STUDIES ON TRANSCRIPTION IN CULTURED RAT MYOBLASTS

by

MARIA SAVERIA CAMPO

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**Acknowledgements**

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SUMMARY

An analysis of several fractions of cellular RNA was performed on cultured rat myoblasts. The study was carried out by means of kinetics of synthesis and decay of the RNA molecules, and by means of DNA-RNA hybridization in DNA excess.

A low molecular weight RNA was isolated from the nucleus and the cytoplasm of rat myoblasts. The RNA has the same base composition and finger-printing pattern of tRNA, but its molecular weight is lower than tRNA. The possible origin and functional role of this RNA fraction are discussed.

It was found that HnRNA is transcribed at a rate of about ten nucleotides per second. The whole population of HnRNA is made up of two classes of RNA with different turnover. One class has a half-life of 30 minutes, and the other has a half-life of 3-4 hours. During a chase experiment, no accumulation of light fractions of HnRNA could be detected, suggesting that giant HnRNA is not cleaved to smaller molecules.

HnRNA greater than 90S contains sequences transcribed from DNA repeated about 300 times, and sequences transcribed from unique DNA. The amount of RNA sequences complementary to repetitious DNA decreases in lighter fractions of HnRNA (40S-80S), and is virtually zero in 20S-40S HnRNA, which is complementary only to unique DNA. mRNA, purified by different techniques, contains sequences complementary to DNA repeated about 300 times, and sequences complementary to single-copy DNA. mRNA hybridizes with the same kinetics as giant HnRNA. The data suggest that mRNA is derived from giant HnRNA.
directly rather than through a process involving many cleavage steps. Experiments performed with cDNA transcribed in vitro from mRNA suggest that some of the sequences complementary to reiterated DNA are covalently linked to the sequences complementary to unique DNA, and are positioned at the 5' end of the RNA molecules.

It was found that 60% of the total mRNA population is transcribed from less than a thousand genes.
INTRODUCTION

Genome organization in higher organisms

A considerable amount of information about the basic problems of Molecular Biology has been obtained from bacterial and viral systems. The processes of replication, transcription, translation and the mechanisms which control them have been thoroughly investigated and are now partly understood in those systems. Even though the conclusions reached on prokaryotes can be extrapolated to a certain extent to higher organisms, it is not always possible to apply the same criteria or to use the same experimental approaches for the two systems.

The main difference between prokaryotes and eukaryotes lays in the organization of the genome.

In prokaryotes the genome is relatively simple, being made up of double-stranded DNA which ranges in amount from \( 10^6 \) to \( 10^9 \) daltons. Each gene is believed to be represented only once per genome, with the exception of the cistrons coding for ribosomal RNA, which are reiterated between five and ten times (Gillespie and Spiegelmann, 1965; Attardi et al, 1965; Kennel, 1968), and the cistrons for tRNA and 5S RNA, which are reiterated from 40 to 50 times, and from 4 to 10 times, respectively (Goodman and Rich, 1962; Giacomoni and Spiegelmann, 1962; Smith et al, 1968; Zehavi-Willner and Comb, 1966). In eukaryotes the genome is much more complex (Mirsky and Ris, 1951) and much less known. In mammals, for instance, there is enough DNA to code for about 4 million different proteins of average MW 30,000 but the actual fraction of DNA transcribed into mRNA
is still unknown. Furthermore, eukaryotic DNA is organized in highly complex structures called chromosomes. The chromosome is made up of double stranded DNA and several different types of proteins; histones (basic) and non-histones (basic and acidic) whose structural and/or regulatory role is not yet very clear.

One of the characteristic features of eukaryotic DNA is the presence of reiterated sequences, that is base sequences occurring more than once per haploid genome (Britten and Kohne, 1966, 1968). On the basis of their reiteration frequency, the DNA sequences fall into 3 natural classes: highly repetitive, intermediate and unique sequences. Highly repetitive sequences are those occurring about a million times per genome. If these sequences have a base composition different enough from the bulk of the DNA, they can be separated on CsCl density gradients as a distinct satellite band (Flamm et al, 1967), as in the case of the mouse satellite DNA and the amphibian oocyte ribosomal cistrons (Birnstiel et al, 1966; Wallace and Birnstiel, 1966). Satellite DNAs, other than ribosomal genes, do not seem to be transcribed into RNA (Flamm et al, 1969) and thus the highly repeated sequences apparently do not play a direct role in protein synthesis. The intermediate fraction is a heterogeneous group of sequences occurring from a few to a few thousand times per genome. The genes coding for rRNA, tRNA and 5S RNA usually fall in this category (Steele, 1968; Quincey and Wilson, 1969; Birnstiel et al, 1971). Unique sequences are those represented only once or a few times per genome and, at least in mammals, account for most of the DNA. At least some of the unique sequences are transcribed into
DNA-like RNA (Gelderman et al., 1971; Davidson and Hough, 1971; Melli et al., 1971; Greenberg and Perry, 1971).

Synthesis and processing of rRNA and tRNA

All cellular RNA, with the exception of some mitochondrial RNA, is synthesised in the nucleus and then transferred to the cytoplasm, (Prescott, 1960, 1964; Perry, 1967). This has been clearly shown in the case of ribosomal RNA in HeLa cells (Penman, 1966; Penman et al., 1966) and in other systems (Perry et al., 1961; Perry, 1962).

The synthesis of rRNA occurs in the nucleolus (Perry, 1964; Penman et al., 1966; Holtzman et al., 1966). The first product of transcription is a single large precursor molecule which undergoes specific cleavage, the result of which is the "loss" of some sequences and the maintenance of others which will give rise to the fully mature rRNAs (Scherrer and Darnell, 1962; Scherrer et al., 1963; Perry, 1964; Penman et al., 1966). Several lines of evidence indicate that in HeLa cells only 50-60% of the original 45S precursor molecule is conserved during processing (Warner et al., 1966). Apparently the other 50% of the molecule is synthesized and then destroyed.

Transfer RNA is synthesized in the nucleus (Scharff and Robbins, 1965; Woods and Zubay, 1965; Perry, 1962; Edstrom and Daneholt, 1967; Ritossa et al., 1966) with the same basic mechanism as rRNA. It appears in fact that a precursor to tRNA is synthesized (Vesco and Penman, 1968; Bernhardt and Darnell, 1969; Rosbash and Penman, 1972), and then is rapidly converted into mature molecules through a process which involves both cleavage and conformational changes.
Characteristics of HnRNA

When HeLa cell pulse labelled nuclear RNA is analyzed on sucrose gradients, in addition to the rRNA species, there is some labelled material, making up about 50% of the rapidly labelled nuclear RNA, which sediments in a heterogeneous fashion ranging from about 20S to greater than 100S (Warner et al, 1966; Soeiro et al, 1966; Houssais and Attardi, 1966).

The heterogeneously sedimenting RNA (HnRNA) has been found not only in cultured cells, but in differentiated cells as well, such as duck erythroblasts (Scherrer et al, 1966; Attardi et al, 1966; Attardi et al, 1970), rat liver cells (Georgie et al, 1963), dipteran salivary glands (Edstrom and Daneholt, 1967), etc.

This RNA is not rRNA or rRNA precursors as shown by its base composition, its size and its stability.

Its base composition resembles very much the base composition of the DNA. In HeLa cells, for instance, the DNA contains 44% GC, and HnRNA contains 48% GC, compared to more than 60% GC for rRNA and 54% GC for tRNA (Soeiro et al, 1966). The DNA-like base composition of HnRNA indicates that this species of RNA is transcribed on DNA stretches markedly different from ribosomal or transfer RNA genes.

Another piece of evidence that HnRNA is not rRNA or rRNA precursors comes from Actinomycin D (AMD) treatment. If further RNA synthesis is blocked by AMD there is no transfer of label to the rRNA species (Sherrer, 1967). In addition, HnRNA is found in erythroblasts, very specialized cells that have already largely suppressed the synthesis of rRNA.
The molecular weight of HnRNA, as estimated from sedimentation coefficient and electron micrographs (Sherrer et al., 1966; Miller et al., 1970) can be as great as $10^7$ daltons - hence the name "giant" RNA. There is a large body of evidence that HnRNA is not an artifact due to experimental procedure. If the giant RNA recovered from a sucrose gradient is run in a sucrose gradient again, it will sediment with the same $S$ value as before (Attardi et al., 1966). DNAase or trypsin treatments do not affect the sedimentation pattern of HnRNA, showing that the giant molecules are not an aggregate of smaller molecules with DNA or proteins (Attardi et al., 1966; Warner et al., 1966).

However, de Kloet and collaborators (1970) found that the treatment of giant RNA from Yeast with the denaturing agent formaldehyde reduces both the polydispersity and the MW of the HnRNA. Similar results have been obtained by Bramwell (1972) with HnRNA from HeLa cells.

When HnRNA from duck erythroblasts is run in a sucrose gradient made in formamide, its sedimentation coefficient ranges from 15S to 30S, while in ordinary sucrose gradients it can be as high as 100S. A similar shift in the $S$ value is observed when the HnRNA is pretreated with formaldehyde (M. Macnaughton - personal communication). These results suggest that, in ordinary conditions, giant RNA is an artifact due to the aggregation of smaller molecules, but the possibility that the changes in the sedimentation pattern are caused by hidden scissions cannot be ruled out.

From genetic and biochemical studies conducted on the giant
chromosomes of Diptera, it has been shown that HnRNA is the transcription product of a complete functional unit. The chromomere "contains only one essential function" (Beerman, 1972) and it is transcribed into HnRNA (Edstrom and Daneholt, 1967), which, at least in the case of BR2 of Chironomus tentans (Daneholt, 1972), is of a defined size, suggesting that the whole chromomere is transcribed in only one class of molecules.

Most of the HnRNA turns over in the cell nucleus and never reaches the cytoplasm, as was already suggested by Harris some years ago (Harris, 1963, 1964) and was shown by Attardi and his colleagues in duck erythroblasts (Attardi et al., 1966). Upon addition of AMD either to duck erythroblasts or to cultured cells, about 90% of the HnRNA decays to the acid soluble pool in about 20'. It seems that, in duck blood cells at least, the other 10% of it goes to the cytoplasm, since a very small but very definite movement of RNA from the nucleus to the cytoplasm has been observed (Warner et al., 1966; Houssais and Attardi, 1966; Sherrer andMarcaud, 1968).

**Characteristics of mRNA**

There is a growing body of evidence that the 10% of the HnRNA which does enter the cytoplasm is a precursor to cytoplasmic mRNA, but before considering the functional role of the HnRNA, one has to examine the characteristics of mRNA. By definition, the mRNA carries the "genetic message", encoded in the DNA. The mRNA molecules of a heterogeneous population are then transcribed from many genes thus reflecting the average DNA base composition. Furthermore, they
must be associated with ribosomes at least while directing protein synthesis.

The RNA extracted from polysomes after a brief labelling period (i.e. not long enough to label rRNA) sediments in sucrose gradients in a heterogeneous fashion ranging from about 10S to 30S and is DNA-like in base composition. Treatment of polysomes with EDTA disrupts the ribosomes and at the same time shifts the radioactivity from the polysomal region of the gradient towards a much lighter region, suggesting that the rapidly labelled RNA is associated with ribosomes (Latham and Darnell, 1965; Penman et al., 1968). On the basis of these considerations the cytoplasmic rapidly labelled polysomal RNA is assumed to be mRNA.

On the other hand, not all the rapidly labelled RNA sedimenting in the polysome region is shifted by EDTA treatment (Penman et al., 1968).

So far, the identity and the role of this RNA, called cytoplasmic heterodisperse RNA, are unknown.

**Relationship between HnRNA and mRNA**

As already said, most of the HnRNA never leaves the nucleus. The question that now arises is: Does the fraction which goes into the cytoplasm act as mRNA?

It should be pointed out again that HnRNA molecules range from 20S to greater than 100S, corresponding to a length of 2000 to 50000 or more nucleotides, or, in other words, to a MW from about $6 \times 10^5$ to about $1.5 \times 10^7$ daltons.
In contrast, mRNA ranges from 10S to 30S, which means molecules containing from 800 to 6000 nucleotides, whose MW varies from about $2 \times 10^5$ to about $2 \times 10^6$ daltons.

HnRNA molecules of average length are then from 5 to 10 times bigger than the average mRNA molecules, while the giant HnRNA can be as big as 20 times the mRNA. If HnRNA is a precursor to mRNA, a mechanism of some kind must then exist which cleaves giant molecules down to much smaller size. Such a process would involve the removal of RNA sequences up to 45000 nucleotides long or about 13 million MW, if there is a 1:1 precursor:product relationship, that is if there is one molecule of messenger per molecule of HnRNA. It is also clear that transcription does not necessarily imply translation, since more RNA is synthesized than is actually needed for protein synthesis.

This situation of course raises the vital problem of why a cell transcribes such long stretches of DNA, if only a small portion of the transcript is then conserved.

Another problem is the processing mechanism itself: does the cleavage occur stepwise, originating molecules of progressively smaller size? Or is it a one-step process which frees the mRNA molecule from its precursor and leaves the rest of the molecule behind?

The existence of such a wide range of HnRNA molecules would favour the first possibility, even if simultaneous processing of HnRNA molecules of different sizes could not be ruled out.

Two main approaches have been used to tackle the problem of the origin of mRNA: kinetics of incorporation of labelled precursors into HnRNA and mRNA, and DNA-RNA hybridization and competition.
On kinetic grounds alone, it is not possible to draw an unequivocal conclusion, as pointed out by the experiments done by Soeiro and collaborators (Soeiro et al, 1966) in HeLa cells. The authors measured the relative amounts of labelled RNA in various cellular fractions after different periods of exposure to the label. If HnRNA were related to mRNA by a simple precursor-product relationship one would expect an increase in HnRNA followed after a certain time by an increase in mRNA. On the contrary, the two RNA species followed different kinetics. On the other hand, mRNA appears in the cytoplasm with a lag of about 15', thus suggesting the existence of a pool (Penman et al, 1968). Chase experiments have shown that HnRNA and mRNA decay with different rates. In avian erythroblasts, a large fraction of HnRNA decays very rapidly with a half life of 20' (Sherrer and Marcaud, 1968) leaving behind molecules of smaller size (Sherrer et al, 1970). This relative accumulation of molecules of lower molecular weight suggests a cleavage mechanism whereby the giant HnRNA is processed to smaller products which might enter the cytoplasm. Yet such reduction in size could not be observed during a chase in HeLa cells, where the decay of HnRNA follows a biphasic pattern, with an initial rapid decay followed by a slower turnover. The light HnRNA decays four times slower than the heavy HnRNA, suggesting that the shorter chains are formed from heavy HnRNA as a result of processing (Sherrer et al, 1970).

The same conclusion has been reached by Ryskov and Georgiev (1970) who found that only the heavy HnRNA contains a nucleoside triphosphate at its 5' end while lighter HnRNA molecules contain only
one phosphate group at that end. It is known from other sources that only nascent RNA contains a nucleoside triphosphate residue at the 5' terminus and that the β and γ phosphates are absent in mature molecules. So it appears that only heavy HnRNA is the primary transcription product and all the other molecules are derived from it as a consequence of a cleaving mechanism.

Another approach employed to study the nature of HnRNA and its relationship to mRNA is given by hybridization competition experiments. The general conclusion drawn from these experiments, is that all the nucleotide sequences present in mRNA are also present in HnRNA while the latter contains sequences which are not present in mRNA, thus suggesting once again a precursor-product relationship (Hoyer et al, 1963; Birnboim et al, 1967; Shearer and McCarthy, 1967; Georgiev, 1967; Church and McCarthy, 1967, 1970; Soeiro and Darnell, 1970).

However, the hybridization experiments were carried out under conditions which allowed the formation of hybrids involving only transcripts from the highly reiterated sequences, while the transcripts from single or seldom repeated sequences were not detected (Melli and Bishop, 1969, 1970). If any differences in nucleotide sequences between HnRNA and mRNA were brought about by non-repeated sequences, these differences would be undetected. Furthermore, since redundant DNA sequences are similar but not identical (Britten and Kohne, 1968), RNAs transcribed from these sequences will be similar enough to compete against each other for the same DNA sites, without actually being identical.
More recent experiments, performed in conditions in which the hybridization of unique sequences can be monitored, have shown that the majority of HnRNA is transcribed from non-repeated sequences and only a small amount of it is transcribed from reiterated DNA sites (Melli et al, 1971; Gelderman et al, 1971; Davidson and Hough, 1971; Greenberg and Perry, 1971; Georgiev et al, 1972).

In addition, Georgiev and collaborators (1972) showed that in rat liver the transcripts from the reiterated sequences are on the 5' end of the HnRNA molecule and that polysomal mRNA, while competing only slightly with total HnRNA, strongly inhibits the hybridization of the 3' end of the HnRNA molecule, indicating that this end contains at least one molecule of mRNA which will be transferred on polysomes.

Another indication of the derivation of mRNA from HnRNA has been given by Melli and Pemberton (1972) and by Williamson et al (1973).

Melli and Pemberton transcribed in vitro 9S haemoglobin mRNA from duck erythroblasts into complementary RNA. This so-called "antimessenger" was then annealed to duck HnRNA and the reaction product was an RNAse-resistant hybrid, suggesting the presence of 9S Hb mRNA sequences in the HnRNA molecule.

Williamson and collaborators found that mouse erythroblast HnRNA injected into Xenopus oocytes could stimulate haemoglobin synthesis in the host cell, supporting the idea that HnRNA contains messenger sequences. In both cases, however, a contamination of HnRNA with 9S mRNA cannot be ruled out.

Presence of poly(A) sequences in HnRNA and mRNA

Recently, the relationship between HnRNA and mRNA has been further clarified by the finding of poly(A) stretches in both types of RNA (Darnell et al 1971a; Edmonds et al 1971; Darnell et al 1971b; Lee et al, 1971; Sheldon et al 1972). The poly(A) sequences are covalently bound to the 3' end of the molecule, are about 150-200 nucleotides long and are rather homogeneous judging by their electrophoretic mobility.

They are not found in the nucleolus, eliminating rRNA as their
possible source. They make up about 0.5\% of HnRNA and from 2.5\% to 5\% of mRNA in HeLa cells (Edmonds et al., 1971; Darnell et al., 1971a). The size and amount of poly(A) sequences in HnRNA and mRNA suggest that many of the RNA molecules within each class contain one poly(A) sequence. This observation, together with the finding that HnRNA contains poly(A) in lower proportion than mRNA, leads once again to the conclusion that mRNA arises from HnRNA as a consequence of a post-transcriptional processing mechanism. The poly(A) sequences may act as recognition sites for a specific nuclease (Edmonds et al., 1971) or be involved in the transport of mRNA from the nucleus to the cytoplasm (Darnell et al., 1971b) or perform both functions.

The occurrence of poly(A) sequences seems to be a very general phenomenon. Poly(A) sequences have been found in mRNA of mouse sarcoma ascites cells (Lee et al., 1971), in duck Hb mRNA (Pemberton and Baglioni, 1972), in rabbit globin mRNAs (Burr and Lingrel, 1971), in mouse immunoglobulin mRNA (Swan et al., 1972) and in slime mould mRNA (Firtel et al., 1972). Nevertheless, one exception exists - histone mRNA does not contain any poly(A), (Adesnik and Darnell, 1972).

The hypothesis of the derivation of mRNA from HnRNA has received more support by the work done in virus-transformed cells in several laboratories. In such systems, the viral genome is integrated into the cellular DNA; the first transcription product is a giant RNA molecule, much larger than the viral DNA itself, containing viral sequences covalently linked to sequences transcribed on cellular DNA, as has been shown by RNA-DNA hybridization (Wall and Darnell, 1971). Subsequently, these molecules are processed and transported
to the cytoplasm, where they are found on polysomes (Lindberg and Darnell, 1970; Acheson et al., 1971). Moreover both viral HnRNA and viral mRNA contain poly(A) stretches, indistinguishable from the cellular ones (Philipson et al., 1971), suggesting that the poly(A) is added post-transcriptionally, since viral DNA does not contain any long sequence of A.

The picture which emerges from these results is the following: The DNA is transcribed into a very long RNA molecule, whose 5' end is transcribed from reiterated sequences and whose 3' end is linked to poly(A). This molecule undergoes specific breakage, at the end of which a much smaller RNA sequence, containing poly(A) at the 3' terminus, moves to the cytoplasm and becomes engaged in protein synthesis on polysomes. All the rest of the original molecule is very rapidly destroyed in the nucleus. The role of this rapidly decaying fraction is still obscure; presumably it is involved in transcription control mechanism(s) but there is no clear answer available yet.

**Purpose of the work**

When this thesis was started, many of the problems concerning HnRNA and its relationship to mRNA were still unsolved.

The derivation of mRNA from HnRNA was suggested by a certain number of experiments, but the overall picture was rather confused. As stated before, the experiments based on kinetics of synthesis and decay of these two classes of RNA were not decisive and the experiments based on DNA-RNA hybridization and competition were done in
conditions such that only RNA transcribed on repeated DNA sites would hybridize. Furthermore, little or nothing was known about the reiteration frequency of the DNA sequences coding for HnRNA and mRNA.

The hybridization technique in vast DNA excess worked out by Melli and collaborators (Melli et al, 1971) provided a more reliable method and a powerful tool to the study of RNA in relation to the DNA which encodes it.

Rat myoblasts are a fairly unexplored territory as far as the problems mentioned above are concerned, so it was decided to analyse their different RNA populations, in order to relate them to each other (i.e. precursor-product relationship) and to the DNA sequences from which they have been transcribed (i.e. reiteration frequency of coding DNA).

This task has been accomplished by kinetics of synthesis and decay of RNA molecules and by the new technique of DNA-RNA hybridization in DNA excess. The work was conducted on a permanent line of rat myoblasts grown in monolayer culture. The cells have a generation time of 24 hours. Unlike other established lines of myoblasts, they never undergo the cellular fusion which leads to the formation of myotubules, but divide until they become confluent and then eventually die. However, their appearance is the same as normal myoblasts, that is, spindle-shaped cells, with a big nucleus and two or three prominent nucleoli. The reasons why these cells do not fuse are unknown and no attempt has been made to investigate this point.

All experiments were performed on cells in exponential growth.
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<td>trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>PCA</td>
<td>perchloric acid</td>
</tr>
<tr>
<td>PPO</td>
<td>2-5-diphenyloxazole</td>
</tr>
</tbody>
</table>
POPOP
Butyl PBD
DOC
Tween 40
MEM
BSA
GF filters
DEP

**Buffers and Solutions**

TKM
50mM TRIS pH 7.5
25mM KCl
5mM MgCl₂

RSB
10mM TRIS pH 7.5
10mM NaCl
1.5mM MgCl₂

NTE
100mM NaCl
5mM TRIS pH 7.5
1mM EDTA

NTES
NTE + 0.5% SLS

SSC
150mM NaCl
15mM Na citrate

Column buffer
0.3M NaCl
0.01 M Na acetate
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding buffer</td>
<td>0.4M NaCl &lt;br&gt;10mM TRIS pH 7.5 &lt;br&gt;1mM EDTA &lt;br&gt;0.1% SLS &lt;br&gt;10% glycerol</td>
</tr>
<tr>
<td>Eluting buffer</td>
<td>10mM TRIS pH 7.5 &lt;br&gt;1mM EDTA &lt;br&gt;0.1% SLS</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>36 mM TRIS pH 7.6 &lt;br&gt;30mM NaH₂PO₄·2H₂O &lt;br&gt;1mM EDTA &lt;br&gt;0.2% SLS</td>
</tr>
<tr>
<td>&quot;Kirby&quot; solution</td>
<td>1% Na-triisopropylnaphtalene sulphonate &lt;br&gt;6% Na-4-amino salicylate &lt;br&gt;1% NaCl &lt;br&gt;6% n-Butanol &lt;br&gt;0.5% SLS</td>
</tr>
<tr>
<td>Phenol-cresol</td>
<td>500 gr phenol=550 ml water-saturated phenol &lt;br&gt;70 gr m-cresol &lt;br&gt;0.5 gr 8-hydroxyquinoline</td>
</tr>
<tr>
<td>PB</td>
<td>Equimolar phosphate buffer</td>
</tr>
<tr>
<td>PEB</td>
<td>1.2 M PB + 5mM EDTA</td>
</tr>
<tr>
<td>KB3</td>
<td>0.15 M Na acetate pH 4.5 &lt;br&gt;0.003 M Zn SO₄</td>
</tr>
</tbody>
</table>
KB3 (continued)

0.1 M NaCl
0.25 M HCl

Counting fluids

Toluene-PPO-POPOP
3 gr PPO and
0.3 gr POPOP per litre of toluene

Butyl PBD fluid
8 gr of Butyl PBD per litre of toluene

Butyl PBD-ethoxyethanol
3 vols of Butyl PBD fluid plus
2 vols Ethoxyethanol

Materials

Male albino rats, weighing 170-200 gr, were obtained from the Small Animal Breeding Station - Bush House - Midlothian.

\( ^3 \text{H}-\text{Uridine triphosphate, } ^3 \text{H}-\text{adenosine triphosphate and } ^{32} \text{P-orthophosphate} \)
were purchased from the Radiochemical Centre, Amersham, England.

MEM was obtained from Wellcome Research Laboratories, Beckenham, England.

Foetal Calf Serum was obtained from Flow Laboratories, Irvine, Scotland.

Plastic Disposable Petri dishes were obtained from Dunc - Denmark.

Dulbecco saline was obtained from Oxoid Ltd., London.

Trypsin was purchased from Difco Laboratories, Detroit, Michigan, U.S.A.

Electrophoretically-pure DNase, pancreatic RNAse, Protease, BSA, TRIS and Tween 40 were obtained from Sigma Biochem. Inc., London.

GF/C filters and paper discs were obtained from Whatman.

Sephadex was purchased from Pharmacia - Uppsala - Sweden.

Cellulose-oligodT was obtained from Merck.

Butyl PBD was obtained from Intertechnique Ltd.
PPO and POPOP were obtained from Packard Ltd.

All the other chemicals were obtained from British Drug Houses Ltd.

Cell cultures

Rat myoblasts were grown in monolayers in vented plastic Petri dishes at 37°C in an incubator gassed with 5% CO₂.

Cells were grown in Eagle's MEM (Hank's based plus non-essential aminoacids), 0.1% NaHCO₃, 10% foetal bovine serum, 120 µg/ml penicillin and 150 µg/ml streptomycin.

When confluent, the cells were harvested with 0.25% trypsin in Dulbecco A, spun down at 500 rpm for 2', resuspended in fresh medium and plated at 1/3 of their previous concentration.

Experimental procedures

About 20 hr before each experiment, the cells were plated at a concentration of 5 x 10⁵ cells per dish.

The cells were labelled with either 100 or 50 µC/ml H³-Uridine (spec. act. greater than 20 Ci/mM) for the desired length of time. In most experiments the cells were treated with 0.04 µg/ml AMD for 30' before the addition of the radioactive precursor, in order to inhibit rRNA synthesis.

For chase experiments, the cells were labelled for 2 hrs with 100 µC/ml H³-Uridine and then any further RNA synthesis was blocked by the addition of 4 µg/ml AMD.

In the case of pulse-labelling and pulse-chase experiments, as many small dishes as the time-points desired were inoculated with 3 x 10⁵ cells/dish. The amount of cells was carefully measured
after harvesting and the experimental results were corrected for the recovery of cells.

**Extraction and purification of DNA from rat liver**

DNA was extracted from purified nuclei, obtained by a modification of the method of Chauveau *et al* (1956).

Rats were starved overnight and killed by decapitation. The livers were removed and dropped into ice-cold 0.2 M sucrose in TKM. After two or three washes with fresh buffer, the livers were chopped and homogenized in ten volumes (v/w) of ice-cold 2.2 M sucrose, 3 mM MgCl$_2$ with a Teflon homogenizer. The homogenate was spun at 27000 rpm for 60' at 2$^\circ$C in a No. 30 Spinco rotor. The supernatant was discarded, and the pelleted nuclei were suspended in 1% SLS in NTE, and stirred at room temperature until all lysed. 1/4 vol. of 5 M NaCl and 1/10 vol of 3 M TRIS pH 8.5 were added to the lysate, which was extracted with an equal volume of phenol-chloroform 1:1.

The mixture was shaken for about 10' in a shaker at room temperature and then centrifuged at 10,000 rpm for 10'. The water-phase was collected; the proteic interphase was re-extracted with fresh NTE and phenol-chloroform and the pooled water-phases were re-extracted with fresh phenol-chloroform. The final water-phase was collected and dialysed against three changes of 2 x SSC at room temperature. The dialysed solution was incubated with 50 µg/ml of pancreatic RNAse for 3 hrs at 37$^\circ$C and then with 400 µg/ml of protease for the same time at the same temperature. The DNA was extracted two more times with phenol-chloroform and then dialysed against two
changes of 2 x SSC and two changes of 0.1 x SSC at room temperature. The DNA was pelleted overnight at 27000 rpm at 2°C. The supernatant was discarded and the pellets resuspended in 0.1 x SSC. The presence of contaminating RNA was checked by alkaline digestion of a sample of DNA.

A solution containing approximately 50 μg/ml of DNA was treated with 1/10 vol of 6 N KOH at 37°C for 1 hr. 1/10 vol of concentrated PCA was then added and the sample was left in ice for 20'.

After centrifugation of 10000 rpm for 10 to remove precipitated DNA and potassium perchlorate, the supernatant was very gently decanted and its ODs measured.

Usually the ODs after alkaline digestion and acid precipitation of the DNA sample were negligible, indicating a considerable purity of the DNA solution.

The DNA was sonicated in pulses of 15" for a total of 1'30", with a Dawe sonicator set at position 8. The size of sonicated DNA was measured in alkaline sucrose gradients and was found to be 1.5 x 10^5 daltons. The sonicated DNA was precipitated with 1/10 vol of 2 M Na acetate pH 5.0 and two vols of absolute alcohol.

The DNA was collected by centrifugation at 10000 rpm for 20' and the pellet suspended in column buffer. The DNA was loaded onto a 2.5 cm x 45 cm column of Sephadex SE 50, swollen in the same buffer. The fractions were read at 260 μm and the peak fractions were pooled and precipitated with 2 vols of alcohol.

The precipitate was collected by centrifugation and dissolved in 0.1 x SSC at a concentration usually higher than 20 mg/ml.
**Extraction and purification of RNA from rat myoblasts**

Cells were harvested by treatment with trypsin at 37°C for about 10' and washed twice with ice-cold Dulbecco. All the operations were carried out at 0°C - 4°C.

The washed cells were lysed by gentle shaking in TKM + 1% Triton x 100 (1 ml per 1 x 10⁶ cells). Nuclei were pelleted at 10000 rpm for 10'. The supernatant was decanted and kept in ice. The nuclei were resuspended in RSB and "washed" according to Penman's procedure (Penman, 1966): 0.15 ml of a solution 2:1 10% Tween 40:10% DOC were added to each ml of nuclear suspension, the nuclei were gently shaken and centrifuged at 10,000 rpm for 10'. The double-detergent procedure removes unbroken cells and the outer nuclear membrane.

The supernatant was added to the previous one. The pooled supernatants were called "cytoplasmic fraction".

The RNA from the nuclear and the cytoplasmic fractions was extracted by a modification of Kirby's method (Kirby, 1965; Parish and Kirby, 1966). The nuclei were lysed with 1 ml of "Kirby" solution per 1 x 10⁶ cells, and gently homogenized with a few strokes in a small Teflon homogenizer. Kirby solution was added to the cytoplasmic fraction in a 1:1 ratio. An equal volume of phenol-cresol was added to both the nuclear and the cytoplasmic fraction and the mixtures were shaken at room temperature for 20'. The samples were centrifuged at 10,000 rpm for 10' and the water-phases were carefully removed by pipetting and kept in ice. The phenol- and the proteic-phases were re-extracted with an equal volume of distilled water, shaken for 20' and centrifuged at 10,000 rpm for 10'. The second water phase was
added to the previous one and half volume of phenol-cresol was added.
The samples were shaken and centrifuged as before, and the final water-phases were washed with one volume of chloroform to remove remaining phenol.

The samples were precipitated with 1/10 vol 2 M Na acetate pH 5.0 and 3 vol of absolute alcohol and stored at -20°C until needed.
The nuclear RNA was collected by centrifugation at 10,000 rpm for 20' and the pellet was washed with 1:1 alcohol-ether to remove the last traces of phenol. The RNA was suspended in 7 mM mgCl₂, 50 mM TRIS pH 7.5 and incubated with 50 µg/ml of repurified RNAse- free DNase for 30' at 0°C. The RNA was alcohol-precipitated again and stored at -20°C. When total-cell RNA was required, the cells were homogenized in Kirby solution with a Teflon homogenizer and the RNA was extracted with phenol-cresol, following the standard procedure.

The purity of radioactive RNA was checked by measuring the acid-soluble cpm after treating aliquots of RNA with 20 µg/ml pancreatic RNase and 50 µg/ml electrophoretically pure DNase.

All the solutions and the glassware used during RNA extraction were either autoclaved or sterilized by DEP treatment at 60°C for 3 hrs.

**Extraction and purification of RNA from the cytoplasm of rat liver**

Rat livers were obtained as previously described, and homogenized in TKM with a Teflon homogenizer. The nuclei were pelleted by centrifugation at 10,000 rpm for 10' and the cytoplasmic fraction was treated with the same volume of Kirby solution and then extracted with an equal volume of phenol-cresol, as described in the previous
section. After the first alcohol-precipitation, the RNA was collected by centrifugation and the pellet was suspended in column buffer.

The RNA was loaded onto a 1.5 cm x 25 cm column of Sephadex G100 in the same buffer, and the eluted fractions were read at 260 μ in a Beckmann spectrophotometer. The peak fractions were pooled and the RNA was precipitated with 2 vols of alcohol and stored at -20°C.

Extraction and purification of polyribosomal RNA from the cytoplasm of rat myoblasts.

Cells were lysed with 1 ml of RSB + 1% Triton x 100 per 1 x 10^6 cells. Nuclei were pelleted at 10000 rpm for 10' and the cytoplasmic supernatant was stratified on a 30 ml 15-30% sucrose gradient in RSB. The gradient was centrifuged at 26000 rpm for 3 hr. 1 ml fractions were collected with an Isco Fraction collector. The fractions containing the bulk of the polysomes were pooled, made 0.5% SLS and 0.2 M Na acetate and precipitated with 3 vols of alcohol. The polysomal RNA was collected by centrifugation at 10,000 rpm for 20'.

Purification of DNase

10 mg of electrophoretically pure DNase were suspended in 2 ml of 0.0025 N HCl and dialysed for 2 days against 2 litres of 0.0025 N HCl. 2.5 ml of 0.2 M Na acetate pH 5.3 and 0.75 ml of 1 M Na iodoacetate were added to the DNase solution. The mix was incubated at 55°C for 60', and then dialysed overnight against 1 litre of 0.0025 N HCl. A precipitate formed which was spun down at 10,000 rpm for 30'. The supernatant contained DNase at the approximate concentration of 2 mg/ml.
In vitro synthesis of labelled poly(U)

Labelled polyU was synthesized in vitro by polymerization of $^3$H-UDP by the enzyme polynucleotide phosphorylase, in presence of 0.05 M TRIS pH 8.5, 0.01 M KCl and $5 \times 10^{-4}$ M MgCl$_2$. The reaction was carried on for 60' at 37°C, and stopped by adding Na acetate pH 5.0 to the final concentration of 0.2 M, and SLS to the final concentration of 0.5%. The reaction product was extracted twice with phenol-chloroform. The final water-phase was loaded onto a Sephadex SP 50 column and the fractions containing the peak of radioactivity were frozen. The specific activity of $^3$H-poly(U) was determined by reading the extinction at 260 m$m$, and by hybridizing an aliquot of it to a known amount of unlabelled poly(A). The specific activity of the labelled poly(U) was found to be between $100 \times 10^3$ and $150 \times 10^3$ cpm/µg.

Sucrose density gradients

Sucrose gradients were run either in the 6 x 16 rotor of a MSE 65 centrifuge, or in the SW 27 rotor of a Spinco L2 centrifuge. Gradients were usually run at 1°C, except when SLS was present; in this case the running temperature was 25°C.

The gradients were collected with an Isco fraction collector in 0.5 ml fractions. The absorbance at 260 m$m$ was continuously monitored with an Isco flow analyser and recorded with a Brian recorder. Radioactive profiles of preparative gradients were obtained by counting aliquots from each fractions in Butyl PBD-ethoxyethanol. Radioactive profiles of analytical gradients were obtained by adjusting each fraction to 10% TCA with 50% TCA and adding 250 µg of BSA as co-
precipitant. The precipitate was collected by filtration on GF/C filters or Millipore filters and washed with cold 5% TCA. The filters were dried in a vacuum oven at 80°C for 20', allowed to cool and then counted in Toluene PPO-POPOP in a Tricarb Scintillation Spectrophotometer.

**Polyacrylamide gel electrophoresis**

Polyacrylamide gel electrophoresis was performed according to the method of Loening (1967).

Acrylamide was recrystalized from chloroform and bisacrylamide from acetone. A stock solution was made by dissolving 15 gr of recrystalized acrylamide and 0.75 gr of recrystalized bisacrylamide in water to a total volume of 100 ml.

Gels were made up at the desired concentration of acrylamide by adding an appropriate amount of 5 x gel buffer. 25 μl of N,N,N,N tetramethyl ethylene diamine and 0.25 ml of 10% ammonium persulphate were added as polymerising agents. The gels were allowed to stand for about 1 hr at room temperature and then pre-run for 30' at 3 mA per gel, for gels of 0.25 cm diameter, and 5 mA per gel, for gels of 1 cm diameter. The RNA dissolved in gel buffer plus 20% sucrose was layered on the gels and run for the appropriate length of time. The gels were scanned at 260 μm in a Joyce-Loeble UV recorder and sliced in slices of 1 mm with a Mickle gel slicer.

The radioactivity profile of analytical gels was obtained by digesting each slice with 0.5 ml NH₃ overnight at room temperature. The NH₃ was allowed to evaporate and 20 ml of Butyl PBD-ethoxyethanol
were added before counting in a Tricarb Scintillation Spectrophotometer. For preparative purposes, the RNA was eluted from the slices with 0.5 ml 0.1 M Na acetate pH 5.0 per slice, overnight at room temperature. An aliquot of the eluate was either counted in Butyl PBD-ethoxyethanol, or spotted on paper discs. The discs were washed with cold 5% TCA, rinsed with alcohol, then with alcohol:ether 1:1, dried with ether and counted in Toluene PPO-POPOP.

**Sepharose-poly(U) columns (Wagner et al, 1971)**

1 ml of Sepharose-poly(U) in 2 x SSC was poured in a 1 cm x 9 cm jacketed column and repeatedly washed with 2 x SSC. The RNA sample in 2 x SSC was loaded onto the column. The RNA molecules lacking poly(A) were eluted with 10 ml of 2 x SSC at room temperature. The RNA molecules with short poly(A) stretches were eluted with 5 ml of 10 mM TRIS pH 7.5 at room temperature. The poly(A)-containing RNA was eluted with 5 ml of 10 mM TRIS pH 7.5 at 40°C (Kates, 1970). Fractions of 0.5 ml were collected at all stages and the radioactivity continuously monitored.

**Cellulose-oligo(dT) columns (Aviv and Leder, 1972)**

30 mg of Cellulose-oligo(dT) were suspended in water and poured in a Pasteur pipette. After repeated washes with water, the column was equilibrated with binding buffer. The RNA sample in binding buffer was layered on the column. Poly(A)-lacking molecules were eluted with 10 ml of binding buffer. The RNA molecules containing poly(A) were released by the addition of 5 ml of eluting buffer. 0.5 ml fractions were collected and the radioactivity of each fraction was monitored.
DNA renaturation

Rat liver DNA denaturation was carried out in 2 x SSC at 70°C (Bishop, 1972a). The DNA dissolved in 0.1 x SSC pH 5.5 was heated for 5' in a boiling water bath, in order to achieve complete denaturation. A control sample was withdrawn and greatly diluted with ice-cold 0.1 x SSC pH 7.5. The remaining solution was put in an oil bath kept constantly at 70°C, and after 30" (to allow the temperature to equilibrate) the salt concentration of the DNA solution was adjusted to 2 x SSC by adding a convenient amount of diluted 20 x SSC pH 5.0.

Liquid paraffin was layered on the mixture and the reaction tube was tightly stoppered to avoid evaporation.

Samples were withdrawn at different times, and diluted in ice-cold distilled water or in ice-cold 0.1 x SSC, at a final DNA concentration of 50 µg/ml.

Usually, two annealing mixtures were made, at a DNA concentration of 400 µg/ml and 10 mg/ml. SLS was present in the mixtures at the final concentration of 0.2% and 1% respectively.

Estimation of the proportion of renatured DNA as a function of time (Melli and Bishop, 1970)

The absorbance spectrum of each sample was recorded from 320 to 230 µm at 50°C in a Unicam SP 800 Spectrophotometer equipped with a temperature control system. The temperature was then raised to 90°C to melt the DNA duplexes, and after 3' the samples were scanned again. The increment in extinction at 260 µm between 50°C and 90°C is due to the denaturation of the DNA duplexes formed during the incubation at 70°C.
By dividing $E_{260}(90^\circ C)$ by the hyperchromocity factor (1.34 for rat DNA), one obtains $E_{260}(50^\circ C)$ for native DNA. The ratio between the increment $E_{260}(50^\circ C) - E_{260}(90^\circ C)$ for renatured DNA, and the increment $E_{260}(50^\circ C) - E_{260}(90^\circ C)$ for native DNA gives the proportion of total DNA in duplex form. The percentage of renatured DNA was plotted as a function of Cot (DNA concentration at zero time expressed in moles of nucleotide per liter multiplied by the time expressed in seconds) according to Britten and Kohne (1966; 1968).

**DNA-RNA hybridization**

Molecular DNA-RNA hybridization was carried out according to the procedure of Mellix et al (1971) and Bishop (1972a).

The reaction mixtures made up in 0.1 x SSC contained 400 $\mu$g, 10 mg or 20 mg of DNA per ml, very small amounts of $^3$H-labelled RNA and 0.2%, 1% or 2% SLS respectively.

The mixtures were heated for 5 min in a boiling water bath to denature the DNA; a control sample was taken and frozen immediately or largely diluted with ice-cold 2 x SSC. The mixtures were then placed in an oil bath at the desired temperature and, after 30 sec., the salt concentration was adjusted to 2 x SSC. Samples were withdrawn and diluted in ice-cold 2 x SSC to reach a final SLS concentration of 0.005% in order not to interfere with RNAse digestion (see below).

**Estimation of the proportion of hybridized RNA as a function of time**

Each sample was divided into four equal portions. Two of these were treated with 20 $\mu$g/ml of pancreatic RNAse to digest non-
hybridized RNA and mismatched hybrids, and all four portions were incubated at 37°C for 30', then chilled in ice. 50 μg/ml of BSA were added as co-precipitant and cold 50% TCA to a final concentration of 10%. The samples were left 20' in ice and filtered on GF/C filters. The collected precipitate was washed with cold 5% TCA. The filters were placed in scintillation vials and dried in a vacuum oven at 80°C for 20'. 10 ml of Toluene PPO-POPOP were added and the samples were counted in a Packard scintillation counter. The percentage of total RNA annealed at a given time was calculated by the ratio of the RNAse treated samples to the untreated samples. The percentage of hybrid was plotted as a function of Cot (Melli et al, 1971).

Annealing of cDNA to rat liver DNA

cDNA was annealed to rat liver DNA according to the procedure of Bishop and Rosbash (1973).

The reaction mixtures contained 10 mg or 20 mg of liver DNA per ml and trace amounts of H³-labelled cDNA in 0.012 M PB, and SLS at the final concentration of 0.1% or 0.2%.

The mixtures were heated in a boiling water bath for 5' to denature the DNA, a control sample was taken and diluted with ice-cold water. The mixtures were then placed in an oil bath at 70°C and, after 30" the salt concentration was adjusted to 0.24 M PB. Aliquots were withdrawn at different times and diluted in ice-cold water.

Estimation of the proportion of annealed cDNA as a function of time

The extent of cDNA annealing was measured either by using the enzyme nuclease S₁ which digests single-strand DNA (Sutton, 1971).
or by fractionation on HAP.

In the first case, the samples withdrawn from the incubation mixture were diluted with water to a final concentration of 0.06 M PB. 1/5 vol of KB3 was added and the samples were divided into two equal portions. One portion was treated with nuclease S1, and both portions were incubated at 50°C for 40'. The samples were placed in ice and 50 μg of BSA per ml were added and TCA to 10% final concentration.

After 20' in ice the samples were filtered on GF/C filters and the filters were washed, dried and counted as previously described. The percentage of cDNA annealed at a given time was calculated by the ratio of the nuclease treated samples to the untreated ones, and plotted as a function of Cot.

When the double-strand DNA was fractionated on HAP, the samples taken from the incubation mixtures were diluted with water to 0.02 M PB.

The samples were loaded on HAP (1 gr of HAP/mg of DNA) in 0.02 M PB at 65°C. Single-strand DNA was eluted with 3 washes of 0.12 M PB and double-strand DNA with 3 washes of 0.4 M PB at 65°C.

The fractions were TCA precipitated, filtered and counted as described. The percentage of annealed DNA was calculated by the ratio of the cpm released with 0.4 M PB to the total cpm, and plotted against Cot.

Saturation curve of cDNA with cytoplasmic RNA

A fixed amount of 3H-labelled cDNA was hybridized to increasing amounts of unlabelled cytoplasmic RNA in 0.24 M PB at 70°C for 3 days.
The samples were diluted in ice-cold water to 0.06 M PB. 1/5 vol of KB3 was added and the samples were split into two equal portions. One portion was treated with nuclease S1, and all the samples were incubated at 50°C for 40'. After TCA precipitation, the samples were filtered, washed and counted as described.

The ratio of the nuclease-treated samples to the untreated ones gave the percentage of hybridized cDNA.

Hybridization of H3-labelled poly(U) along a gradient of RNA.

Aliquots from each fraction of a sucrose gradient were made 2 x SSC by adding an appropriate volume of 20 x SSC. A large excess of H3-labelled poly(U) (specific activity = 150000 cpm/µg) was added. The fraction were incubated for 30' at 40°C and then chilled in ice. 20 µg/ml of RNAse were added to each sample and the digestion was carried out in ice for 20'.

The samples were TCA precipitated, filtered and counted as described.
RESULTS

Incorporation of $H^3$-uridine into rat myoblasts

The first step in the analysis of RNA from rat myoblasts was to follow the incorporation of radioactive precursors into various fractions of RNA. In a typical experiment, cells were labelled with $H^3$-uridine for 30', 1 hr, 2 hrs, 4 hrs and 6 hrs. The overall synthesis of RNA was followed by monitoring the incorporation of $H^3$-uridine into acid-insoluble material in whole cells, in the nuclear fraction, and in the cytoplasmic fraction.

The incorporation of $H^3$-uridine into total cell RNA becomes linear with time after one hour (Fig. 1a). The incorporation into nuclear RNA shows a lag of about one hour, and the synthesis of RNA becomes linear only after two hours of exposure to the precursor (Fig. 1a). The incorporation into cytoplasmic RNA is linear up to two hours and then its rate increases (Fig. 1a). At this stage the radioactivity in cytoplasmic RNA becomes greater than in nuclear RNA due to the continuous transfer of RNA from the nucleus to the cytoplasm.

The nuclear and cytoplasmic RNA were analysed in more detail on sucrose gradients.

(a) Nuclear RNA

Fig. 2 shows the sedimentation profile of nuclear RNA extracted from cells pulse-labelled for different times.

After a 30' pulse (Fig. 2a), most of the label is in the 4S region; the 35S ribosomal RNA precursor is still visible and there are only traces of the mature 28S and 18S rRNA.
Legend to Fig. 1

(a) Incorporation of $^3$H-uridine into whole cells (■■), nuclear fraction (●○) and cytoplasmic fractions (▲▲). Cells were labelled with 100 μC/ml $^3$H-uridine for 30', 1 hr., 2 hrs, 4 hrs, 6 hrs. The cpm were corrected for the recovery of cells. The data are the average from 3 experiments.

(b) The cpm of the nuclear RNA fractions of the sucrose gradients in Fig. 2 were corrected for the recovery of cells and plotted against time. Average from 3 experiments

■■ = LMW RNA
●○ = 18S RNA
▲▲ = 28S RNA
□□ = HnRNA

(c) The cpm of the cytoplasmic RNA fractions of the sucrose gradients in Fig. 3 were corrected for the recovery of cells and plotted against time. Average from 3 experiments

■■ = LMW RNA
●○ = 18S RNA
▲▲ = 28S RNA
FIGURE 1

(a) 

(b) 

(c) 

[Graph showing data points and lines with cpm values on the y-axis and time (hrs) on the x-axis.]
Legend to Fig. 2

5-20% sucrose gradients in NTE of nuclear RNA extracted from cells labelled with 100 μC/ml H\textsuperscript{3}-uridine for 30', 1h., 2 hrs, 4 hrs, 6 hrs.

(a) Δ-Δ = 30'  o-o = 1 hr
(b) Δ-Δ = 2 hrs, Θ-Θ = 4 hrs, o-o = 6 hr.

The gradients were spun for 12 hrs at 24K, 1\textdegree C in the 6 x 16 rotor of an MSE 65 ultracentrifuge, and fractionated in 0.5ml fractions by an Isco fraction collector. The fractions were TCA-precipitated and filtered on GF/C filters. The filters were dried and counted in Toluene PPO-POPOP.
The material at 21S probably corresponds to the 22S RNA described by Steele (1968) in the nucleolar fraction of rat liver, which is thought to be one of the by-products of the processing of rRNA precursors. The low radioactivity in the heavy part of the gradient is due to the fast sedimenting fractions of HnRNA, part of which pelleted in the bottom of the tube.

After 1 hour labelling (Fig. 2a), the nuclear RNA shows a sedimentation profile which remains essentially unchanged even after a 6 hr pulse (Fig. 2b): the 35S prRNA has disappeared and the 28S and 18S rRNA are now distinct peaks.

RNA sedimenting in the 4S region (Low Molecular Weight RNA) is synthesized very rapidly, and the rate is linear (Fig. 1b). The amount of label in 28S and 18S rRNA increases continuously from 30' to 4 hrs, and reaches a plateau when as much RNA is exported to the cytoplasm as is synthesized (Fig. 1b). The amount of radioactive HnRNA (> 80S) increases slowly over the first two hours, but between 2 and 6 hrs of labelling the synthesis of this RNA is accelerated (Fig. 1b and inset).

(b) **Cytoplasmic RNA**

The synthesis of cytoplasmic RNA was followed in a similar manner to that described for nuclear RNA.

After 30' only LMW RNA is labelled. After 1 hr the 18S rRNA is strongly labelled and some label appears in 28S rRNA (Fig. 3a). The more rapid transport of 18S RNA than 28S RNA has been previously described by Joklik and Becker (1965), Girard *et al* (1965) and Penman *et al* (1966). The amount of labelled 18S rRNA is greater than that in 28S rRNA even after a 2 hr pulse (Fig. 3a), but the situation is
Legend to Fig. 3

5-20% sucrose gradients in NTE of cytoplasmic RNA extracted from cells labelled with 100 μC/ml H$^3$-uridine for 30', 1 hr, 2 hrs, 4 hrs, 6 hrs.

(a) △-△ = 30', o-o = 1 hr, □-□ = 2 hrs
(b) o-o = 4 hrs, □-□ = 6 hrs.

The gradients were centrifuged at 24K for 20 hrs at 1°C, in the 6 x 16 rotor of the MSE 65 centrifuge. Subsequent steps as in Fig. 2.
FIGURE 3

(a) and (b) show the distribution of cpm (counts per minute) across various fraction numbers. The graphs depict different patterns with peaks labeled 18 and 28, indicating specific areas of interest. The x-axis represents fraction No., while the y-axis shows cpm values, with markers indicating significant points.
reversed after 4 hrs (Fig. 3b), and the label in 28S RNA present in
the cytoplasm is greater than that in 18S rRNA after 6 hrs (Fig. 3b).
In these experiments the appearance of mRNA in the cytoplasm is
obscured by the large quantities of LMW RNA and rRNA. The rate of
labelling of cytoplasmic RNAs is compared with nuclear RNAs in Fig. 1c.
The delay of about 30' before label appears in the rRNA species (Fig. 2a)
is presumably due to the processing of the rRNA precursors (Scherrer and
Darnell, 1962; Scherrer et al, 1963; Perry, 1964). After this delay
the amount of label increases continuously. Whereas the 18S rRNA is found
in the cytoplasm in greater amounts than 28S rRNA during the first
2 hrs of pulse, the quantity of 28S rRNA increases more than 18S rRNA
in the subsequent period of exposure to the label. As in the nucleus,
the amount of LMW RNA in the cytoplasm increases steadily throughout
the labelling period.

Incorporation of $^3$H-uridine in AMD-treated cells

Similar experiments were carried out on cells treated with low
doses of Actinomycin D (AMD). In low concentration this drug
inhibits the synthesis of rRNA, without greatly affecting the synthesis
of other cellular RNA (Perry, 1963). The purpose of this series of
experiments was to follow the synthesis of HnRNA and the appearance of
mRNA, whose pattern is normally obscured by the presence of rRNA.

The kinetics of incorporation of uridine into whole cells and
nuclei are basically the same in the presence and absence of AMD
(compare Fig. 4a with Fig. 1a). The only apparent difference is in
the kinetics of appearance of cytoplasmic RNA. The level of cyto-
plasmic RNA always remains lower than nuclear RNA for two reasons:
Legend to Fig. 4

(a) Incorporation of $^3$H-uridine into the cellular fractions of cells treated with 0.04 µg/ml AMD. The cells were labelled with 100 µC/ml $^3$H-uridine in presence of the drug for 30', 1 hr, 2 hrs, 4 hrs, 6 hrs. The data are the average from 2 experiments.

- incorporation into whole cells.
- incorporation into the nuclear fraction.
- incorporation into the cytoplasmic fraction.

(b) Incorporation of $^3$H-uridine into nuclear LMW RNA ( ), 10S-40S HnRNA ( ) and 40S-100S HnRNA ( ) in presence of 0.04 µg/ml AMD.

The data have been processed as in Fig. 1b. Average from 2 experiments.

(c) Incorporation of $^3$H-uridine into cytoplasmic heterodisperse RNA in presence of 0.04 µg/ml AMD. The data are the average from 2 experiments and have been processed as in Fig. 1a.
FIGURE 4

(a) 

(cpm x 10^3)

(b) 

(cpm)

(cpm)

(cpm)

(time (hrs))

1 2 4 6

1 2 4 6

1 2 4 6
first, AMD has prevented the synthesis of the major RNA species, 28S and 18S rRNA, and second, it is now apparent that most of the HnRNA decays within the nucleus (Harris, 1963, 1964; Attardi et al., 1966) without ever reaching the cytoplasm.

(a) Nuclear and cytoplasmic RNA

No radioactive rRNA is present in nuclear RNA from drug treated cells (Fig. 5a). The only clear peak of radioactivity is given by LMW RNA. The rather broadly distributed radioactivity is due to HnRNA, which ranges from 10S to greater than 100S (Warner et al., 1966; Soeiro et al., 1966). The very heavy fractions of HnRNA form a pellet at the bottom of the tube. The rate of synthesis of LMW RNA is unchanged (Fig. 4b), showing that this species of RNA is insensitive to low doses of AMD.

Because the synthesis of rRNA has been suppressed, it is now possible to follow the kinetics of incorporation of radioactive uridine into HnRNA molecules of different sizes.

60S-100S HnRNA and 40S-60S HnRNA are synthesized at the same rate as giant HnRNA from untreated cells (Fig. 4b and inset). On the contrary, 10S-40S HnRNA is synthesized at a faster rate and its synthesis is linear with time (Fig. 4b). This result would be expected if 10S-40S HnRNA and 40S-100S HnRNA were synthesized from uridine pools of different sizes. This possibility seems unlikely in view of the results obtained by Wu and Soeiro (1971), who could not detect the presence of different nucleotide pools in Hela cells.

Another explanation of the observed result is that in rat myoblasts the nucleoplasmic RNA polymerase works at a slower rate than previously reported (Soeiro et al., 1968), and takes from one to two
Legend to Fig. 5

(a) 5-20% sucrose gradient in NTE of nuclear RNA labelled with 100 μC/ml H³-uridine in presence of 0.04 μg/ml AMD for 6 hrs. Gradient run for 12 hrs at 24K, 1¹C. 10μl aliquots from each fraction of the gradient were counted in Butyl PBD-Ethoxyethanol. The HnRNA was divided into four fractions for hybridization experiments.

Fraction I = 20-40S RNA  
Fraction II = 40-60S RNA  
Fraction III = 60-90S RNA  
Fraction IV = greater than 100S RNA

(b) 5-20% sucrose gradient in NTES of cytoplasmic RNA labelled with 50 μC/ml H³-uridine in presence of 0.04 μg/ml AMD for 90'. Gradient run for 24 hrs at 24K, 25⁰C. 10μl aliquots from each fraction were counted in Butyl PBD-ethoxyethanol
hours to transcribe completely the longest HnRNA molecules. The radioactivity detected during the first two hours of pulse in the heavy region of the gradient would then by due to giant HnRNA molecules labelled only in their 3' end whereas part of the radioactivity in the 10S-40S region would be due to the RNA molecules which were in the process of being elongated when extracted. After two hours, the newly synthesized giant HnRNA molecules are uniformly labelled and start accumulating.

The sedimentation pattern of radioactive cytoplasmic RNA from AMD-treated cells (Fig. 5b) is rather different from that reported for other systems. In HeLa cells for instance (Penman et al., 1970), labelled cytoplasmic RNA extracted from AMD-treated cells sediments with a sharp peak in the 4S region and with a very broad peak between the 18S and the 28S regions. In contrast, labelled cytoplasmic RNA from rat myoblasts gives a peak in the 4S region only; the rest is very heterogeneous in size and part of it pellets.

The kinetics of appearance in the cytoplasm of RNA other than 4S RNA (Fig. 4c) are remarkably similar to the kinetics of synthesis of HnRNA. Label in both types of RNA increases slowly during the first 2 hrs of exposure to H$^3$-uridine and more rapidly in the subsequent 4 hrs. This similarity is consistent with the relationship thought to exist between cytoplasmic RNA and HnRNA.

**Inhibition of RNA synthesis by high concentrations of AMD**

If cytoplasmic mRNA derives from HnRNA, the latter must be broken before entering the cytoplasm, since cytoplasmic mRNA is much smaller than HnRNA. An experiment was designed to look for a shift in the size of HnRNA, after its synthesis.
Cells were labelled for 2 hrs and further synthesis of RNA was stopped by adding high doses of AMD to the cultures. Samples were taken at 30', 1 hr, 2 hrs, and 4 hrs after the addition of AMD and the RNA extracted as usual. In the first hour of AMD treatment the amount of nuclear RNA decreased and this loss was accompanied by a corresponding increase in cytoplasmic RNA (Fig. 6a), due to the flow from the nucleus to the cytoplasm of RNA synthesized prior to the addition of the drug. After one hour, the nuclear and cytoplasmic RNAs followed parallel decay curves.

(a) Nuclear RNA

Nuclear RNA analysed on sucrose gradients (Fig. 7a and 7b) was arbitrarily divided into "giant" or "heavy" HnRNA (90S to greater than 100S) and "lighter" HnRNA (40S to 80S).

The LMW RNA rapidly disappears from the nucleus (Fig. 6b); by contrast both 18S and 28S rRNA increase during the first hour of chase, because the rRNA precursors synthesized before the addition of AMD continue to be processed, but subsequently the level of 28S and 18S RNAs decreases rapidly (Fig. 6b).

The decay of both heavy and light HnRNA is biphasic (Fig. 6b), suggesting the presence of at least two classes of HnRNA molecules. One class is made up of molecules with a very rapid turnover whose half-life is about 30'. The first part of the curves shows the rapid decay of these molecules. The other class contains more stable molecules with a half-life of 3-4 hrs, whose decay can be seen in the second part of the curves.

There is no accumulation of lighter HnRNA molecules, even if it
Legend to Fig. 6

(a) Cells labelled with 100 μC/ml H3-uridine for 2 hrs were treated with 4 μg/ml AMD, and samples were taken at 0 time, 30', 1 hr, 2 hrs, and 4 hrs. The decay of labelled RNA was followed in whole cells ( ), in the nuclear fraction ( ), and in the cytoplasmic fraction ( ).

(b) The cpm of the nuclear RNA fractions of the gradients in Fig 7 were corrected for the recovery of cells and plotted against time.

- - = LMW RNA
- - = 18S RNA
- - = 28S RNA
- - = 90S-100S HnRNA
- - = 40S-80S HnRNA

(c) The cpm of the cytoplasmic RNA fractions of 5-20% sucrose gradients in NTE were corrected for the recovery of cells and plotted against time.

- - = LMW RNA
- - = 18S RNA
- - = 28S RNA
Legend to Fig. 7

5-20% sucrose gradients in NTE of nuclear RNA extracted from cells labelled with 100 μC/ml H³-uridine for 2 hrs and then treated with 4 μg/ml AMD for 30', 1 hr, 2 hrs and 4 hrs.

(a) Δ—Δ = zero time chase
     ●—● = 30' chase

(b) □—□ = 1 hr chase
     ○—○ = 2 hr chase
     ▲—▲ = 4 hr chase

The gradients were run for 12 hrs at 24K, 1°C and analysed as in Fig. 2.
would appear that the rate of turnover of the more stable class of 40S-80S HnRNA is slower than the corresponding class of giant RNA.

(b) Cytoplasmic RNA

In the cytoplasm (Fig. 6c), the level of both LMW RNA and rRNA increases during the first 2 hrs in the presence of AMD, presumably due to the exit of these RNA species from the nucleus. The amount of LMW RNA and rRNA starts to decrease after 2 hrs, because there is no more synthesis of RNA and because eventually AMD interferes with the transport of RNA from the nucleus to the cytoplasm (Girard et al., 1964; Penman, 1966). It should be pointed out that this first series of experiments was carried out to acquire a general knowledge about the RNA metabolism in the system used. The data are useful insofar as they give general information, but do not allow accurate quantitative measurements, which would require more detailed experiments than the ones performed here.

LMW RNA from nucleus and cytoplasm of rat myoblasts

A large amount of low molecular weight RNA (LMW RNA) sedimenting in the 4S region of a sucrose gradient both in nuclear and cytoplasmic RNA was found in all experiments. The size distribution of this RNA was measured more accurately by polyacrylamide gel electrophoresis according to Loening (1967).

Labelled nuclear and cytoplasmic LMW RNA was purified on sucrose gradients and run on 15% polyacrylamide gels, with unlabelled Xenopus tRNA as a marker. The nuclear LMW RNA showed (Fig. 8a) a small amount of RNA co-migrating with Xenopus tRNA and two other discrete peaks of radioactivity, one of which is almost coincident with the dye
Legend to Fig. 8

15% polyacrylamide gel electrophoresis of (a) nuclear LMW RNA and (b) cytoplasmic LMW RNA. The electrophoresis was carried out for 5 hrs at 5mA/gel.

The gel-slices were digested with NH₃ overnight at room temperature. The NH₃ was allowed to evaporate and 20 ml of Butyl PBD-ethoxyethanol were added to each slice before counting.

x-x = radioactivity

_ = absorbance
Figure 8

(a) and (b) show the distribution of counts per minute (cpm) across different slice numbers. The graphs indicate peaks at specific slice numbers, with labels indicating areas of interest such as '4S' and 'dye'. The x-axis represents slice number, and the y-axis represents counts per minute (cpm) with scales of $10^2$ and $10^3$. The data suggests variations in the distribution across the slices.
bromophenol blue.

Cytoplasmic LMW RNA (Fig. 8b) on the other hand consists mainly of tRNA together with a small amount of 5S RNA, and the same two peaks already found in the nuclear material. The possibility that these two peaks were mononucleotides was checked by eluting the RNA from each fraction of a gel and precipitating it with TCA. The profile of radioactivity obtained in this way was the same as the one shown in Fig. 8b, eliminating contaminating nucleotides as a possible source of the fast-migrating material. The two peaks in question are clearly not related to rRNA because they are present in both the nucleus and cytoplasm of cells whose synthesis of rRNA has been inhibited by low doses of AMD. It was thought that this fast-migrating material might originate from some breakdown process occurring during cell fractionation and extraction of the RNA. Total RNA was extracted from whole cells without fractionation into nucleus and cytoplasm, and the LMW RNA was again analysed by gel electrophoresis. The two peaks of low molecular weight material are still present (Fig. 9a). In polyacrylamide gel, the migration distance is inversely proportional to the log of the MW. From this relationship, the MW of the fast-migrating RNA was calculated to be about 6400 daltons, corresponding to 21 nucleotides. Such RNA would have an S value of about 3.

To see whether this small RNA is in any way related to tRNA, it was analysed by finger-printing. Cells were labelled with $^{32}$P, the tRNA and the fast-migrating RNA were eluted from a polyacrylamide gel and digested with ribonuclease $T_1$, and the digestion products were
Legend to Fig. 9

(a) 15% polyacrylamide gel electrophoresis of LMW RNA extracted from whole cells.

Conditions of electrophoresis as in Fig. 8.

\[ x-x = \text{radioactivity} \]

\[ \_\_\_ = \text{absorbance} \]

(b) Base composition of LMW RNA.

A sample of LMW RNA was digested with alkali at 37°C overnight and analysed by paper electrophoresis.

The paper-strip was cut into 1 cm-long pieces, and counted in Toluene PPO-POPOP.
Plate I

Separation of a RNAse T1 digest of 4S tRNA by ionophoresis on cellulose acetate and chromatography on polyethyleneimine-cellulose (Southern and Mitchell, 1971).
Plate II

Separation of a RNAse T₁ digest of 3S RNA by ionophoresis on cellulose acetate and chromatography on polyethyleneimine-cellulose (Southern and Mitchell, 1971).
subjected to finger-printing (Southern and Mitchell, 1971): The maps of the 3S RNA and of the tRNA are almost superimposable (Plate I and II). There are differences in the relative abundance of some spots, but the two chromatographic patterns are essentially the same. The relatedness of the two RNAs is also indicated by the similarity of their base composition. A sample of 3S RNA was digested with alkali and the nucleotides analysed by paper electrophoresis (Fig. 9b). 3S RNA contains 30.4% C, 21.0% A, 28.0% G, and 20.4% U; close to the value for tRNA (Hatlen and Attardi, 1971).

The conclusions drawn from the results described above is that

\[ \text{the fast-migrating RNA/nucleus and cytoplasm of rat myoblasts is in some way related to tRNA, as already reported by von Heyden and Zachau (1971). The 3S RNA might be a breakdown product of tRNA as suggested by its small size, and by the observation that some of the spots in the finger-print of this RNA are less abundant than the correspondent spots in the finger-print of tRNA. Its role, however, has not been further investigated.} \]

**The kinetics of renaturation of rat liver DNA**

The renaturation kinetics of rat liver DNA has been analysed by Melli et al (1971). The analytical complexity of the haploid rat genome is $1.8 \times 10^{12}$ daltons (Steele, 1968). For renaturation studies the DNA was sonicated to a MW of $1.5 \times 10^{5}$. Reassociation experiments were carried out in 2XSSC at $70^\circ C$, $27^\circ C$ below the Tm. These conditions are also particularly suitable for DNA-RNA hybridization (Bishop, 1972 a and b). The renaturation kinetics of rat DNA, measured optically, is shown in Fig. 10a. The zero-time value is rather high in spite
of the rapid chilling of the denatured sample in low salt (0.1XSSC). Bishop and Rosbash (1973) suggested that the hypochromicity of the zero-time point is partially due to the very rapid reassociation of the highly repeated sequences (Britten and Kohn, 1966, 1968), but is mainly due to single-stranded DNA folding and free-stacking (Studier, 1969) and to the process Geiduschek (1962) calls 'type I reversibility', that is, a very rapid intramolecular reaction occurring even at low ionic strength, where 'true renaturation' is blocked by electrostatic repulsion.

If the zero-time point is subtracted from all the other values, then about 25% of rat DNA renatures before Cot 100. This fraction of the DNA is made up of sequences reiterated from 100000 to 100 times. The wide range of Cot values covered by the first part of the reassociation curve shows the presence of many heterogeneous families of sequences whose reassociation kinetics overlap. The bulk of the DNA renatures at higher Cot values, and exhibits slower but more homogeneous kinetics. The reaction seems to be complete at Cot $30 \times 10^3$. The main transition has a Cot $^{1\over 2}$ of 1000, which corresponds to the value expected for unique sequences.

The kinetics of rRNA hybridization in DNA excess

Uniformly labelled rRNA was extracted from cells grown for four generations in medium containing $^3$H-uridine. 28S and 18S rRNAs were purified by polyacrylamide gel electrophoresis (Loening, 1967) (Fig. 11). The specific activity of the RNA was between $2 \times 10^6$ and $3 \times 10^6$ cpm/μg. Hybridization was carried out in 2XSSC at 75°C instead of 70°C, to make allowance for the higher G-C content of
Legend to Fig. 11

(a) 2.5% polyacrylamide gel electrophoresis of rRNA extracted from cells labelled for four generations with 50 μC/ml 3H-uridine. The electrophoresis was carried out for 3 hrs at 3 mA/gel. The gel was sliced in 1 mm-thick slices and the rRNA was eluted from the slices with 0.5 ml of 0.6M Li acetate 0.5% SLS, overnight at room temperature. 10 μl aliquots were counted in Butyl PBD-ethoxyethanol

- - - - - radioactivity
       - - - absorbance

(b) Effect of increasing rRNA/rDNA ratios on the hybridization of rRNA in 2XSSC at 75°C. Fixed amounts of DNA and labelled rRNA were used and increasing amounts of unlabelled rRNA were added.
ribosomal genes (Marmur and Doty, 1961). Total DNA was in 350,000-fold excess of rRNA, giving an excess of ribosomal genes of 50-fold over rRNA. As expected, the curves for the two rRNA species are coincident (Fig. 10b), showing that 28S and 18S rRNA have the same reiteration frequency.

The reaction is half-completed at Cot 30, which corresponds to a reiteration frequency of 180 genes. This value was calculated from a comparison with the hybridization rate of E. coli cRNA with E. coli DNA and taking the analytical complexity of rat DNA to be $1.8 \times 10^{12}$ daltons and of E. coli DNA to be $2.7 \times 10^{9}$ daltons (Melli et al., 1971). From the reiteration frequency the amount of total single-stranded DNA complementary to 18S + 28S rRNA sequences is calculated to be 0.022%.

The kinetics of HnRNA hybridization in DNA excess

HnRNA was extracted from nuclei of cells labelled for 6 hrs with $\text{H}^3$-uridine, and the RNA was purified on a sucrose gradient. The fraction greater than 100S was taken to avoid contamination from rRNA precursors. In rat, the heaviest prRNA has an S value of 55 (Steele, 1968), so the Hn RNA greater than 100S (giant RNA) should be reasonably free from all rRNA species.

Because giant RNA can be detected only by radioactivity, its specific activity is unknown. To be sure that hybridization was performed in true DNA excess, large amounts of DNA (1 mg of DNA per sample) and the minimum amount of radioactive RNA were used.

The kinetics analysis of giant RNA (Fig. 12a) shows a remarkable heterogeneity; there is very little hybridization if any in the region of fast renaturing DNA; about 15% of the RNA hybridizes with
Legend to Fig. 12

(a) Hybridization of giant HnRNA in 2XSSC at 70°C. HnRNA was extracted from nuclei of cells labelled for 6 hrs with 100μC/ml H³-uridine and was purified on a 5-20% sucrose gradient in NTE.

(b) ▲-▲ = Hybridization of HnRNA greater than 100S in 2XSSC at 70°C. HnRNA was extracted from nuclei of cells treated with 0.04 μg/ml AMD for 30' and then labelled with 100 μC/ml H³-uridine. The HnRNA purified on a 5-20% sucrose gradient in NTE was divided into four fractions according to its S value (see Fig. 5a).

○-○ = Hybridization of HnRNA greater than 100S in presence of large amounts of unlabelled rRNA in 2XSSC at 70°C.

\[
\frac{rRNA}{rDNA} = 200.
\]
intermediate kinetics with Cot $\frac{1}{2}$ 20, corresponding to a reiteration frequency of about 300. This component was first observed by Melli et al (1971) in HnRNA from both cultured rat cells and rat liver.

The major part of giant RNA is complementary to the slow-renaturing DNA sequences. Since the reaction is only 40% complete, it is difficult to assess the Cot $\frac{1}{2}$ of the second component. The apparent Cot $\frac{1}{2}$ is about 3,000, suggesting hybridization to single DNA sequences. The reasons why the reaction does not go to completion are not clear. Probably the failure to hybridize 100% of the RNA is in part due to degradation of the RNA during the long reaction period. Another possible reason is that some hybrids are sensitive to RNase because of a certain degree of mismatching.

The S value of the chosen fraction of the HnRNA and the Cot $\frac{1}{2}$ of the first hybridizing component suggest that the hybridization recorded at low Cots is not due to contaminant rRNA.

To test this point more directly, cells were labelled in the presence of low doses of AMD to inhibit the synthesis of rRNA (Perry, 1963).

The HnRNA, extracted as above, was fractionated in a sucrose gradient into four fractions (Fig. 5a): Fraction 1 is RNA greater than 100S, fraction II is 60S-90S RNA, fraction III is 40S-60S RNA and fraction IV is 20S-40S RNA. The giant RNA (greater than 100S) hybridized under the same conditions showed a rate curve essentially the same as the one obtained with giant RNA from untreated cells (Fig. 12b). The hybridization of the first component is reduced
somewhat and the hybridization of the slow component (Cot 1000-10,000) has been left unaltered. To rule out completely that some rRNA synthesis had occurred during AMD treatment and was responsible for the hybridization at Cot 10-300, the experiment was repeated in presence of great amounts of unlabelled rRNA. The ratio between ribosomal RNA and ribosomal cistrons was greater than 200. Bishop (1972b) has shown that as the DNA/RNA complementary sequences ratio falls, the percentage of hybridization decreases till it reaches values close to zero when the RNA is in excess of the complementary DNA sequences.

At a rRNA/rDNA ratio of 100 the hybridization of ribosomal RNA is only 5% (Fig. 11b). At a rRNA/rDNA ratio of 200, the hybridization of any trace of labelled rRNA should then be virtually suppressed. A 200-fold excess of unlabelled rRNA did not alter at all the hybridization pattern of giant RNA (Fig. 12b).

The results of this series of experiments show that, as the recorded hybridization is not due to contaminant rRNA, HnRNA is complementary to, and hence transcribed from, both reiterated and unique DNA sequences.

In the case of the lighter fractions of HnRNA, less hybrid is formed between Cot 10 and 300, while the hybridization of the unique component remains the same. Less than 10% hybridization is reached by 60S-90S RNA at Cot 10-300 (Fig. 13a), even less by 40S-60S RNA, and there is no hybridization of 20S-40S RNA till Cot 100 (Fig. 13b). It is quite clear that the observed results reflect a true situation rather than being due to experimental variation.

Each HnRNA fraction was hybridized either two or three times and the differences in the hybridization level between Cot 10 and 300 were
found to be very reproducible.

From these experiments it is not possible to establish whether the HnRNA sequences transcribed on repetitive DNA and the sequence transcribed on single-copy DNA are part of the same molecule or belong to different molecules. Since RNAse digests the non-hybridized RNA molecules as well as "tails" which may be present at early stages of hybridization, its use in the measurement of the extent of hybrid formation does not allow a distinction to be made between the two possibilities.

The Kinetics of Cytoplasmic RNA hybridization in DNA excess

Total cytoplasmic RNA was extracted from cells labelled with \(^3\)H-uridine in the presence of low doses of AMD, as described in the methods section. The RNA was purified on a sucrose gradient and the 10-30S fractions were collected. It has been previously shown that in HeLa cells (Penman et al 1970) and L cells (Greenberg and Perry, 1971) mRNA sediments on sucrose gradients as a broad peak ranging from 10S to 30S. When rat myoblast cytoplasmic RNA is run on a sucrose gradient the only defined peak of radioactive material is found in the 4S region, in contrast to the broadly distributed radioactivity in the heavier portion of the gradient (Fig. 5b). The 10-30S RNA was used as the most likely to be mRNA, and was hybridized under standard conditions. As in the case of HnRNA the specific activity of cytoplasmic RNA is unknown, so to reach an excess of DNA over the RNA the same criteria applied to HnRNA hybridization were followed, namely maximum amount of DNA and minimum level of radioactive RNA.
Cytoplasmic RNA also hybridizes with heterogeneous kinetics (Fig. 14a). The hybridization of the first component reaches 15% between Cot 1 and Cot 100, with a Cot$^{1/2}$ of about 10, corresponding roughly to 300 copies. The major component hybridizes up to 50% at Cot 20,000. Since the reaction is not complete, the Cot$^{1/2}$ of the second hybridization step cannot be unequivocally defined. It is about 3,000, as expected for the hybridization of unique sequences.

Once again, the possibility of contamination by rRNA and/or tRNA responsible for the first hybridization step was taken into account. The labelled cytoplasmic RNA was run on polyacrylamide gels together with rRNA as a marker (Fig. 14b). The labelled RNA is distributed along the gel, with a great part of it migrating, as expected, slower than 10S. There are no clear peaks of radioactivity under the 18S and 28S absorbance peaks, indicating that the rRNA species have not been labelled, at least not to any appreciable extent.

Nevertheless, the presence of labelled 5S and 4S RNA is noticeable. According to Quincey and Wilson (1969), the number of genes per cell in rat liver for 5S RNA and 4S RNA is 1600 and 13,000 respectively. The first hybridization step might, therefore, be due to 5S RNA and/or tRNA, although it is unlikely that the DNA cistrons encoding these RNAs are in excess over the RNAs themselves. A competition experiment was carried out with unlabelled 4S and 5S RNA, and unlabelled rRNA in excess of the DNA, at the relevant Cot values. In spite of the very high RNA/DNA ratio, the percentage of hybridization at Cot 10, 30 and 100 is not different from the control values, suggesting strongly that the recorded hybridization is not due to contaminant cytoplasmic RNAs. (Table 1) The possibility that nuclear RNA is responsible for the first hybridization step is an unlikely one since 20S-40S RNA extracted
Legend to Fig. 14

(a) Hybridization of 10-30S cytoplasmic RNA in 2XSSC at 70°C.
Cytoplasmic RNA was extracted from the cytoplasmic fraction of cells labelled for 6 hrs with 100 μC/ml H³-uridine, and was purified on a 5-20% sucrose gradient in NTES.

(b) 2.5% polyacrylamide gel electrophoresis of 10-30S cytoplasmic RNA.
The electrophoresis was carried on for 1 hr 30' at 3 mA/gel. Gels were sliced in 1 mm-thick slices, digested with NH³ and counted in Butyl PBD-ethoxyethanol.

●● = radioactivity       _____ = absorbance
FIGURE 14

(a) Percentage Hybridization vs. log Cot.

(b) Count per minute (cpm) vs. slice No.
Effect of an excess of unlabelled tRNA and unlabelled rRNA on
the hybridization of $^3$H-labeled cytoplasmic RNA.

$$\frac{\text{trNA}}{\text{tDNA}} = 40 \quad \frac{\text{rRNA}}{\text{rDNA}} = 200$$

The hybridization was performed in 2 x SSC at 70°C.
from the same cells as cytoplasmic RNA exhibits only one hybridization transition (Fig. 13b) and the transcripts from reiterated DNA are present only in HnRNA heavier than 60S, (Fig. 13a).

Poly(A) sequences in rat myoblast RNA

The discovery of poly(A) stretches in mRNA (Kates, 1970; Lee et al., 1971; Edmonds et al., 1971; Darnell, et al., 1971) has recently enabled a better purification of this RNA. Virtually pure mRNA has been isolated on Millipore filters (Lee et al., 1971) exploiting the capacity of poly(A) to stick to nitrocellulose; and through columns of cellulose-poly(U) (Kates, 1970) or columns of cellulose-poly(dT) (Edmonds et al., 1971) and columns of cellulose-oligo(dT) (Aviv and Leder, 1972), exploiting the formation of stable hybrids between poly(U) or poly(dT) and poly(A).

In view of the possibility that the hybridization obtained with 10-30S cytoplasmic RNA was due to RNA different from mRNA, it was decided to purify poly(A)-containing RNA by means of one of the available methods.

The presence of poly(A) in rat myoblast RNA was checked by labelling the cells with H$_3$-adenosine. The cells were labelled for two hours with and without low doses of AMD. The cytoplasmic RNA was extracted following the usual procedure and analysed on a sucrose gradient (Fig. 15a). Once again when the RNA is labelled in the presence of AMD, there are no radioactive peaks coincident with the absorbance peaks, except for 4S RNA. The presence of poly(A) stretches in the RNA was detected by treating an aliquot from each fraction of the gradient with pancreatic RNAse which does/...
Legend to Fig. 15

(a) 5-20% sucrose gradients in NTE of cytoplasmic RNA extracted from cells labelled with 25 μC/ml H₃-adenine for 2 hrs with (△-△) and without (○-○) 0.04 μg/ml AMD.

_____ = absorbance

The gradients were run for 20 hrs at 24,000 rpm, 24°C and analysed as in Fig. 2.

(b) 5-20% sucrose gradient in NTES of cytoplasmic RNA labelled in presence of 0.04 μg/ml AMD. Aliquots from each fraction of the gradient were made 2XSSC and treated with 20 μg/ml pancreatic RNase at 37°C for 30'. Correspondent aliquots were kept as control. All the samples were TCA ppt and filtered on GF/C filters. The filters were counted in toluene PPO-POPOP.

△-△ = Control
○-○ = RNase treated
●-● = percentage of RNase resistance.
not digest long A-sequences, and monitoring the fraction of radioactive material unaffected by the treatment (Fig. 15b). Corresponding aliquots were kept as controls. 4S and 5S RNA are partially resistant to RNAse treatment because of their secondary structure, but the undigested portion is not more than 10%. The 10S-30S RNA is only partially digested by the RNAse, and the RNAse resistance reaches a maximum of 37% at about 26S. Such high resistance to RNAse treatment is indicative of the presence of poly(A) sequences in the RNA. An aliquot of the A-labelled RNA was loaded on a sepharose-poly(U) column, according to the method of Kates (1970). The poly(A) stretches of the RNA hybridize to the poly(U) complexed with the sepharose. Any non-hybridized RNA is eluted with high salt buffer; lowering the salt and increasing the temperature causes the melting of the poly(A)-poly(U) hybrids and the release of mRNA. The elution profile of A-labelled RNA from a sepharose-poly(U) column is shown in Fig. 16a. The RNA lacking poly(A) was eluted with 2XSSC at room temperature, and made up 53% of the total cpm (Table II). The RNA containing short poly(A) sequences poorly hybridizing to poly(U) was eluted with 10mM TRIS at room temperature and constituted 15% of the total cpm (Table II). Raising the temperature to 45°C caused the release of the remaining 32% of the counts representing RNA molecules which had formed stable hybrids with poly(U).

The three eluted fractions were tested for RNAse resistance (Table II). The RNAse resistance of the high salt fraction was 18%, somewhat higher than the value found for the 4S fraction of a sucrose gradient (Fig. 15b). The resistance of the low salt fractions eluted at room temperature and at 45°C was 37% and 34% respectively, in agreement with
Legend to Fig. 16

(a) Elution profile of cytoplasmic RNA labelled with $^3$H-adenine in the presence of 0.04 $\mu$g/ml AMD from a sepharose-poly(U) column.

The first arrow indicates the shift in buffer from 2XSSC to 10mM TRIS, and the second arrow indicates the shift in temperature from room temperature to 45°C.

0.5 ml fractions were collected and 10 $\mu$l from each fraction counted in Butyl PBD-ethoxyethanol.

(b) Elution profile of cytoplasmic RNA labelled with 50 $\mu$C/ml $^3$H-uridine for 90' in the presence of 0.04 $\mu$g/ml AMD from a sepharose-poly(U) column.

The first arrow corresponds to the shift in buffer from 2XSSC to 10 mM TRIS and the second arrow to the shift in temperature from room temperature to 45°C.

10 $\mu$l aliquots from each 0.5 ml fraction were counted in Butyl PBD-ethoxyethanol.
### TABLE II

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<th>2 x SSC</th>
<th>10mMTRIS 20°C</th>
<th>10mMTRIS 45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Total cpm</td>
<td>53</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>% of RNase Resistance</td>
<td>18</td>
<td>37</td>
<td>34</td>
</tr>
</tbody>
</table>

H\(^3\)-Adenine labelled cytoplasmic RNA eluted from a Sepharose-poly(U) column with 2 x SSC, 10m M TRIS at room temperature and 10m M TRIS at 45°C; and percentage of RNAse resistance of the three fractions. The digestion with pancreatic RNAse was carried on in 2 x SSC at 37°C for 30' with 20μg/ml RNAse.
the results obtained with the heavier fractions of a sucrose gradient (Fig. 15b).

In a further experiment $H^3$-uridine-labelled cytoplasmic RNA was purified on sepharose-poly(U) column (Fig. 16b). 63% of the labelled RNA was eluted with high salt, in agreement with the finding that from 60 to 70% of the RNA labelled in presence of AMD bands in the 4S region of a sucrose gradient (Fig. 5b).

13% of the RNA was eluted with 10 mM TRIS at room temperature and 15% at 45°C. The high salt fraction and the 45°C fraction were analysed on sucrose gradients (Fig. 17a). As expected, the high salt fraction was made up essentially of 4S RNA, and there was virtually no material in the heavier regions. The RNA of the 45°C fraction (mRNA?) sedimented as a rather broad peak with a mean S value of 10-11. This RNA is smaller than expected on the basis of the previous results for a heterogeneous population of mRNA. One possible reason for the decrease in size is thermal degradation due to the shift in temperature from 20°C to 45°C.

Hybridization kinetics of mRNA purified on a sepharose-poly(U) column

Birnstiel et al (1972) investigated the effect of the size of RNA molecules on the rate of DNA-RNA hybridization in liquid and found that the rate of the reaction is independent of the RNA size. Since the reduction in size of RNA from rat myoblasts would not affect the hybridization rate, the presumptive mRNA purified on sepharose-poly(U) was hybridized to DNA in 2XSSC at 70°C.

The mRNA hybridized with the same kinetics as 10-30S cytoplasmic RNA (Fig. 17b and Fig. 14a). One possible objection to this result is that the presumptive mRNA may be broken down and that the consequent
Legend to Fig. 17

(a) 5-20% sucrose gradient in NTES of the high-salt RNA and the low-salt RNA eluted from a sepharose-poly(U) column. Gradients run for 20 hrs at 24K, 24°C and analysed as in Fig. 2.

Δ-Δ = High-salt RNA
●-● = Low-salt RNA

(b) Hybridization of poly(A)-containing RNA eluted from a sepharose-poly(U) column in 2XSSC at 70°C.
loss of some sequences may alter the hybridization pattern. If the lost sequences are complementary to unique DNA, then the percentage of RNA complementary to repeated DNA would be over-estimated.

**Hybridization kinetics of mRNA purified on a cellulose-oligo(dT) column**

The previous result was confirmed however by experiments performed with mRNA purified on a cellulose-oligo(dT) column (Edmonds et al., 1971). It was decided to use cellulose-oligo(dT) rather than sepharose-poly(U) to avoid the increase in temperature and the consequent breakage of the mRNA, since lowering the salt is enough to melt oligo(dT)-poly(A) hybrids and to cause the release of mRNA.

From 10 to 15% of the labelled cytoplasmic RNA was eluted with low salt from a cellulose-oligo(dT) column, in agreement with the 15% released at higher temperature from sepharose-poly(U) (Table II). The size of the two RNA fractions eluted from cellulose-oligo(dT) was analysed on sucrose gradients (Fig. 18b). As in the case of RNA from sepharose-poly(U), the fraction eluted with high salt consisted mainly of 4S RNA. On the contrary, mRNA from cellulose-oligo(dT) sedimented in a much more heterogeneous way and its average size was greater than mRNA from sepharose-poly(U) (Fig. 17a and Fig. 19b), suggesting that the smaller size of the latter was due to breakdown.

To make sure this RNA did contain poly(A) and was not the result of unspecific binding of other RNAs to the cellulose, aliquots from each fraction of a sucrose gradient of presumptive mRNA were hybridized to labelled poly(U) (Fig. 18b). The hybridization was performed in 2XSSC at 40°C for 30' and the unreacted sequences were digested with RNase, so that only the hybrid formed between labelled poly(U) and unlabelled
Legend to Fig. 18

(a) Elution profile of cytoplasmic RNA labelled with 50 µC/ml \( {\text{H}}^3 \)-uridine for 90' from a cellulose-oligo(dT) column. The arrow indicates the shift in buffer from binding buffer to eluting buffer. 0.5 ml fractions were collected and 10 µl from each fraction was counted in Butyl PBD-ethoxyethanol.

(b) 5-20% sucrose gradients in NTES of the high-salt RNA and the low-salt RNA from a cellulose-oligo(dT) column and hybridization of labelled poly(U) to the low-salt RNA. Aliquots from each fraction of the gradient of poly(A) containing RNA were made 2XSSC and incubated with labelled poly(U) at 40°C for 30'. The samples were treated with 20 µg/ml pancreatic RNAse for 20' at 0°C, TCA ppt and filtered. The filters were counted in toluene PPO-POPOP. Corresponding aliquots were used as control.

- = High-salt RNA
- = Low-salt RNA
- = poly(U) hybridized
poly(A) would be detected.

The poly(U) hybridized to all the fractions of the gradient, showing that the RNA eluted with low salt contains indeed poly(A) stretches. The higher amount of hybridization of poly(U) to the light region of the gradient is presumably due to the accumulation of old mRNA molecules of smaller size (Singer and Penman, 1973). The hybridization of poly(U) would thus reflect the size-distribution of stable molecules, whereas the radioactive mRNA profile would reflect the size-distribution of newly synthesized molecules.

The mRNA from cellulose-oligo(dT) was hybridized to DNA under standard conditions (Fig. 19a). The hybridization kinetics of this RNA are essentially the same as the one obtained with mRNA from sepharose-poly(U) and the one obtained with 10-30S cytoplasmic RNA (Fig. 14a, 17b and 19a).

One possible interpretation of the results so far obtained is that the hybridization recorded is the hybridization of true mRNA. If this is the case, the two-step hybridization pattern can be explained by either an intramolecular heterogeneity of the mRNA (i.e. part of each molecule is transcribed from repeated DNA and part from unique DNA) or an intermolecular one (i.e. some molecules are transcribed from reiterated DNA sequences and some from single sites).

Another possible interpretation of the data is that one of the two hybridizing components is not mRNA. In this case, it is either some cytoplasmic RNA, containing poly(A) stretches but not performing messenger functions, or some nuclear RNA, containing poly(A), present in the cytoplasmic fraction as the result of nuclear leakage and
In order to discriminate between the two interpretations discussed above, it was decided to purify polysomal RNA, on the assumption that the last is true mRNA engaged in protein synthesis.

**Hybridization Kinetics of polysomal mRNA**

Polyribosomes were isolated from cells labelled in the presence of low AMD, and purified on a sucrose gradient (Fig. 20a). Polysomal RNA was extracted by SLS treatment and loaded onto a cellulose-oligo(dT) column, to isolate poly(A)-containing mRNA. Aliquots from the high salt fraction and the low salt fraction of the cellulose column were run in sucrose gradients (Fig. 20b). The radioactive RNA which did not bind to oligo(dT) is 4S RNA, while the poly(A)-containing RNA is heterodisperse and its sedimentation profile is very similar to the one obtained with total mRNA (Fig. 18b). The polysomal mRNA was hybridized to DNA under standard experimental conditions (Fig. 19b). Clearly the isolation of mRNA from polysomes has failed to suppress either of the two hybridizing components. Polysomal mRNA hybridizes with kinetics almost identical to those of 10-30S cytoplasmic RNA, poly(U)-sepharose mRNA and oligo(dT)-cellulose mRNA. This result eliminates the possibility of nuclear RNA, or some other RNA different from mRNA, being responsible for one of the two hybridization steps, and allows the conclusion that mRNA of rat myoblasts does indeed contain sequences transcribed from reiterated DNA, as well as from unique DNA.
Legend to Fig. 20

(a) 15-30% sucrose gradient in RSB of the cytoplasmic fraction of cells labelled with 50 μC/ml H³-uridine for 90' in presence of 0.04 μg/ml AMD. The gradient was run at 26K for 3 hr 30' at 0°C, and 1 ml fractions were collected. 50 μl aliquots were counted in Butyl PBD-ethoxyethanol. The fractions containing the bulk of the polysomes were made 0.5% SLS and alcohol ppt.

- o-o = radioactivity
- - - - - - = absorbance

(b) Sedimentation profile of polysomal RNA from a cellulose-oligo(dT) column.

- ▲▲ = High-salt RNA
- ●● = Low-salt RNA

5-20% sucrose gradients in NTES run for 22 hrs at 24,000 r.p.m., 24°C.

0.5 ml fractions were collected, TCA-precipitated and filtered. The filters were dried and counted in Toluene PPO-POPOP.
In vitro synthesis and characterization of cDNA

Since the discovery of the RNA-dependent DNA-polymerase (reverse transcriptase) in oncogenic viruses (Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman et al., 1970), this enzyme has been used to transcribe specific mRNAs into complementary DNA (Verma et al., 1972; Kacian et al., 1972). The cDNA so obtained has been successfully used in hybridization experiments (Bishop and Rosbash, 1973).

The advantages of cDNA over its RNA template are its very high specific activity and its greater resistance to thermal degradation. Moreover, the excess of unlabelled DNA over cDNA does not need to be as great as the excess of DNA over RNA.

Unlabelled mRNA was extracted from the cytoplasmic fraction of rat myoblasts, purified through cellulose-oligo(dT), and transcribed into cDNA of very high spec. activity (about $6 \times 10^6$ cpm/pg) by the reverse transcriptase of avian myeloblastosis virus. The size of the mRNA template was measured by running an aliquot in a sucrose gradient and hybridizing labelled poly(U) to each fraction of the gradient (Fig. 21a). As found previously (Fig. 18b) poly(U) hybridized to every fraction of the gradient. The similarity of the results allows the assumption that the size of the unlabelled RNA is the same as the size, measured directly, of $^3H$-labelled mRNA.

The size of the cDNA transcript was measured in an alkaline sucrose gradient. $^{14}C$-poly(dT) with an S value of 8.4 was used as a marker in a parallel gradient (Fig. 21b). The cDNA has a mean sedimentation coefficient of 5.4; the peak is rather broad suggesting the presence of transcripts of different sizes.
Legend to Fig. 21

(a) Hybridization of labelled poly(U) to the fractions of a 5-20% sucrose gradient in NTES of unlabelled mRNA eluted from a cellulose-oligo(dT) column.

The gradient was run at 33,000 r.p.m. for 7 hrs. at 24°C in the Spinco SW41 rotor.

The 0.5 ml fractions were made 2XSSC and incubated with labelled poly(U) at 40°C for 30'. The samples were treated with 20 µg/ml pancreatic RNAse at 0°C for 20' and TCA ppt and filtered.

The filters were counted in Toluene PPO-POPOP.

\[\text{-} = \text{hybridized poly(U)}\]

\[\text{-----} = \text{absorbance of marker RNA}\]

(b) 5-20% sucrose gradients in 0.1N NaOH, 0.5% SLS of C\textsuperscript{14}-poly(dT) and H\textsuperscript{3}-cDNA run at 33K for 20 hrs at 24°C in the Spinco SW41 rotor.

0.5ml fractions were counted in 3ml of Instangel (Packard).

\[\text{-} = \text{poly(dT)}\]

\[\square = \text{cDNA}\]
In agreement with previous results (Imizumi et al., 1973; Diggelmann et al., 1973), the reverse transcriptase has not copied the whole template, but only a portion of it. Since the enzyme works from the 3' end to the 5' end of the RNA molecules (the poly(A) stretch at the 3' terminus acting as a primer), the RNA sequences at the 5' ends of the molecules will not have been copied.

The single-strandness of cDNA was tested by incubating an aliquot at 70°C for 24 hrs, and then digesting single-strand DNA with Nuclease S₁ (Sutton, 1971). Even after a 24 hrs incubation, only 2% of the cDNA is resistant to the nuclease, indicating that it is virtually all single-stranded (Table III).

The complementarity of the cDNA to mRNA was tested by hybridizing a fixed amount of cDNA with increasing amounts of unlabelled cytoplasmic mRNA and digesting the unreacted sequences with Nuclease S₁. An apparent saturation plateau is reached at 60% hybridization (Fig. 22), but it seems that the reaction could continue at higher inputs of RNA. The failure to reach 100% hybridization may then be due to the very low concentration of some RNA molecules in the whole population of mRNA. The reaction is half-complete at a mRNA input of 0.04 μg, corresponding to a Rot₁² (Rot = RNA concentration/time) of 1. It is possible to obtain an estimate of the number of the different reacting RNA sequences by comparing the Rot₁² of the reaction with the Rot₁² of known systems, such as haemoglobin mRNA-cDNA (Bishop and Rosbash, 1973). It has been calculated that 60% of the total mRNA population is made up of at least 500 different mRNA molecules.
Test for single-strandness of cDNA.

3 mixtures were made in 0.06 M PB. One mixture was kept as zero-time control (0'). The other two mixtures were placed in a boiling water-bath for 5'. One was immediately chilled in ice and kept as control (0'). The remaining mix was made 0.24 M PB and placed in an oil-bath at 70°C for 24 hr.

All three mixtures were divided into two equal aliquots. One set of aliquots was treated with nuclease S₁ and all the fractions were incubated at 50°C for 40'. The fractions were TCA ppt and filtered and the filters were counted in Toluene PPO-POPOP.
FIGURE 22

0% Hybridization vs. µg RNA

0.2  0.5  1  

70  60  50  40  30  20  10
The kinetics of cDNA-DNA renaturation

The cDNA was annealed to an excess of DNA in 0.24M PB at 70°C for different times, and any unreacted DNA sequences were digested with Nuclease S1 (Fig. 23a). The cDNA reached 70% annealing at Cot 30,000, with only one major transition. However, the spread of the curve is indicative of the presence of sequences with different degrees of repetition. If the curve is considered as a whole, its Cot1/2 is 200, corresponding to an average reiteration frequency of about 5. Since single-copy DNA reassociates with Cot1/2 1000 (Fig. 10a), the above results show that both the "repeated" RNA sequences and the "unique" ones have been copied into cDNA.

The lack of a clearly identifiable first transition in the annealing of the cDNA indicates that the repetitive RNA sequences are under-represented in the cDNA, possibly because they are located near or at the uncopied 5' end of the RNA molecule.

The experiment was repeated measuring the extent of annealing by HAP fractionation (Fig. 23b) rather than by nuclease digestion.

This procedure should give an answer as to whether or not the repeated sequences and the unique ones are part of the same molecule. HAP binds any amount of double-strand DNA irrespective of "tails", so that if the two kinds of sequences are covalently linked, the reaction will appear faster than when monitored by the use of the enzyme.

15% of the cDNA binds to HAP even at zero time (Fig. 23b). When a parallel control is made with E. coli DNA instead of rat DNA, only 2% of the cDNA binds to HAP (Fig. 23b), suggesting that the binding observed in presence of rat DNA may be due to a very little amount of
Legend to Fig. 23

(a) Annealing of cDNA to liver DNA in 0.24M PB at 70°C.
The extent of annealing was measured by the use of nuclease $S_1$.

(b) Annealing of cDNA to liver DNA in 0.24 MPB at 70°C.
The extent of annealing was measured by fractionation on HAP.

■ = zero-time control with liver DNA
● = zero-time control with *E. coli* DNA
duplex which has formed even in non-optimal conditions (i.e. low salt and rapid quenching). Any unreacted sequences linked to the sequences that have already annealed would then be bound to HAP.

Such a very little amount of duplex would be undetected by using the nuclease, which would completely digest the unreacted sequences (see the zero-time point of Fig. 23a).

The overall shape of the annealing curve obtained by HAP fractionation is the same as the one obtained by nuclease treatment.

The percentage of bound cDNA increases slowly up to Cot 30, and reaches 85% at Cot 5,000, when the reaction is essentially complete.

If the zero-time point is not subtracted from all the other points, the Cot$\frac{1}{2}$ of the curve is 100. As expected, the reaction monitored by HAP is twice as fast as the reaction monitored by nuclease. The shift of the curve towards lower Cots is however not big enough to conclude that all the repetitive sequences are covalently linked to the unique sequences, rather suggesting a certain intermolecular heterogeneity, in addition to the intramolecular one.

These conclusions must however be regarded as tentative, since more experiments are needed before they can be fully substantiated.
DISCUSSION

The aim of the work described in this thesis was to characterize the RNA fractions of a given cell type, to establish their place in the metabolic pathway (from synthesis in the nucleus, to processing, and to appearance in the cytoplasm) and to analyse their relationship to defined fractions in the DNA. Particular attention has been paid to the study of the relationship occurring between the several size-fractions of HnRNA and between HnRNA and mRNA.

Low Molecular Weight RNA

The presence of LMW RNA in rat myoblasts was detected while the metabolism of other cellular RNA fractions was under study. The LMW RNA is present both in the nucleus, where it is rapidly synthesized, and in the cytoplasm, to which it is rapidly transferred. Its base composition is rich in G-C (58.4%), and its finger-print resembles very much the finger-print of tRNA. Although its association with chromosomal proteins and its content in dihydropyrimidine bases have not been investigated, its characteristics tend to rule out the possibility that this RNA is the chromosomal RNA detected by many workers in several animal and plant tissues (Bonner et al., 1968; Huang and Bonner, 1965; Dahmus and McConnel, 1969), and thought to be involved in the fine control of gene expression (Bonner and Widholm, 1967; Bechor et al., 1969; Mayfield and Bonner, 1972). In fact, the "chromosomal RNA" is highly stable, is nucleus-restricted and has a DNA-like base composition.

The presence of LMW RNA in cells whose rRNA synthesis has been suppressed, shows that this RNA fraction is not a degradation product of rRNA, as suggested by Artman and Roth (1971) and Arnold and Young (1972). LMW RNA seems rather to be related to tRNA, as already reported
for calf thymus chromatin-associated RNA (von Heyden and Zachau, 1971). It is difficult, however, to compare the properties of rat myoblast LMW RNA with those described for other low molecular weight RNA fractions, because of the many differences in extraction procedures and techniques of analysis. Other authors were specifically concerned with the RNA of chromatin, whereas this work has been conducted on total RNA, and the association of the RNA with proteins has not been investigated.

Recently, the involvement of RNA in DNA replication has become apparent. The conversion of M13 DNA to its replicative form is primed by a short RNA chain (Wickner et al., 1972), and DNA synthesis in E. coli proceeds by the extension of small RNA fragments (Sugino et al., 1972; Lark, 1972). The DNA polymerase from KB cells is able to replicate single-stranded circular TX DNA when primed by short RNA chains (Keller, 1972) and it has been shown (J.M. Bishop - in press) that the synthesis of AMV provirus requires 4S tRNA as a primer.

In the light of these recent discoveries, it is tempting to speculate that LMW RNA is not a mere breakdown product of tRNA, but takes active part in the control of DNA replication. If this is the case, it should be expected that suppression of transcription would cause inhibition of DNA replication, and it should be possible to isolate LMW RNA in the form of a RNA-DNA complex.

**Characterization of HnRNA**

(a) **Rate of synthesis**

The rate of incorporation of $^3$H-uridine into rat myoblast HnRNA becomes maximal after about two hours of exposure to the isotope,
presumably when new complete molecules start to accumulate.

Most of the labelled giant HnRNA molecules detected during the first two hours of the experiment may then be labelled only at their 3' end. If this interpretation is correct, the nucleoplasmic RNA polymerase takes from one to two hours to synthesize a RNA molecule about $50 \times 10^3$ nucleotides long. The rate of transcription is thus about 10 nucleotides per second.

This estimate is in disagreement with the one made for HnRNA in HeLa cells. It is known that HeLa cell precursor rRNA is synthesized at a rate of 100 nucleotides per second (Greenberg and Penman, 1966). Soeiro and collaborators (Soeiro et al., 1968) taking into account the larger size of HnRNA and its shorter half-life, worked out that the rate of transcription of HnRNA is about four times as fast as that of rRNA.

On the other hand, Gall and Callan (1962) found that the giant loops of Triturus lampbrush chromosomes become uniformly labelled only after ten days of exposure to $^3$H-uridine. The giant loops are transcribed into RNA molecules of at least $200 \times 10^6$ daltons (Miller et al., 1972). The RNA polymerase then takes $800 \times 10^3$ seconds to synthesize molecules $600 \times 10^3$ nucleotides long, working at a rate of less than one nucleotide per second.

Since, however, the RNA is a minimum estimate, the rate of transcription could be faster.

It could be speculated that the rate of HnRNA synthesis is related to the physiological state of a particular cell type. The fast rate of transcription in HeLa cells is probably related to the relatively undifferentiated tumoral nature of these cells, whereas the
slowly maturing amphibian oocytes or the partially differentiating myoblasts synthesize HnRNA at a slower rate.

(b) Pattern of decay

The decay-curve of HnRNA shows the presence of at least two populations of molecules with different half-life. The short-lived component turns over in about 30', while the more stable molecules have a half-life of 3-4 hours. The situation parallels the one found in HeLa cells (Scherrer et al., 1970), where a rapid wave of decay is followed by a slower one.

This pattern of decay is common to both giant (90->100S) and 40S-80S HnRNA, and no accumulation of HnRNA molecules of small size could be detected during a chase experiment. This finding would argue against a processing mechanism which cleaves giant RNA molecules to molecules of lower molecular weight, as suggested by Scherrer et al., (1970) and Georgiev et al. (1972).

The chase experiments, however, were performed in the presence of AMD. It cannot be excluded that AMD interferes with the normal cleavage of giant RNA, for instance by disrupting the HnRNA-protein complexes (Samarina et al., 1968), in the same way as it destabilizes polysomes (Murphy and Attardi, 1973; Singer and Penman, 1972).

(c) Hybridization properties

Previous studies have shown that the majority of HnRNA is complementary to unique RNA, and that a small portion of it is complementary to repeated DNA sequences. This is the case for rat HnRNA (Melli, et al., 1971), L cell HnRNA (Greenberg and Perry, 1971) and HeLa cells HnRNA (Pagoulatos and Darnell, 1970).
Whereas these studies were performed on total HnRNA, or on the
heaviest fractions of it, in the work reported here rat myoblast
HnRNA was divided into size-classes and all the classes were analysed
separately.

It was found that about 70% of the reacting giant (>100S) HnRNA
is complementary to unique DNA, and that the remaining fraction is
complementary to DNA sequences of intermediate repetition frequency.
No hybridization has been detected at high Cot values corresponding
to the renaturation of the highly repetitive sequences, suggesting
that, as in the mouse (Flamm et al., 1969), these sequences are not
transcribed in vivo.

It was consistently found that the fraction of HnRNA hybridizing
to repetitious DNA decreases progressively with the decrease of the
molecular weight of the RNA. In contrast with the giant HnRNA, the
lighter HnRNA fractions (20S-60S) react only at high Cot values,
expected for the renaturation of single-copy DNA.

The reproducibility of these results rules out the possibility
that the differences in the hybridization pattern are due to experimen-
tal variation.

One possible interpretation of the results is that the HnRNA
molecules greater than 100S undergo a maturation process with gradual
cleavage of the sequences complementary to repeated DNA, till the
smaller molecules are complementary only to rare DNA sequences.
An alternative explanation is that many classes of HnRNA molecules
of different sizes are synthesized. The longer molecules are tran-
scribed from both unique and repeated DNA sites, while the shorter
ones are transcribed from single-copy DNA only.
Since the processing of giant HnRNA to molecules of lower molecular weight could not be detected, the alternative of many independent transcripts is at the moment to be preferred.

Another implication of the results should be pointed out. If the giant HnRNA is an aggregate of smaller molecules, as suggested by some authors (de Kloet et al., 1970; Bramwell, 1972), then the aggregation process would be a non-random one. It would in fact involve only sequences transcribed from reiterated DNA, which are presumably both structurally and functionally different from the other sequences.

**Relationship between HnRNA and mRNA**

As in the case of giant HnRNA, 70% of the reacting mRNA hybridizes to single-copy DNA, and the remaining 30% to moderately repeated DNA sequences. The possibility that the fraction of mRNA hybridizing at low Cots represents HnRNA lost from the nucleus during cell fractionation, is made unlikely by two considerations: HnRNA of the same size as mRNA does not contain transcripts from repeated DNA; and poly-somal RNA, that is, true mRNA engaged in protein synthesis, still exhibits a heterogeneous hybridization kinetics.

These results, together with the finding that the kinetics of appearance of labelled cytoplasmic RNA are very similar to the kinetics of synthesis of giant HnRNA, strongly suggest that only >60S HnRNA contains sequences destined to become mRNA.

In fact, the lighter HnRNA molecules are complementary only to unique DNA, while mRNA hybridizes to the same DNA sequences, or at least to DNA sequences falling in the same range of repetitiveness,
to which the heavier fractions of HnRNA hybridize.

These findings suggest that mRNA is liberated by the first, or one of the first cleavage steps in the metabolic pathway of giant HnRNA. The non-messenger sequences of HnRNA apparently do not enrich the population of molecules of lower molecular weight but are metabolized. The HnRNA transformation scheme differs from that proposed by Georgiev and collaborators (1972), according to which the giant HnRNA is cleaved in a stepwise fashion, giving rise to progressively smaller molecules, which eventually are transferred to the cytoplasm.

The pathway of HnRNA cleavage then changes from

\[
\begin{align*}
giant \text{ HnRNA} & \rightarrow \text{ light HnRNA} \rightarrow \text{ mRNA} \\
giant \text{ HnRNA} & \rightarrow \text{ mRNA} \rightarrow \text{ light HnRNA}
\end{align*}
\]

It is interesting to note that no fraction of HnRNA has been detected which is transcribed from DNA sequences more repeated than those from which mRNA is transcribed, and that, contrary to what found by Greenberg and Perry (1971) in L cells, there is no preferential loss of the transcripts of reiterated DNA during the maturation process of HnRNA to mRNA. It would rather appear that there is a certain enrichment of repeated sequences in mRNA.

These observations, together with the finding that the lighter fractions of HnRNA are not precursors to mRNA, lead to the conclusion that most, if not all, of the nucleus-restricted HnRNA derives from unique DNA sequences. Therefore, in a given cell type and in a particular physiological state, a considerable fraction of single copy
DNA does not code for proteins, but is transcribed into RNA which may be involved in regulatory mechanisms, or may perform an informational role in another cell type or in another moment of the cellular cycle.

**Molecular heterogeneity of RNA**

The two-step hybridization pattern common to giant HnRNA and mRNA is clearly indicative of a molecular heterogeneity. The use of RNAse in the measurement of the extent of hybrid formation makes it impossible to distinguish between an intermolecular heterogeneity and an intramolecular one. If an RNA molecule is internally heterogeneous, the part of it complementary to multiple DNA copies hybridizes at low Cot values while the portion transcribed from unique DNA forms a "tail" which is digested by RNAse. Only at higher Cot values does the second part of the molecule hybridize to its complementary DNA, forming RNAse-resistant hybrids. The resulting hybridization profile is a two-step curve. The same profile is obtained if the heterogeneity is an intermolecular one. In this case, RNA molecules complementary to repetitious DNA hybridize at low Cot values while the molecules complementary to rare DNA sequences form RNAse-resistant hybrids only at higher Cot values.

Recently Dina *et al* (1973) found that mRNA molecules from developing embryos of *Xenopus laevis* are internally heterogeneous. According to their model, each RNA molecule contains a short sequence complementary to repeated DNA covalently linked to a longer sequence transcribed from unique DNA.

The experiments performed with cDNA transcribed from rat myoblast mRNA suggest that some of the mRNA molecules are internally heterogeneous.
The rate of annealing of cDNA is faster than expected for single-copy DNA and shows that cDNA has been copied on both repeated and unique RNA sequences. The observation that the RNA sequences complementary to repetitious DNA are under-represented in the cDNA, leads to the tentative conclusion that most of the repeated sequences are located near the 5' ends of the mRNA molecules, which have not been copies into cDNA.

The problem, however, needs further clarification. A possible experimental approach towards its solution is the separation of the so-called "Cot 10 DNA" (i.e. repetitious DNA) from unrepetitive DNA, and the subsequent hybridization of the same RNA molecules to both DNA fractions.

A confirmation of the preliminary results reported here would provide some insight into sequence arrangement in the DNA.

If the RNA molecules contain a common sequence transcribed from repeated DNA linked to sequences transcribed from different unique sites, then the repetitious DNA sequences must be interspersed along the genome with single-copy DNA.

**Presence and biological significance of RNA sequences transcribed from repeated DNA**

The occurrence of RNA sequences transcribed from moderately reiterated DNA seems to be a general phenomenon.

Greenberg and Perry (1971) in mouse L cells, and Georgiev et al (1972) in rat liver found that a proportion of both HnRNA and mRNA hybridizes to repeated DNA.

Lambert (1972) showed that BR2 DNA of *Chironomus tentans* is
reiterated about 200 times and is transcribed into a high molecular weight RNA, which is found both in the nucleus and in the cytoplasm (Daneholt and Hosick, 1973).

HnRNA and mRNA from HeLa cells exhibit the same hybridization kinetics as rat myoblast RNAs (Bishop, personal communication). The repeated DNA sequences expressed in mRNA are then part of structural genes, although it seems unlikely that they code for aminoacid sequences. They may perform some regulatory functions at the transcription level, as suggested by Britten and Davidson (1969) or at the translation level, for instance as signals for ribosome or tRNA binding.

However, the finding that some RNA is complementary to repetitious DNA sequences does not necessarily imply that those sequences are all transcribed. It may be that only a fraction of them is expressed and the remaining ones are "silent".

An example of the differential control in the expression of repeated genes is given by the sequence heterogeneity of 5S RNA in kidney cells and ovaries of Xenopus (Ford and Southern, 1973).

There is only one major sequence for 5S RNA in kidney, while there are at least four different sequences in ovaries, indicating that more 5S genes are transcribed in ovaries than in kidney.

**Analytical complexity of rat myoblast mRNA**

An estimate of the complexity of the rat myoblast mRNA population can be obtained by the hybridization kinetics of mRNA to cDNA. Since the reaction is performed in RNA excess, it is RNA-driven, and the rate and extent of hybridization depend on the RNA concentration. If all
the different RNA sequences are present at the same concentration in the whole population, the reaction has a single rate-constant. If, on the other hand, some RNA sequences are less frequent than others, they will hybridize with a lower rate than the more common ones, and the reaction will reach completion only at very high RNA inputs.

The hybridization of mRNA to cDNA does not go to completion even at an mRNA:cDNA ratio of 5000, but reaches an apparent saturation plateau at 60%, suggesting that the concentration of the unreacting sequences is too low to allow their hybridization in the experimental conditions used.

A measure of analytical complexity of the reacting sequences is given by the Rot value (Rot = concentration of RNA/time) at which the reaction is half complete (Rot$^{1/2}$). The Rot$^{1/2}$ of the mRNA-cDNA hybridization is 1, from which the rate constant is calculated to be 0.69. By comparing this value with the rate constants obtained for simple systems, such as $\lambda$ DNA-cRNA (Bishop, 1972b) and haemoglobin mRNA-cDNA (Bishop and Rosbash, 1973), one calculates that rat myoblast mRNA is transcribed from the equivalent of $13\lambda$ genomes or 4000 Hb genes.

From the average molecular weight of rat myoblast mRNA ($0.5 \times 10^6$ daltons), the complexity of the $\lambda$ genome ($30 \times 10^6$ daltons) and the molecular weight of Hb mRNA ($0.2 \times 10^6$ daltons) one can calculate that between 500 and 800 different genes are transcribed into 60% of the total mRNA population.

The relative simplicity of the rat myoblast system makes it a suitable material for studies of genome complexity, sequence arrangement and transcription.
With the knowledge acquired during the pursuit of this project, it should be possible in the near future to answer at least some of the questions still left open.
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