-induced ovulation. When injected into Stage 4 oocytes, this molecule specifically inhibited rRNA synthesis. There was no evidence of comparable inhibitors in the experiments with isolated nuclei described here. However, the assay systems for these inhibitors involved the use of intact cells rather than isolated nuclei. The two approaches might therefore identify agents acting in whole embryos, possibly in a concerted fashion.

Quite a number of other workers have found factors which stimulate specific RNA polymerases. Most of these have used isolated RNA polymerases and purified DNA templates or chromatin. Examples are the polymerase I specific factors of Goldberg *et al.* (1973) from rat liver, and of Nagamina *et al.* (1976) from Ehrlich ascites cells. There are also examples of specific polymerase II stimulatory factors (Link and Richter, 1977; Sekemizur *et al.*, 1976). There are fewer reports of specific factors which stimulate RNA synthesis in isolated nuclei. Maclean and Hilder (1977) have reported that cytoplasmic extracts from *Xenopus* do stimulate RNA synthesis in isolated erythrocyte nuclei. This stimulation depends on added *E. coli* polymerase and the factor involved has not been purified greatly or its specificity analysed.

The finding that the activity of factor IIA follows the general patterns of rRNA in Xenopus early development gives some hope that it has an in vivo role in the control of transcription. The problem of separating cause and effect is, however, a difficult one. First steps toward this goal must be to analyse the mode of action of the purified factors so far described. Experiments attempting to clarify this point are described in the next chapter.

Chapter 7

Mode of action of the factors

(a) Introduction

The mode of action of factors influencing RNA synthesis may be analysed in several ways. One of these would be to determine the identity of the factor. Certain molecules are known to affect the rates of RNA synthesis at various points. An example here is DNAase which introduces single strand breaks in the DNA, which in turn increases the number of initiation sites available for the polymerases (Chambon *et al.*, 1970). Other examples are BSA (Rutter *et al.*, 1970) and, of course, RNA polymerase. Another possible agent which might modify RNA synthesis in isolated nuclei is protein kinase. Maller and Krebs (1977) have put forward a plausible case suggesting that the maturation of the oocyte to form an egg is caused by a change in the activity of a protein kinase. The first part of this chapter is concerned with experiments which assay for the presence in the purified factors of the activities or proteins described above.

The remainder of the chapter describes attempts to analyse the actual effects of the factors. Transcription in isolated nuclei may be altered in a variety of ways. Factors may alter how tightly the polymerases bind to the DNA, their initiation rate and/or the rates of elongation and termination. They may also act indirectly by altering the conformation of the DNA or chromatin. Experiments described in the second half of this chapter investigate how the factors affect some of these properties of transcription and also whether they are active in more defined RNA synthesising systems.

(b) Possible identity of the factors

The most obvious candidates for the identity of the factors ^{are the} RNA polymerases. Experiments already described (Chapter 4) have shown that added polymerases have little effect on the transcriptional activity of XTC-2 nuclei. Also, that the stimulatory activity is located in the cytoplasm of the oocyte, whereas the polymerase is in the oocyte nucleus (Roeder, 1974). These observations tend to rule out the possibility that

the factors are RNA polymerases. However, the Sephadex G-100 column and the DEAE-cellulose column of Stage 5/6 oocyte extracts were assayed for RNA activity by the method of Roeder (1974). Fig. 7.1 shows the result for the Sephadex column. It indicates that the polymerase activity coincides with Peak 1. Fig. 7.2 shows the polymerase assay applied to the DEAE-cellulose fractions of Sephadex Peak 1. The polymerase activity does not exactly coincide with either Peak IA or IB. No polymerase activity was detected in fractions from DEAE-cellulose chromatography of Peak 2. Thus polymerase activity may be involved in IA and/or IB stimulation.

Further analysis used the observation, mentioned above, that the polymerases are confined to the oocyte nucleus. Fig. 7.3 shows enucleated oocyte extracts that were chromatographed on Sephadex G-100 and assayed for their activity in stimulating RNA synthesis by isolated nuclei. Peaks 1 and 2 were present in the same proportions and to the same extent in enucleated as in intact oocytes. Negligible RNA polymerase activity was present in the extract or column fractions. Therefore, the effect of neither Sephadex Peak 1 or 2 depends on its own polymerase activity. It is interesting to note, however, that Peak 3 is reduced or absent. This may imply a nuclear location for this activity or that polymerase activity is involved here. However, it is too small for any known native RNA polymerases.

As mentioned in the introduction, protein kinase activity may be the cause of the stimulation in RNA synthesis. The Sephadex fractionated oocyte extract was assayed for protein kinase activity using calf thymus histone as substrate. Fig. 7.4 shows that a single peak of cyclic-AMP-activated protein kinase was detected, but that this did not coincide with either Peak 1 or 2. Neither of these stimulatory fractions is therefore a kinase of the type studied by Maller and Krebs.

The activity of RNA polymerases in vitro is greatly affected by the introduction of nicks into the template. In association with Dr. Alan Colman, all the purified factors from oocytes were assayed for endonuclease activity, using supercoiled bacterial plasmids. No activity was detected.

Various other proteins were assayed for their effect on RNA synthesis in isolated nuclei. Calf thymus histones can stimulate yeast RNA poly-

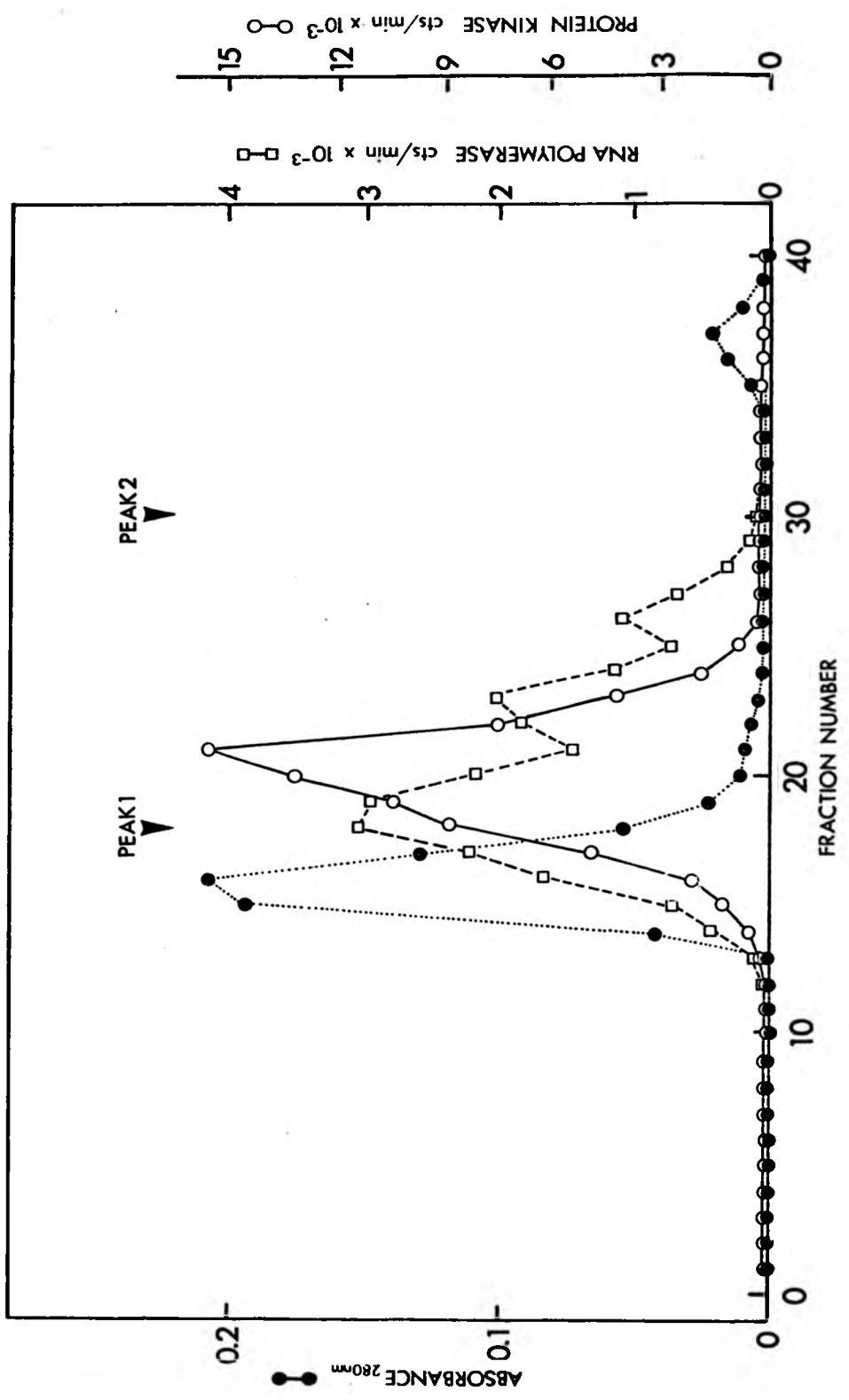
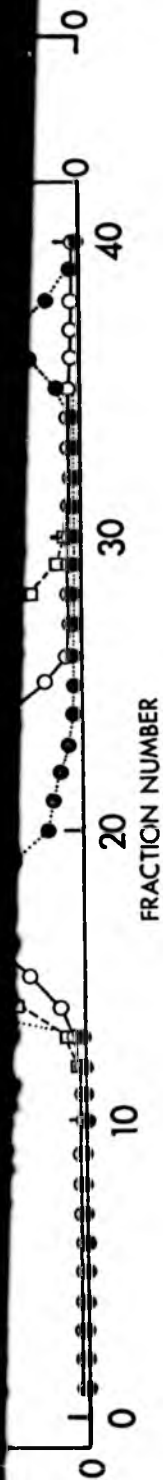


Figure 7.1 G-100 fractionated oocyte cell extract assayed for RNA polymerase and protein kinase activity.

A cell extract from 200 oocytes was fractionated on G-100 Sephadex as described in Chapter 2, and the A_{280} of each fraction measured (●.....●). 30 μ l of each fraction was assayed for RNA polymerase activity by the method of Roeder (1974) in a final assay volume of 100 μ l which was incubated for 20 minutes at 30^o C (□—□). 20 μ l of each fraction was also assayed for cyclic AMP-activated protein kinase activity (○—○) by the method of Maller and Krebs (1977). The profiles for protein kinase were the same in the presence and absence of cyclic AMP. The markers indicate the peaks of stimulation of RNA synthesis in the nuclear assay.



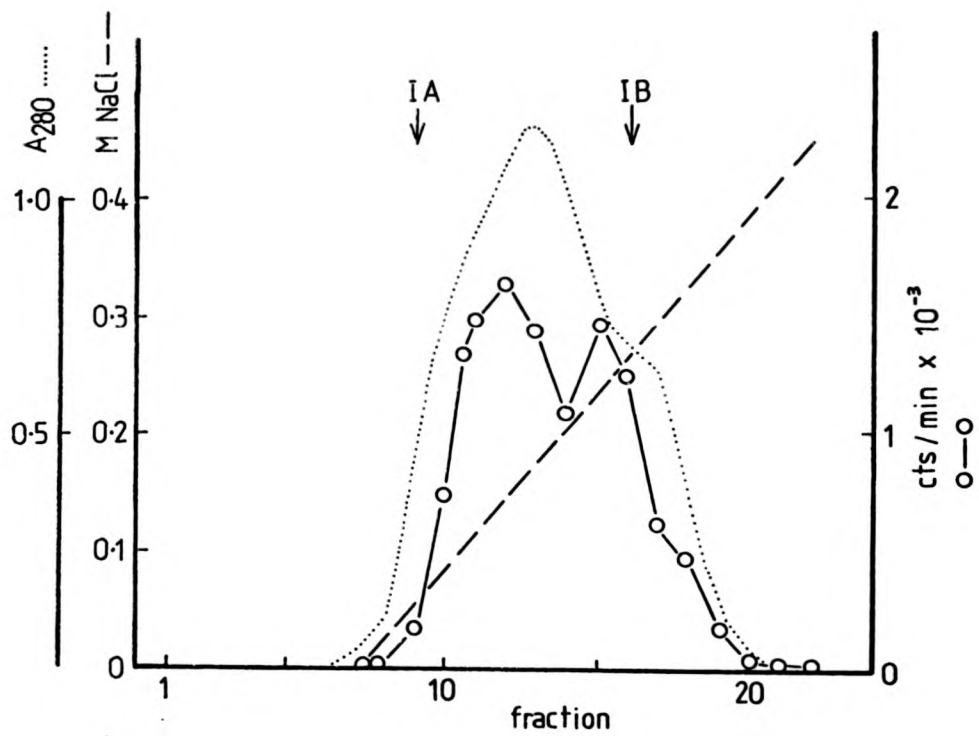


Figure 7.2 DEAE-cellulose fractions of oocyte Sephadex Peak 1 assayed for RNA polymerase activity.

Peak 1 pooled from an oocyte Sephadex column was run on DE52 as described in Figure 5.1B. 30 μ l of each fraction was assayed for the presence of RNA polymerase activity (O) as described in Figure 7.1. (.....), A_{280} ; (— —), salt concentration.

cts/min x 10^{-3}
O—O

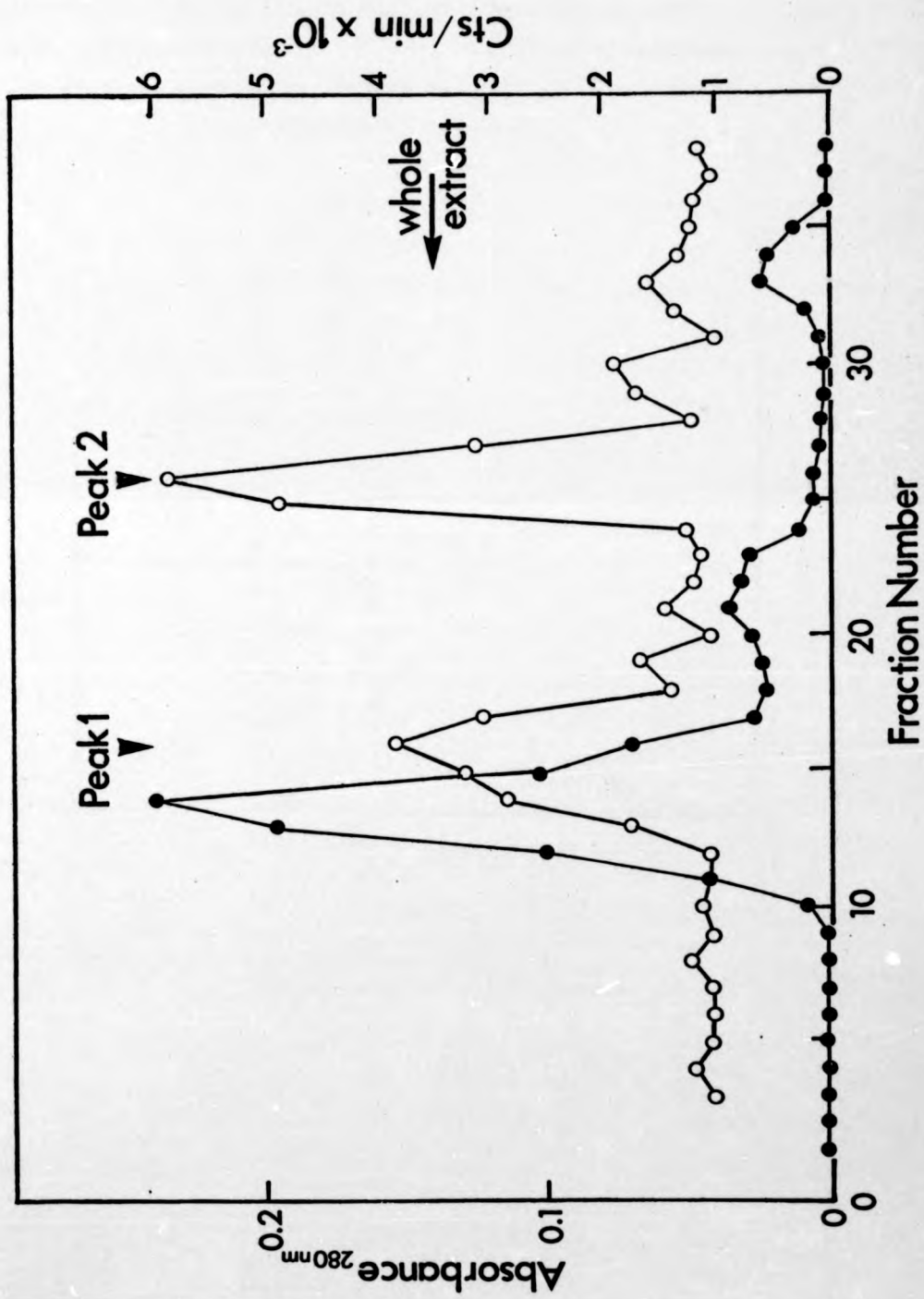
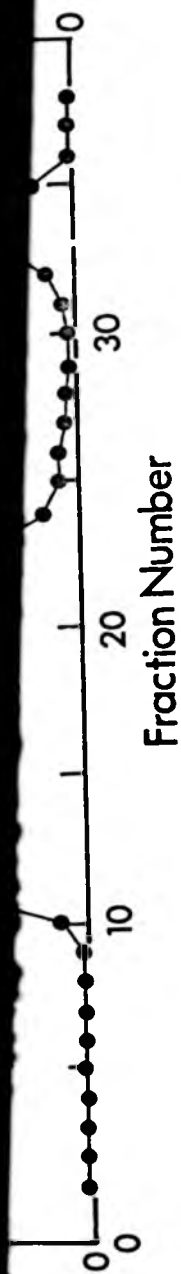


Figure 7.3 Nuclear assays using an enucleated extract fractionated on G-100 Sephadex.

An extract was prepared from 200 oocytes enucleated by the method of Ford and Gurdon (1977). This extract was fractionated on G-100 Sephadex and assayed with XTC-2 nuclei as described in Figure 5.1A. (●—●), A_{280} ; (○—○), [^3H] UTP incorporation in the nuclear assay. The arrow indicates the incorporation by XTC-2 nuclei preincubated with the initial whole extract.



merase activity (Hall *et al.*, 1973), but had no effect on XTC-2 nuclear transcription rates. As already described (Chapter 4), BSA also did not alter the incorporation in the nuclear assay, indicating that total protein is not the cause of the stimulation.

(c) Mode of action of the factors

It would be interesting to know how the modulatory effect of the factors are achieved. There are two obvious questions to be answered. First, is the effect on initiation or elongation of RNA chain formation? Second, is the effect directly on the RNA polymerases or on some other component of the nucleus?

Effects on initiation and elongation are not easily distinguished in the nuclear system. The experiments described in Chapter 4 using rifampicin and γ -³²P indicate that the cell extracts may have some effects on both initiation and elongation. However, these results are not conclusive, for example polymerases may be limiting within the nuclei. The subject is best approached by simplifying or dissecting the system to some extent.

Experiments with XTC-2 chromatin

The first step towards this goal was to prepare chromatin from isolated XTC-2 nuclei and see if the factors affected its transcription in vitro. There are a number of problems here. Extracts or factors may not affect the transcription of chromatin because something, such as the nuclear membrane or some chromosomal proteins, may be lost during chromatin preparation which is essential for the effect.

The fidelity of transcription in isolated chromatin varies considerably according to the worker and the gene products being analysed (Parker and Roeder, 1977; Konkel and Ingram, 1978). De Pomerai *et al.* (1974) have shown that the template properties are dependent on the method of preparing the chromatin. Many workers also add RNA polymerase to the chromatin, and these are either non-homologous or at a low specific activity. This must cast further doubt on the meaning of some results from experiments concerning in vitro transcription of chromatin. Nevertheless, the possibility of showing that the factors have a direct effect on transcription prompted the following experiments with chromatin.

Chromatin was prepared by method VI, as described by De Pomerai *et al.* (1974), which is based on the original method of Reeder (1973). De Pomerai *et al.* (1974) have shown that this form VI chromatin best resembled nuclei in terms of its DNA:RNA:protein ratio, polymerase activity and RNA product. This method, which consists of gently lysing the nuclei (described in Chapter 2) was used to prepare chromatin from previously prepared XTC-2 nuclei.

Chromatin was prepared fresh from nuclei for each experiment and was used immediately. The method produced chromatin with a DNA:RNA:protein ratio of 1:0.3:4 which corresponded well with the ratio for isolated nuclei (Chapter 3). In parallel transcription assays chromatin was found to be slightly less (about 90%) active (without added polymerase) for a given amount of DNA than the original nuclear preparation.

The effect of factor IIA on transcription by isolated chromatin was assayed directly (*i.e.* with no preincubation) in the presence and absence of crude *Xenopus laevis* RNA polymerase. Factor IIA was used in these experiments as it had the most clear cut and specific effect on transcription in isolated nuclei. Fig. 7.4 shows the time course of transcription by the chromatin. Addition of either *Xenopus* polymerase or factor IIA alone to the chromatin caused twice the endogenous incorporation after 2 hours. However, when the two were added together, there was an immediate stimulation which was 6 times the endogenous level after 2 hours. This stimulation is much more than the addition of the individual effects of polymerase or factor IIA. Thus the factor affects transcription in chromatin as in isolated nuclei. The effect is an immediate one, and it seems to involve the added RNA polymerase and therefore probably involves initiation, at least in part. Similar experiments involving the crude oocyte and egg extracts and chromatin indicated that the oocyte extract stimulated transcription as in the nuclear assay. The egg extract caused a slight stimulation also over endogenous levels, but less than the oocyte extract. This difference was maintained even in the presence of added polymerase which had an overall stimulatory effect. Clearly, many more experiments with chromatin have to be carried out before the action of the factors may be fully clarified.

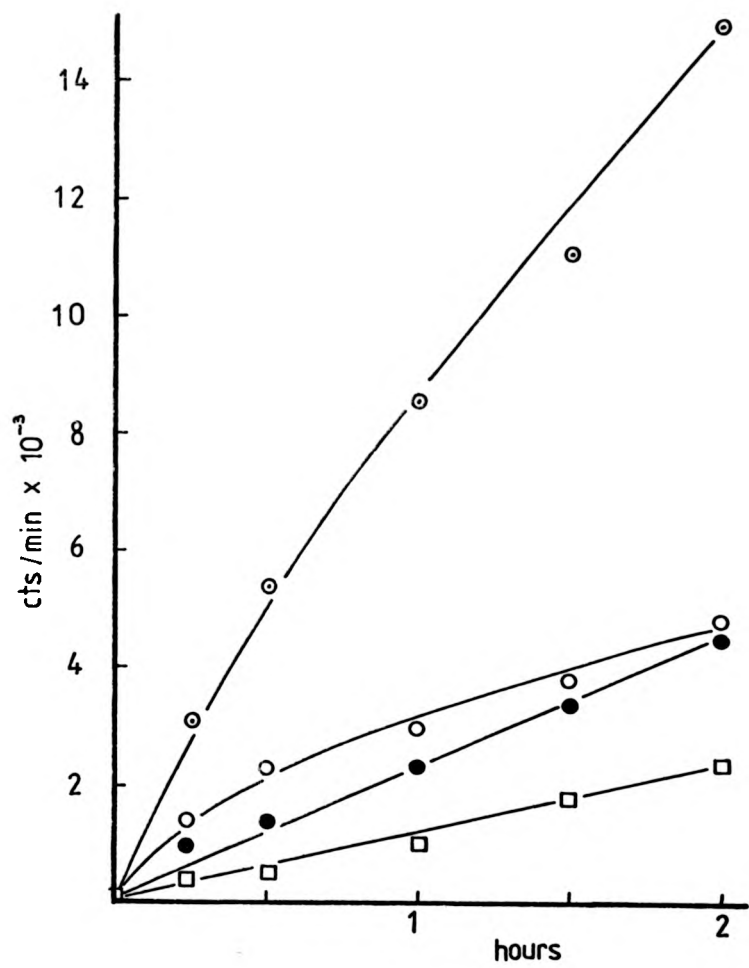


Figure 7.4 The effect of factor IIA on transcription by isolated XTC-2 chromatin in vitro.

Chromatin was isolated from XTC-2 nuclei and assayed without pre-
incubation for RNA synthesis/^{at 25° C}with [³H] UTP as described in Chapter 2.
200 μ l assays were prepared without addition (\square); with 80 μ l of factor
IIA (from oocytes), (\bullet); with 0.05 units of Xenopus RNA polymerases,
(\circ); with factor IIA +0.05 units of Xenopus RNA polymerases (\odot).
25 μ l aliquots were removed at the times indicated and measured for the
presence of acid insoluble radioactivity.

The experiments above indicate that the factors may affect initiation of the polymerases to some extent. If the effect is confined to the RNA polymerase molecules, the factors may be expected to act on purified polymerases and a DNA template. Briefly, the factors have been assayed in this way with purified Xenopus oocyte polymerases and liver DNA (as well as poly d(A-T)) and the factors have no effect in the case of the Peak 2 factors. Peak 1 factors contain polymerase activity and so a less clear result was obtained. Of course, RNA polymerase works absolutely unspecifically on a pure DNA template, so it is difficult to know what result to expect with a putative regulatory factor under these conditions. On the face of it, the result suggests that some other component or mechanism is involved in the factor stimulation of transcription.

Other possibilities

There are several possible mechanisms which may be investigated. The factors may act in a similar way to protein H₁ described by Crepin et al. (1975). In vitro transcription of the lactose operon is stimulated in the presence of this protein. It appears to act by binding to the DNA in the promoter region of the operon. Binding causes a conformational modification of the promoter which increases its functional state in some way.

Stimulation in transcription may also be caused by direct gene de-repression, possibly by altering the chromatin conformation such that it is more 'available' for transcription. Various workers have reported that genes which are being actively transcribed are more susceptible to nuclease digestion than those which are not (Garel and Axel, 1976; Weintraub and Groudine, 1976). This possibility, especially in the case of factor IIA, is a testable one in this system.

RNA polymerases in nuclei are known to exist in two forms; one, the functional, active or 'engaged' state, the other, the inactive, unbound or 'free' enzyme. These two pools were first reported by Yu (1974) in rat hepatocytes, and more recently analysed in detail by Kellas et al. (1977). A reasonable possibility is that the factors act by mobilising one or more of the pools of 'free' polymerase, thus increasing the available functional RNA polymerase.

These possibilities were investigated using nuclei preincubated in the

presence or absence of some of the purified factors.

DNA binding assays

The DNA binding capacity of the Sephadex fractions and purified factors were assayed by Dr. Hugh Woodland and Miss A. Wyllie, using the method of Carrara *et al.* (1977). This method involved incubation of labelled DNA with the proteins or fractions in question in the absence of DMSO. The incubates were then filtered through nitrocellulose filters which were then washed with buffered DMSO. If protein had bound to the DNA, the DNA was retained by the filter. This could be measured by estimating the radioactivity present on the filter. Calf thymus histone was used as a maximum binding control. In these assays, [³H] DNA from XTC-2 cells was used. Binding assays of Sephadex and DEAE column fractions indicated no specific binding of proteins present in the major stimulatory peaks. The main DNA-binding peak coincided with the main protein A₂₈₀ peak in the case of the Sephadex fractions. Similar binding experiments with various species of labelled *Xenopus* RNA showed that none of the factors bound to these RNA's.

DNAase sensitivity of factor treated nuclei

The DNAase Isensitivity of XTC-2 nuclei preincubated without and with cell extracts from oocytes, eggs and blastulae, was tested by the method described by Garel and Axel (1976). This involved preincubating the nuclei with cell extract as usual, then resuspending the nuclei in 20 µg/ml DNAase I, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂. The nuclei were incubated at 37°C and the kinetics of digestion followed by measuring the A₂₆₀ absorbing material which was soluble in 1 M HClO₄, 1 M NaCl. Fig. 7.5 shows the per cent of the DNA remaining acid insoluble in the control and extract preincubated nuclei. Clearly, the extracts alter the DNAase Isensitivity of the nuclei, but this says nothing about individual gene sensitivity in the nuclei.

This was further analysed using the Sephadex Peaks 1 and 2 and purified factors. All were dialysed against Column buffer before use in the preincubation assay. Nuclei were preincubated with these factors as before, then digested with DNAase I at 37°C so that 5% of the original DNA was digested. The reaction was stopped with EDTA (to 5 mM) and

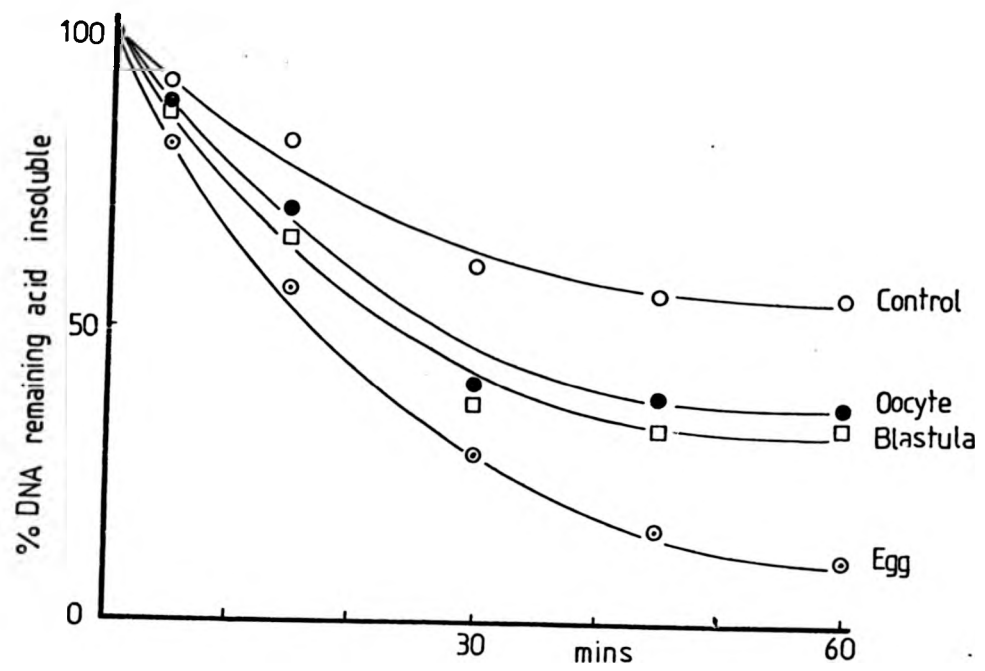


Figure 7.5 DNAase I sensitivity of XTC-2 nuclei preincubated with cell extracts.

XTC-2 nuclei were preincubated in 500 μ l assays with oocyte, egg and blastula cell extracts at 25^o C for 1 hour in the normal way. The nuclei were reisolated and incubated with 20 μ g/ml DNAase I as described by Garel and Axel (1976) at 37^o C. 50 μ l aliquots were removed at the times indicated and made to 1 M HClO₄, 1 M NaCl. The insoluble material was pelleted by centrifugation and the A₂₆₀ of the supernatant measured. This is expressed as a percentage of the original A₂₆₀ of the DNA in a similar undigested aliquot.

- (○) Control treated nuclei (Column buffer).
- (●) Oocyte treated nuclei.
- (⊙) Egg treated nuclei.
- (□) Blastula treated nuclei.

rol

te
tula

the DNA extracted and ethanol precipitated as described in Chapter 2. Purified DNA from each incubation was assayed for the presence of ribosomal gene sequences on filters (see Chapter 2) using purified XTC-2 [^3H]-labelled rRNA as a probe. The level of hybridization occurring is shown in Table 7.1 and suggests that the factors have only a small effect (if any) on the DNAase I sensitivity of the ribosomal genes. This may rule out a gross effect by the factors on the conformation of the ribosomal genes in isolated XTC-2 nuclei. The number of counts involved make it difficult to conclude anything further from these results. A comparison of the effects of factors IB and IIA is, however, particularly interesting. They stimulate total RNA synthesis to the same extent. There is a considerable difference in the stimulation of rRNA and yet the ribosomal genes have similar endonuclease sensitivity.

Free and template-engaged RNA polymerases

As already indicated (see above), nuclei contain distinct pools of free and engaged RNA polymerases. It is difficult to obtain an absolute measure of the amounts of RNA polymerase in each pool or the total RNA polymerase activity in nuclei because of difficulties of totally solubilising the enzyme, loss of activity and varying enzyme efficiencies on different templates. However, an estimate may be made of the activity of the free polymerases in control and factor treated nuclei. A reduction in the free pool may indicate that there has been a shift of free polymerase to the engaged form.

Free polymerase was assayed in nuclei preincubated with factors IB and IIA (see Chapter 2). After preincubation the nuclei were reisolated and assayed for [^3H] UTP incorporation in the presence of 200 $\mu\text{g}/\text{ml}$ Actinomycin D and 100 $\mu\text{g}/\text{ml}$ poly d(A-T) as synthetic template. The activity of each form of the free RNA polymerase was measured by using 1 $\mu\text{g}/\text{ml}$ and 1 mg/ml α -amanitin as usual. Incorporation was allowed to proceed at 25 $^{\circ}\text{C}$ for 30 minutes and then aliquots were assayed for the presence of acid insoluble labelled material as usual.

Table 7.2A shows the incorporation for each of these assays and the breakdown of the total incorporation into that due to each form of the polymerases. Tables 7.2B and 7.2C show these results as percentages normalised with respect to the total incorporation and the control

Table 7.1

Hybridization of [³H] rRNA to DNAase I digested DNA, derived from factor treated nuclei

<u>Treatment of nuclei</u>	<u>% hybridization</u>
Control incubation - digestion	0.37%
Control incubation + digestion	0.36%
Peak 1 incubation + digestion	0.36%
Peak 2 incubation + digestion	0.34%
Factor IA incubation + digestion	0.37%
Factor IB incubation + digestion	0.36%
Factor IIA incubation + digestion	0.35%
Factor IIB incubation + digestion	0.35%
pCr 101	32.8%
pMB 9	0.02%

The nuclei were preincubated with the Stage 5/6 oocyte factors indicated (controls contained Column buffer instead of extracts). The nuclei were reisolated, treated with 20 µg/ml DNAase I at 37° C until 5% of the input DNA was digested. The reaction was stopped with EDTA and the DNA extracted. 10 µg of DNA was loaded on to filters and incubated with 10,000 cpm of [³H] rRNA from XTC-2 cells as described in Chapter 2. The figures give the percentage of the input counts hybridizing after subtraction of background. pCr 101 is the plasmid PMB 9 containing a Xenopus rDNA gene repeat. The plasmid DNA was a gift from Dr. A. Colman.

Table 7.2

Free and template-engaged RNA polymerases in factor treated nuclei

(A)	Incorporation in the 'Free' polymerase assay (counts/minute)			
	Total	Polymerase I	Polymerase II	Polymerase III
Control	6430	3670	1925	835
Peak IB	5665	3210	1610	845
Peak IIA	4810	1725	1790	1295

(B)	Total	Polymerase I	Polymerase II	Polymerase III
Control	100	57	30	13
Peak IB	100	57	28	15
Peak IIA	100	36	37	27

(C)	Total	Polymerase I	Polymerase II	Polymerase III
Control	100	100	100	100
Peak IB	88	87	84	101
Peak IIA	74	47	93	155

XTC-2 nuclei were preincubated with the factors in the standard way. They were then assayed for the presence of free polymerase (see Chapter 2). Actinomycin D was added to 200 $\mu\text{g}/\text{ml}$, poly d(A-T) was added to 100 $\mu\text{g}/\text{ml}$. α -amanitin was used to select for the different polymerase forms. Part (A) shows the incorporation by 50 μl assays, part (B) shows the percentage of the total for each treatment which is due to the activity of the different polymerase forms and part (C) shows the results as percentages normalised with respect to the control level in each case.

incorporation respectively. They indicate that both factors reduce the total, active, free RNA polymerase, factor IIA more so than factor IB. Factor IB appears to reduce the free polymerase I and polymerase II activity but leaves the polymerase III activity unaltered. Factor IIA reduces the free polymerase I activity considerably (by about half), hardly affects free polymerase II but increases the free polymerase III. Another point to note here is that the percentages of the total activity resulting from the different forms of the free polymerases using α -amanitin is very similar in the controls to the percentages obtained for the engaged polymerases in Chapter 3(d) as assayed under the standard conditions, without Actinomycin D and poly d(A-T).

The factors may cause a reduction in total free activity without the polymerase becoming engaged. This assay measures all the free RNA polymerase activity not engaged on templates containing guanosine in the presence of Actinomycin D and poly d(A-T) (Goldberg, 1964). The reduced free polymerase activity caused by both factors (especially polymerase I by factor IIA) may result from loss of polymerase during the preincubation. This was controlled for by assaying the preincubation supernatants in the presence of [^3H] UTP, unlabelled triphosphates and poly d(A-T). There was no activity in the IIA/nuclear supernatant, but there was in the IB/nuclear supernatant (IB already contains RNA polymerase activity).

Another possibility was that the factors may inhibit the individual free polymerase species. However, as already described, they had no effect on purified Xenopus RNA polymerases acting on pure DNA templates.

These experiments may indicate that the factors act partly by reducing the free polymerase pool and thus increasing the level of engaged RNA polymerases. In the case of factor IIA, the considerable reduction in free polymerase I which it causes correlates with its observed effect on rRNA synthesis. This cannot be the only effect of the factor. There is half as much free polymerase I in nuclei treated with this factor, but a 40-50-fold increase in the rRNA synthesis by the nuclei. The reduction in free polymerase may merely be an effect of the stimulation rather than part of its primary cause.

The requirement for triphosphates during preincubation

Some information about the way the factors act comes from a study of the assay conditions involving preincubation. In this procedure the nuclei are reisolated from the incubation medium before adding the radioactive precursor, and no additional factor is added. Results described in Chapter 4 indicate that all four triphosphates were required during the preincubation step for the whole extract to exert any effect on transcription; presumably RNA synthesis must occur during the preincubation. Table 7.3 shows that the same is true of the four separated activities. The small stimulation observed may be associated with a small amount of CTP present in the isolated nuclei. This was taken further in the case of factor IIA. As described already, virtually all the transcription in nuclei preincubated with this factor was α -amanitin insensitive. Thus by using different concentrations of this toxin during the preincubation step, it was possible to ask more questions about how the factor worked. This experiment could indicate firstly, again whether RNA synthesis was necessary for this factor's effect, and secondly, if RNA synthesis was required, which polymerase activity was involved. This experiment was not really practicable with the other factors as it was not clear to what extent α -amanitin was reversible, or could be 'washed out' after the preincubation.

Nuclei were preincubated as usual in the presence and absence of factor IIA, and with and without α -amanitin at 1 μ g/ml and 1 mg/ml. After 1 hour's preincubation the nuclei were reisolated and incubated in the normal way in the presence of α -amanitin at the same concentrations as the preincubation and higher, up to 1 mg/ml. Table 7.4 shows the results of the experiment. This indicates that inhibition of polymerase II activity during the preincubation step eliminates the stimulation of rRNA synthesis by factor IIA. It is therefore possible that the effect of factor IIA is dependent on a polymerase II product or the transit of RNA polymerase II molecules along the DNA.

To give further support to the above observations, oocyte Sephadex fractions were assayed as usual with isolated XTC-2 nucleoli as the RNA synthesising system. Many workers have shown that there is good

Table 7.3

The effect of omitting CTP during the preincubation step on the stimulatory activity of the purified factors

	<u>+CTP</u> <u>counts/n.inute</u>	<u>Stimulation</u> <u>over control</u>	<u>-CTP</u> <u>counts/minute</u>	<u>Stimulation</u> <u>over control</u>
Control	2931	1	2696	1
IA	7932	2.7	3642	1.36
IB	19946	6.7	4911	1.84
IIA	20549	6.9	5970	2.2
IIB	13737	4.6	4029	1.5

XTC-2 nuclei were preincubated for 1 hour with purified factors in the usual way, one set of assays having all four non-radioactive triphosphates, the other all except CTP. After 1 hour the CTP levels were made equal in both sets of assays and RNA synthesis assayed as usual.

Table 7.4

The effect of α -amanitin on factor IIA stimulation

<u>Preincubation step</u>	<u>Labelling step</u> <u>(α-amanitin concentration)</u>		
	<u>0</u>	<u>1 μg/ml</u>	<u>1 mg/ml</u>
-IIA, 0 α -amanitin	4260	1995	408
-IIA, 1 μ g/ml α -amanitin		1248	276
-IIA, 1 mg/ml α -amanitin			258
+IIA, 0 α -amanitin	22,824	20,172	18,354
+IIA, 1 μ g/ml α -amanitin		2,280	474
+IIA, 1 mg/ml α -amanitin			429

XTC-2 nuclei were preincubated as usual in the presence or absence of factor IIA and α -amanitin as indicated in the Table. They were then reisolated and incubated in the presence of [3 H] UTP as usual, and with varying concentrations of α -amanitin as indicated.

fidelity of rRNA synthesis by isolated nucleoli in the absence of added RNA polymerase (Ballal *et al.*, 1978; Grummt and Lindigkeit, 1973). Beebee and Butterworth (1977) have supported the conclusion that isolated nucleoli synthesise predominantly rRNA. However, they have also shown that the nucleoli are severely disrupted during preparation by sonication, and that they probably do not reinitiate. If the Sephadex Peak 2 stimulates RNA synthesis in isolated nucleoli, this may indicate that the result with low levels of α -amanitin during preincubation is artefactual.

Nucleoli were prepared by the method of Grummt and Lindigkeit (1973) from previously prepared XTC-2 nuclei. They were then assayed in the usual way with oocyte Sephadex fractions with 5 mM $MgCl_2$ replacing the $MnCl_2$ and α -amanitin to 1 $\mu g/ml$. Fig. 7.6 shows the incorporation by the nucleolar assay with the different fractions. There is a pronounced peak of incorporation corresponding to Peak 1. Similar results were obtained in the absence and in high concentrations (to 100 $\mu g/ml$) of α -amanitin. This indicates that the incorporation is largely due to RNA polymerase I activity. Clearly, Peak 2 has no effect on the incorporation by isolated nucleoli. Peak 1 corresponds to the peak of RNA polymerase activity in the Sephadex column fractions and this may cause the stimulation in the nucleolar transcriptional activity. Crude *Xenopus* oocyte polymerase was added to the isolated nucleoli and the column fractions reassayed as above. Fig. 7.6 shows the resulting incorporation by these assays. It indicates that RNA polymerase effectively increases the incorporation in most of the assays, and probably caused the stimulation in the original assay. The significance of the slightly inhibitory fraction possibly relates to the presence of bulk protein in these assays. In the nucleolar assays with added polymerase, the incorporation was reduced only 10-13% by α -amanitin concentrations up to 100 $\mu g/ml$ in comparison to assays without α -amanitin added. However, in assays of the crude polymerase preparation with DNA under the same salt conditions, it was found that it consisted of about equal proportions of the three polymerase forms. This would seem to indicate some specificity of the polymerase mixture for the nucleolar template.

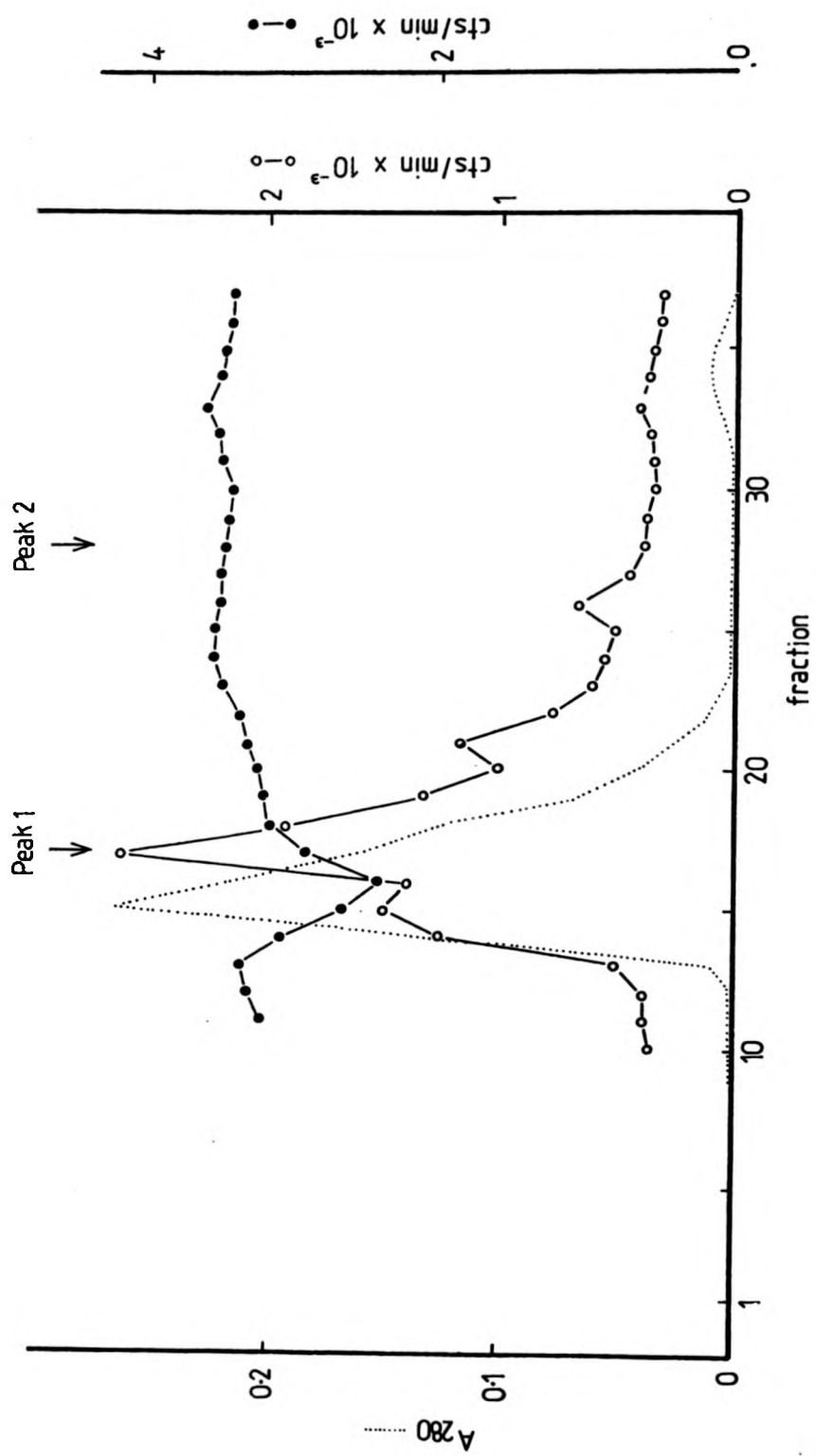


Figure 7.6 The effect of Sephadex fractionated oocyte extract on transcription in isolated XTC-2 nucleoli.

Isolated nucleoli were prepared and incubated with Sephadex fractions of an oocyte extract as described in Chapter 2. The 100 μ l incubates included 40 μ l of column fraction (\circ) or 40 μ l of column fraction and 0.05 units of RNA polymerase from Xenopus ovary (\bullet). α -amanitin was present to 1 μ g/ml, incubation was at 25^o C for 30 minutes, A_{280} (.....).

Peak 2 had no effect on the transcription of nucleoli even in the presence of added RNA polymerase. This may indicate that stimulation by Peak 2 is dependent on a non-nucleolar intermediate step.

(d) Discussion

Most of the eukaryotic factors reported to date which stimulate RNA synthesis have been identified using purified polymerase and DNA templates as the RNA synthesising system. These factors may be grouped under three headings:-

- (1) Non-specific factors
- (2) Factors which are purified independently of RNA polymerases and stimulate synthesis on dsDNA but not on ss DNA.
- (3) Factors which co-purify with RNA polymerase.

As already mentioned, examples of type 1 are DNAase (Chambon et al., 1970) and BSA (Rutter et al., 1973). There are many examples of type 2 factors (Lentfar and Lezins, 1972; Seifart et al., 1973) and of type 3 factors (Sugden and Keller, 1972; Froehner and Bonner, 1973). In general the mode of action of these factors has not been fully clarified except for some of the type 1 factors. Type 2 factors are thought to act like the H_1 DNA-binding protein from E. coli described by Crepin et al., (1975) (see earlier this chapter) or the E. coli unwinding proteins (Molineaux and Gefter, 1974). Because type 3 factors co-purify with RNA polymerase they are thought to be sub-units or factors lost from the polymerases during purification. Results described in this chapter indicate that the factors purified from oocyte cell extracts (and assayed in the isolated nuclei system) appear to act in a specific manner. They either do not consist of, or the nuclei are unaffected by, RNA polymerases, protein kinases, endonuclease, histones or actin. Also, the factors do not exhibit any great DNA-binding capacity and are thus unlike type 1 or 2 factors.

Unless made from enucleated oocytes, the Sephadex Peak 1 contains RNA polymerase activity, but its effect on nuclear transcription is not dependent on this activity entering the nuclei. This may suggest, but by no means proves, that Peak 1 in the nuclear assay is caused by a polymerase sub-unit of some sort. Experiments with purified polymerases

and DNA template do not rule out this possibility. Further purification of factors IA and IB may clarify their mode of action.

Peak 2 factors certainly seem to act in some other way. In the presence of added homologous polymerases, factor IIA is effective on chromatin but not on a pure DNA template. This implies that a more complex mechanism is involved rather than a direct effect on either the RNA polymerase, the DNA or both.

DNAase I digestion has been used to investigate the conformation of the ribosomal genes in IIA treated and control nuclei. After preincubation the two sorts of nuclei differ considerably in terms of their RNA synthetic rate, but their ribosomal genes are equally susceptible to DNAase digestion.

Garel and Axel (1976) have shown that active globin genes are more sensitive to DNAase I than is total DNA. Other workers (Weintraub and Groudine, 1976; Reeves and Jones, 1976) reported that an active gene in one cell was more susceptible to endonucleases than the same, inactive gene in another cell type. None of these prove an absolute correlation between the endonuclease sensitivity of a gene and its rate of transcription. In fact, Garel *et al.* (1977) have shown that genes within a cell which are being transcribed at different rates have similar conformation within the chromatin.

These reports seem to indicate that a change in the conformation of a gene (resulting in its altered endonuclease sensitivity) is a gross form of control of gene expression; possibly the form of control likely to be important in highly differentiated cells. It seems less appropriate to the control of 'house-keeping' genes in relatively undifferentiated cells. Perhaps, not surprisingly, it seems that factor IIA exerts its effect not by a gross conformational change in the ribosomal genes in XTC-2, but by some other, finer mechanism.

Factor IIA reduces the amount of free polymerase I in the isolated nuclei. The level of the change induced, however, is incompatible with the stimulation of rRNA synthesis observed. This suggests that the change in free polymerase, and hence an increase in initiation rate, is not the primary cause of this stimulation.

Experiments involving the omission of triphosphates indicate that RNA synthesis may be necessary for the full effect of the factor to become apparent. At least in the case of factor IIA, a polymerase II product seems to be necessary for stimulation to occur.

There is increasing evidence for the extranucleolar control of rRNA synthesis. Fiume and Laschi (1965) first reported that low concentrations of α -amanitin cause nucleolar fragmentation in KB cells. Other workers (Jacob *et al.*, 1970; Tata *et al.*, 1972) have shown that mRNA synthesis plays a potential role in the maintenance of rRNA synthesis *in vivo*. More recent work has shown that the low concentrations of Actinomycin D known to inhibit rRNA synthesis *in vivo* do not inhibit nucleolar RNA synthesis, but rather polymerase II activity, *in vitro* (Lindell, 1976; Lindell *et al.*, 1978). The possibility that factor IIA acts in a similar fashion is an interesting one. The fact that nucleolar RNA synthesis is unaffected by this factor, while being inconclusive, supports the idea of an intermediary RNA polymerase II product. It also provides the possibility of being able to isolate and identify this intermediary which finally alters transcription of the ribosomal genes.

A problem still remaining is whether the factors affect the rate of initiation in the isolated nuclei. For the whole extracts there was some indication that the rate of initiation of new chains is changed (see Chapter 4 (g)), but there is no certainty that the effect is primarily on initiation. The best evidence for an increase in initiation by the purified factor comes from a consideration of the efficiency of RNA synthesis in the isolated nuclei. This is shown in Table 7.5. These figures represent a minimal estimate, since it is assumed that the specific activity of the [^3H]UTP in the assay is the same as the added precursor, *i.e.*, no UTP is carried over from the preincubation. Even so, Sephadex Peak 2 stimulates the rate of RNA synthesis to 0.67 pg/nucleus/hour. This is close to the normal *in vivo* rate calculated by Davidson (1976) for *Xenopus* neurula cells. Since this continues linearly for at least 4 hours, new chain formation seems likely to occur.

The same argument is extended to the ribosomal genes in Table 7.5, except with Factor IIA, about 10% of the RNA made is ribosomal,

Table 7.5

Total RNA and 18S and 28S rRNA synthesis per hour by isolated XTC-2 nuclei

<u>Added agents</u>	<u>RNA synthesised pg/nucleu/hr</u>	<u>18S & 28S rRNA synthesised pg/nucleus/hr</u>	<u>Transcripts/ ribosomal gene/hr</u>
Control	0.084	0.0085	4
Oocyte extracts	0.21	0.021	10
Egg extracts	0.085	0.0034	2
Blastula extracts	0.184	0.0184	9
Sephadex Peak 2	0.67	0.067	32
DEAE Peak IIA	0.40	0.370	177

The rates of total RNA synthesis are worked out from typical assays, assuming that there is no non-radioactive UTP carried over from the preincubation step. The rates of rRNA synthesis are worked out as above and assuming that the RNA synthesis surviving α -amanitin at 100 μ g/ml is all rRNA.

under the assay conditions used. Thus, when stimulated with Sephadex Peak 2, each ribosomal gene produces about 30 transcripts per hour. Since a maximum of about 100 polymerases could be packed on to a ribosomal gene (Miller and Beatty, 1969), it is hard to avoid the conclusion that new chains must be initiated in a 4 hour incubation. The argument is even more conclusive for factor IIA. This stimulates rRNA synthesis by over 20-fold, giving at least 170 transcripts per gene per hour. Fig. 6.4 shows that incorporation in the presence of this factor continues linearly for at least 2 hours. The rate of initiation of rRNA transcripts must therefore be increased. However, since half of the polymerase is template-engaged in its absence, the prime effect of peak IIA must be on elongation or termination.

Given the presence of these factors in Xenopus oocytes, the question arises why does the egg differ in terms of its content of these factors? Experiments involved with this question are described in the next chapter.

Chapter 8

Micro-injected factors and their fate in the nuclear assay

(a) Introduction

A crucial question posed by the results described so far is why unfertilised egg extracts lack some of the stimulatory activity. This absence, especially of factor IIA, may be due to an artefactual loss or maybe a real phenomenon. There are essentially three possibilities: (1) it may be lost during extract preparation, (2) it may be present but inactive, and (3) it may be absent from eggs. The last two possibilities are difficult to analyse. Some simple experiments may, however, be done to see what happens to oocyte factors injected into eggs and subsequently recovered. Experiments of this sort are described in the first half of this chapter.

Another interesting question is what happens to the factors in the nuclear assay? Again this is a difficult question to tackle. Preliminary experiments are described in the second half of this chapter.

(b) Recovery of injected labelled factors

The fate of the factors in vivo and during extract preparations may be analysed in a variety of ways. The basic question here is why the egg differs from the oocyte and blastula in terms of its factor composition. Simple recovery experiments, adding activity or labelled factors during extract preparation, may give an indication of losses at the preparation step. The injection of activity or labelled factors into oocytes, eggs or embryos and subsequent analysis of their cell extracts after incubation may give further information about the fate of the factors in vivo.

Recovery experiments of oocyte factor IIA during egg extract preparation were carried out as follows. Cell extracts were prepared from 100 unfertilised Xenopus eggs in the presence and absence of 250 μ l of oocyte factor IIA (containing 4 μ g/ml total protein). The resulting extracts were then fractionated on Sephadex G-100 and assayed with nuclei as usual. Fig. 8.1 shows the result of these assays for an egg extract with added Column buffer (A), with added factor IIA (B) and 250 μ l of factor IIA chromatographed by itself (C). The result indicates that factor IIA rechromatographs, as it does originally; also, that there is no great change in the stimulation present in Peak 2 of eggs with oocyte factor IIA

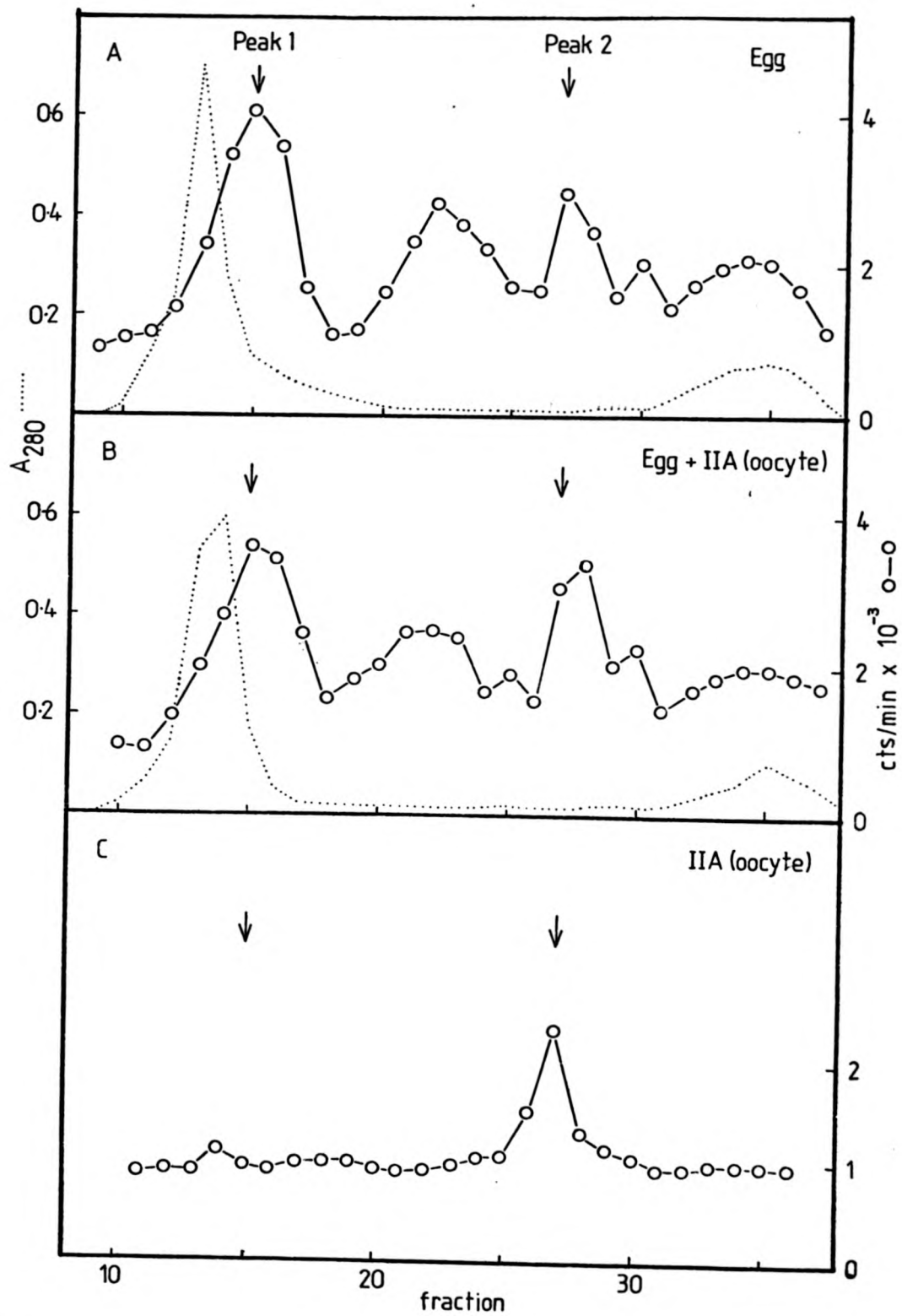


Figure 8.1 Recovery of factor IIA activity added during preparation of unfertilised egg extract.

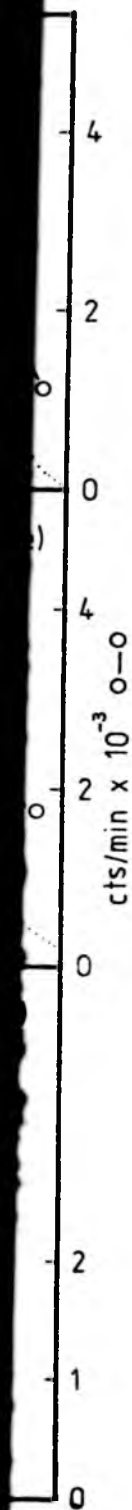
Cell extracts were prepared from 100 unfertilised eggs in the presence and absence of 250 μ l of oocyte factor IIA. The extract was prepared and fractionated on G-100 in the standard way. 20 μ l of each fraction was assayed for its effect on RNA synthesis with isolated XTC-2 nuclei, using [3 H] UTP in the standard nuclear assay.

(A) In the absence of factor IIA.

(B) In the presence of factor IIA.

(C) 250 μ l of factor IIA fractionated and assayed alone.

(O-O), [3 H] UTP incorporation in the nuclear assay; (.....), A_{280} .



added. This suggests that the added factor was lost or inactivated, but with this amount of factor added and at this dilution, a maximum of a further 2-fold stimulation may be expected. Thus the result is an inconclusive one.

It was not feasible to micro-inject sufficient activity of any of the factors into oocytes, eggs or embryos to give a meaningful result in terms of stimulation in the nuclear assay. Factors of the required concentrations could not be prepared, as already described. (This, if it were possible, would be an interesting way to investigate the effect of the factors in vivo). For this reason it was decided to use labelled factors for the micro-injection and recovery experiments.

Isolated oocytes were labelled overnight with [^3H] lysine as described in Chapter 2. Cell extracts were prepared and chromatographed on Sephadex G-100 as usual. Fractions were not assayed with nuclei, but were pooled where peaks 1 and 2 were known to elute from Sephadex G-100. The two [^3H]labelled factors were concentrated to 4-8,000 counts per minute per μl in the following manner.

The labelled preparations were used to swell dry Sephadex G-10 in a 5 ml plastic syringe barrel which was resting inside a centrifuge tube. Swelling was allowed to continue for about 5 minutes when the centrifuge tube and syringe were centrifuged at 1000 rpm for 1 minute. The labelled factors were recovered from the centrifuge tube. This process was repeated until the required concentration had been achieved. Between 80 and 90% of the input radioactivity was recovered after each round of concentration.

Oocyte peaks 1 and 2 were microinjected into oocytes, unfertilised eggs and 2 cell stage embryos of Xenopus laevis. The oocytes and eggs were incubated in Barth X for 3 hours and then quickly frozen on dry ice. These were stored at -70°C . The 2 cell embryos were incubated at 18°C after injection until they had reached Stage $10\frac{1}{2}$ when they were quickly frozen and stored as before. Cell extracts were prepared from these frozen samples as usual and then subjected to gel filtration on Sephadex G-100. The resulting fractions were assayed for radioactivity using Insta-Gel (Packard) scintillation fluid. Samples were also counted from

each stage of the cell extract preparation. 90% of the input radioactivity was recovered in the final cell extracts prepared from oocyte and Stage 10½ embryos injected with either Peak 1 or Peak 2. Only 75-80% was recovered in the egg extract preparations.

Fig. 8.2 shows the Sephadex analysis of cell extracts prepared from oocytes (A), eggs (B) and Stage 10½ embryos (C) which had been injected with labelled Sephadex Peak 1. Also shown in Fig. 8.2A is a similar analysis of a sample of the injected material as a marker. Clearly, the oocyte and Stage 10½ run similarly, and in essentially the same way as the marker Peak 1. However, the Peak 1 injected into the egg behaves differently and elutes with the main A₂₈₀ peak. A similar analysis of Peak 2 is shown in Fig. 8.3. Also shown in part A of this figure is an analysis of the uninjected Peak 2 material. Much of Peak 2 rechromatographs with the main A₂₈₀ peak, possibly because of aggregation. However, the egg extract appears to lack the protein which rechromatographs at Peak 2, and it contains more, lower molecular weight material (which is acid soluble). This may be due to degradation or the presence of [³H] lysine-tRNA degraded to free amino acid.

A recovery experiment was carried out to estimate how much loss of labelled Peak 2 occurred during the preparation of egg cell extracts. [³H] Peak 2 was added to 25 unfertilised eggs and a cell extract prepared and fractionated on Sephadex G-100 as usual. Fig. 8.3B shows this result. Clearly, Peak 2 is lost in this sample also.

These results are difficult to interpret. It is not known how much of the radioactivity in each preparation actually represents the factors. The shift in the elution of Peak 1 to the A₂₈₀ peak in the egg extract is odd, especially as there is little loss in Peak 1 activity in the egg extracts. It may indicate aggregation or some more interesting mechanism of sequestration. The loss of Peak 2 from the egg extract may be due to a recovery problem. There is a greater total loss of radioactivity in the preparation of egg extracts than for the oocyte or Stage 10½ extracts. However, 34% of the uninjected radioactivity rechromatographs in the Peak 2 position. This is a larger percentage than the maximum 20% loss of radioactivity in the egg extract preparation (see above). This may indicate that the factors are still in the egg, but possibly inactive. Again,

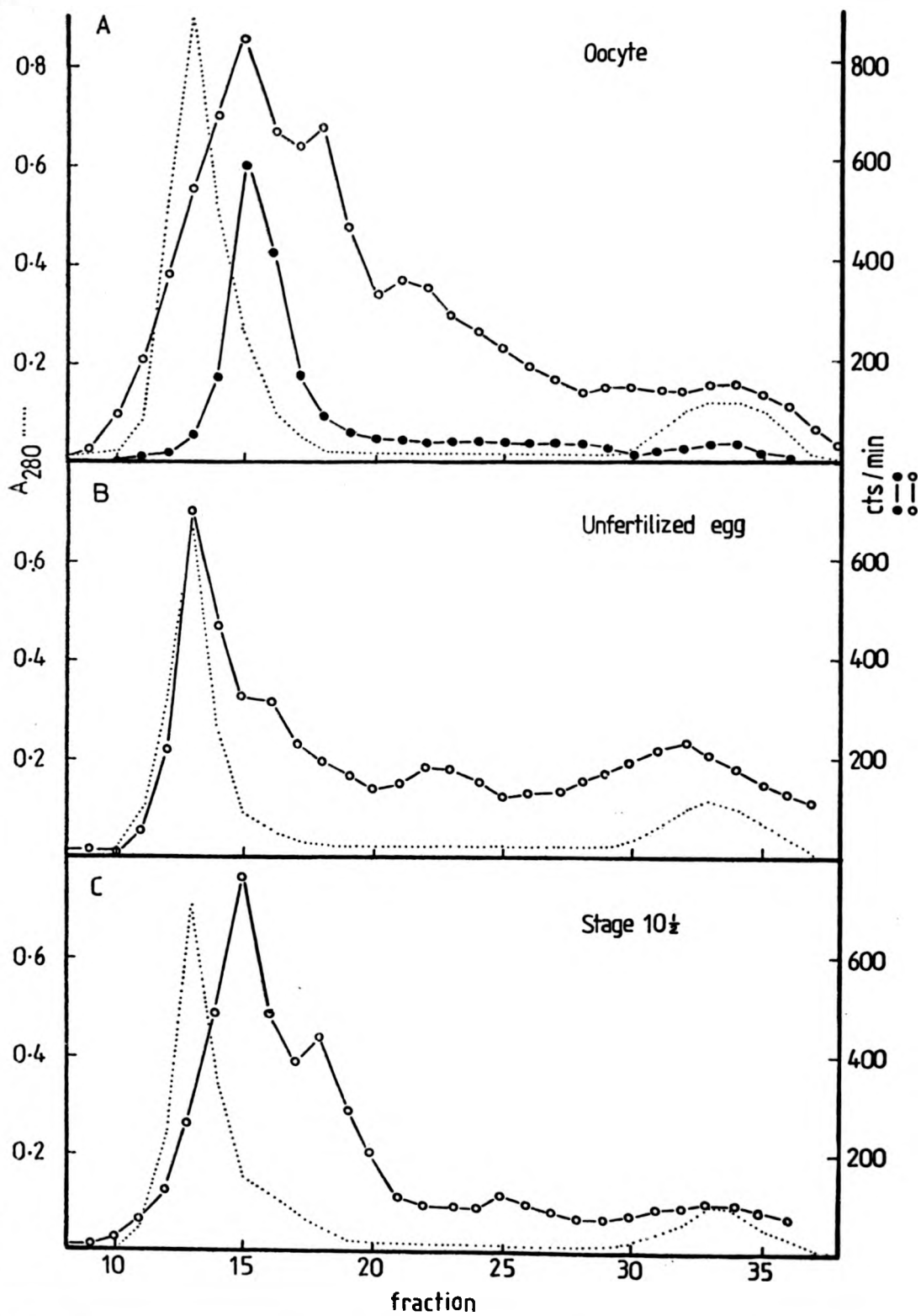
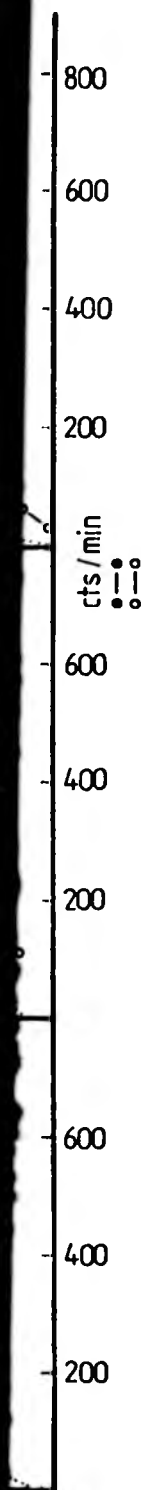


Figure 8.2 Recovery of labelled Sephadex Peak 1 injected into oocytes, eggs and embryos.

50 oocytes, eggs or 2 cell stage embryos were microinjected with a total of 2.5 μ l of [3 H] lysine labelled Peak 1 purified from Stage 5/6 oocytes (see Chapter 2). The injected cells were incubated for 3 hours at 18 $^{\circ}$ C, in the case of the oocytes and eggs, and until the injected embryos had reached Stage 10 $\frac{1}{2}$. The injected cells were quick frozen until they were analysed by preparing a cell extract and fractionating on G-100 Sephadex as usual. The A_{280} of resulting fractions was measured and then the total radioactivity in the sample was estimated.

- (A) Peak 1 injected into oocytes (O). A sample of the injected material (\bullet).
- (B) Peak 1 injected into unfertilised eggs (O).
- (C) Peak 1 injected into 2 cell stage embryos and analysed when the embryos had reached Stage 10 $\frac{1}{2}$ (O). (.....), A_{280} .



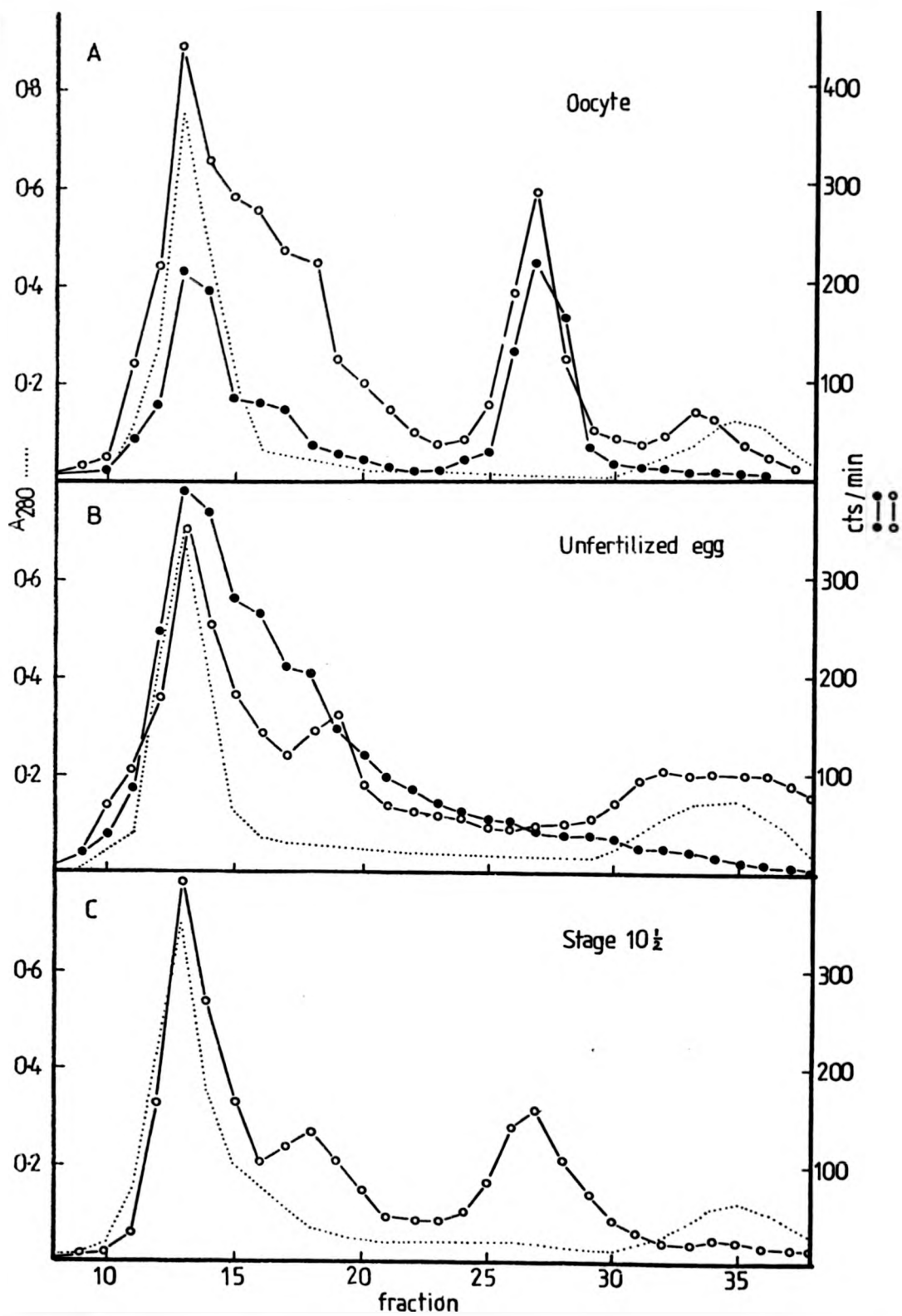


Figure 8.3 Recovery of labelled Sephadex Peak 2 injected into oocytes, eggs and embryos.

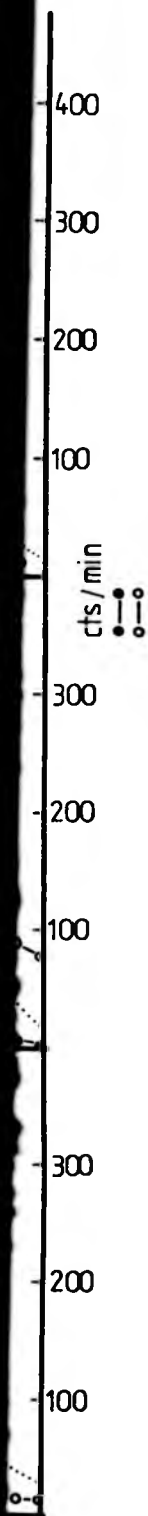
[³H] lysine labelled Peak 2 from Stage 5/6 oocytes was injected into oocytes, eggs and 2 cell stage embryos, and analysed as described in Figure 8.2.

(A) Peak 2 injected into oocytes (○). A sample of the injected material (●).

(B) Peak 2 injected into unfertilised eggs (○). A similar sample added during extract preparation rather than being injected (●).

(C) Peak 2 injected into 2 cell stage embryos and analysed when the embryos had reached Stage 10½ (○).

(.....), A₂₈₀



however, the result is not a clear one and the fate of Peak 2 factors in the unfertilised eggs remains unresolved. Clearly, eggs behave differently from other stages.

(c) What happens to the factors in the nuclear assay ?

An interesting question, related to how the factors work, is what happens to the factors during the preincubation with nuclei. As described earlier, their effect persists even when the nuclei are removed from the preincubation medium. They may exert their effect at the nuclear membrane or may enter the nucleus and remain there.

An initial experiment was to re-assay the supernatants removed from the preincubation step of a nuclear assay of Sephadex factors. XTC-2 nuclei were preincubated with Sephadex peaks 1, 2 and 3 as usual. After 1 hour's preincubation the nuclei were removed and assayed for [^3H] UTP incorporation as usual. The supernatants were assayed for the presence of the stimulatory activities with fresh nuclei as before. Table 8.1 shows the results of these two sets of assays. There was an overall lower incorporation by the second set of nuclear assays compared with the first (possibly due to the addition of further unlabelled triphosphates to the second preincubation or the loss of some other substance from the preincubation medium). This data suggests either that most of Peak 1 enters the nuclei during the first preincubation and is isolated with the first set of nuclei, or that it is destroyed during the first preincubation. The same is true, to a lesser extent, for Peak 2. Peak 3 activity, however, seems to remain in the supernatant and is almost as stimulatory in the second assay as in the first.

It is difficult to separate loss of activity due to degradation and loss due to uptake by the nuclei. To investigate this point further, labelled oocyte proteins fractionated on Sephadex G-100 were again employed. Nuclei were preincubated with fractions from this G-100 column. After 1 hour, each incubation mixture was layered over a small pad of centrifugation medium and the whole was centrifuged in a Beckman Microfuge for 1 minute to pellet the nuclei. This effectively separated the nuclei from the surrounding, labelled incubation medium. The acid-insoluble radioactivity in the nuclear pellets and resulting supernatants were measured.

Table 8.1

The fate of oocyte factors during their preincubation with isolated nuclei

	Incorporation by the 1st nuclear assay (counts/minute)	Stimulation over control	Incorporation by the 2nd nuclear assay (counts/minute)	Stimulation over control
Control	4031	1.0	3843	1.0
Peak 1	21215	5.2	4935	1.3
Peak 2	32070	7.9	9722	2.5
Peak 3	12052	3.0	11528	2.8

XTC-2 nuclei were preincubated with Sephadex peaks 1, 2 and 3 from oocytes in the standard way. After reisolation, the nuclear pellet was assayed as usual (1st nuclear assay) and the supernatant was used in a second standard assay (2nd nuclear assay).

A comparison of the input acid-insoluble radioactivity with the pellet and supernatant total insoluble radioactivity indicated that degradation (to the point of acid solubility) had not occurred during preincubation. Fig. 8.4 shows the radioactivity present in the nuclear pellet and supernatant after preincubation with labelled protein fractions. Also shown is the percentage of the input radioactivity present in each nuclear pellet. This indicates the proportion of the total labelled protein taken up by the nuclei during the preincubation. This value averages about 10% but there is variation. Clearly, proteins eluting with Peak 1 are taken up by the nuclei and this is also true for Peak 2 although the amount of radioactivity involved is much smaller. The percentage uptake is, however, never very high. These data support the view that these two stimulatory activities migrate into the nuclei.

Is this increased uptake a general or a preferential one? To answer this question, the experiment was repeated except that the separated pellets and supernatants were subjected to gel electrophoresis on 18% SDS slab gels. The gels were subsequently dried and autofluorographed (see Chapter 2). Fig. 8.5 shows densitometer traces of the corresponding pellet and supernatant tracks of the fractions around Peak 1 and at Peak 2. Clearly, the traces for each pellet and supernatant are not the same. It indicates that the nuclei are taking up a defined subset of the total labelled proteins in each fraction. The trace of the Peak 2 nuclear pellet shows two major proteins and these have similar mobilities to proteins A1 and A2 described in Chapter 4. Thus, it appears that the uptake of the labelled proteins is selective to some extent and not just a certain percentage of the total protein present. However, the proteins in the nuclear pellet may just be sticking to the nuclear envelope and not entering the nuclei.

(d) Discussion

The experiments described in part (b) of this chapter are inconclusive. They indicate that the difference observed in the factor complement of egg extracts may be due to loss of Peak 2 during extract preparation. It seems that this is a more general phenomenon as both labelled Peak 1 and 2 when injected into eggs rechromatograph differently from the input radioactivity. This, however, may be a manifestation of

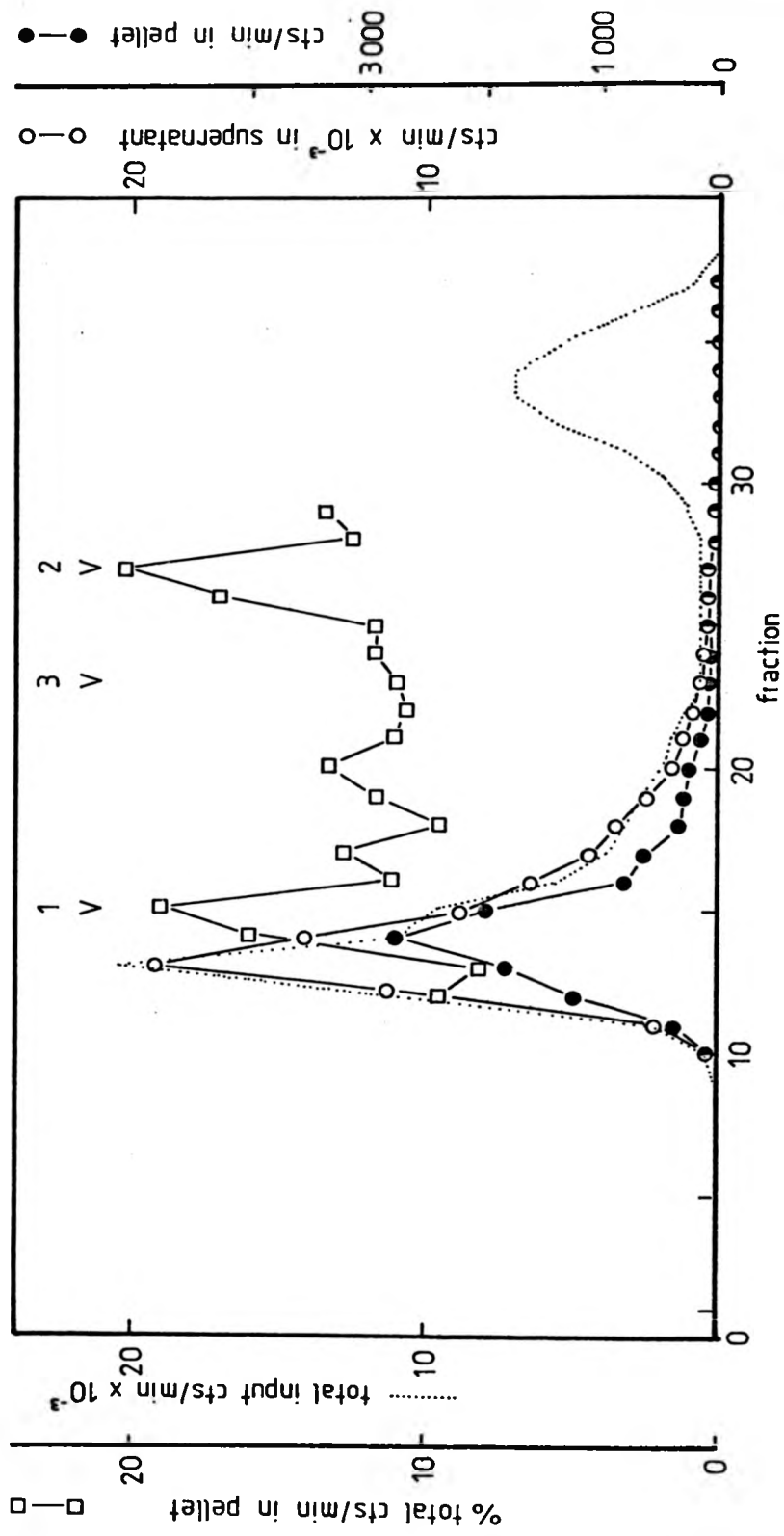


Figure 8.4 Migration of labelled oocyte protein into XTC-2 nuclei in vitro .

XTC-2 nuclei were preincubated with Sephadex G-100 fractionated, [^3H] lysine labelled oocyte extract for 1 hour at 25°C in the standard way. After this time, the incubates were layered over a pad of Centrifugation medium and the nuclei pelleted by centrifugation. The acid insoluble radioactivity present in the nuclear pellet and supernatant was measured (see Chapter 2).

(.....) Total input radioactivity in the assay; (●), radioactivity present in the nuclear pellet; (○), radioactivity present in the incubation medium; (□), percentage of the total radioactivity present in the nuclear pellet. The positions where Peaks 1, 2 and 3 elute are indicated.

fraction

20

10

0

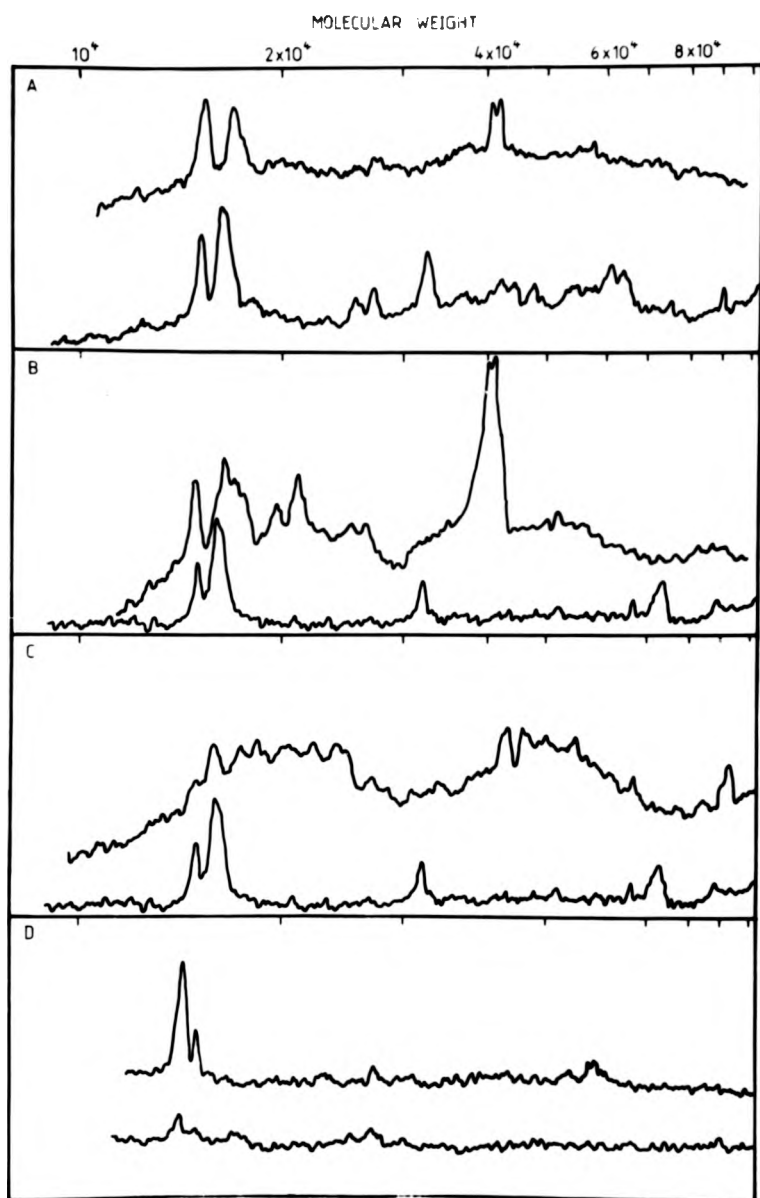


Figure 8.5 Densitometer scans of labelled proteins present in isolated nuclei and their incubation medium after preincubation with labelled oocyte protein.

XTC-2 nuclei were preincubated with G-100 Sephadex fractions of [^3H] labelled oocyte cell extract as described in Figure 8.4. The resulting pellets and supernatants were precipitated with TCA and subjected to gel electrophoresis on 18% SDS gels as described in Chapter 2. The figure shows densitometer traces of the autofluorographed gels. The upper trace shows the labelled proteins present in the nuclear pellet; the lower trace those present in the incubation medium in each case.

(A) Fraction 14, (B) Fraction 15, (C) Fraction 16, (D) Fraction 27.



the mechanism for changing the activity of the factors in the egg.

Apparently, activity of Peaks 1 and 2 is taken up by the nuclei during the preincubation step. The preliminary experiments involving the uptake of labelled proteins are quite exciting. The nuclei seem to be selective in terms of the proteins taken up, and this may give some indication of the integrity of the isolated nuclei.

There has not been a great deal of work in the field of protein uptake by nuclei. Merriam (1969) showed that brain nuclei, when injected into prelabelled oocytes and eggs, acquired cytoplasmic proteins from the hosts during swelling. Recently, Gurdon *et al.* (1976) have shown that HeLa nuclei injected into *Xenopus* oocytes lose large amounts of their proteins during swelling. They also take up histone and non-histone proteins from the surrounding oocyte cytoplasm in proportion to the increase in nuclear volume. Other work has been concerned with protein uptake by oocyte nuclei inside the oocyte. Bonner (1975a), using ¹²⁵I labelled marker proteins injected into oocytes, showed that histones were accumulated by GV's but that entry of proteins with molecular weights of 69,000 and over was hindered. Lower molecular weight proteins (< 20,000 MW) equilibrated between GV and cytoplasm within 24 hours. Other work (Bonner, 1975b), using labelled proteins derived from GV's and oocyte cytoplasm indicated that there are three classes of protein, those that accumulate in the GV, cytoplasmic proteins and those that are found in both GV and cytoplasm. Recent work by Feldherr and Pomerantz (1978) has suggested that protein accumulation by GV's *in situ* is not controlled by the GV envelope but possibly by selective binding within the nucleoplasm.

The system using isolated nuclei described in this chapter could aid the identification of the stimulatory factors and may be useful in the study of the control of protein transport between the nucleus and cytoplasm.

Chapter 9

The effect of *Xenopus* cell extracts on RNA synthesis in isolated BHK and *Xenopus* ^{red cell} blood nuclei

(a) Introduction

All the work described so far in this thesis has involved nuclei derived from one source, namely XTC-2 tissue culture cells. The results described may thus be an artefact of the nuclei used. To overcome this criticism, nuclei from other sources have been prepared and assayed in a similar way to the XTC-2 nuclei.

Nuclei prepared from a Syrian hamster kidney cell line (BHK-21/13) have been used to assay for the effect of the *Xenopus* cell extracts and factors. These experiments are described in the first part of this chapter.

Avian and amphibian erythropoiesis is an extensively used model system in the field of transcriptional control (e.g. Attardi *et al.*, 1976). These species retain a transcriptionally inactive nucleus in their mature erythrocytes. It is an interesting system to study, not only for the mechanisms involved in closing-down nuclear transcription, but also because the inactivation can be reversed by introducing the inactive erythrocyte nucleus into a transcriptionally active cell (Appels and Ringertz, 1975; Harris, 1970).

Work with heterokaryons (for review see Harris, 1970) and nuclear transplantation experiments (for review see Gurdon, 1974) has stressed the importance of the cytoplasm in this reactivation process. These experiments have led to various attempts to reactivate isolated chick erythrocyte nuclei with cell extracts from active cells (Thompson and McCarthy, 1968). Some reactivation (stimulation of DNA and RNA synthesis) was observed in these experiments but no further analysis of its cause was carried out.

More recent work has concentrated on *Xenopus* rather than avian, erythrocytes because of their lower endogenous transcriptional activity (Madgwick *et al.*, 1972; Maclean *et al.*, 1973). Hentschel and Tata, (1978) have shown that in *Xenopus* transcriptionally inactive mature erythrocyte nuclei retain only RNA polymerase II activity. The polymerases are present as engaged transcription complexes,

with a restricted ability to elongate RNA chains in vitro. Maclean and Hilder (1977) have investigated the effects of various cytoplasmic extracts on transcription in Xenopus erythrocyte nuclei in the presence of added E. coli RNA polymerases. Rat liver and Xenopus immature blood cell cytoplasm stimulated transcription in these nuclei. They found that the rat liver active factors were probably proteins of molecular weight of about 40,000 daltons. In this study, they also reported that Xenopus embryonic cell extracts inhibited RNA synthesis in this system.

It was of obvious interest to see if transcription in isolated Xenopus ^{red} blood ^{cell} nuclei was affected in any way by the cell extracts and fractionated factors already described. Experiments of this nature occupy the second half of this chapter.

(b) Results with BHK nuclei

Nuclei were isolated from BHK-21/13 cells and assayed using the methods and conditions described by Marzluff et al. (1973). The salt conditions were optimised as described for the XTC-2 nuclei (see Chapter 2). The optimal conditions for RNA synthesis by these nuclei were similar to the XTC-2 nuclei except that the BHK nuclei showed a somewhat higher K^+ optimum at 150 mM. This concentration was used in all subsequent BHK assays.

Isolated BHK nuclei were found to contain about 7 pg DNA per nucleus. In general, the transcriptional activity of these nuclei, when incubated at 25°C (their optimal incubation temperature) was lower than the XTC-2 nuclei. Even taking into account the difference in DNA content of the nuclei, the incorporation of [3H] UTP was about 75% of XTC-2 nuclei. Addition of purified E. coli or Xenopus polymerases had little effect on this incorporation. Experiments with different α -amanitin concentrations indicated that all three RNA polymerases were active. Under the standard optimised conditions, 35% of the polymerase activity was polymerase III, 40% polymerase II and 25% polymerase I. The RNA product of isolated BHK nuclei, when run on 2.4% polyacrylamide gels under denaturing conditions, was found to be

heterogeneous in nature with a predominance of high molecular weight (approximately 45S) species. It was not necessary to add any inhibitors of RNAase to these nuclear incubations as they contained no detectable nuclease activity.

BHK nuclei and XTC-2 nuclei were preincubated in parallel with oocyte and egg cell extracts, Peaks 1 and 2 from Sephadex and the purified factors. After 1 hour's preincubation the nuclei were reisolated and incubated for 30 minutes under the optimal conditions for each nuclear type. Table 9.1 summarises the data obtained. The incorporation is normalised with respect to the DNA in the incubations. Clearly the incorporations by BHK nuclei are lower than by the XTC-2 nuclei. The extracts, Sephadex peaks and factors are, however, effective in stimulating (or inhibiting) RNA synthesis in the BHK nuclei although the extent is reduced in comparison with XTC-2 nuclei.

These results indicate that the purified factors are not artefacts of the nuclear source. The reduced effectiveness of the factors in stimulating RNA synthesis in BHK nuclei may, however, indicate some sort of species specificity, although this reduction is not a large one.

(c) Results with ^{red}Xenopus^{cell} blood/nuclei

Whole Xenopus blood was obtained by cardiac puncture from freshly killed frogs. Nuclei were isolated and stored as already described (see Chapter 2). The cells were not fractionated into their different erythroid maturation stages before nuclear preparation, but the great majority would have been mature erythrocytes.

Xenopus^{red} blood^{cell} nuclei were assayed as described for the XTC-2 nuclei. Their transcriptional activity was about one-tenth that of the tissue culture nuclei per unit DNA in the assay. However, addition of E. coli polymerase (0.1 units/ 10^6 nuclei) or crude Xenopus oocyte polymerases (0.05 units/ 10^6 nuclei) increased their transcriptional activity by 15-20-fold and 6-8-fold respectively.

Fig. 9.1 shows the time course of incorporation of [3 H] UMP by Xenopus^{red} blood cell nuclei in the presence of oocyte and unfertilised

Table 9.1

The effects of cell extracts and oocyte factors on RNA synthesis in isolated XTC-2 and BHK nuclei

	<u>XTC-2 nuclei</u>		<u>BHK nuclei</u>	
	<u>pmol UMP incorp./ μg DNA/30 minutes</u>	<u>Stimulation over control</u>	<u>pmol UMP incorp./ μg DNA/30 minutes</u>	<u>Stimulation over control</u>
Control	2.5	1.0	1.8	1.0
Oocyte	5	2.0	3.4	1.9
Egg	1.9	0.8	1.4	0.8
Sephadex Peak 1	11.2	4.5	6.8	3.8
Sephadex Peak 2	20.3	8.2	10.1	5.6
Factor IA	6.7	2.7	3.8	2.1
Factor IB	17	6.8	10.3	5.7
Factor IIA	18.2	7.3	10	5.5
Factor IIB	12.5	5.0	8.1	4.5

XTC-2 and BHK nuclei were assayed with cell extracts and factors from Stage 5/6 oocytes in the standard way with preincubation.

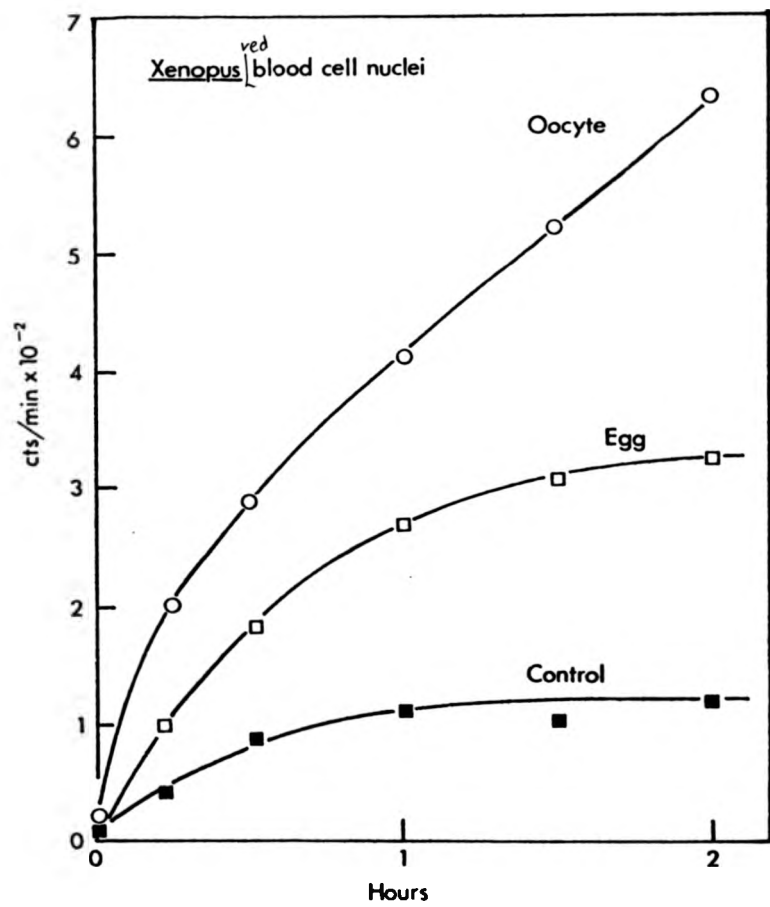


Figure 9.1 Time course of RNA synthesis in *Xenopus* ^{red cell} blood nuclei incubated with and without cell extracts.

Xenopus ^{red cell} blood nuclei were incubated with oocyte (○) and unfertilised egg (□) cell extracts, and in the absence of (■) extracts as described in Chapter 2 (without preincubation). Standard 400 μl incubations were carried out as usual, and duplicate 25 μl aliquots removed at the times indicated.

egg extracts (no preincubation and no added RNA polymerase). These nuclei show a much greater stimulation over control by the oocyte extract (5-fold) than do cultured cell nuclei. There is also the difference that the egg extract stimulates RNA synthesis, rather than inhibiting it. However, the ratio of the oocyte to egg incorporation is similar for both types of nuclei (~ 2). The cell extract effects on ^{ved} blood ^{cell} nuclei transcription are more immediately obvious in the time course than with XTC-2 nuclei.

Clearly, the oocyte extract stimulated transcription in these nuclei. An interesting point was to measure which RNA polymerases were active in the control and extract treated nuclei (remembering that both cell extracts contain about equal amounts of RNA polymerase activities and that mature erythrocytes exhibit only polymerase II activity). Blood ^{cell} nuclei were incubated as above with and without cell extracts and with and without α -amanitin to $1 \mu\text{g/ml}$ and $100 \mu\text{g/ml}$ final concentrations. After 2 hours, samples were withdrawn from the incubates and assayed for their TCA insoluble radioactivity. Table 9.2 summarises these results. In part A, the per cent of the total activity due to each polymerase activity is given with the fold stimulation by the extracts. Part B shows these percentages normalised with respect to the total control incubation as an arbitrarily defined unit of enzyme activity. From this the units of activity for each polymerase form has been calculated for the three incubations.

These data suggest, first, that the main polymerase activity in the control incubation is polymerase II. Secondly, that the egg extract stimulates all three polymerases about equally (2-fold). Thirdly, the oocyte extract increases the polymerase I and III activity considerably (35-fold and 7-fold) and the polymerase II activity about 2-fold. This might imply some form of reactivation of the ^{ved} ^{cell} blood nuclei is occurring in the presence of oocyte extract, especially with the considerable increase in polymerase I activity. All the polymerases are active in the oocyte incubates, not just polymerase II as in the control incubate. The low levels of polymerase I and III activities in the control incubation may result from white cell and erythroblast nuclei in the whole blood nuclear preparations.

Table 9.2

The effect of cell extracts on the RNA polymerase activities of
isolated *Xenopus* ^{red}blood^{cell} nuclei

(A)	Fold stimulation	% of total activity		
		Polymerase I	Polymerase II	Polymerase III
Control	1	4	81	15
Oocyte	4.3	32	44	24
Egg	2	6	83	11

(B)	Total activity	Polymerase I activity	Polymerase II activity	Polymerase III activity
Control	1	0.04	0.81	0.15
Oocyte	4.3	1.40	1.90	1.00
Egg	2	0.10	1.70	0.22

Isolated *Xenopus* ^{red}blood^{cell} nuclei were assayed for 2 hours with [³H] UTP and in the presence of cell extracts and α -amanitin to select for the different polymerases as usual. Part (A) shows the fold stimulation over control (Column buffer) in these assays, and the percentage of the total activity due to each polymerase. Part (B) shows these figures in arbitrary polymerase units taking the control total as 1 unit.

The stimulatory effect of the oocyte extract was further analysed by using Sephadex G-100 fractionation. Sephadex column fractions were assayed for their effect on transcription in isolated ^{ved} blood ^{cell} nuclei in the presence and absence of added Xenopus crude polymerases. The nuclei were incubated under standard assay conditions (no pre-incubation) with the column fractions and the incorporation of [³H] UMP was assayed after 2 hours at 25° C. RNA polymerases were added to a concentration of about 0.05 units/10⁶ nuclei. Fig. 9.2 shows the results of these assays. In the absence of added polymerases there are two peaks of incorporation corresponding to Peaks 1 and 3 (giving a 3-fold stimulation). In the presence of added RNA polymerase, only Peak 3 was present (giving a 1.8-fold stimulation). Peak 1, in the absence of added polymerase, probably results from the polymerase activity in these fractions (see Chapter 7). There was no sign of any Peak 2 activity.

(d) Discussion

Experiments with BHK nuclei indicate that the factors purified using XTC-nuclei are almost equally effective in stimulating transcription in nuclei from both sources. Thus they are probably not species specific.

The isolated blood nuclei appear to exhibit mainly polymerase II activity which agrees well with the data of Hentschel and Tata (1978) using mature erythrocyte nuclei. Whereas egg extracts increase the endogenous polymerase activity by about 2-fold, oocyte extracts increase mainly the polymerase I and III activity. These effects become apparent earlier in the incubation than with XTC-2 nuclei. This observation and that added polymerase increases the template activity of ^{ved} blood ^{cell} nuclei suggests that they are more permeable to proteins etc. than the cell culture nuclei. Comparable effects of cell extracts have been reported by Thompson and McCarthy (1968) using chick erythrocyte nuclei.

Sephadex Peaks 1 and 2 have no effect on RNA synthesis in isolated Xenopus blood nuclei. This is an unexpected result. Peak 2 stimulated polymerase I activity in XTC-2 nuclei but had no effect on blood nuclei with added polymerase. However, the effect of the total oocyte extract includes a large increase in polymerase I activity in

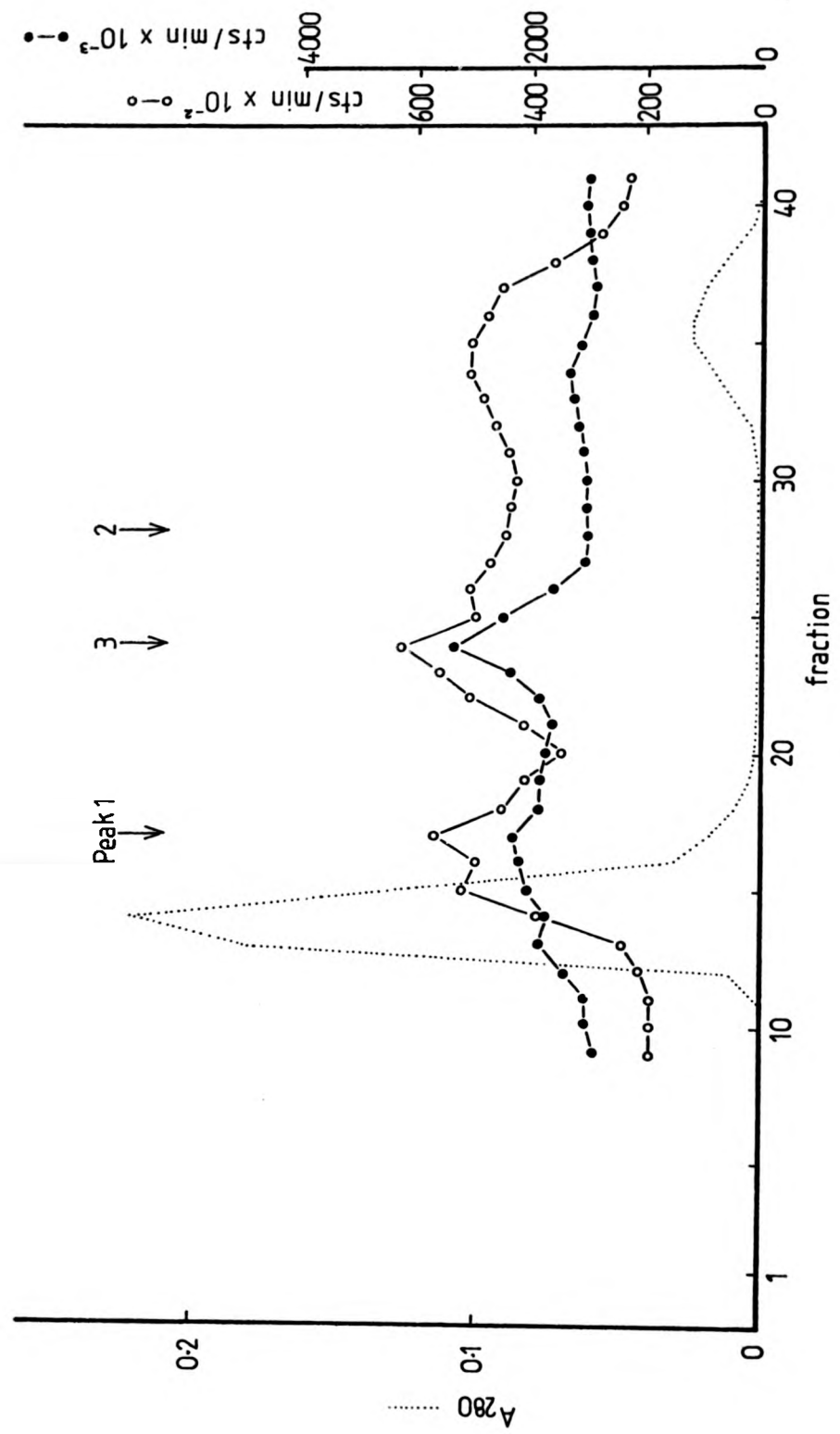


Figure 9.2 RNA synthesis in *Xenopus* ^{red} blood ^{cell} nuclei incubated with Sephadex column fractions of oocyte extract.

Isolated *Xenopus* ^{red} blood ^{cell} nuclei were incubated with G-100Sephadex column fractions of an oocyte cell extract. Incubation was for 2 hours at 25° C in the presence of [³H] UTP. A parallel set of 50 μl assays included 0.05 units of *Xenopus* RNA polymerase also. After 2 hours, the acid insoluble radioactivity present in the incubates was measured.

(.....), A₂₈₀; (○ — ○), [³H] UTP incorporation in the nuclear assay in the absence of RNA polymerase; (● — ●), [³H] UTP incorporation in the nuclear assay in the presence of RNA polymerase.

the ^{red} ^{cell} blood nuclei. Further analysis using α -amanitin in the assays of the Sephadex fractions may help to clarify this point.

Sephadex Peak 3 appears to be active on isolated ^{red} ^{cell} blood nuclei. Other experiments (see Chapter 6) suggested that this factor stimulated polymerase II considerably. Again, α -amanitin experiments would substantiate this effect on blood nuclei. The factor may be involved in activation of the polymerase II transcription complexes present in erythrocyte nuclei (Hentschel and Tata, 1978). It may also correspond to the rat liver factor reported by Maclean and Hilder (1977); both factors elute from Sephadex with a molecular weight of 35-40000 daltons. This stimulatory factor is discussed further in the next chapter.

the ^{red} ^{cell} blood nuclei. Further analysis using α -amanitin in the assays of the Sephadex fractions may help to clarify this point.

Sephadex Peak 3 appears to be active on isolated ^{red} ^{cell} blood nuclei. Other experiments (see Chapter 6) suggested that this factor stimulated polymerase II considerably. Again, α -amanitin experiments would substantiate this effect on blood nuclei. The factor may be involved in activation of the polymerase II transcription complexes present in erythrocyte nuclei (Hentschel and Tata, 1978). It may also correspond to the rat liver factor reported by Maclean and Hilder (1977); both factors elute from Sephadex with a molecular weight of 35-40000 daltons. This stimulatory factor is discussed further in the next chapter.

Chapter 10

Sephadex Peak 3

(a) Discussion

This chapter is designed to briefly summarise the results so far obtained concerning Sephadex Peak 3. The main conclusions are listed below:-

- (1) It is a minor stimulatory peak in the original Sephadex G-100 fractionation of oocyte extracts analysed with XTC-2 nuclei (maximum stimulation ~ 3-fold) (see Chapter 5).
- (2) The activity of this factor changes little between cell extracts made from stages during oogenesis and early development (see Chapter 5).
- (3) α -amanitin studies indicated that the factor stimulates mainly polymerase II activity in XTC-2 nuclei (see Chapter 6).
- (4) Most of the activity remains in the incubation medium after pre-incubation, suggesting that the factor is either recycled, does not enter the nucleus, or is lost from the nuclei during their re-isolation (see Chapter 8).
- (5) Activity stimulating RNA synthesis in isolated Xenopus blood nuclei (with and without polymerase) is present in the same fractions as Peak 3 (XTC-2) (see Chapter 9).
- (6) Preliminary experiments involving fractionation of this Sephadex peak using DEAE-cellulose chromatography and XTC-2 nuclei has yielded at least 5 separate activities (one of which elutes in the wash through fractions) giving stimulations of between 1.4 and 2-fold (data not shown).

All these observations point to this factor being worthy of much greater attention and further analysis in terms of its possible role in controlling RNA polymerase II activity and thus the synthesis of particular mRNA's. Important experiments concerning this factor and using the isolated nuclei system may include:

- (i) α -amanitin sensitivity (i.e. polymerase II specificity) of the DEAE fractionated activities from Sephadex Peak 3.
- (ii) Further analysis of this peak using extracts from different developmental stages.
- (iii) Analysis of the DEAE fractionated Peak 3 using Xenopus^{red} ^{cell} blood nuclei as the transcription system.

(iv) Further investigation of the mode of action of this factor (the red blood^{cell} nuclei may be particularly useful in this respect).

(v) Analysis of the new RNA products from Peak 3 treated nuclei using gel electrophoresis, but more importantly hybridization studies using specific mRNA probes (e.g. for globin or histone sequences). This may give an indication of whether the synthesis of specific sequences is being altered by the presence or absence of such factors.

Experiments of this sort hold exciting possibilities for clarifying the control of gene expression by cytoplasmic factors, if this is the mechanism involved.

Chapter 11

Conclusions and Prospects

Before going on to discuss how far the aims of this thesis, set out in Chapter 1, have been achieved, it will be helpful to briefly review the main conclusions drawn in the preceding chapters.

Results described in Chapter 3 showed that isolated XTC-2 nuclei fulfilled, as far as could be determined, the main criteria required of a transcription system to assay for cytoplasmic controlling factors (see Chapter 1). The rate of RNA synthesis in isolated XTC-2 nuclei was changed when they were incubated with oocyte and egg cell extracts in the optimised assay system, the oocyte extract being stimulatory and the egg inhibitory. Cell extracts prepared from other developmental stages assayed in the same way indicated that extracts of early cleavage stages were also inhibitory, whilst Stages 7½ to 13 became increasingly stimulatory. ^{The causes of} These effects were shown not to be trivial.

Four main stimulatory factors were isolated from Stage 5/6 oocytes to varying degrees of purity. Three were found to be relatively non-specific in their action, the fourth specifically stimulated polymerase I and the synthesis of rRNA. The activity of this factor was found to vary during oogenesis and early development (though possibly because of poor recovery) and its level of activity correlated reasonably with the known rate of rRNA synthesis occurring in vivo. The factors were not species specific.

A fifth minor stimulatory factor was found mainly to affect RNA polymerase II activity. The factor's activity changed little between extracts from different developmental stages. It was the only purified factor which also affected RNA synthesis in isolated Xenopus ^{ved cell} blood nuclei.

The mode of action of these stimulatory factors were less clear. There were some indications that both initiation and elongation of RNA chains were affected. A general requirement for their activity was found to be the presence of triphosphates during the preincubation step of the assay. In the case of the polymerase I-specific factor, stimulation of RNA synthesis depended on polymerase II activity during preincubation.

The factors stimulated RNA synthesis when assayed with isolated chromatin and added RNA polymerases. However, they were ineffectual when assayed with a purified DNA template and RNA polymerases.

To the extent of devising an assay system and using it to purify factors affecting RNA synthesis in vitro, the goals set out in Chapter 1 have been to some extent attained. There remain some basic questions about the system itself and about the stimulatory factors.

One of the essentials of the isolated nuclei system was that initiation of new RNA chains should occur. The data described here regarding to what extent initiation occurs is perhaps inconclusive. In the case of factor IIA, calculations on the kinetics of rRNA synthesis imply that initiation must be occurring in nuclei treated with this factor. However, direct evidence concerning this question is limited.

The integrity of the RNA product may also be a cause for concern. Although the XTC-2 nuclei incorporate [³H] precursors into rRNA-like sequences, the ratio of the 28S:18S rRNA species is different from that observed in vivo. This may be a feature of isolated nuclei, or it may be the result of degradation, aberrant initiation or termination of the polymerase molecules. An exception is the rRNA product of factor IIA treated nuclei which has a reasonably normal 28S:18S ratio. This might indicate that this factor is involved in increasing the integrity of transcription as well as stimulating it.

There are several questions concerning the factors which have been partially purified here. Probably the most interesting aspect of such factors is how they actually work. Data on this problem is sparse. Indeed, even the presence of low levels of endonuclease in the factor preparations can not be totally excluded in this respect. Further analysis depends critically on using purified factors and a simpler, more defined assay system. There is some hope of this in the case of factor IIA.

Another unanswered question is, if the factors are involved in controlling RNA synthesis in XTC-2 cells, why do the isolated nuclei not already contain the factors? Obvious answers might be that they do, but at lower levels than very transcriptionally active cells like large oocytes or gastrulae, or that they contain the factors in vivo but these are lost

during the nuclear isolation procedure. This last might be a strong possibility, especially if the factors are directly involved in nuclear-cytoplasmic interactions. The question may only be answered by looking for similar factors in whole XTC-2 cell extracts, their separated cytoplasm and possibly even the isolated nuclei themselves.

This, however, brings us to the major problem. Do these factors have any physiological relevance? Although their total activity in the extracts from different developmental stages roughly follows the in vivo RNA synthetic rate, this is not proof for a role controlling transcription in vivo. The most obvious problem in any such system is to show that the factors are the primary cause of the changes observed rather than a subsidiary effect of these changes. Experiments with the factors in vivo, possibly involving their micro-injection into Stage 1/2 oocytes or unfertilised eggs, may make a start in answering this question.

Given the criticisms outlined above, how useful or relevant is such an in vitro assay system? Such a complex system as the control of gene expression in eukaryotic cells can, at present, only be analysed by simplification to the in vitro situation. In vitro transcription systems and modulating factors are themselves interesting phenomena and may lead to a greater understanding of particular aspects of gene expression. This alone makes them worthy of further investigation. Results obtained in this way must ultimately be applied to the coupled, in vivo situation if the complex problems of development and differentiation are to be fully understood.

What are the prospects for the nuclear assay system described in this thesis? A number of important questions relating to the system itself have already been discussed to some extent. Some further general areas of investigation using this system are given below:-

- (1) Investigation of the nature of the reactivation process of blood nuclei exposed to oocyte extracts and how it occurs.
- (2) Analysis of the control of protein migration between the nucleus and its cytoplasmic environment (this may give a further indication of the identity of the stimulatory factors and possibly their primary target).

(3) The most exciting possibility is the investigation of what new gene products the nuclei synthesise when they are exposed to a different cytoplasmic environment. Use of heterologous nuclei (e.g. HeLa) with the Xenopus oocyte extract may aid identification of the new gene products. This would repeat in vitro the work of De Robertis et al. (1977) who found that new HeLa proteins were present in oocytes which had been injected with HeLa cell nuclei. Methods for purifying newly initiated RNA chains are available and are usable in this system (Reeve et al., 1977). Analysis of these new RNA products could be by hybridization studies using specific gene probes or even by translating the RNA's in an in vitro translation system such as the reticulocyte lysate (Pelham and Jackson, 1976). This may indicate the existence of regulatory factors controlling the synthesis of a particular mRNA or group of mRNA's. Sephadex Peak 3 may prove particularly interesting here because of its effect on polymerase II activity.

A further approach in this area might be to analyse the 5S RNA synthesised by untreated and oocyte treated isolated nuclei. Oocytes are known to synthesise 5S RNA's containing different sequences from those synthesised by somatic cells (Ford and Southern, 1973; Brown et al., 1977). It would be interesting to see if an oocyte extract induced such a change in somatic cell nuclei and, if so, what caused it.

REFERENCES

- Appels, R. and Ringertz, N.R. (1975), Current Topics in Dev. Biol., 9, 137.
- Attardi, G., Parnas, H. and Attardi, B. (1976), Exptl. Cell Res., 62, 11.
- Ballal, N.R., Choi, Y.C., Mouche, R. and Busch, H. (1978), Proc. Natl. Acad. Sci. USA, 74, 2446.
- Barth, L.G. and Barth, L.J. (1959), J. Embryol. Exp. Morph., 7, 210.
- Beebee, T.J.C. and Butterworth, P.H.W., (1977), Eur. J. Biochem., 77, 341.
- Benbow, R.M. and Ford, C.C. (1975), Proc. Natl. Acad. Sci. USA, 72, 2437.
- Benecke, B-J. and Penman, S. (1977), Cell, 12, 939.
- Bird, A.P. and Birnstiel, M.L. (1971), Chromosoma (Berl.), 35, 300.
- Birnstiel, M.L., Chlpchase, M.I.H. and Flamm, W.G. (1964), Biochem. Biophys. Acta, 87, 111.
- Birnstiel, M.L., Sells, B.H. and Purdon, I.F. (1972), J. Mol. Biol., 63, 21.
- Biswas, B.B., Ganguly, A. and Das, A. (1975), Prog. in Nucleic Acid Res., 15, 145.
- Bombik, B.M. and Baserga, R. (1974), Proc. Natl. Acad. Sci. USA, 71, 2038.
- Bonner, W.M. (1975a), J. Cell Biol., 64, 421.
- Bonner, W.M. (1975b), J. Cell Biol., 64, 431.
- Brown, D.D. and Littna, E. (1964), J. Mol. Biol., 8, 688.
- Brown, D.D. and Littna, E. (1966a), J. Mol. Biol., 20, 81.
- Brown, D.D. and Littna, E. (1966b), J. Mol. Biol., 20, 95.
- Brown, D.D. and Gurdon, J.B. (1978), Proc. Natl. Acad. Sci. USA, 75, 289.
- Brown, D.D., Carrol, D. and Brown, R.D. (1977), Cell, 12, 1045.
- Burton, K. (1968), In 'Methods in Enzymology', XII B, p. 163. Academic Press, London.
- Busiello, E. and Di Girolamo, M. (1975), Eur. J. Biochem., 55, 61.
- Butterworth, P.H.W., Cox, R.F. and Chesterton, C.I. (1971), Eur. J. Biochem., 23, 229.
- Carrarra, G., Gattoni, S., Mercanti, D. and Tocchini-Valentini, G.P. (1977) Nucleic Acid Res., 4, 2855.
- Chambon, P., Glssinger, F., Mandel, J.L., Kedingler, C., Gniazdowski, M. and Meilhac, M. (1970), Cold Spring Harbour Sym. Quan. Biol., 35, 693.
- Chuang, R.Y. and Chuang, L.F. (1975), Proc. Natl. Acad. Sci. USA, 72, 2935.
- Colman, A. (1974), J. Embryol. Exp. Morph., 32, 515.

Colman, A. and Cook, P. (1977), Eur. J. Biochem., 76, 63.

Colman, A. and Gadian, D.G. (1976), Eur. J. Biochem., 61, 387.

Crepin, M., Cukler-Kahn, R. and Gros, F. (1975), Proc. Natl. Acad. Sci., 72, 333.

Crippa, M. (1970), Nature (Lond.), 227, 1138.

Davidson, E.H. (1976), 'Gene Activity in Early Development', 2nd Edn., Academic Press, N.Y.

Dawid, I.B. (1965), J. Mol. Biol., 12, 581.

De Pomerai, D.I. and Butterworth, P.H.W. (1975), Eur. J. Biochem., 58, 185.

De Pomerai, D.I., Chesterton, C.J. and Butterworth, P.H.W. (1974), Eur. J. Biochem., 46, 461.

De Robertis, E.M., Partington, G.A., Longthorne, R.F. and Gurdon, J.B. (1977), J. Embryol. Exp. Morph., 40, 199.

Detke, S., Stein, J.L. and Stein, G.S. (1978), Nucleic Acid Res., 5, 1515.

Ecker, R.E. and Smith, L.O. (1971), Develop. Biol., 24, 559.

Emerson, C.P. and Humphreys, T. (1971), Science, 171, 898.

Ernest, M.J., Schutz, G. and Feigelson, P. (1976), Biochemistry, 15, 824.

Feldherr, C.M. and Pomerantz, J. (1978), J. Cell Biol., 78, 168.

Fiume, L. and Laschi, R. (1965), Sper. Arch. Biol. Norm. Pathol., 115, 288.

Ford, C.C. and Gurdon, J.B. (1977), J. Embryol. Exp. Morph., 37, 203.

Ford, P.J. (1971), Nature (Lond.), 233, 561.

Ford, P.J. (1972) In 'Oogenesis', Eds. Biggers, J.D. and Schuetz, A.W., p.167, Butterworth.

Ford, P.J. and Southern, E.M. (1973), Nature New Biol., 241, 7.

Froehner, S.C. and Bonner, J. (1973), Biochemistry, 12, 3064.

Gallien, L. (1956), Biol. Bull. France Belg., 90, 163.

Garel, A. and Axel, R. (1976), Proc. Natl. Acad. Sci. USA, 73, 3966.

Garel, A., Zolan, M. and Axel, R. (1977), Proc. Natl. Acad. Sci. USA, 74, 4867.

Gilbert, W. and Muller-Hill, B. (1970) In 'The Lactose Operon', Eds. Beckwith, J.R. and Zipser, D., Cold Spring Harbour Laboratory, N.Y.

Gilboa, E., Soreq, H. and Aviv, H. (1977), Eur. J. Biochem., 77, 393.

Gillespie, D. and Spiegelman, S. (1965), J. Mol. Biol., 12, 829.

Gilmour, R. and Paul, J. (1973), Proc. Natl. Acad. Sci. USA, 70, 3440.

- Goldberg, M.I., Perriard, J.C. and Rutter, W.J. (1977), Biochemistry, 16, 1648.
- Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963), Biochem. J., 89, 114.
- Grummt, I. and Grummt, F. (1976), Cell, 7, 447.
- Grummt, I. and Lindigkelt, R. (1973), Eur. J. Biochem., 36, 244.
- Gurdon, J.B. (1968), J. Embryol. Exp. Morph., 20, 401.
- Gurdon, J.B. (1974) 'The Control of Gene Expression in Animal Development', Clarendon Press, Oxford.
- Gurdon, J.B. (1976), J. Embryol. Exp. Morph., 36, 523.
- Gurdon, J.B. and Woodland, H.R. (1969), Proc. Roy. Soc. B., 173, 99.
- Gurdon, J.B. and Woodland, H.R. (1975) In 'Handbook of Genetics', Ed. King, R.C., Plenum Press, U.S.A.
- Gurdon, J.B., Partington, G.A. and De Robertis, E.M. (1976), J. Embryol. Exp. Morph., 36, 451.
- Hagopian, H.K. and Ingram, V.M. (1978), Cell, 13, 213.
- Hall, B.D., Brzezinska, M., Hollenberg, C.P. and Schultz, L.D. (1973) In 'Molecular Cytogenetics', Eds. Hamkalo, B.A. and Papaconstantinou, J., p. 217, Plenum Press, N.Y.
- Harris, H. (1970), 'Nucleus and Cytoplasm', 2nd Edn. Clarendon Press, Oxford.
- Harris, S.E., Rosen, J.M., Means, A.R. and O'Malley, B.W. (1975), Biochemistry, 14, 2072.
- Hentschel, C.C. and Tata, J.R. (1978), Develop. Biol., 65, 496.
- Hilder, V.A. and Maclean, N. (1974), J. Cell Sci., 16, 133.
- Hill, R.W. and McConkey, E. (1972), J. Cell Physiol., 79, 15.
- Hollinger, T.G. and Smith, L.D. (1976), Develop. Biol., 51, 86.
- Honjo, T. and Reeder, R.H. (1974), Biochemistry, 13, 1896.
- Jacob, F. and Monod, J. (1961), J. Mol. Biol., 3, 318.
- Jacob, S.T., Sadjel, E.M. and Munro, H.N. (1970), Nature (Lond.), 225, 60.
- Jeffreys, A.J. and Flavell, R.A. (1977), Cell, 12, 1097.
- Kellas, B.L., Austoker, J.L., Beebee, T.J.C. and Butterworth, P.H.W. (1977), Eur. J. Biochem., 72, 583.
- Knowland, J.S. and Graham, C.F. (1972), J. Embryol. Exp. Morph., 27, 167.
- Konkel, D.A. and Ingram, K.M. (1978), Nucleic Acid Res., 5, 1237.

- Kressmann, A., Clarkson, S.G., Pirrotta, V. and Birnstiel, M.L. (1978), Proc. Natl. Acad. Sci. USA, 75, 1176.
- Laemmlli, U.K. (1970), Nature (Lond.), 227, 680.
- Lamarca, M.J., Smith, L.D. and Strobel, M.C. (1973), Develop. Biol., 34, 106.
- Laskey, R.A. and Mills, A.D. (1975), Eur. J. Biochem., 56, 335.
- Laskey, R.A., Gerhart, J. and Knowland, J.S. (1973), Develop. Biol., 33, 241.
- Laskey, R.A., Mills, A.D. and Morris, N.R. (1977), Cell, 10, 232.
- Lentfer, D. and Lezins, A.G. (1972), Eur. J. Biochem., 30, 278.
- Lindell, T.J. (1976), Nature (Lond.), 263, 347.
- Lindell, T.J., O'Malley, A.F. and Puglisi, B. (1978), Biochemistry, 17, 1154.
- Link, G. and Richter, G. (1977), Eur. J. Biochem., 75, 119.
- Loening, U.E. (1967), Biochem. J., 102, 251.
- Loening, U.E. (1969), Biochem. J., 113, 131.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951), J. Biol. Chem., 193, 265.
- MacGregor, H.C. (1972), Biol. Rev., 47, 177.
- Maclean, N. and Hilder, V.A. (1977), J. Cell Sci., 24, 119.
- Maclean, N., Hilder, V.A. and Baynes, Y.A. (1973), Cell Differentiation, 2, 261.
- Macpherson, I.A. (1963), J. Nat. Cancer Inst., 30, 795.
- Madgwick, W.J., Maclean, N. and Baynes, Y.A. (1972), Nature New Biol., 238, 137.
- Maller, J.L. and Krebs, E.G. (1977), J. Biol. Chem., 252, 1712.
- Marzluff, W.F. and Huang, R.C.C. (1975), Proc. Natl. Acad. Sci. USA, 72, 1082.
- Marzluff, W.F., Murphy, E.C. and Huang, R.C.C. (1973), Biochemistry, 12, 3440.
- Marzluff, W.F., Murphy, E.C. and Huang, R.C.C. (1974), Biochemistry, 13, 3689.
- McNamara, D.J., Racevskis, J., Schumm, D.E. and Webb, T.E. (1975), Biochem. J., 147, 193.
- Meilhac, M., Tysper, Z. and Chambon, P. (1972), Eur. J. Biochem., 28, 291.
- Merriam, R.W. (1969), J. Cell Sci., 5, 333.

- Miller, L. (1974), Cell, 3, 275.
- Miller, O.L. Jr. and Beatty, B.R. (1969), Genetics, Suppl. 61, 1.
- Molineux, J.J. and Gefter, M.L. (1974), Proc. Natl. Acad. Sci. USA, 71, 3858.
- Nagamine, T., Natori, S. and Mizuno, D. (1976), FEBS Letters, 67, 198.
- Nakahashi, T. and Yamana, T. (1976), Develop. Growth and Differ., 18, 329.
- Nieuwkoop, P.D. and Faber, J. (1956) 'Normal Table of Xenopus laevis (Daudin)', North Holland, Amsterdam.
- O'Malley, B.W. and Means, A. (1974), Science, 183, 610.
- O'Malley, B.W., Tsai, M-J., Tsai, S.Y. and Toule, H.C. (1977), Cold Spring Harbour Sym. Quan. Biol., 42 (part 2), 605.
- Orkin, S.H. (1978), Biochemistry, 17, 487.
- Parish, J.H. and Kirby, K.S. (1966), Biochem. Biophys. Acta, 129, 554.
- Parker, C.S. and Roeder, R.G. (1977), Proc. Natl. Acad. Sci. USA, 74, 44.
- Paterson, B.M., Roberts, B.E. and Yaffe, D. (1974), Proc. Natl. Acad. Sci. USA, 71, 4467.
- Pelham, H.R.B. and Jackson, R.J. (1976), Eur. J. Biochem., 67, 247.
- Penman, S. (1966), J. Mol. Biol., 17, 117.
- Perez, A.G., Klm, J.H., Gelbard, A.S. and Djordjevic, B. (1972), Exptl. Cell Res., 70, 301.
- Price, R. and Penman, S. (1972), J. Mol. Biol., 70, 435.
- Pudney, M., Varma, M.G.R. and Leake, C.J. (1973), Experientia, 29, 466.
- Ramage, P.R. and Barry, J.M. (1975), Biochem. Biophys. Acta 395, 152.
- Randerath, K. and Randerath, E. (1967), 'Methods in Enzymology', XII A, p. 327, Academic Press, London.
- Read, R.S.D. and Mauritzen, C.M. (1970), Can. J. Biochem., 48, 559.
- Reeder, R.H. (1973), J. Mol. Biol., 80, 229.
- Reeder, R.H. and Roeder, R.G. (1972), J. Mol. Biol., 67, 433.
- Reeve, A.E., Smith, M.M., Pigiet, V. and Huang, R.C.C. (1977), Biochemistry, 16, 4464.
- Reeves, R. and Jones, A. (1976), Nature (Lond.), 260, 495.
- Rickwood, D. and Klempner, H.G. (1971), Biochem. J., 123, 731.
- Roeder, R.G. (1974), J. Biol. Chem., 249, 249.
- Rutter, W.J., Morris, P.W., Goldberg, M., Paule, M. and Morris, R.W. (1973) In 'Biochemistry of Gene Expression in Higher Organisms', Eds. Pollak, J.K. and Lee, J.W., p. 89, Australia and New Zealand Book Co., Australia.

- Sarma, M.H., Feman, E.R. and Baglioni, C. (1976), Biochem. Biophys. Acta, 418, 29.
- Seifart, K.H., Juhasz, P.P. and Benecke, B.J. (1973), Eur. J. Biochem., 33, 181.
- Sekemizur, K., Kobayashi, N., Mizuno, D. and Natari, S. (1976), Biochemistry, 15, 5064.
- Shiokawa, K. and Yamana, K. (1967), Develop. Biol., 16, 389.
- Sklar, V.E.F. and Roeder, R.G. (1977), Cell, 10, 405.
- Smith, M.M. and Huang, R.C.C. (1976), Proc. Natl. Acad. Sci. USA, 73, 775.
- Sugden, B. and Keller, W. (1973), J. Biol. Chem., 248, 3777.
- Tata, J.R., Hamilton, M.J. and Shields, D. (1972), Nature New Biol., 238, 161.
- Thompson, E.B. and Gelehrter, T.D. (1971), Proc. Natl. Acad. Sci. USA, 68, 2589.
- Thompson, L.R. and McCarthy, B.I. (1968), Biochem. Biophys. Res. Comm., 30, 166.
- Tilghman, S.M., Curtis, P.J., Tlemeier, D.C., Leder, P. and Weissmann, C. (1977), Proc. Natl. Acad. Sci. USA, 74, 4406.
- Tsai, R.L. and Green, H. (1973), Nature New Biol., 243, 168.
- Veber, J., Jellnek, W. and Darnell, J.E. Jr. (1977), Cell, 10, 611.
- Wei, C.M. and Moss, B. (1977), Proc. Natl. Acad. Sci. USA, 74, 3758.
- Weinmann, R. and Roeder, R.G. (1974), Proc. Natl. Acad. Sci. USA, 71, 1790.
- Weintraub, H. and Groudine, M. (1976), Science, 193, 848.
- Widnell, C.C. and Tata, J.R. (1966), Biochem. Biophys. Acta, 87, 531.
- Williams, A.R. (1973), Biochem. Biophys. Acta, 307, 58.
- Wilson, G.N., Steggle, A.W. and Niehuis, A.W. (1975), Proc. Natl. Acad. Sci. USA, 72, 4835.
- Woodland, H.R. and Adamson, E.D. (1977), Develop. Biol., 57, 118.
- Woodland, H.R. and Graham, C.F. (1969), Nature (Lond.), 221, 327.
- Woodland, H.R. and Pestell, R.Q.W. (1972), Biochem. J., 127, 597.
- Yu, F-L. (1974), Nature (Lond.), 251, 344.