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THE EFFECT OF HYPER TROPHY ON 
mRNA OF RAT LIVER

by

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Thesis presented for the degree of
Doctor of Philosophy, Faculty of Science,
at the University of Glasgow.

October, 1981.
To my Parents.
Acknowledgements

I wish to express my gratitude to a number of people without whom this thesis would not have been possible.

Professor R.M.S. Smellie and Professor A.R. Williamson for providing the facilities of the Department of Biochemistry for this research.

Dr. J.T. Knowler for unceasing moral support and invaluable discussions.

Dr. G.D. Birnie for discussions and for providing the research facilities at the Beatson Institute for Cancer Research. Also, to Dr. P.R. Wilkes and Mrs. R. Schott, of the same Institute, for their help.

To all my friends and colleagues in A3, A16 and 20 for their help.

My parents and family members for their encouragement, love and for financial support.

Finally, Mrs. A. Mosson for patiently typing the Manuscript.
Abbreviations

Standard abbreviations are in general as recommended in the "Instructions to Authors", of the Biochemical Journal (Biochem. J. (1978) 169, 1-27). Enzyme commission numbers are not used. Additional abbreviations used are :

I. Nucleic acids :
   a : RNA        Ribonucleic acid
   b : DNA       Deoxy-ribonucleic acid
   c : mRNA     Messenger RNA
   d : hnRNA    Heterogeneous nuclear RNA
   e : Poly(A)⁺RNA Polyadenylated messenger RNA
   f : cDNA     Complementary DNA

II. Phosphoric acid residues (left side = 5', right side = 3')
   Terminal : p; e.g. pppN .... is a polynucleotide
   with a 5'-triphosphate at one end; Ap is adenosine
   3'-phosphate; c...... e.g. cAMP..... is Cyclic-3'-5'-
   AdenosineMono.phosphate.

III. Synthetic polymers.
   a : Poly(A)ₙ     Polyadenylate homopolymer
   b : Poly(dA)ₙ    Poly deoxyadenylate homopolymer

   Some times prefix poly is replaced by oligo.
The subscript "n" is replaced by numerals indicating actual size, e.g. Oligo(dT)₁₂-₁₈.
IV. Miscellaneous Abbreviations.

a : RNase  Ribonuclease
b : DNase  Deoxy-ribonuclease
c : Bisacrylamide  NN'methylene bis acrylamide
d : c.p.m.  Counts per minute
SUMMARY
SUMMARY

This thesis describes a study of hypertrophy in rat liver and presents data on the effects of hypertrophy on liver mRNA populations.

Results are presented on:

i. The effects of partial hepatectomy on liver polysomal Poly(A)\(^+\) RNA populations.

ii. Effects of adrenalectomy on liver polysomal Poly(A)\(^+\) RNA populations.

iii. Effects of glucocorticoids on the liver polysomal Poly(A)\(^+\) RNA populations of adrenalectomized rat livers.

Liver mRNA was prepared from total polysomal RNA and was fractionated on Oligo(dT)-cellulose to obtain polysomal Poly(A)-containing species. The purified mRNA was extensively characterized under denaturing and non-denaturing conditions.

The diversity and complexity of mRNA populations were examined by cDNA hybridization and unique DNA hybridizations. Differences between the populations were also compared by heterologous hybridization to total and fractionated cDNA and unique DNA preparations.

Normal liver was found to contain 12,400 diverse Poly(A)\(^+\) RNA sequences when analysed by cDNA hybridization and 29,000 sequences when analysed by unique DNA hybridizations. These results compare favourably with those of others and illustrate the well documented findings that analysis by unique DNA hybridization tend to produce values 2 to 2\(\frac{1}{2}\) times greater than those derived by cDNA hybridization. Reasons are discussed
for believing that the latter method produces underestimates.

Analysis of mRNA populations of liver responding to partial hepatectomy showed little change when compared with normal rat liver. Both cDNA and unique DNA hybridization revealed a total complexity similar to that of the intact tissue. Heterologous hybridizations, however, showed that in the later stages of the hypertrophy phase of regeneration some changes in the relative abundances of the Poly(A)$^+$RNA species could be detected.

Complementary DNA hybridization analysis of the mRNA population of adrenalectomized rat liver revealed a massive change in the complexity such that total number of sequences obtained was reduced to one quarter. This unlikely result was found to be repeatable but is thought to reflect the changes in the low abundance, high complexity class of mRNA species. Thus, the large number of sequences which appear to disappear from the mRNA population of the adrenalectomized rat liver have probably just become much rarer and are therefore difficult to detect by Rot hybridization. These concepts are supported by unique DNA hybridizations which revealed substantial but very much smaller differences in the complexities of the two mRNA populations. Within 4-6 hrs of the treatment of adrenalectomized rats with glucocorticoids, the mRNA complexity became very similar to that of normal rat liver. Prolonged treatment with glucocorticoids did not result in a significant increase in the mRNA complexity.

The possible significance of these findings is discussed.
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1. **INTRODUCTION**

This thesis is concerned with the study of populations of mRNAs expressed as a result of hypertrophy in rat liver. Before presenting the results obtained, it is intended to review the salient features of the chromatin, DNA, pre-mRNA and mRNA of eukaryotes which may be of importance in the controlled expression of genes. It will also be of interest to review, in brief, what is known as the "control of gene expression" in rat liver.

1.1: **STRUCTURE AND FUNCTION OF CHROMOSOME**

The study of living organisms at the biological level has led to four great generalizations:

i. The theories of evolution: by natural selection as proposed by Darwin and Wallace. Proposing that today's complex plants and animals are derived by a continuous evolutionary progress from the first primitive organisms.

ii. The cell theory: the realization that all organisms are built up of cells.

iii. The chromosomal theory of heredity: the understanding that the function of chromosomes is the control of heredity.

iv. Gene theory: the molecular components concerned with the transmission of heredity characteristics.

The genetic information for the development and proper functioning of the organism is encoded in the linear sequence
of the deoxyribonucleotide molecules. This DNA is present, with the exception of polytene chromosomes, at one molecule/chromosome (Balmain et al., 1972, Ravenoff and Ziman, 1973). The DNA of eukaryotic cells is present in complex form with nucleoproteins and is known as a chromatin.

1.1.1: CHROMATIN

Regulation at the transcriptional level is one of the mechanisms involved in the control of gene expression in eukaryotic cells. How the structure of the chromatin is related to this regulation and whether the chromatin conformational changes are a pre-requisite for transcription or, are secondary to the transcriptional events, have been long standing problems.

1.1.1.1: Structure and Function of Nucleosomes

The fundamental unit of the chromatin is the nucleosome composed of a stretch of 146 base pairs (Bryan et al., 1979; Simpson and Kunzler, 1979) of DNA wound around an octamer histone core which contains two copies of each of the histones H2A, H2B, H3 and H4. These roughly cylindrical core particles, with a diameter of 11nm and a height of 5.5nm, are linked by 10 to 70 base pairs of 'linker' DNA. One copy of the remaining histone, H1, is associated with the linker DNA, and outside the nucleosome (Thomas & Khabaza, 1980).

The amino acid sequences of the histones H3 and H4 are highly conserved within all organisms, thus implying a critical function. It is now clear that these arginine-rich histones are sufficient and necessary for assembly of DNA
into a nucleosome-like structure (Stockley & Thomas, 1979). Two proteins which facilitate the assembly have been found in eukaryotic nucleus.

Nucleosome formation increases the packing ratio of the DNA to 7. However, this is far below the packing ratio required in vivo. The next order level is a 10nm diameter filament consisting of linear arrays of nucleosomes. A thicker (30nm diameter) fibre appears to be generated by the coiling of the thin fibre so packing nucleosomes into a "solenoid" structure (Thomas et al., 1979). Histone H1, which has the most species-dependent amino-acid composition, is implicated in this packing. However, it is still uncertain or unknown or undetermined whether H1 is on the inside or the outside of the solenoid (Thomas et al., 1979; Klug et al., 1980).

The above picture is largely static and implies that nucleosomes are merely vehicles for packing DNA with no functional role in replication and transcription. Contrary to earlier suggestions, recent evidence suggest that nucleosomes are not randomly located but have a specific phase relationship with respect to certain base sequences (Witting & Witting, 1979; Wu et al., 1979). Thus, a model has been proposed for nucleosome segregation at the replication fork (Seidman et al., 1979) and nuclease digestion studies of transcriptionally active chromatin shows their nucleosome-like structures to be conformationally distinct from those of bulk chromatin.
1.1.2: Active and Inactive Forms of Chromatin

Two forms of interphase chromatin have been known for more than 50 years (Heitz, 1928). Heterochromatin is inactive, transcription being localized in the diffuse euchromatin regions (Littau et al., 1964). The quantity of euchromatin varies widely from tissue to tissue. In mouse thymus lymphocytes, it represents 20% of the total nuclear DNA component (Littau, et al., 1964), while in the mouse hepatocyte it forms 90% (Yamineh and Yunis, 1970).

The extent to which euchromatin can be strictly equated with chromatin which is actively transcribed is controversial. The small euchromatin content of the thymus lymphocyte nucleus is approached by the quantity of DNA believed to be expressed as RNA (10-20%) in these cells. Several workers have shown, however, that in many cells most of the euchromatin is transcriptionally inactive. In the mouse hepatocyte, only 6-7% of the total genome is expressed, whereas nearly 90% of the genome is present as euchromatin (Grouse et al., 1972). Transcriptionally active chromatin is thus a relatively small fraction of the nuclear chromatin, and may represent as little as 2% of the total genome.

1.1.2.1: Structure of Active Chromatin

The chromatin of a differentiated cell is probably fixed structurally in the early stages of differentiation, and this conformation is maintained during the "life" of that cell. Several workers have been able to detect significant differences between the structure of transcriptionally active
genes and transcriptionally inert genes by using a variety of methods.

An early approach to differentiate between active and inactive chromatin employed cell free chromatin transcription studies. It was suggested that the regions which are transcribed selectively by RNA polymerase were in the active regions of chromatin (Axel et al., 1973).

Nucleases have been extensively used to determine the structure of active chromatin. Staphylococcal nuclease fails to distinguish any difference between active and inactive chromatin (Axel et al., 1973), but pancreatic DNase (DNase I) and Spleenic-DNase (DNase II) appears to digest active regions of chromatin selectively. This in turn indicates that active regions of chromatin exhibit a structural difference when compared to inactive regions (Weintraub & Groudine, 1976).

DNase I probing of chromatin structure provides convincing evidence, that, indeed, "active" chromatin has an altered and more accessible ("open") configuration. Weintraub & Groudine (1976) showed that the globin gene sequences are hypersensitive to digestion by DNase I in the nuclei of the cells actively expressing these regions. Similar results have been obtained for several other "active" genes; the ovalbumin gene in hen oviduct (Bellard et al., 1977), the ribosomal genes in various organisms (Weintraub, 1975; Stadler et al., 1973), and the induced heat-shock loci in Drosophila tissue culture cells (Wu et al., 1979). In addition, chromatin sequences complementary to the total population of nuclear RNA (Weintraub & Groudine, 1976), nuclear Poly(A)^+ RNA (Garel et al., 1977) and cytoplasmic Poly(A)^+ RNA (Levy-W and Dixon, 1977) are hypersensitive to
DNase I digestion.

Electronmicroscopy studies by Foe et al., (1976) and DNA/chromatin packing ratio determinations (Foe, 1977) have shown that the portion of genome which codes for ribosomal RNA (rDNA) is not packed into nucleosomes. Nevertheless, the nuclease sensitivity of this fraction is similar to that of DNA organised as nucleosomes (Mathis and Gorovsky, 1976). It therefore remains possible that the differences are more artifactual than real. Other regions of high transcriptional activity, like amphibian lampbrush chromosomes, also appear to be devoid of nucleosomal structures (Scheer, 1978). There may be correlation, therefore, between the degree of transcriptional activity and frequency of nucleosomes.

1.1.3: PROTEINS ASSOCIATED WITH "ACTIVE" CHROMATIN

Nuclease digestion has led to the conclusion that transcribed genes are complexed with proteins to form a periodic structure similar to, but more accessible than, that of nucleosome-packaged bulk chromatin (Kornberg, 1977).

Recent efforts have been directed at elucidating those factors which might be responsible for this active configuration. Biochemical and electronmicroscopic evidence suggest that the presence of transcriptional machinery is not necessary (Foe et al., 1976; Scheer et al., 1977). One other hypothesis suggested is that "active" chromatin regions exhibit an atypical histone composition; depleted in some or all of the core histones (Varshavsky et al., 1974; Chae, 1974), missing histone H1 (Pedersen, 1978), or possessing specific primary
sequence histone variants (Newrock et al., 1977). Another view is that template-active chromatin exhibits a typical histone complement, but that post-synthetic histone modifications effect an alteration in "active" chromatin structure that facilitates transcription (Johnson & Allfrey, 1978). Finally non-histone proteins have often been suggested as factors that could modulate chromatin transcription (Stein et al., 1978).

1.1.3.1: The Histones

The histones constitute a set of low molecular weight, highly basic proteins arranged into five different classes. It was first proposed that histones act as repressor molecules in eukaryotic gene expression by Stedman & Stedman (1950) and in support of these concepts, Allfrey et al., (1963) and Huang et al., (1964) provided evidence that activation or repression of RNA transcription depended upon the amount of histone complexed to DNA. Recently, it has been shown that histones severely reduce the template capacity of the ovalbumin gene (Tsai et al., 1976a, 1976b), and globin gene (Gilmour & MacGillivray, 1976). This may be due to its effect on RNA chain elongation rate (Koslov & Georgiev, 1970). Structural modifications such as phosphorylation, acetylation, ribosylation by poly (ADP-ribose) and methylation, which could lead to changes in electrostatic interaction with DNA, may be responsible for inducing or repressing transcription of specific genes (Tsai et al., 1976a; Axel et al., 1975; Stein & Stein, 1976).
It has also been suggested that acetylation of histones is responsible for the mechanism by which inert chromatin is activated for RNA transcription (Allfrey et al., 1964). This hypothesis appears attractive for two reasons. Firstly, a striking correlation between the enhancement of histone acetylation and enhancement of RNA synthesis has been detailed in a number of systems (Johnson & Allfrey, 1978; Allfrey, 1977). Secondly, since acetylation is normally found in the basic NH$_2$-terminal "arms" of core histones, it is easy to visualise how this modification could alter DNA-histone binding to allow RNA-polymerase read through (Yamamoto & Alberts, 1976). Similarly, other histone modifications can activate transcription associated with various biological processes, for example, proliferation (Gurley et al., 1974), stimulation by hormones and transformation by oncogenic viruses (Gottesfeld et al., 1975; Stein et al., 1978b).

Thus histone may play an important role in maintenance of genome structure (Korenb erg, 1977) and in the non-specific repression of DNA-dependent RNA synthesis (Stein et al., 1978a).

1.1.3.2: a) Non-histone Proteins

The chromosomal non-histone proteins (NHCPs) are a heterogeneous group, consisting of macromolecules that vary in molecular weight, amino acid composition, half-life and DNA binding properties.

This group is known to contain proteins with an enzymic role (Stein et al., 1978b). Contractile proteins, which may form structural components (Dauvas et al., 1975; LeStourgen et al.
1975) and regulatory molecules (Beserga, 1974; Stein & Klein-smith, 1975; Stein et al., 1975a).

Like the histones, NHCPs can undergo post-translational modifications including acetylation (Suria and Liew, 1974), methylation (Friedman, 1969) and phosphorylation (Kleinsmith, 1975). These modifications increase the potential of NHCPs for functioning in the fine control of gene expression. Additional changes in the composition and metabolism of NHCPs accompany the alterations that occur during eukaryotic gene expression associated with development (Johnson & Hnilica, 1972), cellular differentiation (Platz et al., 1975), the cell cycle (Gerner & Humphrey, 1973), stimulation of cell proliferation (Stein & Burtner, 1974; 1975), response to steroid hormones (O'Malley & Means, 1974; O'Malley et al., 1977) and in carcinogenesis (Stein et al., 1978b). Quantitative and qualitative changes to NHCPs have also been observed in euchromatin of transcriptionally active tissues (Gottesfeld et al., 1974; 1975).

NHCPs may specifically control the expression of a given gene and it has been shown that, only in the presence of those NHCPs normally associated with a given active gene, was that gene transcribed. Such experiments have implicated NHCPs in the specific expression of globin genes (Barret et al., 1974; Gilmour & MacGillivray, 1976), the oestrogen-induced transcription of ovalbumin genes (Tsai et al., 1976a; 1976b; 1976c; Masaki et al., 1976) and cell cycle stage specific transcription of histone genes (Jansing et al., 1977; Stein et al., 1975b).
Some doubts have been expressed at the validity of these experiments, due to the presence of contaminating endogenous RNA sequences and the origin of transcribed RNA (Zasloff & Felsenfeld, 1977; Shih et al., 1977).

1.1.3.2: b) High-Mobility-Group Proteins

The "high-mobility-group" (HMG) proteins are now a well characterized subset of non-histone protein population (Goodwin et al., 1979). They are low molecular weight species which bind to chromatin and histones (Goodwin et al., 1975) and have an unusual amino acid composition, containing approximately 25% basic and 30% acidic amino acids. Five such fractions from calf-thymus chromatin have been studied extensively; HMGs 1, 2, 14, 17 and 20 (or ubiquitin). Proteins with somewhat similar properties have been identified in the trout testis (Goodwin et al., 1979): "H6" (analogous to HMG 14 and 17), "T" (analogous to HMGs 1 and 2) and "S" (ubiquitin). In addition, an erythrocyte specific HMG (E) has been described (Sterner et al., 1978). The discovery of HMG proteins in yeast (Spiker et al., 1978) has established their wide distribution in eukaryotes; in fact, some similarity has been noted between HMGs and the prokaryotic "HU" proteins (Spiker et al., 1978). The lack of substantial tissue and species specificity (Sterner et al., 1978; Rabbani et al., 1978), however, has suggested that the HMGs, like histones, play a predominantly structural role in chromatin organization. Nevertheless, they are present in amounts accounting for 1-5% of the DNA content.
It has been shown that HMG proteins 14 and 17 are associated with actively transcribing genes (Weisbord & Weintraub, 1979; Weisbord et al., 1980). Various reconstitution experiments using HMG depleted nucleosome preparations, and purified HMG 14 and 17, have shown that the DNA of nucleosomes containing these proteins are more sensitive to DNase I digestion than those without, thus indicating a more open ("active") confirmation. This is the first indication of direct involvement of a defined NHCPs in any aspect of the control of gene expression.

1.2 : THE GENOMIC DNA

Many reports over the last five years have shown that the majority of genes that have so far proved accessible to detailed analysis are not colinear with their final product. The coding regions of genes (exons) are frequently interspersed with non-coding sequences (introns). The role of introns remains to be elucidated, but it is clear that the initial transcript of gene consists of coding and non-coding portions and that the non-coding portions are subsequently edited out.

The first discontinuous sequences observed were in ribosomal genes (Wellauer & Dawid, 1977), but the most interesting observation was the discontinuous sequences in the mRNA coding sequences in genomic DNA of SV40 (Celma et al., 1977; Alloni et al., 1977), Adenovirus (Klessig, 1977), Ovalbumin (Jeffreys & Flavell, 1977), Hemoglobin (Jeffreys & Flavell, 1977) and Immunoglobulins (Brack & Tonegawa, 1977).
From the above and other recent studies, it became clear that the combined length of the introns can often exceed the combined length of the coding sequences (exons).

Some eukaryotic and viral genes appear to lack intron sequences, for example, sea urchin histone genes (Schaffner et al., 1978), the yeast mitochondrial gene for cytochrome C (Smith et al., 1979) and adenovirus polypeptide 1X (Alestrom et al., 1980).

Recently, with the development of DNA sequencing technique (Sanger et al., 1977; Maxam & Gilbert, 1977) it has become possible to investigate the nucleotide sequence of specific genes and to look for sequences which might be involved in the controlled expression and maturation of mRNA. Three main sites appear to be particularly important in this respect. They are the 5'-terminus, and flanking sequences, the intron/exon boundary and 3'-terminus of the genes.

The available evidence that the coding and non-coding sequences of a gene are transcribed into large precursor mRNA species from which intron transcripts are subsequently deleted is reviewed in section 1.3.3.4. A possible mechanism for this editing process is presented in section 1.3.3.5.

1.2.1: THE 5'-TERMINUS AND FLANKING SEQUENCES

Extensive biochemical and genetic studies have indicated that 5' termini of ovalbumin, conalbumin and adenovirus early and late genes have some common features (Benoist et al., 1980). At about 10 b.p. before the initiation site there is an AT-rich region, known as "Hogness Box" (typically
5'--- TATAAT ---3', Rosenberg & Court, 1979). Centred around -35 b.p. there is a second sequence, the "recognition site" (typically 5'---- TGTTGACAATTT ----3', Rosenberg & Court, 1979).

Mutation of Hogness box, be it total deletion or, in the case of ovalbumin gene, mutation of the sequence TATA to TAGA, reduces transcription drastically. However, some viral genes which are transcribed by the RNA polymerase II of infected cells do not have a Hogness box to the 5' side of the transcription sequence. Furthermore, the importance of the Hogness box as a promoter region has been called into question by experiments employing recently developed system in which rDNA genes are transcribed in vitro. A number of observers, working with defined gene sequences, have shown that deletion of region upstream from the gene initiation site, including the Hogness box, does not eliminate in vitro transcription (Flavell, 1980). However, it does reduce transcription and results in heterogeneity of the 5' end of the transcript. Chambon's group have shown that the TATA box region, in an SV40 early gene, is involved in fixing initiation precisely within a narrow area, but it is dispensable for gene expression, while the sequences located more than 150 b.p. upstream are indispensable (Benoist & Chambon, 1981; Mathis & Chambon, 1981). Similarly Wasylyk and Chambon (1981) have shown that small deletions of 2, 3 and 4 nucleotides in the "TATA" box drastically decrease specific initiation of transcription. They have further suggested that a "T" to "A" transition drastically decreases transcription, showing that
the transcription is altered mainly by the alteration of nucleotide recognition site rather than helix stability.

It has also been observed that sequences more remote to the gene may modulate RNA polymerase II activity. A number of globin genes have a "CAAT" box sequence 80 nucleotides upstream from the coding sequences. Deletion of these sequences drastically reduced in vivo globin mRNA synthesis but was ineffective on in vitro transcription (Minty & Newark, 1980). Even further from initiation site, but apparently influencing transcription are "AT" rich sequences 184 to 520 nucleotides on the 5'-end side of the histone H2A gene and a sequence approximately 200 nucleotides upstream of a Drosophila gene which encodes a protein produced in response to the insect hormone, ecdysone (Wasylyk et al., 1980).

1.2.2: INTRON/EXON BOUNDARY

It is likely that the conserved sequences around the intron/exon boundary of gene transcripts are important in the accurate removal of intron sequences. A proposal by Steitz and co-workers is that snRNA hybridization to the splice point provides a mechanism for the "splicing". This is described in section 1.3.4 (snRNA).

1.2.3: SEQUENCE AT 3'-END

Recently Benoist et al., (1980) have sequenced the 3'-end of chicken ovalbumin gene and reported some features of interest. They have demonstrated the presence of two or three residues of the poly A tail which could be transcribed
from the genomic DNA. This phenomenon appears to be general for all 3' sequences studied to date. Thus the first adenosine at 3' end of SV40 early protein mRNA (Reddy et al., 1979) and mouse β-globin major mRNA (Konkel et al., 1979) are encoded in the gene. The first two 3' adenosines are gene transcripts in rabbit β-globin mRNA (Reddy et al., 1979), SV40 late protein mRNA (Reddy et al., 1978) and some adenovirus early mRNAs (Alestrom et al., 1980). The first three adenosines of the ovalbumin mRNA and the first four adenosines in mouse dihydrofolate reductase mRNA (Nunberg et al., 1980) poly A tail could also be transcribed from the genomic DNA.

Apart from this feature, comparison of the available genomic sequences immediately downstream from messenger coding sequences show little homology. These regions are often "AT" rich, however, and contain some runs of dT residues of varying lengths. Furthermore, a sequence close to the 3' end of the transcript appears to be based on a model sequence:

$$5'\text{-}\text{TTTCACCTGC}\text{-}3'$$

The sequence upstream from the 3' end of messenger coding portion of transcripts has also been the subject of considerable investigation. A comparison of sequences at the 3' end of messengers transcribed by E. coli RNA polymerase has led to a model for transcription termination involving a G-C rich region capable of forming a hairpin, followed by a run of uridine residues terminating the message (Reddy et al., 1979). No equivalent of these sequences have been detected at the 3'-end of those eukaryotic mRNAs some of which have been investigated. These include human α and β-globin mRNAs (Wilson et al., 1979; Proudfoot, 1977), mouse β-globin (Konkel
et al., 1978), mouse dihydrofolate reductase mRNA (Nunberg et al., 1980) and silk fibrion mRNA (Hagenbuchle et al., 1979).

It has been pointed out that the only feature common in these and other eukaryotic mRNAs is the sequence 5'---AAUAAA---3'; 14-30 residues upstream from the poly A tail. This sequence may be associated with specifying the site of poly-adenylation. The sequence of 10 nucleotides immediately preceding the poly A tail were also related in some of mRNA studied.

Recently, Fitzerald & Shenk (1981) have shown that all of the known SV40 late mRNAs also have the 5'---AAUAAA---3' sequence 12 nucleotides from their poly(A) site. They have further demonstrated that mutants having small deletions between the AAUAAA and the normal poly(A) site produce mRNAs poly-adenylated at new, downstream site.

1.3 : TRANSCRIPTION AND MATURATION OF EUKARYOTIC mRNA

1.3.1 : TRANSCRIPTIONAL ENZYMES

The synthesis of eukaryotic RNA molecules is catalysed by multiple forms of RNA polymerases which fall into three major classes designated I, II and III (Roeder et al., 1976).

Chambon (1975) and Roeder et al. (1976, 1977) have shown that each class of RNA polymerase has distinct catalytic properties, distinct subunit structure and specific function in the synthesis of major classes of RNA.

The class I enzyme is nucleolar in origin and catalyses the synthesis of ribosomal RNA (Blatti et al., 1970; Weinman and Roeder, 1974). Class II enzyme catalyses the synthesis of
the heterogenous nuclear RNA (Hn RNA) in the nucleoplasmic fraction of the cells (Reeder & Roeder, 1972) and the class III enzyme catalyses the synthesis of the precursors of 5S and tRNA (Roeder et al., 1977; Weil & Blatti, 1976).

The catalytic activity and the quantitative changes which occur during some stages of gene expression appear to be modulated by some unstable factors which have been shown to be structurally similar (Benson et al., 1978) to the subunits of the prokaryotic polymerases (Burges, 1969). Thus, protein factors, such as sigma factor and other low molecular weight protein factors (Chamberlain, 1974) which are required for proper initiation and specificity of prokaryote gene expression, may also be present in eukaryotic transcription system. However, it appears likely that the ability of the RNA polymerases to gain access to specific genes is primarily due to structural modifications of the chromatin (see section 1.1.2).

1.3.2 : THE HETEROGENOUS NUCLEAR RNA

Warner et al., (1966) used the term heterogenous nuclear RNA (Hn RNA) for RNA species present in nucleus and having heterogeneity in respect to their molecular weight. Various other authors have used different terms for the designation of this nucleoplasmic RNA species. Earlier it was called nuclear AU-rich RNA or dRNA (Georgiev and Mantieva, 1962) due to its DNA like base composition (Williams et al., 1968; Soeiro et al., 1966, 1968). This base composition, rapid synthesis, turnover and its nuclear location gave rise to the
concept that Hn RNA was the nuclear precursor to cytoplasmic mRNA.

1.3.3: PRECURSOR-PRODUCT RELATIONSHIP BETWEEN HnRNA and mRNA

Certain structural features common to both HnRNA and mRNA provide persuasive evidence in support of the biogenesis of mRNA from large hnRNA precursors. Such features include low abundance of internal methylated nucleotide sequences, adenylc acid polymers at the 3' ends, cap structures at 5' ends and various internal sequence homologies.

1.3.3.1: Polyadenylation of HnRNA and mRNA

The existence of polyadenylic acid in eukaryotic RNA was first demonstrated by Hadjivasilov and Brawerman (1966). It has since, become established that the poly(A) is associated with mRNA (Adesnik et al., 1972; Sheldon, 1972) and hnRNA (Adesnik et al., 1972; Sheiness & Darnell, 1973). Experimental evidence further shows that the poly(A) exists on 3'-OH ends of both RNA species (Molloy et al., 1972; Sheldon et al., 1972) and that it arises by a post-transcriptional addition to existing hnRNA (Perry et al., 1974; Brawerman, 1974; Knochel et al., 1978). Inhibitors such as cordycepin, which do not effect the synthesis of hnRNA, block poly-adenylation of hnRNA and subsequently prevent the appearance of mRNA in the cytoplasm (Darnell et al., 1971; Penman et al., 1970). These observations suggest that poly-adenylation may represent a part of nuclear RNA modifications and processing although nuclear poly-adenylation may not represent an absolute pre-requisite for
mRNA transport to the cytoplasm (Brawerman, 1976; Herman et al., 1976). Poly-adenylation is not, however, exclusively a nuclear event as poly-adenylation of pre-existing cytoplasmic mRNA molecules has also been demonstrated (Brawerman and Diex, 1975; Slater and Slater, 1974).

Size measurements based on mobility relative to that of polynucleotides of known length have yielded values as high as 300 nucleotides for the nuclear poly(A) of mammalian cells (Sheiness et al., 1975). A value of about 200 nucleotides was observed by comparing the yields of adenosine and AMP after alkaline hydrolysis (Mendki et al., 1972). However, the latter estimates did not take into consideration the turnover that occurs at the end of poly(A) sequences. When corrections were made to account for this turnover, a minimum value of 260 nucleotides was obtained for the average length of poly(A) in the nucleus (Brawerman & Diex, 1975).

The newly synthesized poly(A) in the cytoplasm is less homogeneous than its nuclear counterpart, but a small reduction in size is apparent after labelling periods as short as 5 mins. The steady-state of poly(A) has been studied by using cells labelled for extended periods (Shieness et al., 1975; Shieness & Darnell, 1973; Greenberg & Perry, 1972) or by assaying for the unlabelled poly(A) by annealing with radioactive poly(U) (Shieness et al., 1975). Both approaches have shown that cytoplasmic poly(A) is highly heterogeneous in size, most of the segments being considerably shorter than those of the nucleus.
It has been suggested that not all the eukaryotic mRNAs are poly-adenylated. The histone mRNA lacks poly(A) (Adesnik & Darnell, 1972; Greenberg & Perry, 1972). Similarly some of the polysomal mRNA also lacks poly(A) stretches, for example 30% of mRNA in HeLa cells, mouse L-cells (Milcarek et al., 1974; Greenberg, 1976) and mouse brain (VanNess et al., 1979).

Recently, however, it has been demonstrated that histone H5 mRNA is poly-adenylated (Molgaard et al., 1980). Similarly, it has been shown that in amphibian oocytes most of the histone mRNA is poly-adenylated (Ruderman & Pardue, 1977, 1978) and the poly-adenylate is probably removed during the maturation of the oocyte (Ruderman et al., 1979).

While the evidence available at present does not conclusively demonstrate a specific role for poly(A), experiments have indicated that de-adenylated poly(A) mRNA is inefficient in protein synthesis by cell-free systems and in frog oocytes (Williamson et al., 1974; Sippel et al., 1974; Doel and Carey, 1976), thus implying greater stability for poly(A) containing mRNAs. However, not all experimental evidence supports a role for poly(A) in mRNA stability. The non-poly-adenylated histone mRNA is very stable in the cytoplasm (Perry & Kelly, 1973) and a short lived class of poly-adenylated mRNA has been detected in HeLa cells (Pucket et al., 1975; Darnell et al., 1976).

Recently it has been proposed that the poly(A) facilitates the splicing event by promoting triple-stranded structures within the mRNA precursors (Bina et al., 1980).
1.3.3.2: Methylation of HnRNA and mRNA

Methylation of ribonucleotides was initially observed in rRNA, tRNA and their precursors (Maden & Salim, 1974; Burdon, 1975; Perry, 1976). Perry & Kelly (1974), however, demonstrated methylated mRNAs in mouse L-cells, and Desrosiers et al., (1974) reported similar structures in Novikoff hepatoma cells. At the same time methylation of viral mRNA was also reported (Shatkin, 1974, Wei & Moss, 1974) and it was noted that a large proportion of the methyl-nucleotides were present in these RNA molecules as an alkali-stable oligonucleotide at the 5' terminus of the message. Based on the data obtained from several groups, a general structure for this nucleotide was proposed in which a 7-methylguanosine residue was joined by 5'-5' pyrophosphate linkage to a 2'-O-methylnucleoside (Rotman et al., 1974). The 5' terminal "Cap" can be either m^7GpppNm\* (cap I) or m^7GpppNm-N"m-N" (cap II), containing one and two 2'-O-methylnucleosides, respectively (Adam and Cory, 1974: Desrosiers et al., 1975)

Additional methylation of cellular mRNA molecules occur internally, between 5'-cap and 3'-poly(A) segment, yielding 6-methyladenosine (m^6A) (Desrosiers et al., 1974, Wei et al., 1976). Studies on hnRNA indicate that these molecules also contain 5'-caps and internal m^6A (Perry et al., 1975; Salditt-Georgieff et al., 1976).

Furuichi (1978) has suggested that the formation of cytoplasmic polyhedrovirus mRNA requires capping as the first step in RNA synthesis. The result obtained by Salditt-Georgieff et al., (1980) suggests that the RNA polymerase II
may initiate chains most frequently but not always with purines, and that the caps are added very early in the transcription.

Recently Gedamu et al., (1981) have indicated the possibility of heterogeneity in the capping of protamine mRNA from trout testis. They have suggested that mRNA coding for protamine components CI and CIII have a cap I structure while mRNAs coding for CII have no cap and are unmethylated. This indicates that cap structures are not always required for the processing or translation of mRNA.

It has also been observed that capped oligonucleotides in ovalbumin mRNA have varying abundances. Their structure and relative abundances are $m^7$GpppAUACAG, 3%; $m^7$GpppACAUACAG, 61%; and $m^7$GpppGUACAUACAG, 36% (Malek et al., 1981). These differences could be associated with differences in the relative abundances of mRNA and also in the different rates of translation.

1.3.3.3 Transcribed Homologous Oligonucleotides in HnRNA and mRNA

Besides the poly(A) sequences in hnRNA and mRNA, additional homologies were revealed when two shorter internally located sequences were found within the RNA of a number of cultured cells. These comprise a stretch of oligo adenylic acid of about 25 AMPS (Edmonds et al., 1976) which are transcribed from DNA (Jacobson et al., 1974) and a stretch of about 30 uridylate nucleotides (Burdon & Shenkin, 1972) transcribed from 2-3 repetitive DNA sequences (Molloy et al., 1972, 1974).
Oligo(U) sequences in mRNA are found in both polyadenylated and non-polyadenylated species (Korwek et al., 1976) and the non-polyadenylated Oligo(U) rich mRNA seems to represent a separate class of functionally active mRNA (Korwek et al., 1976; Edmonds et al., 1976). The function of Oligo(U) in hnRNA is not clear but they may serve as cleavage sites for the production of mRNA species (Molloy et al., 1974).

1.3.3.4 Demonstration of mRNA sequences in HnRNA

A sensitive technique employed to demonstrate the precursor-product relationship between hnRNA and mRNA is molecular hybridization.

In most experiments, this involves the synthesis of single stranded, complementary DNA (cDNA) using mRNA as a template (Kacian et al., 1972; Ross et al., 1972; Verma, 1977) and its hybridization to hnRNA.

Melli and Pamberton, (1972) first demonstrated the presence of globin mRNA sequences in hnRNA and Bostas and Aviv (1977) demonstrated pre-mRNA species which sedimented at 28S, 15S and 10S. Furthermore, although they were not employing fully denaturing conditions, they presented clear evidence that there was a precursor-product relationship between these species such that the 28S gave rise to the 15S precursor which in turn matured into a 10S species. These results have been confirmed and extended in a number of different species (Strair et al., 1977; Kwan et al., 1977) using pulse chase experiments (Strair et al., 1977; Crawford & Wells, 1978) and R loop analysis.
(Jefferys & Flavell, 1977) and sequence analysis.

It has now become clear that the initial transcripts of genes include transcripts of the non-coding introns and that these are edited out during the maturation of mRNA. Thus the 15S β globin pre-mRNA contains a transcript of one of the two globin introns which is spliced out during the maturation to the 10S mRNA (Jefferys & Flavell, 1977). Similarly, the initial product of the ovalbumin gene contains transcripts of all seven introns which are spliced out in a preferred though not obligatory order (Tsai et al., 1980). This processing of precursor mRNA species has now been described for a number of other gene products including chicken conalbumin (Cochet et al., 1979), mouse, rabbit and human globin (Van Ooyen et al., 1979), Xenopus vitellogenin (Wahli et al., 1980), rat insulin Lomedico et al., 1979), Adenovirus 2 genes (Maat & Van Ormondt, 1979).

cDNA copies of entire populations of mRNA have also been employed to study mRNA abundance in hnRNA. Herman et al., (1976) found sequence homology between hnRNA and mRNA of HeLa cells and indicated the presence of mRNA sequences in large polyadenylated hnRNA. Similar results have also been obtained by a number of other investigators, from a variety of tissues, for example Aziz et al., (1979) have shown the large hnRNA, in immature and mature rat uterus, is precursor of polysomal poly-adenylated mRNA. Similarly, large precursor molecules of mRNA have been reported in guinea-pig mammary gland (Bathurst et al., 1980), mouse brain (Hahn et al., 1978), sea urchin embryo (Wold et al., 1978), normal and neoplastically transformed cells (Moyzis et al., 1980), Friend cells
(Balmain et al., 1980), chick oviduct (Nordstrom et al., 1979; Tsai et al., 1980), Xenopus liver (Ryffel et al., 1980; Jost et al., 1978), rat liver (Wilkes et al., 1979; Scholla et al., 1980) and in Petroselium sativum (parsley) (Kiper et al., 1979). All these results strongly suggest that the first transcript of the DNA is a large precursor, which is then processed to form mature mRNA. However, the demonstration that hnRNA includes mRNA sequences does not prove that all hnRNA is pre-mRNA. The statement that "some hnRNA possesses other roles" cannot at present be excluded. Indeed, some repeat sequences which are known to occur between genes (Coggins et al., 1980) are also known to be transcribed into nuclear DNA still interspersed between gene transcripts (Costrantini et al., 1980). The very existence of hnRNA, together with that of the nuclear membrane and hnRNA particle proteins has suggested to many that eukaryote cells may use the maturation of nuclear precursor molecules as a means to control gene expression.

1.3.3.5 The Intron-Exon Boundary and its Role in pre-mRNA Maturation

The mechanism by which the intron transcripts are spliced out of mRNA precursors is the subject of intense speculation. Among the most favoured schemes is one put forward by Steitz and her colleagues (Lerner et al., 1980). This proposal rests on two main findings. Firstly, the nucleotide sequence of the intron-exon boundaries show considerable homology both within a given message and between different mRNA species (Benoist et al., 1980; Lerner et al., 1980; Sharp, 1981).
The homology is such that the following "Consensus sequence" can be derived, from which, the splice points of any known intron differs by not more than a few nucleotides.

\[ 5'---\text{AGGUAGU}---------\text{UYUYUXCAGG}----3' \]

splice point of $5'$ consensus sequence

splice point of $3'$ consensus sequence.

$Y = \text{pyrimidine} \;; \; X = \text{variable}$.

The second finding that Steitz and co-workers (Lerner et al., 1980), and a number of other groups, have drawn attention to is the fact that the small nuclear RNA known as U1 has a nucleotide sequence at its 5' end which is capable of extensive base pairing with 5' and 3' consensus sequences in succession. Thus, it is suggested that U1 RNA binds to both splice points drawing together two adjacent exons and looping out the intervening sequence (Lerner et al., 1980). In this form the intron transcript could easily be removed by ribonuclease and the ends of the two exons annealed with a ligase. Both U1 RNA and hnRNA (pre-mRNA) are normally found complexed in ribonucleoproteins. The protein components, which include ribonuclease activities, are assumed to assist in the specificity of base pairing and splicing (Lerner et al., 1980).

A number of authors have suggested that the extra sequences in eukaryotes are an evolutionary remnant that prokaryotes have been able to discard because of their rapid evolution (Doolittle 1978; Darnell, 1978). That this might be occurring, though at a slow rate, in eukaryotes is supported by the finding that the simple, rapidly dividing eukaryote, yeast, tends to have
smaller intervening sequences than higher cells. There is some evidence that introns can be lost during the evolution of higher eukaryotes. Thus, all known insulin genes have two introns except one of the two rat insulin genes which has only one (Lomedico et al., 1979). Sequence homology studies indicate that the two rat genes have resulted from a gene duplication and that the inference is, therefore, that one intron was lost from one copy during the duplication event (Lomedico et al., 1979).

An extension of these ideas, that introns represent an early stage of evolution, is that split genes result from a type of recombination event whereby the various exons of a gene have been recruited into the same transcriptional unit. The evolutionary advantage of this concept is that, a particular DNA sequence coding, for example, for a protein domain able to find a particular coenzyme, could become a part of many genes. Each exon might thus be expected to correspond to a functional domain in the protein product. Craik et al., (1980) lent support to this theory by demonstrating that the product of the central exon of the globin gene is a complete functional domain with a specific binding site for haem. Such an exon could conceivably have its evolutionary origins in a primative haem binding protein. Stein et al., (1980) have recently shown that the intervening sequences of chicken ovomucoid gene separate coding portions which correspond to the functional domains of the protein. Similarly, Tonegawa and co-workers (Sakano et al., 1979) have demonstrated a functional relationship between the domains of immunoglobulin heavy chain and the exons of its gene. All these ideas imply that introns are functionless
sequences and the fact that two introns - and one intron - genes for rat insulin are both equally well expressed suggests that the larger intervening sequence can be lost with no ill effects. On the other hand, a number of recent studies show that introns may after all have a structural function. Hamer & Leder (1979) have shown that absence of splice junctions results in the loss of transcription. Similarly, results presented by Grass & Khoury (1980) suggest that introns are necessary for the maturation of transcripts into stable mRNA.

1.3.4: HETEROGENEOUS RIBONUCLEOPROTEIN PARTICLES

From a very early stage in its transcription, hnRNA exists as ribonucleoprotein extractable as ribonucleoprotein particles (hnRNP). Miller & Hamkalo (1972) have shown that protein associates with hnRNA while it is still being transcribed, and free hnRNA has not been detected in the cell. Characteristically, the particles consist of approximately 20% RNA and 80% protein and can be isolated as polymers arranged like beads on a string of hnRNA.

Studies on hnRNP have not led to a clear picture of the interrelationship of protein and RNA. The early model, proposed by Samarina et al., (1968), placed RNA around the particle in a manner analogous to the way in which DNA is thought to coil around the nucleosome. Other models place the RNA coiled within the protein of the particle and partially protected between the particles by other proteins (VanVenrooij and Janssen, 1978; Heinrich et al., 1978). There is no
conclusive evidence on the interrelationship of the molecular species within the particle. It is conceivable, for instance, that particles might initially form only on those portions of hnRNA which are to be conserved, leaving the transcripts of the introns without protection. However, the regular spacing of particles on hnRNA (Miller & Hamkalo, 1972) compared with the irregular spacing of introns argues against this possibility.

In order to study the role of hnRNP particles in mRNA maturation it is important to study first the protein species themselves. The dominant protein species are those known as core proteins; approximately four species of 38,000 - 45,000 molecular weight. It is assumed that these polypeptides are the structural proteins of the particle. They appear to be universally present and, in parallel with other nucleic acid binding proteins, i.e. histones and ribosomal proteins, they are basic (Suria & Liew, 1979) and have a low turnover rate (Martin & Billings, 1979).

LeStourgeon et al., (1977) have suggested that, because they have abundance of glycine, they would be expected to contain a high percentage of β-sheet in their structure, and might thus be suited for intercalating with double-stranded portions of RNA. Suria & Liew (1979) and Wilkes & Knowler (1981) have shown that the major proteins exhibit charge heterogeneity. Wilkes & Knowler (1981) have suggested that, at least in some cases, heterogeneity is the result of phosphorylation. Such modifications might permit variation in the role or strength of binding of the protein and the RNA in the same way that modification of histone or protamines
effects their association with DNA.

Several enzymic activities have been detected in hnRNP particles, for example, an Mn$^{2+}$ dependent poly(A)-polymerase (Niessing & Sekeris, 1973), "capping" enzymes (Bajszar et al., 1978), Ribonuclease (Niessing & Sekeris, 1970), Protein Kinase (Karnet et al., 1977; Wilks & Knowler, 1981) have been demonstrated in particles from rat liver, similarly double stranded RNA specific RNase (Molnar et al., 1978; Rech et al., 1979), Protein Kinase (Blanchard et al., 1977) and Phosphoprotein phosphatase (Periasamy, 1977) have been detected in particles from HeLa cells.

It is conceivable that some of these proteins could have a role in the control of messenger RNA maturation (Knowler & Wilks, 1980; Karn et al., 1977; Blanchard, 1977). Thus, Wilks & Knowler (1981) have shown that the activity of liver hnRNP kinase(s) is enhanced by cyclic AMP or polyamines. They have also reported that the extent of phosphorylation of hnRNP proteins is altered in adrenalectomized rat liver, but returns to normal after the administration of glucocorticoids (Knowler & Wilks, 1980). Such effects may be related to the known role of glucocorticoids in increasing the active amounts of mRNA coding for liver gluconeogenic enzymes, and in stimulating tissue hypertrophy.

1.3.5: SMALL NUCLEAR RIBONUCLEOPROTEIN PARTICLES (SnRNP particles)

Discreet, stable, small RNA molecules are found in the nuclei of cells (Weinberg & Penman, 1968) from a wide variety
of eukaryotic organisms (Hellung-Larsen & Frederiken, 1977). There are six major species designated U1 to U6, some of which have minor structural variants. Of these, U3 and its variants occur only in the nucleolus (Zieve & Penman, 1976). They range in size from about 90 to 220 nucleotides, have been well-characterized (Zieve & Penman, 1976; Jelink & Kinwand, 1978; Benecke & Penman, 1977) and some of them have been sequenced (Shibata et al., 1975; Lerner & Steitz, 1979).

At their 5'-end they have a "cap like" structure, which is different from that of mRNA. It consists of the first two 5' nucleotides which are transposed N²,N²-dimethyl-7-methyl guanosine followed by 2'-O-methyladenosine (Cory & Adams, 1975).

The most abundant snRNA species exist as a closely related set of RNA-protein complexes called small nuclear ribonucleoproteins (SnRNP) (Lerner & Steit, 1979). Sekeris & Niessing (1975) included them in a model of hnRNP particle structure.

Lerner & Steitz, (1979) have shown that some patients with systemic lupus erythematosus, an autoimmune disease, produce antibodies which specifically complex with SnRNP particles. These particles contain seven polypeptides and the five nucleoplasmic snRNAs. The polypeptides have molecular weight ranging from 12000 - 32000 and are unrelated to either hnRNP core proteins or histones.

The possible role of snRNA, particularly the species U1, in the maturation of mRNA has already been discussed in section 1.3.3.5. Attention has also been drawn to the possible role of snRNP proteins in this maturation process. Lerner
et al., (1980) showed that in metabolically active cells where snRNA is most abundant, snRNP sediments with hnRNA at 30S. When, however, the particles contained U1 RNA from which the 5'-end was missing, annealing to the consensus sequence of exon-intron boundaries could not occur and the particle sedimented at 10S. Lerner et al., (1980) suggest that the splicing enzyme might be the ribonucleoprotein complex of snRNP.

1.4: SEQUENCE COMPLEXITY AND DIVERSITY OF mRNA AND hnRNA

Britten and Kohn (1968) Britten and Davidson (1969,1971) observed that the DNA of eukaryotic genome consisted of 10-20% repetitive and unique sequences. The unique sequences contain most of the structural genes which give rise to sequences represented in hnRNA and mRNA. At any one time during cellular differentiation, development or cell proliferation, a small fraction of the genomic unique sequences is transcribed as mRNA (Davidson & Britten, 1973; Lewin, 1975c). Thus the cytoplasmic mRNA population will include transcripts of diverse genes in the form of various mRNA species. Hence sequence complexity can be defined as the molecular weight of unique DNA transcribed to give rise to the RNA population.

A number of experimental approaches have been devised to measure the number of different mRNA sequences and total complexities in an RNA population. The number and distribution of mRNA sequences can be determined from the kinetics of hybridization of complementary DNA to poly-adenylated RNA (Bishop et al., 1974). Another method is saturation hybrid-
ization in which trace amounts of radioactive single-copy DNA are annealed to saturation with excess unlabelled RNA. The amount of DNA driven into RNA-DNA hybrid gives a direct measure of the percentage of the unique sequence DNA transcribed. Although some reports show similar measurement of complexity using both techniques (Axel et al., 1976; Hereford & Rosbash, 1977) and others find a wide disparity in values for the same tissue (Aziz et al., 1979; Wilkes et al., 1979). In general, cDNA measurement tends to underestimate complexity because of difficulty in estimating the kinetic transition of the high complexity, low abundance class of mRNA. In addition, cDNA hybridization is usually limited to poly(A)$^+$RNA sequences so that complexity contained in non-polyadenylated RNAs is overlooked.

Complexity and diversity of mRNA and hnRNA, by both of the above methods, have been determined from a variety of animal and plant tissues and has been summarized in Table 1. It is noteworthy, however that these measurements represent the complexity of poly-adenylated species and hence do not reflect the true complexity of total cellular mRNA.

The complexity of non-polyadenylated mRNA has also been determined. Grady et al., (1978) have shown about 8000 different non-polyadenylated mRNA species in mouse liver and 12000 different polyadenylated mRNA sequences, thus giving a total of 20,000 diverse mRNAs. In a similar study Chikaraishi (1979) and VanNess et al., (1979) using highly labelled mouse brain unique DNA sequences have shown that polysomal poly(A)$^-$ and poly(A)$^+$ mRNA from mouse brain contain a non-overlapping
<table>
<thead>
<tr>
<th>Cell/ Tissue</th>
<th>Source of mRNA or hnRNA</th>
<th>Total Base sequence complexity mRNA-cDNA</th>
<th>mRNA-reaction Unique DNA reaction</th>
<th>Total Nos of mRNA/hnRNA mRNA-cDNA mRNA-reaction Unique DNA reaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Total poly(A)^+RNA</td>
<td>1.8x10^9 daltons</td>
<td>3,000</td>
<td>4,000</td>
<td>Hereford &amp; Rosbash, 1977</td>
</tr>
<tr>
<td><em>N. crassa</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td>2.4x10^6 daltons</td>
<td>1,869</td>
<td></td>
<td>Wong &amp; Marzluf, 1980</td>
</tr>
<tr>
<td></td>
<td>Nuclear poly(A)^+RNA</td>
<td>3.1x10^6</td>
<td>2,356</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. sativum</em></td>
<td>root cells polosomal poly(A)^+RNA</td>
<td>1.9x10^7</td>
<td>13,700</td>
<td>10,000</td>
<td>Kiper et al., 1979</td>
</tr>
<tr>
<td></td>
<td>leaf cells &quot; &quot;</td>
<td>1.9x10^7 nucleotides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chicken oviduct</em></td>
<td>Cytoplasmic poly(A)^+RNA</td>
<td>14,000</td>
<td>15,700</td>
<td></td>
<td>Davidson et al., 1973</td>
</tr>
<tr>
<td><em>Lacting Guinea-pig</em></td>
<td>Cytoplasmic poly(A)^+RNA</td>
<td>4.5x10^6 daltons</td>
<td>3,250</td>
<td></td>
<td>Bathursh et al., 1980</td>
</tr>
<tr>
<td><em>Rat uterus</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td>4.7x10^9 daltons</td>
<td>7.56x10^9 daltons</td>
<td>8,000</td>
<td>Aziz et al., 1979</td>
</tr>
<tr>
<td>Immature (4hr treated)</td>
<td>Polysomal poly(A)^+RNA</td>
<td>3.2x10^10 daltons</td>
<td>3.53x10^10 daltons</td>
<td>36,000</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td><em>Mouse liver</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td>8.4x10^9 daltons</td>
<td>9.1x10^10 daltons</td>
<td>8,000</td>
<td>Grady et al., 1978</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td></td>
<td></td>
<td>12,000</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td><em>Rat liver</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td></td>
<td></td>
<td>23,000</td>
<td>Savage et al., 1978</td>
</tr>
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<td></td>
<td>Polysomal poly(A)^+RNA</td>
<td></td>
<td></td>
<td>9,200</td>
<td>Sippel et al., 1977</td>
</tr>
<tr>
<td></td>
<td>Polysomal poly(A)^+RNA</td>
<td></td>
<td></td>
<td>50,000</td>
<td>Wilkes et al., 1979</td>
</tr>
<tr>
<td></td>
<td>Polysomal poly(A)^+RNA</td>
<td></td>
<td></td>
<td>15,000</td>
<td>Jacobs &amp; Birnie 1980</td>
</tr>
<tr>
<td></td>
<td>Polysomal poly(A)^+RNA</td>
<td></td>
<td></td>
<td>26,000</td>
<td>Towle et al., 1979</td>
</tr>
<tr>
<td><em>Euthroid rat liver</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td>1.7x10^7 daltons</td>
<td>11,400</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hypothyroid rat liver</em></td>
<td>Polysomal poly(A)^+RNA</td>
<td>1.4x10^7 daltons</td>
<td>9,900</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
set of sequences, with poly(A)$^-$mRNA representing about 50% of the mRNA sequence complexity. If the results of Nemer et al., (1974), Galau et al., (1974a, 1974b) and McColl & Aronson, (1978) are combined, it would appear that about 90% of the sea urchin mRNA sequences are non-polyadenylated. However, it is important to note that this situation does not hold true in every cell type examined so far. In the cells of plant *Petroselinum sativum* (Kiper et al., 1979) and in yeast (*Saccharomyces* (Hereford & Rosbash, 1977) polysomal poly(A)$^+$RNA and poly(A)$^-$RNA appears to share the same sequences.

Several workers have observed that the complexity of nuclear RNA is at least 4-10 fold greater than that of cytoplasmic RNA, and complementary to 1-4% of unique DNA (Getz et al., 1975; Kleiman et al., 1977; Chikaraishi et al., 1978; Aziz et al., 1979). The less complex eukaryotes however, for example, protista and fungi, do not have a separate class of hnRNA molecules (Hereford & Rosbash, 1977; Rozek et al., 1978). This is demonstrated by the similarities in the sequence complexities of their polysomal, nuclear and total cellular RNA. This implies that the probable function of hnRNA in the biogenesis of mRNA is unique to higher eukaryotes.
1.5: GLUCOCORTICOIDS

A study of the changes in mRNA complexity and translation is obviously an important approach to the study of differentiation, growth and the development of a tissue.

One system which lends itself to such a study, and which is the subject of the work in this thesis, is the glucocorticoid-induced hypertrophy in rat liver.

Glucocorticoids are secreted by the cells of the inner layer of the adrenal cortex in response to environmental stress. A chain reaction is set off by unique signals in the environment. The limbic system of the brain appears to be the initial signal bearing mechanism. The hippocampus may be the most important structure in this system which carries the message, probably by the changes in electrical transmission, to the hypothalamus. In response, the hypothalamus secretes the corticotrophic releasing factor (CRF) into a closed portal system (Mezey & deWied, 1979) connecting the hypothalamus with the anterior pituitary. The corticotrophic cells of anterior pituitary have specific membrane receptors for CRF (Martini et al., 1970). Binding probably results in an elevation of intracellular cAMP levels (Hutson et al., 1976; Kneer et al., 1974), culminating in the release of preformed ACTH into the blood stream.

Studies initiated two decades ago by Haynes et al., (1960) implicated cAMP as an intracellular mediator in the action of ACTH on the adrenal cortex. Grahame-Smith et al., (1967) reported that ACTH enhanced adenylate cyclase activity in adrenal homogenates and it was subsequently established that adrenal
adenylcyclase is selectively stimulated by ACTH (Touton et al., 1969; Kelly & Koritz, 1971).

Recently, this mechanism of action of ACTH has been queried. Studies using perfused isolated cat adrenals, showed that the increase in the tissue cAMP alone was not sufficient to elicit steroid release (Carchman et al., 1971). Hudson & McMartin (1975) investigated the kinetics of steroidogenesis and cAMP accumulation in response to ACTH, and concluded that their findings did not support an obligatory role for cAMP as a mediator of ACTH action on the adrenal. A similar conclusion has also been reached by others (Moyle et al., 1973; Ramachandran & Moyle, 1977).

More recent studies, using $^{125}$I-labelled ACTH, have provided evidence for the existence of two populations of adrenal ACTH receptors; occupation of one receptor appeared to be related to cAMP production, and occupancy of the other was related to steroidogenesis (McIlhinney & Schulster, 1975). The presence of two mechanisms for ACTH activation, only one of which involves the necessary production of cAMP has also been proposed by Bristow et al., (1980).

The terminal hormone, glucocorticoids, feeds back negatively on corticotrophic cells of the limbic system to terminate the release of hormones and altered electrical stimulation which was set off by the stress signal (Knobil & Sawyer, 1974).

1.5.1: MECHANISM OF ACTION OF GLUCOCORTICOIDs

The study of the mechanism of action of steroid hormones at the molecular level has attracted many investigators and
extensive data is now available about the many aspects of glucocorticoid hormone action. However, the fundamental mechanisms through which these hormones act remains obscure. It is thought that most of the inhibitory and stimulatory actions of the glucocorticoid hormones are initiated by the binding of the steroid to specific glucocorticoid receptors.

1.5.1.1: Glucocorticoid Receptors and Other Binding Proteins

The first indication that macromolecular binding of the adrenal glucocorticoid hormone occurred in a prime target tissue was given by Litwack et al., (1965). Macromolecular binding of radioactive material derived from the labelled hormone was observed as quickly as the measurement could be made after injection. Ultimately this approach led to the characterization of five new cytoplasmic proteins. Some of these proteins proved to be cortico-steroid metabolite-binding proteins of importance in context other than hormone action (Litwack et al., 1971; Steeger & Litwack, 1978). Among the proteins which bind unmetabolized glucocorticoids, the major one of interest is designated as the glucocorticoid receptor (Litwack et al., 1971). Its identity was based upon the similarity with the nuclear form (Koblinsky et al., 1972; Litwack et al., 1973). In addition to these proteins in target cells, cytosol a serum globulin (CGB) had previously been described which is synthesized and exported from hepatocytes (Westphal, 1971).

Receptors for steroid hormones have been studied in many tissues and organisms. Perhaps the best studied, from a structural and functional standpoint, has been the chicken
oviduct progesterone receptor. This receptor has been purified to apparent homogeneity and found to consist of two non-identical progesterone-binding components. One, progestophilin A, has a molecular weight of 79,000 (Coty et al., 1979) and binds avidly to DNA (Schrader et al., 1972). The other, progestophilin B, has a molecular weight of 115,000 (Schrader et al., 1977) and binds weakly to DNA but strongly to chromatin from target cells (Schrader et al., 1972). Isolation of high molecular weight aggregates of these proteins (Schrader et al., 1975), crosslinking studies (Birnbaumer et al., 1979) and functional tests of chromatin transcription (Buller et al., 1976) have suggested that A and B are subunits of a larger AB complex. Recently, Schrader et al., (1980) have isolated rat liver glucocorticoid receptor. The purification procedure used by them was the same as that for progesterone receptor.

The functional form of the receptor is envisioned to be a dimer of two dissimilar steroid-binding subunits. The sedimentation coefficient of the dimer is about 6S, whereas the subunits are about 4S each. The subunit has a molecular weight of 70,000 (Schrader et al., 1980)

The dimer has nuclear binding activity following association with its ligand, thus causing a net accumulation of the complexes in the nucleus (Buller et al., 1975). As with other steroid hormone-receptor complexes, this nuclear accumulation is thought to take place after "activation" (Higgins et al., 1973). The activated complex binds to regions of chromatin defined by the presence of a subclass of non-histone chromosomal proteins (Spelsberg et al., 1977), an interaction which is presumed,
by mechanisms that are currently unknown, to modulate levels of specific mRNAs (Schultz et al., 1973; Ringold et al., 1975; Martial et al., 1977a; Nakanishi et al., 1977). The translation products of these mRNAs then mediate the glucocorticoid response.

It is likely that the diversity of glucocorticoid responses elicited in various cell types result from differences in cellular elements distal to the receptor. This notion derives from currently available data that the receptors for these hormones are identical in the various glucocorticoid target tissues. For instance, somatic cell fusion experiments show that the capability of receptors from a responsive cell to elicit a response is determined by the tissue source of the nuclei (Gehring & Thompson, 1979). In addition, no tissue-specific differences have been observed in the receptor's binding affinity of various glucocorticoid analogs (Rousseau & Baxter, 1979) or in the physiochemical characteristics of the receptor (Litwack & Singer, 1972). Thus nuclear elements dictated by the differentiation of a cell apparently determine the selection of genes to be influenced by the receptors once they bind to the chromatin.

1.5.2: ACTION OF GLUCOCORTICOIDS IN NUCLEUS

1.5.2.1: Nuclear Acceptor Sites for the Binding of the Receptor-Glucocorticoid Complexes

The most popular model to explain steroid hormone action assumes that they regulate the transcription of specific mRNAs by binding specifically at a limited number of sites on target cell chromatin. The specific localization of the
limited number of sites on target cell chromatin. The specific localization of the glucocorticoid-receptor complex would result either from specific DNA sequences that bind the complexes, or from chromatin proteins located at specific sites that bind or influence binding of receptor-steroid complexes. Such a model is an attractive analogy to the well-studied examples of prokaryotic gene regulation. It has also been assumed that such "relevant operator sites" for receptor action may be undetectable owing to an enormous background of non-specific binding of the receptor in nuclei (Yamamoto & Alberts, 1975). Baxter (1976) has shown that, over a wide concentration range, a constant proportion (about 50% of the total) of receptor-glucocorticoid complexes is found in a bound form in the nucleus. Many of these sites may be considered "non-specific" and it has been assumed that the "specific operators for steroid action" would be saturated at lower concentrations of complexes but would be a small proportion of the total binding (Yamamoto and Alberts, 1975).

The chemical nature of the nuclear acceptor sites that bind receptor-steroid complexes has been studied by numerous investigators, and the general conclusion is that DNA is in some manner involved in the nuclear localization of receptor. The evidence for this includes the following: (1) Nuclei predigested with DNase I lose all capacity to bind hormone-bound receptors (Baxter et al., 1972). (2) Glucocorticoid-receptor complexes bind to DNA under cell-free conditions (Rousseau et al., 1975) (3) Receptors from a glucocorticoid-resistant, S49 lymphoma cell mutant, show a lower affinity for DNA,
compared to S49 N⁺ (nuclear transfer increase) receptors which grow a higher affinity for binding to DNA - cellulose in vitro (Yamamoto et al., 1974). (4) "Activation" of the receptor-glucocorticoid complex by salt or heat treatment enhances binding of the complex to isolated DNA or Nuclei (Rousseau et al., 1975). Receptors do not bind to RNA although they fail to distinguish between DNA isolated from a variety of eukaryotes, prokaryotes and bacteriophages (Rousseau et al., 1975).

Nuclear binding of receptors could also be influenced by specific nuclear proteins as suggested by work in systems responsive to sex-steroids (Spelsberg et al., 1971; Mainwaring et al., 1976).

The "acceptor" activity was first linked to a non-histone protein in prostate nuclear chromatin (Tymoczko and Liao, 1971). In the presence of purified DNA, the acceptor protein can form a nucleoprotein complex that can specifically bind the prostate DHT-receptor complex. Many properties of the acceptor activity of the nuclear proteins, in liver, are very similar to those of prostate nuclei. But the liver nuclear protein preparations appear to contain a much smaller amount of acceptor-protein-like material (Tymoczko & Liao, 1971).

It has been suggested that a steroid-binding protein (α-protein) in the cytosol fraction can inhibit the DHT-receptor complex from binding to the nuclear chromatin (Fang & Liao, 1971). Such a protein may also play a key role in the regulation of receptor binding to the chromatin, and has been reported in the cytosol of rat uterus (Chamness et al.,
1974), chick oviduct (Buller et al., 1975), rat liver (Milgrom & Atger, 1975), and rat hepatoma cells (Simons et al., 1976).

1.5.3: SPECIFICITY OF GLUCOCORTICOID RESPONSES

Proteins synthesized under the influence of glucocorticoids are usually specific to given target cells and encompass a small subset of the expressed genes. It has been observed that in rat pituitary tumour cells (Martial et al., 1977b) and in rat hepatoma cells (HTC) (Ivarie & O'Farrel, 1978), the domain of the glucocorticoid response encompass less than 1% of the detectable gene products.

The specificity of glucocorticoid response has recently been examined at the level of mRNA in a line of cultured pituitary cells, where the synthesis of growth hormone and its mRNA are stimulated by glucocorticoids. Martial et al., (1977b) have shown that in this cell line the effect of glucocorticoids is only on the synthesis of pre-growth hormone, while the synthesis of other cellular protein is unaffected. Furthermore, no mRNA species was induced that constituted a large proportion of the total cellular mRNA. Thus glucocorticoids only increase certain mRNAs and models for receptor function must ultimately account for such selectivity.

Furthermore, glucocorticoids are generally not the primary inducers of the gene that they influence. Although one exception to this general rule may be the induction of mouse mammary tumor virus mRNA (Stallcup et al., 1978). The evidence to date indicates that in most systems glucocorticoids
modulate the levels of specific mRNAs, which are also synthesized to a different extent in their absence. This contrasts with the sex steroids, which induce the synthesis of proteins which were either not made or made in minute quantities in their absence. Furthermore, sex steroids even bring about the differentiation of the protein producing cells. Thus oestrogen in young chicks causes the precocious development of the gland cells of the magnum region of the oviduct and the production by these cells of egg white proteins.

Glucocorticoid hormones exert varied and often contrasting effects on cellular metabolism and on the regulation of specific gene products. For example, in muscle, skin, adipose, lymphoid and connective tissue, glucocorticoids produce inhibitory influences on protein, fat, nucleic acid and carbohydrate metabolism (Cahill, 1971; Fain & Czech, 1975; Fauci & Dale, 1974). In liver the response is characteristically stimulatory and specific enzymes involved in gluconeogenesis, particularly those involved in the gluconeogenic breakdown of amino acid are synthesized. Such enzymes include Tyrosine amino transferase (Nickol et al., 1978), alanine amino transferase (Webe, 1963; Swaraj et al., 1974) tryptophan pyrolase (Beverly & Tomkins, 1968), glucose-6-phosphatase (Greengard & Dewey, 1970), pyruvate carboxylase (Rosen et al., 1959), glycogen synthetase (LeLoir & Goldberg, 1960). The overall effect of the hormone is thus an inhibition of reincorporation of amino acids released by protein breakdown and an advanced degradation of amino acids to the gluconeogenic precursors. All these result in elevated liver glycogen and blood glucose and are particularly important to animals suffering prolonged fasting.
In addition to the above effects, the liver responding to glucocorticoids exhibits profound hypertrophy. All classes of RNA are synthesized in increased quantities. Ribosomal RNA is stimulated 3 fold (Feigelson & Feigelson, 1963) and is accompanied by increased production of tRNA (Turkington, 1969), polysome formation and a stimulated rate of protein synthesis (Schimke & Doyle, 1970). In other cells such as fibroblasts, both stimulatory and inhibitory effects on DNA synthesis have been reported (Pratt & Aronow, 1966; Thrash & Cunningham, 1973).

Such bidirectional glucocorticoid responses are by no means limited to general effects on cellular metabolism. For example, in the cultured rat pituitary cell line GH3, two major gene products, growth hormone and prolactin are oppositely regulated by glucocorticoids. Growth hormone synthesis is stimulated, while prolactin production is inhibited (Dannies & Tashjian, 1973). In HTC cells, there are proteins whose rates of synthesis are inconsistently repressed by the steroid and others whose synthesis is always stimulated (Ivie & O'Farrel, 1978). Thus, the reported biological responses of glucocorticoid target cells range from effects on general cellular metabolism and have morphological influences on specific gene products; these may be stimulatory or inhibitory.
1.5.4: REGULATION OF THE CELLULAR RESPONSES TO GLUCOCORTICOIDs

It has been observed that cellular environment plays a major role in modulating the programme of hormone responses in a target cell.

In primary cultures of hepatocytes, glucocorticoids are unable to induce the synthesis of tyrosine amino transferase mRNA unless both glucagon and cyclic AMP are present in the culture media (Ernest & Feigelson, 1979). Gospodarowicz & Moran (1974) have shown that in the 3T3 fibroblast cells, glucocorticoids were unable to induce thymidine incorporation in the absence of fibroblast (FGF) and epidermal (EGF) growth factors.

The cellular milieu not only modifies the magnitude of the response to glucocorticoids but, in fact, determines whether the response is stimulatory or inhibitory. Johnson et al., (1979) demonstrated that, in fibroblasts, the nature of glucocorticoid effect on thymidine incorporation could be profoundly modified when the metabolic status of the target cell was altered, in this case by media depletion or by addition of growth factors.

The effect of media conditions on the killing of lymphoid cells by glucocorticoids has also been examined. Nicholson & Young (1978) have shown that a receptor-containing strain of cells that is glucocorticoid resistant in vivo becomes susceptible to the nucleolytic effects of glucocorticoids when incubated in vitro. They have suggested that hormone
resistance in vivo is the result of the development of more resilient nuclear membranes, which are overcome in vitro by the stress of incubation. Thus, the sensitivity of lymphoid cells to the "killing" actions of glucocorticoids can also be significantly influenced by the cellular environment. Johnson et al., (1980) have also shown that media conditions can profoundly effect the induction of a specific gene product by glucocorticoids.

1.6 : LIVER REGENERATION

The regeneration of mammalian liver following partial hepatectomy is characterized by marked morphological and metabolic changes, which have been considered to be indicative of changes in gene expression (Bucher & Malt, 1971). Many capacities of resting liver are working at levels below their maxima. Protein synthesis is promoted by natural or experimental increases in the portal amino acids and the replicative machinery is idling and/or deficient in, for example, DNA synthesis.

1.6.1 : BIOCHEMICAL CHANGES IN REGENERATION INTERPHASE OF LIVER

It is known that immediately after partial hepatectomy there are major changes in haemodynamics (Bucher & Swaffield, 1973). Portal blood, which was previously distributed throughout the whole liver, is now channelled through the residual liver lobes. Transporters and receptors functioning below saturation capacity in normal liver can respond by an
increase in solute transport and hormone binding. Increased intracellular concentrations of cations (Koch & Leffert, 1979), amino acids and nucleotides (Baserga, 1976; Bresnick, 1971) are partially consequential on the altered blood flow, but, where altered kinetic parameters are observed, pleiotropic effects from increased binding by hormones and other regulatory factors are implicated. The membrane effects are immediate and independent of protein synthesis.

Classic studies following the introduction of partial hepatectomy as an experimental procedure established that hormones were not essential for regeneration (Harkness, 1957), although their absence usually extends the first G1-phase and delays the time by which the liver weight is restored. Normal metabolic constraints to growth are overcome by increased intracellular concentrations of a variety of factors. These include rate limiting essential amino acids such as tryptophan and methionine (McGowan et al., 1979), increased cyclic AMP concentrations (McManus et al., 1973; Thrower & Ord, 1974) and, almost certainly, increased $Ca^{2+}$ (Rixon & Whitfield, 1976). Four hours after the operation new rRNA is detectable in the cytoplasm (Baserga, 1976).

The hypertrophic phase of regeneration, from 0 to 9 hours, involves increased production of constitutive proteins and, at the time of fastest growth, a 50% decrease in the average rate of protein degradation (Scornik & Batbol, 1976). This latter process is still not completely understood. Current hybridization studies indicate that the fraction of non-repetitive genome transcribed remains almost unchanged,
although some new nuclear and polysomal poly-adenylated species appear (Scholla et al., 1980; Wilkes et al., 1979; Wilkes & Birnie, 1981; Grady et al., 1981). By 12 hrs post-operation, the concentration of cytoplasmic and polyribosomal poly(A)\(^+\)RNA species increases by 120% (Atryzek & Fausto, 1979) suggesting that this phase of regeneration is principally due to increased efficiency in the procedures by which the transcribed RNA is processed and passed into cytoplasm for translation. Cell-fusion experiments in the 1960's indicated the importance of the nucleolus in these processes (Harris, 1974).

As well as the increased synthesis of constitutive proteins, the hypertrophic phase of partial hepatectomy results in the production of some enzymes that are otherwise facultatively expressed; for example, tyrosine amino transferase and ornithine decarboxylase. Model studies with the former have established that the appearance of protein is associated with an increase in the cytoplasmic levels of its mRNA (Russell & Haddox, 1979). Ornithine decarboxylase has been intensively studied in regenerating liver and in many other systems where growth has been promoted (Russell & Haddox, 1979). In liver, three peaks of ornithine decarboxylase occur, coincident with peaks in cAMP levels (Baserga, 1976). The appearance of inducible enzymes follows induction/de-repression of the genome, though no inducers have yet been identified.

Between 6 hr and 9 hrs post-operatively the sensitivity of the regenerative process to actinomycin is minimal (Thrower & Ord, 1974), but between 9 hr and 12 hrs, sensitivity returns and the cells become committed to replication. In many animal cells this occurs about 6 hrs before the start
of DNA synthesis, as measured by thymidine incorporation (Kizer & Howell, 1979). In regenerating liver, the period is coincident with the second peak of cAMP and ornithine decarboxylase activity. Studies with hepatomas and other cell lines that lack adenylate cyclase make it clear however, that cAMP is facilitatory, not essential. In liver, the period is also detectable by co-ordinate changes in the activities of a number of enzymes, leading to the production of deoxynucleoside triphosphates. Commitment to replication is sensitive to diverse inhibitors, whose action cause the onset of DNA synthesis to be delayed. Examples include sub-lethal exposure to X-radiation (Holmes & Mee, 1952), Be\(^{2+}\) (Witschi, 1970) dimethylnitrosamine (Carddock, 1975), interferon (Frayssinet et al., 1973) and \(\alpha\)-blockers, such as phenoxybenzamine (Thrower et al., 1973). These agents must be administered within the first 9 hrs of the operation, to be effective.

Products of the hypertrophic phase that could promote changes in chromatin structure are discussed in the following section.

1.6.2: CHANGES IN NUCLEAR PROTEINS DURING REGENERATION

(in \(G_1\) and S-phase)

One immediate result of partial hepatectomy on liver nuclei is that compounds that intercalate with DNA, such as Acridine orange show increased binding (Truitt & Alvarez, 1976). This may result from changes in membrane permeability which cause alterations in intracellular Na\(^+\) or Ca\(^{2+}\). Increased movement of Na\(^+\) into chromosomal "puffs", and into
nuclei transplanted into the cytoplasm of transcriptionally active cells has been known for some time (Harris, 1974). The rapidity with which the increased dye binding can be detected suggests that ion changes are involved.

Changes in optical properties of DNA follow which indicate that proteins intercalate into the helix (Baserga, 1976). It has been suggested that NHCP is trapped within nuclei as a result of increased phosphorylation by protein kinase I (Fonagy et al., 1977). Phosphorylated NHCPs are concentrated into transcriptionally active regions of the genome and produce altered histone binding and induce phosphorylation of histone H1 (Fonagy et al., 1977). Increased transcriptional activity from chromatin in vitro is detectable 6 hrs operatively and correlates with the appearance of altered NHCP profiles (Kostraba & Wang, 1973).

Phosphorylation (D'Anna et al., 1978) and Acetylation (Nadler, 1976, Pogo et al., 1968) of nuclear-proteins is rapidly promoted after partial hepatectomy. This may be a consequence of ion or non-histone protein changes altering histone conformation, or as indicated by the kinetics of the response, because the proportion of inhibitor for the deacetylase rises.

The phosphate content of the histones falls with the decline in cAMP concentration, but rises very markedly by about 15 hrs, just before the peak of DNA synthesis and immediately after the second, smaller, peak in cAMP concentration. At least 25% of histones H2A and H4 may be modified (Ord & Stocken, 1975). N-phosphorylation does not rise markedly during this
period (Chen et al., 1977).

Coinciding with these modifications, and possibly dependent on them, new non-histone proteins enter the nuclei. Ord & Stocken (1980) have proposed that the changed non-histone protein and histone complement and/or their modifications appear to serve three functions:

i. The protein-protein and protein-DNA interactions are altered. Thermal stability of DNA in the total chromatin from S-phase liver nuclei is lowered and there are changes in the optical properties of DNA (Baserga, 1976).

ii. Phosphorylated NHCP promote transcription of histone mRNA. The phosphorylation of the proteins is essential for this function (Thompson et al., 1976). As the cells pass through S-phase, synthesis is also increased of a number of proteins whose expression is required in replication; including ribonucleotide reductase. The amounts of these enzymes remain high as the cells enter a second division cycle.

iii. A third function of the modification of new histones postulated, is that they prevent random interaction of the newly synthesized histone with new DNA (Sung & Dixon, 1970). This, however, was postulated before the nucleosomal organization of histones and DNA was established.

There is no alteration in nucleosomes during G₁ and S-phase of liver regeneration (Caplan et al., 1978) with DNA
synthesis, changes in histone-histone and histone-DNA interactions are observed, resulting in an increased accessibility to the nucleases. Simpkins et al., (1981) have suggested that at times of maximum DNA synthesis (18-24 hrs after partial-hepatectomy) the accessibility of the probe is increased markedly (40-50%).

The processes outlined above fail to explain how the massive changes in the nuclear structure are initiated as the cells become committed to replication. The relevant signals are presumably consequential to the growth process and are likely to include non-specific amplificatory effects as well as specific inducers.
MATERIALS AND METHODS
2. MATERIALS

2.1: ENZYMES, HORMONES AND METABOLIC INHIBITORS

2.1.1: ENZYMES

S₁ Nuclease was purchased from Sigma Chemical Co., London, U.K. or from Boehringer Corporation, London, U.K. T₁ ribonuclease and pancreatic ribonuclease A were obtained from Sigma Chemical Co., London, U.K. AMV reverse transcriptase was supplied by Dr. J.W. Beard of Life Sciences Inc., Florida, U.S.A. through the viral cancer programme, National Cancer Institute, Bethesda, U.S.A. Proteinase K was purchased from Boehringer Corporation, London, U.K.

2.1.2: HORMONES

Dexamethasone Sodium Phosphate Succinate was a gift from Merck, Sharp and Dohme Ltd., Hoddesdon, Herts, U.K., and hydrocortisone was purchased from Sigma Chemical Co., London, U.K.

2.1.3: INHIBITORS

Cycloheximide and diethyl pyrocarbonate (D.E.P.) were obtained from Sigma Chemical Co., London, U.K. Actinomycin D and heparin (5000 Units/ml) were purchased from Calbiochem Ltd., London, U.K.
2.2: REAGENTS FOR RNA PURIFICATION

Phenol, bentonite and Sodium dodecyl Sulphate (SDS) were purchased from British Drug House Chemicals Ltd., Dorset, U.K.

Phenol was redistilled before use and bentonite was purified by the method of Fraenkel-Conrat et al., (1961), CsCl was obtained from Hopkins and Williams Ltd., Dorset, U.K. and before use, was passed through Dowex Chelating resin, obtained from Sigma Chemical Co., London, U.K. to remove heavy metal ions.

2.3: REAGENTS FOR ELECTROPHORESIS

Acrylamide electrophoresis grade and N,N,N',N'-Tetramethylethlenediamine (TEMED) were purchased from Koch-Light Laboratories Ltd., Bucks, U.K.

Ethylendiacrylate was purchased from Kodak Ltd., Kirby, Lancs., U.K.

N,N'-methylene bisacrylamide was supplied by the British Drug House Chemical Ltd., Dorset, U.K.

FORMAMIDE PURISS, purchased from Fluka, Bush, Switzerland was deionised for 3 hours with AG-501-X8(D) mixed bed resins (Bio-rad Laboratories, Watford, U.K.) at 3-4g 100ml⁻¹. The deionised formamide was recovered by filtration through glass fibre filters. The formamide was also used in hybridization buffers and Eluent buffers in Poly(U) Sepharose affinity chromatography.
2.4: NUCLEIC ACIDS

_E.coli_, Calf thymus and Salmon sperm DNA were obtained from Sigma Chemical Co., London, U.K.
Poly (U), Poly (C) and Poly (A) were obtained from Miles Laboratories, Stoke, Pooges, U.K.
dATP, dCTP, dGTP, dTTP and oligo (dT)\textsubscript{12-18} were purchased from P-L Biochemicals Inc., Wisconsin, U.S.A.

2.5: BUFFERS

Trizma-HCl and Trizma-Base, HEPES (N\textsuperscript{\textprime}-2 hydroxy ethyl-piperazine-N-2-Ethane Sulphonic acid) were purchased from Sigma Chemical Co., London, U.K.

2.6: COLUMN CHROMATOGRAPHY

2.6.1: SEPHADEX COLUMN CHROMATOGRAPHY

Sephadex G-25, G-50 and G-100 were obtained from Pharmica (G.B.) Ltd., London, U.K. Before use gels were allowed to swell at room temperature overnight or at 90\degree for 1 hr in 15-20 volumes of distilled water containing 0.02\% (v/v) diethyl pyrocarbonate (D.E.P.). Swollen gels were autoclaved and columns were 25ml packed volume over a pad of Dowex chelating resin.

2.6.2: AFFINITY COLUMN CHROMATOGRAPHY

2.6.2.1: Poly (U) - Sepharose Chromatography:

Poly (U)-Sepharose was supplied by Pharmacia (G.B.) Ltd., London, U.K. 0.3g gel was swelled in 15ml 1m NaCl
pH 7.5, containing 0.02% (v/v) diethyl pyrocarbonate (D.E.P.) at room temperature for 30-45 mins., and layered in a sterile disposable syringe to 2ml packed gel volume. The column was washed extensively with 0.4m NETS (0.4m NaCl, 0.001 M EDTA, 0.01m TRIS-HCl pH 7.4; 0.02% w/v sodium dodecyl sulphate (SDS) and equilibrated with a concentrated salt buffer prepared in 25% (v/v) formamide in 0.7m NaCl, 50mM Tris-HCl pH 7.5, and 10mM EDTA.

2.6.2.2: Oligo (dT)-Cellulose Chromatography

Oligo (dT) Cellulose Chromatography was based on the method of McGrath (1978), which was a modification of the method of Aviv and Leader (1972). For this, 0.2 - 0.5g of oligo (dT) cellulose, obtained from Caboratine Research Labs., was suspended in sterile water and packed into a sterile disposable syringe (5ml) up to 2ml packed volume. The column was washed with 5 volumes of 2% (w/v) SDS and then equilibrated with loading buffer (0.5M LiCl, 10mM Tris-HCl pH 7.5, 1mM EDTA and 0.1% (w/v) SDS). This equilibrated column was used for Poly(A)$^+$RNA preparations as described in Section 3.4.2. of Methods section.

The same column was regenerated for reuse by washing it with 10-15ml 0.1N NaOH, followed by washing with 2.0% (w/v) SDS and then equilibrated with loading buffer as described before. By using this method each column was used 10-12 times without effecting the yield of Poly(A)$^+$RNA.
2.6.2.3: Thiol - Sepharose Chromatography

Thiol-Sepharose containing 3.0 μ moles ml⁻¹ resin was prepared as described by Dale and Ward (1975) and was a gift from Dr. P.R. Wilkes of the Beatson Institute for Cancer Research, Glasgow. Before use, it was activated by treatment with 50mM dithiothreitol (DTT) in 0.5M Tris-HCl pH 8.0 for 30 mins at room temperature and washed extensively with 0.1M NETS buffer (0.1M NaCl, 0.01M Tris-HCl pH 7.4, 1mM EDTA and 0.5% (w/v) SDS). This activated thiol sepharose was used for the chromatography of mercurated RNA (see Section 3.7.6.2).

2.6.2.4: Hydroxyapatite Chromatography

Bio-Rad, Biogel HTP (DNA grade) was supplied by Bio-Rad Laboratories Ltd., Watford, U.K. Preparation and characterization is described in Section 3.7.7.1.

2.7: PREPARATION OF DOWEX CHELATING RESIN:

Dowex Chelating Resin was obtained from Sigma Chemical Co., London, U.K. Before use, the resin was suspended in 5 volumes of sterile water and allowed to swell at room temperature, overnight. The suspension was stirred with gradual addition of 1N HCl until pH 7.0.

2.8: PREPARATION OF CsCl FOR RNA PREPARATION:

CsCl was obtained from Hopkins and Williams, Dorset, U.K. Before use CsCl was deionized. For this 100g of CsCl was dissolved in 400ml water and then passed through 10ml packed volume of Dowex Chelating resin, pH 7.0, under
vacuum. Solution of CsCl was then boiled till the crystals of CsCl started to form. This CsCl was dried at 100° and used for RNA extraction.

2.9: **ISOTOPES AND LIQUID SCINTILLATION COUNTING**

[\(^3\text{H}\)]-dCTP, 19.0 Ci/m mole was purchased from Radiochemicals Centre, Amersham, Bucks, U.K.

[\(\alpha^{32}\text{-P}\)]-dCTP 500 Ci/m mole was obtained from Amersham, Bucks, U.K.

Triton/Toluene scintillator was prepared by dissolving P.P.O. (2,5, Diphenyloxazole) at 0.5% (w/v) in solution containing 35% (w/v) Triton X-100 and 65% (v/w) Analar Toluene.

P.P.O. was purchased from International Enzymes, Windsor, U.K.

2.10: **GLASSWARE**

Glassware used to collect radioactive fractions were coated with "Replecote", supplied by Hopkins and Williams Ltd., Dorset, U.K., and autoclaved.

All other glassware was D.E.P. treated and/or autoclaved.

2.11: **MISCELLANEOUS**

Albumin, Bovine serum was purchased from Sigma Chemical Co., London, U.K. Glassfibre filters (GF/C) were product of Whatman, Kent, England. Nitro-cellulose fibres (0.45\(\mu\)m) were obtained from Millipore.

Cellulose nitrate and Polyallomer tubes were a product of Beckman Spinco Ltd., Paloalta, California, U.S.A.

Ziptrol dispenser used in dispensing less than 1\(\mu\)l samples was
purchased from Aberdeen Glass Co., Aberdeen, Scotland, U.K. All other chemicals were, wherever possible of AnalaR grade and were obtained from the British Drug House Chemicals Ltd., Dorset, U.K.

3. METHODS

3.1: EXPERIMENTAL ANIMALS

Male Wistar rats (bred at Glasgow University) weighing 200-250g were used in all experiments. The animals were kept in temperature controlled rooms under 12 hrs alternating light-dark cycles.

Two-third partial hepanectomies were performed according to the method of Higgins and Anderson (1931), using ether anaesthesia. Control rats were Sham-operated by laparotomy without removal of liver lobes.

Bilateral adrenalectomy was performed through a dorsal approach under ether anaesthesia. Adrenalectomized rats were maintained on 0.9% saline. In Sham adrenalectomized rats the adrenals were exposed as above but left intact and tap water was available ad libitum as for unoperated rats.

After 7 or 14 days of Adrenalectomy, the rats were anaesthetized, the thoracic cavity opened and blood withdrawn by heart puncture. The serum was collected after centrifugation (4°C, 15 min, 3000 x gav) and used for the determination of corticosterone. The liver was quickly excised and used for Polysome preparation. In some cases, adrenalectomized rats received dexamethazone before sacrifice.
Dexamethazone derivative was dissolved in 0.9% saline and injected intraperitoneally as 1mg per 100g body weight 4, 6, 8 and 12 hours before death. Control animals received 0.9% saline only.

3.2: POLYSOME PREPARATION:

3.2.1: LIVER POLYSOME PREPARATION

Livers were washed in 0.25M sucrose in 0.025M KCl, 0.05M Tris-HCl pH 7.6, 0.003M MgCl₂, containing 50μg/ml cyclohexamide and 0.02% (v/v) D.E.P. They were then minced in the same buffer (20ml/liver) and homogenized with a motor driven loose fitting teflon-glass homogenizer. The homogenate was adjusted to 1.0% (w/v) with Triton X-100 and centrifuged at 800 x gav for 15 min in HB4 rotor of Sorval RC-5 centrifuge at 4°. The post mitochondrial supernatant was layered over 1ml cushion of a solution containing 50mM Tris-HCl (pH 8.5), 50mM KCl, 3mM MgCl₂ in 1M sucrose containing 5μg/ml cyclohexamide, 7mM 2-mercaptoethanol and 0.02% (v/v) D.E.P., and was sedimented at 17,0000 x gmax in cellulose nitrate tubes of the SW40.L Ti rotor in L2-65B Beckman Ultra-Centrifuge. The supernatant was discarded and the pellet washed and suspended in appropriate buffer for polysome profile analysis or RNA extraction. To analyse the polysome profile, the pellet was washed and suspended at 5.0 O.D. units ml⁻¹ in 50mM Tris HCl (pH 7.6), 250mM KCl, 5mM MgCl₂ containing 5.0μg/ml cyclohexamide, 50μg ml⁻¹ heparin. Aliquots of 0.2ml were layered over 15-45% (w/v) sucrose density gradients in wash buffer, and centrifuged at 50,000 r.p.m. for 35 mins in SW50.1 rotor of the L2-65B Beckman Ultracentrifuge.
Gradients were scanned with a Gilford gradient scanner attached to the Gilford 240 spectrophotometer. All steps were carried out at 0-4°C.

Polysomal RNA was prepared as described in Section 3.3.

3.2.2: RETICULOCYTE POLYSOME PREPARATION

Polysomal pellets were prepared from the blood of rabbits, made anaemic with phenylhydrazine, and were provided by Mrs. J. Beaumont of Biochemistry Department, University of Glasgow. Blood was collected in capped centrifugation tubes and spun at 2,000 r.p.m. for 10 min in a M.S.E. Mistral Centrifuge. Supernatant and the white cells at the top of the red pellet were removed by suction. The red pellet, so obtained, was suspended in ice cold B.S.S. and spun as before. Again the supernatant and white cells were removed and the pellet was suspended in B.S.S. This procedure was repeated twice more. 10ml of buffer A (20mM NaCl, 50mM Tris-HCl (pH 7.4), 5mM Mg AC, 0.88M sucrose, containing 7mM mercaptoethanol, 1mg ml⁻¹ heparin, 100µg ml⁻¹ cycloheximide and 0.08% (w/v) Triton X-100) was added to 5ml of pellet and homogenized with a loose fitting pestle (6 strokes) to disrupt cells. The homogenate was transferred to polyallomer M.S.E. tubes and centrifuged at 20,000 r.p.m. for 10 min in SS 34 rotor of Sorval RC-5 centrifuge. The supernatant was transferred to another tube and kept on ice. The pellet was resuspended in 10ml of buffer B (As buffer A but containing 0.2M KCl and 0.06% (w/v) SDS), homogenized with tight fitting pestle (10 strokes) and centrifuged as above. Supernatant from this
spin was pooled with the first supernatant, layered over 8ml cushion of buffer C (1.5M sucrose in 25mM NaCl, 5mM Mg Ac, 50mM Tris-HCl (pH 7.4); containing 7mM mercaptoethanol) in 50Ti polyallomer tubes and spun at 60,000 r.p.m. for 2 hrs at 4°C. P•ellet obtained from this step contained polysomes, DNA and debris. DNA and debris were removed by gentle washing with sterile distilled water. Polysomal RNA and polysomal Poly(A)+RNA were extracted as described in the following section.

3.3: EXTRACTION OF RNA

3.3.1: PHENOL EXTRACTION OF RNA

Polysomal pellets were suspended in 6ml of 0.1M NETS buffer [0.1M NaCl, 0.001M EDTA, 0.01M Tris HCl pH 7.5, 0.2% (w/v) SDS] containing 200µg proteinase K and were incubated at 37°C for 30 mins. 10ml of Phenol:Chloroform:Iso-amyl-alcohol (1:1:0.1) was then added, vortexed briefly and the RNA was extracted on a mechanical shaker for 10 min at room temperature. The phases were separated by centrifugation at 6,000 r.p.m. for 6 min in the HB4 rotor of the Sorval RC-5 centrifuge at 4°C. The phenol phase and interphase were subjected to two further extractions with 0.1M Tris HCl buffer pH 9.0 containing 1mg.1ml⁻¹ Bentonite and 1% (w/v) SDS. The combined aqueous phases were centrifuged at 9,000 r.p.m. for 15 min, in the HB4 rotor of the Sorval RC-5 centrifuge, to remove bentonite and were subsequently precipitated with 2 volumes of ethanol in the presence of 0.65M NaCl at -20°C, overnight.

In some cases addition of Proteinase K and subsequent incubation at 37°C for 30 mins was omitted.
3.3.2: **CsCl EXTRACTION OF RNA**

The procedure used was a modification of the method described by Glisin et al., (1974), the major difference being that prolonged centrifugation in CsCl was avoided.

Polysomal pallets were dissolved in buffer I (0.15M NaCl, 0.05M Hepes (pH 7.5), 0.01 M EDTA, 1% (w/v) SDS); (1-2mg of polysomes per ml). Solid chelaxed CsCl was then added (1.4g. ml⁻¹ of the original solution) and dissolved by vortex mixing. The solution was spun at 10,000 r.p.m. for 25 min in the HB4 rotor of the Sorval RC-5 centrifuge.

The CsCl solution was then collected by inserting a long needle past the pellicle of protein (which should be firmly packed and on the top of the gradient). This solution containing deproteinized RNA was filtered through a GF/A filter in a millipore Swinnex-13 filter holder.

Once the solution containing RNA was recovered, 2.5 to 3 volumes of sterile distilled water and 7-8 volumes of cold ethanol were added, RNA was precipitated by storage at -20°C for at least 4 hrs.

3.4: **PREPARATION OF POLYADENYLATED RNA**

3.4.1: **POLY (U) - SEPHAROSE CHROMATOGRAPHY**

0.8 - 1.0mg polysomal RNA was suspended in a high salt buffer (0.7M NaCl, 50mM Tris-HCl pH 7.0, 10mM EDTA) containing 25% (v/v) formamide and denatured by incubation for 5 min in a 55°C water bath. The RNA was then carefully layered on a 2ml column of Poly (U) sepharose and allowed to drain through
under gravity. The initial eluate was recycled through the column to ensure complete binding of the Poly (A)$^+$ RNA species to the Poly (U) homopolymers immobilised on the sepharose matrix.

The unbound RNA was recovered by washing with the high salt buffer containing 25% (v/v) formamide. The bound (Poly) A containing RNA species was eluted differentially with 2 x 1ml followed by 2 x 2ml and 3 x 1ml pH 7.5 elution buffer consisting of 90% (v/v) formamide, containing 10mM KH$_2$PO$_4$, 10mM EDTA and 0.02% (w/v) SDS. The Poly (A)$^+$ RNA was further purified by two passages through a fresh 2ml column of Poly (U)-sepharose, precipitated with ethanol, overnight at -20$^\circ$ in the presence of 0.15M NaCl.

The precipitated Poly (A)$^+$ RNA was spun at 10,000 r.p.m. for 15 min, in the HB4 rotor of the Sorval RC-5. Palleted RNA was dissolved in sterile water and desalted by gel filtration through a column Sephadex G-25 or G-50 underlaid with Dowex chelating resin and equilibrated with sterile water. The salt free Poly (A)$^+$ RNA was freeze-dried, dissolved at 1-2mg ml$^{-1}$ in sterile water and kept at -70$^\circ$. Purity and integrity of polysomal RNA and Poly (A)$^+$ RNA was checked by electrophoresis either on 2.7% (w/v) aqueous polyacrylamide gels or on 3.5% (w/v) polyacrylamide gel containing 96-98% (v/v) formamide. Globin mRNA was prepared as described above from reticulocyte polysomes.

3.4.2: Oligo (dT) - Cellulose Chromatography

1.0 - 2.0mg polysomal RNA was suspended in 1ml of loading buffer (0.5M LiCl, 10mM Tris-HCl pH (7.5), 1mM EDTA, 0.1%
(w/v) SDS), warmed up for 3 mins in a 55°C water bath, and rapidly cooled to room-temperature. The RNA was then carefully layered on a 2ml column of oligo (dT)-cellulose and allowed to drain through gravity. The initial elute was recycled, twice through the column to ensure complete binding of Poly (A)⁺ RNA species to the oligo (dT)-homopolymers immobilized on the cellulose matrix. The unbound RNA was removed by washing with loading buffer, until the A₂₆₀ of the elute less than 0.01. The bound Poly (A) containing RNA species was eluted differentially with 2 x 1ml followed by 2 x 2ml and 4 x 1ml of elution buffer (10mM Tris- HCl pH 7.5, 1mM EDTA), made 0.5M LiCl, 10mM Tris- HCl pH 7.5, 1mM EDTA, 0.1% w/v SDS and loaded into a fresh column of oligo (dT)-cellulose. The washing and elution procedure was repeated and Poly (A)⁺ RNA was made 0.15M with respect to NaCl and precipitated with 2 volumes of ethanol at -20°C overnight or at -70°C for 4 hr.

Poly (A)⁺ RNA was desalted, characterized and stored as described in Section 3.4.1.

3.5: FRACTIONATION OF RNA

3.5.1: FRACTIONATION OF RNA ON AQUEOUS POLYACRYLAMIDE GELS.

Gels were prepared as described by Knowler and Smellie (1971). 2.7% gels which were used for the fractionation, contained 2.7% (w/v) acrylamide, 0.25% (w/v) ethylene-diacyrate and 1.0% (v/v) N, N, N'N' tetra-methylenediamine (TEMED). They were prepared in electrophoresis buffer (36mM Tris-HCl pH 7.7 - 7.8, 30mM NaH₂PO₄ and 1mM EDTA),
pH was adjusted with phosphoric acid as described by Loening (1969) and polymerization was catalysed by the addition of ammonium per sulphate to 0.1% (w/v).

The prepared solutions were mixed and 3.0ml aliquots were rapidly pipetted into vertical 0.5 x 12.0cm plexiglass tubes. Water was carefully layered over the solution using a Hamilton syringe. The gels were allowed to set for 30 mins at room temperature. The water layer was removed and replaced by electrophoresis buffer. All gels were pre-electrophoresed at 2.5mA gel\(^{-1}\) for 15-30 mins before samples (30-40 \(\mu\)g), dissolved in 40-50 \(\mu\)l electrophoresis buffer, containing 20% (w/v) sucrose and 0.2% (w/v) SDS, were layered on the gels. Electrophoresis was 2-5 hrs at 5mA gel\(^{-1}\). After separation, the gels were carefully ejected from the tubes using water pressure gently applied from a 10ml syringe.

The gels were soaked in water for 10-15 mins before they were scanned at 260nm in the linear transport attachment for the Gilford 240 recording spectrophotometer. Gels containing radioactive RNA were frozen in powdered solid CO\(_2\) and sliced in 1mm or 2mm sections using a Mickle gel slicer. Slices of acrylamide/ethylene diacrylate gels containing radioactive RNA were digested individually in vials with 0.5ml of 2M aqueous NH\(_4\)OH at 60\(^\circ\). After evaporation to dryness, the gel residues were broken up in 0.3ml water and left for 1 hr. Radioactivity was counted by scintillation counting in 10ml of Triton-toluene base scintillation fluid. Where acrylamide/bisacrylamide gels were used, the individual slices were dried in scintillation vials by incubation at 60\(^\circ\) for 2 hr,
followed by digestion by a further incubation at 60° overnight in the presence of 0.5ml 30% (v/v) hydrogen peroxide. Radioactivity was assayed as above.

3.5.2: FRACTIONATION OF RNA ON DENATURING POLYACRYLAMIDE GELS

The integrity and purity of polysomal Poly (A)⁺ RNA was analysed by fractionation on 3.5% polyacrylamide gels containing 98% formamide. The gels were prepared using a modified version of the procedure of Staynov et al., (1972), as described by Maniatis et al., (1975). 3.5% (w/v) acrylamide and 0.42% (w/v) bisacrylamide was prepared in deionized formamide. 74ml of this solution was mixed with 1ml of a solution containing 100mg of ammonium per sulphate, 170mg of dibasic sodium phosphate and 40mg of monobasic sodium phosphate. After mixing, the solution was polymerized by the addition of 150μl of TEMED. 3ml aliquots were rapidly pipetted into 0.5 x 12cm plexiglass tubes. Water was carefully layered over the solution as described before and the gels allowed to polymerize for 20-30 mins at room temperature. The water layer was replaced with 98% (v/v) formamide and the gels were left as such for 24 hr. Prior to electrophoresis, the formamide was replaced with electrophoresis buffer (0.02M sodium phosphate pH 7.5). The gels were pre-electrophoresed for 1hr at 5mA gels⁻¹. Ethanol precipitated Poly (A)⁺ RNA samples (30-40μg), suspended in 50μl 98% (v/v) formamide, were placed in a boiling water bath for 3-4 mins, quickly cooled in ice and applied to gels. The gels were run at room temperature at a constant current of 5mA gels⁻¹ for 3hr. After electrophoresis, gels were carefully extruded into the test tubes containing warm water
(40-50°) and after two changes of water, over 15-30 mins, they were scanned at 260nm as previously described. The treatment with warm water considerably reduced background absorption due to farmamide.

3.6: PREPARATION OF NUCLEASE RESISTANT RNA NUCLEOTIDES

The method used to prepare Poly(A) segments from polysomal polyadenylated RNA was that described by Adesnik et al., 1972. The polysomal RNA was extracted as described before (section 3.3.1 and 3.3.2), and ethanol precipitated polysomal RNA from two separate experiments were pooled, washed twice with 95% (v/v) ethanol; 1-2mg of this RNA was then digested in 1ml of a high salt buffer (400mM NaCl, 10mM Tris-HCl, pH 7.5, 5mM EDTA) with RNase A (0.4mg ml⁻¹) and RNase T1 (250mg Egami units ml⁻¹) at 37° for 30 mins. This was followed by the addition of pronase (nuclease free, 10µg ml⁻¹) and mixture was incubated at 37° for 30 mins. The resulting Poly(A) segments were recovered by affinity chromatography on oligo(dT) cellulose, or on Poly(U) sepharose as described in section 3.4.

Purified Poly(A) segments obtained by this method were precipitated with 2 volumes of 95% ethanol, in the presence of 0.15M NaCl and 40µg yeast 4S tRNA.
3.6.1: DETERMINATION OF POLY (A) SIZE AND CONTENT OF LIVER POLYADENYLATED POLYSOMAL RNA.

Purified Poly (A) segments obtained as described above were resolved on 2.7% polyacrylamide gels for 2-3 hrs at 5mA gel⁻¹ and sliced into 2mm slices. Each slice was dissolved in water and incubated for 24 hrs at room temperature to extract adenylated nucleotides. The extract was spun at 2,500 r.p.m. in MSE Mistral centrifuge for 5 min and the supernatant adjusted to 2 x SSC.

The position of Poly (A) in gel was determined by hybridization to an excess of [³H]-Poly (U) (> 50,000 c.p.m.), and followed by analysis of RNase resistance as described below. Size of Poly (A) so located was estimated by reference to Poly (A) (220 and 90 nucleotides) and oligo (A) (28 nucleotides) marker homopolymers. From the position of these markers, a least square analysis was used to calculate a line of best fit for the log molecular weight versus electrophoretic mobility. From this plot the number average nucleotide length was calculated using the relationship:

\[
\frac{\sum \text{Ni} \cdot \text{Li}}{\sum \text{Ni}}
\]

\(\text{Ni} \) :- the Number

and \(\text{Li} \) :- the length of individual molecules of a given size class.

The Poly (A) content of polysomal polyadenylated RNA was estimated by determination of the amount of pancreatic RNase resistant [³H] -Poly (U) radioactivity hybridized to 4-10μg of unlabelled RNA compared to known amounts of synthetic
Poly (A).

Each standard 100\mu l assay mixture contained 20\mu l of 10 x SSC (1.5M NaCl, 0.15M sodium citrate pH 7.2), \[^{3}H\]-Poly (U) at 20 \mu g/ml and varying amounts of Poly (A) in water. Mixtures were incubated at 37\degree for 30 mins and cooled to 4\degree before 1ml of pancreatic RNase at 20\mu g ml\(^{-1}\) in 2 x SSC was added. Digestion was at a 37\degree for 30 mins. Acid insoluble material was prepared as in section 3.9.

Precipitates were collected onto GF/C filters, dried at 60\degree overnight and radioactivity assayed by scintillation counting in 10ml toluene based scintillation fluid.

3.7: COMPLEMENTARY DNA - mRNA HYBRIDIZATION

The effects of adrenalectomy and partial hepatectomy on the quantitative and qualitative regulation of liver mRNA was investigated by nucleic acid hybridization technique. The experimental procedure consists essentially of:

i. Isolation of mRNA;

ii. The synthesis of Complementary DNA (cDNA) probes and

iii. An assay procedure to measure the kinetics of hybridization of the RNA with cDNA probe.

cDNA used in this investigation was synthesized \textit{in vitro}, by purified RNA directed DNA polymerase (Reverse transcriptase) using RNA as template. The cDNA product can therefore hybridize with complementary bases of mRNA by the Watson-Crick base pairing. By using radiolabelled precursors, the cDNA can be labelled to a high specific activity so that the
hybridization reaction can be monitored (Bishop et al., 1974; Birnie et al., 1974). Thus any effect on mRNA concentration or on mRNA sequences can be determined by measuring the rate of hybridization of cDNA probe with RNA samples isolated from different sources. The procedure for the isolation of liver polysomes and polysomal polyadenylated RNA has been described in section 3.4.

3.7.1: SYNTHESIS OF DNA COMPLEMENTARY TO POLYSOMAL POLYADENYLATED mRNA

Synthesis of cDNA was essentially by the method of Getz et al., (1975). Template polysomal Poly (A)\(^+\) RNA (5-20 \(\mu\)g) was incubated at 37\(^\circ\) for 2 hr in a mixture (0.25 - 1.0ml) containing 50\(\mu\)g ml\(^{-1}\) oligo (dT) 12-18, 400 \(\mu\)m each of dATP, dGTP, dTTP and \(^3\)H\(-\)dCTP (4.85 Ci m mole), 100 \(\mu\)g ml\(^{-1}\) Actinomycin D, 200 \(\mu\)g ml\(^{-1}\) Bovine serum albumin, 50mM Tris-HCl (pH 8.2), 50mM KCl, 10mM dithiothreitol, 5mM magnesium acetate and 250 units ml\(^{-1}\) of reverse transcriptase. After incubation, the mixture was adjusted to 10mM EDTA and chilled in ice. The entire mixture was chromatographed on Sephadex G-50 columns. 100\(\mu\)g E. coli DNA was added to the excluded fraction which was freeze-dried and dissolved in a minimal volume of water.

cDNA, complementary to globin mRNA, was prepared in a similar manner.

3.7.2: CHARACTERIZATION OF COMPLEMENTARY DNA.

cDNA prepared and isolated as described above was freeze-dried, dissolved in 0.9ml of 0.9M NaCl, 0.1M NaOH and
fractionated on a linear 20ml, 4-11% (w/w) alkaline sucrose gradients in 0.9M NaCl, 0.01M NaOH. Centrifugation was for 24 hrs in the 3 x 25ml MSE swing out rotor at 29,000 r.p.m. and 20°. 1ml fractions were collected and radioactivity in 5μl of each fraction was counted in 5ml Triton-toluene based scintillation fluid. Sedimentation coefficients and molecular weights were determined by computer programme as described by Steensgard et al., (1978). cDNA of desired molecular weight was recovered by precipitation in the presence of 2 volumes of ethanol at -20° and 100 μg E. coli DNA. Precipitated cDNA was spun at 10,000 r.p.m. in the HB4 rotor of the Sorval RC-5 centrifuge, dissolved in 0.5ml of sterile water and desalted by Sephadex G-50 column chromatography. The excluded fractions were freeze-dried, dissolved in a minimal volume of water and kept at -20°.

3.7.3: RNA - cDNA HYBRIDIZATION

The technique has been described in detail by Birnie et al., (1974). Appropriate volumes of RNA at various concentrations and cDNA solutions, in sterile, distilled water were mixed, lyophilized and redissolved in hybridization buffer (0.5M NaCl, 25mM HEPES pH 6.8, 5 mM EDTA, 50% (v/v) formamide). Before addition of formamide the solutions were desalted by passage through Chelex-100 resin, treated with diethylpyrocarbonate and autoclaved. Portions of solutions (0.4 - 1.0 μl) were dispensed with a Ziptrol dispenser and sealed in glass capillaries. The capillaries were heated at 70° for 5 mins, then incubated at 43° for various lengths of time. The Rot (moles, sec. litre⁻¹) value of each hybridization time point was calculated. A Rot of 1 moles, sec. litre⁻¹ is attained
when RNA is incubated at \(83\mu g\) ml\(^{-1}\) for 1 hr (Britten and Kohne, 1975).

Thus Rot = \(\frac{\text{RNA (\(\mu g\) ml\(^{-1}\)) \times t (h)}}{83}\)

### 3.7.4: ASSAY OF RNA - cDNA HYBRIDIZATION REACTION

#### 3.7.4.1: Assay of S\(_1\) - Nuclease Activity

It was found necessary to check the activity of commercial S\(_1\) nuclease as the activity varies from batch to batch. Aliquots of globin mRNA-cDNA hybrids, purified by hydroxyapatite chromatography, or single stranded globin \(^3\text{H}\)-cDNA were incubated at 37\(^o\) in the presence of 14\(\mu g\) ml\(^{-1}\) denatured calf thymus DNA, with 8-20 units of S\(_1\) nuclease, in nuclease buffer (70mM sodium acetate pH 4.5, 2.8mM ZnSO\(_4\), 0.14M NaCl). At various times of incubation, a portion of the incubation mixture was taken to determine the total radioactivity and a further portion was acid precipitated as described in the following section. S\(_1\) nuclease activity is expressed as percentage digestion.

### 3.7.5: ASSAY OF RNA - cDNA HYBRIDIZATION BY S\(_1\) NUCLEASE

The hybridization mixture from each capillary was flushed out with 0.25ml of buffer containing 70mM sodium acetate pH 4.5, 2.8mM ZnSO\(_4\), 140mM NaCl and 14\(\mu g\) ml\(^{-1}\) heat-denatured calf thymus DNA. The extent of hybridization was determined by digesting the non-hybridized probe and represented as a percentage of the total cDNA. 100\(\mu l\) of S\(_1\) nuclease (10-20 units) in nuclease assay buffer was added to each capillary flushing and incubated at 37\(^o\) for 1\(\frac{1}{2}\) hours. The incubations
were chilled, 100µl removed and radioactivity determined by scintillation counting with 10ml of Triton-toluene base scintillation fluid to determine total radioactivity present in cDNA \( (T) \). A further 200µl was removed and precipitated by addition of 50µl of carrier (consisting of 1mg ml\(^{-1}\) BSA and 150µg ml\(^{-1}\) calf thymus DNA) and 50µl of ice cold 3N perchloric acid. After standing in ice for 15 min, the precipitate containing undigested cDNA - mRNA hybrids was removed by centrifugation at \( 4^\circ \) and 2,500 r.p.m. for 15 mins in the MSE Mistral centrifuge. 200µl samples of the clear supernatant were removed and radioactivity counted as described above to determine acid-soluble radioactivity \( (AS) \). The amount of nuclease added was sufficient to ensure complete degradation of unhybridized cDNA within 1 hour. The percentage of cDNA in hybrid was calculated as:

\[
\% \text{ hybrid} = 1 - \frac{0.75 \times AS(c.p.m.)}{T(c.p.m.)} \times 100
\]

To double check the extent of hybridization, the precipitate remaining after the removal of acid-soluble fraction for radioactivity determination, was subjected to alkaline digestion. For this, 1ml of 1N perchloric acid was added to the precipitate, vortexed and sedimented at 2,500 r.p.m. as described above. The supernatant was removed by suction, 300µl of 1N NaOH was added and digestion was completed by incubation at \( 37^\circ \) for 2 hours. After incubation, the mixture was chilled and neutralized by the addition of 300µl 1N HCl and 10ml Triton-toluene based scintillation fluid was added to the whole mixture to determine radioactivity \( (T_2) \).
Percentage of hybridization was determined by the formula:

\[
\% \text{ Hybrid} = \frac{T_2}{T_1 \times 2} \times 100
\]

3.7.6: **FRACTIONATION OF ABUNDANCE CLASSES OF cDNA**

In brief, this was achieved by mercurating mRNA, hybridizing it to cDNA to a defined Rot and then using thiol-sepharose chromatography to isolate hybrids from which the cDNA could be recovered.

3.7.6.1: **Mercuration of mRNA and Hybridization to cDNA**

Polysomal Poly (A)\(^+\) RNA from rat liver, under different conditions of stress, was extracted as described in section 3.4. The polysomal Poly (A)\(^+\) RNA was mercurated as described by Dale and Ward (1975), by dissolving at a final concentration of 100\(\mu\)g ml\(^{-1}\) in 100\(\mu\)l sodium acetate buffer pH 6.0, containing mercuric acetate at 1mg ml\(^{-1}\). The mixture was incubated at 50\(^\circ\)C for 90 min, after which one tenth of the volume of 100mM EDTA pH 7.0 was added and the entire mixture passed over a Sephadex G-50 column. The eluted fractions were freeze-dried and dissolved in a minimal volume of sterile water.

Appropriate amounts of mercurated RNA (Hg-RNA) and total cDNA mixed in sterile water were freeze-dried and taken up in appropriate volume of hybridization buffer as described in section 3.7.3. Hg-RNA was hybridized in excess (10\(^2\) to 10\(^3\) fold to 10\(^6\) counts/min) of polysomal cDNA to a Rot of 10 moles
-1

sec.1 in hybridization buffer containing 1mM mercaptoethanol. The hybridization mixture was diluted with NETS buffer (0.1M NaCl, 1mM EDTA, 10mM Tris-HCl, pH 7.5, 0.1% (w/v) SDS) and applied to an activated thiol-sepharose column.

3.7.6.2: Thiol-Sepharose Chromatography

The retention of mercurated RNA cDNA hybrids on thiol-sepharose was measured by using two procedures. One procedure involved chromatography at ambient temperature and the other included a washing step at 60°.

1.5ml columns of thiol-sepharose were prepared in 2ml plastic syringes and were activated with dithiothreitol as described in section 2.6.2.3.

In the ambient temperature the sample of mercurated RNA-cDNA hybrid was applied to the column in 0.5ml of NETS buffer and left for 10 minutes. The column was then washed with 30ml of NETS and retained material, cDNA-Hg mRNA hybrid, was eluted with 5 x 1ml NETS containing 100mM 2-mercaptoethanol.

The procedure at elevated temperature employed a water jacketed column equilibrated at 60°. The sample was applied and left to equilibrate for 10° before the column was washed with 10ml of NETS at 60° and 20ml of NETS at room temperature. The elution of bound material was carried out as described above. This procedure has been found to give a considerable improvement in background compared with the ambient temperature procedure.

The percentage hybrid was calculated as:
% Hybrid = \( \frac{\sum \text{(Bound from)}}{\sum \text{(Bound c.p.m.)} + \sum \text{(Unbound c.p.m.)}} \) \times 100

Complementary DNA which had not hybridized with RNA was not bound and was washed from the column with NETS buffer. Hybridized cDNA was bound to the column and was eluted with 0.1M β-mercaptoethanol in NETS. The bound cDNA, representing the abundant class of sequences, was purified by desalting followed by alkaline hydrolysis of the associated RNA in 0.1M NaOH for 2 hr at 37°C; it was finally desalted on Sephadex G-50. Unbound cDNA was re-isolated and hybridized with its polysomal RNA template to a Rot of 3,000 moles s/l, hybridized sequences were isolated by thiol-sepharose chromatography as described above. Complementary DNA sequences isolated by this second cycle of purification represent the rare class of messenger RNA molecules.

Fractionation of normal rat-liver cDNA into normal rat liver specific and non-specific classes was performed using thiol-sepharose chromatography, as described above. In this case, Adrenalectomized rat liver polysomal Poly (A)^+ RNA was mercurated and hybridized to normal rat liver cDNA to a Rot value of 10,000 moles s/l at a mass ratio of 100:1. The bound cDNA, in this case, was taken to represent the non-specific class of cDNA whereas the unbound cDNA was considered to be the normal rat liver specific sequences.

3.7.7: UNIQUE DNA - mRNA SATURATION HYBRIDIZATION

3.7.7.1: Preparation and Characterization of Hydroxyapatite

Bio-Rad Biogel DNA grade HTP hydroxyapatite (HAP) was used. Its capacity varied from batch to batch in the range...
from 0.5 to 1 mg DNA per gram dry weight. HAP was prepared for use as follows: 40 gm was suspended in 4 volumes of 1 M sodium phosphate buffer pH 6.8 and left for 1 hour at room temperature. The HAP was recovered by centrifugation and washed several times with excess of 0.16 M sodium phosphate buffer pH 6.8. After the final wash, HAP was solubilized by suspending at 1.0 gm/ml in the wash buffer and heating in a boiling water bath for 10-15 min. HAP was then recovered by centrifugation, washed again in two changes of 0.03 M sodium phosphate pH 6.8 and stored in this buffer at 0.1 g/ml at 4°C. Before use, the suspension was again solubilized in a boiling water bath for 10-15 mins.

HAP columns were prepared by layering about 2 ml packed volume in 6" x 5" Dia. pyrex tubes with pyrex glass filters. The tubes were fixed with rubber grommets into a pyrex barrel of internal diameter 7 cm and length 40 cm. Water at 60°C was circulated through the barrel. The HAP column was washed extensively with sterile water, and by 0.03 M sodium phosphate buffer pH 6.8.

Efficient separation of single and double stranded nucleic acid by HAP chromatography depends on the concentration of sodium phosphate buffer, pH 6.8, used and the optimum concentration varied with each batch of HAP.

To determine the effective concentration of the elution buffer, [3H]-cDNA-globin mRNA hybrids and single stranded globin[3H]-cDNA were mixed in 0.15 M NaCl containing 0.03 M sodium phosphate buffer, pH 6.8, and applied to a 2-3 ml packed volume HAP column maintained at 60°C. The column was washed
extensively with 0.03M sodium phosphate buffer pH 6.8, followed by a stepwise elution with sodium phosphate buffer pH 6.8. 0.12, 0.14, 0.2, 0.4 and 0.6M. 6 x 1ml fractions were collected at each step, aliquots removed and radioactivity counted in 10ml Triton-toluene scintillation fluid. The effective concentrations of phosphate buffer that eluted single stranded and double stranded nucleic acids were noted.

The chosen concentrations of the batch employed in the work here described, were 0.14M and 0.4M sodium phosphate buffer pH 6.8 for single stranded and double stranded DNA respectively. When retested with these concentrations, 90-95% of standards could be recovered after chromatography.

3.7.7.2: Preparation of Highly Labelled Rat Liver Unique DNA Sequences

3.7.7.2a: Purification of Unfractionated DNA

Rat liver nuclei were isolated by the method of Chaveau et al., (1956) and DNA was extracted by the method of Hell et al., (1972) with slight modification. Purified nuclei were suspended in R.S.B. (10mM NaCl, 10mM Tris-HCL, pH 7.0 and 1.5mM MgCl₂) at the concentration of 10⁷ nuclei ml⁻¹ and sonicated at a setting of 2 amps for 3 x 10 sec. bursts, allowing 30 sec on ice for cooling between each burst. To monitor the complete lysis of nuclear envelope, the nuclei were examined under phase contrast microscope. After complete lysis of nuclear envelope the suspension was added to 20 volumes of 8M urea, 0.24M sodium phosphate buffer, pH 6.8 (MUP), containing 1% (w/v) SDS and 10mM EDTA. The whole mixture was poured into a thick slurry of 10gm hydroxyapatite in
100ml MUP, stirred for 15 min and left to stand at room temperature for an hour. The slurry was then poured into a sintered glass funnel No. 3 (9.5cm Diameter) and the liquid was drawn off under vacuum. The HAP was washed free of RNA and protein with MUP until no further $E_{260}$ or $E_{280}$ absorbing material was eluted (approximately 1500ml per 30gm HAP). After further precautionary washing with 500ml MUP, urea was removed by washing with 0.014M sodium phosphate buffer, pH 6.8 (approximately 500ml per 30gm HAP). The removal of urea was monitored by refractive index measurement on the effluent. The DNA was eluted with 0.4M sodium phosphate buffer, pH 6.8, and was dialysed for 12-18 hrs against 10 volumes of distilled water at 4°C. Solid NaCl was then added to 0.2M and DNA was precipitated with 2 volumes ethanol at -20°C for 18 hr. The precipitate was collected by centrifugation at 16,300g$_{max}$, for 30 min, dried in air at room temperature and redissolved in distilled water. This DNA was dialysed three times against 100 volumes 50mM NaCl, to remove excess salt.

DNA prepared by this method contained high amounts of Ca$^{++}$ ions. It was decalcified by stirring for 15 min, with Dowex 50Na$^+$ (Bio-Rad analytical grade cation exchange resin AG-50W-X8, 1gm resin per 50gm DNA). The slurry was poured into a column containing 1cm of packed Dowex 50 Na$^+$ and the resin was washed with 3-5ml 50mM NaCl. Ca$^{+2}$ free DNA was recovered quantitatively in the total effluent.
3.7.7.2b: **Fractionation of DNA**

Unique DNA was prepared by fractionation of total DNA on hydroxyapatite according to the procedure of Kleiman et al., (1977). DNA was taken up at a concentration of 1mg ml\(^{-1}\) in 0.15M NaCl and was sonicated with a Dawe sonoprobe type 7530A at a setting of 5 amps for six 30 sec bursts, allowing a minute on ice for cooling between each burst. After this treatment, the size of the DNA was found to be reduced to 5-6S as measured by alkaline sucrose gradients (section 3.7.2). The DNA was desalted on Sephadex G-50 underlaid with Dowex chelating resin, lyophilised and taken up in hybridization buffer HB/F at 1mg/ml.

It was denatured by heating at 70\(^{\circ}\)C for 5 min and allowed to reanneal to a cot value of 250 moles sec. litre\(^{-1}\) at a temperature of 43\(^{\circ}\)C (DNA at 1mg ml\(^{-1}\) in HB/F at 43\(^{\circ}\)C reaches a cot 250 moles sec. litre\(^{-1}\) after 20 hours and 50 min).

The reannealed DNA was diluted 500 fold with 150mM NaCl, 0.03M sodium phosphate, pH 6.8, and applied to hydroxyapatite in a 2ml column at 60\(^{\circ}\)C. Single stranded DNA was eluted with 0.14M sodium phosphate, pH 6.8, freeze-dried and desalted by Sephadex G50 chromatography. The reannealing step was then repeated to give material remaining single stranded after two cycles of fractionation. After the second stage of fractionation, the DNA was lyophilised, decalified and desalted on Sephadex G50. The DNA was then reannealed to a cot value of 25,000 moles sec. litre\(^{-1}\) in HB/F or in 0.18M NaCl and diluted 100 fold with 0.15M NaCl before nick translation.
3.7.7.2c: Nick Translation

Nick translation was carried out according to the procedure of Balmain & Birnie (1979). 10 \( \mu g \) of rat liver unique DNA (u-DNA) was nick translated in a total volume of 1ml containing 100nmoles each of dGTP, dTTP and dATP, 20nmoles of \([^3H]\)-dCTP (25.5 Ci moles), 50 units of DNA Polymerase I, 50mM Tris-HCl, pH 7.9, 10mM MgCl\(_2\), 10mM dithiothreitol, 50\( \mu g \) ml\(^{-1}\) bovine serum albumin. The reaction was allowed to proceed for 20-24 hr at 15\(^0\)C.

After incubation the mixture was chilled, made up to NETS buffer by addition of an equal volume of double concentrated buffer and extracted once with phenol:chloroform:isooamylalcohol (1:1:0.1). The aqueous phase was removed and brought up to 0.12 sodium phosphate and heated for 5 min. After boiling, the sample was diluted to 30mM sodium phosphate by addition of sterile water at 60\(^0\)C and loaded onto a 1ml hydroxyapatite column equilibrated with 30mM sodium phosphate, pH 6.8, at 60\(^0\)C. The column was washed extensively with the same buffer to remove all traces of nucleotide as detected by scintillation counting. Approximately 100ml of buffer were routinely used in this washing. Single stranded material was eluted with 0.14M sodium phosphate, pH 6.8, and these fractions were then pooled, lyophilised and desalted by gel filtration on Sephadex G50.

The specific activity of the unique DNA (u-DNA) prepared in this way was about 7 \( \times \) 10\(^6\) c.p.m. \( \mu g^{-1}\) DNA and its hybridizability with an excess of unlabelled total rat liver was at least 70\%. Further characterization of the DNA probe is
described in detail by Balmain & Birnie (1979).

3.7.8: UNIQUE DNA - mRNA HYBRIDIZATION

Polysomal Poly (A)$^+$ RNA from rat liver, under different conditions (normal, partial hepatectomized or adrenalectomized), was extracted and purified as described in section 3.2.

The RNA was mercurated as described before in section 3.7.6.1.

Appropriate amounts of mercurated mRNA and $[^3H]-u$DNA, mixed in sterile distilled water, were freeze-dried and taken up in the hybridization buffer. Hybridization in solution were performed in formamide hybridization buffer (0.5M NaCl, 0.25mM HEPES pH 6.8, 5mM EDTA and 50% v/v formamide) containing 1-2mM mercaptoethanol, to reduce interaction between mercury subunits, as described by Brown & Balmain (1979).

The extent of hybridization was assayed by thiol-sepharose chromatography as described in section 3.7.6.2.

3.7.8.1: Fractionation of $[^3H]$-Unique DNA Probe

$[^3H]$-uDNA probes specific for both normal and adrenalectomized rat liver polysomal Poly (A)$^+$ RNA were isolated by preparative hybridization of Hg-RNA with$[^3H]$-uDNA to a Rot of 40,000 moles s/l at a mass ratio of 100:1. Hybridized$[^3H]$-uDNA was then isolated by thiol-sepharose chromatography and purified by alkaline hydrolysis and desalting as described for cDNA (section 3.7.6.2).
3.7.9: **COMPUTER ANALYSIS OF EXPERIMENTAL DATA**

**HYBRIDIZATION DATA**

Polysomal Poly (A)$^+$ RNA and cDNA best fitting hybridization curves were drawn using a computer program adopted by Dr. B.D. Young at Beatson Institute of Cancer Research, Glasgow, from that of Monahan et al., (1977).

The fitting was defined by the equation:

$$\frac{d}{D_0} = B + \sum_{i=1}^{n} P_i \cdot 1 - \exp \left( 0.693 \frac{\text{Rot}}{\text{Rot} + \frac{1}{2}} \right)$$

This programme was designed to fit data to a set of curves $n$, where $n = 1, 2, 3$ or $4$: $\frac{d}{D_0}$ is the fraction of hybrid formed for each component of the curve; $P_i$ donates the proportion of hybridizable cDNA within each component. Rot is the number of moles second litre$^{-1}$ of nucleotides of RNA; Rot $\frac{1}{2}$, the number of moles sec. litre$^{-1}$ of RNA at 50% hybridization for that component and B is the zero time hybridization value. The theory of this equation has been described in detail by Bishop (1972). The programme estimates Rot $\frac{1}{2}$ values for hybridization reactions containing one or a number of different hybridizing components. It provides an estimate of the percentage of the total hybridizing material that each component represents and it also estimates background hybridization value.

The method of least squares based on a non-linear regression equation was applied to obtain the best fit to the data on the hybridization between mercurated Poly (A)$^+$ RNA and $^{3}H$-labelled unique DNA.
3.8: SEDIMENTATION COEFFICIENTS

The calculations of sedimentation coefficients of complementary DNA in alkaline sucrose gradients was based on a FORTRAN programme of Steensgaard et al., (1978).

3.9: a) : Preparation of Acid-Insoluble fractions from less than 100μg of material

Small amounts of material, such as the RNA in fraction from sucrose density gradients, were mixed with an equal volume of ice cold 10% (w/v) trichloroacetic acid and a further 5ml of 5% (w/v) trichloroacetic acid. One drop of 2% (w/v) Bovine serum albumin was added as carrier. After mixing, left at 0-4°C for at least 15 mins., the precipitates were collected on 2.5cm diameter Whatman GF/C glass fibre filters set up in the millipore filtration unit. The filters were washed with a further 5ml of 5% (w/v) trichloroacetic acid, dried in scintillation vials at 60°C for 1 hr and radioactivity measured by scintillation counting in 10ml toluene based scintillator.

3.9: b) : Chemical Measurements

i. DNA was assayed as described by Burton (1956).

ii. RNA was estimated as described by Kerr and Seraidarian (1945).
3.10: DETECTION OF ABUNDANCE CLASSES OF mRNA POPULATION
BY CLONED LIBRARY OF COMPLEMENTARY DNA

A partial cDNA library consisting of 300 sequences
derived from normal rat liver polysomal Poly(A)$^+$RNA, and
cloned in pBR322 was prepared and provided by Ms. R. Shott
of Beatson Institute of Cancer Research, Glasgow. The
recombinants were maintained in 96 well microtitre plates
and were used for the detection of various abundance classes
of mRNA population.

This procedure required the following steps:

i. Preparation of filter papers having
clones on them.

ii. Hybridization of cDNA to the clones and
analysis by autoradiography.

3.10.1: SCREENING THE LIBRARY

Agar plates were prepared by pouring 50ml of Agar-broth
(containing 10g Bacto-tryptone, yeast extract 5g, 10g NaCl
and 15.0g Agar in 1 litre of water) in large plates. To
prevent agar from adhering to the nitrocellulose filters
when it was lifted from the plate, plates were set on edge
overnight to drain excess liquid.

Nitrocellulose filters were cut from rolls (HAMP 000 10)
of Millipore filter paper (type HA, pore size 0.45μm) to fit
easily over the agar plate. These filters were autoclaved and
then laid on the agar. Colonies from the 96-well microtitre
plate were transferred onto the filter paper, using a transfer
plate, and were left overnight to allow the colonies to grow on the filter.

DNA was denatured and bound to the filters by the method of Jeffreys and Flavell (1977) as follows:

Colonies grown overnight on filters, were lysed with 3mg/ml lysozyme in 10mM Tris-HCl pH 8.0, 4% (w/v) sucrose for 10 mins. (This lysis and other steps, except where it is mentioned, were carried out by soaking a large Wattman filter paper with the solution and then the filters with colonies were laid on them for the time mentioned). They were then washed twice with 0.1% Triton X-100 in 0.5 N NaOH to remove proteins etc., and neutralized with three 10 min washes of 1M Tris-HCl pH 7.6.

Finally, they were rinsed, first in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.6 for 5 mins (as for previous steps) and then in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). After the rinse, filters were dried in air and baked at 80° for 2 hours.

To prepare the filters for hybridization to a labelled probe, they were wetted in about 30ml per filter of 3 x SSC for 15 mins at 65°C and then washed for 15 mins at 65°C in 20ml per filter of 3 x SSC. Pre-hybridization was performed with continuous agitation for at least 1 hr at 65°C in 10ml per filter of a solution containing 3 x SSC, 10 x Denhardt's solution (1 x Denhardt's contains 0.02% Bovine serum albumin (BSA); 0.02% Ficoll, 0.02% Polyvinyl pyrrolidone titrated to pH 7.0), 0.1% SDS, 10μg/ml poly(A), 10μg/ml poly C and 100μg/ml
salmon sperm DNA. The pre-hybridization and the subsequent hybridization to $^{32}$P-labelled probes were carried out in a thermally sealed plastic bag.

3.10.2: SYNTHESIS OF COMPLEMENTARY DNA.

Complementary DNA was synthesized as already described in section 3.7.1.; reaction mixture was 100μl and contained $[^{32}\text{P}]$dCTP (100μCi) instead of $[^{3}\text{H}]$dCTP and dGTP, dTTP, dATP, concentrations were reduced to 1mM each. Thirty units of reverse transcriptase were used for the reaction and 1μg of polysomal Poly(A)$^{+}$RNA was used as template. After incubation for 1½ hr at 37°C, the reaction was stopped by adding 1/10th the volume of 10mM EDTA pH 7.0, SDS to 0.1%. 50μg salmon sperm DNA was added as carrier and NaOH was added to 0.1 N and incubated for 1 hr at 37°C to digest the mRNA. After the incubation, the reaction mixture was chilled and neutralized with 0.1 N HCl in the presence of phenol red indicator.

This neutralized mixture was layered over Sephadex G-100 column and fractionated with 1 x SSC. Fractions containing cDNA were pooled and used for hybridization.

3.10.3: HYBRIDIZATION AND WASHING OF FILTERS

Hybridization was carried out, as described above, in thermally sealed plastic bags, by the addition of the cDNA probe at 1.0 x 10$^6$ c.p.m./ml to the hybridization mixture. Hybridization was carried out for 16 hours at 65°C.

After hybridization, the filters were washed once by
agitation in 15ml/filter of 3 x SSC, 10 x Denhardt's solution, 0.1% SDS, 50μg/ml salmon sperm DNA at 65°C for 30 mins; three times in a similar volume of 3 x SSC, 0.1% SDS at 65°C for 15 mins; twice in 0.1 x SSC, 0.1% SDS for 15 mins; and once in 3 x SSC at room temperature. In some cases, more stringent washing conditions (0.5 x SSC) were used as a final step. The filters were blotted dry, mounted in a cassette having intensifying screens and exposed to preflashed Kodak XR5 X-Ray films. The exposure was usually for 1-4 days at -70°C.
agitation in 15ml/filter of 3 x SSC, 10 x Denhardt's solution, 0.1% SDS, 50μg/ml salmon sperm DNA at 65°C for 30 mins; three times in a similar volume of 3 x SSC, 0.1% SDS at 65°C for 15 mins; twice in 0.1 x SSC, 0.1% SDS for 15 mins; and once in 3 x SSC at room temperature. In some cases, more stringent washing conditions (0.5 x SSC) were used as a final step.
The filters were blotted dry, mounted in a cassette having intensifying screens and exposed to preflashed Kodak XR5 X-Ray films. The exposure was usually for 1-4 days at -70°C.
RESULTS
1. CHARACTERIZATION OF LIVER POLYSOMAL POLY(A)⁺RNA

1.1: ISOLATION AND CHARACTERIZATION OF LIVER POLYSOMES

The size of liver polysomes was studied by examining their distribution on sucrose density gradients. Fig. 1 shows the profile of a preparation from normal liver and reveals a wide range of sizes including several peaks sedimenting at greater than 100 S. The figure also shows a preparation from adrenalectomized rat liver in which the polysomes were, on average, slightly smaller. The size distribution of polysomes from adrenalectomized rats which had been given dexamethasone and those isolated from regenerating liver did not differ significantly from those of normal animals.

1.2: ISOLATION OF LIVER POLYSOMAL RNA

The occurrence of high levels of RNase in liver presents a serious problem for the isolation of intact mRNA, therefore a number of isolation procedures were tested so that a method could be found for routine preparation of intact Poly(A) containing species. Some of the methods tested included the extraction of cytoplasmic RNA from post-mitochondrial supernatant, from mRNP particles and polysomal pellets with or without proteinase K digestion of the polysomal pellet.

Analysis of methods employed, the yield of polysomal RNA and of polyadenylated species is presented in Table 2. With the procedures involving differential extraction with phenol:chloroform:iso-amyl-alcohol at neutral and alkaline
Figure 1
Polysome Profile of Rat liver

4-8 A$_{260}$ nm Units of liver polysomes were sedimented through 15-45% (w/v) Sucrose gradients in 5mM Tris-HCl, pH 7.6, 250 mM KCl, 5 mM MgCl$_2$, 50 µg/ml heparin and 5µg/ml cycloheximide at 234,000 x gav for 35 min at 4°C. Gradients were scanned at 260nm with a gradient scanning attachment to the Gilford 240 Spectrophotometer.

---
: Normal rat liver
-.............. : Adrenalectomized rat liver
Table 2: Extraction of Polysomal RNA and Isolation of Poly(A)$^+$RNA.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>Total Polysomal RNA (μg)</th>
<th>Poly(A)$^+$RNA by Poly(U) Sepharose Chromatography, % of Polysomal RNA</th>
<th>Poly(A)$^+$RNA by Oligo(dT) Cellulose chromatography, % of Polysomal RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol extraction of Post-mitochondrial RNA</td>
<td>480.0</td>
<td>2.6%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Phenol extraction of RNA in polysomal pellet without Prot. K digestion</td>
<td>560.0</td>
<td>2.5%</td>
<td>2.8%</td>
</tr>
<tr>
<td>Phenol extraction of RNA in polysomal pellet with Prot.K digestion</td>
<td>500.0</td>
<td>3.0%</td>
<td>2.9%</td>
</tr>
<tr>
<td>CsCl extraction of RNA in polysomal pellet with Prot.K digestion</td>
<td>600.0</td>
<td>3.0%</td>
<td>3.0%</td>
</tr>
<tr>
<td>CsCl extraction of RNA in polysomal pellet without Prot.K digestion</td>
<td>750.0</td>
<td>3.0%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

The Purification of Liver Poly(A)$^+$RNA from Polysomes

Polysomal RNA was prepared from liver as described in Materials and Methods section. Poly(A)$^+$RNA was purified by Poly(U) sepharose or Oligo(dT) cellulose chromatography. RNA was measured by taking absorbance at 260nm and taking 1.0 O.D. Unit equal to 40μg of RNA.
pH, a large proportion of RNA from the post-mitochondrial supernatant was obtained. There are, however, several drawbacks to procedures using phenol as the deproteinizing agent. First, there is evidence to suggest that phenol causes aggregation of RNA (McNaughton et al., 1974), which has important consequences when the covalent molecular weight is determined; namely, that aggregation gives rise to a spuriously high molecular weight. Second, there are indications that phenol removes Poly(A) sequences from the 3'-end of polyadenylated mRNA molecules (Perry et al., 1972). Third, phenol procedures involve several steps and manipulations, so increasing the time during which ribonucleases may possibly attack RNA.

Glisin et al. (1974) described a technique for preparing RNA by centrifugation in CsCl. The principle of technique lies in the fact that protein, DNA and RNA have different buoyant densities in CsCl. If the density of CsCl solution is chosen correctly, centrifugation causes proteins and polysaccharides to float and DNA to be banded in the solution, while RNA forms a pellet.

The procedure chosen to prepare RNA from polysomal pellet is a variation of the method of Glisin et al. (1974), (as described in Methods section 3.3.2).

The technique offers several advantages over others:

1. It is a rapid, one step purification;
2. Poly(A) sequences are not removed from the 3'-end of mRNA molecules;
iii. The RNA is not aggregated, and
iv. Yields are extremely high, usually in the region of 90%.

The least efficient of methods tested for recovery of polyadenylated RNA was that which involved the isolation of Poly(A) rich mRNP particles. The dissociation procedures using EDTA and lauroyl-sarcosine which had been used successfully in isolating polyadenylated RNA species in a number of cultured cells (Paderson and Lindberg, 1972; Adesnik et al. 1972) also proved inefficient for dissociating liver polysomes and resulted in low recovery of polyadenylated mRNA.

1.3: ELECTROPHORETIC ANALYSIS OF LIVER POLYSOMAL RNA

The integrity of total polysomal RNA prepared by CsCl extraction was verified by polyacrylamide gel electrophoresis. (Figure 2), which shows that all the major polysomal RNA species were intact. Figure 3 illustrates analysis by polyacrylamide-formamide gel electrophoresis of the polyadenylated RNA of liver polysomes after one and two passages through Oligo(dT)-cellulose. The product contained no visible contamination with ribosomal RNA, even after only one passage through Oligo(dT)-cellulose, provided the affinity column was extensively washed as described in Methods section. As a precaution, however, the polyadenylated RNA was routinely purified by two passages over Oligo(dT)-cellulose.

Polysomal RNA was prepared from adult normal, regenerating or adrenalectomized rat liver, weighing 200 - 250 gm.

Approximately 40μg of RNA was electrophoresed through 2.7% polyacrylamide aqueous gels for 2½ hrs at 5mA/gel. Gels were soaked in distilled water for 15 min before scanning at 260nm with a Gilford 240 Spectrophotometer gel scanning attachment.
Size distribution of Polysomal Poly(A)$^+$RNA from liver purified by Oligo (dT)-Cellulose column chromatography.

Poly(A)$^+$RNA was purified by oligo (dT)-cellulose chromatography and further purified by a second passage through regenerated or fresh oligo (dT)-cellulose columns.

Approximately 40μg of RNA was used for electrophoresis, and RNA was resolved on 3.5% polyacrylamide gels containing 98% formamide, for 2½ hr at 5 mA/gel. Gels were washed with two changes of water before scanning at 260nm with a Gilford 240 Spectrophotometer gel scanning attachment.

--- : Polysomal Poly(A)$^+$RNA after one step purification;  
-----: Polysomal Poly(A)$^+$RNA after two step purification through oligo (dT)-cellulose columns.
1.4: POLY(A) SIZE AND CONTENT OF LIVER POLYADENYLATED POLYSOMAL RNA

To estimate size and content of Poly(A) in liver polysomal Poly(A)$^+$RNA (mRNA), preparations, derived from various stress conditions, were digested with T$_1$ RNase plus pancreatic RNase A under conditions that prevent over-splitting of adenylate residues in Poly(A) (Dubroff and Nemer, 1975). The products were purified by CsCl extraction and resolved on 2.7% aqueous gels for 3 hours. Figure 4 shows the profile of RNA extractable from the gels and able to form hybrids with $[^3]$H-Poly(U). The peak of radioactivity in both preparations was around the 5.8S rRNA marker of 150-160 nucleotides. To determine the number of average Poly(A) length, the positions of homopolymeric markers and the 5.8S rRNA on parallel and identical gels were joined by a line of best fit by the method of least squares and a standard calibration curve of the log molecular weight versus distance of electrophoretic mobility drawn (Figure 5). From the plot it could be estimated that the number average Poly(A) length for the normal liver mRNA (and regenerating rat liver mRNA, data not shown) was 160 nucleotides, while the adrenalectomized rat liver was 150 nucleotides.

This estimate of mRNA size was confirmed by employing sucrose density gradient sedimentation of Poly(A) homopolymers in 15-35% (w/v) sucrose gradients in 0.1M NETS buffer (0.1M NaCl, 1mM EDTA, 10mM Tris-HCl pH 7.4, 0.2% (w/v) SDS) in a Beckman SW40 rotor. Centrifugation was at 20,000 r.p.m. for 6 hrs at 4°C and fractions containing Poly(A) nucleotides were again located by $[^3]$H-poly(U) hybridization.
Figure 4: Size distribution of Poly(A) from Liver Polysomal Poly(A)^+RNA

Poly(A) tracts were prepared from normal, and 14 days adrenalectomized rat livers polysomal RNA by digesting Poly(A)^+ RNA with Ribonucleases as described in Materials and Methods section.

The nuclease-resistant adenylate core was resolved by 2.7% polyacrylamide gel electrophoresis for 3 hrs at 5mA/gel. After electrophoresis, gels were sliced and fractions were eluted and hybridized to [3H]-Poly(U) to quantitate the Poly(A). Formamide-denatured 32P-labelled 5.8S rRNA was resolved on a parallel gel to provide 5.8S markers.

(A) : Poly(A) from normal rat liver polysomal Poly(A)^+RNA.

(B) : Poly(A) from 14 days adrenalectomized rat liver polysomal Poly(A)^+RNA.

---: [3H]-Radioactivity per slice.

---: [32P]-Radioactivity per slice.
Figure 5: **Electrophoretic Mobilities of Nucleotide Markers on Polyacrylamide Gels**

The figure illustrates the relationship between logarithm of average molecular weight of homopolymeric nucleotides and 5.8 S rRNA, and electrophoretic mobility on 2.7% polyacrylamide gel. Each homopolymeric nucleotide and formamide-denatured 5.8 S rRNA was resolved on separate gels at 5mA/gel for 3 hr. The peaks were located by measuring extinction at 260nm and are indicated by arrows.

a : Poly A_____ (220)

b : 5.8 S rRNA

c : Poly A_____ (95)

d : Oligo A_____ (28)
FIGURE 6: THE SIZE DISTRIBUTION OF POLY(A) FROM LIVER
POLYSOMAL POLY(A)RNA DETERMINED BY SUCROSE GRADIENTS

Poly(A) tracts were prepared from normal rat liver polysomal RNA by digesting Poly(A)RNA with Ribonuclease as described in materials and methods section.

The nuclease-resistant adenylate core was resolved by 15 - 35% (W/V) Sucrose gradients at 20,000 rpm for 6 hr at 4°C and fractions containing Poly(A) nucleotides were located by $^{3}$H-Poly(U) hybridization.

Poly(A) of known length were used as markers on separate gradients and their positions were also determined by $^{3}$H-Poly(U) hybridization.

A: Poly(A) \[ \text{220} \]
B: Poly(A) \[ \text{95} \]
### Table 3. Size and Poly(A) Content of Polysomal Poly(A)$^+$RNAs from Liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Polysomal RNA Bound to Oligo(dT)-Cellulose (a)</th>
<th>Number Average Length of Poly(A) Tract (Nucleotides)(b)</th>
<th>Poly(A) Content % (c)</th>
<th>Molecular Weight (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Liver</td>
<td>2.8%</td>
<td>160</td>
<td>8.0</td>
<td>6 x 10$^5$</td>
</tr>
<tr>
<td>Regenerating Liver</td>
<td>3.0%</td>
<td>160</td>
<td>8.5</td>
<td>6 x 10$^5$</td>
</tr>
<tr>
<td>Liver from Adrenalectomized</td>
<td>2.2%</td>
<td>150</td>
<td>7.0</td>
<td>6 x 10$^5$</td>
</tr>
</tbody>
</table>

(a): RNA was quantified by absorbance at 260 nm assuming that 1.0 OD = 40 μg RNA. Percentage of polysomal RNA as poly(A)$^+$RNA was determined at 260 nm from the fraction which bound second time to Oligo (dT)-cellulose.

(b): Number average size was determined by hybridization of $[^3]$H-poly(U) to extract of gel slice containing adenylate nucleotides. The size was referred to poly(A)$^+$poly(A)$^{95}$ and Oligo(A)$^{28}$.

(c): Poly(A) content was determined by hybridization of 4-10 μg poly(A)$^+$RNA to excess $[^3]$H-poly(U) as in Bantle et al., (1976).
Homopolymers of known length were used as markers on separate gradients and their position was also located by the hybridization to $^{3}$H-poly(U). The distribution of hybridizable $^{3}$H-poly(U) across the gradient gave the molecular weight distribution of the Poly(A) and the length of unknown segments was determined by comparison with the position of known length homopolymers as described by Spirin (1963).

Results obtained by this procedure are shown in Figure 6. It is clear from this method that the average length of Poly(A) segment, obtained from normal liver mRNA, is 160. While the adrenalectomized rat liver mRNA has on average 145-150 nucleotides. Results obtained by this method are thus similar to those obtained by electrophoretic mobility procedure.

The Poly(A) content of intact mRNA was quantified by hybridization of liver mRNA to excess $^{3}$H-poly(U). Comparison with $^{3}$H-poly(U) hybridization to authentic Poly(A) homopolymers gave a Poly(A) content for 3 separate preparations of mRNAs in the range of 7.5% to 8.2%.

These results, along with the estimated average size of Poly(A) sequences are summarised in Table 3. The average molecular weight of mRNA is estimated at $6 \times 10^5$.

2. CONDITIONS FOR HYBRIDIZATION STUDIES BETWEEN LIVER POLY(A)$^+$ RNA AND COMPLEMENTARY DNA.

The effect of hypertrophy induced by partial hepatectomy and the effect of adrenalectomy on the number of genes transcribed was investigated by mRNA-cDNA hybridizations. This technique was also used to investigate sequence homology of
Figure 7: Alkaline Sucrose Gradients of cDNA

cDNA derived from Poly(A)^+RNA of normal rat liver (▲▲▲▲); 12 hr regenerating liver (■■■■); 14 days adrenalectomized liver (●●●●) and globin mRNA (○○○○) were sedimented through 4-11% (w/w) sucrose gradients in 0.9 M NaCl, 0.1M NaOH at 95,000 x g_av for 24 hr at 20°C. 1ml fractions were collected and radioactivity counted as described in Materials and Methods section. Sedimentation coefficients were determined by the method of Steensgard et al. (1978).
Characterization of complementary DNA

Radioactivity $[^{3}H] - cpm \times 10^3$

Sedimentation coefficient

Fraction number
mRNA to determine the distribution of sequences into different mRNA abundance classes.

2.1: PREPARATION OF COMPLEMENTARY DNA

For the preparation of cDNA, purified and salt free Poly(A)\(^+\) RNA was incubated with purified reverse transcriptase in a buffered medium containing KCl, Mg\(^{2+}\), dithiothreitol, the deoxy nucleotide triphosphates, \(^{3}\text{H}\)-dCTP and Oligo(dT) primer, 12-18 residues long. Actinomycin D was also included to ensure that the product was single stranded together with RNase inhibitor, which was found to improve the yield and quality of the product. The yield of cDNA prepared by this method was 6-10\% by weight of the template Poly(A)\(^+\)RNA. The concentrations of deoxynucleotide triphosphate and \(^{3}\text{H}\)-dCTP used, resulted in the incorporation into cDNA of 5-7 x 10\(^7\) c.m.p/\(\mu\)g at 30\% counting efficiency for \(^{3}\text{H}\).

When the products were characterized on alkaline sucrose gradients (Figure 7) the mean size of the cDNA transcribed from Poly(A)\(^+\)RNA was approximately 6.0S, which corresponds to nearly 1.4 x 10\(^5\) daltons or 500 nucleotides. Only cDNA's sedimenting between 5S and 10S were recovered and used in hybridization studies of sequence complexities and diversity. Although the number average molecular weight of liver mRNA was on average at least three times the length of globin mRNA, the cDNA transcripts were of similar length.
Assay of S1 Nuclease Activity

S1 Nuclease was obtained from Sigma. Hybrids of Globin mRNA - \( [^{3}H]cDNA \), were isolated as described in materials and methods from hydorylpatite. The hybrid or single stranded globin \( [^{3}H]cDNA \) was incubated with 8(●) and 20(○) Units of nuclease in 100 μl digestion buffer containing 70mM Sodium acetate pH 4.5, 2.8 mM ZnSO₄, 0.14 M NaCl and 14 mg denatured calf thymus DNA at 37°. At various time of incubation, an aliquot was removed to determine total radioactivity and a further portion to determine acid-soluble radioactivity, as described in materials and methods section. S1 Nuclease activity is expressed as percentage digestion.

- : Double stranded Nucleic acid with 8 Units of S1.
- : Double stranded Nucleic acid with 20 Units of S1.
- : Single stranded Nucleic acid with 8 Units of S1.
- : Single stranded Nucleic acid with 20 Units of S1.
2.2 : REVERSE TRANSCRIPTION OF POLY(A)$^+$RNA.

The observation that the yield of cDNA was 6-10% by weight of template and that the mean length of cDNA was 20% the size of the liver Poly(A)$^+$RNA suggested that the bulk of the mRNA species were represented in the population of cDNA molecules synthesized. This satisfied the condition that for hybridization experiments, it is essential that all mRNA representative of each of the frequency classes is transcribed into cDNA. It has been shown, by others, that reverse-transcriptase is capable of copying most, if not all, Poly(A)$^+$RNA (Harrison et al. 1974; Bishop et al. 1974).

2.3 : ASSAY OF HYBRIDIZATION REACTIONS

The proportion of cDNA which hybridizes to RNA can be estimated in a variety of ways of which digestion with a single-stranded specific nuclease was the method chosen for the current analysis (Birnie et al. 1974; Young et al., 1974).

Since commercial S1 nuclease is known to vary in quality, it was felt necessary to characterize the enzyme with respect to optimum concentrations and digestion time so that double stranded cDNA-mRNA hybrids were not digested. S1 nuclease from Sigma Chemical Co., London, has been used for all the experiments here described but it was still found necessary to characterize every new batch. Figure 8 shows the characterization of a current batch of enzyme which is used at 8 units per assay at 37°C and digestion time of 2 hours.
3. KINETICS OF HYBRIDIZATION

3.1: HYBRIDIZATION OF cDNA WITH HOMOLOGOUS RNA

The cDNA derived from different liver Poly(A)$^+$ RNAs, as well as that derived from globin mRNA, were hybridized to their own templates and the kinetics of hybridization assayed by resistance to S1 nuclease digestion. Under the hybridization conditions used, Harrison et al. (1974) and Birnie et al. (1974) showed that the RNA-cDNA represented reasonably faithful copies of its own template.

It is noteworthy, however, that under these conditions a variable proportion of cDNA comprising 15-25% of the cDNA was non-hybridizable. This did not affect complexity determinations since $R_0^{\frac{1}{2}}$ (where $R_0$ is initial concentration of RNA in moles of nucleotides per litre and $t_\frac{1}{2}$ is time of half reaction in seconds) values were calculated on the basis of hybridizable proportions. Under conditions of large RNA excess, such a reaction has pseudo first-order kinetics and the rate of hybridization, when measured in terms of $R_0$, has been shown to be proportional to the base sequence complexity of the RNA population (Birnsteil et al. 1972; Young and Paul, 1973; Young et al. 1974). The base sequence complexity of an unknown RNA population may be determined by deriving $R_0^{\frac{1}{2}}$ values for the reaction between the RNA and its cDNA and comparing it with the $R_0^{\frac{1}{2}}$ obtained with a kinetic standard of known base sequence complexity (Birnsteil et al. 1972; Bishop et al. 1974; Getz et al., 1975; Birnie et al. 1974; Sippel et al. 1977; Balmain et al. 1980). The standard used was globin mRNA consisting of $\alpha$ and $\beta$ globin sequences with a
Legend to Figure 9: Kinetics of Hybridization of cDNA
With Homologous Poly(A)+RNA

Homologous hybridization of the cDNA to its template
mRNA was described in experimental section.

(▲▲▲): Hybridization of globin cDNA with excess
of template.

(●●●●): Hybridization of normal rat liver cDNA with
excess of template.

The RNA concentrations used were (log Rot -4 to -2)
1μg/ml; (log Rot -2 to 0) 0.5 mg/ml; (log Rot 0-2) 1mg/ml;
(log Rot 2-3) 5mg/ml. Each point contained 2000 c.p.m. of
[^3]H-cDNA recovered from gradients fractions 5S - 10S (Fig.7).
% hybridization was assayed by resistance to S1 nuclease
digestion (see Materials and Methods section), and Rot ½
values produced by computer analysis are indicated by:→.
Figure 10: Kinetics of Hybridization of Regenerating Liver cDNA.

All the experimental conditions are as described in the Legend for Figure 9.

○ ○ ○: Hybridization of 12 hr regenerating rat liver cDNA with excess of template.

-----: Homologous hybridization of normal rat liver cDNA, redrawn from Figure 9.
Hybridization of regen. Liver cDNA to own template

% HYBRIDIZATION

LOG Rot (mole/sec. l^-1)
Figure 11: Kinetics of Hybridization of Adrenalectomized Rat Liver cDNA.

■ ■ ■: Hybridization of 14 days adrenalectomized rat liver cDNA to excess of own template; broken line represents the normal rat liver homologous curve, redrawn from Figure 9.

All other experimental conditions are as described in the Legend to Figure 9.
Hybridization of adrenalectomized rat liver cDNA to own template
combined sequence complexity of $4 \times 10^5$ daltons (Williamson et al. 1971). The globin mRNA hybridized to its cDNA within 1.5 to 2 log units (Figure 9), a value typical of a single abundance class. Computer analysis of the data gave a $R_{\text{A}}^{1/2}$ value of $4 \times 10^{-3}$, which was consistent with the findings of Birnie et al. (1974), Young et al. (1974), Getz et al. (1975) and Brown and Balmain (1979).

The computer fitted curves for the hybridizations of the liver cDNAs to their own template Poly(A)$^+$ RNAs are illustrated in Figure 9, 10 and 11 and covered a range of at least 6 log units. These prolonged hybridization kinetics imply that a heterologous population of liver mRNA sequences were present in varying concentrations (Figure 9). Under these conditions Bishop et al. (1974) and Hastie and Bishop (1976) have shown that polysomal Poly(A)$^+$ RNA could be resolved into three abundance classes. In a similar manner, computer analysis of the data for normal liver (Figure 9) revealed that a good fit to the experimental data points was obtained by a three component curve. Hybridization between Poly(A)$^+$ RNA derived from 12 hr regenerating liver and a homologous cDNA population was also best fitted by a three component curve (Figure 10).

Conversely, when the data obtained from the hybridization of adrenalectomized rat liver polysomal Poly(A)$^+$ RNA to its own cDNA, was analysed on the computer, the best fit to the experimental data points was obtained by a two component system (Figure 11).
3.2: SEQUENCE COMPLEXITY AND DIVERSITY OF LIVER POLY(A)*

RNA.

Numerical evaluation of the hybridization kinetics of the figures 9 to 12 are summarised in Table 4.

The base sequence complexity of an RNA population can be calculated from the kinetics of its hybridization with a cDNA copy of the RNA in comparison with the rate of a standard mRNA-cDNA reaction. The standard used in these experiments was the reaction of globin mRNA with its cDNA. This reaction has a Rot of 4 x 10^-3 moles sec./l (Figure 9). The base sequence complexity of unknown RNA population = Rot (of unknown RNA) x 4 x 10^5, assuming a base sequence complexity of 4 x 10^5 daltons for globin mRNA.

In order to calculate the base sequence complexity of one component from a reaction involving two or more components, the proportion of the total hybridizable cDNA which is contained in that component must be taken into account. Hence, the base sequence complexity of the abundant component of the polysomal Poly(A)*RNA (Table 4) = 26.841 x 4 x 10^5 x .80 = 1.0 x 10^6 daltons.

The number of different sequences contained in this population is derived by dividing the base sequence complexity by the average molecular weight of the mRNA, which is taken to be 0.6 x 10^6 daltons (Table 3). The number of average size sequences present in the normal rat liver abundant component = 1.0 x 10^6 = 1.66.
Table 4: Sequence Complexity of Polysomal Poly(A)^+RNA from Rat Liver

The data represents computer analysis of the hybridization kinetics in Figures 8, 9 and 10.

a: Denotes the value of Rot \( \frac{1}{2} \) corrected for the components were analysed as a single class and 100% pure.

b: Taking the molecular weight of the globin mRNA as \( 4 \times 10^5 \) daltons (Williamson et al., 1971).

c: Taking average molecular weight of liver polysomal Poly(A)^+RNA to be \( 6 \times 10^5 \).
Table 4: Sequence Complexity of Polysomal Poly(A)^+RNA from Rat Liver

The data represents computer analysis of the hybridization kinetics in Figures 8, 9 and 10.

a: Denotes the value of Rot $\frac{1}{2}$ corrected for the components were analysed as a single class and 100% pure.

b: Taking the molecular weight of the globin mRNA as $4 \times 10^5$ daltons (Williamson et al., 1971).

c: Taking average molecular weight of liver polysomal Poly(A)^+RNA to be $6 \times 10^5$. 
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Transition</th>
<th>% Hybrid. cDNA</th>
<th>Rot$^{1/2}$ (moles·s·l$^{-1}$) Observed Corrected</th>
<th>Seq. Comp.</th>
<th>No. of div. Seq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Liver</td>
<td>I</td>
<td>8.50</td>
<td>0.120</td>
<td>0.010</td>
<td>1.0x10$^6$</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>24.87</td>
<td>5.00</td>
<td>1.20</td>
<td>1.2x10$^8$</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>36.60</td>
<td>203.30</td>
<td>74.50</td>
<td>7.4x10$^9$</td>
</tr>
<tr>
<td>Regen. Liver</td>
<td>I</td>
<td>8.120</td>
<td>0.140</td>
<td>0.110</td>
<td>1.1x10$^6$</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>27.80</td>
<td>6.50</td>
<td>1.80</td>
<td>1.8x10$^8$</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>29.30</td>
<td>257.500</td>
<td>75.400</td>
<td>7.5x10$^9$</td>
</tr>
<tr>
<td>Liver 14d after adrenalectomy</td>
<td>I</td>
<td>18.600</td>
<td>1.900</td>
<td>0.360</td>
<td>3.6x10$^7$</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>33.500</td>
<td>58.50</td>
<td>22.90</td>
<td>1.9x10$^9$</td>
</tr>
<tr>
<td>Adrenalectomized rat liver</td>
<td>I</td>
<td>28.5</td>
<td>2.35</td>
<td>0.66</td>
<td>6.6x10$^7$</td>
</tr>
<tr>
<td>a) 2 hr after hormone-treatment</td>
<td>II</td>
<td>33.5</td>
<td>138.5</td>
<td>46.40</td>
<td>4.6x10$^9$</td>
</tr>
<tr>
<td>b) 6 hr after hormone-treatment</td>
<td>I</td>
<td>29.10</td>
<td>2.68</td>
<td>0.77</td>
<td>7.7x10$^7$</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>32.90</td>
<td>200.60</td>
<td>65.80</td>
<td>6.5x10$^9$</td>
</tr>
</tbody>
</table>
Figure 12: Kinetics of Hybridization of Hormone Treated Adrenalectomized Rat Liver cDNA with Homologous mRNA.

Homologous hybridization of the cDNA to its own template was described in experimental section.

▲——▲: Hybridization of liver message cDNA of 2 hr corticosteroid (Dexamethasone) stimulated adrenalectomized rats to an excess of template.

○——○: Hybridization of liver message cDNA of 6 hr corticosteroid (Dexamethasone) stimulated adrenalectomized rats to an excess of template. Broken line represents the adrenalectomized rat liver homologous curve, redrawn from Figure 11.

All other experimental conditions are as described in the Legend to Figure 9.
Similar calculations were carried out to determine the number of average size sequences present in intermediate abundant and rare classes in normal, regenerating and adrenalectomized rat livers, and are summarized in Table 4.

It is seen that normal rat liver polysomal Poly(A)⁺RNA contains approximately 2 sequences in very high abundance, 210 sequences of moderate abundance and about 12,400 sequences in the rare or low abundance class of mRNA.

The same data, when analysed by two component systems, gave the total base sequence complexity of about 12,000. A value in close agreement with that observed using a three component system. Best fit curves for both i.e. two and three component systems were obtained after 11 computer iterations.

Figure 12 shows the homologous cDNA:mRNA hybridizations of preparations from the livers of adrenalectomized rats which had received dexamethasone 2 hr and 6 hr before death. The numerical evaluation of these hybridizations is presented in Table 4. The base sequence complexities obtained after 2 hr and 6 hr of hormone-treatment were 7,800 and 11,000 respectively. The results indicate that adrenalectomized rat liver mRNA populations increase after hormone treatment and 6 hr after hormone treatment the complexity of the adrenalectomized rat liver mRNA population was in close agreement to the sequence complexity of normal rat liver.

3.3: BASE SEQUENCE COMPLEXITY DETERMINATIONS BY UNIQUE DNA HYBRIDIZATION

Another of the limitations on the determination of mRNA
complexities by cDNA hybridization technique is that the final frequency class representing the rare or unique sequence is difficult to measure and, because high mRNA concentrations and long periods of time are necessary to ensure complete hybridization, can easily be underestimated. Thus, many of the 6,000 plus mRNA sequences which appear, from Figure 11 and Table 4, to disappear from liver after adrenalectomy, might simply become very much rarer.

A more sensitive method for measuring the complexity of rare mRNA sequences is one that employs unique DNA hybridization. In this method, labelled unique DNA sequences are hybridized to a large excess of mRNA (Gelderman et al., 1971; Galau et al., 1974, 1976).

In view of the findings with cDNA hybridization, it was felt essential to complement the results by evaluating the extent to which unique rat liver DNA sequences hybridized with polysomal Poly(A)$^+$RNA from normal, regenerating and adrenalectomized rat liver.

3.3.1: Isolation of Unique DNA

To determine the extent of unique DNA hybridization to total DNA hydroxyapatite was used to fractionate single and double stranded reaction products. To ensure the reliability of the procedure it was considered necessary to characterize hydroxyapatite before use.
Figure 13: Characterization of Hydroxyapatite Chromatography (HAP)

HAP chromatography columns were set up as described in the Materials and Methods section. Purified, double-stranded and single stranded $[^3H]$-labelled nucleic acids mixture were chromatographed on HAP columns at 60°C. The nucleic acids were eluted in a stepwise fashion with increasing concentrations of sodium phosphate buffer as indicated, 1ml fractions were collected, aliquots removed and radioactivity in each fraction was assayed as described in Materials and Methods section.

a: Chromatographic fractions of 0.14M sodium phosphate buffer.

b: Chromatographic fractions of 0.4M sodium phosphate buffer.
\( ^3 \text{H} \)-radioactivity c.p.m.

Fraction Number
Figure 14: Kinetics of Reassociation of $[^3\text{H}]-\text{uDNA}$

Unique DNA was prepared as described in Methods section, the extent of hybridization was determined by HAP chromatography at 60°C.

The $[^3\text{H}]-\text{uDNA}$ was annealed to total rat liver DNA (● ●). For comparison a renaturation profile of $[^3\text{H}]-\text{total rat liver-DNA}$ is shown (◆◆). The arrows indicate the Cot $\frac{1}{2}$ values for the uDNA component of each hybridization curve.

The hybridizations were performed in HB/F at 43°C at DNA concentrations of 10mg/ml and 20mg/ml. The mass ratio of driver DNA to $[^3\text{H}]-\text{uDNA}$ was 10,000:1.
3.3.1.1: Characterization of Hydroxyapatite.

The hydroxyapatite (HAP) was characterized with respect to its binding capacity and the phosphate buffer concentration effective in eluting single-stranded and double-stranded nucleic acids. Figure 13 shows that almost all single-stranded material was eluted with 0.14 M sodium phosphate buffer pH 6.8 and the double-stranded material was effectively eluted with 0.4 M sodium phosphate pH 6.8. In excess of 90% of each fraction of radioactive standards was recovered. The low radioactivity recovered in the wash fraction implied that there was no degradation during the chromatography.

3.3.2: Reassociation of Unique Labelled DNA

A sample of isolated labelled unique DNA was annealed with a 10,000 fold excess of unlabelled whole rat-liver DNA. The percentage of unique DNA hybridized at each Cot (moles. sec. l^-1) value was determined by hydroxyapatite chromatography. Figure 14 shows the resulting curve compared with the reassociation curve of unfractionated DNA.

At Cot values of up to 30 the [^3H]-unique DNA probe shows no hybridization, while some of the repetitive sequences in the total DNA have hybridized by this Cot value. The unique DNA (uDNA) probe was able to hybridize to about 72%. The Cot_1 of 1600 moles. sec. l^{-1} compares favourably with the Cot_1 of the unique portion of the whole DNA (1200 moles. sec. l^{-1}), and the difference between the two values can be explained by the average size of the [^3H]-uDNA (200 nucleotides) being smaller than that of total DNA preparation (300-400 nucleotides).
Figure 15: Ultraviolet Absorption Spectra of Mercurated Poly(A)^+RNA.

An aliquot of salt-free, mercurated Poly(A)^+RNA was dissolved in H₂O and u.v. spectra was recorded using a Unicam-sp 2000 Spectrophotometer.
Figure 16: Saturation Hybridization of Unique[^3H]-Labelled DNA to Polysomal Poly(A)^+RNA.

[^3H]-Labelled unique DNA was hybridized to saturation with mercurated polysomal Poly(A)^+ mRNA and analysed by thiol-sepharose chromatography as described in Methods section.

Curve A: Shows the hybridization of normal rat liver mRNA to unique DNA (○). It also represents the background values obtained when E. coli RNA was hybridized to rat liver unique DNA (△).

Curve B: Shows 12 hr regenerating rat liver mRNA (○). The broken line represents E. coli RNA reproduced from Curve A.

Curve C: Shows the hybridization curve of 14 days adrenalectomized rat liver mRNA (○) and dashed line represents the curve of normal rat liver mRNA reproduced from curve A.

Hybridizations were performed in HB/F at 43°C at RNA concentrations of 10mg/ml and 20mg/ml. RNA:[^3H]-unique DNA ratios were 500:1 and 1000:1 respectively.
Table 5

Sequence complexity of polysomal Poly(A)$^+$RNA of rat liver determined by hybridization to unique DNA.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Observed % saturation of $[^3H]^{-}$ Unique DNA</th>
<th>Corrected$^{(a)}$ saturation value</th>
<th>Complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat liver</td>
<td>1.00</td>
<td>1.38</td>
<td>29,000</td>
</tr>
<tr>
<td>12-hr Regenerating liver</td>
<td>1.00</td>
<td>1.38</td>
<td>29,000</td>
</tr>
<tr>
<td>14-days adrenalectomized rat liver</td>
<td>0.75</td>
<td>1.04</td>
<td>22,000</td>
</tr>
</tbody>
</table>

The above data represent the numerical evaluations of the kinetics of saturation hybridization of $[^3H]^{-}$-DNA with Poly(A)$^+$RNA from figure .

a,b = Taking the molecular weight of the haploid rat genome to be $1.8 \times 10^{12}$ and assuming 70% of the DNA consists of unique sequences. Correction was also made for the 71% hybridizability of the $[^3H]^{-}$-unique DNA probe.
3.3.3: Hybridization of Unique \(^3\text{H}\)-Labelled DNA to Liver Polysomal Poly(A)\(^+\)RNA

The RNA used for hybridization was mercurated. The intactness of RNA, after mercuration, was analysed by an absorbance scan. The absorption maxima remained at 260nm (Figure 15), thus indicating the intactness of bases of the mercurated polysomal Poly(A)\(^+\)RNA.

The mercurated mRNA was hybridized to \(^3\text{H}\)-uDNA in vast excess (10\(^3\) to 10\(^4\) fold). The kinetics of the hybridization reactions are shown in Figure 16 as computer generated least-square fit of the data and the numerical evaluation of Figure 16 is given in Table 5. Saturation hybridization of the liver Poly(A)\(^+\)RNA from normal, 12 hr regenerating and 14 days adrenalectomized rat liver occurred with 1.0%, 1.0% and 0.75% of the unique DNA respectively. Since the \(^3\text{H}\)-uDNA was 72% hybridizable (Figure 14) when reassociated with total rat liver DNA, these saturation values were corrected to 1.38%, 1.38% and 1.04% respectively.

The average molecular weight of the rat liver haploid genome is 1.8 x 10\(^{12}\) (Sober, 1968), 70% or 1.26 x 10\(^{12}\) of which is unique sequences. Thus, the molecular weight of unique DNA expressed in the normal and regenerating liver is 0.0138 x 1.26 x 10\(^{12}\) which is equal to a molecular weight of 1.73 x 10\(^{10}\) or the equivalent 1.73 x 10\(^{10/6}\) x 10\(^5\) = 29,000 diverse sequences.

In the adrenalectomized rat liver the total base sequence complexity is 0.0104 x 1.26 x 10\(^{12}\) which is equal to a molecular weight of 1.31 x 10\(^{10}\) or the equivalent of 1.31 x 10\(^{10/6}\) x 10\(^5\) = 22,000 diverse sequences.
3.3.4. The Comparison of Polysomal Poly(A)$^+$RNA Populations from Normal and Adrenalectomized Rat Livers by Hybridization to Fractionated Unique DNA.

As discussed above, unique DNA hybridization may give a more accurate assessment of the complexity of mRNA populations. They suffer, however, from the disadvantage that the extent of hybridization, of any given mRNA preparation to total unique DNA, is low and differences between different populations are correspondingly small. One way of avoiding these deficiencies is to isolate the unique DNA sequences which are complementary to one population and to use these when comparing with other populations. It was considered that the above data was of sufficient importance to warrant such isolations.

Normal rat liver polysomal Poly(A)$^+$RNA was hybridised to $[^3H]$-unique DNA and the hybrids were isolated by thiol-Sepharose chromatography (see Methods section 3.7.8.1).

This purified $[^3H]$-uDNA probe was then used for homologous and heterologous reactions against Poly(A)$^+$RNA from normal rat liver and from adrenalectomized rat liver respectively. Figure 17 illustrates the results obtained and it is seen that the two curves are initially very similar but then diverge such that mRNA from normal rat liver is eventually, at a Rot of 11,000, able to saturate 67% of the unique DNA while that from adrenalectomized rat liver is only able to saturate 50%.

The number of RNA sequences determined from this experiment is very close to that obtained by using total unique DNA (Table 5). Normal rat liver polysomal Poly(A)$^+$RNA shows 33,000 sequences while the adrenalectomized rat liver shows 24,000 Poly(A)$^+$RNA
Table 6

Determination of base sequence complexity of Polysomal Poly(A)$^+$RNA by fractionated unique DNA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% of Hybridization</th>
<th>Rot $\frac{1}{2}$ of the curve</th>
<th>Sequence (a) complexity</th>
<th>Number (b) of diverse sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat liver</td>
<td>67%</td>
<td>198.5</td>
<td>$1.98 \times 10^{10}$</td>
<td>33,000</td>
</tr>
<tr>
<td>Adrenalectomized rat liver</td>
<td>50%</td>
<td>144.0</td>
<td>$1.44 \times 10^{10}$</td>
<td>24,000</td>
</tr>
</tbody>
</table>

The above data represent the numerical evaluations of the kinetics of hybridization of fractionated $^3$H-unique DNA with Poly(A)$^+$RNA from figure 17.

(a) : Taking the molecular weight of the globin mRNA as $4 \times 10^5$ daltons (Williamson et al., 1971)

(b) : Taking average molecular weight of liver polysomal Poly(A)$^+$RNA to be $6 \times 10^5$. 

sequences. Thus a difference of 9,000 sequences is again observed (Table 6).

These results indicate that the base sequence complexities of liver Poly(A)$^+$RNA determined by cDNA-mRNA hybridization were indeed underestimated. The data did confirm the finding that the Poly(A)$^+$RNA from the adrenalectomized rat liver was less complex than that from normal rat liver although the difference was nowhere near as dramatic as that observed with cDNA hybridization. Thus the 8,000 sequences missing from adrenalectomized rat liver mRNA when analysed by cDNA hybridization represent 67% of the total population while the 7,000 sequence difference between normal and adrenalectomized rat-liver mRNA populations analysed by unique DNA hybridization represent 25% of the population. As discussed above, this may be due to the different sensitivities of the two systems in the detection of rare sequences and to the fact that cDNA hybridization might lead one to believe a sequence was missing when it had simply become much rarer.

4. POLYSOMAL POLY(A)$^+$RNA SEQUENCES COMMON IN RAT LIVER UNDER DIFFERENT CONDITIONS.

4.1: HOMOLOGY BETWEEN NORMAL AND REGENERATING RAT LIVER mRNA POPULATIONS.

The extent of sequence homology between polysomal Poly(A)$^+$RNA populations of normal and regenerating livers was investigated by heterologous hybridization. A vast excess of mRNA from the regenerating liver was hybridized to cDNA prepared
Figure 18: Kinetics of Hybridization of Normal Rat Liver cDNA with Heterologous mRNA.

Heterologous hybridization of the normal rat liver cDNA to regenerating rat liver mRNA was described in experimental section.

□ ○ ● : Hybridization of normal rat liver cDNA to polysomal Poly(A)+RNA from 12 hr regenerating rat liver.

---- : Represents homologous curve of normal rat liver redrawn from Figure 9.

All other experimental conditions were the same as described in the Legend of Figure 9.
Hybridization of regen. Liver Polysomal poly(A)^+ RNA to normal rat liver cDNA
Figure 19: Kinetics of Hybridization of Regenerating cDNA with Heterologous mRNA.

Heterologous hybridization of the regenerating rat liver cDNA to normal rat liver mRNA was described in experimental section.

◊◊◊: Hybridization of 12 hr regenerating rat liver cDNA to polysomal Poly(A)$^+$RNA from normal rat liver.

-----: Represents homologous curve of 12 hr regenerating rat liver cDNA to own template, redrawn from Figure 9.

All other experimental conditions were the same as described in the Legend of Figure 9.
Hybridization of normal rat liver Polysomal poly(A)$^+$ RNA to regen. Liver cDNA
from the normal rat liver polysomal Poly(A)$^+$RNA and vice versa.

The result of these experiments are shown in Figures 18 and 19. It was observed that the overall extent of hybridization was the same in homologous and heterologous reactions. This indicates that the bulk of the polysomal Poly(A)$^+$RNA in both normal and regenerating rat liver is composed of sequences held in common between them. It was also clear from the data in Figure 18 that the regenerating polysomal Poly(A)$^+$RNA drives the normal rat liver cDNA into hybrid slower than the normal rat liver polysomal Poly(A)$^+$RNA. This indicates that some of the sequences held in common between the two populations are at a lower abundance in the 12 hr regenerating liver. The reverse cross-hybridization of normal liver polysomal Poly(A)$^+$RNA to regenerating liver cDNA might therefore be expected to be faster than the homologous reaction. However, Figure 19 shows that it proceeds at about the same rate. Since it is the abundant sequences which have the greatest influence on the overall kinetics of hybridization, this apparent anomaly could be explained if some sequences which were rare in normal liver become abundant in regenerating liver.

4.2: HOMOLOGY BETWEEN NORMAL AND ADRENALECTOMIZED RAT-LIVER mRNA Populations.

The homology between normal and adrenalectomized rat liver polysomal Poly(A)$^+$RNA was determined by heterologous hybridization, as explained for regenerating rat liver.

Complementary DNA (cDNA) was prepared against polysomal Poly(A)$^+$RNA of normal rat liver. The dashed curve in Figure 20
Figure 20: Kinetics of Hybridization of cDNA with Homologous and Heterologous RNA.

A: Hybridization of liver message cDNA from normal rats to: ◊ ◊ mRNA from adrenalectomized rat livers (◊ ◊ represent two different mRNA preparations), mRNA from normal rat liver (homologous).

Each point contained 2000 c.p.m. $[^3H]$-cDNA and RNA concentrations were the same as Figure 9.
Hybridization of adrenalect. Polysomal poly(A)\textsuperscript{+}RNA to normal liver cDNA
represents the hybridization of this cDNA to its template and has already been described (section 3.2 and Figure 9). The remaining curve in Figure 20 represents a heterologous hybridization in which the cDNA from normal rat liver was hybridized to Poly(A)\(^+\)RNA from adrenalectomized rats. It is seen that the kinetics of hybridization of the heterologous reaction were considerably shifted. At lower Rot values the heterologous reaction is faster than the homologous reaction indicating that some abundant sequences are proportionately even more abundant after adrenalectomy. At higher Rot values, the rate of the heterologous reaction is much slower, implying that common sequences present in the heterologous mRNA population were present in much lower concentrations.

The plateau level attained in such heterologous reactions reflects the extent of common sequences between the different mRNA populations. In the homologous reaction, the mRNA was able to hybridize with its cDNA to maximum value of 77%. Polysomal Poly(A)\(^+\)RNA from 14 day adrenalectomized rat liver was able to form hybrids with the cDNA to about 67%. The number of diverse sequences obtained, by determining the Rot\(^1/2\) values for the heterologous reaction, was about 6,000. This implied that a high proportion of the 14 day adrenalectomized rat liver mRNA population were common but that many sequences were not present in the post-operative animals. This result revealing that up to 6,500 sequences are missing from the liver mRNA population of adrenalectomized rats and that many more sequences become much less abundant is perhaps a way to rationalize the differences between the cDNA hybridization (Figure 11) and unique DNA hybridization results (Figures 16
Figure 21. **Kinetics of Hybridization of Adrenalectomized Rat Liver cDNA to Heterologous mRNA.**

- Hybridization of 14 day adrenalectomized rat liver cDNA to normal rat liver polysomal Poly(A)^+RNA.
- Hybridization of 14 day adrenalectomized rat liver cDNA to own template, redrawn from Figure 11.

All other experimental conditions were as described in the Legend to Figure 9.
and 17).

Figure 21 represents the reverse heterologous reaction, in which normal rat liver mRNA was hybridized to 14 day adrenalectomized rat liver cDNA. It is seen that the overall rate of the heterologous reaction is slightly faster than the homologous reaction, but the plateau levels attained by both reactions were very similar. Thus, although Figure 20 indicates that there are a large number of sequences present in normal liver that are not present in adrenalectomized rat liver, the reverse is not the case. Figure 21 shows that there are few, if any, sequences present in the adrenalectomized rat liver that are not present in the normal rat liver though the different rate of hybridization does suggest the possibility of some quantitative changes.

4.3: HOMOLOGY BETWEEN NORMAL AND HORMONE TREATED ADRENALECTOMIZED RAT LIVER mRNA POPULATIONS.

The homology between normal and hormone treated adrenalectomized rat-liver polysomal Poly(A)^+RNAs was determined by heterologous hybridization, as explained for regenerating rat liver.

Complementary DNA (cDNA) was prepared against polysomal Poly(A)^+RNA of normal rat liver. The dashed curves in Figures 22 and 23 represent the hybridization of this cDNA to its own template and has already been described (Section 3.2 and Figure 9). The remaining curve in Figures 22 and 23 represent heterologous hybridizations in which the cDNA from normal rat liver was hybridized to polysomal Poly(A)^+mRNA.
Figure 22: Kinetics of Hybridization of cDNA with Homologous and Heterologous RNA.

Hybridization of liver message cDNA from normal rats to:

- • • •: mRNA from 2 hr hormone-stimulated adrenalectomized rat livers.

--------: mRNA from normal rat liver (homologous).

Each point contained 2000 c.p.m. [\(^3\)H]-cDNA and RNA concentrations were the same as Figure 9.
Figure 23: Kinetics of Hybridization of cDNA with Homologous and Heterologous RNA.

Hybridization of liver message cDNA from normal rats to:

▲ ▲ ▲: mRNA from 6 hr hormone-stimulated adrenalectomized rat livers.

-----: mRNA from normal rat liver (Homologous).

Each point contained 2000 c.p.m. $[^3]H$-cDNA and RNA concentrations were the same as Figure 9.
from 2 hr and 6 hr hormone treated adrenalectomized rat-livers, respectively.

It is seen that the kinetics of hybridization of the heterologous reaction, in Figure 22 were similar to the heterologous hybridization with mRNA from untreated adrenalectomized rats (Figure 20). Thus, as previously indicated in Figure 12, 2 hr treatment with hormone has not restored mRNA populations to normal. Conversely, after 6 hr hormone treatment the heterologous curve closely resembles the homologous curve (Figure 23) and indicates that, by this time, the mRNA complexity is substantially restored. This again confirms the results of Figure 12.

4.4: **PREPARATION OF ABUNDANT AND RARE CLASSES OF cDNA**

To further investigate the diversity of mRNA populations the cDNAs derived from normal 12 hr regenerating and 14 day adrenalectomized rats were used to prepare fractions enriched in abundant and rare sequences. The fractions were then used in further homologous and heterologous hybridizations.

The cDNAs representing abundant and rare sequences were isolated and purified by thiol-Sepharose chromatography.

The thiol-Sepharose was first characterized with respect to its binding capacity and the temperatures effective in eluting single-stranded and double-stranded nucleic acids. Figure 24 shows that a clean separation of single-stranded and double-stranded nucleic acids was obtained by the procedure which employed a washing step and elution of single-stranded material at 60° followed by the elution of double-stranded
Figure 24: Characterization of Thiol-Sepharose

Thiol-sepharose was prepared and characterized as described in Materials and Methods section.

M: Isolation of single-stranded and double-stranded nucleic acids was performed at ambient temperature.

A - Washing and elution of single-stranded nucleic acids with NETS buffer.

B - Elution of double-stranded nucleic acids with NETS buffer containing 100mM β-mercaptoethanol.

N: Isolation of single and double-stranded nucleic acids was performed at elevated temperature.

A - Washing step at 60°C with NETS buffer.

B - Washing step at 20°C with NETS buffer.

C - Elution of double-stranded nucleic acids at 20°C with NETS buffer containing 100mM β-mercaptoethanol.
material at room temperature. (see Methods section 3.7.6.2). In excess of 90% of each fraction of radioactive standards was recovered. The low radioactivity recovered in the wash fraction implied that there was no degradation during the chromatography.

4.4.1: Kinetics of Hybridization of Fractionated 'Abundant' Polysomal cDNAs.

Abundant sequences of cDNA preparations were isolated by allowing total cDNA to hybridize to total mRNA to a Rot value of 10 moles. sec. 1⁻¹. The single-stranded and double-stranded nucleic acids were then isolated by thiol-Sepharose chromatography. The bound fraction represented the 'abundant' class of cDNA since all the abundant sequences in a homologous reaction are expected to form hybrids at Rot 10 (Figure 9).

The abundant cDNA was freed of mRNA by alkaline digestion and desalted by sephadex chromatography. This purified and desalted abundant cDNA was used in homologous and heterologous hybridizations. The results are shown in Figures 25 and 26. The homologous curve (Figure 25A) where abundant sequences derived from normal rat liver are hybridized to polysomal Poly(A)⁺RNA of normal rat liver reveals a lowered Rot ½ (1.20 moles. sec. 1⁻¹, as compared with 7.2 moles. sec. 1⁻¹ for the abundant component of total cDNA) and earlier completion of hybridization when compared to total cDNA which is consistent with a six fold enrichment in abundant sequences.
Figure 25: **Kinetics of Hybridization of Abundant cDNA Sequences to Homologous and Heterologous Poly(A)$^+$RNA.**

Abundant cDNA sequences were isolated and purified by thiol-sepharose chromatography as described in Methods section.

**A:** The normal rat liver abundant cDNA was hybridized to an excess of rat liver Poly(A)$^+$RNA from ◊ ◊ ◊ normal rats, ◆ ◆ ◆ regenerating rat liver.

Each point contained 2000c.p.m. $^3$H-cDNA and RNA concentrations used were 0.05 mg/ml (up to Rot 1.0) 0.5 mg/ml (from Rot 1.0 to 600)

**B:** Regenerating 'Abundant' cDNA hybridized to: ◆ ◆ ◆ Normal rat liver polysomal Poly(A)$^+$RNA, and ▲ ▲ ▲ Regenerating rat liver polysomal Poly(A)$^+$RNA (Homologous).
Hybridization of normal liver abundant cDNA to own template (0), regen. Liver RNA (+)

A

B
4.4.2: **Kinetics of Hybridization of Regenerating Liver mRNA**

Also shown on Figure 25 is a curve of the hybridization of the cDNA enriched in abundant sequences of normal rat liver polysomal Poly(A)$^+$RNA to total polysomal Poly(A)$^+$RNA from 12 hr regenerating rat liver. The kinetics of hybridization were very similar to the homologous curve but slightly slower, again indicating that the same sequences were present in regenerating tissue but at lower abundance (compare with Figure 10). The very similar extent of saturation reached in the homologous and heterologous curves indicate that few, if any, qualitative differences exist in the two populations. The reverse hybridization, in which abundant cDNA of 12 hr regenerating rat was employed, also revealed no differences (Figure 25B). Thus these experiments with cDNA enriched in abundant sequences confirm the results obtained with total cDNA (Figures 18 and 19).

4.4.3: **Kinetics of Hybridization of 14 day Adrenalectomized Rat Liver mRNA.**

The kinetics of homologous and heterologous hybridizations with the abundant cDNA from normal and 14 day adrenalectomized rat liver polysomal Poly(A)$^+$RNA are shown in Figures 26A, B. It was observed that when normal rat liver cDNA was hybridized to adrenalectomized rat liver polysomal Poly(A)$^+$RNA the rate of reaction was faster than the homologous reaction employing normal rat liver mRNA (Figure 26A).

The kinetics of two reactions (Figure 26A) were one log unit of $R_0$ different, indicating 10 fold, on average, increase
Figure 26: Kinetics of Hybridization of Abundant cDNA Sequences to Homologous and Heterologous mRNA.

All experimental conditions are as for Figure 25, except that:

A: The normal rat liver abundant cDNA was hybridized in excess of liver Poly(A)^+ RNA from: ◦ ◦ ◦ normal rat liver (Fig. 25), and from: ○ ○ ○ 14 days adrenalectomized rat liver.

B: The adrenalectomized rat liver abundant cDNA was hybridized in excess of liver mRNA from: ▲ ▲ ▲ adrenalectomized rat liver, and from ○ ○ ○ normal rat liver.
Hybridization of normal liver abundant cDNA to own template (A); adrenalect. RNA (B)
in concentration of these sequences in adrenalectomized tissue.

The reverse hybridization of adrenalectomized rat liver cDNA to normal rat liver mRNA would be expected to produce the opposite result to Figure 26A, i.e. a slower reaction than the homologous reaction. Figure 26B shows that this is the case; thus re-enforcing the contention that the concentration of abundant sequences are less in the liver of normal rats than in the liver of adrenalectomized rats.

4.5: KINETICS OF HYBRIDIZATION OF FRACTIONATED "RARE" POLYSOMAL cDNAs.

Rare sequences of cDNA preparations were isolated by allowing total cDNA to hybridize to total mRNA to a Rot of 10 moles sec. 1⁻¹. The single-stranded and double-stranded nucleic acids were then isolated by thiol-Sepharose chromatography. Unbound fraction, which was single-stranded, represented the "rare" class of cDNA since the abundant sequences would form hybrids at Rot 10 and would be bound to thiol-Sepharose. The 'rare' sequences were treated with alkali, to remove mRNA, desalted by Sephadex chromatography and further purified by rehybridization to its own template mRNA up to Rot 3,000 followed by isolation of double-stranded material by thiol-Sepharose chromatography. This step removed all the non-hybridizable cDNA, which represent 15-20% of the total population. The bound fraction from the second hybridization was again treated with alkali to remove RNA and was desalted by Sephadex Chromatography.
Figure 27: Kinetics of Hybridization of Rare cDNA

With Homologous and Heterologous Poly(A)$^+$RNA

Preparation and purification of rare cDNA sequences are described in Methods section.

A: The rare normal rat liver cDNA was hybridized to excess normal rat liver polysomal Poly(A)$^+$RNA (● ● ●), and to excess 12 hr regenerating rat liver polysomal Poly(A)$^+$RNA (◇ ◇ ◇).

B: The rare 12 hr regenerating cDNA was hybridized to 12 hr regenerating Poly(A)$^+$RNA (◇ ◇ ◇) and to normal rat liver Poly(A)$^+$RNA (● ● ●). Each point contained 2000 c.p.m. cDNA and 1mg/ml (up to Rot 300) and 5.0mg/ml (Rot 200 onwards).
Hybridization of Polysomal poly(A)+RNA to rare cDNA from normal rat liver, hom(+), het(0) RNA

A

Hybridization of Polysomal poly(A)+RNA to rare cDNA from regen. Liver, hom(0), & het(+)

B
Purified and desalted "rare" cDNA was used in homologous and heterologous hybridizations. The results are shown in Figures 27 to 28. The homologous curve (Figure 27) where rare sequences derived from normal rat liver are hybridized to polysomal Poly(A)$^+$RNA of normal rat liver reveals a lowered Rot$^1_2$ and earlier completion of hybridization when compared to total cDNA which is consistent with a six to seven fold enrichment in 'rare' sequences.

The rare sequences were purified in such a way that they should theoretically hybridize completely to the homologous message. However, all of the rare cDNA fractions prepared in this work and those described by others (Wilkes et al., 1979) contain a substantial component which does not hybridize. The reason for this is not understood.

4.5.1. : Kinetics of Hybridization of Regenerating Rat Liver mRNA

The heterologous curve of Figure 27a and 27b show that whether the 'rare' cDNA was derived from normal liver or regenerating liver it hybridized to polysomal Poly(A)$^+$mRNA from both sources with very similar kinetics. The fact that the rate of these reactions and the final plateau values were very similar, re-enforces the previous finding of Figure 18 that mRNA population of the normal and regenerating tissue are qualitatively and quantitatively very similar. However, both curves indicate that some sequences may be rarer and some more common in the regenerating tissue.
Figure 28: Kinetics of Hybridization of Normal Rat Liver Rare cDNA to Adrenalectomized Liver mRNA.

Preparation and purification of rare cDNA sequences are described in Methods section.

A: Normal rat liver rare cDNA was hybridized to 14 days adrenalectomized rat liver polysomal Poly(A)$^+$RNA (o o o o ), broken line represents the hybridization of normal rat liver rare cDNA to homologous RNA (Redrawn from Fig. 27). Open and closed circles represent two different polysomal Poly(A)$^+$RNA preparations.

B: 14 days adrenalectomized rat liver cDNA was hybridized to homologous RNA (o), and to polysomal Poly(A)$^+$RNA derived from normal rat liver (▲). Conditions for hybridization were as for Figure 27.
Hybridization of normal liver rare cDNA to adrenalect RNA

A

Hybridization of Polysomal poly(A)RNA to 14 days adrenalect Rat liver rare cDNA (Hom = Het = )

B
4.5.2. : Kinetics of Hybridization of Adrenalectomized Rat Liver mRNA.

Figure 28A shows the hybridization of the "rare" cDNA sequences derived from normal rat liver polysomal Poly(A)$^+$RNA to its own template and also to polysomal Poly(A)$^+$RNA of adrenalectomized rat liver. The heterologous hybridization was considerably slower than that of homologous reaction. This indicates that the sequences held in common between normal and adrenalectomized rats are less abundant in adrenalectomized rats. Rather more significant, however, is the finding that at, a Rot of 5,000 moles. sec. $1^{-1}$, the mRNA from adrenalectomized rats is unable to saturate the cDNA to the same extent as that from normal animals.

The heterologous reaction reaches only 42% saturation value, which after correction is equivalent to 21% of the sequence complexity being absent from the adrenalectomized rat liver mRNA population. This is in good agreement with the data from unique DNA hybridizations.

Figure 28B represents the reverse cross-hybridization and the curves are exactly those which would be predicted from Figure 28A. Rare cDNA from adrenalectomized rat liver was hybridized to own template and to the normal rat liver polysomal Poly(A)$^+$RNA. Normal liver mRNA was able to drive this cDNA into hybrid at a faster rate than the homologous mRNA preparation from adrenalectomized rat liver. The result, again shows that sequences common to the two preparations were more abundant in normal liver.

These data together with the results of the hybridization
Figure 29: Kinetics of Hybridization of Normal Rat Liver 'rare' cDNA to hormone treated adrenalectomized rat liver mRNA

Preparation and purification of 'rare' cDNA sequences are described in Methods section.

A: Normal rat liver 'rare' cDNA sequences were hybridized to liver mRNA isolated from rats which had been adrenalectomized 14 days before death and given corticosteroids 2 hr before death (●●●●●). Open and closed circles represent data from two different polysomal Poly(A)$^+$RNA preparations.

B: Normal rat liver 'rare' cDNA sequences were hybridized to liver mRNA isolated from rats which had been adrenalectomized 14 days before death and given corticosteroids 6 hr before death (●●●●●). Open and closed circles represent data from two different polysomal Poly(A)$^+$mRNA preparations.

The other curves (in A & B) represent the hybridization of normal rat liver rare cDNA to

--- : Normal rat liver mRNA (homologous, redrawn from Figure 27A).

---- : 14-days adrenalectomized rat liver mRNA (heterologous, redrawn from Figure 28A).
of abundant class cDNA (Figures 26 and 28) indicate that the original differences observed between the liver mRNA populations of normal and adrenalectomized rats reside primarily in the rare class.

4.5.3: Kinetics of Hybridization of Hormone Treated Adrenalectomized Rat Liver mRNA.

Figure 29 shows the hybridization of the 'rare' cDNA sequences derived from normal rat liver polysomal Poly(A)$^+$RNA to polysomal Poly(A)$^+$RNAs isolated from the liver of hormone treated adrenalectomized rats. Once again it reveals that corticosteroids can reverse the effects of adrenalectomy and that this effect is much less apparent 2 hrs after hormone administration than it is after 6 hr (compare with Figure 12, 22 and 23).

5. LOSS OF mRNA SEQUENCES AFTER ADRENALECTOMY

In an attempt to confirm that most profound differences in the mRNA population isolated from rat livers before and after adrenalectomy were in the rare sequences, the following experiment was performed.

Mercurated adrenalectomized rat liver polysomal Poly (A)$^+$ RNA was hybridized to normal rat liver cDNA to a Rot of 3,000 (moles. sec. $1^{-1}$). Fractionation by thiol-Sepharose chromatography produced a bound fraction, which comprised 67% of the cDNA, and an unbound fraction (see Methods section). The unbound cDNA, which should be considerably enriched in sequences present in normal liver but absent from adrenalectomized
Figure 30: Hybridization of Normal Rat Liver Specific cDNA

cDNA enriched in sequences not present in the liver of adrenalectomized rats was hybridized to normal rat liver polysomal Poly(A)$^+$ RNA (○) and 14 days adrenalectomized rat liver polysomal Poly(A)$^+$ RNA (▲) (see Materials and Methods section). Hybridizations were performed in HB/F at 43°C at RNA concentrations of 1mg/ml and 10mg/ml; RNA:cDNA ratios were 2,500:1 and 25,000:1, respectively. 2,000 counts/min (0.5ng) of cDNA were used for each data point.
animals, was recovered. It was then hybridized to both normal and adrenalectomized rat liver polysomal Poly(A)\textsuperscript{+}RNA (Figure 30). This cDNA fraction was able to hybridize to about 30% of the total mRNA. This is to be expected since the cDNA preparation would also be enriched in that 23% of the total cDNA preparation which never forms hybrid. The results of Figure 30 clearly show that a considerable enrichment has been achieved and many cDNA sequences have been isolated which hybridize to normal liver cDNA but not to that from adrenalectomized rats. Nevertheless, it is worth noting that Poly(A)\textsuperscript{+}RNA from adrenalectomized rat liver is able to hybridize to 12% of the cDNA preparation (Figure 30). This can be explained as follows: the cDNA fraction was prepared by isolating these sequences which at a Rot of 3,000 did not form hybrids with a 100 fold excess of mRNA from adrenalectomized rat liver. The curves of Figure 30 were performed at the presence of up to 10,000 fold excess of the same mRNA. It is clear, therefore, that the cDNA fraction contains sequences which could not find partners at a mass ratio of 100:1 but could at 10,000:1. These findings support the previously raised suggestions that some of the differences observed between the mRNA populations of normal and adrenalectomized rat liver are not strictly qualitative but reflect large differences in abundance.

Calculations of the number of sequences forming hybrids in the reaction with polysomal Poly(A)\textsuperscript{+}RNA of normal liver in Figure 30 gives a value of 8,000. This is very close to the results obtained either by total cDNA or unique DNA hybridization.
Figure 31: Hybridization of Normal Rat Liver Non-Specific cDNA

cDNA enriched in sequences common to normal and adrenalectomized rat liver was hybridized to polysomal polyadenylated mRNA from both sources.

○ ○ ○: Normal rat liver mRNA (homologous)

▲ ▲ ▲: Adrenalectomized rat liver mRNA (heterologous)

All experimental conditions are as described in the Legend to Figure 30.
As a comparison with hybridizations of Figure 30 involving the cDNA fractions which were unique to normal liver, the same experiments were performed with those sequences which formed hybrids and therefore are common to normal and adrenalectomized liver (Figure 30). It was expected that the abundant sequences in this fraction would hybridize most rapidly to mRNA from adrenalectomized rats but that no differences should be detected in the rate of hybridization of rarer sequences whether to mRNA from normal or adrenalectomized rats. It is seen from Figure 31 that this is exactly the result obtained.
DISCUSSION
1. **RELIABILITY OF HYBRIDIZATION TECHNIQUES FOR THE ANALYSIS OF mRNA COMPLEXITY.**

Polyadenylated RNA-DNA hybridization has become a useful analytical technique for evaluating the extent of genetic information expressed in different eukaryotic cell types and tissues (Table 1). By the application of either saturation hybridization of single-copy DNA (Brown and Church, 1971; Galau et al., 1974, 1976) or the analysis of the hybridization kinetics between cDNA and its template Poly(A)^+RNA (Birnie et al., 1974; Bishop et al., 1974), the base sequence complexity and abundance classes of mRNA can be estimated.

The interpretation and validity of analysis of total mRNA populations are, however, subject to a number of variables which must be carefully monitored and taken into account. Among these are the following:

1. **Source and Nature of mRNA**

In this work mRNA was extracted from purified polysomes. This avoids contamination with nuclear pre-mRNA species but also results in the loss of cytoplasmic mRNA not associated with polysomes. However, mRNA not on polysomes is not being translated, indeed considerable evidence exists that at least some of it is not available for translation.

Since Triton X-100 was employed in the preparation of the polysomes, those associated with membranes will have been recovered, so the estimates of total mRNA complexity will not have been affected by any fractionation between membrane bound and free polysomes.
It must be emphasized that this study concerns only polyadenylated mRNA and any species which is non-polyadenylated, or which became non-polyadenylated after adrenalectomy or during regeneration, would not be detected by this analysis.

ii. **Measurement of mRNA Concentration**

Since Rot is a function of concentration, it is essential that mRNA concentration is accurately established in Rot curve analysis or totally erroneous data can be generated. Considerable care was exercised in ensuring the reliability of the measurements obtained in this work. Spectrophotometers, used to estimate quantity by absorbance at 260nm were frequently checked with standards.

iii. **Size of Complementary DNA**

Complementary DNA must be large enough to hybridize with its template after the effect of Poly(A) tail is taken into account. In the work described here, cDNA was prepared by the method of Birnie et al. (1974). This method had the advantage of being well characterized with the chosen standard, globin mRNA (Young et al., 1974). However, it does not make cDNA as long as some of the more recently published methods. The synthesized cDNAs were on average, greater than, 450 bases long (Figure 7), whether the template was globin or the liver polysomal Poly(A)^+RNA which were, on average, three times longer than globin mRNA. The cDNA prepared were, however, of sufficient length for the hybridization studies described and were of high specific activity. They were routinely sized and only those longer than 5S (450 bases) were retained.
iv. Hybridization Conditions

RNA-cDNA hybridizations were carried out at 43°C in salt buffer containing formamide. The low temperature was chosen because RNA is much more stable at 43°C (Young et al., 1974) than at the higher temperatures (usually about 70°C) more commonly used for this type of reaction. This resulted in lower reaction rates (Young et al., 1974), but was compensated for by longer incubation times (up to 18 days).

2. THE mRNA POPULATION OF NORMAL RAT LIVER

Analysis of cDNA hybridization to homologous polysomal polyadenylated RNA from normal rat liver suggest that there are 12,500 sequences of 600,000 average molecular weight (Table 4). This value was in close agreement to the values obtained for rat liver Poly(A)⁺RNA population by several other workers (Table 1). However, estimates of diversity and complexity by this method are known to suffer from the drawback that the unique sequences were most refractory to analysis by cDNA hybridization assay and their complexity is likely to be under-estimated (Young et al., 1976; Ryffel, 1976; Aziz et al., 1979; Jacobs and Birnie, 1980). It was therefore considered imperative that the above findings were re-evaluated by alternative methodology.

The method of Galau et al., (1974, 1976) which measures the total sequence complexity by saturation hybridization between [³H]-labelled unique DNA sequences and purified mRNA, gives a more precise measurement of the complexity of least abundant mRNA species. However, it is not sensitive enough
to analyse changes in mRNA abundance.

Table 5 shows that when normal rat liver mRNA is assayed by this method, 29,000 average size sequences are expressed indicating that the analysis of cDNA hybridization was indeed an underestimate. These results are in agreement with the findings in other tissues. Thus complexity determinations in mouse brain (Hastie and Bishop, 1976; Young et al., 1976), in rat liver (Sippel et al., 1977; Jacobs and Birnie, 1980) and in mature rat uterus (Aziz et al., 1979) have all recorded lower values when complexity was measured by cDNA hybridization relative to determinations with unique DNA.

In unique DNA hybridization with excess unlabelled RNA, self annealing of sequences present in the probe can lead to an overestimation of the percentage hybridized at saturation. Several workers have overcome this difficulty by carrying out a low salt RNase treatment of the mixture of RNA-DNA hybrids followed by hydroxyapatite chromatography to obtain an estimate of the amount of RNA-DNA hybrids (Galau et al., 1974, 1976; Kleiman et al., 1977). In these studies this step was not required since the affinity binding to thiol-Sepharose ensured that radioactive DNA was only retained as a hybrid with mercurated RNA.

3. EFFECT OF REGENERATION ON POLYSOMAL POLYADENYLATED RNA POPULATION

The regeneration of mammalian liver following partial hepatectomy is characterized by marked morphological and metabolic changes which have been considered to be indicative
of changes in gene expression (Bucher and Malt, 1971).

During the first 36 hours of this process, events occur in a relatively synchronous manner and two distinct phases can be recognised: hypertrophy, lasting for 12-16 hours and hyperplasia, characterized by the initiation of DNA synthesis 14-18 hours after operation (Grisham, 1962). During the hypertrophic phase the important switch from a non-growing to a growing tissue takes place.

Qualitative and quantitative alterations in mRNA populations have so far been carried out mainly on liver in the hyperplasia phase of regeneration while the work here presented is confined to the hypertrophic phase, particularly the 12 hr time point.

The findings of this study, however, appear to parallel the findings of other workers, looking at later time points in the regeneration process. No qualitative differences were detected in the 12 hr regenerating liver. A slower rate of hybridization of the total mRNA population indicated that some sequences, at least, were present at lower abundance in the regenerating tissue and studies with fractionated cDNA indicated that these were largely in the abundant sequences. The complexity of the rarer sequences were very similar in regenerating and normal liver although there were indications that some sequences were more abundant while others were less so.

As previously discussed in the Introduction to this thesis, very similar results were obtained with the 16 hr regenerating liver (Wilkes et al., 1979), the 24, 48 and 72 hr regenerating tissue (Scholla et al., 1980). The only major
change in regenerating liver RNA population was recently reported by Wilkes et al., (1981) who found a complexity change in nuclear RNA of 16 hr regenerating liver equivalent to 5,000 to 10,000 sequences of 2,000 nucleotides in length. They conclude, however, that these were probably not potential mRNA species.

4. **THE EFFECT OF ADRENALECTOMY ON POLYSOMAL POLY(A)^+ RNA POPULATION**

Steroid hormones enter their target cells, bind to specific cytoplasmic receptor proteins and the hormone-receptor complex then moves into the nucleus where it stimulates transcription.

Glucocorticoids exert their effects in this way on a number of target tissues. In rat liver, transcriptional responses lead to the production of specific enzymes. Particularly those associated with gluconeogenesis from amino acids (Nickol et al., 1978; Webe, 1963). They also cause general tissue hypertrophy which has been followed as stimulated synthesis of stable RNA species (Feigelson and Feigelson, 1963; Turkington, 1969), formation of new ribosomes and stimulation of total protein synthesis (Schimke and Doyle, 1970). In this work, the effect of glucocorticoids has been monitored in a different way. The effect of adrenalectomy on the total mRNA population of rat liver has been monitored and the ability of dexamethasone to overcome these effects.

After adrenalectomy considerable qualitative and quantitative changes occur in the liver mRNA population. The qualitative changes appeared to be very dramatic when analysed by
Table 7: Base Sequence Complexity of mRNA of Rat Liver by Different Hybridization Methods.

<table>
<thead>
<tr>
<th>Section</th>
<th>Tissue</th>
<th>Number of diverse sequences observed by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cDNA (a)</td>
<td>Fractionated &quot;rare&quot; cDNA (b)</td>
</tr>
<tr>
<td>I</td>
<td>Normal rat liver</td>
<td>12,500</td>
</tr>
<tr>
<td>II</td>
<td>Adrenalectomized rat liver</td>
<td>4,000</td>
</tr>
<tr>
<td>III</td>
<td>Differences observed</td>
<td>8,500</td>
</tr>
</tbody>
</table>

The above data represent numerical evaluation of kinetics of Hybridization of Poly(A)$^+$RNA to cDNA or Unique DNA (fractionated and total).

a): Taken from Table 4; b): Calculated from Figure 28; c): Calculated from Figure 30; d): Taken from Table 5; e): Calculated from Figure 17.
total cDNA-mRNA hybridization and to involve 3/4 of the total
liver population. Reasons, have been presented, however, for
believing that the change is less dramatic than this response
indicated and that quantitative differences also contribute to
the result. This reasoning was supported by further hybrid-
ization analysis with fractionated cDNA and with both total
and normal liver specific unique DNA. Nonwithstanding this,
however, it is clear from Table 7 that some 7,000 - 9,000 mRNA
sequences disappear from rat liver after adrenalectomy. It
is also clear from Figures 11, 17, 20 and 28 that there are,
in addition, considerable quantitative changes.

5. THE FURTHER ANALYSIS OF mRNA ABUNDANCE

The results presented above show that mRNA can exhibit
considerable diversity in abundance and that this abundance
can vary through several orders of magnitude.

In all eukaryotic cells so far examined, the Poly(A)$^+$RNA
on the polysomes occur in a wide range of abundances despite
the vast majority of structural genes being present in one,
or at most, a few copies per haploid genome (Minty and Birnie,
1981). The rat liver contains a spectrum of mRNAs ranging
in abundance from several thousand to less than 10 copies per
gene per cell (Savage et al., 1978; Wilkes et al., 1979)
and in rat uterus there is at least a thousand fold difference
between the concentrations of the most and least abundant
polysomal Poly(A)$^+$RNA (Aziz et al., 1979a).

It is unlikely that this enormous range of abundance is
generated by a single mechanism. Studies of systems such as
that of the globin, ovalbumin and vitellogenin genes have revealed that genes can be switched on by transcriptional events and their mRNA can appear at thousands of copies per cell when they were previously almost absent. These proteins are not necessarily typical however and many mRNAs may not exhibit this on/off situation but may show varying abundance under different physiological situations. The result of this thesis indicates that this may well be the case in the liver.

The further analysis of these possibilities will however require more sensitive analysis than the hybridization of total mRNA population. The techniques of genetic engineering provide the opportunity to select specific mRNA species for study and a recent report by Birnie et al., (1981) show that sequences can be selected which show a deviation in abundance under different physiological circumstances. Furthermore, some indications can be derived as to whether the abundance is controlled at a transcriptional or post-transcriptional level.

Birnie et al., (1981) have used four clones selected from a cDNA library derived from normal rat liver polysomal Poly(A)$^+$ RNA. They used these clones as probes to quantitate, by filter binding assay, the amount of complementary message in the nuclear and polysomal RNA of liver from normal and partially hepatectomized rats as well as in HTC hepatoma cells. They have shown in this way that the control of mRNA abundance can be regulated at many levels. Thus clone pBR 117 was complementary to a message which appeared to be under transcriptional control. Its levels were induced in HTC cells and this could be shown
Plate 1: Analysis of Abundance Classes by cDNA Library.

The complementary DNA synthesized to polysomal Poly(A)*RNA was hybridized to colonies of cDNA library in pBR322 grown on nitrocellulose filter papers (as described in Methods section).

A: Hybridization observed with normal rat liver cDNA.

B: Hybridization observed with 14 days adrenalectomized rat liver cDNA.
in both nuclear and cytoplasmic RNA. Conversely, the depressed levels of polysomal sequences complementary to clone pBR 5B in regenerating liver appeared to be a post-transcriptional response as the sequences accumulated in the nucleus, presumably because of inadequate processing. Sequences complementary to pBR 83 almost disappeared from the polysomes of HTC cells despite a continued presence in the nucleus. Presumably this reflected a change in the half-life of the message.

At the end of the work described in this thesis, similar work to that described by Birnie et al. (1981) was initiated. A partial cDNA library (300 cloned sequences) to normal rat liver mRNAs was screened by filter binding methods, for specific sequences which differed in abundance after adrenalectomy.

Plate 1 represents the initial results obtained. The following differences might be pointed out as the most dramatic:

i. Clone 137 (position C4 on Plate 1) is complementary to sequences which virtually disappear after adrenalectomy.

ii. Clone 44 (position E2 on Plate 1) is complementary to sequences which become more abundant after adrenalectomy.

iii. Clones 142 and 145 (positions F6 and F7 respectively on Plate 1) are complementary to sequences which become much less abundant after adrenalectomy.

These four clones have been selected for an expanded study on the basis of that described by Birnie et al. (1981). Thus they will be used to quantitate the mRNA, as a mass ratio,
in nuclear and cytoplasmic RNA of normal rats, of adrenalectomized rats at various times post-operatively and of adrenalectomized rats at various times after the administration of dexamethasone. Unfortunately, the time has not allowed the completion of these experiments prior to the end of my Studentship.
REFERENCES
Allström, P., Akusjarvi, G., Perricaudet, M., Mathews, M.B.,
Allfrey, V.G. (1971). In: Histones and Nucleohistones (Phillips,
Aloni, Y., Bratosin, S., Dhar, R., Laub, O., Horowitz, M. and
42, 559-570.
69, 1408-1412.
Axel, R., Cedar, H. and Felsenfeld, G. (1975). Biochemistry,
14, 2489-2495.
100, 85-94.
100, 95-100.


Baser, R. (1976). In "Multiplication and division in Mammalian Cells; The Biochemistry of Diseases", (Farber, E. and Pitot, H.C., eds.) vol. 6, Marcel and Dekker, New York.


Haynes, Jr., R.C., Sutherland, E.W. and Rall, T.W. (1960).


Nuc. Acid Res. 5, 4781-4793.


Biochemistry, 16, 1218-1223.


Nature 287, 509-516.


Malek, L.T., Eschenfeld, W.H., Munns, T.W. and Rhoads, R.E.
    Biochemistry, 14, 3787-3794.
Martial J.A., Baxter, J.D., Goodman, H.M. and Seeburg, P.H.
    5, 37-42.
    Chem. 251, 1137-1146.
Mathis, D.J. and Gorovsky, M.A. (1976). Biochemistry, 15,
    750-756.
    74, 560-564.
    11, 792-798.
Milgrom, E. and Atger, M. (1975). J. Steroid Biochem. 6,
    487-496.
    1-25.


Nature 283, 502-504.


Cell 1, 46-53.


Monahan, J.J., Harris, S.E. and O'Malley, B.W. (1977). In:

Chem. 248, 2409-2417.

Moyzis, R.K., Grady, D.L., Li, D.W., Mirvis, S.E. and Ts'o,


82, 735-754.


Nicholson, M.L. and Young, D.A. (1978). Cancer Res. 38,
3673-3680.

253, 4009-4015.
Nuc. Acid Res. 5, 4195-4211.


Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and
Salditt-Georgieff, M., Jelinek, W., Darnell, J.E., Funichi, Y.,
Samarina, O.P., Lukandin, E.M., Molnar, J. and Georgiev, G.P.
Biochemistry 17, 462-467.
Schaffner, W., Kunz, G., Daetwler, H., Telford, J., Smith, H.O.
Scheer, U., Trendelenburg, M.F., Kehne, G. and Franke, W.W.
929-956.
Chem. 255, 2855-2860.
Chem. 247, 2401-2407.
Schrader, W.T., Socher, S.H. and Buller, R.E. (1975). In:
"Methods in Enzymology," (O'Malley, B.W. and Hardman, J.G.


Steensgaard, J., Muller, R.N.P. and Funding, L. (1978). In:
"Centrifugal Separations in Molecular and Cell Biology" 
(Birnie, G.D. and Rickwood, D. eds). pp. 115-167, 
Butterworth, London.
18, 439-449.
319-326.
390, 56-58.
Stein, G.S. and Kleinsmith, L.J. (1975) in "Chromosomal Proteins 
and their role in the regulation of Gene Expression"; 
Stein, G.S., Mans, R.J., Gabby, E.J., Stein, J.L., Dowis, J. 
Stein, G.S., Park, W., Thrall, C., Mans, R. and Stein, J.L. 
Stein, G.S., Stein, J.L. and Kleinsmith, L.J. (1978a). In: 
"Methods of Cell Biology: Chromatin and Chromosomal 
38, 1181-1201.
Stein, J.P., Catterall, J.F., Kristo, P., Means, A.R. 
253, 3830-3836.


Young, B.D., Harrison, P.R., Gilmour, R.S., Birnie, G.D.