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THE EFFECT OF OESTRADIOL-17\beta
ON THE SYNTHESIS OF UTERINE
HnRNA and mRNA

by

SHAHARUDDIN AZIZ B.Sc.(Hons.), M.Sc.

Thesis presented for the degree of
Doctor of Philosophy, Faculty of Science,
at the University of Glasgow, February 1979.
Acknowledgements

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Abbreviations

Standard abbreviations are in general as recommended in the "Instruction to Authors", revised edition of the Biochemical Journal (Biochem. J. (1978) 169, 1-27). Enzyme commission numbers are not used since many of the enzymes referred to in this thesis are incompletely characterized and are inadequately described by the numbering system. Additional abbreviations used are:

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<td>NN'-methylene bisacrylamide</td>
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<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegration per minute</td>
</tr>
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<td>HnRNA</td>
<td>Heterogenous nuclear RNA</td>
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<tr>
<td>Oestradiol-17β</td>
<td>1,3,5(10)-estratrien-3, 17β -diol.</td>
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<td>poly(A)+HnRNA</td>
<td>polyadenylated heterogenous nuclear RNA</td>
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<tr>
<td>poly(A)+mRNA</td>
<td>polyadenylated messenger RNA</td>
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<tr>
<td>rRNA</td>
<td>RNA containing the ribosomal RNA genes</td>
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Evidence collected in this laboratory showed that an early action of oestrogen on the immature rat uterus was a dramatic stimulation in the synthesis of heterogenous nuclear RNA (HnRNA). Subsequently, mRNA, which is presumably derived from the HnRNA, accumulates in the cytoplasm and brings about the aggregation of pre-existing ribosomes into polysomes. This early response, and the translation of the mRNA into a small number of ill-characterized proteins, appears to be a prerequisite for the subsequent striking increase in synthesis of rRNA, the accumulation of new ribosomes and the subsequent uterine hypertrophy and hyperplasia.

The work in this thesis describes a continuing study of oestrogen-induced changes in uterine transcription and concentrates on 1) the effects of oestrogen on uterine HnRNA synthesis, 2) the effect of oestrogen on the population of mRNA in uterine cell cytoplasm, 3) the relationship of the HnRNA synthesis to the changes in mRNA diversity.

Uterine HnRNA was extensively purified and characterized under denaturing as well as non-denaturing conditions. The purified high molecular weight HnRNA species were fractionated on poly(U) Sepharose into species differing in their poly(A) content. Each fraction was also found to differ in its size profile in polyacrylamide gels and sucrose gradients. Oestradiol treatment of the rats stimulated the synthesis of all three chromatographic fractions of high molecular weight HnRNA, but the kinetics of synthesis, degree of stimulation and size distribution of the newly synthesized RNA differed in each fraction. The stimulation of HnRNA synthesis was detectable as early as 30 min after the
administration of oestradiol and preceded the synthesis of mRNA and the aggregation of ribosomes into polysomes. The stimulated synthesis of HnRNA at early times after oestradiol administration to immature rats was most striking in the polyadenylated fraction. Since evidence is accumulating that HnRNA contains polynucleotide sequences which ultimately become messengers, it is suggested that the stimulated production of this species in the uterus of oestrogen-stimulated rats may reflect hormone-induced mRNA synthesis.

The diversity and complexity of the uterine poly(A)+mRNA population has been compared at two different stages of uterine growth and development in the rat. Analysis by cDNA hybridization to homologous mRNA indicates that there are 8000 different sequences expressed in the immature rat uterus responding to just 4h of oestradiol-17β induced growth while the fully developed uterus expresses 36,000 sequences. Reasons are discussed for believing that the analysis by this may lead to underestimates and for this reason and as a check on results obtained by cDNA-RNA hybridization kinetics, the complexity was reanalysed by hybridization of mercurated mRNA to total unique ³H-labelled DNA and hybridization assayed by thiol-Sepharose chromatography. Analysis by this method indicates that the hormone stimulated immature rat uterus contains 12,000 poly(A) containing mRNA sequences while the fully differentiated adult tissue contains 53,000 diverse sequences.

The mRNA population of immature rat uteri, responding to different lengths of oestradiol-induced differentiation, have been compared by the technique of DNA hybridization with heterologous RNA. Over the first 4h of hormone-induction there are continuing qualitative and quantitative changes in the poly(A)+mRNA such that by 4h after
Oestradiol treatment the population bears little resemblance to that of the unstimulated animal. Between 2h and 4h after oestradiol administration, the most striking changes are in sequences of intermediate abundance. The possible significance of these findings is discussed.

cDNA complementary to mRNA was separated into the abundant sequences by HAP chromatography and hybridized to oestradiol-stimulated poly(A)+HmRNA. Hybridization data revealed the presence of mRNA sequences in poly(A)+HmRNA sedimenting at 40-30S under denaturing conditions. The mRNA sequence content increased several fold after 2h of oestradiol treatment.
INTRODUCTION
1. **Introduction**

1.1 **Eukaryotic Gene Material and Transcription**

The factor concerned with the transmission of hereditary characteristics, be it an individual cell or a multicellular organism is the broadest working definition of a gene. The genetic information for the development and proper functioning of the organism is encoded in the linear sequences of the deoxyribonucleotide molecules present in chromosomes at one DNA molecule per chromosome (Kavenoff & Ziman, 1973; Pates & Pangman, 1972; Blamier *et al.*, 1972). The DNA of eukaryotic cells is present in complex with nucleoproteins, namely histone and the acidic non-histone proteins. This nucleoprotein complex containing genetic material was identified in 1882 (Flemming, 1882) as a nuclear material which stained with a basic stain and in recent years has come to be known as chromatin.

The structure of chromatin in relation to the arrangement of histones along the DNA molecule and localization of the non-histone proteins has been the central theme of a number of research projects. Electron microscope data (Olins & Olins, 1974), neutron diffraction data (Balwin *et al.*, 1975) and biochemical evidence (Thomas & Kornberg, 1975; Kornberg 1977; Finch *et al.*, 1977) has shown that histones are complexed with the DNA in the form of clusters interspersed with stretches of nucleotides that are more vulnerable to nucleases (Felsenfeld, 1976; Kornberg, 1977). Such histone-DNA complex or nucleosomes look like beads on a string when examined under the electron microscope and these have been observed in many plant and animal cells.
(Stein et al., 1976; Felsenfeld et al., 1976; Kornberg, 1977) and in some DNA tumour viruses (Stein et al., 1978a; Poliski & McCarty, 1975; Germond et al., 1975).

1.1.1 The histones

The histone constitute a set of low molecular weight highly basic proteins arranged into five different classes. Histones were first proposed to act as repressor molecules in eukaryotic gene expression by Stedman & Stedman (1950) and in the 1960's Huang et al. (1964) and Alfrey et al., (1963) provided evidence that the activation or repression of RNA transcription depended upon the amounts of histone complexed to DNA. In recent studies 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 effects on RNA chain elongation rate (Koslov & Georgiev, 1970). Structural modifications of histones such as phosphorylation, acetylation and methylation which could lead to changes in the electrostatic interaction with DNA, may be responsible for inducing or repressing transcription of specific genes (Tsai et al., 1976a; Gottesfeld et al., 1975; Axel et al., 1975; Stein & Stein, 1976). Such changes occur concomitantly with the activation of transcription associated with various biological processes e.g. cellular proliferation (Balhorn et al., 1971; 1972a,b,c; Gurley et al., 1974), stimulation by hormones and transformation by oncogenic viruses (Gottesfeld et al., 1975; Langan & Hohmann, 1975; Hnilica, 1972; Stein et al., 1978b). Thus histones appear to play an important role in the maintenance of
genome structure (Kornberg, 1977) and in the non-specific repression of DNA-dependent RNA synthesis (Stein et al., 1978a).

1.1.2 Non-histone chromosomal proteins (NHCP)

The term non-histone chromosomal proteins defines all chromosomal proteins except the histones. Thus the NHCP includes proteins which form structural components or genome associated contractile proteins (Douvas et al., 1975; Le Stourgeon et al., 1973, 1975), enzymes including those associated with transcription and replication (Stein et al., 1978b) and regulatory molecules (Baserga, 1974; Elgin & Weintraub, 1975; Paul et al., 1975; Stein & Kleinsmith, 1975; Stein et al., 1975a; Thomas & Patel, 1976).

Like the histones, the NHCP can undergo post-translational modifications including acetylation (Suria & Liew, 1974), methylation (Friedman, 1969) and phosphorylation (Kleinsmith, 1975). These modifications confer the potential for increased specificity on NHCP. Additional changes in the composition and metabolism of NHCP accompany the alterations that occur during eukaryotic gene expression associated with development (Johnson & Hnilica 1972; Shelton & Weel, 1971), cellular differentiation (Platz et al., 1975; Stellwagen & Cole, 1969; Zornetzer & Stein, 1975), the cell cycle (Stein & Borun, 1972; Germer & Humphrey, 1973; Bhorjee & Pederson, 1972), stimulation of cell proliferation (Levy et al., 1973; Stein & Burtner, 1974, 1975; Tsuboi & Baserga, 1972), in response to various steroid hormones (Swaneck et al., 1970; O'Malley & Means, 1974; O'Malley et al., 1977), during viral infection and transformation (Cholon & Stuzinski, 1974; Krause et al., 1975a, 1975b) and in tumourigenesis (Stein et al., 1978b). Quantitative and
qualitative changes of the NHCP have also been observed in euchromatin of transcriptionally active tissues (Wilhem et al., 1973; Gottesfeld et al., 1974, 1975).

That NHCP may specifically control the expression of a given gene has been indicated by reconstitution experiments in which it was shown that, only in the presence of those NHCP normally associated with a given active gene, was that gene transcribed. Such experiments have implicated NHCP in the specific expression of globin genes (Barret et al., 1974; Chiu et al., 1975; Gilmour & MacGillvary, 1976) the oestrogen-induced transcription of ovalbumin genes (Tsai et al., 1976a, 1976b, 1976c; Masaki et al., 1976) immunoglobulin kappa light chain genes (Smith & Huang, 1976) and the cell cycle stagespecific transcription of histone genes (Jansing et al., 1977; Stein et al., 1975b).

However, doubts have been expressed in the validity of some of these experiments, because of the influence of contaminating endogenous RNA sequences on the interpretation of specificity and origin of transcribed RNA (Zasloff & Felsenfeld, 1977; Shih et al., 1977).

1.1.3 Transcriptional Enzymes

In all living cells, the expression of genetic information involves the synthesis of RNA molecules from DNA and the functioning of these RNA molecules in protein synthesis. The synthesis of RNA molecules is catalysed by multiple forms of RNA polymerases which fall into three major classes designated Class I, II and III (Roeder et al., 1976). Each class has distinct catalytic properties, distinct subunit structures and specific functions in the synthesis of the major classes.
of RNA (Chambon, 1975; Roeder et al., 1976, 1977). The class I enzyme is nucleolar in origin and catalyses the synthesis of ribosomal RNA (Blatti et al., 1970; Zylber & Penman, 1971; Reeder and Roeder, 1972; Weinman & Roeder, 1974). Class II enzyme catalyses the synthesis of the heterogenous nuclear RNA in the nucleoplasmic fractions of cells (Reeder & Roeder, 1972; Blatti et al., 1971; Roeder & Rutter, 1970). The class III enzyme catalyses the synthesis of the precursors of 5S and tRNA (Weinman & Roeder, 1974; Schwartz et al., 1974; Roeder et al., 1977; Udvardy & Seifart, 1976; Weil & Blatti, 1976; Weil et al., 1977). Changes in catalytic activity of these enzymes have been correlated with alterations in the biological states of cells and tissues that reflect modifications in gene expression that is during cell proliferation (Schwartz et al., 1974; Jaehnings et al., 1975; Mauck & Green, 1975); embryonic development (Roeder, 1974), viral infection of host cells (Weinman et al., 1975) and during oestrogen induced changes in transcription (O'Malley & Means, 1974; O'Malley et al., 1977).

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 (Hondo & Blatti, 1977; Benson et al., 1978) to the subunits of the prokaryotic polymerases (Burgess, 1969). Thus dissociable protein factors such as sigma factor and other low molecular weight protein factors (Chamberlain, 1974) which are required for proper initiation and specificity of gene expression may also be present in eukaryotic transcription systems. Recently such factors have been purified to homogeneity from calf thymus (Sekizu et al., 1976; Benson et al., 1978).
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 chromosomal chromatin. This concept, originally propounded by Bonner et al., (1968) has found considerable support in the work of Axel & Felsenfeld (1973); Gilmour & Paul (1973; Steggles et al., (1974) and Towle et al., (1976).

2. The products of gene transcription

Eukaryotic DNA is more complex than that of prokaryotes and appears to include entities of differing abundance. One abundance class, apparently coding for the majority of proteins are present in single or a small number of copies — the so called unique sequences. Another class which are present in reiterated copies is known to include the genes for mRNA, tRNA and histones which are clustered in a few sites in the genome. A third class, the highly repetitive class includes satellite DNAs of very short repeat lengths. In a wide range of organisms, from insects to mammals, repeated DNA sequences also occur in a highly ordered arrangement in which they are interspersed with unique sequences (Angerer & Evans, 1977). A model, first proposed by Britten and Davidson, suggests that these repetitive sequences may represent control regions for the expression of adjacent structural genes (Britten & Davidson, 1969). A number of structural genes, here defined as the DNA sequences from which a functional polysomal mRNA is transcribed, have been identified, namely, ovalbumin (Harris et al., 1976; Monahan et al., 1976; Woo et al., 1976, 1977) which has recently been prepared by amplification with bacterial plasmids (McReynolds et al., 1977)
immunoglobulin genes (Smith and Huang, 1976), the silk fibroin genes
(Suzuki et al., 1972), histone genes (Kedes & Birnsteil, 1971;
Weinberg et al., 1972; Stein et al., 1975b; Jansing et al., 1977),
and globin genes (Tilghman et al., 1977). The mechanism of transcription
of structural genes is still not clear. However, mRNA is probably
derived in a similar fashion to rRNA from large precursor molecules
(for review, see Perry, 1976) which are then cleaved in the nucleus
to yield mRNA. Considerable evidence suggests that these precursors
form at least a part of heterogeneous nuclear RNA (Lewin, 1975b and 1975c).

2.1. The Heterogenous Nuclear RNA (HnRNA)

HnRNA, a designation proposed by Warner et al., (1966),
refers to its physical characteristics of polydispersity or heterogeneity
in respect to molecular weight. Various authors have used different
terms for designation of this nucleoplasmic RNA species. Earlier it
was called nuclear AU-rich RNA or dRNA (Georgiev & Mantieva, 1962a,
1962b) due to its DNA-like base composition (Williams et al., 1968;
certain structural features, in common with mRNA which fortifies the
concept that the HnRNA are precursors to cytoplasmic mRNA.

2.1.1 The high molecular weight nature of HnRNA

Depending on time of pulse-labelling of radioactive
precursors the distribution of labelled HnRNA species ranged between
10-90S. For example, pulse labelling of HeLa cells with $^{32P}$ or $^{3H}$
labelled precursors for 10-45 seconds resulted in HnRNA transcripts
The uteri of four 18–21 day old rats, weighing 25–30g were incubated in 2ml of Eagle's medium which had its phosphate content reduced to 10% of the normal concentration but contained 2mCi (³²P) orthophosphate. After washing the incubated uteri thoroughly, the total RNA was purified and separated on a 2.7% polyacrylamide gel for 5h. The extinction at 260nm and the radioactivity were determined throughout the gel and the RNA was extracted from selected slices. This was precipitated in the presence of unlabelled RNA, hydrolysed and its base composition analysed.

--- = Extinction at 260nm

.... = Radioactivity per slice in cpm
**Base Composition of Uterine RNA**

**3^2\text{P} radioactivity (cpm) \times 10^{-3}**

<table>
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<th>Slice</th>
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</tr>
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<td>51.1</td>
<td>56.6</td>
<td>56.5</td>
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**Extinction 260nm**
being equal to sedimentation rate of 16S. However, at steady state, with a labelling time of 3.5 hours the radioactivity was contained in molecules 10-15 kilobases corresponding to larger than 45S RNA species (Derman & Darnell, 1974; Derman et al., 1976). The relative size distribution of HnRNA varies from organism to organism. In the sea-urchin embryos, there appear to be discrete molecular classes of HnRNA with a mean sedimentation value as large as 36S (Dubroff & Nemer, 1975; Kung, 1974). In insects, the Drosophila HnRNA has modal sedimentation coefficient of 26S with small amounts of RNA species sedimenting at 60S and Aedes HnRNA shows a distribution of species sedimenting at 32S to 60S with a modal S value of about 35S (Lengyel & Penman, 1975). The average sedimentation rate of rat liver nuclear RNA was 33-34S with molecules as large as 40-45S (Sippel et al., 1977a). The nuclear RNA of mouse brain contain molecules that sediment at between 16S-60S (Bantle & Hahn, 1976). In the immature rat uterus, HnRNA sedimenting at values greater than 45S has been identified (Knowler & Smellie, 1973). The high molecular weight HnRNA has often been viewed as non-specific aggregates resulting from phenol extraction of RNA (Hagenbuchle et al., 1975; Kohne et al., 1977), or the annealing of complementary structures in the HnRNA population. It has been noted that exposure of HnRNA to denaturing conditions effects a moderate to extreme reduction in size, as judged by sedimentation rate or mobility on polyacrylamide gels containing formamide or dimethylsulphoxide (deKloet et al., 1970; Mayo & deKloet, 1971; Imaizumi et al., 1973; Morrison & Busch, 1973; McNaughton et al., 1974; Birnie et al., 1974; McKnight & Schimke, 1974; Derman & Darnell, 1974; Spohr et al., 1976;
Levis & Penman, 1977). However, even under these conditions, HnRNA still exhibits a heterogeneous size distribution and include molecules of greater than 45S in sedimentation values. In some cases, it has been observed that when HnRNA recovered from denaturing sucrose gradients was reanalysed on non-denaturing sucrose gradients some of it again sedimented with increased sedimentation coefficients. This finding is thought to be due to regions of the HnRNA capable of base pairing in the form of intermolecular partial duplexes (McNaughton et al., 1974; Ryskov et al., 1975; Fedoroff & Wall, 1976; Fedoroff et al., 1977). Electron microscope studies substantiate this finding showing the presence of intra and intermolecular duplexes in HnRNA (Fedoroff et al., 1977). Whether these molecules interact in this manner in vivo is not known. Pulse labelling experiments and the use of various inhibitors to study HnRNA synthesis (Derman et al., 1976; Eggyazi, 1975; Sehgal et al., 1976a, 1976b) revealed large initial transcripts. Thus like the precursors to rRNA, HnRNA is considerably larger than the average size of mRNA molecules.

2.1.2 The kinetics of labelling of HnRNA

One of the fundamental characteristics of HnRNA is its rapid synthesis and breakdown in the nucleus. Experiments utilising drugs such as actinomycin D to suppress rRNA synthesis, gave values for the half life/HnRNA of approximately 3 minutes (Soeiro et al., 1968). Since the time for transcription of an average HnRNA molecule approaches 3 minutes and no simultaneous transcription-translation complexes (such as are found in prokaryotes) have been detected in eukaryotic
chromatin (Miller & Baken, 1972), this would indicate that most of the HnRNA is degraded as it is made and is never transported to the cytoplasm (Harris, 1962). However, Brandhorst & McKonkey (1974) without using any inhibitors estimated a much longer first-order decay rate for L cell HnRNA of 23 minutes. Similar estimates of HnRNA half-life have been made in HeLa cells (Penman et al., 1968) and in duck reticulocytes (Attardi et al., 1966). These values would allow processing of a portion of the HnRNA to the cytoplasm. Brandhorst & McKonkey (1974) estimate that about 2% of labelled HnRNA may exit to the cytoplasm during longer chase periods. Recent studies on pulse-chase experiments in cultured Drosophila cells (Levis & Penman, 1977) revealed the presence of several different populations of HnRNA each with different half-lives. The nonpolyadenylated HnRNA, which constituted 80% of the pulse-labelled material, decays with a half-life of 10–15 minutes. The polyadenylated HnRNA behaved as two kinetic components, with half-lives of 20 and 80 minutes. Most of the cytoplasmic mRNA labelled during 30 minutes of chase was largely derived from the pulse-labelled nuclear transcript. Berger & Cooper (1978), working with human lymphocytes provided similar findings in that two types of functionally different polyadenylated HnRNA were labelled. One was labelled predominantly with exogenous radioactive precursors during the pulse and most cytoplasmic mRNA was derived from these molecules. The other was labelled almost exclusively with reutilised precursors made available during chase incubations which did not contribute any cytoplasmic mRNA. These findings would appear to confirm previous
postulates (Jelinek et al., 1973; Perry et al., 1974) that not all polyadenylated HnRNA could be precursors to mRNA and that there ought to exist two or more classes of precursors each with differing half-lives to explain the kinetic relationship between HnRNA and mRNA.

2.2 Evidence for a precursor-product relationship between HnRNA and mRNA

Apart from the kinetic analysis implicating HnRNA as precursor to mRNA, there are a number of direct and indirect findings which fortify the hypothesis.

2.2.1 Indirect evidence

Certain structural features common to both HnRNA and mRNA provide persuasive evidence in support of the biogenesis of mRNA from large HnRNA precursors. These common structural features include methylated nucleotide sequences, adenylic acid polymers at the 3' ends and various internal sequence homologies.

2.2.1.1 Polyadenylation of HnRNA and mRNA

The existence of polyadenylic acid (polyA) in eukaryotic RNA was first demonstrated by Hadjivasilov & Brawerman (1966). It is now known that the poly(A) is associated with mRNA (Lim & Canelakis, 1970; Darnell et al., 1971; Lee et al., 1971; Adesnik et al., 1972; Sheldon et al., 1972; Mendecki et al., 1972) and HnRNA (Edmonds et al., 1971; Adesnik et al., 1972; Greenberg & Perry, 1972; Stevenson et al., 1973; Nakazoto et al., 1973; Sheiness & Darnell, 1973). Experimental evidence indicate that the poly(A) exists on 3'OH ends of both RNA species
and that it arises by a post-transcriptional addition to existing HnRNA (Perry et al., 1974; Brawerman, 1974). Inhibitors such as oroidyopenin, which do not alter the synthesis of HnRNA, block polyadenylation of HnRNA and subsequently prevent the appearance of mRNA in the cytoplasm (Adesnik & Darnell, 1972; Darnell et al., 1971; Penman, 1970). These observations suggest that polyadenylation may represent an intrinsic part of nuclear RNA modifications and processing although nuclear polyadenylation may not represent an absolute prerequisite for mRNA transport to the cytoplasm (Herman et al., 1976; Brawerman, 1976). Polyadenylation is however not exclusively a nuclear event as Poly(A) has been observed to be added onto preexisting cytoplasmic mRNA molecules (Diez & Brawerman, 1974; Brawerman & Diez, 1975; Slater & Slater, 1974). Poly(A) sequences can also become shorter by removal of adenylate residues from the 3' terminus through ageing of the mRNA (Brawerman, 1976), thus resulting in a heterogeneous population of poly(A) sequences in the cytoplasm (Jeffrey & Brawerman, 1974; Brawerman & Diez, 1975). Heterogeneity of poly(A) sequences have also been observed in HnRNA preparations, with large HnRNA molecules containing short stretches of poly(A) and smaller HnRNA molecules containing longer stretches of poly(A) (Bantle & Hahn, 1976), also polyadenylation occurs not only on the primary transcript but also on older molecules resulting from a cleavage of higher molecular weight precursors (Derman & Darnell, 1974).

The majority of mRNA isolated in eukaryotic cells contains stretches of poly(A) about 200 nucleotides long (Greenberg, 1975). The
only identified specific mRNA lacking poly(A) is histone mRNA (Adesnik & Darnell, 1972; Greenberg & Perry, 1972). However, some polysomal mRNA lacks poly(A) stretches, for example 30% of mRNA in HeLa cells and mouse L cells lacks polyA (Milcarek et al., 1974; Greenberg, 1976). Similarly some mRNA of developing sea-urchin embryos, excluding histone mRNA lacks poly(A) (Nemer et al., 1974; Fromson & Duchastel, 1975) and these include the mRNA of non-histone chromosomal proteins (Nemer et al., 1974; 1975; Nemer, 1975). It was suggested that there were different sets of genes namely, the repetitive histone genes which yield nonpolyadenylated histone mRNA, the unique non-histone genes which yield polyadenylated non-histone mRNA and the unique non-histone genes which yield non polyadenylated mRNA (Nemer, 1975). It was further suggested that each gene set performed different functions and was susceptible to different mechanisms of control during embryonic development (Nemer, 1975; Nemer et al., 1974; 1975; McColl & Aronson, 1974).

While the evidence available at present does not support a specific role for poly(A), experiments have indicated that deadenylated polyA mRNA is inefficient in protein synthesis (Williamson et al., 1974; Sippel, et al., 1974; Huez et al., 1974; Doel & Carey, 1976; Marbaix et al., 1975) either in cell free systems or in frog oocytes, thus implying greater stability for poly(A) containing mRNAs. However, not all experimental evidence supports a role for poly(A) in mRNA stability. The nonpolyadenylated histone mRNA is very stable in the cytoplasm (Perry & Kelly, 1973) and besides, a short-lived class of polyadenylated mRNA has been detected in HeLa cells (Pucket et al., 1975; Darnell et al., 1976). Thus it seems that the poly(A) stretches
in mRNA, like many other accessory polynucleotides in precursor molecules and genes may play some as yet undefined function(s) in the regulation of gene expression in the eukaryotic systems.

2.2.1.2. Methylation of HnRNA and mRNA

Methylation of ribonucleotides was known only in rRNA, tRNA and their precursors (Maden & Salim, 1974; Burdon, 1975; Perry, 1976) until Perry & Kelly (1974) demonstrated methylated mRNAs in mouse L cells and Desrosiers et al., (1974) reported similar findings in Novikoff hepatoma cells. Since then, methylated constituents have been identified in mRNA and HnRNA of viral and mammalian origin (Furuichi et al., 1975a; 1975b; Furuichi & Miura, 1975; Wei & Moss, 1975; Abrahams et al., 1975; Urishibara et al., 1975; Perry et al., 1975; Perry & Kelly, 1976; Salditt-Georgieff et al., 1976). There are two general kinds of methylated nucleotides in mRNA, namely those that occur at the 5' ends in the form of 7-methylguanosine in a 5'-5' pyrophosphate linkage to a 2'-O-methyl nucleotide (cap I) and an internal N^6 methyl adenine moiety (Darnell et al., 1976). A more highly modified 5' terminus, designated cap II, contains an additional 2'-O-methylated nucleotide and has been found in many cultured mammalian cells as well as tissue mRNA of known coding functions. The silk fibroin mRNA contain exclusively the cap II type structure (Yang et al., 1976); globin mRNA has in addition to cap II structure at its 5' end (Perry & Scherrer, 1975) a 7-methylated guanosine group at its 5' terminus (Muthutkrishman et al., 1975).

Removal of this group is sufficient to prevent the translation of
the mRNA (Muthukrishnan et al., 1975). Ovalbumin mRNA similarly has cap II type structure at its 5' end. Cap II is not found in HnRNA (Adams & Cory, 1975; Perry & Kelly, 1976) and is added to mRNA in the cytoplasm. Since cap I and the internal M$^6$Ap methylated nucleotides occur in HnRNA, these may represent cleavage sites and conserved regions of putative precursors during the processing reactions. Schibler & Perry (1976) showed that initial transcripts had the structure pppXp ('X' is any nucleotide) at the 5' terminus and that other structures, for example m$^7$GpppXp and cap I structure represented processing derivatives. From this data, the authors proposed a model in which some mRNA sequences were located at transcriptionally initiated portions and others in internal regions of the HnRNA precursors.

2.2.1.3 **Transcribed homologous oligonucleotides in HnRNA and mRNA**

Besides the poly(A) sequences in HnRNA and mRNA, additional sequence homologies were revealed when two shorter internally located sequences were found within the RNA of a number of cultured cells. One is a short stretch of oligo adenylic acid of about 25 AMPs (Edmonds et al., 1976) which in contrast to the poly(A) is transcribed (Nakazoto et al., 1974; Jacobson et al., 1974) and the other, a stretch of about 30 uridylic nucleotides (Burdon & Shenkin, 1972), was transcribed from repetitive DNA sequences (Molloy et al., 1972). Oligo(A) sequences were postulated to serve as primers in poly(A) synthesis (Scherrer, 1973; Jacobson et al., 1974; Edmonds et al., 1976).
Oligo(U) sequences occur as 2–3 units of 30–40 nucleotides in large polyadenylated HnRNA (Molloy et al., 1974). Oligo(U) sequences in mRNA are found in both the polyadenylated and nonpolyadenylated species (Korwek et al., 1976) and the nonpolyadenylated oligo U rich mRNA represents 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). Some sequences derived from repetitive DNA occur in HnRNA molecules (Kronenberg & Humphreys, 1972; Jelinek et al., 1974) take the form of double stranded sequences (Ryskov et al., 1973; Jelinek & Darnell, 1972; Jelinek et al., 1974) of about 500 nucleotides in length. The bases that are contained in the intramolecular loops are largely of two general kinds, the longer A+U rich and shorter C+G rich, neither types were detectable in ribosomal rRNA precursors (Ryskov et al., 1973). Such highly ordered sequences of secondary structures in HnRNA may serve as signals for the post-transcriptional modification of these molecules (Molloy et al., 1974).

2.2.2 Direct evidence for a precursor–product relationship between HnRNA and mRNA

Direct evidence for the biogenesis of mRNA from putative HnRNA precursors should be able to demonstrate the presence of mRNA sequences in HnRNA or the ability of HnRNA to code for specific proteins.

2.2.2.1 Translational approach
2.2.2.1 **Translational approach**

This requires that HnRNA containing mRNA sequences should be able to be translated into proteins in cell free systems, thus a translational approach has been followed by several workers. Williamson et al., (1973) microinjected HnRNA from mouse foetal liver cells into oocytes and showed globin synthesis. The RNA used sedimented faster than 35S. Stevens & Williamson, (1973) used HnRNA from mouse myeloma cell lines which directed the synthesis of antibody heavy chain proteins when injected into oocytes. Pulse-chase experiments showed that this HnRNA precursor is polyadenylated and is finally processed into mRNA which is later transported to the cytoplasm. Riuza-Carrilo et al., (1973) demonstrated the synthesis of duck globin in Krebs II ascites lysates by fully denatured nuclear RNA sedimenting at greater than 45S RNA. The evidence demonstrating messenger coding sequences in HnRNA has to be interpreted with some caution. For example, the high molecular weight nature of the mouse foetal liver cell HnRNA used by Williamson et al., (1973) may be a consequence of aggregation. Translation in frog oocytes may be complicated by contamination of the nuclear RNA with mRNA; especially as the oocyte system is extremely sensitive to nanogram quantities of mRNA (Lane et al., 1973).

2.2.2.2 **mRNA sequences in HnRNA demonstrated by molecular hybridization experiments**

A more sensitive technique employed to demonstrate the precursor-product relationship between HnRNA and mRNA is by molecular hybridization experiments. In most experiments, this involves the synthesis of single stranded, complementary DNA (cDNA) using mRNA as
a template and avian myeloblastosis viral RNA-dependent DNA polymerase (reverse transcriptase) as a catalyst (Kacian et al., 1972; Ross et al., 1972; Varma et al., 1977). The existence of globin mRNA sequences in HnRNA was first demonstrated by Melli & Pemberton (1972) using the molecular hybridization technique and in further studies Imaizumi et al., (1973) made cDNA from duck globin mRNA and showed that it hybridized with HnRNA molecules sedimenting at larger than 28S. In a similar fashion McNaughton et al., (1974) indicated the presence of globin mRNA in nuclear RNA of a discrete size. Ross (1976) detected a 15S nuclear precursor to globin mRNA of mouse foetal liver cells and in Friend erythroleukaemic cells induced dimethylsulphoxide, globin mRNA was detectable in HnRNA sedimenting at 15S (Curtiss & Weisman, 1976) and 27S (Bastos & Aviv, 1977). Similar size classes of HnRNA contained globin mRNA in cells of anaemic mice and spleen (Kwan et al., 1977). Pulse-chase experiments have provided direct evidence that globin mRNA is derived from a large precursor molecule (Strair et al., 1977; Crawford & Wells, 1978) and subsequently processed to the cytoplasmic 10S globin mRNA. cDNA, copied from whole populations of polyadenylated mRNA, has been used to probe for complimentary sequences 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. Perry et al., (1976) prepared DNA probes complimentary to poly(A)+mRNA and used these unique 'mDNA' to probe for mRNA sequences in HnRNA fractions of mouse L cells. They found mRNA sequences in several size classes of polyadenylated as well as non polyadenylated HnRNA. Hybridization of cDNA, prepared
against rat liver cytoplasmic poly(A)RNA to total rat liver nuclear RNA, showed the presence of the cytoplasmic RNA sequences in large nuclear precursor molecules sedimenting between 37S - 44S (Sippel et al., 1977a, 1977b).

Infection of cells of DNA virus results in the integration of the viral genome into the host cell DNA the transcription of which produces large HnRNA molecules containing viral specific sequences (Craig et al., 1974; Sharp et al., 1974; Craig & Raskas, 1976). Hybridization of endonuclease (EcoRI) restriction fragments of viral genome to newly synthesised HnRNA revealed viral specific mRNA sequences. The size of the HnRNA containing viral sequences decreased with size, indicating actual processing of the HnRNA into cytoplasmic virus-specific mRNA (Bachenheimer & Darnell, 1975; Craig & Raskas, 1976).

2.2.2.3 Gene 'Inserts' and the Relationship between HnRNA and mRNA

It appears that a much greater understanding of the relationship between HnRNA and mRNA may come from the discovery of gene inserts.

Studies initially with the globin gene, led to the discovery that the coding sequence of genes may be interrupted by intragenic DNA sequences generally termed 'inserts' (Jeffrey & Flavel, 1977; Tilghman et al., 1978; Kinniburgh et al., 1978). Inserts have subsequently been observed in a number of other genes, including a 1250 base insert separating the variable and constant part of the gene for the immunoglobulin light chain of a mouse myeloma cell line.
(Brack & Tonegawa, 1977) and a total of seven different intervening sequences within the ovalbumin gene (Dugaiczyk et al., 1978), Interrupted DNA sequences were also found in rRNA and tRNA genes (Glover & Hogness, 1977; Wellauer et al., 1977; Goodman et al., 1977). Recently it has been shown that the 15S nuclear precursor of globin mRNA contains a transcript of the globin insert which must be ligated during its maturation to the 10S globin mRNA (Kinniburgh et al., 1978). Transcripts of the inserts within tRNA\textsubscript{tyr} are also found in pre-tRNA\textsubscript{tyr} (O'Farrel et al., 1978) and evidence also exists for ovalbumin mRNA precursors containing transcripts of the inserts (Dugaiczyk et al., 1978).

2.3 Diversity and Complexity of mRNA and HnRNA

Britten & Kohn (1968) and Britten & Davidson (1969, 1971) observed that the DNA of the eukaryotic genome consisted of 10-20% repetitive sequences and 80-90% intermediate 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 sequence is transcribed as mRNA (Davidson & Britten, 1973; Lewin, 1975c). Thus the polysomes or generally the cytoplasm will contain 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
Table 1: Sequence Complexity and Diversity of mRNA and Nuclear RNA population

<table>
<thead>
<tr>
<th>Eukaryotic cell types and tissues</th>
<th>Total sequence complexity</th>
<th>Total nos. of mRNA/nRNA species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source of mRNA/nRNA</td>
<td>mRNA-unique mRNA reaction</td>
<td>mRNA-unique mRNA reaction</td>
</tr>
<tr>
<td>S. cerevisae</td>
<td>total polya RNA</td>
<td>1.8x10^3</td>
<td>30000</td>
</tr>
<tr>
<td>Rainbow trout testis</td>
<td>total nuclear RNA</td>
<td>3.6x10^3</td>
<td>4000</td>
</tr>
<tr>
<td>Sea urchin embryos</td>
<td>total nuclear RNA</td>
<td>3.4x10^3</td>
<td>6000</td>
</tr>
<tr>
<td>Hela cells</td>
<td>erytoplasmic polya RNA</td>
<td>1.7x10^3</td>
<td>14,000</td>
</tr>
<tr>
<td>Mouse Friend cells</td>
<td>Nuclear polya RNA</td>
<td>1.6x10^3</td>
<td>14,000</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>cytoplasmic polya RNA</td>
<td>5x10^3</td>
<td>35,000</td>
</tr>
<tr>
<td></td>
<td>nuclear polya RNA</td>
<td>1.2x10^3</td>
<td>31,000</td>
</tr>
<tr>
<td>Rat liver</td>
<td>9x10^3 kilobases</td>
<td>12,000</td>
<td>23,000</td>
</tr>
<tr>
<td>Tobacco leaves</td>
<td>total nuclear RNA</td>
<td>1.5x10^4 kilobases</td>
<td>7000-8000</td>
</tr>
<tr>
<td>Embryonal Carcinoma cell line</td>
<td>nuclear polya RNA</td>
<td>1.2x10^4 kilobases</td>
<td></td>
</tr>
</tbody>
</table>

Rat kidney                       | polya RNA                 | 11,500 | 11,500 | Hantis & Bishop 1976 |
Rat brain                        | Total nuclear RNA         | 8,200  | 10,000 | Chikarashiki et al 1976 |
Rat liver                        | nuclear mRNA              | 5.8x10^8 | 130,000 | Chikarashiki et al 1976 |
Rat kidney                       | nuclear mRNA              | 4x10^8  | 91,000 | Chikarashiki et al 1976 |
Rat spleen                       | nuclear mRNA              | 2x10^8  | 44,000 | Chikarashiki et al 1976 |
Rat thymus                       | nuclear mRNA              | 1.2x10^8 | 35,000 | Chikarashiki et al 1976 |
Rat prostate                     | Total polya RNA           | 1.7x10^8 | 39,000 | Chikarashiki et al 1976 |
Gastrulated Rat prostate         |                            | 7853   | 7853 | Parker & Mainwaring 1977 |
Egg-laying hen oviduct           | polya mRNA                | 9.5x10^9 | 13,000 | Hynes et al 1977 |
Hormone-withdrawn oviduct        | 9.5x10^9 daltons           | 13,000 | 13,000 | Hynes et al 1977 |
Egg-laying hen liver             | 9.5x10^9 daltons           | 13,000 | 13,000 | Hynes et al 1977 |
Mouse embryo                     | 7x10^9 daltons             | 14,000 | 15,000 | Axel et al 1977 |

Young et al 1976
in an RNA population. The percentage rendered double stranded in unique DNA-mRNA saturation hybridization experiments, after appropriately correcting for average size of mRNA and genome size of the organism under study, can be related to the total number of mRNA species and their complexities (Galau et al., 1974). The number and distribution of mRNA sequences has also been determined from the kinetics of hybridization of cDNA to polyA+RNA (Bishop et al., 1974). In principle the radioactive cDNA is complimentary to the different mRNA sequences and thus form hybrids with its complement. Analysis of the kinetics of the hybridization reaction permits a determination of the number of different sequences present as mRNA and the relative abundance of these sequences within the messenger population (Bishop et al., 1974; Birnie et al., 1974). A number of experiments of this nature have been carried out on a variety of cell types, animal and plant tissues revealing the quantity and diversity of unique DNA transcripts. Table 1 summarizes data of mRNA and HnRNA complexity and diversity estimated by both of the above methods.

It is noteworthy, however, that these measurements represent the complexity and diversity of polyadenylated mRNA species and hence do not reflect the true complexity of total cellular mRNA. In a recent investigation the complexity of nonpolyadenylated mRNA has been reported (Grady et al., 1978). These investigators showed the presence of about 8000 different nonpolyadenylated mRNA species in mouse liver and 12,000 different polyadenylated mRNA sequences, thus giving a total of over 20,000 diverse mRNAs. Close examination of the complexity of nuclear RNA and mRNA (Table I) shows that the nuclear RNA of all cell
types and tissues studied were at least 4-10 fold more complex than the cytoplasmic RNA, and complimentary to about 1-4% of unique RNA (Getz et al., 1975; Hough et al., 1975; Liarakos et al., 1973; Ryffel et al., 1976; Bantle & Hahn, 1976; Levy et al., 1976; Kleiman et al., 1977; Chikaraishi et al., 1978). The less complex eukaryotes however, for example the protista and fungi, do not have a separate class of HnRNA molecules (Firtel & Lodish, 1973; Hereford & Rosbash, 1977; Timberlake et al., 1977; Rokek et al., 1978). This is demonstrated by the similarities in the sequence complexities of their polysomal, nuclear and total cellular RNA (Hereford & Rosbash, 1977; Rokek et al., 1978; Timberlake et al., 1977) and this implies that the probable function of HnRNA in the biogenesis of mRNA is unique to higher eukaryotes. Hence, from this review, the data presented is in accordance with the hypothesis that only a small fraction of HnRNA molecules transcribed from structural genes are conserved and processed to the cytoplasm where they function as mRNA.

3. Oestrogens

A study of the changes in HnRNA synthesis and maturation and of mRNA complexity and translation is obviously an important approach to the study of differentiation and development. One system which lends itself to such a study and which is the subject of the work in this thesis is the growth of the uterus in response to oestrogens.

3.1 Physiological Characteristics of Oestrogens
3.1 **Physiological Characteristics of Oestrogens**

The major oestrogens produced by women are oestradiol, oestrone and oestriol, oestradiol being the major secretory product of the ovary.

Oestrogens are produced in the theca-cells of the Graafian follicle during the early stages of menstrual cycle and after ovulation the oestrogens are produced by the granulosa cells of the corpus luteum. The oestrogens stimulate the growth and development of female reproductive organs and secondary sex characteristics characterised by proliferation of the epithelium of the fallopian tubes, endometrium, cervix and vagina. During pre-ovulatory phase, oestrogen induces changes in the tubular mucosa and mobility of the fallopian tubes and, as a consequence of stimulated contractions, promotes the transport of the ovum. In rabbits, rats and guinea pigs, water content of the uterus and its weight increases through water inhibition. Oestrogen together with pituitary factors and progesterone also stimulates mammary growth.

3.2 **Blood oestrogen levels**

Oestrogens are carried in the bloodstream from their sites of synthesis to the target organs. Plasma proteins carry about 60% of the oestrogen in blood (Szego *et al.*, 1953) and weak binding occurs to every plasma fraction (Sandberg *et al.*, 1957). At normal blood oestrogen levels, most of the steroid is attached to the steroid binding globulin (SBG) fraction while binding to albumin and $\alpha_2$ globulin predominates at higher concentrations such as during pregnancy and during proestrus: in rats (Rosenbaum *et al.*, 1966; DeMoor *et al.*, 1957).
SBG is present in plasma from a number of species (Murphy et al., 1968) but rat plasma appears to contain a protein with a somewhat different ligand specificity as it will bind oestradiol and oestrone but not oestradiol-17α or testosterone (Soloff et al., 1971; Baulieu et al., 1971). The sex steroid binding protein of human pregnancy serum has been purified to homogeneity and the amino acid composition presented (Mickelson et al., 1978). Steroid hormones can passively pass the cell membrane but only target tissues are able to accumulate and respond to the hormone because only they contain specific cytoplasmic receptor proteins (Clark & Peck, 1977).

3.3 **The mode of action of oestrogens**

Subsequent to entry into target cells, steroid hormones bind to cytoplasmic receptor molecules and undergo translocation to nuclear sites. The nuclear binding of these steroid-receptor complexes is considered to be an important step in the stimulation of nuclear-mediated events that result in the biologic response. These interactions have been studied extensively in the rat uterus, and a general picture of the mode of action of oestrogen has emerged:

3.3.1 **Oestrogen Receptors**

Substantial evidence has indicated that oestradiol initiates its biological action by forming a complex with an oestrogen-binding protein referred to as an oestrogen receptor (Jensen & DeSombré, 1973; Jensen et al., 1974; Gorski & Gannon, 1976; Notides, 1978).

In the rat uterus, the cytoplasmic oestrogen-receptor sediments as an 8S oestrogen-binding protein in sucrose gradients.
without KCl, while in the presence of 0.3M - 0.6M KCl, the receptor dissociates into a 4S oestrogen-binding protein (Korenman & Rao 1968; Jensen et al., 1969).

As the oestrogen complexes with the cytoplasmic receptor there appears to be a temperature-dependent transformational change in the protein with a concomitant translocation of the complex into the nucleus (Gorski et al., 1973). Translocation is a dose-dependent phenomenon (Clark et al., 1972; 1973). Coincident with transformation and nuclear uptake of the oestrogen receptor complex, the sedimentation coefficient of the receptor increases from 4S to 5S in salt containing gradients (Jensen et al., 1969; Shymala & Gorski, 1969; Giannopoulos & Gorski, 1961). The 5S, but not the 4S oestrogen-binding protein shows a high affinity for uterine nuclei (DeSombre et al., 1972) and has been shown capable of increasing nuclear RNA polymerase activity (Raynaud-Jammet & Baulieu, 1969; Mohla et al., 1972). The 5S activated or transformed receptor is formed by the association of the 4S protein with another macromolecule, independently of the action of an enzyme (Notides & Nielsen, 1974, 1975; Notides et al., 1975; Notides, 1978).

3.3.2 Oestrogen-receptor binding to 'Acceptor Sites' in the Nucleus

The activated receptor interacts with 'acceptor' components of the cell's nuclear structure (Spelsberg et al., 1972; Buller & O'Malley, 1976). Initial studies have examined the role of DNA in binding hormone-receptor complex. Treatment of target tissue DNA, previously exposed to $^3$H-oestradiol, with DNase released bound radiolabelled oestradiol (Harris et al., 1971; King & Gordon, 1972;
Toft, 1972) thus implicating DNA as an acceptor site.

Chromatin regions to which oestrogen binds are highly susceptible to DNase digestion (Chamness et al., 1974; Yamamoto & Alberts, 1974) suggesting that hormone–receptor complex is binding directly to exposed regions of the DNA. This interaction proceeded rapidly at high temperatures (Yamamoto, 1974; Yamamoto & Alberts, 1974; 1975) and the hormone–receptor complex interacted non-specifically to a variety of homologous and heterologous DNA (Yamamoto & Alberts, 1975; Yamamoto, 1976).

Other investigators have shown that the oestrogen–receptor complex binds to euchromatin (Heminki, 1977; DeBoer et al., 1978) and heterochromatin (Sala-Trepat et al., 1977; DeBoer et al., 1978) of nuclear fractions and have suggested that chromatin proteins might participate in a cooperative fashion with DNA in defining nuclear acceptor sites. Spelsberg et al., (1971, 1972) showed that histones are not acceptor molecules and subsequently Puca et al., (1974) have shown more definitively the non histone proteins are prime components of nuclear acceptor sites.

The acceptor role of nonhistone proteins is best illustrated in the studies involving progesterone receptors of chick oviduct chromatin. The progesterone receptor is a dimer composed of 'A' and 'B' subunits that have different and unique properties (Schrader et al., 1975; 1977). The B subunit binds to the oviduct nonhistone protein–DNA complex but poorly to pure DNA, whereas the A subunit binds non-specifically to DNA but poorly to chromatin (O'Malley et al., 1976; Schrader et al., 1977). It is suggested that the B subunit acts as a binding site specifier to
localise the dimer in certain regions of chromatin, and the A subunit may destabilise a portion of the chromatin DNA so that new sites are available to RNA polymerase for the initiation of RNA synthesis (O'Malley et al., 1976).

The general scheme of events as reviewed in this section holds good for a variety of steroid hormones including progesterone (Schwartz et al., 1976; Buller et al., 1976), aldosterone (Edelman et al., 1968; 1975), glucocorticoids (Baxter et al., 1972; Higgins et al., 1973; Beato et al., 1973) and androgens (Bullock & Bardin, 1974; King & Mainwaring, 1974).

3.4 **Biochemical Effects of Oestrogen**

Target tissues respond to steroid hormones in many different ways and changes in target cells have indicated that steroids may exert their regulatory role at the transcriptional level. This conclusion was derived from results illustrated by the action of oestrogens on transcriptional enzymes, chromatin proteins and its activation of specific genes (Gannon & Gorski, 1976; O'Malley et al., 1977).

3.4.1 **Effects of oestrogen on Chromatin template Capacity**

Over a decade ago it was demonstrated that isolated chromatin could serve as template for RNA synthesis (Huang & Bonner, 1962; Baker & Warren, 1966; Sonnenberg & Subay, 1965; Marushige & Bonner, 1966) in the presence of exogenously added RNA polymerase and ribonucleotide triphosphates. The level of RNA synthesised by a given amount of chromatin is generally referred to as its template capacity.
Administration of oestradiol to ovarioctomised rats resulted in an increase in the chromatin template capacity (Barker & Warren, 1966; Warren & Baker, 1967; Teng & Hamilton, 1968) when this was measured using bacterial RNA polymerase. Church & McCarthy (1970) showed that within 2h of hormone treatment the chromatin template activity of rabbit endometrium rose by 500% using the endogenous RNA polymerase. Glasser et al., (1972) also showed an increase in the template capacity of rat uterine chromatin which was demonstrable by 30min after hormone treatment, was maximal at 1 hour and remained constant for a further 4h before declining to control levels by 8h.

The synthesis of RNA on chromatin largely depends on the binding of the RNA polymerase to the template, initiation of RNA synthesis, elongation of the nascent RNA chain, termination and release of the completed chain. Oestrogen may stimulate the transcriptional process by altering any of these parameters. Its effect on RNA initiation sites has been demonstrated with oviduct chromatin. The rifampicin-nucleotide challenge assay (Schwartz et al., 1975; Tsai et al., 1976c) showed an increase by several fold in the level of chromatin initiation sites as early as 30min (Tsai et al., 1975) after restimulation of previously oestrogen treated chick oviduct. The level of increase correlates well with estimates of the total polyA containing RNA sequence complexity of stimulated chick oviduct RNA (Monahan et al., 1976). The kinetics of oestrogenic stimulation of initiation sites for RNA synthesis in chick oviduct chromatin was also shown to correlate with the changes in the endogenous levels of nuclear oestrogen receptor (Kelimi et al., 1976).

Increased template capacity as a consequence of oestrogen treatment may reflect an alteration of the composition of non-histone
chromatin proteins. This was first suggested by Teng & Hamilton (1968) and a number of workers have demonstrated the rapid stimulation in the synthesis of nonhistone chromosomal proteins in the uterus of rats and mice (Teng & Hamilton, 1969; 1970; Smith et al., 1970; Cohen & Hamilton, 1975a, 1975b). In the chick oviduct, the reconstitution of nonhistone proteins from the chromatin of oestrogen-stimulated chicks with other chromatin components from withdrawn chicks resulted in a chromatin able to synthesize substantial amounts of ovalbumin mRNA (Tsai et al., 1976a, 1976b). Thus the NHCP were implicated as specific regulators of oestrogen-induced changes in chromatin template activity. However, the care necessary in interpreting reconstitution experiments has been mentioned in Section 1.1.2.

3.4.2 Effects of oestrogen on RNA-dependent RNA polymerases

An important aspect of gene transcription is the modulation of RNA synthesis by RNA polymerases. Thus the regulation of gene expression by oestrogen may occur by altering the catalytic properties or the levels of the transcriptional enzymes. Early reports on the effects of oestrogen on RNA polymerases in target tissues (Gorski, 1964; Hamilton et al., 1965; Maul & Hamilton, 1967; Barry & Gorski, 1971) suggested that oestrogen evokes an increase in the rate of chain elongation, thus implying that oestrogen stimulates the activity but not the number of active enzyme molecules. All the three major enzyme activities are affected by oestrogen treatment. RNA polymerase I activity in uteri is increased during the first 6h following oestradiol treatment (Glasser et al., 1972; Borthwick & Smellie, 1975; Hardin et al., 1976; Webster & Hamilton, 1976; Weil et al., 1977). As far as the action
of oestrogen on polymerase II is concerned, there are conflicting reports. Glasser et al. (1972) and Hardin et al. (1976) observed an increase in the enzyme activity as early as 15 min after oestradiol treatment and declining to control levels at 60 min before a second increase 3–6 h after oestradiol treatment. Similar rises in RNA polymerase II activity have been reported in nuclei isolated from 30 min–4 h oestradiol treated rabbit uterus (Borthwick & Smellie, 1975). These results are in agreement with findings on the oestrogen-stimulated synthesis of HnRNA (Knowler & Smellie, 1973; Knowler, 1976). RNA polymerase III activity was shown to increase by several fold within 6 h of oestradiol treatment (Weil et al., 1977) and the increase persisted after 24 h of hormone treatment (Webster & Hamilton, 1976). The increase in polymerase II activity was affected by actinomycin D and administration of this inhibitor 30 min before oestradiol abolished the biphasic increase in its activity (Glasser et al., 1972). Administration of cycloheximide did not affect the primary increase in activity but completely abolished the secondary increase in activity. This result, taken together with the findings of Nicolette & Babler (1974) and Lindell et al. (1978) demonstrated that the full effect of oestrogen on RNA synthesis depended on protein synthesis.

It is noteworthy that all of the above enzyme activities were measured in isolated nuclei and may therefore reflect increased template availability rather than increased enzyme activities. Indeed when Borthwick & Smellie (1975) extracted and fractionated uterine RNA polymerase I and II, no oestrogen induced effects were observed.
3.4.3 Transcriptional and translational responses to oestrogen

The oestrogen-induced stimulation of chromatin template capacity and RNA polymerase activity manifests itself in transcriptional and translational events which vary in the different target tissues. It is proposed to compare and contrast these responses in the three target tissues which have been the subject of most study, namely the avian oviduct, the avian and amphibian liver and the mammalian uterus.

3.4.3.1 Transcriptional and translational responses in the oviduct

The magnum region of the oviduct is responsible for the secretion of the egg white proteins which it does under the influence of oestrogen and progesterone.

When immature chicks are given daily injections of oestradiol or diethylstilbestrol, the oviduct begins to grow and differentiate into several new cell types (Oka & Schimke, 1969; Kohler et al., 1969; O'Malley, et al., 1969) with a preponderance of the tubular gland cells which actively produce ovalbumin (Kohler et al., 1969; Rosenfeld et al., 1972; Means et al., 1972; Palmiter & Smith, 1973). Harris et al. (1975), using cDNA hybridisation techniques showed that the daily oestrogen treatment resulted in an increase in the ovalbumin mRNA from essentially zero to 48,000 molecules/cell and upon withdrawal of oestrogen treatment for 12 days caused a decrease of mRNA to about 0-10 molecules/cell (Harris et al., 1975; 1976; Monahan et al., 1976). The number of structural genes expressed in the hen oviduct has been determined. There are 13000–15000 (Axel et al., 1976; Hynes et al., 1977) diverse mRNA species in the egg-laying hen oviduct including, besides ovalbumin mRNA,
ovomucoid mRNA and lysozyme mRNA (Shutz et al., 1977; Groner et al., 1977) and oestrogen affects an increase in abundance of these mRNAs by 3000 fold (Hynes et al., 1977).

A number of findings in the oviduct reveal that oestrogen exhibits subtle differences in the way it effects the different genes which it influences. The egg white protein conalbumin is synthesized with a much shorter lag than ovalbumin and Palmiter et al., (1976) have shown that these differences reflect similar lags in the transcription of the mRNAs. A third egg white protein, avidin is only made after oestrogen has brought about the initial tissue growth and development and progesterone has activated mRNA synthesis (O'Malley et al., 1967; Chan et al., 1973). With all of the egg white proteins, synthesis is directly related to the accumulation of their mRNAs (Palmiter et al., 1976; Harris et al., 1976; Lee et al., 1978; McKnight, 1978). Evidence has already been presented which suggests that egg white protein mRNA is represented in the target cell nuclei as large HnRNA which may contain transcripts of gene inserts.

3.4.3.2 Transcriptional and translational responses in liver

The liver of birds and amphibians synthesizes vitellogenin which is transported in the blood to the ovaries where it is cleaved into egg yolk proteins phosvitin and lipovitellin (Tata, 1976; Jost & Pehling, 1976). The synthesis of vitellogenin in response to a single injection of oestradiol to these animals occurs with a lag phase which depends on whether vitellogenesis is induced in vivo or in tissue culture (Dolphin et al., 1971; Clemens et al., 1975; Green & Tata, 1976; Waugh & Knowland, 1975). In vivo, the peak of stimulation
occurred after 10 days of oestradiol treatment (Tata, 1976) and the continuous presence of oestradiol is required for the maintenance of vitellogenin synthesis (Green & Tata, 1976). Oestradiol induction of vitellogenin in liver explants is a dose-dependent phenomenon which suggests the presence in Xenopus liver of an oestradiol receptor (Waugh & Knowland, 1975; Green & Tata, 1976) similar in properties to that found in oviduct and other mammalian target tissues. The appearance and extent of vitellogenesis is coincidental to the accumulation and abundance of vitellogenin mRNA (Ryffel et al., 1977). In chicken liver, besides vitellogenin, oestrogen stimulates the synthesis of transferrin (Lee et al., 1978), the major serum iron-binding protein and its synthesis is also dependent on the appearance and accumulation of transferrin mRNA.

The induction of vitellogenin synthesis in the liver is associated with other growth responses. Oestrogen has been shown to enhance the synthesis in liver of high-molecular-weight poly(A) containing MmRNA (25-60S) though it has not yet been shown that these are precursors of vitellogenin mRNA. Vitellogenin mRNA is a large messenger species and its appearance in the liver cytoplasm is associated with very large polysomes (Berridge et al., 1976).

3.4.3.3 Transcriptional and translational responses in uterus

The oestrogen induced transcription and translational responses in the uterus are not characterized by the appearance of major new proteins. Rather the hormone initiates a cycle of growth and development which prepares the tissue for implantation and possible pregnancy. This manifests itself at the biochemical level by a mobilization of the protein synthesizing machinery of the cell and by
subsequent hypertrophy and hyperplasia.

The first transcriptional response observed is a stimulation in the synthesis of HnRNA which is detectable by 30min after the administration of oestradiol-17β to immature rats (Knowler & Smellie, 1971; 1973) and which mirrors the stimulation of nucleoplasmic RNA polymerase recorded at this time (Glasser et al., 1973; Borthwick & Smellie, 1975). The stimulation of HnRNA synthesis, and its possible maturation into RNA, can be followed by the appearance of extractable HnRNP particles (Knowler, 1976) and the aggregation of pre-existing ribosomes into polysomes containing newly made mRNA (Teng & Hamilton, 1967; Merryweather & Knowler, 1978; Merryweather & Knowler, in preparation).

By 1 hour after the administration of oestradiol to immature rats, the synthesis of rRNA is stimulated and peaks at 10-12 times unstimulated levels by 2-4h after hormone treatment (Knowler & Smellie, 1973). Inhibitor studies strongly suggest that this stimulation of rRNA synthesis is dependent on the prior stimulation of HnRNA synthesis and on protein synthesis. Thus, if α-amanitin, a specific inhibitor of nucleoplasmic RNA polymerase is administered to rabbits 30min before oestradiol-17β, the stimulated synthesis of rRNA as well as HnRNA is inhibited. Conversely, if the inhibitor is given 30min after oestrogen, i.e. after the stimulation of HnRNA synthesis, then rRNA synthesis is unaffected (Borthwick & Smellie, 1975). Similar time dependent effects on the stimulated synthesis of rRNA have been observed with protein synthesis inhibitors (Knowler & Smellie, 1971; Borthwick & Smellie, 1975).
The above findings have given rise to the suggestion that oestrogen initiates in the uterus a sequence of interdependent events as follows:

\[ \text{oestrogen binding} \rightarrow \text{stimulation of HnRNA synthesis} \rightarrow \text{maturation of some HnRNA to mRNA} \rightarrow \text{synthesis of small number of proteins} \rightarrow \text{stimulation of rRNA synthesis} \]

The nature of the small number of proteins is completely unknown. They could be non-histone proteins, hormone specific factors for RNA polymerase, ribosomal proteins, pre-ribosomal particle proteins, etc. New species of non-histone protein have been detected in the oestrogen stimulated uterus (Cohen & Hamilton, 1975a; 1975b). The best known protein which is synthesized in stimulated levels at this time is the oestrogen-induced protein 'IP' (DeAngelo & Gorski, 1971; Katzenellenbogen & Gorski, 1972; Baulieu et al, 1972). The function of this protein is presently unknown, though in a recent review, Garland et al, (1978) suggested that it may play a role in the replenishment of receptor.

At the same time or perhaps slightly earlier than the stimulated synthesis of rRNA, the synthesis of tRNA and 5S RNA is also stimulated (Knowler & Smellie, 1971) and this is followed by the accumulation of new ribosomes, the mobilization of the protein synthesizing machinery, and a stimulation of total protein synthesis leading to cellular hypertrophy. At later times still DNA synthesis, mitosis and hyperplasia occur (Stormshak et al., 1978). It is significant, that these later events require the continued presence of oestrogen and short lived
oestrogens such as oestradiol to show slight stimulation of rRNA synthesis (Knowler, 1978) and do not produce hyperplasia (Clark et al., 1978).

**Aim of this Project**

As outlined above, a prerequisite for the oestrogen-stimulated production of new ribosomes appears to be the prior synthesis of HnRNA, the maturation of the HnRNA to mRNA and the expression of the mRNA as a small number of ill defined proteins. As part of a continuing study of these processes, the work described in this thesis aims to further elucidate some of these processes, namely the stimulated synthesis of HnRNA, the associated changes in the mRNA population and the relationship of the HnRNA to the mRNA.
MATERIALS AND METHODS
MATERIALS AND METHODS

1. Materials

1.1 Isotopes and Materials for liquid scintillation counting

\[ ^{3}H \]Uridine (5 Ci/mmol); \[ ^{8}H \] Guanosine (10 Ci/mmol - 25 Ci/mmol) \[ ^{2}H \]Adenosine (30 Ci/mmol), \[ ^{3}H \]dGTP 4.85 Ci/mmol - 25 Ci/mmol) \[ ^{3}H \]poly(U) (35 Ci/mol Pi) were purchased from the Radiochemical Centre, Amersham, Bucks, England. Toluene/PPO scintillator was prepared by dissolving PPO at 0.5% (w/v) in AnalR Toluene. Triton/Toluene scintillator was prepared by dissolving PPO at 0.5% (w/v) and Bis-MSB or POPOP at 0.5% and 0.3% (w/v) respectively in a solution containing 3% (v/v) Triton X-100 and 6% (v/v) AnalR toluene. PPO (2, 5diphenyloxazole) (scintillation grade) was purchased from International Enzymes, Windsor, or from Koch Light Laboratories, Colnbrook, Bucks, England. Bis-MSB(p-bis(0-methylstyryl) benzene) was supplied by Eastman Kodak, Kirby, Lancs, England. POPOP(scintillation grade) (1, 4 bis 2-(5-phenyloxazolyl) benzene) was supplied by Nuclear Enterprises, Edinburgh, Scotland. Glassfibre filters (G F/C) were supplied by Whatman, Kent, England. Kieselguhr (Hydro-Supercell) was purchased from Koch-Light Ltd., Colnbrook, Bucks, England.

1.2 Column Chromatography

a) Sephadex G50 or G25 Column Chromatography

Sephadex was obtained from Pharmacia (G.B.) Ltd., London, England. When used, they were stirred into 15 - 20 volumes of water containing 0.02% (v/v) diethyl pyrocarbonate (sterile water), and
allowed to swell at room temperature, overnight. The swollen gels were autoclaved at 5psi for 2 hours. Columns were 25ml packed volume over a pad of Dowex-chelating resin. The columns were equilibrated with the appropriate buffers or sterile water.

b) **Dowex-chelating resin**

Dowex-chelating resin was obtained from Sigma Chemicals, London, England. When used, 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.

c) **Hydroxyapatite**

Bio-Rad, Biogel HTP (DNA grade) was supplied by Bio Rad Laboratories Ltd., Watford, England. Preparation and characterization for chromatography are described in section 9.5.1 of the Methods section.

d) **Poly(U) Sepharose and Thiol-Sepharose affinity Chromatography**

Poly(U) Sepharose was supplied by Pharmacia (C.B.) Ltd., London, England. 0.1g Poly(U) Sepharose was swelled in 15ml 1M NaCl pH 7.5, containing 0.02% (v/v) diethyl pyrocarbonate at room temperature for 30-45 mins. The gel was layered into Pasteur pipettes plugged with glass fibre filters and the 2-4cm columns washed extensively with 0.1M NETS (0.1M NaCl, 0.001M EDTA 0.01M Tris, 0.02% (w/v) sodium dodecyl sulphate) pH 7.4. These columns were equilibrated with 0.4M NETS pH 7.4 (as above but NaCl at 0.4M) and used to fractionate HnRNA. For the
fractionation of polysomal RNA, 0.3g gel was swelled in 15ml 1M NaCl as above and layered in sterile disposable syringes up to 2ml packed gel volume. The column was washed extensively with 0.4M NETS and equilibrated with a concentrated salt buffer prepared in 25% (v/v) formamide in 0.7M NaCl, 50mM Tris-HCl pH 7.5, 10mM EDTA. Thiol-Sepharose, containing 3 μmole SH/ml resin was prepared as described by Dale & Ward (1975) and was the generous gift of Dr. Alan Balmain of the Beatson Institute for Cancer Research, Glasgow. Before use, it was activated by treatment with 50mM dithiothreitol in 0.5M Tris-HCl pH 8.0 for 30 min. at room temperature and washed extensively with 0.1M NETS buffer (0.1M NaCl, 0.01M tris-HCl pH 7.4, 1mM EDTA and 0.7% w/v sodium dodecyl sulphate).

1.3 Buffers

Trizma-HCl and Trizma base, HEPES (N-2-hydroxyethyl-piperazine-N-2 ethanesulphonic acid) were obtained from Sigma Chemical Co., London, England.

1.4 Nucleic Acids

E. coli DNA, calf thymus DNA and yeast tRNA were obtained from Sigma Chemical Co., London, England. Poly(U), Poly(A) and oligo A28 were obtained from Miles Laboratories, Stoke Poges, England. dATP, dGTP, dTTP and oligo dT (12–18) were supplied by P-L Biochemicals Inc., Wisconsin, U.S.A. Generous gifts of other naturally occurring nucleic acids are gratefully acknowledged as follows:

Rat liver unique DNA and (3H)-labelled nick-translated unique DNA from Dr. Alan Balmain, Beatson Institute for Cancer Research, Glasgow.
Erythrocye globin mRNA from Dr. George Birnie, Beatson Institute for Cancer Research, Glasgow. $^{32}$P-labelled 5.8S, 28S, 32S and 45S HeLa cell nucleolar RNA from Dr. Keith Vass of this department.

1.5 Enzymes, Hormones and Metabolic Inhibitors

a) Enzymes

$S_1$ Nuclease was purchased from Sigma Chemical Co., London, England or from Boehringer Corporation, London, England. $T_1$ ribonuclease and pancreatic ribonuclease A were obtained from Sigma Chemical Co., London, England. AMV reverse transcriptase was supplied by Dr. J.W. Beard of Life Sciences, Inc., Florida, U.S.A. through the Viral Cancer Program, National Cancer Institute, Bethesda, U.S.A. Proteinase K was purchased from Boehringer Corporation, London, England.

b) Hormones

Oestradiol-17$\beta$ was obtained from Sigma Chemical Co., London, England.

c) Metabolic Inhibitors

Cycloheximide was obtained from Sigma Chemical Co., London, England. Actinomycin-D and heparin were purchased from Calbiochem Ltd., London, England.

1.6 Reagents for the Purification of RNA

Bentonite, phenol and sodium dodecyl sulphate (specially pure) were purchased from British Drug House Chemicals Ltd., Dorset, England and bentonite was purified essentially by the method of Fraenkel-Conrat et al., (1961). 20g bentonite was suspended in 400ml of distilled water
and mixed thoroughly by homogenization with an Ultra-Turrax. The paste was centrifuged at 800g for 15 mins. The supernatant fraction was recentrifuged at 800g for 20 min and the resulting sediment was re-suspended in 0.1M disodium EDTA (pH 7.0) for 48 hours at room temperature. The suspension in EDTA was recentrifuged differentially and the 8000g sediment suspended in sterile distilled water was autoclaved at 15 psi for 30 min. When required, an aliquot was removed, dried down, and suspended in the appropriate buffer at 20mg/ml. Phenol was redistilled before use.

1.7 Reagents for Electrophoresis

Electrophoretically pure acrylamide and NNN'N'-tetramethylethylenediamine were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. NN'-methylene bisacrylamide was supplied by the British Drug House Chemical Ltd., Dorset, England. Ethylene diacrylate was purchased from Kodak Ltd., Kirby, Lancs., England. Formamide puriss, purchased from Fluka, Busch, Switzerland was deionised for 3 hours with AG-501-X8(D) mixed bed resin at 3-4g/100ml purchased from Bio-Rad Laboratories, Watford, England. 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.

1.8 Reagents for In Vitro incubations

Eagle's medium (Glasgow University Modification) of Busby et al., (1964) containing 100 µg/ml of streptomycin, 100 units/ml of penicillin and 0.02% (w/v) phenol red was the usual medium in which
excised uteri were incubated in vitro.

1.9 Glassware

Glassware used to collect fractions of highly radioactive material was coated with 'Repelcoate' supplied by Hopkins and Williams Ltd., England, boiled in diethyl pyrocarbonate treated water, and baked at 200\(^\circ\) overnight. Corex tubes for use in RNA preparations were baked as above or autoclaved at 15 psi for 30 mins. Capillaries for hybridization experiments were supplied by the Aberdeen Glass Company, Aberdeen, Scotland. The capillaries were repelcoated, washed with sterilized water.

1.10 Miscellaneous

Cellulose nitrate and polyallomer centrifuge tubes were a product of Beckman Spinco Ltd., Palo Alto, California, U.S.A.

Ziptrol dispenser used in dispensing less than 1\(\mu\)l samples was purchased from the Aberdeen Glass Company, Aberdeen, Scotland. All other chemicals were, wherever possible AnalalR grade and were usually obtained from the British Drug House Chemicals Ltd., Poole, Dorset, England.

2. Biological Methods

2.1 Experimental Animals

The rats, which were derived from Wistar Strain and bred at Glasgow University, were fed ad libitum on diet 413 (Bruce & Parkes 1956). Immature females were 18 – 21 days old and in all experiments were limited to a weight of 25 – 35g. Adult females were 170 – 200g.
and were used at proestrous. Proestrous was confirmed by microscopic 
examination of vaginal smear. The rats were anaesthetized with ether 
and killed by cervical dislocation. The excised uteri were carefully 
dissected free of connective tissues and rapidly frozen in 'drikold'/ 
methanol bath before further treatment. In in vitro experiments, 
freezing was avoided. Uteri were collected into Eagle's medium (Busby 
et al., 1964) at 37° under an atmosphere of 95% O₂/5% CO₂. 

2.2 Administration of Hormone, Inhibitors and Radioactive 
precursors.

a) Hormone

Oestradiol-17β was solubilized at 10μg/ml in 0.3M NaCl/0.5% 
(v/v) ethanol by the method of Roberts & Szego (1947). All immature 
rats received 1μg oestradiol by intraperitoneal injection in 0.1ml of 
carrier. Control animals received carrier only.

b) Inhibitors

Actinomycin D were injected intraperitoneally in 0.5 ml of 
0.9% NaCl.

c) Radioactive precursors

Radioactive precursors were administered intravenously via 
the lateral tail vein in 0.1 or 0.2ml of saline. To facilitate easier 
handling, animals were kept under light ether anaesthesia during the 
 injection and their tails were pre-warmed in water at 40°C for 1 - 2 mins. 
Tritiated ribonucleosides were administered either as an equal mixture 
of [5-³H] uridine and [8-³H] guanosine or [5-³H] uridine alone. When 
uteri were used for the preparation of total uterine Acid-Soluble and 
Acid Insoluble fractions, the rats received 20μCi of the [5-³H] uridine.
When they were to be used for the purification of uterine RNA or the preparation of sub-cellular components, they received 100μCi of $^{[5-^3H]}$ uridine. However, precise conditions are described in the legends to each figure.

2.3 *In vitro incubations*

Groups of uteri from 8 animals which had received oestradiol treatment or otherwise were incubated in 5ml conical flasks under an atmosphere of 9% O₂/5%CO₂ at 37°C in a shaking water bath. Incubations were usually in 4ml Eagle's medium containing 10 - 20μCi/ml of $^{[5-^3H]}$ uridine. Incubations were for 30 min after which the uteri were washed twice in cold saline, blotted dry and frozen in solid CO₂/methanol bath. RNA extractions, acid-soluble and acid-insoluble fractions were then prepared. In one experiment, when nuclease-resistant polyadenylate core of uterine HnRNA was investigated, 6 - 8 uteri from oestrogen treated rats were removed, dissected free of adipose and connective tissues and incubated in Eagle's medium containing 125μCi of $^{[2-^3H]}$ adenosine/ml for 1 hour. When nuclei were to be prepared, uteri from 12 rats were incubated for 30 min in 4ml Eagle's medium which contained 10μCi/ml $^{[5-^3H]}$ uridine.

3. *Preparations of Acid-Soluble and Acid Insoluble Materials*

3.1 *Acid-Soluble and Acid-Insoluble Materials of whole uteri.*

Acid-Soluble and Acid-Insoluble materials were prepared by a modification of the method of Billing *et al.* (1969a) and as described
by Knowler & Smellie (1971). Uteri, removed from hormone treated animals which had received radioactive precursors 30 min before death were placed individually in universal containers and rapidly frozen in a solid CO$_2$/methanol bath. They were then either stored for up to 3 days at $-60^\circ$ or used immediately. The uteri were thawed, chopped with scissors and homogenised in 2.5ml ice-cold distilled sterile water using a glass homogenizer with a motor driven teflon pestle. All subsequent steps were performed at 0 - 4$^\circ$. The homogenate plus a further 2ml of water, used to rinse the homogenizer, were added to 0.5ml of 50% (w/v) trichloroacetic acid and mixed. After standing for 15 min in an ice-bath, 1.25ml was removed and both the remainder and the aliquot were sedimented at 800g for 5 mins. The supernatant from the smaller portion was discarded and the pellet retained for IHA assay. The supernatant from the larger fraction was retained and the pellet washed with a further 2ml of 5% (w/v) trichloroacetic acid. The washings were combined with the supernatant and this constituted the acid-soluble fraction. An 0.4ml aliquot of the acid-soluble fraction was counted in 10ml of triton/toluene scintillator. The pellet from the larger fraction, after washing, was suspended in a small volume of 5% (w/v) trichloroacetic acid and mixed with 2ml of 2% (w/v) kieselguhr suspension in 5% (w/v) trichloroacetic acid and mixed. An additional 2ml of kieselguhr was collected as a pad on a 2.5cm Whatman No. 1 filter paper disc. The acid-insoluble pellet bound to kieselguhr was collected as a second layer on this pad and washed with 3 x 15ml portions of 5% (w/v) trichloroacetic acid, 1 x 15ml portion of absolute alcohol and 2 x 5ml portions of diethyl ether. The pad was extracted with 0.5ml 1M hyamine hydroxide for 10 min at 60$^\circ$ and the radioactivity measured by
scintillation counting in 10ml toluene based scintillator.

3.2 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 7% (w/v) trichloroacetic acid. One drop of 2% (w/v) bovine serum albumin or 40µg yeast RNA was added as carrier. After mixing and standing at 0 - 4°C for at least 15 min, 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 7% (w/v) trichloroacetic acid, dried in scintillation vials at 50°C for 1 hour and the radioactivity measured by scintillation counting in 10ml toluene based scintillator.

4. Chemical Measurements

4.1 DNA determination

DNA was assayed as described by Burton (1956). Samples were mixed with an equal volume of 0.5N perchloric acid and heated for 15 min at 70°C. The Burton reagent was prepared fresh before use by mixing 0.1ml of acetaldehyde solution (16mg/ml) with 20ml of diphenylamine solution (1.5 g in 100ml of glacial acetic acid and 1.5ml of concentrated sulphuric acid). 1ml of sample was mixed with 2ml of Burton reagent and left to stand overnight at room temperature in the dark. The absorbance was read at 600nm and a calibration curve, using calf thymus DNA as
standard was constructed over a concentration range of 10 - 200μg/ml.

4.2 RNA determination

RNA was measured as described by Kerr & Seraidarian (1945). The orcinol reagent was 60mg orcinol in 10ml of 0.02% (w/v) FeCl₃ in concentrated HCl. 3ml of orcinol reagent was mixed with an equal volume of RNA sample and heated for 30 min at 95°C. The mixture was cooled to room temperature and the absorbance read at 665nm.

5. Preparation of Subcellular Components

5.1 Uterine Nuclei Preparation (Method A)

The method has been described by Knowler (1976). 12 uteri from in vitro incubations were rapidly frozen in solid CO₂/methanol bath and broken up with a footed-glass rod. These were suspended in 2ml RSB (0.01M tris-HCl pH 7.4, 0.01M NaCl, 0.001M MgCl₂) containing 1% (w/v) triton X-100 and homogenized in an Ultra Turrax set at 60V for 30 - 50 sec. This and all subsequent steps were carried out at 0 - 4°C.

The homogenate was filtered through two thicknesses of muslin and washed with a further 2ml of the homogenization medium. The filtrate was sedimented at 300g for 15 min and at 4°C to obtain the crude nuclear pellet. This was suspended in 2ml of the homogenization buffer and a further 2ml of homogenization buffer, containing 0.5M sucrose was mixed with the suspension. The purified nuclei was sedimented at 800g for 15 min and at 4°C through a 2ml cushion of 0.32M sucrose in homogenization buffer.
5.2 Nuclei Preparation (Method B)

The method was essentially that described by Knowler et al. (1973) and Knowler & Smellie (1973). 12 uteri from in vitro incubations were frozen and broken up as in method A, were homogenised in 2ml of 1mM MgCl₂ in an Ultra Turrax homogenizer set at 60V for 20 – 30 sec. This, and all subsequent steps were carried out at 0 – 4°C. The homogenate was filtered through two thicknesses of muslin. The filtrate was mixed with 2ml of cold 0.1M citric acid in 1mM MgCl₂, this having first been used to wash the homogenizer and muslin. The nuclei were collected by sedimentation at 300g for 15 mins and the pellet resuspended in 1mM MgCl₂ containing 0.05M citric acid and 1% (w/v) Triton X-100. The mixture was made 0.25M with respect to sucrose as in method (A) and underlaid with 2ml of 1mM MgCl₂ containing 0.32M sucrose, 0.05M citric acid and 1% (w/v) Triton X-100. The nuclei were collected by sedimentation at 800g for 5 mins.

5.3 HeLa cell subfractionation

5.3.1 Preparation of HeLa cell nuclei (Penman et al., 1968).

³²P-labelled HeLa cell pellets, the generous gifts of Dr. K. Vass were washed once with 5ml of RSB buffer (Reticulocyte Standard Buffer containing 0.01M NaCl, 0.0015M MgCl₂ and 0.01M tris-HCl pH 7.4) pelleting the cells at 450g for 5 min and at 4°C. The cells were then resuspended in 4ml of RSB and left to swell. After 5 min, the cells were broken at 4°C by 15 – 20 strokes in a stainless steel ball homogeniser with a clearance of 0.003" diameter. The homogenate was centrifuged at 800g for 2 min. The supernatant was kept aside at -60°C for the
subsequent preparation of HeLa cell rRNA for use as markers. The
pellet was suspended in 4ml of RSB and 0.6ml of 'Magik' detergent
mixture (1 part 10% sodium deoxycholate and 2 parts 10% Tween 80). This procedure removed the outer nuclear membrane. When the nuclei
was examined under the phase contrast microscope, there were insignificant
amounts of cytoplasmic debris and the nuclei appeared clumped. The
mixture was vortexed for 10 sec and centrifuged at 2000 rpm for 10 min.
at 4° in the HB 4 rotor of the Sorvall centrifuge. The supernatant
was discarded and the purified nuclei were washed with another 4ml of
RSB and centrifuged as before.

5.3.2 Preparation of HeLa nucleoli (Penman et al., 1968)
HeLa cell nucleoli was prepared from 32P labelled nuclei
which were gifts from Dr. Keith Vass or from the nuclei as prepared
above. Purified nuclei were suspended in 2ml of HSB (0.5M NaCl,
0.05M MgCl2 and 0.01M Tris-HCl pH 7.4) containing 100µg DNase and
repeatedly forced through a pasteur pipette until viscosity and
visible particles had disappeared. The whole preparation was layered
onto a 15 - 30% (w/v) sucrose gradient in HSB and centrifuged at
22,000 rpm for 15 min. in a SW27 rotor for the Beckman L2-65B ultra-
centrifuge. The supernatant nucleoplasm was kept aside for the extraction
and purification HeLa HnRNA. The pellet consisted of purified nucleoli.
All steps were carried out at 0 - 4°.
5.4 Preparation of uterine polysomes

Uterine polysomes were prepared by a procedure developed in this laboratory by my colleague Mr. M.J. Merryweather and it is described here with his permission. The procedure was developed from the published method of Berridge et al. (1976).

24-72 oestradiol-treated immature rat uteri or 6-8 adult uteri were finally chopped with scissors and homogenized in 5 ml of 200 mM Tris-HCl, 50 mM KCl, 15 mM MgCl₂ pH 8.5 containing 5 μg/ml cycloheximide, 7 mM 2-mercaptoethanol and 0.02% (w/v) diethyl pyrocarbonate with a motor driven loose-fitting teflon-glass homogenizer. The homogenate, adjusted to 2% with Triton X-100 was centrifuged at 10,000 rpm for 10 min in the SS-34 rotor of the Sorvall centrifuge. The post-mitochondrial supernatant was layered over a 1 ml cushion of solution containing 50 mM Tris-HCl, 50 mM KCl, 15 mM MgCl₂ pH 8.5 in 1 M sucrose containing 5 μg/ml cycloheximide, 7 mM 2-mercaptoethanol and 0.02% diethyl pyrocarbonate which was spun at 47,000 rpm in cellulose nitrate tubes of the SW 50.1 rotor in the L2-65B Beckman ultracentrifuge. The supernatant was discarded and the polysomal pellet washed once with 50 mM Tris-HCl pH 7.6, 250 mM KCl, 5 mM MgCl₂ containing 50 μg/ml heparin and 5 μg/ml cycloheximide. To analyse the polysome profile, the pellet was suspended in the wash buffer as above and 0.2 ml aliquots were centrifuged in a 15-45% (w/v) sucrose gradient, made in the wash buffer at 50,000 rpm, for 35 min in the SW 50.1 rotor of the L2-65B Beckman ultracentrifuge. Gradients were scanned with a Gilford gradient scanner attached to the Gilford spectrophotometer. All steps were carried out at 0 - 4°C.
6. **Preparation of RNA**

6.1 **Preparation of HeLa cell heterogenous nuclear RNA**

During the preparation of the nucleoli (section 5.3.2) the nucleoplasmic fraction was kept aside. The volume was noted and then precipitated with 2 volumes of ethanol in the presence of 100μg yeast RNA, overnight at -20°. The pellet was dissolved in 1.5ml LEITS (0.01M Tris-HCl pH 7.4, 0.1M LiCl, 1mM EDTA 0.02% (w/v) SDS) buffer and an equal volume of phenol was added. The HnRNA was extracted at 60° with vortexing every 2-3 min. The mixture was centrifuged at 20,000g for 6 mins, at 4° to separate the phases. The aqueous phase was removed and the phenol phase re-extracted with fresh LEITS buffer at 60°. The phases were again separated as before and the aqueous phase combined with the previous one. The volume of the combined aqueous phases were noted and precipitated with 2 volumes of ethanol in the presence of 60μg yeast RNA and 0.15M NaCl. The RNA was pelleted, washed once with ethanol and dissolved in 1ml sterile water and re-precipitated with 2 volumes of ethanol.

6.2 **Preparation of HeLa ³²P-labelled nucleolar RNA**

³²P nucleolar RNA (45S and 32S) were generously provided by Dr. Keith Vass or extracted from nucleolar pellet as described by Fraser (1974). The nucleoli was dissolved in 2ml LEITS buffer pH 7.4 and vortexed until properly dispersed. 2ml of LEITS buffer saturated phenol was added and the nucleolar RNA extracted at room temperature (22°) by occasional vortexing for 2-3 min. The aqueous phase was
obtained by centrifugation at 20,000g at 4°C for 10 min. The phenol interphases were re-extracted with fresh LETS buffer and the aqueous phases from each extraction step were precipitated with 2 volumes of ethanol at -20°C overnight in the presence of 0.15M NaCl and 20μg yeast RNA.

6.3 Preparation of uterine high molecular weight RNA

6.3.1 Preparation of uterine RNA by phenol extraction at various temperatures under differing pH conditions

Sets of 8-12 uteri, removed from treated animals or from in vitro incubations, were rapidly frozen in a solid CO₂/methanol bath. These were broken up with a footed glass rod and transferred into a 'Kontes' all-glass homogenizer containing 10ml of a mixture of 0.05M sodium acetate buffer pH 5.2, 1mg bentonite/ml and 1% (w/v) sodium dodecyl sulphate. The tissue was homogenised at 0-4°C and the homogenate transferred into a 50ml corex tube containing 10ml 88% phenol in acetate buffer pH 5.2. The mixture was then blended for 1 min. in an Ultra Turrax homogenizer set at 60V at room temperature (22°C).

Phenol extraction was carried out by rapid stirring of the homogenate at 55°C for 3 min and, after cooling in ice for 2 mins., was centrifuged at 20,000g for 6 min at 4°C to separate the phases. The phenol layer was discarded and the aqueous layer and interface were made 1mg/ml with respect to bentonite and re-extracted with a further 5ml 88% (v/v) phenol in the respective buffers. After re-centrifugation, the aqueous phase of this second extraction was removed and set aside at
0\textdegree{} to 4\textdegree{} in the presence of 4mg bentonite. The phenol layer and interface were re-extracted for a third time with a further 2ml of buffer containing 2mg bentonite 0.5\% (w/v) sodium dodecyl sulphate. The aqueous layer from this extraction, combined with that from the second extraction, was centrifuged at 20,000g for 15 min to remove most of the bentonite. RNA and DNA was then precipitated from the resulting supernatant in the presence of 0.15M NaCl and 2 volumes of ethanol at -20\textdegree{} overnight.

The precipitated nucleic acid was collected by centrifugation, washed twice with 95\% (v/v) ethanol and dried in a gentle stream of nitrogen. The pellet was digested with 0.3mg ribonuclease-free deoxyribonuclease dissolved in 1.0ml of 0.01M Tris-HCl (pH 7.5) containing 1mM MgCl$_2$. Digestion was effected by incubations at 10\textdegree{} for 5 min and at 25\textdegree{} for 10 min. After cooling in ice, the digest was made 1mg/ml with respect to bentonite and to 1\% with sodium dodecyl sulphate. 0.5 volumes of 80\% (v/v) phenol in Tris-HCl buffer pH 7.5 was added and the mixture extracted at 4\textdegree{} on a mechanical shaker. The phases were separated as before and 1mg bentonite was added to the aqueous phase which was retained at 0 - 4\textdegree{}. The phenol and interphase were re-extracted with 0.5ml of 0.01M Tris-HCl (pH 7.5) containing 1mM MgCl$_2$ and the combined aqueous phases centrifuged as before to remove bentonite.

RNA was precipitated from the supernatant at -20\textdegree{} for 2 hr in the presence of 0.1M NaCl and 2 volumes of ethanol. The RNA pellet was recovered by centrifugation and DNA oligonucleotides were removed by reprecipitation in 1mM MgCl$_2$, 2M potassium acetate and 2\% ethanol at -20\textdegree{}. The reprecipitation was performed twice and the final precipitate washed once with cold 95\% (v/v) ethanol.
The above method was a modification of the method of Joel & Haggerman (1969) and resulted in the isolation of good quality high molecular weight RNA species from which the HnRNA could subsequently be isolated. In some early experiments, the method was modified in an attempt to employ extraction at varying pH or at varying temperatures as described by Georgiev (1967) and Brawerman (1976). In one set of experiments, the initial phenol extraction was carried out successively at 10°, 40° and 55° and a further set employed sequential extractions in 0.1M Tris-HCl buffer containing 2mg bentonite/ml and 1% (w/v) sodium dodecyl sulphate at pH 7.6 and pH 8.3.

The crude R12T1A, DNA pellet obtained from the extractions in Tris-HCl buffer pH 8.3 and pH 7.6 at 55° were repurified by repeating the extraction with 5ml of 0.05M acetate buffer pH 5.0 containing 0.001M EDTA, 2mg bentonite/ml and 1% (w/v) sodium dodecyl sulphate and an equal volume of 85% (w/v) phenol. Extraction was carried out at 55° with intermittent vortexing. This procedure was repeated twice more and the aqueous phases from the three extractions were precipitated as described earlier.

6.4 Preparation of uterine RNA from subcellular components

6.4.1 Preparation of uterine nuclear RNA

Nuclear RNA was prepared as described for total RNA in Section 6.3 except that phenol was not added until after the initial homogenization in 0.1M Tris-HCl pH 8.3 containing 1mg/ml bentonite and 1% (w/v) sodium dodecyl sulphate. This was necessary as phenol can protect nuclei from lysis. It was only necessary to perform one
potassium acetate/ethanol precipitation in order to remove the DNA oligonucleotides remaining after the deoxyribonuclease digestion. In extracting these small amounts of RNA, reagent volumes were reduced by half and it was necessary to add unlabelled cytoplasmic uterine RNA to effect precipitation.

6.4.2 Preparation of polysomal RNA

Polysomal pellets were suspended in 2ml 0.1M NETS buffer (0.1M NaCl, 0.001M EDTA, 0.01M Tris-HCl pH 7.5 0.2% sodium dodecyl sulphate) containing 200μg proteinase K and incubated at 37° for 30 min...

2ml of phenol: chloroform: isoamylalcohol (1:1:0:1) was then added, vortexed briefly and RNA was extracted on a mechanical shaker for 10 min at room temperature. The phases were separated by centrifugation at 20,000g for 6 min at 4°. The phenol and interphase were subjected to 2 further extractions with 0.1M Tris-HCl buffer pH 9.0 containing 1mg bentonite/ml and 1% SDS (w/v). The combined aqueous phases were centrifuged at 20,000g for 15 min to remove bentonite and subsequently precipitated with 2 volumes ethanol at -20° overnight. The polysomal RNA was washed twice with ethanol, dried in a gentle stream of N₂ and dissolved in sterile water. The virtual absence of DNA in these preparations precluded the need for a DNase digestion step.

In the early development of this method, comparative experiments were conducted in which proteinase K digestion was not employed while others were performed on post-mitochondrial supernatants rather than polysomal pellets. In these latter experiments the initial extraction followed the addition of an equal volume of phenol: chloroform: isoamylalcohol (1:1:0:1) to the supernatant but subsequent extraction
steps were as above.

6.5 **Preparation of polyadenylated RNA species**

Poly(A)\(^+\) RNA species from uterine high-molecular-weight HnRNA and polysomal RNA populations were purified by poly(U) Sepharose affinity chromatography.

6.5.1 **Poly(U)-Sepharose chromatography of uterine high molecular weight HnRNA**

RNA samples dissolved in 1ml of 0.4M NETS (0.4M NaCl, 0.01M EDTA, 0.01M Tris-HCl, 0.2\% (w/v) buffer pH 7.4) were applied to columns of poly(U) Sepharose packed in Pasteur pipettes. To ensure complete binding the initial eluate was recycled through the column once. Poly(A)-free HnRNA was eluted with the same buffer, whereas poly(A) rich HnRNA was eluted in a stepwise fashion with 15 x 1ml of increasing concentrations of formamide in ETS buffer pH 7.4 (0.01M EDTA, 0.01M Tris-HCl, 0.2\% (w/v) sodium dodecyl sulphate). Samples were removed from each fraction and the radioactivity was measured in the acid-insoluble material as described in section 3.2. Results show that (see Figure 9 and Table 4 in the Results section) the amounts of bound HnRNA released from poly(U) Sepharose are eluted into two peaks at 15\% (v/v) and 90\% (v/v) formamide/ETS buffer. In subsequent experiments these two concentrations of formamide were used so that two bound fractions of HnRNA were collected together with an unbound fraction. The HnRNA fractions were precipitated with 2 volumes of ethanol in the presence of 0.15M NaCl and 40\mu g unlabelled uterine cytoplasmic RNA, overnight at \(-20^\circ\). The precipitates were recovered by centrifugation,
washed twice with ethanol, dissolved in sterile water and re-precipitated with ethanol.

6.5.2 Poly(U) Sepharose chromatography of polysomal RNA

0.8-1mg polysomal RNA was suspended in a high salt buffer (0.7M NaCl, 50mM Tris-HCl, 10mM EDTA pH 7.5) containing 25% (v/v) formamide and denatured by incubation for 5 mins in a 55° waterbath. 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 rigorously removed 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% formamide (v/v) containing 10mM KH₂PO₄, 10mM EDTA, and 0.02% (w/v) sodium dodecyl sulphate. 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°C in the presence of 0.15M NaCl. The poly(A)^+RNA was redissolved in sterile water and desalted by gel filtration through Sephadex G25 or G50 underlaid with Dowex chelating resin and equilibrated with water. The salt-free poly(A)^+RNA was freeze dried, dissolved in a minimum volume of water and kept at -70°. Purity and integrity of polysomal RNA and poly(A)^+RNA was checked by electrophoresis on 3.7% polyacrylamide gels containing 98% formamide.
6.5.3 Preparation of poly(A)^+RNA from dissociated polysomal ribonucleoprotein particles.

17–20 OD_{260} units of polysome pellet was suspended in 0.5ml of dissociating solution made up on 1% (w/v) N-lauryl-Sarcosine and 0.03M EDTA (Lindberg & Persson 1972). The suspension was mixed and diluted 5 times with high salt buffer containing 25% (v/v) formamide and the diluted sample was incubated for 30 mins at 37° and immediately layered over a 2ml packed volume poly(U) Sepharose. The sample was allowed to drain through under gravity and the initial eluate recycled once through the affinity column. The unbound RNA was rigorously removed by washing with the concentrated salt buffer containing 25% (v/v) formamide and the bound polyadenylated RNA species were eluted with 2 x 1ml followed by 2 x 2ml elution buffer containing 10mM KH_2PO_4, pH 7.5, 0.2% (w/v) N-lauroyl sarcosine and 90% (v/v) formamide. RNA in each chromatographic fraction was precipitated with 2 volumes of ethanol at -20° overnight in the presence of 0.15M NaCl and 30μg unlabelled uterine RNA. The RNA pellet was recovered by centrifugation, washed twice with ethanol, dissolved in sterile water and reprecipitated with ethanol.

7. Fractionation of RNA

7.1 Fractionation of RNA on aqueous polyacrylamide gels

Gels were prepared as described by Knowler & Smellie (1971). 2.7% gels, which were used for the fractionation of high molecular weight RNA or when RNA was to be recovered from gel slices, contained 2.7% (w/v) acrylamide, 0.2% (v/v) ethylene diacrylate and 1% (v/v) NaN_3N.
tetramethylethlenediamine. They were prepared in the electrophoresis buffer (36mM Tris, 30mM NaH₂PO₄ and 1mM EDTA adjusted to pH 7.7–7.8 with phosphoric acid) as that described by Loening (1969) and polymerization was catalyzed by the addition of ammonium persulphate to 0.1% (w/v). 10% gels were prepared in the same manner but the acrylamide concentrations were 10% (w/v) and they contained bisacrylamide at 0.26% (w/v) instead of ethylene diacrylate.

The prepared solutions were carefully mixed and 3ml aliquots were rapidly pipetted into vertical 1/4 x 5" plexiglass tubes. Water was carefully layered over the solution using a Hamilton syringe. The gels were allowed to set for 30 min at room temperature. The water layer was removed and replaced by the electrophoresis buffer. All gels were pre-electrophoresed at 2.5mA/gel for 15–30 min before RNA samples (80–100µg), dissolved in 40–50µl electrophoresis buffer containing 20% (w/v) sucrose and 0.2% (w/v) sodium dodecyl sulphate. Electrophoresis was 2–5hrs at 5mA/gel. After separation, the gels were carefully ejected from the tubes using water pressure gently applied from a 10ml syringe. For 10% gels, a 10% (w/v) sodium dodecyl sulphate solution was used in place of water. The gels were soaked in water for 15 min 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 CO₂ and sliced in 1mm or 2mm sections using a Mickle gel slicer. Slices of acrylamide/ethylene diacrylate gels containing ³H or ³²P labelled RNA were digested individually in vials with 0.5ml of aqueous 2M NH₄OH at 60°. After evaporation to dryness, the gel residues were taken up in 0.3ml water.
and left for 60 mins. 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° 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.

7.2 Fractionation of RNA on denaturing polyacrylamide gels

The integrity and purity of polysomal and polyadenylated polysomal 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). 3.2% (w/v) acrylamide and 0.42% (w/v) bisacrylamide was prepared in deionised formamide. 74ml of this solution was mixed with 1ml of a solution containing 100mg of ammonium persulphate, 170mg of dibasic sodium phosphate and 40mg of monobasic sodium phosphate. After mixing, the solution was polymerized by the addition of 150μl NNN'N' tetramethylethylenediamine. 3ml aliquots were rapidly pipetted into plexiglass tube. Water was carefully layered over the solution as described before and the gels allowed to polymerize for 20-30 min at room temperature. The water layer was replaced with 98% formamide and the gels are 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 1 hr at 5mA/gel. Ethanol precipitated RNA samples, (20-40μg) suspended in 50μl 98% formamide were placed in a boiling water bath for 3-4 min.
quickly cooled in ice and applied to gels. The gels were run at room temperature at a constant current of 5mA/gel for 3 hr. After electrophoresis, the gels were carefully extruded into test tubes containing warm water (40-50°C) and after 2 changes of water, over 15-30 min, they were scanned at 260nm as described before. The treatment with warm water considerably reduced background absorption due to formamide.

7.3 Fractionation of RNA on Sucrose Density Gradients

7.3.1 The fractionation of high molecular weight HnRNA

The method used was a modification of the method of Girard et al. (1965) and as described by Knowler & Smellie (1973). 80-160μg of ethanol precipitated RNA in 0.1-0.5ml of LETS buffer (0.01M Tris-HCl pH 7.4, 0.1M LiCl, 0.001M EDTA and 0.2% sodium dodecyl sulphate (w/v) was layered on a linear 15-30% (w/v) sucrose density gradient in LETS buffer in cellulose nitrate tubes for the SW40 rotor. Centrifugation was for 16hr at 31,800g and at 20°C in the Beckman model L2-65B ultracentrifuge. Gradients were fractionated by the use of a peristaltic pump, through the flow cell of a Gilford 240 spectrophotometer and the extinction at 260nm was continuously monitored. Approximately 0.4ml fractions were collected and aliquots were precipitated with an equal volume of 10% (w/v) trichloroacetic acid and the acid insoluble material collected on glass fibre filters as described in section 3.2.
Section 7.3.2 Fractionation of high molecular weight HnRNA on denaturing sucrose gradients (Ross, 1976)

High molecular weight HnRNA was prepared as described in section 6.3.1. RNA, pelleted out of ethanol and dried in a gentle stream of nitrogen, was suspended in 0.08ml 0.002M EDTA, 0.01M Tris-HCl pH 7.5 and then 0.12ml of 98% (v/v) formamide in 0.002M EDTA, 0.01M Tris-HCl pH 7.5 was added. The dissolved RNA was heated for 1 min in a 90° water bath and immediately cooled in ice. Four sucrose solutions were prepared by dissolving 8, 12, 16 or 20g sucrose in a final volume of 100ml with 98% formamide, 0.002M EDTA and 0.01M Tris-HCl pH 7.5. The sucrose solutions were carefully layered in polyallomer tubes for the SW56 rotor as follows: 0.7ml 20% sucrose in the bottom then 1ml 16% followed by 1ml 12% and finally 0.75ml 8% sucrose. The tubes were then tightly sealed with parafilm and set aside at room temperature for 3 hr before use.

Centrifugation was for 21 hr at 30° in the SW56 rotor at 32,000rpm in the Beckman L2-65B model ultracentrifuge. Approximately 0.2ml fractions were collected by puncturing the tubes from the bottom. Acid-insoluble material from each fraction was prepared and collected on glass fibre filters by Millipore filtration as described earlier. Radioactivity was assayed in 10ml Toluene based scintillation fluid.

For preparative purposes, a larger gradient system was employed as described by McNaughton et al. (1974). RNA was denatured in 90% formamide in a boiling water bath and sedimented in a 13ml 5-20% (w/v) sucrose gradient containing 85% formamide, 0.002M EDTA, 0.01M Tris-HCl pH 7.5. Centrifugation was at 28,000 rpm for 20 hr.
and at 30° in the SW40 rotor of a Beckman model L2-65B ultracentrifuge.

Fractions were collected using a peristaltic pump and aliquots were precipitated with an equal volume of 10% trichloroacetic acid and acid-insoluble material collected on glass fibre filters as described in section 3.2. Radioactivity was assayed by scintillation counting in 10ml Toluene base scintillation fluid.

8. Preparation of Nuclease-resistant RNA nucleotides

8.1 Preparation of polyadenylate cores of poly(U) Sepharose fractionated HnRNA

6–8 uteri from oestrogen-treated rats were removed, dissected free of adipose and connective tissues and incubated in vitro as described in section 2.3. in Eagle's medium containing 125µCi [2-3H] adenosine/ml for 1 hr. High molecular weight HnRNA was prepared as described earlier (Section 6.3.1) and fractionated on poly(U) Sepharose into three fractions as described in Section 6.5.1. The fractionated RNA from two separate experiments were pooled and precipitated in 95% ethanol overnight at —20° in the presence of 0.15M sodium chloride. The RNA from each fraction was pelleted, dissolved in 1ml sterile distilled water and reprecipitated in ethanol. The RNA was subsequently pelleted, freeze-dried and dissolved in digestion buffer (0.3M NaCl, 0.005M MgCl₂ and 0.01M Tris-HCl, pH 7.4) containing 100 units/ml T₁ RNAse and 50µg/ml pancreatic RNAse A (Dubroff & Nemer, 1975). Digestion was at 37° for 90 min and terminated by the addition of an equal volume of digestion buffer—saturated phenol and 0.5% (w/v) sodium
dodecyl sulphate. The adenylate core was extracted twice at room
temperature and precipitated with 2 volumes of 95% ethanol. 0.15M
NaCl in the presence of 40μg yeast 4S tRNA.

8.2 Determination of the poly(A) size and content of
uterine polyadenylated polysomal RNA

Purified Poly(A) segments obtained as described above were
resolved on 2.7% polyacrylamide gels for 3 hours at 5mA/gel and
sliced into 2mm slices. Each slice was dissolved in water and incubated
for 24 hr at room temperature to extract adenylated nucleotides. The
extract was centrifuged at 2,500 rpm for 5 min and the supernatant
adjusted to 2 x SSC.

The position of poly(A) in the gel was determined by hybrid-
ization to an excess of 3H poly(U) (> 50,000cpm), followed by analysis
of RNase resistance as described below. Size of the poly(A) so located
was estimated by reference to 5.8S rRNA, poly(A) (90 nucleotides) and
oligo A (28 nucleotides) homopolymers. From the position of these
markers, a least squares 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:

\[
\frac{\sum N_i L_i}{\sum N_i} \quad \text{where } N_i \text{ is the number and } L_i \text{ the length of individual}
\text{molecules of a given size class. The poly(A) content of polysomal}
\text{polyadenylated RNA was estimated by determination of the amount of}
\text{pancreatic RNase-resistant } ^3\text{H} \text{-poly(U) radioactivity hybridized to}
\text{4-10μg of unlabelled RNA compared to known amounts of synthetic poly(A).}
\]
Each standard 100μl assay mixture contained 20μl 10 x SSC (1.5 M NaCl, 0.15M Sodium citrate pH 7.2), 1H poly(U) at 20μg/ml and varying amounts of poly(A) in water. Mixtures were incubated at 37° for 30 min., cooled to 4°, 1ml pancreatic RNAse at 20μg/ml in 2 x SSC added and then left in a 37° water bath for a further 30 min. Acid-insoluble material was prepared as described in Section 3.2. Precipitates were collected onto GF/C filters, dried at 60° overnight and radioactivity assayed by scintillation counting in 10ml toluene based scintillation fluid.

9. **cDNA–mRNA hybridizations**

The effect of oestradiol-17β on the quantitative and qualitative regulation of uterine mRNA at differing hormonal status was investigated by nucleic acid hybridization technique. The experimental procedures consist essentially of (1) isolation of RNA (2) the synthesis of complementary DNA (cDNA) probes and (3) an assay procedure to measure the kinetics of hybridization of the RNA with cDNA probe. cDNA used in this investigation is synthesized in vitro using RNA-directed DNA polymerase (reverse transcriptase) purified from RNA tumour virus particles and uterine polysomal poly(A) + RNA as template. The cDNA product therefore can 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 the effect of steroid hormones on mRNA concentration and determination of common sequences can be determined by measuring the rate of hybridization of the cDNA probe with RNA samples isolated from
tissues of differing hormonal status. The procedure for the isolation of uterine polysomes and of polysomal polyadenylated RNA has been described (section 5.4 and 6.5.2 respectively).

9.1 Synthesis of cDNA complementary to uterine polysomal poly(A)⁺RNA

Synthesis of cDNA was essentially the method of Birnie et al. (1974). 8-10µg of template poly(A)⁺RNA was incubated at 37° for 2 hr in 0.5ml mixture containing 15µg oligo dT₁₂₋₁₈, 0.2µmole each of dATP, dGTP, dTTP, 20nmole of ³H-dGTP (4.85 Ci/mM), 50µg actinomycin-D, 100µg BSA, 50mM Tris-HCl pH 8.2, 50mM KCl, 10mM dithiothreitol, 5mM Mg²⁺ acetate and 250 units/ml of reverse transcriptase. After incubation, the mixture was adjusted to 0.1M EDTA and chilled in ice. The entire mixture was chromatographed on Sephadex G50 columns underlaid with Dowex-chelating resin. The excluded fraction was adjusted to contain 100µg/ml E. coli DNA, freeze-dried and dissolved in a minimal volume of water. cDNA complementary to globin mRNA was prepared in a similar manner. Globin mRNA was the generous gift of Dr. George Birnie.

9.2 Characterization of complementary DNA

cDNA prepared and isolated as described above was freeze-dried, then dissolved in 1ml of 0.9M NaCl, 0.1M NaOH and fractionated a linear 25ml 5-12% (w/w) alkaline sucrose gradients in 0.9M NaCl, 0.01M NaOH. Centrifugation was for 24 hr in the 3 x 25ml MSE swing-out rotor at 29,000 rpm and 20°. 1ml fractions were collected and radioactivity in 5µl of each fraction were counted in 5ml Triton-Toluene base scintillation fluid. Sedimentation coefficients and molecular weights
were determined by computer programme as described by Steensgaard et al. (1978). cDNA of desired molecular weight was recovered by neutralization and precipitation with ethanol. It was then desalted by passage through Sephadex G50 columns underlaid with Dowex-chelating resin. The excluded fraction was adjusted to 100μg E. coli DNA and freeze-dried. The cDNA was subsequently dissolved in a minimal volume of water and kept at -70°.

9.3 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 re-dissolved in hybridization buffer (0.5M NaCl, 25mM HEPES, 0.5mM EDTA, pH 6.8, 50% (v/v) formamide). The salt solutions, before addition of formamide were passed through Chelex-100 resin, treated with diethyl-pyrocarbonate and autoclaved. Portions of the solutions (0.4-1μl) were dispensed with a Ziptrol dispenser and sealed in glass capillaries. The capillaries were heated at 90° for 5 min 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 mole sec. litre⁻¹ is attained when RNA is incubated at 83μg/ml for 1 hr (Britten and Kohne 1975). Thus Rot =

\[
\frac{RNA (\mu g/ml)}{83} \times t (h)
\]

9.4 Assay of RNA-cDNA hybridization reaction
9.4 Assay of RNA-cDNA hybridization reaction

9.4.1 Assay of S₁ Nuclease activity

It was found necessary to check the activity of commercial S₁ Nuclease. Aliquot of globin mRNA-cDNA hybrids, purified by hydroxyapatite chromatography, or single stranded globin ³H-cDNA were incubated at 37° in the presence of 14µg/ml denatured calf-thymus DNA with from 8-20 units of S₁ nuclease in nuclease assay buffer (70mM sodium acetate pH 4.5, 2.8mM ZnSO₄, 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. S₁ Nuclease activity is expressed as percentage digestion.

9.4.2 Assay of RNA-cDNA hybridization by S₁ Nuclease

The hybridization mixture in each capillary was flushed out with 0.25ml of buffer comprised of 0.07M sodium acetate pH 4.5, 2.8mM ZnSO₄, 0.14M NaCl and 14µg/ml of heat-denatured calf thymus DNA. The percentage of cDNA which formed hybrids was determined by digesting the non-hybridized probe. 0.1ml of S₁ nuclease (8-20 units) in nuclease assay buffer was added to each capillary flushing and incubated at 37° for 1 ½ - 2 hours. The incubations were chilled and 0.01ml removed and radioactivity determined by scintillation counting with 10ml of Triton-Toluene based scintillation fluid to determine total radioactivity present in cDNA(T). A further 0.2ml was removed and precipitated by addition of 0.05ml of a solution containing 1mg/ml BSA and 150µg/ml calf thymus DNA as carrier and 0.05ml ice cold 3N perchloric acid. After standing in ice for 15 min, the precipitate containing undigested
cDNA-DNA hybrids was removed by centrifugation at 4°C and 2,500 rpm for 15 min. An 0.2ml sample of the clear supernatant was removed and radioactivity counted as described above to determined acid-soluble radioactivity (AS). The amount of nuclease added was sufficient to ensure complete degradation of unhybridized cDNA within 1 hr. The percentage of cDNA in hybrid was calculated as:

\[
\% \text{ hybrid} = 1 - \frac{0.75 \times \text{AS cpm}}{\text{T cpm}} \times 100
\]

9.5 Fractionation of abundance classes of cDNA

9.5.1 Preparation and Characterization of Hydroxyapatite (HAP)

Bio-Rad Biogel DNA grade HTP was used and the capacities vary from batch to batch from 2-1mg DNA/gm dry weight. HAP was prepared for use as follows. 20-40gm HAP was suspended in 4 volumes of 1M sodium phosphate buffer pH 6.8 and left for 1 hr at room temperature. The HAP was recovered by centrifugation and washed several times with excess of 0.16M sodium phosphate buffer pH 6.8. After the final wash HAP was suspended in the wash buffer and placed in a boiling water bath for 10-15 min, to solubilize the HAP. The buffer was discarded by centrifugation and the HAP washed again in two changes of 0.03M sodium phosphate buffer pH 6.8 and stored in this buffer at 4°C. Before use the suspension was again placed in a boiling water bath for 15-30 min.

HAP columns were prepared by layering about 2ml packed volume in 6" x 0.5" dia. pyrex tubes fitted with pyrex microfilters. The tubes were fixed with rubber grommets into a perspex barrel of internal diameter 7cm and length 21cm. Water at 60°C was circulated
through the barrel. The HAP column was washed extensively with sterile water and followed by 0.03M sodium phosphate buffer pH 6.8.

Efficient separation of single stranded and double stranded nucleic acid by HAP chromatography depended on the concentration of sodium phosphate buffer pH 6.8 used and this varied for each batch of HAP. To determine the effective concentration of the elution buffer, \(^3\)H-cDNA-globin mRNA hybrids and single stranded globin \(^3\)H-cDNA were mixed in 0.15M NaCl containing 0.03M sodium phosphate buffer pH 6.8 and applied to a 2-3ml 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 increasing concentrations of sodium phosphate buffer, pH 6.8 in the range of 0.14-0.4M and in steps of 0.12, 0.14, 0.2, 0.4, 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. These two phosphate buffer concentrations were re-tested and results (see results section for details) showed that 90-95% of radioactivity of starting material was recovered as single-stranded and double-stranded material when they were eluted with the chosen concentration of 0.14M and 0.4M sodium phosphate buffer pH 6.8 respectively.

9.5.2 Isolation of abundant and rare sequence cDNA

Total cDNA was prepared from polysomal poly(A)\(^+\)RNA isolated from oestradiol treated immature animals and the abundant and rare cDNA sequences were fractionated by hydroxyapatite chromatography. The predominantly abundant sequences were isolated from a large batch of
the total cDNA after hybridization in excess to its own template to a Rot of 1 (moles sec. litre\(^{-1}\)). To fractionate the rare sequences, hybridization was extended to Rot 12 and the single-stranded cDNA remaining constituted the predominantly rare cDNA sequences. The hybrids were expelled from hybridization capillaries with 0.15M NaCl in 0.03M sodium phosphate buffer pH 6.8 and the mixture loaded into a 2-3ml packed volume HAP column maintained at 60\(^\circ\) in the diluent buffer. The column was washed extensively with 0.03M sodium phosphate buffer pH 6.8 followed by a stepwise elution with 6 x 2ml 0.14M sodium phosphate buffer pH 6.8 which effectively eluted the single-stranded cDNA. The double-stranded cDNA-RNA hybrids were effectively eluted with 2 x 2ml 0.4M sodium phosphate buffer pH 6.8. The abundant and rare sequence cDNAs were purified by boiling for 5 min. in 0.5N NaOH, neutralised using equimolar HCl and passed over a Sephadex G50 column underlaid with Dowex chelating resin. The cDNAs were recovered in the excluded volume, freeze-dried in the presence of 40\(\mu\)g E. coli DNA, then dissolved in a minimal volume of water and kept at -20\(^\circ\).

10. **Unique DNA-mRNA saturation hybridization**

10.1 **Preparation of highly labelled rat liver unique DNA sequences**

The \(^3\)H-labelled nick-translated unique DNA sequences were the generous gift of Dr. Alan Balmain of the Beatson Institute, Glasgow, and prepared as follows: Unique DNA was obtained from total rat liver DNA by the method of Hell et al (1972) and purified by two cycles on hydroxyapatite columns. In preparation for nick translation it was dissolved at 10mg/ml in 0.5M NaCl, 25mM HEPPS, 0.5mM EDTA and
50% (v/v) formamide, allowed to anneal to a high Cot (greater than 20,000 moles sec. litre\(^{-1}\)) and the double-stranded DNA stored in 0.15M NaCl at -20\(^\circ\). 1-2\(\mu\)g of double-stranded unique DNA were incubated in a final volume of 100\(\mu\)l containing 50mM Tris-Cl pH 7.9, 10mM MgCl\(_2\), 10mM DTT 50\(\mu\)g/ml BSA, 35\(\mu\)M \(^3\)H-dCTP (25 Ci/m mole) and 80mMdATP, dGTP and dTTP. DNA polymerase I was added at 5 units/\(\mu\)g DNA and the mixture incubated at 12\(^\circ\) for 20 hr. The nick translated product was deprotei-
ised by extraction with phenol/chloroform (1:1) and excess radioactive triphosphate, together with any 'hairpins' or rapidly reannealing DNA present in the probe, were removed by hydroxypaptite chromatography.

The specific activity of the unique DNA prepared in this way was about 7 \times 10^6 CPM/\(\mu\)g DNA. Its size, as determined by rate zonal centrifugation through 5-11\% (w/w) alkaline sucrose gradients was 4S and its hybridizability with an excess of unlabelled total rat liver DNA was at least 70\%. Further characterization of the DNA probe is described in detail by Balmain & Birnie (1979).

10.2 Preparation of mRNA for unique DNA-mRNA saturation hybridization

Polysomal poly(A)\(^+\)RNA from the uteri of immature rats stimulated with oestrogen for 4 hours or from the uteri of adult proestrous animals was extracted and purified as described in sections 6.4.2 and 6.5.2. The RNA was mercurated, as described by Ward & Dale (1975), by dissolving at a final concentration of 100\(\mu\)g/ml in 0.1M sodium acetate buffer pH 6.0 containing mercuric acetate at 1mg/ml. The mixture was incubated for 90 min. at 50\(^\circ\) after which one tenth the volume of 0.1M EDTA pH 7.0 was added and the entire
mixture passed over a Sephadex G50 column. The excluded volume was retained, freeze-dried and dissolved in a minimal volume of sterile water. Aliquots were removed and the intactness of the mercurated-RNA was checked by determining its absorption maximum on a Unicam SP-2000 spectrophotometer.

10.3 Preparation and assay of unique $[^3H]$ DNA-mercurated mRNA hybridization

Appropriate amounts of mercurated-mRNA and unique $[^3H]$ DNA (7.0 x $10^6$ cpm/µg DNA) mixed in sterile distilled water were freeze-dried and taken up in hybridization buffer (0.2M sodium phosphate buffer pH 6.8 containing 5mM EDTA, 1mM mercaptoethanol and 0.1% (w/v) SDS). Suitable volumes were sealed in glass capillaries, denatured at 100° for 5 min and incubated at 60° for various time periods. Each sample was then flushed from the capillaries with 0.5ml of NETS buffer and applied to a 200µl packed volume of activated, washed thiol-sepharose in an Eppendorf tube. The tubes were vortexed, incubated at 60° for 10 min and centrifuged at 800 gav. on the Mistral MSE centrifuge for 2 min. at 30°. The supernatants were transferred to scintillation counting vials. The above washing procedure was repeated with further 1ml quantities of NETS buffer at 60° until background values of 20–30 cpm were obtained. The bound material, consisting of mercurated mRNA-unique $[^3H]$DNA hybrids was then eluted by several washes with 1ml quantities of NETS buffer containing 0.1M mercaptoethanol at 20°. The percentage hybrid is calculated as:

$$\frac{\Sigma (\text{Bound cpm})}{\Sigma (\text{Bound cpm}) + \Sigma (\text{unbound cpm})} \times 100$$
The percentage hybrid at zero time was subtracted from each point. Appropriate controls were carried out with mercurated *E. coli* ribosomal RNA to show that non-specific hybridization did not increase after hybridization for different times.

11. **Computer Analysis of Experimental Data**

11.1 **Hybridization data**

The data obtained in hybridization experiments between cDNA and polysomal poly(A)*RNA was analysed using a computer programme devised by Monahan et al. (1977) and designed to fit the data to a set of curves n, where n = 1, 2, 3 or 4. The fitting was defined by the equation:

\[
\frac{d}{d_0} = B + \sum_{i=1}^{n} p_i \left(1 - \exp\left(0.693 \frac{R_i}{R_{i0}}\right)\right)
\]

where \(\frac{d}{d_0}\) is the fraction of hybrid formed. For each component curve, \(p_i\) denotes the proportion of hybridizable cDNA within each component. \(R_i\) is the number of moles second litre\(^{-1}\) of nucleotides of RNA; \(R_{i0}\) the number of moles second litre\(^{-1}\) of RNA at 50% hybridization for that component and \(B\) is the zero time hybridization value. The theory for this equation has been described in detail by Bishop (1972). The programme estimates the \(R_{i0}\) 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.

11.2 Sedimentation coefficients

RESULTS
RESULTS

1. Characterization of uterine HnRNA and the effects of oestradiol-17β on its synthesis

1.1. Fractionation and characterization of uterine RNA

Investigations of RNA metabolism in a differentiating tissue, such as the immature uteri responding to oestradiol treatment, requires reliable and adequate isolation and purification procedure, effective in the extraction of all species of RNA and avoiding concomitant degradation. To this end, a number of isolation procedures were tested.

The principle of the fractionation procedure described by Georgiev (1967), which employs various temperatures to sequentially extract different RNA species, was adapted for the isolation of high molecular weight uterine RNA. The pH of the extraction media was kept at pH 5.2 in acetate buffer (Joel & Haggerman, 1969).

Figures 2a, b, and c show the electrophoretic resolution of immature rat uterine RNA extracted at 10°, 40° and 55° respectively. Each rat had received 100μCi of radioactive precursors as described in the methods section. Extraction at 10° isolated mainly 28S and 18S ribosomal species (figure 2a). It was seen that these RNA species carried the bulk of the radioactivity while there was very little radioactivity in the high molecular weight species, which are refractory to extraction at 10°. Radioactivity due to pre-rRNA precursors was barely discernible. Extraction at 40° removed a substantial proportion of high molecular weight RNA species and showed some reduction in the yield of rRNA species compared to extraction at 10°. At 55°, more of
18-21 day old rats, weighing 25-30g, received 100 μCi of each of (5-^3H) uridine and (8-^3H) guanosine intravenously 30 min before death. Uterine RNA was extracted and purified by the method of Joel & Haggerman (1969) (see Material & Methods) at various temperatures. The purified uterine RNA was separated for 5h in 2.7% polyacrylamide gels at 5mA/gel in the presence of unlabelled uterine RNA.

(A) = 10°
(B) = 40°
(C) = 55°

— = Extinction at 260nm
.... = Radioactivity per slice in cpm
the high molecular weight RNA was isolated. However, the fraction isolated at 55° still contained 28S and 18S species, the latter prevailing over the former. Extracts at this temperature were enriched in HnRNA which were dispersed across the gel and particularly noticeable as radioactive species of very high molecular weight. These have been previously characterized (Knowler & Smellie, 1973). The data thus indicated that heavy and heterogenous RNA from immature rat uteri appeared in the aqueous phase of a phenol extraction only at elevated temperatures (55°). Higher temperatures were not considered as this can lead to aggregation of RNA molecules (Wagner et al., 1967). These results are consistent with previous findings (Brawerman et al., 1965; Georgiev 1967; Joel & Haggerman 1969; Olzanska et al., 1974). However, it was not considered that the temperature dependent effects were complete enough to form the basis of a fractionation of uterine RNA.

The hot phenol-sodium dodecyl sulphate(SDS) extraction procedure appeared well suited for the extraction of uterine heterogenous nuclear RNA. However, it was shown by Brawerman (1976) that the hot phenol-SDS is most effective at alkaline pH and had a preferential effect on the recovery of poly(A) containing nuclear RNA. Hence, RNA from oestradiol-stimulated and nonstimulated immature rat uteri, into which radioactive precursors were incorporated in vitro, were extracted at 55° with buffers at pH 5.2, 7.6 and 8.3. Table 2 shows the recovery of uterine RNA and the DNA content at each purification step. It is seen that DNA contamination of the initial RNA pellet was considerable at pH 8.3 and least in the RNA extracted at acidic pH. However, in all cases, a considerable proportion of the contaminating DNA could be
TABLE 2

The DNA content in the aqueous phase at each extraction step (μg DNA).

<table>
<thead>
<tr>
<th>Aqueous phase</th>
<th>pH 8.3</th>
<th>pH 7.6</th>
<th>pH 5.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. After first NaCl/EtoH precipitation</td>
<td>540</td>
<td>400</td>
<td>180</td>
</tr>
<tr>
<td>2. After reextraction at pH 5.0</td>
<td>235</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>3. After INαse treatment</td>
<td>5</td>
<td>1</td>
<td>not detectable</td>
</tr>
<tr>
<td>4. After final EtoH/potassium acetate</td>
<td>not detectable</td>
<td>not detectable</td>
<td></td>
</tr>
<tr>
<td>5. Recovery of RNA (as % of total/uteri)</td>
<td>55-60%</td>
<td>50-55%</td>
<td>50-55%</td>
</tr>
</tbody>
</table>

The uterine RNA of eight 18-21 day old rats, weighing 25-30g was purified by the Method of Joel & Haggerman (1969) (see Materials & Methods) and also using alkaline and neutral extraction buffers (see Materials & Methods). At each precipitation step an aliquot was removed, precipitated and washed with 5% (w/v) trichloroacetic acid at 0-4°C, digested in 0.5M perchloric acid at 70°C for 1 hr and used to assay RNA and DNA as described in the Materials & Methods section. RNA content is expressed as a percentage of total uterine content (105μg/uteri).
FIGURE 3

The Nature of purified RNA extracted at alkaline pH from the uterus of Immature Rats

18-21 day old rats, weighing 25-30g, received 1µg oestradiol by intraperitoneal injection 4h before death, 100µCi each of (5-^3H) uridine and (8-^3H) guanoside by intravenous injection. Uterine RNA was extracted at pH 8.3 and purified by the method of Joel & Haggerman (1969) (see Materials and Methods). Purified uterine RNA was separated for 5h in 2.7\% polyacrylamide gels at 5mA/gel.

(A) = Radioactive Precursor administered 15 min before death

(B) = Radioactive Precursor administered 30 min before death

--- = Extinction at 260nm

•••• = Radioactivity per slice in cpm
removed from the initial extract by a second extraction at pH 5.0 and any residual DNA was then eliminated by DNase treatment and selective precipitation as described by Joel & Haggeman (1969).

Performing the initial extraction at alkaline pH resulted in a small improvement in the overall yield of RNA. However, there was a marked improvement in the recovery of high molecular weight HnRNA. Table 3 shows that RNA derived from 4 hr oestradiol-stimulated immature rat uteri, extracted at pH 8.3 and at 55° contained 47% of its incorporated radioactivity in high molecular weight RNA.

When the RNA was fractionated by aqueous 2.7% polyacrylamide gel electrophoresis (figure 3), a number of distinct radioactivity peaks migrating at greater than 45S were evident. In figure 3, the RNA was derived from the uteri of rats which had been treated for 4 hr with oestradiol and had received radioactive precursors intravenously 15 min or 30 min before death. After 30 min incorporation, radioactivity is clearly discernible in the 45S and 32S precursor species and incorporation is also apparent in the RNA of the ribosomal species. After 15 min incorporation, a high proportion of the radioactivity is incorporated into very high molecular weight species migrating at greater than 45S and the number of radioactivity peaks of RNA species are not as clear cut as when rats received a 30 min incorporation of radioactive precursors. Although labelled radioactivity peaks were usually observed migrating at the 45S and 32S pre-RNA marker positions, these two peaks were not always clearly separated from the radioactivity corresponding to higher molecular weight species. The amounts of radioactivity incorporated in the higher molecular weight RNA species were rather low compared to that observed after 30 min incorporation. The
TABLE 3
The proportion of recovered radioactivity incorporated in uterine RNA
extracted at 55°C but with buffers at different pH.

<table>
<thead>
<tr>
<th>Method of Extraction</th>
<th>Total radioactivity as acid-insoluble material (dpm)</th>
<th>Total radioactivity as acid-insoluble material (dpm) in high molecular weight RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.3</td>
<td>43,600</td>
<td>20,000 (47%)</td>
</tr>
<tr>
<td>pH 7.6</td>
<td>34,700</td>
<td>9,400 (30%)</td>
</tr>
<tr>
<td>pH 5.2</td>
<td>35,000</td>
<td>11,300 (32%)</td>
</tr>
</tbody>
</table>

The uteri of eight, 18-21 day old rats, were incubated for 30 min in 4 ml Eagle's medium containing 100μ Ci 5^-3H uridine. The uterine RNA was extracted with buffers at the indicated pH and purified by the method of Joel & Haggaman (1969)(see Materials & Methods). An aliquot was removed and the total acid-insoluble radioactivity determined as described in the Materials & Methods section. The RNA of each preparation was sedimented in 15-30% sucrose gradient in LETS buffer as described in the Methods section (7.3.1). The high-molecular-weight HnRNA was recovered from the gradient fractions 1-24 by ethanol precipitation in the presence of unlabelled uterine RNA as carrier. The recovered high-molecular-weight HnRNA was acid-precipitated and radioactivity measured as described in the Materials & Methods section. The number in parenthesis indicates the high-molecular-weight RNA content of each preparation.
positions of the uterine 45S and 32S precursors in the gel system were identified from $^{32}$P-labelled nucleolar RNA prepared from HeLa cell nucleoli and fractionated in parallel gels under identical conditions.

The uterine RNA species migrating at greater than 45S have been identified as HnRNA on the basis of base composition (figure 1), its nuclear origin, its lack of methylation and by its rate of synthesis and decay (Knowler & Smellie 1973).

It should be stressed here that for the purpose of preparing defined RNA species, as opposed to total cellular RNA, it is usually necessary to isolate the appropriate subcellular structures. However, in the uterus the problem of degradation by ribonucleases is particularly serious and it has not proved possible, for instance, to adequately protect nuclear RNA from the effects of intranuclear RNAses during the preparation of uterine nuclei. The citric acid method (Knowler & Smellie, 1973; Knowler, 1976) is only partially successful in this respect. Figure 4a shows that when nuclei are prepared in the presence of citric acid, high molecular RNA is preserved but the profiles still reveal some degradation. In the absence of citric acid (figure 4b) degradation is much more complete.

One other possible method of preparing RNA in which HnRNA could be selectively studied was to inhibit the synthesis of rRNA prior to incorporation of radioactive precursors and RNA isolation. Figure 5 shows the results of such an attempt where actinomycin D was used to selectively inhibit rRNA synthesis. It is seen that the inhibitor did effect rRNA synthesis more strongly than HnRNA synthesis. However, the effect was insufficiently pronounced to form the basis of subsequent investigations.
The nature of RNA synthesized in the nuclei of immature rat uteri

A) The uteri of 12, 18-21 day old rats, weighing 25-30g were incubated in 4ml of Eagle's medium which contained 10μCi/ml [5-3H] uridine. The nuclei were then prepared by Method B in the Materials & Methods section. RNA was extracted at pH 8.3 and at 55° from the pellet, purified and separated on 2.7% polyacrylamide gels for 5 h.

B) The experimental details were as above and the nuclei were prepared by Method A in the Materials & Methods section.

--- = Extinction at 260nm

..... = Radioactivity per slice in cpm
FIGURE 5

Effect of Actinomycin D on oestradiol-17β stimulated HnRNA synthesis

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol-17β and actinomycin D simultaneously 4h before death. 100μCi [5-3H] was administered intravenously 30 min before death. Purified RNA was separated on 2.7% polyacrylamide gels for 5 hr at 5mA/gel. 32P labelled HeLa cell nucleolar RNA was separated in a parallel gel to provide the 45S and 32S markers.

(A) = no actinomycin D
(B) = 15μg/Rat of Actinomycin D
(C) = 30μg/Rat of Actinomycin D

--- = Extinction at 260nm
.... = Radioactivity per slice in dpm
1.2. **Isolation and characterization of uterine high-molecular-weight HnRNA**

1.2.1. **Isolation from sucrose gradients**

An early response to oestrogen stimulation is the increased synthesis of high molecular weight RNA (Knowler, 1972; Knowler & Smellie, 1971, 1973; Teng & Hamilton, 1972) and identified by Knowler & Smellie (1973) as HnRNA. To further understand the role of HnRNA in oestrogen action, it was desirable to purify it from the total RNA preparations and this was achieved by sucrose density gradient separation based on the method of Girard et al. (1965) and as modified by Knowler & Smellie (1973). Total uterine RNA was resolved on a 15-30% (w/v) sucrose gradients in LETS buffer (0.01M tris-HCl, pH 7.4; 0.01M LiCl; 1mM EDTA and 0.2% (w/v) SDS) under conditions such that rRNA species underwent little migration and the heavier HnRNA species occupied most of the gradient. Figure 6 illustrates the radioactivity profile of such a sucrose density gradient analysis of total $^3$H-labelled uterine RNA and a parallel gradient of $^{32}$P HeLa nucleolar RNA which provided the 45S and 32S markers. It is seen that the gradient system separates the high molecular weight HnRNA from the pre-ribosomal and ribosomal species. The profile of $^3$H-radioactivity revealed that most of the labelled RNA was located at the top of the gradient. Only material sedimenting at greater than 45S entered the lower two thirds of the gradient (fractions 1-24). After 4 hr of oestradiol treatment, and 30 min incorporation of radioactive precursors, about 47% (table 3) of the total radioactivity incorporated was contained in RNA species sedimenting between fractions 1-24 (figure 6). In the routine preparation of uterine HnRNA, fractions
FIGURE 6
Separation of high molecular weight uterine RNA on sucrose density gradients

18–21 day old rats received 50μCi of each of $[\text{5-}^3\text{H}]$ uridine and $[\text{8-}^3\text{H}]$ guanosine by intravenous injection 30 min before death. Purified RNA was fractionated on 15–30% (w/v) sucrose density gradients as described in the Materials & Methods section. The gradients were pumped through the flow cell of a Gilford 240 recording spectrophotometer and the extinction at 260nm was continuously monitored. Approx. 0.4ml fractions were collected and assayed for acid-insoluble radioactivity. $^{32}\text{P}$-labelled HeLa cell nucleolar RNA was centrifuged in a parallel gradient to provide the 45S and 32S markers.

- - - - - - - = $^{32}\text{P}$-labelled HeLa cell nucleolar RNA
- - - - - - - = $^3\text{H}$-labelled uterine RNA
- - - - - - - = extinction at 260nm
containing RNA of a size greater than 45S were pooled from 2-4 identical gradients and precipitated overnight at -20°C, in the presence of 40μg unlabelled uterine rRNA as carrier.

1.2.2. Characterization of high molecular weight RNA species in various composite fractions of the sucrose gradient

Part of the same RNA preparation used to derive the result of figure 6 was layered onto a similar gradient and sedimented in the same way as described in figure 6. The whole gradient was then divided into 3 composite fractions as indicated in figure 7a. Fraction 1 consisted of gradient fractions from 1-12, fraction 2 was pooled from gradient fractions 13-24 and fraction 3 contained gradient fractions 25-36. Constituent RNA of each fraction was precipitated by 2 volumes of ethanol in the presence of 40μg unlabelled uterine RNA and 0.15M NaCl. Recovered RNA was separated on 2.7% gels for 5 hours. Figure 7b shows the distribution of labelled RNA from the composite fraction 1. There were two distinct peaks at the gel origin which were larger than the 45S RNA and it is obvious from this result that RNA species contained in the lower third of the gradient retained their size characteristics. The middle 12 fractions (figure 7c) also contained heavy RNA species showing a single peak of radioactivity larger than 45S pre-rRNA marker. The top fractions (figure 7d) contained the ribosomal species as well as most of the 45S and 32S RNA species. The RNA species sedimenting in the lower 2/3 section of the whole gradient was previously identified as uterine high molecular weight HnRNA (Knowler & Smellie, 1973). It appeared therefore the gradient system provided a suitable way of recovering the heavy HnRNA species. A disadvantage of this method is that low molecular
FIGURE 7

The distribution of uterine RNA on sucrose density gradients

The RNA, which formed part of the preparation used in Figure 6, was layered onto a 15-30% sucrose density gradient in LETS buffer and sedimented for 16hr at 31,000g and at 20°C. The gradient fractions 1-12 (I) 13-24 (II) and 25-36 (III) were pooled, and the constituent RNA precipitated by two volumes of ethanol in the presence of 40µg of unlabelled uterine RNA. Recovered RNA was separated on 2.7% polyacrylamide gels for 5h at 5mA/gel.

(A) = the sucrose density gradient indicating the positions of the three composite fractions

(B) = Polyacrylamide gel resolution of the RNA in the gradient fractions 1-12

(C) = Polyacrylamide gel resolution of the RNA in the gradient fractions 13-24

(D) = Polyacrylamide gel resolution of the RNA in the gradient fractions 25-36.

----- = Extinction at 260nm

..... = Radioactivity per slice in dpm
weight HnRNA species are not recovered.

1.2.3. Sedimentation of uterine high molecular weight HnRNA in denaturing sucrose density gradients

Non-specific aggregation of RNA molecules which occurs during phenol extraction (Hagenbuchle et al. 1975; McNaughton et al. 1974; Kohne et al. 1977) or the self-annealing of complementary structures of HnRNA (Fedoroff et al. 1977) may contribute to the high molecular weight characteristics of the uterine HnRNA as observed in figure 7. A number of investigations have indicated that exposure of HnRNA molecules to denaturing conditions either in sucrose gradients or polyacrylamide gels containing formamide or dimethylsulphoxide (deKloet et al. 1970; McKnight & Schimke, 1974; Spoehr et al. 1976; Levis & Penman, 1977) results in a reduction of HnRNA sizes.

To test whether the high molecular weight nature of the uterine HnRNA (figures 3 and 7) was genuine, the RNA was denatured in formamide at 90° and sedimented in a 8–20% (w/v) sucrose gradient containing 98% deionised formamide at 30°. Figure 8 illustrates the result of such a fractionation of uterine HnRNA and HeLa cell nucleolar RNA. When the profile of 3H-labelled uterine HnRNA was compared with that of purified HeLa cell 45S and 32S pre-rRNAs it was seen that the greatest part still sedimented more rapidly than 45S RNA, but a proportion appeared in the 45–35S region. Only a small part sedimented more slowly than 35S RNA. The apparent decrease in sedimentation rate of this otherwise very large HnRNA could be attributed to the denaturation of duplex regions of HnRNA molecules, as observed by Fedoroff et al. (1977) in their investigation of the physical basis of HnRNA complex-formation.
Sedimentation of uterine high molecular weight HnRNA under denaturing conditions

Uterine RNA was purified from 18–21 day old immature rats that had received radioactive RNA precursors as described in the legend to Figure 6. The high molecular weight HnRNA was then isolated, denatured at 90°C in 98% formamide, cooled rapidly and fractionated on an 8–20% (v/v) sucrose gradient containing 98% deionized formamide, 0.002M-EDTA, 0.01M tris-HCl pH 7.5. The gradients were run in polyallomer tubes for the SW56 rotor and centrifuged in the Beckman Model L2 65B ultracentrifuge for 21h at 30°C and 32,000 rpm. Fractions (approx. 0.2ml) of the gradients were collected dropwise, and acid-insoluble material was prepared from each fraction. 32P-labelled HeLa cell nucleolar RNA was centrifuged in a parallel gradient to provide 45S and 32S markers. Fractions are numbered from the bottom of the gradient.

- = 3H-labelled uterine high molecular weight HnRNA

- = 32P-labelled HeLa cell nucleolar RNA
This gradient system is, however, not suitable to serve as a standard procedure for the preparation of denatured HnRNA molecules as it was small. A method described by McNaughton et al. (1974) was adopted for preparative purposes. In these experiments, high molecular weight HnRNA was denatured in 90% (v/v) formamide at 90° for 5 min and sedimented in a 13 ml 5-20% (w/v) sucrose gradient containing 85% (v/v) formamide in the Beckman SW 40 rotor as described in the Methods section. The results are described in a later section.

1.3. Fractionation of uterine high molecular weight HnRNA

1.3.1. Poly(U)Sepharose chromatography

HnRNA is the most abundantly synthesized RNA in eukaryotes and on the basis of the evidence as described in the Introduction, is presumed to serve as a precursor to mRNA. One of the chemical characteristics of these large RNA molecules is the covalently linked polyriboadenylic acid (poly(A)) sequences at the 3' end (see Introduction for review). The poly(A) segments on some HnRNA molecules has permitted these molecules to be separated from those lacking polyA (Molloy et al. 1974; Nakazato & Edmonds, 1974) by exploiting the property that poly(A) hybridizes with complementary homopolynucleotides immobilized upon a suitable matrix such as poly(U)Sepharose.

In the present study poly(U)Sepharose affinity chromatography was used to demonstrate three distinct fractions of uterine HnRNA which differed in the extent to which they bound to poly(U)Sepharose. When uterine high molecular weight HnRNA (15,000-20,000 dpm) was applied to poly(U)Sepharose columns, a high percentage of the labelled RNA did not
High molecular weight HnRNA was purified from groups of eight 18-21 day old rats, each having received 100 μCi (5-³H) uridine and (5-³H) guanosine intravenously 30 min before death. A total of 15,500 dpm ³H-labelled HnRNA was applied to poly(U)-Sepharose columns. Unbound RNA was washed with 0.4M NETS buffer, and the bound HnRNA was eluted in stepwise fashion with increasing concentrations of formamide in ETS buffer. 1ml fractions were collected, aliquots removed and radioactivity was measured in the acid-insoluble material. ³²P-labelled HeLa cell HnRNA was fractionated in a similar fashion on a parallel column.

- - - = ³H-labelled uterine HnRNA
□ □ □ = ³²P-labelled HeLa cell HnRNA
### TABLE 4

Variable affinity of uterine high molecular weight HnRNA to poly(U) Sepharose column chromatography

<table>
<thead>
<tr>
<th>Concentration of Formamide (%, v/v)</th>
<th>RNA eluted from column (acid-insol. dpm)</th>
<th>Percentage of total HnRNA input eluted from column</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8850</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>165</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>3940</td>
<td>25.4</td>
</tr>
<tr>
<td>35</td>
<td>96</td>
<td>0.63</td>
</tr>
<tr>
<td>90</td>
<td>2050</td>
<td>13</td>
</tr>
</tbody>
</table>

Experimental details are as described in the legend to figure 9. Acid-insoluble material of HnRNA in each chromatographic fraction was prepared and radioactivity assayed as described in the Materials & Methods section.
bind and was eluted with the wash buffer (table 4). The remaining
15-25% was bound to poly(U) Sepharose and was eluted only by the
addition of increasing concentrations of formamide to the eluting
buffer. Figure 9 shows the profile of elution of bound HnRNA eluted
by 5, 15, 35 and 90% (v/v) formamide. Some RNA was eluted at each
concentration of formamide but the bulk was eluted by 1% and 90%
formamide. Conversely, HeLa cell HnRNA prepared and fractionated in
the same way was largely eluted by 33% and 90% formamide.

It seemed likely that the HnRNA eluted by low concentrations
of formamide contained only short oligoA stretches as reported by
Molloy et al. (1974) and Dubroff & Nemer (1975). The other HnRNA
fraction presumably bound to the poly(U) Sepharose through duplexes
containing long stretches of poly(A) and therefore required elution
with buffer containing high formamide concentration. It was therefore
decided that further fractionation would employ a two stage elution of
bound HnRNA, one eluted by 1% buffered formamide and the second by
90% buffered formamide. Table 4 shows that such an elution regime would
be expected to yield 50% of the HnRNA unbound, approximately 26.5%
bound but eluted with 1% formamide and approximately 13.5% bound but
eluted with 90% formamide. The bound fractions were completely retained
by a second passage through fresh poly(U) Sepharose columns and could be
re-eluted with the same concentrations of formamide.

1.3.2. The size distribution and integrity of poly(U) Sepharose
fractionated uterine HnRNA

As seen in figure 9 and table 4, uterine HnRNA could be
fractionated into three components each with variable affinities for
FIGURE 10

Separation of poly(U)-Sepharose fractionated uterine high molecular weight HnRNA on polyacrylamide gels

HnRNA was purified from 8, 18–21 day old rats weighing 20–25g each having received radioactive ribonucleoside precursors as described in the legend to Figure 9. The purified HnRNA was fractionated on poly(U)-Sepharose into the components which were unbound, bound but eluted with 15% formamide and bound but eluted with 90% formamide. The constituent RNA was precipitated by 2 volumes of ethanol in the presence of 40μg of unlabelled uterine RNA. Recovered RNA was dissolved in electrophoresis buffer containing 90% formamide and denatured at 90° for 5 min., rapidly cooled and separated on 2.7% polyacrylamide gels for 5h at 5mA/gel.

(A) = Unbound HnRNA fraction
(B) = Bound HnRNA but eluted with 15% formamide
(C) = Bound HnRNA but eluted with 90% formamide

--- = Extinction at 260 nm
..... = Radioactivity per slice in cpm
poly(U)Sepharose. However, it had to be demonstrated that each fraction represented separate and distinct RNA components as opposed to artificial derivatives as a consequence of aggregation or degradation of complex high molecular weight molecules. To evaluate the range of molecular weights of each of the HnRNA fractions, the purified components were denatured and fractionated on both polyacrylamide gels and denaturing sucrose gradients.

Figure 10 demonstrates that the various HnRNA species from unstimulated animals when resolved on aqueous gels, after a prior denaturation step, possessed both a distinctive average migration size and migration range. The unbound fraction (figure 10a) possessed the broadest radioactivity profile in a size ranging from larger than 45S to about 20S with a distinct peak at about 50S. The presence of low molecular weight species in RNA that was previously all greater than 45S could, in part, be explained by the formamide denaturation as in figure 8. However, the possibility of some degradation during the affinity chromatography could not be excluded.

The HnRNA species eluted by 15% buffered formamide (figure 10b) occupied the narrowest range of molecular sizes with a high proportion of its HnRNA migrating at the 45S position. This finding indicated that this fraction of the HnRNA was composed of a different set of molecules to the unbound fraction on the basis of size and migration characteristics. The HnRNA species eluted by 90% buffered formamide was as heterogenous in size as the unbound fraction. Most of this fraction migrated around the 32S marker after denaturation and species at around the 45S RNA marker were barely discernible. Figure 11
FIGURE 11
Sedimentation of poly(U)-Sepharose fractionated uterine HnRNA in sucrose-formamide gradients

HnRNA was isolated and purified from immature rats which were treated with 1 µg oestradiol intraperitoneally 2 hr before death and radioactive ribonucleosides as described in the legend to Figure 9. The purified HnRNA was fractionated on columns of poly(U)-Sepharose into the unbound, bound but eluted with 15% formamide and bound but eluted with 90% formamide. The constituent RNA was precipitated by 2 volumes of ethanol in the presence of 40 µg unlabelled uterine RNA. Recovered RNA was dissolved in 8% formamide, denatured at 90° for 2-4 min and cooled immediately at 0-4°C. The RNA was sedimented in a 13 ml, 5-20% (w/v) sucrose gradient containing 8% formamide in the Beckman SW40 rotor as described in the Methods section. Radioactivity was measured in the acid-insoluble material. 32P-labelled HeLa cell nucleolar and 28S RNA was sedimented in a parallel gradient to provide the 45S, 32S and 28S markers.

(A) = Unbound HnRNA fraction
(B) = Bound HnRNA but eluted with 15% formamide
(C) = Bound HnRNA but eluted with 90% formamide
illustrates the sedimentation of the three HnRNA fractions, in denaturing sucrose gradients. It was seen that the results mirrored those obtained on gels and showed that the poly(U)Sepharose fractionated HnRNA species had different molecular weight profiles. The three fractions also corresponded closely to the components seen in a fractionation of total high molecular weight HnRNA on denaturing sucrose gradients (figure 8). The preparation used in figure 11 was derived from oestradiol treated rats while that used in figure 10 was from untreated animals. This shows that the poly(U)Sepharose chromatography produces broadly similar fractionation patterns from hormone treated and untreated animals.

1.3.3 Characterization of nuclease-resistant adenylate core from HnRNA fractions

The binding of the uterine HnRNA to poly(U)Sepharose depends on duplex formation between the immobile homopolymers and the poly(A) segments of the bound RNAs. Because the bound uterine HnRNA was eluted by buffers containing two different formamide concentrations, it seemed probable that differences in size of adenylate segments in the HnRNA fractions were responsible for the fractionation. However, Molloy et al. (1974) showed that not all poly(A)+RNA binds to poly(U)Sepharose; therefore the presence of poly(A) containing HnRNA in the unbound fraction could not be ruled out. In order to detect poly(A) segments, HnRNA labelled with $^{3}H$-adenosine was digested with nucleases under conditions which have been shown to spare adenylate segments (Dubroff & Nemer, 1975). Analysis of the adenylate cores from total unfractinated HnRNA and in chromatographic fractions was carried out by recovering trichloacetic acid-precipitable
Measurements of Nuclease-resistant adenylate cores of 2h oestradiol-stimulated \textsuperscript{3}H-adenosine labelled HnRNA fractions.

6-8 uteri of 18-21 day old rats, weighing 25-30gm each having been treated intraperitoneally with 1\mu g oestradiol-17\beta 2hr before death were incubated in Eagle's medium containing 125\mu Ci of \textsuperscript{\textit{\textit{3}}}H adenosine/ml for 1 hr. High molecular weight HnRNA was prepared and fractionated on Poly(U)Sepharose as described in the Methods section. The fractionated RNA from two separate experiments was pooled and precipitated with 2 volumes of ethanol. Each chromatographic fraction was digested with a mixture of T\textsubscript{1} and pancreatic ribonuclease as described in the Materials and Methods section. The amount of radioactivity incorporated and of nuclease-resistant adenylate core was determined in the acid-insoluble material.
Table 5

Measurements of nuclease resistant adenylate cores of 2 hours oestradiol stimulated $^{3}\text{H}7$-adenosine labelled HnRNA fractionated on poly(U) Sepharose.

<table>
<thead>
<tr>
<th>HnRNA</th>
<th>% (dpm) of total (64,844 dpm)</th>
<th>% (dpm) nuclease resistant material relative to total unfractionated HnRNA</th>
<th>% (dpm) nuclease resistant material relative to fractionated HnRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbound</td>
<td>59% (38,300 dpm)</td>
<td>&gt; 0.06</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Bound but eluted with 1% formamide</td>
<td>8% (5,120 dpm)</td>
<td>&gt; 0.045</td>
<td>&gt; 0.3</td>
</tr>
<tr>
<td>Bound but eluted with 90% formamide</td>
<td>12% (7,800 dpm)</td>
<td>0.8</td>
<td>7.0</td>
</tr>
</tbody>
</table>
material. The contribution of small nucleotides was thereby minimized because they are inefficiently precipitated in acid.

Table 5 shows the percentage incorporation of label precursors into the various fractions. Before nuclease digestion the bulk of the radioactivity (59%) occurs in the unbound fraction, followed by the HnRNA fraction eluted by 90% formamide which contained 1% while the fraction eluted by 15% formamide contained 3% of the label. These results effectively confirm those of table 4 in which ³H-uridine was the precursor. However, there is a substantial difference in the percentage of the precursor found in the fraction eluted by 15% formamide. It is not known whether this difference reflects a high uridine/adenine ratio in this fraction.

Acid-precipitable nuclease resistant adenylate cores of ³H-adenosine-labelled HnRNA represented about 1% of the total high molecular weight and unfractionated HnRNA, a value which is consistent with most HnRNA of various animal tissues, cultured cells and viral nuclear RNA (Lewin 1975b, 1975c). After 1 hr of in vitro labelling with ³H-adenosine, nuclease resistant material in oestradiol-stimulated HnRNA of all three chromatographic fractions differed greatly in accordance with the concentrations of formamide present in the eluting buffers. That is, the homopolymeric adenylic acid content was least (0.1%) in the unbound fraction; in the HnRNA fraction eluted by 15% formamide, it was 0.3% and in the fraction eluted by 90% formamide it was 7%. These results are summarised in table 5 and consistent with the observed poly(A) content of HnRNA fractions from HeLa cells (Edmonds et al. 1971; Jelinek et al. 1973) and sea-urchin embryos (Dubroff & Nemer, 1975).
1.3.4 Size distribution of polyadenylate cores of uterine HnRNA fractions

One of the most persuasive indications of a precursor–product relationship between HnRNA and mRNA is the evidence showing the presence of polyadenylic acid in both types of RNA (Edmonds et al. 1971; Lee et al. 1971; Adesnik et al. 1972; Jelinek et al. 1973). There are also internally located oligo adenylic acid residues of about 25 AMps (Edmonds et al. 1976) in HnRNA and these would constitute nuclease resistant material. As shown in Table 5 all HnRNA fractions isolated by poly(U)Sepharose contained adenylic acid polymers as evidenced by the recoverable acid-precipitable nuclease resistant material. Analysis of purified adenylic acid polymers of chromatographic fractions of HnRNA from HeLa cells and uteri are shown in figure 12 as a radioactivity profile on 10% aqueous gels. Figure 12a shows the electrophoretic profile of $^{32}$P-labelled poly(A) sequences of polyadenylated HnRNA derived from HeLa cells. The poly(A) sequences migrated fairly heterogenously and conformed to previous findings that poly(A) from cells labelled for a very long time is heterogenous, with a substantial proportion of relatively large segments migrating more slowly than tRNA (Sheiness & Darnell 1973). The method employed thus preserved this segment. Figure 12b illustrates the electrophoretic profile of the ribonuclease-resistant adenylic acid polymers of the uterine HnRNA eluted by 90% formamide. The radioactivity profile was heterodisperse and indicated the presence of poly(A) sequences with a size range of 80–150 nucleotides relative to a 4S marker. Significant peaks due to poly(A) sequences in either the unbound fraction or in the HnRNA eluted with 15% formamide could not be detected
6-8 uteri of 18-21 day old rats, weighing 25-30gm each having been treated intraperitoneally with 1μg oestradiol 2h before death, were incubated in Eagle's medium containing 125μCi of $[2\textsuperscript{3}H]$ adenosine/ml for 1hr. High molecular weight HnRNA was prepared and fractionated on poly(U)-Sepharose as described in the Methods section. The fractionated RNA from two separate experiments was pooled and precipitated with 2 volumes of ethanol. The recovered RNA was digested with a mixture of T1 and pancreatic ribonuclease as described in the Methods section. The nuclease-resistant material was phenol extracted and precipitated in the presence of 40μg 4S tRNA and separated on 10% polyacrylamide gels for 3hr at 5mA/gel with the carrier 4S RNA as marker.

The figure illustrates the ribonuclease-resistant core from:

(A) $= {\textsuperscript{32}P}$-labelled HeLa cell HnRNA
(B) $= {\textsuperscript{3}H}$-labelled uterine HnRNA eluted with 90% formamide
(C) $= {\textsuperscript{3}H}$-labelled uterine HnRNA in the unbound fraction
(D) $= {\textsuperscript{3}H}$-labelled uterine HnRNA eluted with 15% formamide.

—— = extinction at 260nm
•••• = Radioactivity per slice in cpm
However, shorter nucleotides of less than 15 bases which might have been expected in the latter fraction (Dubroff & Nemer, 1975) would not have been detected in the gels used.

Previous reports (Molloy et al. 1974; Jelinek et al. 1974) have shown that not all HnRNA molecules that bind to poly(U)Sepharose contain long stretches of poly(A). However, if the columns were eluted with an increasing concentration of formamide, as has been done in these studies, the long poly(A) sequences can be separated from other AMP-rich sequences that bind to the column with lower affinity. The bound HnRNA fraction eluted by 15% formamide may be just such HnRNA molecules. Furthermore, internally located oligo(A) sequences of 15-25 bases (Edmonds et al. 1976) and double stranded RNA segments containing AMP-rich nucleotide segments (Jelinek & Darnell, 1972; Ryskov et al. 1973) that are resistant to the action of ribonucleases may have contributed to the percentage of nuclease-resistant acid-precipitable material observed for the HnRNA fraction eluted by 15% formamide (Table 5).

Thus, the fractionation by poly(U)Sepharose chromatography of uterine HnRNA into three main fractions each with their own size distribution and differing poly(A) content suggest separate classes of HnRNA in the uterine nucleus. This also raises the possibility of distinguishable functions for each class.

It is also worthy of note that Edmonds et al. (1971) and Darnell et al. (1971) observed an inverse relationship between the proportion of poly(A) and the size of the nuclear RNA. Thus, for the total high molecular weight HnRNA sedimenting at between greater than 45S to 32S (figure 8) with an estimated molecular weight of 2-4 x 10^6 (Spohr et al., 1976), a poly(A) sequence of 80-150 nucleotides (figure 12b)
would lead to a poly(A) content of 0.6–2.7%. This prediction is in agreement with the experimentally estimated poly(A) content of 1% for HnRNA size distribution as seen in figure 8. However, a similar calculation made for the poly(A) containing HnRNA fraction gives a predicted poly(A) content of 1.2–2.3% which is considerably lower than the estimated figure of 7% (table 5). This implies that the polyadenylated HnRNA probably contain internally located oligo(A) sequences and other AMP-rich regions in the form of secondary structures (Jelinek et al. 1973; Ryskov et al. 1973, 1975) to account for the high poly(A) content. Based on a similar assessment, the HnRNA fraction eluted by 15% formamide would contain adenylated sequences but considerably smaller than 80 nucleotides. That this is the case with this HnRNA fraction is indicated in figure 12d.

1.4  The effects of oestradiol-17β on uterine RNA synthesis

1.4.1  Time course for the effect of oestradiol-17β on precursor incorporation into acid-insoluble and acid-soluble material

The increased incorporation of radioactive precursors into RNA has usually been interpreted to mean an increase in the rate of RNA synthesis, but it could be accounted for by other factors such as changes in the sizes of precursor pools or changes in the transport of radioactive precursors into the oestradiol-stimulated uterus (Billing et al. 1969a, b). Before initiating a study of the effects of oestradiol on uterine HnRNA synthesis it was considered desirable to exclude the possibility that the effect of oestrogen on transcription could be
FIGURE 13

Time course for the effect of oestradiol-17β (1µg/Rat) on the incorporation of RNA precursor into 18-21 day old rat uteri.

Hormone was administered intraperitoneally for various time period and 20µCi [5-3H] uridine given intravenously 30 min before death. Results were expressed as dpm/µg DNA and expressed as a percentage of the uptake in non-hormone treated control animals. Each point represents a mean of four animals. Bars indicate the maximum and minimum value at each time point.

- - - - - = Acid Insoluble fractions
- - - - - = Acid Soluble fractions
caused by stimulated precursor uptake of the radioactive ribonucleoside precursors.

In this study, a standard system was used in which 18-21 day old rats weighing 25-35g were treated with 1 µg of oestradiol administered intraperitoneally and each immature rat received 20 µCi $^3$H-uridine 30 min before death. Figure 13 illustrates the results of this experiment. Oestradiol treatment resulted in a slight increase in uptake of precursors into the acid-soluble material 1 hour after hormone treatment and reached a maximum of 200% of the control value at 4 hr. Conversely, uptake of radioactive precursor into the acid-insoluble material had reached its peak of 730% of the control value at the same time. By 6 hr of hormone treatment, radioactivity in the acid-insoluble fraction fell to nearly 300% and thereafter declines slowly reaching a value of 220% after 24 hr of hormone treatment. These findings, which are consistent with the observations made by Knowler & Smellie (1971), indicate that the stimulation by oestrogen of uterine RNA synthesis cannot be accounted for by increased precursor uptake.

1.4.2 The effects of oestradiol-17β on the synthesis of high molecular weight uterine RNA species

The effects of oestradiol upon uterine RNA synthesis was determined by following the incorporation of radioactive ribonucleoside precursors which was administered 30 min before death. Purified RNA was analysed on 2.7% aqueous gels.

Figure 14 shows the effect of oestradiol-17β on the incorporation of tritiated ribonucleosides into the RNA species
FIGURE 14

Effect of oestradiol-17β on the synthesis of uterine RNA

18-21 day old rats, weighing 25-30g, received 1μg of oestradiol or vehicle by intraperitoneal injection and 100μCi each of \( \text{\textsuperscript{3}H} \) uridine and \( \text{\textsuperscript{3}H} \) guanosine by intravenous injection 30 min before death. Purified uterine RNA was separated for 5h in 2.7% polyacrylamide gels at 5mA/gel. \( \text{\textsuperscript{32}P} \)-labelled HeLa nucleolar RNA was separated in a parallel gel to provide the 45S and 32S markers.

(A) = control
(B) = 30 min after oestradiol-17β administration
(C) = 1h after oestradiol-17β administration
(D) = 2h after oestradiol-17β administration

--- = Extinction at 260nm
**** = Radioactivity per slice in dpm
at various times after oestradiol treatment. After 30 min (Figure 14b) of oestradiol treatment there was a marked increase in the incorporation into RNA species confined to the first few slices of the gel. At later times the synthesis of ribosomal precursor RNA species and the ribosomal species became obvious but always their peaks were superimposed on a heterogenous profile of radioactive precursor incorporation into species of a wide range of molecular weights. These results repeat those of Knowler & Smellie (1971, 1973) who demonstrated that the synthesis of the very high molecular weight RNA was the first observable transcriptional response after the administration of oestradiol to rats. They went on to show, by base composition, lack of methylation, nuclear location and kinetics of synthesis and decay, that the high molecular weight RNA was HnRNA. The continued investigation of uterine HnRNA forms the basis of the first part of this result section.

1.4.3 The effects of oestradiol-17β on the synthesis of uterine HnRNA

High molecular weight fractions of the HnRNA were purified and isolated as described in the Methods section and subfractionated on poly(U)Sepharose into fractions that differed in their poly(A) content (table 5). These HnRNA fractions have been shown to differ in their size profile on polyacrylamide gels (Figure 10) and on denaturing sucrose gradient (figures 11a, b, c). The kinetic of synthesis, degree of stimulation and size distribution of the newly synthesized RNA in each fraction were investigated after oestradiol treatment.

Figure 13 shows the effects of oestradiol-17β on the synthesis of the various fractions of high molecular weight HnRNA.
FIGURE 15
Effect of oestradiol-17β on the synthesis of total uterine high molecular weight HnRNA and poly(U) Sepharose-fractionated HnRNA.

High molecular weight HnRNA was prepared from groups of immature rats (18-21 days old) that had received 100μCi of $\text{[^3H]}\text{U}$ uridine intravenously 30 min before death and 1μg oestradiol-17β or vehicle at various times before death. In some experiments the RNA was further fractionated by poly(U) Sepharose chromatography as described in the Materials & Methods section. Total high-molecular weight HnRNA or chromatographic fractions were acid-precipitated and prepared for determination of acid-insoluble radioactivity as described in the Materials & Methods section. Each point represents the mean values of two separate experiments and eight animals.

○ = Total high molecular weight HnRNA
△ = High molecular weight HnRNA not bound by poly(U) Sepharose
□ = High molecular weight HnRNA eluted from poly(U) Sepharose with buffered 17% formamide
● = High molecular weight HnRNA eluted from poly(U) Sepharose with buffered 90% formamide.
Time Post Oestradiol Treatment

% Stimulation

- 1000
- 900
- 800
- 700
- 600
- 500
- 400
- 300
- 200
- 100

30 mins 1 hour 1½ hours 2 hours
It was observed that the incorporation of radioactive precursors into total high molecular weight HnRNA was stimulated by nearly 3-fold as early as 30 min post-oestradiol treatment; thus confirming previous observations (figure 14) and consistent with the findings of Knowler & Smellie (1973) and Knowler, (1976). By 2 hr after hormone treatment, the stimulated synthesis of total HnRNA had increased to 10 fold unstimulated levels. On fractionating the HnRNA on poly(U)Sepharose, it was observed that the increase in HnRNA synthesis at 30 min after oestrogen administration was reflected in an increase in the precursor incorporation of poly(A) containing HnRNA whereas the other eluted fractions showed marginal increases. At 2 hr post-oestradiol treatment, however, the synthesis of poly(A) containing HnRNA was least stimulated while there was a 10 fold increase in the rate of synthesis of the unbound HnRNA fraction. It is possible that the poly(A) containing HnRNA was being turned over more rapidly or that there had been a rapid processing of the HnRNA into possible putative messengers. At all time intervals the HnRNA fractions eluted with 15% formamide showed responses to oestradiol-17β which were roughly intermediate between the other two HnRNA fractions.

1.4.4 **Electrophoretic analysis of the oestradiol-stimulated HnRNA species**

Figure 16 demonstrates 2.7% polyacrylamide gel radioactivity profiles of the three HnRNA fractions prepared from untreated and oestrogen treated immature rats and run in the presence of unlabelled uterine ribosomal RNA as carrier. Parallel gels contained 32P-labelled HeLa nucleolar RNA. Each fraction exhibited a profile containing a
FIGURE 16

Oestrogen effect on the electrophoretic profile of uterine high molecular weight HnRNA fractions

High molecular weight HnRNA was prepared from groups of immature rats (18–21 days old) that had received 100μCi of $^{32}$H uridine intravenously 30 min before death and 1μg oestradiol-17β, or vehicle only, intraperitoneally 2h before death. The HnRNA was prepared and fractionated on poly(U) Sepharose as described in the Materials & Methods section. RNA in the various chromatographic fractions was ethanol precipitated in the presence of unlabelled uterine rRNA as carrier and subsequently resolved on 2.7% polyacrylamide gels for 5h at 5mA/gel. The positions of the 45S and 32S RNA were identified by resolving $^{32}$P-labelled HeLa cell nucleolar RNA on a parallel gel, and the carrier RNA provided 28S and 18S markers.

A: Unbound fraction — control
B: Fraction eluted with 15% buffered formamide — control
C: Fraction eluted with 90% buffered formamide — control
D: Unbound fraction — 2h oestradiol-17β treatment
E: Fraction eluted with 15% buffered formamide — 2h oestradiol-17β treatment
F: Fraction eluted with 90% buffered formamide — 2h oestradiol-17β treatment

—— = Extinction at 260nm
.... = Radioactivity per slice in dpm
number of peaks of radioactive precursor incorporation and, as has been observed earlier, the profiles differed between fractions (figures 10 & 11). They also differed between preparations derived from untreated and oestrogen-treated animals. The fraction not retained by poly(U)Sepharose shows the greatest increase in incorporation of radioactivity in response to 2 hours of oestradiol treatment. Conversely, the polyadenylated HnRNA was the least stimulated as evidenced by the comparatively low radioactivity incorporation. This observation confirmed the kinetic experiment (figure 15). The nonpolyadenylated HnRNA fraction also contained the greatest concentration of RNA in high molecular weight species. However, all three fractions of HnRNA showed substantial radioactivity in species of lower molecular weight than originally isolated. In the poly(U)Sepharose bound fractions, this change in molecular weight could be attributed to the denaturing effects of formamide and paralleled the observation in figures 10 and 11. The fact that lower molecular weight species were also detected in the unbound fraction implies that some degradation during the column chromatography could not be ruled out.

Work with ribosomes has shown that within 30 to 60 min of oestradiol administration to immature rats, ribosomes are aggregating into polysomes (Merryweather & Knowler, 1977) and it is tempting to speculate that they are doing so because of the availability of mRNA derived from the poly(A)+HnRNA fraction. It was hoped that these concepts could be tested by hybridization experiments (see results section 3).
2. Characterization of uterine polysomal poly(A)+mRNA and the effects of oestradiol-17β on its synthesis

2.1 Isolation and characterization of uterine polysomes

Characterization studies of polysomes were carried out on unstimulated immature rat, immature rats stimulated with oestrogen for 4 hr, and adult rats. The adult animals were at proesterous when circulating oestrogens are maximal, and were taken to represent the fully developed tissue. The polsosome profile of preparations at the various hormonal states are shown in figure 17. The preparation from unstimulated immature rats contained few polysomes and only showed peaks of monomers and dimers. 4 hr after the administration of oestradiol to immature rats, the uterine ribosomes had aggregated into polysomes thus implying the availability of newly synthesized mRNA and confirming previous findings (Merryweather & Knowler, 1977). In the adult rat, the polysome profile revealed a wide range of polysome sizes including several peaks sedimenting at greater than 100S.

2.2 Isolation of uterine polysomal RNA

The occurrence of RNAses presents a serious problem for the isolation of intact mRNA, therefore a number of isolation procedures was tested so that a method could be found for routine preparation of intact polysomal RNA and for the subsequent purification of the poly(A) containing species.

Some of the methods tested included the extraction of cytoplasmic RNA from the post-mitochondrial supernatant, from mRNAP particles and from polysomal pellets with or without prior Proteinase K
4-10 A$_{260}$ nm units of uterine polysomes were sedimented through 15-45% (w/v) sucrose gradients in 5mM Tris-HCl pH 7.6, 250mM KCl, 5mM MgCl$_2$, 50μg/ml heparin and 5μg/ml of cycloheximide at 234,000 x g$_{av}$ for 35 min at 4°C. Gradients were scanned at 260nm with a gradient scanning attachment to the Gilford 240 spectrophotometer. (a) unstimulated immature rat uteri (b) 4 hour oestradiol stimulated immature rat uteri (c) adult rat uteri at proestrous.
TABLE 6

The purification of uterine RNA from polysomes of the immature rat.

Sets of 24, 18-21 day old rats, weighing 25-30g received 1μg oestradiol intraperitoneally 4 hr before death and 100μCi [3H] uridine intravenously 1½ hr before death. Polysomal RNA, or ribosomal subunits as mRNP particles, were prepared from the excised uteri as described in the Materials & Methods section. Poly(A)+RNA was purified by poly(U)Sepharose affinity chromatography. Radioactivity was assayed as acid-insoluble material in each RNA preparation. μg RNA was assayed by the method of Kerr & Seraidarian (1945) (see Materials & Methods) and μg RNA in mRNP was assayed by measuring absorbance at 260nm and taking 1 O.D. unit representing 40 μg RNA.
<table>
<thead>
<tr>
<th>Method</th>
<th>Radiosactivity as acid-insoluble (cpm) material in total</th>
<th>Radiosactivity as acid-insoluble (cpm) material of poly(rA)-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phenol extraction of post-mitochondrial RNA</td>
<td>412</td>
<td>923 (5%)</td>
</tr>
<tr>
<td>2. Lauryl sarcosine-polysomes EDTA dissociated</td>
<td>640</td>
<td>132 (0.4%)</td>
</tr>
<tr>
<td>3. Phenol extraction of RNA in polyosomal pellet without Proteinase K digestion</td>
<td>450</td>
<td>640 (2.4%)</td>
</tr>
<tr>
<td>4. Phenol extraction of RNA in Proteinase K digested polysome pellet</td>
<td>600</td>
<td>1,980 (3.6%)</td>
</tr>
</tbody>
</table>

**Table 6**

Extraction of Polyosomal RNA and Isolation of Poly(rA)-RNA
digestion of the polysomal pellet.

Table 6 shows an analysis of methods employed and tabulates the yield of polysomal RNA and of polyadenylated species. With the procedures involving differential extraction with phenol: chloroform: isooamylalcohol at neutral and alkaline pH, a large proportion of RNA from the post-mitochondrial supernatant was obtained. One objection to using phenol alone in the extraction of polysomal RNA is that, besides causing aggregation, phenol preferentially removes poly(A)+RNA sequences from the RNA population into the proteinaceous interphase (Perry et al. 1972). For this reason, the protein content of polysomal pellet was reduced by incorporating a digestion step using proteinase K in the presence of SDS and a differential extraction procedure at pH 7.4 and pH 9.0 (Brawerman, 1976) with phenol: chloroform: isooamylalcohol (1:1:0.1) was carried out.

The least efficient of the methods tested for recovery of polyadenylated RNA was that which involved the isolation of poly(A) rich mRNP particles. Although radioactivity incorporation was substantial, the dissociation procedure using EDTA and lauroyl-sarcosine which had been used successfully in isolating polyadenylated RNA species in a number of cultured cell (Pederson & Lindberg, 1972; Adesnik et al. 1972) proved less efficient in dissociating uterine polysomes. This resulted in low recovery of polyadenylated mRNA.

2.3 Electrophoretic analysis of uterine polysomal RNA

Based on the results tabulated in table 6, the method of choice for the extraction of uterine polysomal RNA was that employing
FIGURE 18

Size distribution of uterine polysomal and polysomal poly(A)+RNA of Polysomes treated with Proteinase K.

Polysomal RNA was prepared from uteri of 24, 18–21 day old rats, weighing 25–30g that received 1μg oestradiol intraperitoneally 4hr before death or from 6–8 adult uteri. Polyadenylated RNA was purified by poly(U) Sepharose chromatography and further purified by a second passage through fresh poly(U) Sepharose columns.

Approximately 20–25μg of RNA was electrophoresed, through 3% polyacrylamide gels containing 98% formamide, for 3h at 5mA per gel. Gels were washed with two changes of water before scanning at 260nm with a Gilford 240 spectrophotometer gel scanning attachment. (a) total polysomal RNA derived from 4 hr oestradiol stimulated uteri; (b) polysomal poly(A)+RNA from 4h oestrogen stimulated uteri; (c) polysomal poly(A)+RNA from adult uteri at prooestrus. Arrows mark the positions of uterine rRNA markers electrophoresed on parallel gels.
FIGURE 19

Separation of 4h oestradiol-stimulated uterine polysomal RNA on polyacrylamide gels

Experimental details were as described in the legend to Figure 18. Each rat received 100μCi of $^3$H-uridine 1½hr before death. Approximately 60μg of total polysomal RNA, or 20μg poly(A)+ RNA, was separated on 2.7% polyacrylamide gels for 3hr at 5mA/gel. Unlabelled uterine RNA was separated on a parallel gel to provide the 28S and 18S rRNA markers.

(A) = Total polysomal RNA

(B) = Polyadenylated RNA after a single passage through poly(U) Sepharose column

(C) = Polyadenylated RNA after a second passage through poly(U) Sepharose column

--- = extinction at 260nm

... = Radioactivity per slice cpm
proteinase K digestion of the polysomal pellet followed by phenol extraction (the method is described in full in the methods section). The integrity of total polysomal RNA prepared by this method was verified by polyacrylamide–formamide gel electrophoresis (figure 18a) which shows that all the major polysomal RNA species were intact.

Figure 18b and 18c illustrates the polyadenylated RNA of both immature and adult rat uteri, purified from the polysomal RNA by two passages through poly(U)Sepharose. The product contained no visible contamination with ribosomal RNA. That this degree of purification did require two passages through two columns of poly(U)Sepharose (see methods section) is illustrated in figure 19 which illustrates the polyadenylated RNA recovered after one and two passages. It is seen that after one passage through the affinity column the polyadenylated product still contained contaminating ribosomal RNA but that this was not noticeable after a second passage through a fresh column.

2.4 Poly(A) size and content of uterine polyadenylated polysomal RNA

For the estimation of poly(A) size and content, uterine polysomal poly(A)+RNA (mRNA) derived from 4 hr oestradiol–stimulated immature rats and from adult rat uteri at proestrus, was digested with T₁ plus pancreatic RNAseA under conditions that prevent oversplitting of adenylate residues in poly(A) (Dubroff & Nemer, 1975). The products were purified by phenol extraction and resolved on 2.7% aqueous gels for 3 hours. Figure 20 shows the profile of RNA extractable from the gels and able to form hybrids with ³H–poly(U).
Slice Number

FIGURE 20

Size distribution of poly(A) from uterine polysomal poly(A)+RNA

Poly(A) tracts were prepared from 4h oestradiol-stimulated and adult proesterus uterine polysomal RNA by digesting poly(A)+RNA with Ribonucleases as described in the Materials & Methods section. The nuclease-resistant adenylate core was electrophoresed on 2.7% polyacrylamide gels for 3hr at 5mA/gel. After electrophoresis, gels were sliced and fractions were eluted and hybridized to \(^{3}H\)-poly(U) to quantitate the poly(A). Formamide-denatured \(^{32}P\)-labelled 5.8S RNA was electrophoresed in a parallel gel to provide the 5.8S marker.

\[(A) = \text{Poly}(A) \text{ from 4h oestradiol-stimulated polysomal poly}(A)+RNA.\]

\[(B) = \text{Poly}(A) \text{ from adult proesterus uterine polysomal poly}(A)+RNA.\]

- - - - = \(^{3}H\) radioactivity per slice

--- = \(^{32}P\) radioactivity per slice
FIGURE 21

Electrophoretic mobilities of nucleotide markers and 5.8S rRNA on polyacrylamide gels.

The figure illustrates the relationship between logarithm of average molecular weight of homopolymeric nucleotides and $^{32}$P-labelled 5.8S rRNA and electrophoretic mobility on 2.7% polyacrylamide gel. Each homopolymeric nucleotide and formamide-denatured 5.8S rRNA was resolved on separate gels at 5mA/gel for 3 hr. The mobility of the peak of homopolymeric nucleotides was located by measuring extinction at 260nm and is indicated by arrows. The mobility of the peak of 5.8S rRNA is indicated by the arrow and was located by measuring $^{32}$P radioactivity in each slice.
A graph showing the relationship between log molecular weight and the distance migrated (cm). The graph includes labels for 5.8S rRNA, POLY(A)₉₀, and OLIGO(A)₂₈.
The analysis demonstrated that the poly(A) derived from adult uterine mRNA migrated heterogeneously and exhibited a wide range of size classes (figure 20b). The polyadenylated sequences of the mRNA from the uteri of 4 hr oestradiol-stimulated immature rats was somewhat less heterogeneous and confined to a narrower size distribution (figure 20a). The peak of radioactivity in both preparations was around the 5.8S rRNA marker of 150–160 nucleotides. Due to the heterogeneity, the number average poly(A) length was determined. To do this, 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 21). From this plot it could be estimated that the number average poly(A) length for the adult uterine mRNA and the 4 hr oestradiol-stimulated immature uterine mRNA was 150 and 160 nucleotides respectively. This estimate was confirmed by employing sucrose density gradient sedimentation of the poly(A) homopolymers in 15–30% (w/v) sucrose gradients in 0.1M NETS buffer (0.1M NaCl, 1mM EDTA, 0.01M tris-HCl pH 7.4, 0.2% (w/v) SDS) in a Beckman SW 40 rotor. Centrifugation was at 20,000 rpm for 6 hours at 4°C. Fractions containing poly(A) nucleotides were located by \(^3\)H-poly(U) hybridization. The distribution of hybridizable \(^3\)H-poly(U) across the gradient gave the molecular weight distribution of the poly(A) and it was therefore possible to calculate the number average molecular weight from a cumulative plot of the fraction of poly(A) molecules against their length (Spirin, 1963).
**TABLE 7**

**Size and poly(A) content of polysomal poly(A)+RNA from rat uterus.**

a: RNA was quantified by absorbance at 260nm assuming that 1 O.D. unit is 40μg RNA. Polysomal RNA was heat-treated prior to chromatography and the percentage of polysomal RNA as poly(A)+ RNA was determined at 260nm from the fraction which bound a second time to poly(U)Sepharose.

b: Number average sizes were determined by hybridization of ^3H-poly(U) to extract of gel slices containing adenylated nucleotides (see Materials & Methods section. The size was referred to a 5.8s (160 nucleotides) slime mold rRNA, synthetic poly(A) (90 nucleotides) and oligo(A) (28 nucleotides). The ^32P labelled marker was analysed on a parallel 2.7% polyacrylamide as in Knowler and Smellie (1973).

c: The poly(A) content was determined by hybridization of 4-10μg poly(A)+RNA to excess ^3H-poly(U) as in Bantle, et al., (1976).
<table>
<thead>
<tr>
<th>Tissue</th>
<th>% polysomal RNA bound to poly(U) sepharose</th>
<th>Number average length of poly(A) tract (nucleotides)</th>
<th>poly(A) content (%)</th>
<th>Average molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult proesterous uteri</td>
<td>3.1</td>
<td>150</td>
<td>8.4</td>
<td>$6 \times 10^5$</td>
</tr>
<tr>
<td>4 hrs oestradiol treated uteri</td>
<td>2.8</td>
<td>160</td>
<td>7.6</td>
<td>$6 \times 10^5$</td>
</tr>
</tbody>
</table>
The poly(A) content of intact mRNA was quantitated by hybridization of uterine 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 4 separate preparations of these RNAs of about 8.4% and 7.6% for the adult uterine mRNA and the 4 hr oestradiol-stimulated immature rat uterine mRNA respectively. These results, together with the estimated average size of the poly(A) sequences are summarised in table 7. The average molecular weight of both uterine mRNAs is estimated at $6 \times 10^5$. Both uterine mRNA preparations isolated and assayed by $^3$H-poly(U) hybridization were 90–95% pure.

2.5 **Conditions for hybridization studies between uterine poly(A)+mRNA and complementary DNA**

The role of oestradiol-17β in regulating uterine polysomal poly(A)+RNA(mRNA) abundance and its influence on the number of average size genes transcribed were investigated by RNA–cDNA hybridization. This technique was also used to investigate sequence homology of mRNA at differing hormonal states and the concentration of classes of sequences in a population of RNA during different stages of uterine development.

2.5.1 **Preparation of complementary DNA (cDNA)**

For the preparation of cDNA, purified and salt free uterine poly(A)+mRNA was incubated with purified reverse transcriptase in a medium containing buffer, KCl, Mg$^{++}$, dithiothreitol, the deoxy-nucleotide triphosphates, $^3$H-dCTP and oligo dT primer 12–18 residues
FIGURE 22
Alkaline sucrose gradients of RNA

cRNA derived from poly(A)+RNA of 4 hours oestradiol
stimulated uteri (○-○-○); adult uteri (○-○-○) and globin mRNA
(□-□-□) were sedimented through 5-11% (w/v) sucrose gradients
in 0.5M NaCl, 0.1M NaOH at 95,000 x g_{av} for 24h at 20°C. 1 ml
fractions were collected and radioactivity counted as described
in the Materials and Methods section. Sedimentation Coefficients
were determined by the method of Steensgard et al. (1978).
long. Actinomycin D was also included to ensure that the product was single-stranded together with RNAse inhibitor (Roth, 1956) which was found to improve the yield and quality of the product. The yield of cDNA prepared by this method was consistently 8-10% by weight of the template poly(A)+mRNA. The concentrations of deoxynucleotide triphosphate and $^3$H-dCTP used, resulted in the incorporation of 5-7 x $10^7$ cpm/µg cDNA at 30% counting efficiency for $^3$H.

When the products were characterized on alkaline sucrose gradients (figure 22), the mean size of the cDNA transcribed from poly(A)+mRNA of adult uteri and oestradiol-stimulated uteri was approximately 5.5S which corresponds to nearly 120,000 daltons or nearly 450 nucleotides. However, cDNA sedimenting between 5S and 10S was recovered and used in hybridization studies of sequence complexities and diversity. Although the number average molecular weight of uterine mRNA was, at least on average, three times the length of globin mRNA, the cDNA transcripts were similar in length to globin cDNA.

2.5.2 Reverse transcription of uterine poly(A)+mRNA

Uterine poly(A)+mRNA preparations were transcribed in the same way as globin mRNA (Harrison et al. 1974). The observation that the yield of cDNA was at least 10% by weight of template and that the mean length of cDNA was 20% the size of the uterine poly(A)+mRNA suggested that the bulk of the mRNA species were represented in the population of cDNA molecules synthesized. This, thus satisfied the condition that for hybridization experiments, it is essential that all mRNA representative of each of the frequency classes is transcribed into
Moreover, evidence has shown that reverse transcriptase is capable of copying most if not all poly(A)+RNA (Harrison et al. 1974; Bishop et al. 1974).

2.5.3 Assay of hybridization reactions

The proportion of cDNA which hybridizes can be estimated in a variety of ways including digestion with a single-strand specific nuclease (Birnie et al. 1974; Young et al. 1974; Hall et al. 1972). In all of these experiments hybridization was assayed by $S_1$ nuclease digestion and since enzymes were obtained from commercial sources, it was felt necessary to characterize the enzyme with respect to optimum concentrations and digestion time such that double-stranded RNA-cDNA hybrids were spared.

Figure 23 shows the effect of $S_1$ nuclease obtained from the two commercial sources designated 'A' and 'B' on the digestion of single-stranded $^3$H-labelled globin cDNA and globin mRNA-cDNA hybrids. It was seen that by 2 hours of incubation with 20 units of enzyme activity from both 'A' and 'B' suppliers, nearly all the single-stranded cDNA had been digested. At earlier times, however, $S_1$ Nuclease 'A' appeared slightly more effective. When pure mRNA-cDNA hybrids, the fidelity of which had been tested by determination of $T_m$, were exposed to $S_1$ nuclease, $S_1$ Nuclease 'B' showed a small amount of digestion of hybrids while under similar conditions, $S_1$ Nuclease 'A' did not. From these observations, $S_1$ Nuclease 'A' at 20 units of enzyme activity and an incubation period of $1\frac{1}{2}$ hours was chosen for the routine assay of hybridization. If, however, $S_1$ Nuclease 'B' was used 8 units of
FIGURE 23

Assay of S₁ Nuclease activity

S₁ Nuclease was obtained from commercial sources designated 'A' (Sigma) and 'B' (Boehringer). Hybrids of globin mRNA–³H–cDNA, prepared as described in the Materials & Methods section was purified by hydroxyapatite chromatography. The hybrid or single stranded globin ³H–cDNA was incubated with 20 units S₁ Nuclease 'A' and 'B' in 100μl digestion buffer containing 70mM sodium acetate pH 4.5, 2.8mM ZnSO₄, 0.14M NaCl and 14μg denatured calf thymus DNA at 37°. At various times of incubation, an aliquot of incubation mixture was removed to determine total radioactivity and a further portion to determine acid-soluble radioactivity as described in the Materials & Methods section. S₁ Nuclease activity is expressed as percentage digestion.

- A-A- = 'A' S₁ Nuclease digestion of double-stranded nucleic acid

- - - - = 'B' S₁ Nuclease, digestion of double-stranded nucleic acid


- O-O-O = 'B' S₁ Nuclease digestion of single-stranded nucleic acid
enzyme activity and an incubation period of 2 hours was employed.

2.6 **Effects of oestradiol-17β and uterine development on uterine polysomal Poly(A)+mRNA complexity**

2.6.1 **Kinetics of hybridization of cDNA with homologous RNA**

The cDNAs derived from adult and oestradiol-stimulated immature rat uterine poly(A)+mRNAs as well as that derived from globin mRNA were hybridized to their own template and the kinetics of hybridization assayed by resistance to S1 nuclease digestion. The results are shown in figure 24 as computer fit to the hybridization data. It was observed that from 75-85% of each cDNA could form nuclease resistant hybrids with its template. Under the hybridization conditions used, Harrison et al. (1974) and Birnie et al. (1974) showed that the RNA-cDNA hybrids were of high quality and that each cDNA represented reasonably faithful copies of its own template.

It is noteworthy, however, that under these conditions a variable proportion of cDNA comprising 15-20% of the total cDNA was nonhybridizable. This did not affect complexity determinations since Rot (where Ro = initial RNA concentration in moles of nucleotides per litre and t½ = time for 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 Rot, has been shown to be proportional to the base sequence complexity of the RNA population (Birnstiel et al., 1972; Young & Paul 1973; Young et al., 1974). The base sequence complexity of an unknown
Homologous hybridization of the cDNA to its template mRNA was as described in the experimental section. (△) hybridization of globin cDNA with excess of template; (△□) hybridization of uterine message cDNA of 4h oestradiol-stimulated rat to an excess of template; (●■) hybridization of uterine message cDNA of adult rat to an excess of template. The RNA concentrations used were (△△) 1μg/ml; (●) 5μg/ml; (□) 1mg/ml; (■) 5mg/ml. Each point contained 2000 cpm of \(^3\)H cDNA recovered from gradient fractions 55-105 (Fig. 22).
RNA population may be determined by comparison of Rot for the reaction between the RNA and its cDNA with the Rot obtained with a kinetic standard of known base sequence complexity (Birnstiel et al. 1972; Bishop et al. 1974; Birnie et al. 1974; Getz et al. 1975). The standard used in this investigation was mouse globin consisting of and a globin sequences with a combined sequence complexity of 4 x 10^5 daltons (Williamson et al. 1971). The globin mRNA hybridized to its cDNA (figure 24) within 1.5-2 log units, a value typical of a single abundance class. Computer analysis of the data gave a Rot value of 4 x 10^-3 which was consistent with the findings of Birnie et al. (1974), Young et al. (1974) and Getz et al. (1975).

The computer fitted curves for the hybridization of the two uterine cDNAs to their own template poly(A)+mRNAs each covered a range of at least 6 log units, thus implying that the heterogenous population of uterine mRNA sequences were present in varying concentrations. Under these conditions, Bishop et al. (1974) and Hastie & Bishop (1976) showed that the polyadenylated polysomal RNA could be resolved into at least three abundance classes. In a similar manner, computer analysis of the data in figure 24 revealed that a good fit to the experimental data points was obtained by a three component curve. (See Methods section)

The two uterine mRNA preparations hybridizing to their respective cDNAs, differed in the kinetics of their association. Thus, at a Rot of 0.1 (moles sec. litre^-1) about 20% of the RNA from oestrogen stimulated immature rat uterus had saturated its cDNA. At the same Rot value, only 9% of the adult uterine RNA had hybridized. This suggested that at an early stage in the oestrogen-induced differentiation there
was a greater concentration of highly abundant RNA species than was
found in the fully mature animal. Conversely, the slow overall rate
of hybridization of the adult preparation suggested a greater sequence
complexity in the mature animal.

2.6.2 Sequence complexity and diversity of rat uterine
poly(A)+mRNA

Numerical evaluation of the hybridization kinetics of
figure 24 are summarised in table 8. The proportion of RNA in each
abundance class was calculated as a proportion of hybridizable cDNA,
this being 78% and 71% of the adult and immature preparations respectively.

Observed Rot, for each abundance class was corrected to the value which
would have been obtained if hybridization had been to a pure component
and complexities were derived by comparison with the globin standard.
The calculated values for the complexity and for the average molecular
weight of uterine poly(A)+mRNA were then used to estimate the number
average size genes transcribed in each class (table 8).

It is seen that uterine poly(A)+mRNA from 4 hr oestradiol-
stimulated animals contains about 9 sequences in very high abundance,
approximately 150 sequences of moderate abundance and about 7800 scarce
sequences. The RNA from adult uteri at proestrous exhibits a greater
complexity of expressed genes. Thus it contains approximately 18
abundant sequences, 2100 sequences of intermediate abundance and 34,000
scarce sequences. It should be emphasized that these calculations can
only be regarded as estimates. They will be subject to a number of
limitations, the main one of which is the estimate of the sizes of the
RNA sequences. Nevertheless, it is clear that the adult and immature
| a: | denotes the value of $R_{1}$ corrected for if the components were analysed as a single class and 100% pure. |
| b: | Taking the molecular weight of the mouse globin as $4 \times 10^{5}$ M.W. (Williamson et al., 1971). |
| c: | Taking average molecular weight of both uteri to be $6 \times 10^{5}$. |
Sequence complexity of polysomal poly(A)+RNA from rat uterus

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Transitions</th>
<th>% hybridizable cDNA</th>
<th>Observed Rot$^{1/2}$ (moles•sec$^{-1}$)</th>
<th>Corrected Rot$^{1/2}$</th>
<th>a Complexity $^{b}$</th>
<th>Number of diverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult uteri at proestrous</td>
<td>1</td>
<td>19.34</td>
<td>0.495</td>
<td>0.096</td>
<td>$10.8 \times 10^6$</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>42.15</td>
<td>27.1</td>
<td>11.4</td>
<td>$12.8 \times 10^9$</td>
<td>2100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>38.51</td>
<td>470</td>
<td>181</td>
<td>$20.4 \times 10^9$</td>
<td>34000</td>
</tr>
<tr>
<td>4 hrs oestradiol treated immature uteri</td>
<td>1</td>
<td>28.12</td>
<td>0.171</td>
<td>0.0481</td>
<td>$5.4 \times 10^6$</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.35</td>
<td>2.309</td>
<td>0.794</td>
<td>$8.9 \times 10^7$</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>37.53</td>
<td>111.84</td>
<td>41.84</td>
<td>$4.7 \times 10^9$</td>
<td>7800</td>
</tr>
</tbody>
</table>


$^{a}$ Calculated using the formula: $a = \text{Corrected Rot}^{1/2} \times 10^n$, where $n$ is the number of diverse sequence.

$^{b}$ Calculated using the formula: $b = \text{Observed Rot}^{1/2} \times 10^n$, where $n$ is the number of diverse sequence.
animal show a considerable difference in the complexity of their poly(A)+mRNA population.

It is of interest to note that, although the adult animal expresses a greater diversity of abundant mRNA species, those species expressed by the oestradiol-stimulated immature animal represent a considerably larger percentage of the poly(A)+mRNA population.

2.6.3 Hybridization of unique $^3$H-labelled DNA to uterine poly(A)+mRNA

One of the limitations on the determination of mRNA complexities by cDNA hybridization technique is that the final frequency class representing the unique sequences is difficult to measure and for this reason, the total base sequence complexity of the RNAs could have been underestimated. The technique of measuring the proportion of labelled unique DNA sequences which hybridize at saturation in the presence of a large excess of RNA (Gelderman et al. 1971; Galau et al. 1974, 1976) represents an alternative approach and is a much more sensitive method for measuring the complexity of those mRNA sequences of least abundance. In view of this, it was felt essential to complement the cDNA results by evaluating the extent to which unique rat liver DNA sequences hybridized with poly(A)+mRNA from adult and oestradiol-stimulated uteri.

The RNAs used were mercurated and hybridization was carried out in phosphate buffers at 70°. The extent of hybridization was assayed by thiol-Sepharose chromatography. The effect of mercuration of uterine poly(A)+mRNA is shown in the absorbance scan of figure 25. The absorption maxima remained at 260nm, thus indicating the intactness of
An aliquot of salt-free, mercurated-poly(A) + mRNA was dissolved in water and UV spectra was recorded using a Unicam-SP 2000 spectrophotometer.
Saturation hybridization of unique $^3$H-labelled DNA to poly(A)+RNA

$^3$H-labelled unique DNA was hybridized to saturation with mercurated poly(A)+RNA and analyzed by thiol-sepharose chromatography as described in the Methods section. Each point contains 50,000cpm of $^3$H-labelled DNA and either (●) 1.75mg/ml of Hg-poly(A)+RNA from uteri of rats stimulated with oestrogen for 4 hr, (▼) 4.5mg/ml of Hg-poly(A)+RNA from adult rat uteri or (□) 2.5mg/ml of E. coli Hg-rRNA.
Sequence complexity of polysomal poly(A)+RNA of rat uterus determined by hybridization to unique DNA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Observed % saturation of $^3$H-unique DNA</th>
<th>Corrected saturation value</th>
<th>Complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult proestrous uteri</td>
<td>2</td>
<td>2.8</td>
<td>$3.52 \times 10^{10}$</td>
</tr>
<tr>
<td>4 hrs oestradiol treated uteri</td>
<td>0.45</td>
<td>0.6</td>
<td>$7.56 \times 10^{9}$</td>
</tr>
</tbody>
</table>

The above data represent the numerical evaluations of the kinetics of saturation hybridization of $^3$H-DNA with poly(A)+RNA from figure 26.

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 70% hybridizability of the $^3$H-unique DNA probe.
absorbing bases of the mercurated poly(A)+mRNA. The kinetics of the hybridization reactions are shown in figure 26 as computer generated least-square fit of the data. Saturation hybridization of the uterine poly(A)+mRNA from adults and oestradiol-stimulated immature rats occurred with 2% and 0.4% of the unique DNA respectively. Since the \( ^3H \)-labelled probe was 70% hybridizable (Dr. A. Balmain, personal communications) when reassociated with total rat liver DNA, these saturation values were corrected to 2.3% and 0.6% respectively.

The molecular weight of the haploid genome is approximately \( 1.8 \times 10^{12} \) (Sober, 1968), 70% or \( 1.26 \times 10^{12} \) of which is unique sequences. Thus, the molecular weight of unique DNA expressed in the uterus of immature rat responding to 4 hr of oestrogen treatment, is \( 0.006 \times 1.26 \times 10^{12} \) which equals a molecular weight of \( 7.56 \times 10^9 \) or the equivalent \( 7.56 \times 10^9 / 6 \times 10^5 = 12,000 \) diverse sequences. In the adult uterus at proestrous the total complexity of transcribed genes is \( 0.028 \times 1.26 \times 10^{12} \) which equals a molecular weight of \( 3.53 \times 10^{10} \) or the equivalent of \( 3.53 \times 10^{10} / 6 \times 10^5 = 53,000 \) diverse sequences (table 9).

These results indicated that the base-sequence complexities of the uterine poly(A)+mRNA determined by cDNA-mRNA hybridization were indeed underestimates. However, the data did confirm the finding that the poly(A)+mRNA from the uteri of rats at proestrous was several fold more complex than that from the oestradiol-stimulated immature rats.
2.6.4 Poly(A)+mRNA sequences common to the uteri of both adult and oestrogen-stimulated immature rats

The extent of sequence homology between the uterine poly(A)+mRNA populations of adult and oestrogen treated immature rats was investigated by heterologous hybridization. A vast excess of mRNA from the uteri of 4 hr oestradiol treated immature rats was hybridized to cDNA prepared from the adult uterine poly(A)+mRNA. The result of this experiment is shown in figure 27 as a computer generated curve. It was found that there was considerable homology between the RNA populations. More than 65% of the adult uterine message cDNA could be hybridized by the heterologous RNA at Rot 9000 (moles sec. litre⁻¹). It was also clear from the data in figure 27 that not only was the overall reaction much slower than the homologous reaction but there was a lack of the fast hybridizing component. This implied that the RNA sequences driving the reaction were present in lower abundance than the same sequences in the homologous RNA population. Considering only that cDNA which was reactable, that is 78% for the homologous reaction and 57% for the heterologous reaction (figure 27), there was a difference of 21% of the adult message cDNA that was not complementary to the poly(A)+mRNA from the uteri of oestradiol-stimulated immature rats. At first sight the difference was inadequate to account for the several fold complexity differences observed between these two poly(A)+mRNA population when estimated by the two techniques of cDNA-mRNA hybridization and unique DNA-mRNA hybridization. For instance, cDNA-mRNA hybridization revealed 28,000 sequences present in adult rat uterus and not in the oestradiol-stimulated immature animals. Even if all of
**FIGURE 27**

Kinetics of hybridization of cDNA derived from adult proestrous uterine polysomal poly(A)+RNA against the poly(A)+RNA of 4h oestradiol-stimulated uterus.

Experimental details are as described in the legend to Figure 24 except that total cDNA at 2000cpm per point was used and each point contained 1mg/ml RNA. % hybridization was assayed by resistance to S1 Nuclease digestion (see Materials & Methods).

---

= kinetics of the reaction driven by the poly(A)+RNA from 4h oestradiol-stimulated uterus

= homologous reaction, reproduced from Figure 24.
the 21\% difference observed in figure 27 corresponded to the rare sequences it could only account for 26,000 sequences.

However, a 78/57 or 73\% homology between these two poly(A)+mRNA populations does not mean that 73\% of their poly(A)+mRNA sequences are common because a given species may exhibit different abundances in different populations. This is best illustrated by a hypothetical example. Consider a message which formed part of the abundant class of the adult poly(A)+mRNA population and formed 5\% of the cDNA copied from that population. That species might be part of the intermediate abundant fraction in the 4 hr oestradiol-stimulated immature uterine poly(A)+mRNA and represent 0.01\% of the population. Nevertheless, because the hybridization was conducted with a vast excess of mRNA, the poly(A)+mRNA representing 0.01\% of the population might still be able to saturate 5\% of the cDNA.

This sort of argument leads one to the conclusion that heterologous experiments of the sort illustrated in figure 27 can say very little about the comparative complexities of the two poly(A)+mRNA population involved. Nevertheless, such experiments can give qualitative indications of changes in mRNA populations and proved particularly valuable in this respect in following hormone-induced population changes in the immature rat (see following section).

2.6.5 Oestrogen-induced changes in the immature uterus of poly(A)+mRNA at differing hormonal stages

Complementary DNA(cDNA) was prepared against poly(A) containing mRNA from the uterii of immature rats which had been treated with oestrogen
Kinetics of Hybridization of oDNA with Homologous and Heterologous poly(A)+mRNA

Hybridization of uterine message oDNA from immature rats receiving 4 hr oestrogen treatment to: - - mRNA from untreated immature rats, ▲▲▲ mRNA from immature rats receiving 2 hr oestrogen treatment, --- mRNA from immature rats receiving 4 hr oestrogen treatment (homologous). Each point contained 2000 cpm \(^3\)H-oDNA and 0.1-\(\mu\)g/ml RNA.
for 4 hr. The dashed curve in figure 28 represents the hybridization
of this cDNA to its template and has already been described (section
2.6.1, figure 24). The remaining curves in figure 28 represent
heterologous hybridization in which the cDNA from rats receiving 4 hours
oestrogen treatment was hybridized to poly(A)+mRNA from untreated rats
and from rats receiving 2 hr hormone treatment.

It is seen that the kinetics of hybridization of the
heterologous reaction were shifted to higher Rot values implying that
common sequences present in the heterologous RNA populations were
present in much lower concentrations. With reservation expressed in
section 2.6.4 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 80%. Polysomal poly(A)+mRNA from
unstimulated animals was able to form hybrids with the cDNA to about
11%. In this case, however, the difference in the final percentage
hybridization could not be ascertained as it was difficult to prepare
sufficient quantities of mRNA from the unstimulated immature animals for
high Rot value determinations. The unstimulated immature rat uterus
had very low levels of mRNA and the ribosomes were mainly present as
monomers (figure 17). Nevertheless, it is clear that the mRNA sequences
present in the oestrogen-stimulated animal made up a low percentage of
the mRNA population of unstimulated immature rat uterus.

Animals receiving oestradiol 2 hours before death contained
uterine mRNA sequences that were able to saturate 56% of the cDNA. This
implied that a high proportion of the 4 hr and 2 hr mRNA population were
common but the slower rate of hybridization, relative to the homologous reaction, showed that the common sequences were present in lower abundance in the animals receiving 2 hours oestrogen treatment.

2.6.6 Preparation of abundant and rare classes of uterine messenger cDNA

The level of hybridization of mRNA to the cDNA probe in figure 28 revealed a 2-fold difference depending on whether the mRNA was prepared from the uteri of rats after 4 hr or only 2 hr oestrogen treatment. It was reasonable to assume that this difference represented mRNA species which were synthesized during the second two hours of the hormonal response and as such they were likely to be of considerable importance in the increased production of new ribosomes initiated at this time.

To investigate this mRNA population further the cDNA derived from uterine polysomal poly(A)+RNA of rats treated for 4 hr with oestrogen was used to prepare fractions enriched in abundant and rare sequences respectively. These fractions were then used in further heterologous hybridization. The cDNA representing abundant and rare sequences of the 4 hours oestradiol-stimulated uterine mRNA population were isolated and purified by hydroxyapatite chromatography. The HAP was initially characterized with respect to its binding capacity and the phosphate buffer concentration effective in eluting single-stranded and double-stranded nucleic acids. Figure 29 shows that nearly all single-stranded material was eluted with 0.14M sodium phosphate buffer pH 6.8 and the double-stranded material was effectively eluted with
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 \(^3^H\)-labelled nucleic acids (see Materials and Methods) mixture were chromatographed on HAP columns at 60°. The nucleic acids were eluted in a stepwise fashion with increasing concentrations of sodium phosphate buffer as indicated. 1 ml fractions were collected, aliquots removed and radioactivity in each fraction assayed as described in the Materials and Methods section.

A = Chromatographic fractions of 0.14M sodium phosphate buffer

B = Chromatographic fractions of 0.4M sodium phosphate buffer containing double-stranded nucleic acid.
A  250
0.4 M
3000
2000
1000

0.14 M
0.3 M

\[ { }^{3} \text{H} \text{ Radioactivity cpm} \]

Fraction Number
0.4M sodium phosphate buffer pH 6.8. The recovered radioactivity in each fraction was in excess of 90%. The fact that very low radioactivity was recovered in the wash fraction indicates that degradation during the chromatography could be ruled out.

2.6.7 **Oestrogen-induced changes in uterine abundant mRNA sequences**

The cDNA representing the predominantly abundant sequences of the 4 hours oestradiol-stimulated mRNA populations were recovered by prior hybridization with an excess of its template to a Rot of 1.0 (moles sec. litre⁻¹). The double-stranded material was isolated by HAP chromatography and further purified as described in the Methods section. 36% of the cDNA was recovered as hybrid, a figure coinciding well with 38% hybrid formation at Rot 1.0 in figure 24. The results plotted in figure 30 show the hybridization of this abundant cDNA to uterine poly(A)+mRNA derived from adult rats and from immature rats receiving oestradiol-17β 2 hours or 4 hr before death.

The homologous reaction, employing polysomal poly(A)+RNA from 4 hr treated rats, was almost complete by a Rot of 0.1 (moles sec. litre⁻¹) and achieved a maximum of 70% hybridization. The corrected Rot₂ for this reaction was 0.051 (Table 10) a figure which agreed well with the corrected Rot for the most abundant class in the homologous reaction of the total cDNA with its own template (table 8). This suggested that there was little heterogeneity of poly(A)+mRNA concentrations in the highly abundant class. The kinetics of this hybridization covered a range 2.5 log Rot units, which was broader than the 1.5 units expected for a pseudo-first order reaction. This was probably due to
FIGURE 30

Kinetics of hybridization of abundant cDNA sequences with homologous and heterologous poly(A)+mRNA

Abundant uterine message cDNA sequences transcribed from immature rats receiving 4hr oestrogen treatment was isolated and purified by HAP Chromatography as described in the Methods section. The abundant cDNA sequences was hybridized in excess of uterine poly(A)+mRNA from ● ● ● adult rats, ▲▲▲ immature rats receiving 2hr oestrogen treatment, ○ ○ ○ immature rats receiving 4hr oestrogen treatment. Each point contained 2000 cpm $^3$H-cDNA and 10μg/ml RNA.
### TABLE 10

Characterization of the abundant cDNA sequences

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Abundant cDNA-mRNA hybridization</th>
<th>Total cDNA-mRNA hybridization (abundant component)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Hybridization</td>
<td>70</td>
<td>28</td>
</tr>
<tr>
<td>Observed Rot $\frac{1}{2}$</td>
<td>0.07</td>
<td>0.17</td>
</tr>
<tr>
<td>Corrected Rot $\frac{1}{2}$</td>
<td>0.051</td>
<td>0.048</td>
</tr>
</tbody>
</table>

The cDNA used was complementary to polysomal poly(A)+mRNA from immature rats which had received oestradiol-17β 4 hr before death. It was hybridized to its own template. The abundant fraction was isolated as described in the experimental section and the data is derived by a computer analysis of the kinetics of Fig. 30. The data on the abundant component of the total cDNA is reproduced from table 8.
some variation in the sizes of the reactable cDNA. Previous work has indicated that larger cDNA transcripts hybridize faster than small transcripts and hence the reaction may cover a broader range than the 1.5 log units observed under ideal conditions (Young et al. 1974; Getz et al. 1975).

There was considerable sequence homology between the most abundant mRNA sequences of the template from 4 hr oestrogen-treated rat uteri and poly(A)+mRNA from 2 hr stimulated animals (figure 30). However, the 2 hr poly(A)+mRNA could not fully saturate the 4 hr abundant cDNA implying that the abundant sequences did have slightly different abundances in the two mRNA populations. For much of the curve, the 2 hr poly(A)+mRNA hybridized faster than the 4 hr preparation indicating that some sequences were more abundant at the earlier time.

Also illustrated in figure 30 is a heterologous hybridization between adult poly(A)+mRNA and the abundant cDNA. The slow rate of hybridization observed indicated that the adult mRNA had a low concentration of sequences common to the abundant mRNA of uteri at the earlier stage in oestrogen-induced differentiation. This was in agreement with previous findings (figure 27) indicating that adult uterus had a more restricted population of abundant sequences.

It has been suggested by many workers that the abundant mRNA species, being those which code for the most predominant proteins are responsible for the phenotype of the cell. It is therefore not surprising that progression from the relatively undifferentiated state to the adult stage involved changes in the common abundant mRNA sequences.

2.6.3 Oestrogen-induced changes in uterine rare mRNA sequences
Oestrogen-induced changes in uterine rare mRNA sequences

cDNA derived from 4 hours oestradiol-stimulated uterine poly(A)+mRNA and predominantly complementary to the rare sequences was fractionated by preliminary hybridization with a vast excess of its own template to a Rot of 12 (moles sec. litre\(^{-1}\)) followed by separation of the hybridized and unhybridized fractions by HAP chromatography. The unhybridized single-stranded cDNA eluted by 0.14M sodium phosphate buffer constituted the rare cDNA sequences. Figure 31 shows the hybridization of the rare cDNA sequences to its template and to the 2 hours oestradiol-stimulated immature uterine poly(A)+mRNA. 78% of the \(^3\)H-cDNA hybridized to homologous RNA. The kinetics of this hybridization reaction extend over 3 log units indicating that the fractionated cDNA comprised more than one kinetic component. This was likely to be caused by some contamination by the intermediate abundant cDNA and this conclusion was supported by the curve itself which showed a slight transition at low Rot values and a considerable percentage hybridization at Rot values below Rot 12. The heterologous hybridization, in which 2 hr oestrogen-stimulated uterine mRNA was employed, showed no such inflection at low Rot values. There were therefore few sequences in the 2 hr mRNA preparation which were complementary to these cDNA components of intermediate abundance. The rarest sequences showed considerably more homology between the two mRNA populations though the heterologous reaction was much slower indicating that sequences common to both were in much lower abundance in the animals stimulated for the shorter time.

This data and the results for the hybridization of the
FIGURE 31

Kinetics of hybridization of rare cDNA with homologous and heterologous poly(A)+mRNA

Preparation and purification of the rare cDNA sequences are described in the Methods section. The rare uterine message cDNA from immature rats receiving 4 hr oestrogen treatment was hybridized to excess uterine poly(A)+mRNA from immature rats receiving 2 hr oestrogen treatment, immature rats receiving 4 hr oestrogen treatment. Each point contained 2000 cpm $^3$H-cDNA and 1 mg/ml RNA.
abundant class cDNA (figure 30) indicated that the original differences observed in the level of hybridizability between the homologous 4 hr mRNA-cDNA hybridization and the heterologous 2 hr mRNA-4hr cDNA reaction (figure 28) were likely to reside primarily in the intermediate abundant class.

2.6.9 mRNA sequences synthesized between 2 hours and 4 hours after oestradiol administration

In an attempt to confirm that the most profound differences in the mRNA population isolated from immature rats 2 hr and 4 hr after oestradiol-17β administration were in the sequences of intermediate abundance the following experiment was performed. The cDNA to 4 hr oestradiol-stimulated rat uterine mRNA was hybridized to a Rot of 2000 (Moles sec⁻¹ litre⁻¹) against poly(A)+mRNA from 2 hr oestradiol-stimulated animals. The cDNA which did not form hybrids under these conditions, which should represent the mRNA species present at 4 hr but not at 2 hr after hormone treatment were isolated by HAP chromatography and purified as described in the Methods section. They were then back-hybridized to their own template; namely uterine mRNA from 4 hr oestrogen-stimulated animals.

Figure 32 shows a computer generated curve of this hybridization and compares it with a homologous hybridization using total mRNA (hatched line). It is seen that the unhybridized cDNA contained sequences complementary to abundant, intermediate abundant and rare mRNA species. Table 11 summarizes the computer-fitted numerical interpretation of the hybridization curve and expresses the data as abundant, intermediate
Hybridizable uterine poly(A)+mRNA present 4 hr but not 2 hr after oestradiol stimulation of immature rats.

Uterine mRNA from rats after 4 hr oestradiol treatment was hybridized to ——— homologous cDNA or ——— homologous cDNA from which the sequences complementary to uterine mRNA from 2 hr oestradiol stimulated rats had been removed as described in the Methods section. Each point contained 2000 cpm $^3\text{H}$-cDNA and 1 mg/ml RNA.
TABLE 11

COMPLEXITY OF mRNA CALCULATED FROM HYBRIDIZATION DATA OF FIGURE 32.

The data above represents computer analysis of the hybridization kinetics in figure 32.

The cDNA used represented the unhybridized fraction of the heterologous hybridization between the 4 h oestradiol stimulated uterine message cDNA with the 2 h oestradiol stimulated mRNA (figure 28). The unhybridized cDNA was isolated and subsequently used to rehybridize its own template under conditions as described in the legend to figure 24.

a: denotes the value of Rot$_{2}$ corrected as though the components were analysed as pure.

b: taking the complexity of the mouse globin (hybridization standard) as $4 \times 10^5$ daltons and a Rot$_{2}$ of $4 \times 10^{-3}$ moles sec. liter$^{-1}$ (Williamson et al., 1971 and Birnie et al., 1974). The number average molecular weight of the uterine polysomal poly(A)$^+$RNA is $6 \times 10^5$ (table 7).
<table>
<thead>
<tr>
<th>Source of uterine mRNA</th>
<th>Transition of mRNA species present at 4 h but not at 2 h after oestradiol-17β administration</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corrected Rot$_1^a$ (moles sec. liter$^{-1}$)</td>
<td>0.008</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Corrected Rot$_2^a$ (moles sec. liter$^{-1}$)</td>
<td>0.81</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Number of diverse sequences</td>
<td>91</td>
<td>2533</td>
</tr>
<tr>
<td></td>
<td>% hybridizable DNA</td>
<td>16.4</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>Observed Rot$_1^b$ (moles sec. liter$^{-1}$)</td>
<td>0.0485</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Observed Rot$_2^b$ (moles sec. liter$^{-1}$)</td>
<td>1.5</td>
<td>151</td>
</tr>
</tbody>
</table>

Complexity of mRNA calculated from hybridization data.
abundant, and rare sequences. The mRNA present at 4 hr but not at 2 hr after hormone administration contained representatives of all abundance classes but was considerably enriched in sequences of intermediate abundance. In fact, the number of sequences in the class was 151 and the corrected Rot for this kinetic component was in very close agreement to that estimated in the homologous hybridization with the total mRNA-cDNA populations (table 8). Conversely, of the 9 or so abundant sequences and 7800 rare sequences found in the total poly(A)+ mRNA population of the 4 hour oestrogen-stimulated uterus (table 8), there were only 1–2 and 2500 sequences respectively which were not present 2 hr after oestradiol-17β administration.

It should be noted that approximately 20% of the 4 hr message cDNA never forms hybrids. The cDNA fraction isolated above would be enriched in this element thus explaining why the hybridization only went to 50%.

3. Interrelationship between uterine HnRNA and polysomal poly(A)+mRNA

3.1 Hybridization of abundant cDNA sequences towards poly(A)+HnRNA

Results thus far obtained have revealed that the earliest response, detected within 30 min of oestradiol treatment to immature rats, was a stimulation in the rate of synthesis of uterine HnRNA (figure 15). This early response was reflected most noticeably in the poly(A)+HnRNA (figure 15). It was also noted that the synthesis
of the abundant mRNA species reflected an early response to oestradiol-17β treatment (figure 30). Some of the abundant mRNA sequences of the 4 hr oestradiol-stimulated uterine poly(A)+mRNA population were also shown to be complementary to mRNA sequences synthesised after 2 hr of hormone treatment and appeared to be in greater abundance at earlier times of hormone treatment. Thus, by inference, it was expected that the abundant sequence fraction was also abundant in the oestradiol-stimulated poly(A)+HnRNA population.

To investigate this possible relationship, the abundant cDNA sequences, derived from the 4 hr oestradiol-stimulated poly(A)+mRNA population, was isolated and purified as described in the methods section. It was then used to probe for complementary sequences in poly(A)+HnRNA synthesised at 30 min and 2 hr after oestradiol treatment. Figure 33 shows the kinetics of hybridization of the abundant cDNA driven by uterine poly(A)+HnRNA purified from high molecular weight HnRNA of 30 min oestradiol-stimulated immature rat uterus. Initially, the RNA was purified from high molecular weight HnRNA recovered from aqueous gradients (figure 6) and only denatured in 90% formamide at 90°C for 5 min immediately prior to hybridization. However, in a repeat experiment the poly(A)+HnRNA was purified from HnRNA which had been sedimented through denaturing sucrose gradients containing 87% formamide. The kinetics of hybridization is also shown in figure 33.

The figure clearly shows that the poly(A)+HnRNA synthesized after 30 min of oestradiol treatment did indeed contain mRNA sequences homologous to the abundant mRNA species synthesized after 4 hr of oestradiol treatment. By comparison to the homologous reaction, the hybridization with poly(A)+HnRNA occurred to the same extent but was
Abundant cDNA, isolated and purified as described in the Materials and Methods section, was hybridized with poly(A)+HnRNA of uterus from rats treated with oestradiol-17β 30 min before death. Hybridization was assayed by resistance to S1 Nuclease digestion. Each point contained 2000 cpm $^{3}$H-cDNA and 1 mg/ml RNA.

- - - = hybridization of the abundant cDNA to its own template (4h oestradiol-stimulated poly(A)+RNA) and reproduced from figure 30.

- - - - = heterologous hybridization to formamide-denatured poly(A)+HnRNA purified from HnRNA sedimented through non-denaturing gradients (see figure 6).

▲▲▲ = points containing poly(A)+HnRNA recovered from HnRNA sedimented through denaturing gradients as described by McNaughton et al. (1974) (see Materials and Methods section).
FIGURE 34

Hybridization of oDNA complementary to abundant poly(A)+mRNA of the 4h oestradiol-stimulated uteri against high-molecular-weight poly(A)+HnRNA of uteri from rats stimulated with oestradiol-17β 2 hr before death.

Experimental details are as described in the legends to figure 36.

--- = homologous reaction of the abundant oDNA to its own template (4h poly(A)+RNA) and reproduced from figure 30.

--- = formamide-denatured poly(A)+HnRNA purified from HnRNA sedimented through non-denaturing gradients (see figure 6).  

●●● = points containing poly(A)+HnRNA recovered from HnRNA sedimented through denaturing gradients as described by McNaughton et al. (1974) (see Materials and Methods section).
shifted to higher Rot values and the reaction produced a steeper hybridization curve than the curve seen with total poly(A)+mRNA. This indicated that the abundant mRNA sequences in poly(A)+HnRNA had a narrower frequency range than those present in polysomes.

Figure 34 illustrates an identical experiment to that in figure 33 except that the poly(A)+HnRNA was derived from rats which received oestradiol 2 hr before death. There was a striking difference in the Rot values derived from the cDNA-HnRNA curves which were dependent on whether the HnRNA came from rats stimulated with oestrogen for 30 min or 2 hr. The former has a Rot value of 1.5 moles sec. litre\(^{-1}\) while the latter was 60 moles sec. litre\(^{-1}\). This clearly indicated that the 4 hr oestradiol-stimulated abundant mRNA sequence content was several fold greater in the poly(A)+HnRNA population synthesized at 2 hr after oestradiol treatment. The messenger sequence content in the poly(A)+HnRNA at the two hormonal states could be calculated from the shift of the Rot values obtained as compared to the corrected Rot value (0.051 moles sec. litre\(^{-1}\)) obtained with the homologous mRNA-abundant cDNA hybridization kinetics. Thus, the abundant mRNA sequence content in poly(A)+HnRNA derived from 30 min and 2 hr oestradiol-stimulated uteri was 0.08% and 3.4% respectively. These values are in agreement with the concept that only a small fraction of the HnRNA is destined to serve as mRNA precursor molecules.
DISCUSSION
DISCUSSION

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 (for review see Gorski & Gannon, 1976; Yamamoto & Alberts, 1976). Oestrogens exert their effects in this way on a number of target tissues. In some avian and amphibian tissues, transcriptional responses lead to the production of specific egg protein mRNAs. Thus, as a result of oestrogen stimulation, the hen oviduct synthesizes the mRNAs coding for the egg-white proteins while the livers of both amphibians and birds respond to the hormone by inducing production of the mRNAs for yolk protein precursors (O'Malley et al. 1975; Tata, 1976).

In the mammalian uterus, oestrogens serve a somewhat different function. Here, they initiate a chain of events which leads to overall growth of the tissue and its preparation for the implantation of a fertilised egg. Evidence collected in this and other laboratories shows that, early on in this process oestrogen elicits a dramatic stimulation of the synthesis of heterogenous nuclear RNA (HnRNA). Subsequently, mRNA, which is presumably derived from the HnRNA, accumulates in the cytoplasm and brings about the aggregation of pre-existing ribosomes into polysomes (Figure 17), (Teng & Hamilton, 1967; Merryweather & Knowler, 1977). This early response and the translation of the mRNA into a small number of ill-characterized proteins (Garland, et al. 1978) appears to be a prerequisite for the subsequent striking stimulation in rRNA synthesis, the accumulation of new ribosomes and the subsequent uterine hypertrophy and hyperplasia (Knowler & Smellie, 1971; Borthwick & Smellie, 1975).
The work described in this thesis was designed to further elucidate the role in oestrogen-induced growth of the uterus of

a) Early HnRNA synthesis

b) Changes in mRNA populations, and

c) The relationship of the HnRNA synthesis to mRNA accumulation.

A. Early HnRNA synthesis in oestrogen-stimulated uteri

The earliest response to oestradiol treatment, detected in immature uterus, in the present work was the incorporation of precursor into an RNA of very high molecular weight (figure 14), which parallels the observation made by Knowler & Smellie, (1971). Previous studies on the behaviour of this RNA species showed that it was rapidly synthesized, had a short half-life, lacked methylated bases and had a base composition low in G+C and high A+U (Knowler & Smellie, 1973). These findings supported the conclusion that the material was heterogeneous nuclear RNA and further studies were undertaken to investigate its chemical properties and whether the increased synthesis involved discrete species of this RNA type. Simultaneously with these studies, evidence has been accumulating that HnRNA is a precursor to mRNA species (see Introduction) and that the polyadenylated HnRNA contains substantial complementary mRNA sequences (Lewin, 1975b, c). The study of HnRNA synthesis, in a system in which it can be separated from RNA species of lower molecular weight may provide a way in which the synthesis of HnRNA and its subsequent maturation into mRNA can be studied. In the immature uterus, the process can also be studied in relation to hormone-induced differentiation.
The purification of uterine HnRNA was difficult. It was desirable when preparing a nuclear RNA species to start with purified nuclei but the smooth muscle of the uterus is refractory to the preparation of good nuclei and even the citric acid method (Knowler & Smellie, 1973) did not completely protect the nuclear RNA species from endogenous ribonucleases. Furthermore, the nuclear RNA was always contaminated with cytoplasmic species. The use of metabolic inhibitors provided an alternative and it was observed that the oestradiol-stimulated synthesis of rRNA was more susceptible to actinomycin D inhibition than the synthesis of HnRNA. However, at a higher dose of the inhibitor, although the synthesis of rRNA was almost totally eliminated, the synthesis of HnRNA was invariably affected by the inhibitor (figure 5). Differential extraction methods such as those of Georgiev, (1967) did not produce good results with the uterus.

Because of the above difficulties, HnRNA was purified by virtue of the large size of a proportion of the molecules. Only HnRNA sedimenting at greater than 45S on aqueous sucrose density gradients was isolated. Under denaturing conditions, some of this material exhibited lower molecular weights but most still sedimented at greater than 35S (figures 8 & 11).

It would be of immense value were it to prove possible to isolate the lower molecular weight species of uterine HnRNA. Experiments which measured both the ultraviolet inactivation of HnRNA transcription (Derman et al. 1976) and the effect of DRB(5,6-dichloro-1-B-D-ribofuranosylbenzimidazole), a drug which apparently blocks chain initiation on HnRNA synthesis (Seghal et al. 1976a) indicated that relatively short HnRNA chains were probably not degradation products of
larger chains. The results suggested that many HnRNA molecules containing mRNA sequences may be directly transcribed as molecules about 5000 bases in length. This conclusion was supported by experiments, which demonstrated that the majority of the poly(A)+HnRNA molecules in Ehrlich ascites cells were approximately 28S and were considered primary transcripts because they contained a tetraphosphate (Schiminoke et al. 1976). The possibility exists, therefore, that the HnRNA isolated in the present work had little relationship to the species of lower molecular weight RNA.

The high molecular weight HnRNA was fractionated on poly(U)-Sepharose columns into fractions which differ in their poly(A) content and their size profile on polyacrylamide gels (figures 9, 10). Oestrogen treatment of the rats stimulates the synthesis of all three fractions of HnRNA but the kinetics of synthesis, degree of stimulation and size distribution of the newly synthesized RNA differs in each fraction. An interesting observation is the stimulated synthesis of polyadenylated HnRNA (figure 15). This fraction responds most rapidly to oestradiol treatment of immature rats, its synthesis being stimulated almost 3 fold within 30 min of hormone administration. This finding indicated the possible simultaneous synthesis of mRNA, the translation of which results in proteins that regulate the transcription of rDNA. Therefore, it became imperative to investigate the relationship of HnRNA to mRNA in the rat uterus during oestradiol stimulation and the effect of the hormone on the synthesis of uterine mRNA.

B. Changes in mRNA population in oestrogen-stimulated uteri
Changes in mRNA population in oestrogen-stimulated uteri

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 & Church, 1971; Gelderman et al. 1971; Hahn & Laird, 1971; Grouse et al. 1972; Galau et al. 1974, 1976) or the analysis of hybridization kinetics between cDNA and its template poly(A)+mRNA (Birnie et al. 1974; Bishop et al. 1974), the resulting base sequence complexity and abundance classes of the mRNA population can be estimated. Such determinations have begun to furnish information on the extent of genomic DNA expressed in eukaryotic cells.

The effect of oestrogen on mRNA complexity was first investigated by Monahan et al. (1976). Their results indicated that poly(A)+RNA from oviducts of egg-laying hens and diethylstilbestrol-stimulated chicks contained 20,000-25,000 different RNA sequences present in three classes of different abundance ranging from fewer than 3 to many thousand copies of each sequence per cell. In contrast, there were only 10,000 different sequences in the oviducts withdrawn from oestrogen treatment. In a similar study, I have initiated an investigation of the extent of gene transcription in the rat uterus. Polysomal location has been taken as a criterion for active mRNA. Since the non-polyadenylated mRNA is a small proportion by mass of the polysomal RNA, the poly(A)+mRNA can be considered as representative of the mRNA population. Two time points in the growth and development of the uterus have been studied. Adult rat uterus, taken from rats at proestrus when circulating oestrogens are maximal, was taken to
represent the fully developed tissue. Uteri from immature rats responding to 4 hr oestrogen treatment were taken as tissue at an early stage in oestrogen-induced development when the hormone has stimulated an aggregation of ribosomes into polysomes (Figure 17; Merryweather & Knowler, 1977) and the early oestrogen-induced proteins are accumulating (Galand et al. 1978).

The preparation of uterine polysomes included treatment with tritonX-100 to release any membrane-bound polysomes, which are thought to have a different function from free polysomes (Tata, 1972). The preparation also included cycloheximide which prevents extensive run-off of ribosomes from mRNA. In the presence of RNase inhibitors (heparin and diethyl pyrocarbonate), the polysome profiles (Figure 17) exhibited a number of $E_{260}$ absorption peaks with sizes exceeding 100S, thus it is assumed that the preparation constituted undegraded polysome population. It must be emphasized that this study concerns only the complexity of polyadenylated RNA.

Complementary DNA 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 cDNA were on average 450 bases long (Figure 22) whether the template was globin or the uterine poly(A)+mRNA which were, on average three times longer than globin mRNA (Figure 18). The cDNAs prepared were, however, of sufficient length for the hybridization studies described and were of high specific activity. The more recently published methods producing long cDNAs suitable for cloning require levels of template mRNA which were not practical when working
with the immature rat uterus and producing long cDNAs tend to have lower specific activities.

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

Analysis of cDNA hybridization to homologous poly(A)+RNA from rat uterus suggest that there are 36,000 sequences of 600,000 average molecular size mRNA expressed in the adult uterus at proestrous (Table 8). This value was unexpectedly high, falling within the range of the most active of the mammalian tissues and cells in culture (Bishop et al. 1974; Savage et al. 1978; Kleiman et al. 1977; Chikaraishi et al. 1978). However, estimations of diversity and complexity by this method are known to suffer from the drawback that the unique sequences are most refractory to analysis by cDNA hybridization assay and their complexity is likely to be under-estimated (Campo & Bishop 1974; Weiss et al. 1976; Ryffel & McCarthy, 1976; Young et al. 1976; Ryffel, 1976). 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 the least abundant mRNA species. Table 9 shows that when uterine mRNA from rats at proestrous is assayed
by this method, 53,000 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 & Bishop, 1976;
Ryffel & McCarthy, 1975; Young et al. 1976), in Friend erythroleukemic
cells (Kleiman et al. 1977) and in rat liver (Sippel et al. 1977a) have
invariably resulted in lower values when 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. Previous workers have
circumvented this difficulty by carrying out a low salt RNAse treatment
of the mixture of RNA-DNA and DNA-DNA hybrids followed by hydroxyapatite
chromatography to obtain an estimate of the amount of RNA-DNA hybrids
(Kleiman et al. 1977; Galau et al. 1974, 1976). By the technique used
in these studies this step was not necessary since only mercurated
mRNA-DNA hybrids are retained on the thiol-sepharose. Furthermore, the
percentage of hybrids at very low Rot values (< 10) is less than 0.08%
(fig 26) suggesting that contamination with repetitive sequences is
minimal. The specificity of the reaction was confirmed when unique
3H-labelled DNA was hybridized with mercurated E. coli rRNA and no
significant hybridization above background levels was observed (fig 26).

One source of error in mRNA complexity determinations is
contamination of mRNA preparations with nuclear RNA. Contamination
of mRNA from uterine tissues in this manner is ruled out by the finding
that nuclear RNA labelled by short pulses of tritiated precursors does
not appear in the purified product (see also fig 3). Furthermore,
saturation hybridization taken over Rot values higher than 10,000 moles sec. litre$^{-1}$ did not show a transition for nuclear RNA.

It is clear that the adult uterus transcribes several fold more mRNA sequences than immature animals responding to a short pulse of oestradiol. In the light of these results, it would be desirable to also investigate the unstimulated immature rat uterus. However the fact that, in these animals, uterine ribosomes are virtually all monosomes (figure 17) and mRNA levels are very low, make such a study technically extremely difficult. Similar difficulties accompany mRNA preparations from uteri of overiectomized adult rats where polysomes rapidly disintegrate and mRNA levels fall. However, I have succeeded in preparing sufficient unstimulated immature rat uterine mRNA to conduct heterologous hybridization against cDNA from stimulated animals.

Such heterologous hybridizations were conducted between cDNA and mRNA preparations representative of the uterine poly(A)+mRNA population at various stages in hormone-induced development. They reveal that, in the uterus responding to stimulation by oestrogen, changes are observed in the concentration of existing mRNA species and in the complexity of the population. Changes in the concentration of uterine mRNAs are apparent during early times of oestrogen stimulation and in fully developed tissue. Figure 28 reveals that, in general, sequences common to rats receiving 2 or 4 hr hormone stimulation are less abundant in the animals receiving the shorter treatment. Conversely, figure 30 shows that some of the abundant sequences may be more abundant at the earlier time. These findings mirror the observations on differences between the uterine mRNA population of adult rats and that of immature rats treated with oestrogen for 4 hr (figure 24). Here
again the mRNA population of the more differentiated uteri was
most complex but the uteri at an earlier stage of hormone-induced
development had relatively larger amounts of abundant sequences.

Changes in complexity of uterine mRNAs are profound.
Poly(A)+RNA from the uteri of unstimulated immature rats has so
little in common with that 4 hr after oestrogen treatment to the
rats, that there is very little complementarity between the two.
It must be concluded that of the 8000–12,000 sequences expressed
in the 4 hr oestrogen stimulated rat uterus (Table 8) very few are
expressed to the same extent in the unstimulated animal. Since these
profound changes are known to be associated with a striking stimulation
in the synthesis of HmRNA (figures 14, 15 & 16) and the appearance in
the cytoplasm of newly made mRNA, it would appear that a large number
of uterine genes are switched on by oestrogen or activated to allow a
much faster rate of transcription. For many years, workers studying
oestradiol binding in the uterine nucleus have been puzzled by the
large number of apparent nuclear receptor binding sites. It would now
seem possible that the large number of sites could be associated with
the hormone effect on a large number of gene loci.

A recent investigation by Markaverich et al. (1978) demonstrated
that oestradiol more than doubled the number of initiation sites for
E. coli RNA polymerase in immature rat uterine chromatin over a 12 hr
period. Such studies tend to support the concept of a large increase
in the number of genes transcribed though they must be subject to
reservation over the validity of using a bacterial enzyme to transcribe
a mammalian genome.
It is also worthy of note that the changes in the mRNA population occur over a prolonged period of time. Thus, only some of the complexity changes seen by 4 hr of oestrogen stimulation have occurred by 2 hr (figure 28). Furthermore, the changes are far from complete at 4 hr, indeed the population of the uterine mRNA of adult uterus is several times as complex. While some of the mRNA population of the mature uterus may be induced by other factors, such as progesterone, it seems likely that oestrogen will be responsible for some of these later developments.

It is of interest to speculate what changes in uterine protein synthesis are brought by the observed changes in mRNA synthesis.

The induction of specific proteins, for example ovalbumin, conalbumin in oviduct and vitellogenin and transferrin synthesis in avian and amphibian liver, is coincidental with the stimulated synthesis and accumulation of their respective mRNAs (Green & Tata, 1976; Wahli et al. 1976; Ryffel et al. 1977; Hynes et al. 1977; Palmiter et al. 1976; Lee et al. 1978; McKnight, 1978). This indicates clearly that oestrogen action on protein synthesis is based on their differential effect on gene transcription and is mediated at the level of mRNA production. Other mRNAs in the chick oviduct, present only in very low number of copies, are also stimulated to several thousand fold over control values (Axel et al. 1976). These species may include lysozyme and ovumucoid mRNAs (Hynes et al. 1977).

It seems likely that most of the changes in uterine mRNA populations observed in the results section concern proteins of low abundance, the stimulated synthesis of which would be difficult to
detect. Nevertheless, it is likely that many of them are of considerable importance in the hormone-induced growth of the tissue.

From the point of view of studying these oestrogen-induced changes in protein synthesis, perhaps the most interesting finding in the above results concerned the differences in the uterine mRNA populations between rats receiving oestradiol-17β 2 hr before death and those with 4 hr treatment. Such comparison revealed differences in complexity across the range of a Rot analysis and a general finding that the sequences common to both population were most abundant after the longer hormone treatment. The most striking differences in the two mRNA populations, however, was found in the sequences of intermediate abundance. This finding does not so far have a parallel in any other hormonal system where the most profound changes hitherto observed have been in the most abundant sequences (Hynes et al., 1977; Parker & Mainwaring, 1977; Parker & Scrase, 1978). However, the uterus is not known to synthesize major new proteins in response to oestrogen. Instead one sees an overall growth process which may require moderate quantities of a group of proteins and necessitate the appearance of their mRNAs in the immediate abundance class. Ribosomal proteins may well fall into this category and indeed it is between 2 and 4 hr after oestradiol administration to immature rats that the synthesis of uterine ribosomal proteins begins to increase (Merryweather & Knowler, unpublished observations; Means & Hamilton 1966a, b; Moore & Hamilton, 1964; Greenman & Kenny, 1964; Teng & Hamilton 1967a, b). At this time also the synthesis of uterine HmRNA is peaking at ten times control levels (Knowler & Smellie, 1973; see also figures 16 & 17) and can be
extracted as HnRNP particles (Knowler, 1976). Clearly, the need for proteins associated with these particles may also require a relative abundance of the mRNAs coding for them.

Evidence has accumulated over the last decade for an involvement of non-histone proteins in a number of differentiating systems including those responding to hormone stimulation (O'Malley et al. 1977). It may well be that the stimulation of uterine rRNA synthesis by oestrogen is dependent on protein synthesis because specific non histone proteins require to be synthesized and to associate with rRNA. Such proteins or a protein would be likely candidates for the species of intermediate abundance. Finally, it is known that in the first hours of oestrogen action in the uterus, the receptor protein has to be replenished (Clark et al. 1978). This may also necessitate fairly large quantities of mRNA.

The evaluation of the nature and function of the proteins synthesized in the immature rat uterus during the first 4 hours of oestrogen action are the subject of continuing investigation in this laboratory.

C. The relationship of HnRNA synthesis to mRNA accumulation in the oestrogen-stimulated uteri

The long term aim of the study initiated in this thesis was a study of the relationship between the effects of oestrogen on the three HnRNA fractions characterized in section A and the oestrogen-induced changes in the uterine mRNA population described in section B. In this study it was planned to hybridize the three fractions of HnRNA against
cDNA transcribed from total mRNA.

Such a study has been initiated but has proved difficult. Great care has been taken to avoid contamination of the HnRNA. This has involved isolating only very-high-molecular-weight species of HnRNA and then retaining only those portions which retain their high-molecular-weight characteristics on denaturing sucrose gradients. These precautions have resulted in low yields of the three HnRNA fractions which have been inadequate to completely saturate the cDNA for the completion of Rot curves against the entire mRNA population. However, yields have been adequate for an analysis of the hybridization of HnRNA to abundant mRNA sequences and such study was initiated with the most interesting HnRNA fraction namely that containing poly(A). It was found that for all species of abundant mRNA sequences, there exist large poly(A)+HnRNA transcripts which contain their sequences. Since the group of abundant mRNAs is only a limited fraction of the approximately 8000–12,000 different oestradiol-stimulated immature rat uterine polysomal poly(A)+mRNA and the unknown number of non poly(A) containing mRNA, this finding cannot be generalised for all mRNA sequences in the cell.

The finding that more poly(A)+HnRNA was needed to saturate the cDNA to the same extent as its homologous mRNA, expresses the degree of dilution of nuclear messenger sequences by other non-messenger poly(A)+HnRNA. This finding is in agreement with the concept that not all poly(A)+HnRNA can serve as precursors to mRNA. However, since the plateau of hybridization with the poly(A)+HnRNA was similar to that of the homologous reaction, it is reasonable to assume that all of the
mRNA species in the abundant mRNA component were represented in the poly(A)+HnRNA. This was the case whether the HnRNA was derived from rats treated with oestradiol for 30 min or 2 hr. In addition to this qualitative detection of messenger sequences in the nuclear RNA, it was possible to determine their quantitative distribution from the difference in hybridization rates relative to the homologous reaction. Thus, the 30 min oestradiol-stimulated uterine poly(A)+HnRNA contains 0.08% and the 2 hr oestradiol-stimulated uterine poly(A)+HnRNA 3.4% messenger sequence material. This finding indicates that the relative messenger sequence content increases steadily as time of hormonal stimulation is increased. It is noteworthy, however, that the present study is limited only to poly(A)+HnRNA of a size ranging between 40S-30S in denaturing sucrose gradients (figure 11). It is possible that smaller poly(A)+HnRNA may contain a substantially higher messenger sequence content as has been found in the rat liver nuclear RNA (Sippel et al. 1977b).

Nevertheless, at least 2% of the mass of the examined nuclear messenger sequences of rat liver poly(A)+mRNA is in RNA molecules of a size between 37S and 44S (Sippel et al. 1977b). Furthermore, recent findings have indicated that the ovalbumin mRNA nuclear precursors are on the average 40S in size (Roop et al. 1978). In addition to the above findings, electron micrographs of transcriptionally active non-ribosomal chromatin from amphibian lampbrush chromosomes, (Hamkalo & Miller, 1973) and from the embryo of the milk-weed bug, (Foe et al. 1976) show specific sites for initiation and termination of transcription and suggest rather long primary RNA transcript for at least some structural genes.
The non-polyadenylated HnRNA species may also contain a high messenger sequence content. That this is indeed the case has been demonstrated for rat liver poly(A)+mRNA (Sippel et al. 1977b); globin mRNA (Ross, 1976; Curtis & Weissman, 1976; Kinniburgh & Martin, 1976) and mRNA of the mouse L cells (Hames & Perry, 1977).

Based on the findings reported in this thesis, further studies would conduct a similar analysis with the other HnRNA fractions and examine the possibility that all the three HnRNA fractions contain similar sequences by investigating whether they would compete with each other during hybridization to cDNA.
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