ABSTRACT OF THESIS

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A method is described for the estimation of base sequence complementarity between RNA and DNA. The method involves measuring the rate of formation of stable hybrid molecules by HAP chromatography and for this purpose a batch method for chromatography is described. From the reaction rate the effective concentration of complementary sequences in the nucleic acid species is estimated and the extent of complementarity is discussed.

The procedure described permits of the recovery of the complementary DNA strand from the hybrid molecule. Reannealing complementary strands of DNA with a second RNA from a different source gives a measure of the amount of sequences common to the two RNA's. Also, reannealing complementary strands of DNA with homologous DNA gives a measure of the dispersal of the complementary sequences in the reiterated families of DNA sequences.

It is seen that 12% of the DNA is complementary to sequences in the RNA, under the conditions employed. Of the total RNA sequences about 20% from the nucleus are effective in the hybridization reaction whilst the RNA of the cytoplasm shows an effectiveness of about 1%. Thermal dissociation of the hybrids formed from the two cellular fractions are different though the sequences in the DNA involved in both reactions appear to be identical.

RNA from the kidney nucleus has an effective concentration of almost 40% compared to 20% for the liver nucleus. Though the overall complementarity of the DNA to the RNA from liver and kidney nuclei is the same the sequences involved are, in part, different, and are representative of different sequence families.

A computer simulation of the hybridization scheme is presented. The theoretical results from the computer substantiate the interpretations of the hybridization scheme in which only one of the reactants (the DNA) need be radioactive.

In an appendix, the results of a preliminary study of the resolution of hybrid molecules by buoyant density centrifugation are presented.
RNA : DNA relationships in the Mammalian Genome

by

David B. Malcolm

Thesis presented for the degree of Doctor of Philosophy in the University of Edinburgh, Faculty of Science.

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References cited
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For the completion of this written work I most gratefully acknowledge the perseverance and persistence of my wife, Marian.
The relationship between RNA and DNA is studied by molecular hybridization. This technique estimates nucleotide sequence homology between the two molecules. A method employing unlabelled RNA has been used because it is often difficult to obtain labelled RNA's; also the different rates of synthesis of RNA species is such that the use of isotope labels can often confuse the interpretation of results.

The diversity of RNA species makes comparisons between tissues difficult because nucleotide sequences may occur at different concentrations in different tissues. For this reason comparisons have been made by isolating the hybrid molecules and thereby recovering the complementary DNA strand for hybridization with a second RNA.
ABBREVIATIONS AND TERMS USED

mb DNA - the major component of mouse DNA separated from the lighter satellite component by CsCl buoyant density centrifugation.

rRNA - ribosomal RNA.

RNAase - ribonuclease.

A - Adenylc Acid (AMP)

C - Cytidylic Acid (CMP)

G - Guanylic Acid (GMP)

U - Uridylic Acid (UMP)

T - Thymidylic Acid (TMP)

Reactions -

a) Reassociation; the formation of double stranded DNA molecules by base pairing between single DNA strands of sufficient complementarity to be stable at the conditions employed.

b) Hybridization; the formation of double stranded molecules by base pairing between single strands of RNA and DNA of sufficient complementarity to be stable at the conditions employed.

c) 2nd Cycle Reactions; DNA strands recovered from a hybridization or reassociation reaction (1st cycle reaction) are recovered and again reacted to form reassociated or hybrid molecules.
1. 2nd cycle Homologous Reassociation reaction: \(^{32}\text{P}\) labelled DNA strands recovered from hybrid molecules are used in a 2nd cycle reassociation reaction with unlabelled DNA.

2. 2nd cycle Homologous reaction: DNA strands recovered from hybrid molecules are used in a 2nd cycle hybridisation reaction with RNA from the same source as that used in the first reaction.

3. 2nd cycle Heterologous reaction: DNA strands recovered from hybrid molecules are used in a 2nd cycle hybridisation reaction with RNA from a different source as that used in the first reaction.
Introduction

The genes of an organism largely determine its pattern of chemistry and structure. In addition, the genetic material retains a record of the organism's evolutionary history or phylogeny. Unravelling the pattern of chemistry and structure provides an exciting challenge and the reading of the phylogenetic record a challenge equally as great. (The connection between the two is clearly understood in the theory of natural selection).

A central idea in molecular biology is that the gene is made up of DNA and that DNA carries information encoded in the form of specific sequences of nucleotides, which determine the amino acid sequences of protein. It has been demonstrated that a simple congruence exists between these two sequences and RNA acts as the intermediary in the translation.

DNA is a helical double-stranded polynucleotide molecule made up of long linear sequences of the four nucleotides A, G, C, and T. The two single strands of the DNA molecule are held together by specific interactions between complementary nucleotide pairs. A and T normally pair with each other as G and C pair together; these are referred to as complementary base pairs. The two nucleotide strands (complementary strands) in helical configuration are joined by complementary base pairing.

It is thought that the genetic code relating DNA and protein sequences is universal. Direct evidence is available indicating that the same code for protein synthesis is utilised in bacterial and animal cells (Woese, 1967). What is not known however, is the fraction of the total DNA which codes for protein, or even that which is expressed as RNA, in any organism. From nucleotide sequence studies on a viral genome, (Dahlberg 1968) it is known that the ends of the molecule are devoid of protein initiation or terminating sequences. It is reasonable to suggest that a proportion of all DNA's will
not be translated into proteins and that certain nucleotide sequences may have a structural role or be recognised by a particular protein configuration. The latter requirement is necessary, for example, for the operon hypothesis of Jacob and Monod which has been proven in part by the demonstrations of the protein nature of the repressor. (Gilbert, Muller-Hill, 1968; and Ptashne, 1968).

The work to be described is specifically concerned with the homology between RNA and DNA. There are two levels at which this relationship may be discussed. At the gross level, a considerable body of information exists concerning the overall base composition of the two classes of molecules. A population of RNA molecules transcribed from one particular cistron will be less likely to show overall base complementarity to that of the total DNA, whereas a population of molecules arising from many different cistrons will be more complementary to the DNA. This has proved valuable for classifying RNA's and has made some contribution to assigning metabolic roles for the different molecular species. Base ratio complementarity and sequence homology are however, distinctly different, for there are many possible sequence combinations of any one composition.

The ultimate description of complementarity between two molecules is in their complete base sequences. Although virtually complete sequences are known for the majority of t-RNA's (see review by Philippe 1969) even the RNA bacteriophage informational molecules are a long way from complete determination (Brownlee et al, 1969). There is no general method for the isolation of a single cistron except through the purification of a single messenger molecule which in turn is possible only in simple viral systems, (Bautz and Reilly 1966).

At this date, therefore, the problem of complete sequence determination,
particularly in mammals, is far from being solved. Fortunately, however, the nucleic acids themselves show specificity in sequence recognition when subjected to certain conditions. After the discovery of Doty et al. (1960) and Marmur and Lane (1960) that the two strands of DNA may be separated and reannealed, it became clear that this reaction could be useful in analysing RNA as well as estimating the genome complementarity to purified RNA species. Thus the sites of synthesis of transfer and ribosomal RNA's have been assayed (Goodman and Rich, 1962, Ciacomoni and Spiegelman, 1962, Yanofsky and Spiegelman (1962, a,b), Gillespie and Spiegelman, 1965)) and even isolated (Birnsteil, 1966). Estimates have been made of the recurrence of the cistrons for these species and in the case of rRNA, studies have been made on the organisation of the cistron itself. (Birnsteil et al. 1969).

These studies make use of purified RNA preparations, so that high concentrations of one or a few RNA sequences can be employed. The results of these experiments show that under these conditions all of the DNA sites available for the reaction with the RNA, have reacted. When DNA is reacted with a mixture of RNA species it is difficult to demonstrate a reaction between the DNA and representative RNA molecules from each species, because each forms only part of the total and thus different species will show different effective concentrations in the reaction. This effect has been demonstrated in principle (Bishop, 1968).

When RNA and denatured DNA are reacted in solution, two different reactions can occur: (a), the DNA can reassociate with itself, or (b), it can enter into the hybrid reaction with RNA. The reaction that takes place on a nucleotide sequence will depend upon the relative concentration of that sequence in the RNA and the DNA.

The existence of these competitive interactions can introduce serious
errors, particularly in experiments determining absolute amounts of homology (saturation plateaus).

The problem of the homologous DNA-DNA reaction was initially overcome by immobilising the DNA phase of the reaction, first on nitrocellulose columns (Bautz and Hall 1962) using glucosylated DNA, and subsequently by embedding the DNA in agar gels (Bolton and McCarthy 1962). Britten (1963) fixed DNA by U.V. irradiation to synthetic polymers which were then employed as columns for RNA hybridisation.

From the observation of Nygaard and Hall (1964) that nitrocellulose filters strongly absorbed single stranded DNA along with any hybridized RNA, Gillespie and Spiegelman (1965) designed a technique which proved to be most convenient, both in terms of the small quantities of reactants required and for the handling of many samples. In this, denatured DNA is absorbed on to a nitrocellulose filter disc and the whole incubated with the RNA. Adventitously bound RNA is removed by washing and by RNAase digestion. The latter procedure also removes the unduplexed regions of the RNA, though there is evidence that this is restricted to pyrimidine rich regions (Attardi et al, 1965 and Merits et al, 1966).

In certain instances the complication of the homologous reaction can be surmounted by incubating at temperatures well below the melting temperature (Tm) of the DNA, a strategy used by Yankofsky and Spiegelman (1962, 1963). However, low temperature is not a universally available solution. RNA molecules possessing an extensive secondary structure will not hybridize until their own melting temperature is approached, a situation encountered with t-RNA (Giacomoni and Spiegelman 1963).

In mammalian hybridization experiments the problem of the homologous reaction may not even be circumvented entirely by immobilisation of the DNA.
The reason for this lies in the complexity (number of base pairs) of the DNA, but there are other and possibly more serious problems associated with the complexity. It is clear that the reassociation reaction depends upon effective collisions of complementary strands; thus the rate of the reaction should be inversely proportional to the number of different nucleotide sequences which exist in a given DNA (Wetmur and Davidson, 1968). This implies an inverse proportionality between the rate of reaction and the DNA content per haploid cell of an organism. Clearly the rates of renaturation should be much slower for a bacterial than for a viral DNA and very much slower for a mammalian DNA. It has been known for some years that this is not the case (Hoyer et al, 1964, Walker and McLaren, 1965), and in fact, were it so studies of DNA/DNA and DNA/RNA duplex formation would not have been possible. The expected dependency of the rate of reaction on the complexity of the DNA is observed to the level of the eukaryote, where a rate similar to that of a bacterial DNA is seen (Britten and Kohne, 1966).

The other observation which elucidates the structure of mammalian DNA comes from melting experiments. The melting profiles of duplexes formed with mammalian DNA are very much a function of the annealing temperature. The higher the temperature of incubation, the greater the stability of the duplex structure (Walker and McLaren, 1965, Martin and Hoyer, 1966). This is not so in the profile of renatured bacterial DNA's where the melting profiles are essentially the same, independent of the annealing temperature in the range of 50-75°C (McCarthy and McConaughy, 1968).

The mammalian genome is made up in part of nucleotide sequences which are sufficiently similar to reassociate with each other under the conditions of the incubation. These related sequences may be considered as families, perhaps phylogenetically related through gene duplication as is suggested for the
ribosomal cistrons (Moore and McCarthy, 1968). In mammals at least, different families of sequences may contain variable numbers of individuals (Britten and Kohne, 1966). An extreme example of reiteration is offered by the satellite of mouse DNA (Kit, 1963) in which there appear to be approximately a million similar sequences (Waring and Britten, 1966). The majority of sequences (about 70%) in the mouse genome, occur uniquely and will only react under conditions of high concentration and long incubation times.

As a result of the great similarity of base sequences within a family, the specificity of DNA/DNA and DNA/RNA (Church and McCarthy, 1968) duplex formation is reduced. Thus, the nucleotide sequence is not sufficiently distinct, or alternatively, the specificity of the reaction is not sufficiently great to preclude the reaction of RNA or DNA strands originating from different, yet similar, genetic loci. This will account for a high rate of reaction, and for the failure to form duplexes which have stabilities close to that of the native structure. In addition, since the various members of base sequence families are related to differing degrees, the extent of matching of base pairs will depend on the conditions of the incubation. These conditions have been reviewed for the reassociation reaction (Marmur et al, 1963, McCarthy and McConaughy, 1968) and for the hybrid reaction (Church and McCarthy, 1968).

In view of the lack of complete locus specificity in hybridization reactions with complex DNA's, it is clear that saturation or titration experiments of the type used to calculate the amount of genome complementary to rRNA (Ritossa and Spiegelman, 1965) cannot easily be applied to the entire spectrum of RNA molecules. Thus the experiments described in the publications of Crippa, Davidson and Mirsky (1967), and Widholm and Bonner (1967) require cautious interpretation. The purpose of such experiments is to determine
the fraction of the genome responsible for RNA synthesis in an embryo of a given developmental stage, or for the synthesis of a given type of RNA molecule. Particularly in the case of Crippa, Davidson and Miresky the saturation plateau must be greatly elevated as a result of the high salt concentrations used in the reaction system. In general, most hybridization experiments estimating saturation levels will be subject to over estimations as a result of RNA reacting with related partially complementary DNA sites, and underestimated because with the amounts of RNA used, reaction with unique sequences does not occur. No firm conclusions may be drawn regarding the fractional activity of the genome owing to the sequence similarities and complexity, but it may be supposed that saturation plateaus determined under similar conditions will be reasonable relative estimates of the genome transcribed.

In the same way, the results of competition experiments (Nygaard and Hall, 1964) are completely dependent upon the incubation conditions. There is some disagreement in the literature, not only on the interpretations of competition experiments (Bishop et al, 1969, McCarthy and Bolton, 1964, and Bolle et al, 1968), but also on the outcome of the different means employed. Whiteley et al (1966) demonstrated that the results were identical when the DNA was presaturated with competitor prior to hybridization, and when the competitor was included in the incubation mixture. Church and McCarthy (1968), Melli and Bishop (1969) confirmed these results. Birnboim et al (1967) found that competition was higher on simultaneous incubation. It is note-worthy that the last mentioned authors pre-incubated with rather smaller amounts of RNA than the first and it may be argued that if the messenger or easily hybridizable fraction constitutes only a few per cent of the RNA, then the low level of RNA used as competitor would not be sufficient to
completely saturate the sites. Nevertheless the same amount of RNA was used in both competition systems.

Interpreting competition experiments is very difficult. When the RNA contains a single or uniform distribution of sequences, interpretation of the data is straightforward. When a mixture of many different sequences at different relative concentrations is used serious problems of interpretation arise. a. If there are very many different sequences, effective concentrations of each may be unattainable due to solubility, (for instance non-ribosomal RNA has a lower solubility in salt solutions than rRNA (Samarina et al, 1965)). b. Certain species of RNA may not enter into the reaction because their effective concentration is too low. c. Certain species may occur at high concentrations and be complementary to only a small fraction of the DNA (e.g. rRNA) thus effectively reducing the concentration of other species present.

In competition experiments the shape of the dilution curve is determined by the sequence homology of the competing mixtures, and by their concentrations. In the comparison of the RNA of differentiated tissues, differences could perhaps be accounted for in terms of the effective concentration of the sequences that enter into the reaction. The same sequences may in fact be transcribed in different systems and in the same amounts, yet the effective concentrations of these sequences may be quite different, depending on the amount of other RNA's in the preparation. Competitive saturation does not necessarily overcome this problem. The results from competition experiments clearly have some meaning, but that meaning may not be connected with the common sequences transcribed, or the extent to which they are transcribed. Tissue specific differences have been demonstrated in various systems employing the competition reaction (McCarthy and Hoyer, 1964, Perry et al (1967) Church
and McCarthy, 1967a and b, Drews et al. 1967, Miyagi et al. 1967, Gilmour and Paul 1968a and b, 1969; Hennig 1969). That these hybridizations are restricted to the reiterated part of the genome has been demonstrated by Melli and Bishop (1969).

Considering the lack of specificity in the eukaryote hybridization reaction and the restricted amount of the genome examined, it is surprising that tissue specific molecules can be detected. Some authors have, however, expressed the contrary view that differences are surprisingly small (McCarthy and Royer, 1964, Miyagi et al. 1967).

Comparisons of the rate and time of synthesis of different cell fractions and species of RNA have also been made in this way (Church and McCarthy 1967a, Birboim et al. 1967, Drews et al. 1967, Arion et al. 1967, Melli and Bishop 1969). Many of these experiments involve pulse labelled RNA's and consequently introduce the uncertainty of isotopes distribution. In certain cases in vitro synthesised RNA is used as the competing agent for RNA's from different sources (Gilmour and Paul, 1968a and b 1969, Melli and Bishop, 1969). Though this system will undoubtedly have a uniform distribution of label it may not have a uniform concentration of sequence, for it is known that the polymerase molecule, without its sigma factor determining its sequence start specificity (Burgess and Travers, 1969), will transcribe at random. Furthermore, the possibility of forming RNA sequences complementary to one another cannot be discounted as it can in naturally occurring RNA's in mammals (Harris, 1961) and in bacteria (McCarthy and Bolton, 1964). Possibly the aspect of use of in vitro made RNA in competition experiments, which causes most concern is in the amount of genome transcribed by the polymerase molecule. Clearly if the in vitro made competitor is not representative of the total genome then the results obtained
from a competition experiment will reflect the sequences recognised by the polymerase and not the spectrum of sequences common to the genome and the cellular RNA.

In addition to the problems of specificity and complementary sequence concentration in competition reactions, there are questionable features of any immobilised DNA hybridization method. It is fundamental to the procedure that all of the sequences in the DNA be proportionally represented and free for sequence matching with the RNA. The mere fact that the DNA is in contact with the solid phase means that this cannot be the case unless some property of the solid attracts the ribose phosphate backbone of the molecule or, alternatively, repels the bases. Possibly more controllable however, is the concentration of DNA and salt at which the filter is loaded. The rapid rate of reassociation of small amounts of mammalian DNA may result in some sequences becoming involved in the homologous reaction. Also, the size of the molecule and salt concentrations employed in the process of loading may result, to some extent, in non-specific aggregations. (Studier, 1969).

In the work to be described, the DNA:DNA reassociation reaction has been allowed to proceed and RNA has been employed as a competitor in this reaction. In this way unlabelled RNA may be used since the effect of the RNA sequences is seen in effectively increasing or decreasing the rate and extent of the homologous reaction. The method employed is one in which duplexed material can be recovered. By adjusting the conditions of the reaction it is possible to recover the virtually pure complementary strand of the DNA. This strand can be matched against sequences in a second RNA and the extent of the reaction is a measure of the sequences common to both RNA's. The rate of the reaction is a measure of their effective concentration. Tissue or cell fraction comparisons of RNA carried out in this way give an estimate of the
complementarity, the reiterative value of the complementary sequences and some measure of the concentration of the sequences in the RNA.

Hybrid and reassociated molecules are recovered by salt chromatography on NAP. This material permits fractionation independent of temperature, thus duplex molecules can be recovered at incubation temperature. A simple technique is described which permits rapid fractionation of many samples in such a way that the reaction rate may be measured and the molecules recovered.

Using this technique the extent of homology between DNA and RNA sequence is studied. The continuity of RNA sequences between the nucleus and cytoplasm is considered, firstly in terms of their overall homology with the DNA and their effective concentration in the hybrid reaction. Secondly, direct comparisons are made between the complementary sequences in the DNA derived from nuclear and cytoplasmic RNA hybrid reactions. Using these DNA sequences the proportion of complementarity, or the family size, is measured, and the fractions characterised by estimating their degree of reiteration and also by reannealing sequences derived from the nuclear RNA hybrid with cytoplasmic RNA and vice-versa. In a similar manner the RNA sequences of the liver and kidney nuclei are compared.

A computer simulation of the hybrid reaction in solution, and employing unlabelled complementary sequences, is used to substantiate the interpretations of the hybridization scheme.
MATERIALS AND METHODS

I. The Isolation of Nuclei.

Nuclei were isolated in two ways; 1. By the citric acid procedure originated by Dounce (1943) and 2. By the sucrose procedure (which in principle is credited to Arnesen et al, 1949). The relative merits of the various methods for the isolation of nuclei have recently been reviewed by Roodyn (1969).

The citric acid procedure is quick and easy for bulk preparations and most important, it simultaneously removes the outer layer of the nuclear envelope. This layer is known to be associated with ribosomes and its loss might be expected to significantly raise the concentration of non-ribosomal RNA. The citric acid preparation of nuclei is also appealing because nucleases are inhibited at low pH values. This method was easily applicable to all mouse somatic tissues, though for cells derived from tissue cultures prewashing in isotonic salt solution was necessary to remove the buffering capacity of the growth media.

Nuclear preparations derived from citric acid solutions of decreasing pH were compared in terms of breakage and RNA/DNA ratio. This preliminary study resulted from a consideration of the wide range of pH's employed in various citric acid nuclear preparations; and because RNA may leak from the nuclei with decreasing pH.

At pH 5 and above nuclear breakage was considerable so the RNA/DNA ratio shown in Table I is for preparations below pH 5. No significant difference in ratio is seen at any pH down to pH 2, in keeping with the work of Higashi et al (1968) who employed a 2.5% w/v citric acid solution and obtained clean and intact nuclei. The difference seen between citric acid nuclei and sucrose
nuclei preparations from the same source are probably due to ribosomes associated with the nuclear envelope.

The routine method of preparing citric acid nuclei was as follows:

Five adult mouse livers were homogenised in 100 ml of 0.025M citric acid in an M.S.E. blender at the medium speed setting for 4 minutes. After this time a considerable frothing had occurred, trapping in it much of the fibrous material of the lysate. Using a bone spatula this material was retained in the blender whilst the suspension was filtered through eight layers of cheese-cloth into a Sorval centrifuge bottle. Nuclei were pelleted at 2,000 r.p.m. in the Sorval refrigerated centrifuge for 5 minutes. The entire procedure was carried out at temperatures below 5°C. The nuclear pellet was in some instances cleaned by a second pelleting, after re-suspension in a fresh citric acid solution, whilst in others it was sufficient merely to agitate the last few milliliters on decanting to remove the additional sediment over the nuclei.

The routine method of preparing sucrose nuclei was as follows:

One adult mouse liver (1.5 gms. wet weight) in 15 ml of 0.25M sucrose containing 3.3M CaCl₂ was homogenised by three strokes of a teflon-glass homogeniser. Homogenates were pooled and made up to 2M sucrose 3.3M CaCl₂ prior to straining through eight layers of cheese cloth. Nuclei were pelleted by centrifugation for 2 hours at 18,000 r.p.m. in the M.S.E., or for 1 hour at 23,000 r.p.m. in the Spinco model L. The pellet derived from the slower centrifugation was the cleanest though a lower yield was observed. The entire procedure was carried out at 5°C. Nuclei prepared by the sucrose method contained a considerable amount of cell debris, this is reflected in the nucleic acid content shown in Table I.
Isotopically-labelled L cells were used throughout as the source of mouse DNA. Cells grown for two or three generations in the presence of $^{32}$P sodium phosphate were harvested, lysed, and the DNA extracted according to the procedure described by Walker and McLaren (1965a), which is a modification of that by Marmur (1961). The DNA was simultaneously cleaned and fractionated into its major band and satellite components by centrifuging to equilibrium in CaCl$_2$ after the procedure of Flamm et al (1966). Fractions from the CsCl gradient were pooled and diluted prior to pelleting the DNA by centrifugation for 18 hours at 50,000 r.p.m. The pellet was raised in the buffer required and dialysed overnight to bring the DNA completely into solution and to remove any remaining cesium ion.

Prior to use in annealing, the molecular weight of the DNA was reduced by the ultrasonic treatment described by Walker and McLaren (1965b), which produces fragments of about 500,000 Daltons. DNA was denatured in alkali by adding one tenth volume of IN NaOH to the DNA solution in 0.03M-PO$_4$$^{3-}$ buffer pH 6.8. DNA was maintained (as far as was possible) in solution in the native state in 0.03M-PO$_4$$^{3-}$ buffer in a refrigerator at 4°C.
3. The Preparation of RNA.

A. Nuclear RNA.

A slight modification of the procedure described by Scherrer et al. (1963) was used throughout. Nuclei were taken up in 0.01 M acetate pH 5.1 containing 2ug/ml polvinylsulphate. Sodium lauryl sulphate was added to a final concentration of 0.5%. Acetate buffer saturated phenol, pre-heated in a boiling water bath, was added in equal volume and the whole raised to 70°C with continuous mixing. Emulsification was achieved by plunging the preparation into a dry ice bath at -20°C. Subsequently the emulsion was broken by centrifugation. Phenol extraction was carried out twice on the aqueous and interphases. The aqueous phase was finally extracted with ether, made 0.1M in NaCl, and RNA precipitated by the addition of two volumes of ethanol pre-cooled to -20°C. Precipitation was allowed to continue overnight at this temperature. Sometimes an additional precipitation with 2M potassium acetate and 25% ethanol (Di Girolamo et al., 1964) was employed. In all preparations the precipitated RNA was redissolved in 0.01 M tris H Cl pH 7.0 and the solution made up to 1.70g/cm³ with CsCl. The solution was run to equilibrium in an MSE high speed centrifuge at 20°C. The RNA pellet resulting from this was stored under alcohol at -20°C; when used, the pellet was taken up in .12M PO₄ buffer and dialysed for one hour against three changes of the same buffer at 5°C. A pellet once dissolved, was never re-precipitated and as far as possible fresh RNA pellets were used for each experiment.

Pelleting RNA through CsCl at high speed removed any extraneous matter from the preparation since RNA is the only biological molecule known to have a density greater than 2gm/cm³. Thus the last traces of DNA and protein are
removed from the RNA preparation. This was demonstrated by including $^{32P}$ labelled DNA ($2 \times 10^6$ cpm) to a preparation of RNA to be pelleted through CsCl. After a final dialysis of the RNA pellet the resulting RNA solution contained no detectable radioactivity.

For small scale L-cell preparations, the washed cells were lysed in 0.01M acetate buffer pH5.1, 0.01M EDTA and 0.5% sodium lauryl sulphate. The lysate was lightly homogenised in a loose fitting glass homogeniser and CsCl was added to a final density of 1.70g/ml whilst mixing continuously. At this high salt concentration a dense protein precipitate develops which gradually rises on standing. This precipitate was removed and filtered through Whatman grade 90 filter paper which had been presoaked in CsCl solution. The precipitate was finally washed with CsCl solution and the total eluant centrifuged to equilibrium under liquid paraffin. If the protein precipitate is not removed it forms a gel on top of the CsCl gradient at its liquid paraffin interphase, which becomes a firm elliptical pad on centrifugation. When the tube is removed from the rotor much of the gradient is disturbed by the movements of this pad and the gradient cannot be fractionated. The small diffuse pad derived from the filtered preparation does less damage and DNA can be fractionated past the RNA pellet, which in the angle rotor is on one side of the tube (Flamm et al, 1966).

DNA banded in this preparation scheme can also be recovered from CsCl by absorbing onto HAP after a ten-fold dilution of the CsCl solution. The recovery of DNA in this way compares well with that obtained by pelleting from the diluted CsCl solution. Density gradient centrifugation is described in section 10. (Materials and Methods). Whole mouse tissue RNA was prepared as described for nuclear RNA.
B. The Preparation of Cytoplasmic RNA.

Three different preparation procedures were investigated. In each case the extraction was carried out at 5°C. Redistilled phenol was used throughout.

a) The supernatant from the citric acid preparation of nuclei was adjusted to pH 6 with the addition of tris buffer and NaCl to final concentrations of 0.1N and 0.14N respectively. This was then extracted with an equal volume of phenol and the aqueous phase recovered. After a further two extractions with phenol the RNA was precipitated overnight at -20°C after the addition of two volumes of cold ethanol. The RNA was pelleted and taken up in 0.02N tris buffer pH 6 before extraction with ether and final pelleting through CsCl.

b) A procedure was devised for whole organs based on Georgiev and Mantieva's (1960) observation that, on the extraction of tissues for the preparation of RNA by Kirby's procedure (1956), nuclei remain intact in the phenol and interphase layers.

Tissues were quickly homogenised in the MSE blender in the presence of ten times the tissue weight of 0.14 N NaCl and an equal volume of water saturated phenol, pH 6. After centrifugation the aqueous phase was recovered and retained. The phenol and interphase was re-extracted with 0.14 N NaCl and again the aqueous phase retained. The aqueous phases were pooled and extracted twice with phenol before precipitation and pelleting through CsCl as described previously.

c) Cytoplasm, recovered from the sucrose nuclei preparation already described, was extracted as follows:
The supernatant from a sucrose nuclei preparation was made up to 2 ug/ml P.V.S. and dialysed at 4°C for four hours against four changes of 0.14M NaCl. Dialysis was considered complete after the fourth change of salt solution as this was found sufficient for normal separation of the phenol and aqueous phases on subsequent extraction of the RNA. The extraction procedure was exactly as that described previously for preparation 'b'.

C. Note on the preparations of RNA used.

No technique for the preparation of nuclei is free from criticism with regard to RNA losses or cytoplasmic contaminants (Dounce, 1955, Roodyn, 1969). The acid procedure for the preparation of nuclei was preferred for its obvious applicability to large scale preparations, for nuclease inhibition at the pH employed and for its selective removal of the outer membrane layer. The supernatant from the nuclear pellet is also easily extracted for cytoplasmic RNA. The RNA prepared from the citric acid nuclei supernatant appears identical to that prepared by the method used by Georgiev et al (1963) (preparation 'b' above), on the criteria of effective concentration of RNA sequences in a hybridization experiment. Because of this, these preparations were used together with the citric acid nuclear preparation procedure for the preparation of cytoplasmic and nuclear RNA; thus maintaining the division between the two RNA classes.

D. Preparation of Ribosomal RNA.

Ribosomes were made by the Henshaw (1964) procedure. Liver was homogenised
in five times the tissue weight of a medium containing 0.05 M KCl 0.5x10^{-4} M Mg acetate and 1x10^{-3} M tris pH 7.8. The supernatant obtained after centrifuging at 15,000g for 10 minutes was treated with 0.5% deoxycholate and ribosomes were sedimented by centrifugation at 150,000g for 1 hour. Ribosomal RNA was prepared by extracting the ribosomal pellet with a solution of 0.01 M acetate buffer pH 6 with 1% SDS in a teflon glass homogenizer. An equal volume of phenol was added after homogenization and the phases separated by centrifugation. The aqueous phase was re-extracted twice before the RNA was precipitated with alcohol.
Nucleic Acid Estimations.

I. The estimation of DNA.

The Giles and Myers (1965) modification of the Burton (1956) diphenylamine method was used throughout. DNA estimations of mouse liver and kidney, together with estimations made on preparations of nuclei from those tissues are shown in Table 1. Mouse DNA was taken as having 42% G+C.

2. The Estimation of RNA.

The orcinol procedure of Schneider (1967) was the method used. Base ratio estimations were carried out by the procedure described by Chamberlain (1964). Base ratio estimations of nuclear and total cell RNA were made on L cells and the base distribution assumed to be the same in mouse somatic tissues. RNA estimations of mouse liver and kidney, together with those made on preparations of nuclei from these tissues are shown in Table 1.
### TABLE 1

<table>
<thead>
<tr>
<th>Source</th>
<th>mg/gm</th>
<th>wet wt. tissue</th>
<th>RNA</th>
<th>DNA</th>
<th>Ratio RNA : DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>9.1</td>
<td>2.6</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>1.6</td>
<td>1.5</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Nuclei: Liver**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>A. Citric Acid pH 4</td>
<td>22</td>
<td>108</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric Acid pH 3.5</td>
<td>18</td>
<td>99</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric Acid pH 3.0</td>
<td>20</td>
<td>108</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric Acid pH 2.5</td>
<td>24</td>
<td>110</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric Acid pH 2.0</td>
<td>19</td>
<td>104</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B. Sucrose**

|       | 36   | 64          | 0.56|

**Nuclei: Kidney**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>A. Citric Acid pH 2.5</td>
<td>16</td>
<td>22</td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Sucrose</td>
<td>22</td>
<td>20</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**TABLE 1**

The nucleic acid content of material used for the extraction of RNA.
5. HAP Chromatography.

a. Introduction.

HAP fractionation of deoxypolynucleotides depends on the finding that at certain phosphate buffer concentrations extended single chains of denatured DNA do not remain absorbed onto the column material, whereas duplex molecules are still firmly bound (Miyazawa and Thomas 1965, Bernardi 1965). Evidence presented by Walker and McLaren (1965a), strongly suggested that in DNA these duplex molecules could be native, or renatured, or merely contain stretches of intra-molecular hydrogen bonding, comparable with those found in RNA. McCallum and Walker (1967) demonstrated the techniques of resolving between these three states. Each state can be separated from the single stranded molecule by fractionation procedures in which the previous treatment as well as the phosphate and temperature conditions during fractionation, are varied. The methods are:

1. Thermal chromatography of native DNA at a constant phosphate concentration, which distinguishes DNA fragments by their base composition.

2. Salt chromatography of denatured DNA at high temperatures and after various times of incubation before fractionation, which distinguishes reassociated molecules from the remainder.

3. Salt chromatography at lower temperatures without prior incubation, in which DNA is separated on its ability to form intra-molecular secondary structure, stable at a defined temperature.

Flamm, Walker and McCallum (1969) have employed the salt chromatographic method at high temperatures for estimating the rate of reassociation of DNA. To apply this procedure to estimating the rate of hybrid formation, the salt chromatography of RNA, hybrid and reassocaited molecules must be studied as well as their possible effects on the chromatographic behaviour of each other.
The preparation of Calcium Phosphates (HAP's)

The following procedure, that of Miyazawa and Thomas (1965) is a slight modification of the original design by Tiselius, Hjerten and Levin (1956).

Approximately 400 mls. of distilled H<sub>2</sub>O was run into a 5 L glass beaker. Into this was run separately, 2,000 mls. each of 0.5M Na<sub>3</sub>PO<sub>4</sub> and 0.5M CaCl<sub>2</sub> by means of separating funnels at the rate of 120 drops per minute. The contents of the beaker were mixed with a stainless steel propeller turning at 530 r.p.m. The resulting precipitate was then washed four times with distilled H<sub>2</sub>O and the final wash was allowed to settle overnight. After decanting, the white slurry was raised and thoroughly mixed in 4 L of distilled water and 100 mls. of a 40% w/v solution of Na<sub>2</sub>O was added on mixing. The whole was boiled for one hour and after that time the crystals were allowed to settle and the supernatant was decanted whilst still very hot. A considerable amount of fine material was lost in this process. One further boiling step for 15 minutes in 0.01M PO<sub>4</sub><sup>3-</sup> buffer pH 6.8 was performed with subsequent repeated washings of this buffer until pH 6.8 was attained. The HAP thus obtained can be stored in 0.01M PO<sub>4</sub><sup>3-</sup> in the cold as a 50% w/v slurry. This preparation was termed THAP.

These crystals in the light microscope appear as blade-like structures. Their excellence regarding stability and reproducible chromatographic performance, as well as having a high absorption factor for polynucleotides, suffers slightly in terms of column flow rate. Particularly when large incubation mixes require passage through a column, flow rate can be a crucial characteristic of the material. Possibly more important, however, is the flow rate factor in thermal chromatography.
Main, Wilkins and Cole (1959) studied various modifications of calcium phosphate in terms of polynucleotide absorption, elution from the column, and flow rate. From their studies, a material with a specially good flow rate and moderate retention was prepared as follows:

Calcium phosphate was precipitated by adding dropwise (one drop per second) 100 mls. of 0.5M CaCl₂ to 120 mls 0.5M sodium phosphate buffer pH 6.7 with continuous mixing. The resulting suspension was stirred for a further hour and then allowed to settle. The pH of the solution was determined and adjusted to pH 6.7 if necessary. The precipitate was washed by decantation seven times with 600 mls of 0.005M phosphate buffer pH 6.7. After one wash with distilled H₂O, the material was boiled for 30 minutes in a solution made up of 600 mls. distilled H₂O and 750 mls. of a filtered solution of Ca(OH)₂ (saturated at room temperature). 5 drops of a 0.2% solution of phenolphalein were added prior to boiling. The amount of Ca(OH)₂ added was usually sufficient to maintain the pH of the solution above 8.5. In certain instances, the pH fell below this value, and the preparation was rejected. Calcium phosphate derived from this preparation procedure was termed WHAP.

This preparation, when observed under the microscope, appears more rectangular than blade-like and the majority of the crystals have a shared end with several others, producing a complex three-dimensional rosette.
C. Flow Rate.

The rate of flow of 0.12M phosphate buffer (PB) through the different calcium phosphate preparations (THAP and WHAP) was estimated in the following way. Glass capillary tubing of 1 cm internal diameter and containing at one end a glass wool plug, was used to contain the preparation. The HAP as a 50% slurry was pipetted onto the glass wool to produce a settled column length of 7 cm. A head of 10 cm PB was maintained and the rate of flow through the preparation estimated. Measurements made on the same preparation in different columns agreed to within ± 2%.

Preparations differed markedly, particularly in the case of THAP ± 10%, in LHAP the differences were ± 5%.

The estimations of flow rate are shown in Table 2. The flow rate is not affected by temperature or amount of sheared polynucleotide absorbed. The column material can be disturbed after packing and unlike most column chromatographic materials, WHAP, possibly, because of its low resistance to flow, does not suffer from channeling. Chromatographic performance was found to be independent of flow rate.

d. Nucleic Acid Absorption Characteristics on HAP.

An experiment was designed to examine the effects of RNA on the binding of sheared, native, and reassociated DNA on HAP. The binding capacity of HAP was simultaneously calculated. Both HAP preparations were examined.

Trace amounts of $^{32P}$ labelled sheared native and reassociated DNA's were bound to HAP in the presence of increasing unlabelled amounts of each
TABLE 2A

Flow rate and Nucleic Acid absorption characteristics on HAP

<table>
<thead>
<tr>
<th>HAP MATERIAL</th>
<th>Flow rate through 1x7cm. column ml./hr.</th>
<th>Flow rate mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
<th>mg Nucleic Acid bound per cm. packed vol. HAP mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>THAP</td>
<td>5.5 ml/hr.</td>
<td>5±mg</td>
<td>5±mg</td>
<td>4.25mg</td>
<td>4mg</td>
<td>3.8mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHAP</td>
<td>75.3 ml/hr.</td>
<td>3.6mg</td>
<td>3.8mg</td>
<td>2.4mg</td>
<td>2.25mg</td>
<td>2.25mg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2B

<table>
<thead>
<tr>
<th>HAP MATERIAL</th>
<th>LABELLED CONTROL Nucleic Acid</th>
<th>% labelled control Nucleic Acid displaced by unlabelled competitor</th>
<th>mg. Amounts of competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheared RNA</td>
<td>0.25 0.5 1.0 1.5 2.0 2.5 3.0</td>
<td>total RNA</td>
</tr>
<tr>
<td>THAP</td>
<td>native DNA</td>
<td>0 0 2.5 5 8 10 17</td>
<td>total RNA</td>
</tr>
<tr>
<td>MHAP</td>
<td>renatured DNA</td>
<td>5 10 15 24 32 37 42</td>
<td>total RNA</td>
</tr>
<tr>
<td>THAP</td>
<td>denatured DNA</td>
<td>0 0 2 4 5 10 17</td>
<td>total RNA</td>
</tr>
<tr>
<td>MHAP</td>
<td>total RNA</td>
<td>0 0 0 0 0 0 0</td>
<td>total RNA</td>
</tr>
<tr>
<td>THAP</td>
<td>Sheared DNA</td>
<td>0 0 0 0 0 0 0</td>
<td>Total RNA</td>
</tr>
<tr>
<td>MHAP</td>
<td>native DNA</td>
<td>0 0 0 0 0 0 0</td>
<td>Renatured DNA</td>
</tr>
<tr>
<td>THAP</td>
<td>renatured DNA</td>
<td>0 0 0 0 0 0 0</td>
<td>renatured DNA</td>
</tr>
<tr>
<td>MHAP</td>
<td>denatured DNA</td>
<td>0 0 0 0 0 0 0</td>
<td>renatured DNA</td>
</tr>
<tr>
<td>THAP</td>
<td>total RNA</td>
<td>0 0 0 0 0 0 0</td>
<td>renatured DNA</td>
</tr>
</tbody>
</table>
other and of total RNA from mouse liver. The reaction was carried out in 0.12M PB at 60°C. The conditions for fractionation were as described for kinetic experiments (section 7). Controls of trace amounts of each alone served as a basis for the results (Tables 2a and b) which are expressed in % of the control displaced. The tracer in each sample contained 50,000 c.p.m. Absorption was onto 1ml. packed volume of the HAP preparation.

The possibility of displacing material absorbed onto HAP was also investigated. Trace amounts of single-stranded, (slow fraction, Britten and Kohne, 1966), reassociated and native DNA's and RNA were absorbed onto HAP in 0.12M PB at 60°C. Each nucleic acid/HAP was subsequently incubated for 1 hour at 40°C with increasing amounts of the others as in the last experiment. Prior to incubation each nucleic acid/HAP was washed thoroughly to remove any unbound material. After incubation each was again washed, in each case washes were collected in scintillator vials and radioactive measurements were made by the Cerenkov method. In no case where material absorbed onto HAP in .12M PB was subsequent displacement observed.

The absorption capacity of HAP preparations were not routinely determined, instead suitable controls were included in each experiment to check that the capacity of the column had not been exceeded.
The Chromatographic Behaviour of DNA on HAP.

The behaviour of DNA and HAP is well documented. (Walker and McLaren 1965, McCallum and Walker, 1967). From the above results it is clear that the presence of RNA has no effect on this behaviour provided the binding capacity of the HAP has not been exceeded. Fig.1a shows the salt chromatographic behaviour of sheared native DNA, and Fig.1b that of reassociated DNA under the conditions employed in a kinetic experiment.

f. The Chromatographic Behaviour of RNA on HAP.

Bernardi observed that at low temperatures, DNA elutes from HAP on a salt chromatogram at concentrations between 0.15 and .5M PB. McCallum and Walker (1967) suggested that this result might be explainable in terms of secondary structures stabilised by hydrogen bonding between only a few bases. tRNA is known to be organised in this way, as is much of the rRNA molecule (Spirin, 1963). If this explanation is correct then it is reasonable to expect these RNA's to elute in this salt range. This has already been demonstrated for tRNA (Muench and Berg 1966) and Fig.2a shows the stepwise elution of a $^{32}$P total RNA salt chromatogram together with those of heat denatured RNA and Ribonuclease I digested RNA. Fig.2b shows the elution of $^{32}$P rRNA.

g. The Chromatographic Behaviour of RNA/DNA Hybrids on HAP.

Hybrid molecules were formed between $^{32}$P RNA and DNA under the conditions
Fig. 2A

Salt chromatography of RNA on HAP.

Filled circles and lines: untreated total RNA.

Open circles and broken lines: heat denatured RNA.

Filled squares and dotted line: RNAased RNA.

Fig. 2B

Salt chromatography of rRNA on HAP.
fig. 2

A

B

M. P. B.

M. P. B.
Fig. 2A'
Salt chromatography of hybrid molecules recovered from CsCl density gradients, on HAP.
Open area : untreated.
Hatched area : hybrids treated with RNAase.

Fig. 2B'
Salt chromatography of hybrid molecules on HAP. White dots on black background: hybrid formed with 0.1mg/ml $^{32}$PNRNA and 1x10$^{-3}$mg/ml mb DNA.
Black dots on white background: hybrid formed with 0.25mg/ml $^{32}$PNRNA and 1x10$^{-3}$mg/ml mb DNA. Black small dots on white background: hybrid formed with 0.5mg/ml $^{32}$PNRNA and 1x10$^{-3}$mg/ml mb DNA. White area: hybrid formed with 1mg/ml $^{32}$PNRNA and 1x10$^{-3}$mg/ml mb DNA.
fig. 2

A'

B'

M. P.B.

M. P.B.

c.p.m. x 10^3

c.p.m. x 10^3

c.p.m. x 10^3

c.p.m. x 10^3
presented in section 8. The total nucleic acid in the incubation mixture was absorbed onto HAP and salt chromatogrammed at a column temperature of 0.65°C. The fractions from each were made up to 0.5M KCl and the hybrid molecules, detectable by the presence of the radio label, were trapped on nitro-cellulose filters (Nygaard and Hall 1964). Fig. 2b shows the salt chromatogram of these hybrid molecules. Hybrids recovered from CsCl gradients were also shown to elute from HAP in exactly the same place (Fig. 2a) and under the same conditions as the non CsCl fractionated hybrids. Fig. 2a also shows the effect of Ribonuclease I digestion on this hybrid subsequent to fractionation on HAP.

Fractionation of hybrid molecules on CsCl is the subject of Appendix I.
6. The Column Procedure for HAP Chromatography.

1cm. internal diameter water-jacketed columns, containing a coarse grade sintered glass disc, were used to contain the HAP. It was found that the most reproducible results were obtained by adding the HAP as a 50% slurry in the required PB. Also, the best results were obtained if the HAP was pre-mixed with the solution to be absorbed and added to the column with two, or three washes. Water circulation about the column was maintained by a Cirotherm II (Shandon) circulating pump containing a regulatable heating unit. In this way the temperature of the slurry was maintained at less than 0.5° below that of the pump reservoir in the range 60-75°.

At high temperatures air bubbles sometimes formed in the column, but did not appear to effect the results. Drying out of the material had no effect though it was avoided throughout. The packed HAP can be agitated and mixed with the eluate with no ill effects; in thermal chromatography this was routinely performed by means of a glass rod, with every increase in temperature, prior to elution.

Salt chromatograms in the column procedure were performed by step-wise elution of increasing PB concentrations. 10ml. washes were collected in scintillation vials either by maintaining a constant flow rate with a finger pump on the outlet or by air pressure from the top of the column. It has already been noted that the performance of HAP appears to be independent of flow within practicable flow rates. WHAP showed little more resistance to flow rate than did the glass scinter disc supporting it, and in the main, required no assistance in elution. With 10cm. column bed lengths air pressure from the lungs or a bicycle pump produced very rapid elution. Thermal chromatograms were performed as follows: 10ml. PB solutions were
maintained in the circulating pump reservoir prior to pouring onto the column. The temperature of the eluate was measured in the column and its increase arrested at the temperature required. The column contents were mixed with a glass rod and the whole equilibrated for 1 minute before elution and further increase in temperature with addition of the next preheated wash volume of PB. With up to 1 ml. packed volume of HAP, one 10 ml. wash was found sufficient to remove all of the label eluting at one particular temperature.

As in all HAP column work, prior to the elution programme the HAP was washed in the starting PB; after absorption of the material to be chromatographed it was further thoroughly washed to background with the starting buffer.

Recovery from the column was in the range 90-95% estimated by radioactivity. Estimations of recovery from the column in which the radio label was on the DNA were consistently higher than when the RNA contained the label.

Salt chromatographic fractionation can be performed quickly and easily by simply separating the eluate (supernatant) from the HAP crystals by centrifugation at a chosen temperature. This procedure is equivalent to step-wise salt elution on a column.

A small centrifuge constructed to fit into a thermostatically controlled oven consisted of four swinging buckets which could attain a speed of 2,000 r.p.m. This speed was sufficient to pellet 1 ml. packed volume of HAP crystals through a 5 ml. solution in a siliconised plastic tube of 1.5 cm. internal diameter.

The oven was placed next to a large thermostatically controlled bath which contained, along with the incubation mixture to be chromatographed, various phosphate buffers. Siliconised plastic centrifuge tubes containing a pre-determined sufficient amount of HAP in the required PB were also maintained in the bath. The HAP content of each tube had previously been pelleted in the heated centrifuge through the desired buffer.

The sequence of events in a kinetic experiment were as follows.

Incubation mixtures containing the experimental and control solutions were sampled by pipetting and the aliquot run into the 5 ml HAP PB buffer slurry. After agitation on a Rotamixer (Hook and Tucker) the tube was replaced in the bath in sequence with the other four tubes in the experiment. In the same sequence the tubes were run in the centrifuge and after pelleting the HAP crystals (1 minute), the supernatants were decanted into scintillator vials.

At each PB concentration three washes were found to be sufficient to
remove all unbound label. In kinetic experiments tubes were washed three times in 0.12M, 0.15M and 0.30M PB solutions. It is clear that the temperature of the tube contents is only critical for the first two sets of washes. Thus tubes can be stored and final washes to remove the duplexed material can be done at some later time.

Annealing was estimated by determining the amount of radioactivity in the 0.3M PB set of washes as a fraction of the total. Counting was performed either by the Cerenkov method or, after drying, in toluene based scintillator.

This method gave results which were found to be indistinguishable from those obtained by column chromatography.

8. Initiation of Annealing Reactions.

It is imperative that DNA enters into the reaction initially in the denatured state. To be certain of this, DNA was mixed into the incubations in alkali solution.

In the hybrid incubations, RNA in 0.12M PB at nine-tenths the final volume was heated in the water bath to the temperature of the reaction. DNA in a solution of 0.1N NaOH at a concentration ten times that of the incubation concentration required, was also heated to the incubation temperature. The DNA solution was rapidly added to the RNA solution with a hypodermic syringe. In the control reassociation reactions, volumes of 0.12M PB were used, otherwise the procedure was exactly the same.
9. Recovery of the DNA Strand from Hybrid Molecules.

Hybrid molecules recovered from HAP, generally in 0.3M PB, were dialysed for two hours in the cold against two changes of distilled H₂O. The solution was made up to 0.5M NaOH and incubated at room temperature overnight. This treatment was found sufficient to reduce the RNA to an ineffective state in a hybrid reaction.

After alkaline digestion the solution was neutralised with TM PB and the whole dialysed against distilled H₂O until the dialysate matched distilled H₂O in the refractometer. The contents of the dialysis bag were then condensed either by evaporation in a Rotoevaporater or by hanging the dialysis bag in the draught of a fan. Recovery by the latter procedure was between 80 and 85%.

A consistent background of between 4 and 6% was observed with all mb DNA preparations. This background may be due in part to contaminating satellite sequences and to cross-linked DNA (Walker and McLaren, 1968). This fraction appears to be characteristic, to some extent, of the preparation since it varies between preparations but is consistent within a preparation. Prior to re-cycling DNA after digestion of the RNA of the hybrid, this fraction can be removed to some extent by incubation and passage through HAP.

9. Materials

Adult male Q strain mice were used as the source of RNA. Mouse L cells grown in tissue cultures were used as the source of DNA. E.coli RNA was a gift from Dr. J.W. Robinson.
$^{32}$P orthophosphate was purchased from the Radiochemical Centre, Amersham. CsCl (Analar) was obtained from the British Drug Houses Ltd., Poole, England. Nitrocellulose membrane filters were obtained from Sartorius Membrane Filter GmbH, Gottingen, Germany.

10. Density Gradient Centrifugation.

a. Introduction.

Preparative isopycnic centrifugation of DNA in CsCl or Cs$_2$SO$_4$ density gradients has been applied to a variety of problems. Mammalian DNA has been resolved into major and minor components (Falli et al., 1965; Wit, 1961; Flamm et al., 1966; 1967) separation of native from heat denatured DNA (Rownd et al., 1961) from phage or viral DNA (Erikson, 1964), from newly synthesised DNA (Bubna et al., 1965) and from glycogen (Counts and Flamm, 1966). RNA/DNA hybrids, were initially demonstrated (Hall and Spiegelman, 1961) on density gradients of CsCl (Meselson et al., 1957). This technique has achieved the resolution and recovery of the rRNA cistrons (Birnstiel et al., 1968; Brown and Webster, 1968).

These separations were achieved in high speed swinging bucket rotors. Fisher, Cline and Anderson (1964) showed that the fixed angle rotor could be used for preparative gradients, pointing out that the fluid translation of the density gradient from vertical to horizontal during deceleration, replaces the mechanical transition of the tubes in the swinging bucket rotor. Hersey et al. (1965) showed partial resolution of the single strands of DNA which differ in buoyant density by 0.005 g/cm$^3$ in CsCl solution using the angle rotor. Flamm, Bond and Burr (1966) demonstrated the superior resolution of
the fixed angle rotor by direct comparison with swinging bucket centrifugation. The procedure described by these authors has been used throughout this work.

b. The Fractionation of CsCl Gradients.

Preparative gradients were run in the 8x25 MSE aluminium rotor at 35,000 r.p.m. for 60 hours. Each tube contained 9 ml. CsCl solution, the top of the tube being filled with liquid paraffin.

On completion of the run, tubes were carefully removed from the rotor and clamped in the vertical position. The tube cap sealing screw was removed and replaced by a flexible threaded connection with a liquid paraffin reservoir. Fractions (0.25 ml.) were collected by piercing the bottom of the tube with a 27 gauge needle and displacing the tube contents with liquid paraffin by means of a double syringe device connected to a three way stopcock. A 1 ml. syringe could thus be used to measure the fractions by filling from the reservoir and subsequently displacing into the centrifuge tube slowly in aliquots of 0.25 ml. Collection was in 2ml. glass test tubes.


Optical density measurements of the 0.25 ml. fractions were either measured directly in a Beckman DB spectrophotometer in a microcell, or after dilution with 0.01M tris buffer.

Radioactivity measurements were made by removing an aliquot into glass scintillator tubes (Packard) and drying down the solution prior to adding toluene based scintillator fluid. In hybridization experiments the excess
amount of $^{32}$P RNA in the reaction obscured the hybrid molecules. The excess RNA in this case was removed by taking aliquots from the fractions, diluted to .5M KCl, onto nitrocellulose filter discs which retained hybrid (Nygaard and Hall, 1964) but not RNA, molecules. Thus 'peak' positions according to tube fraction number could be detected and the buoyant density position either measured directly in the refractometer or deduced from calibration curves obtained from different rotors run to equilibrium at different speeds.

'Peak' material recovered by absorbing onto HAP was found to be heavily contaminated with unhybridized RNA. Even digestion with RNAase prior to centrifugation was insufficient to remove this material completely.
RESULTS

The satellite sequences of mouse DNA have been shown to be without a complementary RNA in the cell (Flamm et al., 1969). In the experiments described in this work major band (mb) DNA is used.

a) The Reaction Between RNA and DNA.

A preliminary experiment was performed to examine the reaction kinetics of reassociating $^{32}$P mb DNA in the presence of excess unlabelled RNA. The results of this experiment are shown in Fig. 3 (a, b and c).

To 0.1, 0.5 and 1 mg/ml. amounts of liver N RNA in 0.12M-PB at 65°C, $^{32}$P mbDNA was added in alkali. The rate of the reaction of 1, 2.5 and 5 ug/ul mb DNA in each amount of N RNA, was estimated by the THAP batch method.

It can be seen that the presence of complementary cold sequences accelerates the hybridisation reaction and also inhibits the homologous reaction. The acceleration effect is best seen at low DNA inputs (Fig. 3a). The competitive effect is most clearly demonstrated at higher DNA and RNA inputs. (Fig. 3c).

No significant deviation from the control homologous reaction was seen in the presence of 1 mg/ml. E. coli RNA. Pre-treatment of the liver N RNA with Ribonuclease or alkali gave the same result. The salt concentration and temperature of incubation used in the experiment are those used for annealing DNA prior to its fractionation on HAP (McCallum and Walker, 1967).

There are two interesting aspects of these results. The first of these is the plateau in the RNA/DNA reaction. The reaction in this situation is essentially complete as is made clear from Fig. 3c which shows the homologous control reaction progressing while the heterologous hybrid reaction ceases after only ten minutes of incubation. The Plateau occurs at approximately the same value in each case but the time taken to attain this final value varies
Fig. 3

Reaction of 0.1, 0.5 and 1 mg/ml amounts of NRNA with 1, 2.5 and $5 \times 10^{-3}$ mg/ml mb DNA. Filled circles and continuous line: reassociation control reaction.

Open circles and broken line: hybrid reaction with 0.1 mg/ml NRNA.

Open squares and lines and crosses: hybrid reaction with 0.5 mg/ml NRNA.

Filled squares with dashed line: hybrid reaction with 1 mg/ml NRNA.

Fig. 3A

Reaction with $1 \times 10^{-3}$ mg/ml mb DNA.

Fig. 3B

Reaction with $2.5 \times 10^{-3}$ mg/ml mb DNA.
Figure 3

Graphs showing % Duplex over time (min.) for A and B conditions.
Fig. 3C

Reaction with $5 \times 10^{-3}$ mg/ml mb DNA.

Fig. 3D

Reaction of $1 \times 10^{-3}$ mg/ml mb DNA with 2.5 and $5 \times 10^{-2}$ mg/ml NRNA and $1 \times 10^{-1}$ mg/ml NRNA. Open triangles: hybrid reaction with $2.5 \times 10^{-2}$ mg/ml NRNA. Filled triangles and dashed line: hybrid reaction with $5.0 \times 10^{-2}$ mg/ml NRNA.

Open circles and dashed line: hybrid reaction with $1 \times 10^{-1}$ mg/ml NRNA. Filled circles and continuous line: reassociation reaction of $5 \times 10^{-3}$ mg/ml mb DNA.
fig 3
with the input concentration of the reactants.

The experiment with 1 μg/ml input was repeated with RNA inputs of 25, 50 and 100 μg/ml. These results are shown in Fig. 3d together with a repeat of a homologous 5 μg/ml DNA reaction. The latter DNA conc. was chosen as it best demonstrates how the effective concentration of the RNA may be calculated in the experiment. It can be seen that the reaction of 25 μg/ml RNA with 1 μg/ml DNA almost matches the rate of the 5 μg/ml homologous DNA reaction. Clearly the input RNA has raised the reaction rate of the lower input DNA to a value five times as fast as the homologous reaction. This means that approximately one fifth of the liver RNA is complementary to sequences in the DNA which will reassociate under the conditions used.

The second point of interest in the experiments shown in Fig. 3 is the fairly consistent background value. This value is virtually independent of input, which suggests that it is not totally the effect of contamination by satellite sequences (Flamm et al., 1969). The mb DNA used in these experiments had been passed twice through CsCl with the express purpose of removing satellite sequences. The results of Fig. 3d were obtained from mb DNA where a special step had been introduced to remove this background material. In this step the DNA in alkali was neutralised at 65°C and immediately passed through HAP. Almost 15% of the counts applied to the HAP adhered and this figure was reduced to 5% with four washes of 0.12 M-PB. The fraction remaining must have a considerable duplex or secondary structure. Walker and McLaren (1968) reported a fraction of almost 2% in the mammalian genome which they deduced was cross-linked. The fraction seen as background on these experiments will be in part made up of this fraction and may also contain some material which has reassociated by intra-strand annealing.
Comparison of Fig. 3a with Fig. 3c shows that the level of the plateau in the hybrid reaction is the same in both cases if the background value is subtracted from the former.

It is possible that under the conditions used the effect of the RNA sequences on the reaction of DNA could be due to an event other than base pairing. Therefore the effects of salt concentrations and temperature of incubation on the reaction were studied (for a review see Marmur et al, 1963). Major band DNA (0.5 µg/ml) was incubated in various salt solutions between 0.05 and 1 M-PB at 65°C with 1 µg/ml. N RNA. At salt concentrations greater than 0.1 M-PB suitable dilutions were made before absorption on to M-HAP. The amount of material which did not adhere to the HAP after washing at 0.16 M-PB was estimated prior to the transfer of the crystals to a column where the melting profiles of the reassociated DNA and hybrid molecules were measured by elution with 0.12 M-PB at increasing temperatures. At each salt concentration the extent of the homologous reaction was determined by a control containing no RNA. These results are shown in Figs. 4a and 5a.

In a parallel experiment using exactly the same materials in the same quantities the effect of the incubation temperature was studied at 0.12 M-PB. The results of this experiment are shown in Fig. 4b and Fig. 5b. Incubations were performed at temperatures between 40 and 80°C at 10°C intervals. Fractionation in this case was performed by the M-HAP column method because of difficulties in the batch method at temperatures above 70°C.

The results show that the extent of the interaction (rate) and the stability of the products are dependent upon temperature and salt concentration as shown for eukaryote hybrid molecules by Denis (1966) and Church and McCarthy (1968). These authors also observed the characteristic spread of the hybrid melting
Fig. 4A
Reaction of $5 \times 10^{-4}$ mg/ml mb DNA with 1 mg/ml NRNA at 65°C with increasing salt concentrations.
Open circles and dashed line; hybridization reaction.
Filled squares and continuous line; control reassociation reaction.

Fig. 4B
Reaction of $5 \times 10^{-4}$ mg/ml mg DNA with 1 mg/ml NRNA at different temperatures in 0.12M Hepes.
Open circles and dashed line; hybridization reaction.
Filled squares and continuous line; control reassociation reaction.
**FIG 4**

**A**

- Graph showing the relationship between % Duplex and M.PB.
- Two lines, one solid and one dotted, indicating different conditions or results.

**B**

- Graph showing the relationship between % Duplex and °C.
- Two lines, one solid and one dotted, indicating different conditions or results.

Legend:
- Solid line: % Duplex as a function of M.PB.
- Dotted line: % Duplex as a function of °C.
Fig. 5A

Thermal chromatography of hybrids formed at different salt concentrations at 65°C.

Filled circles; hybrids formed in 0.05MPB.

Open circles; hybrids formed in 0.2MPB.

Open triangles; hybrids formed in 0.4MPB.

Filled triangles; hybrids formed in 0.7MPB.

Thermal chromatography with 0.12MPB.
fig. 5A
Fig. 5B

Thermal chromatography of hybrids formed at different temperatures in 0.12M PB.

Filled triangles; hybrids formed at 40 °C.
Open triangles; hybrids formed at 50 °C.
Open squares; hybrids formed at 60 °C.
Filled squares; hybrids formed at 65 °C.
Open circles; hybrids formed at 70 °C.
Filled circles; hybrids formed at 80 °C.

Thermal chromatography with 0.12M PB.
fig. 5b
profile. This feature predicted by Chamberlin and Patterson (1965) has been demonstrated in the comparison of RNA/DNA hybrids to duplex DNA (Chamberlin and Berg 1964). It is concluded that base pairing between RNA and DNA occurs with increasing specificity at higher temperatures and at decreasing salt concentrations. Exactly the same relationship exists for the DNA/DNA reaction (Walker and McLaren, 1966; Martin and Hoyer, 1966; McCarthy and McConaughy, 1968).

b) The Complementarity of Mouse Liver RNA.

The extent of homology between total liver RNA and mb DNA was studied in the following way: increasing amounts of total liver RNA were incubated in 0.12 M-PB at 65°C with 0.5 ug/ml mb DNA for 1 hour. The result of this experiment is shown in Fig. 6a. The complementary sequences of the DNA were not saturated even at an input of 15mg/ml RNA.

If the DNA complementary strands in the reaction are representative of the distribution of sequences in the genome then these strands should reassociate with unlabelled excess DNA at the same rate as the total DNA. To test this complementary strands recovered from alkali digestion of the hybrid, were made up to 0.01 mg/ml with unlabelled DNA. Reaction rate was measured by the batch procedure on THAP. Estimation of the reassociation
Fig. 6A

Reaction of mouse liver RNA with $5 \times 10^{-4}$ mg/ml mb DNA at 65°C in 0.12MPB.

Filled squares: control reassociation reaction.
Open triangles: cytoplasmic RNA
Filled triangles: total cell RNA.
Filled circles: NT RNA.

Fig. 6B

Homologous reassociation reactions.

Open squares: the reassociation reaction of $1 \times 10^{-2}$ mg/ml mb DNA (Left axis)
Open circles: the homologous reassociation reaction of complementary DNA strands from a first cycle reaction with total cell RNA, with $1 \times 10^{-2}$ mg/ml mb DNA (Right axis).
Filled squares: the reassociation reaction of complementary DNA strands from a first-cycle reaction with total cell RNA. (Right axis).
fig 6
rate of the radioactive complementary strand compared to the rate of reassociation of a labelled control at 0.01 mg/ml gives a measure of the dispersion of the liver RNA complementary sequences in the genome. (Fig. 6b) A scheme similar to this was also used by Kohne (1968) to estimate the number of copies of the ribosomal cistrons in E. coli and other bacterial species.

In the original liver RNA/DNA incubation some DNA/DNA reassociation must have taken place. The maximal reassociated contribution was estimated in a parallel experiment in which no RNA was included. This control was given exactly the same treatment as the experiment. The results are shown in Fig. 6b. In a repeat of this experiment the DNA was pre-fractionated on HAP (as described previously) before incubating with the RNA in the experiment and alone in the control. Using pre-fractionated material the control reaction was undetectable; the hybridization values were the same as with unpre-fractionated DNA.

These results suggest that the sequences of the genome complementary to liver RNA are restricted to the reiterated fraction. Britten and Kohne (1966) describe the relationship between the extent of reiteration and the rate of the reaction; using their calculation, the liver RNA is complementary to sequences of the DNA which exist within a set of families of sequences of which there are between 10,000 and 100,000 members (copies of similar sequences). The size of these families or their fraction of the total genome can also be calculated from the re-cycling homologous reaction. Since only 50% of the label was found in the reassociated fraction it follows that only 50% of the family members have reassociated. Since about 4% mb DNA constituted the liver complementary fraction (double-stranded amount) it must have membership
of a set of families which make up between 8 and 9% of the total DNA. If this deduction is correct, on extending the incubation time or increasing the concentration in the re-cycled homologous reaction, complete reassociation should be seen. The most convenient way of relating concentration and time of incubation for different data, is by the Cot term (initial concentration x time of incubation, expressed as moles nucleotide per litre per second) described by Britten and Kohne (1966).

The liver RNA experiment was repeated and the DNA complementary to the RNA was subjected to a further incubation with unlabelled DNA at Cot values up to a 100. At a Cot of a 100, sequences of only 1,000 copies are partly reassociated. To achieve this Cot value 1mg/ml. unlabelled DNA was incubated for 10 hours. To investigate the possibility of reassociating molecules breaking down over this period a control of pre-fractionated $^{32}$P reassociated DNA was included. Reassociated DNA repeatedly re-chromatographed with a background of between 5 and 10% at 65°C on 1ml packed volume THAP. After 1 hour incubation reassociated DNA re-chromatographed at $34\% \pm 2$ and after 10 hours the background was $92\% \pm 4$. In each case the estimation was made in triplicate. The experiment showed $34\% \pm 5$ reassociation after 10 hours. It may be concluded from this that a maximum of 10% of the liver RNA complementary sequences occur in families with less than a 100 members (copies) and that at least 60% occur within the reiterative range 10,000 to 100,000.
c) The relationship of RNA Sequences in the Nucleus and Cytoplasm.

RNA was extracted from total liver, from nuclei prepared by the citric acid procedure, and from the supernatant of the citric acid nuclear preparation. Increasing amounts of each were incubated with 0.5μg/ml. mb DNA. The results of this experiment are shown in Fig. 6a. This shows that the effective concentration of the nuclear RNA preparation is much higher than those of the total liver and cytoplasm. This is not altogether unexpected since the bulk of the RNA in each case will be made up of rRNA which will be inert in the hybridization reaction. As most of the rRNA occurs in the cytoplasm, the effective concentration of the nuclear should be much higher than the cytoplasmic RNA.

This experiment was repeated using DNA which had been pre-fractionated on HAP. DNA at a concentration of 0.5μg/ml. was incubated with 15 mg. each of total liver and cytoplasmic RNA (in this case prepared as described in Section 3b(b) (materials and methods). Incubation times were extended to give DNA Cot values of up to 0.05. The results of this experiment and the control reaction are shown in Fig. 7a. Also shown in Fig. 7b are the re-cycled homologous reassociation reactions of the complementary strands from each of the three RNA’s. These results indicate the close similarity of the family sequences complementary to the three RNA’s as is seen in the rates of reassociation in the homologous second cycle reaction.

Long incubation times increase the contribution of the simultaneous DNA/DNA reaction to the total duplex measured in the hybrid reaction with total liver and cytoplasmic RNA’s. It is possible that the inability of the cytoplasmic RNA to attain the same levels of saturation as the nuclear, may be due to the reassociation of a fraction of the complementary DNA strands. This
Fig. 7A

Reaction of mouse liver RNA's with $5 \times 10^{-4}$ mg/ml mb DNA at 65°C in 0.12 MPB.

Filled squares; control reassociation reaction.

Open triangles; cytoplasmic RNA.

Filled triangles; total cell RNA.

Filled circles; NRNA.

Fig. 7B

Homologous reassociation reactions.

Filled squares; the reassociation reaction of $1 \times 10^{-2}$ mg/ml mg DNA (Left axis) also, filled squares; lower line, the reassociation reaction of complementary DNA strands from a first cycle reaction with cytoplasmic RNA (Right axis).

Filled circles, filled triangles and open triangles; homologous reassociation reaction of complementary DNA strands from first cycle reactions with NRNA, total cell RNA, and cytoplasmic RNA respectively. (Right axis).
Fig 7

A

% DUPLEX vs. time (hrs.)

B

% DUPLEX vs. time (min.)
fraction might account for the difference in the extent of the reactions but it cannot account for the overall similarity of the homologous second cycle reassociation reactions. In order to avoid the transfer of reassociated strands into the homologous second cycle, DNA from the first cycle after digestion of the RNA in alkali was incubated at the same effective concentration as on the first cycle reaction. Strands which reassociated in the first cycle should reanneal and therefore be separable on HAP leaving only the strands complementary to the RNA unreassociated. There is however, one important consideration that must be made in this procedure. The amount of material reassociated may only be a fraction of a family of sequences with a certain effective concentration. If the first cycle reassociation reaction is confined to a family making up 20% of the total DNA, then the effective concentration for the reaction is one fifth of the total DNA. In the annealing reaction to remove this fraction it is not sufficient merely to adjust the concentration to compensate for the family sequence concentration in the initial reaction. If the effective concentration of the family was known and adjusted to that value in the re-cycling reaction then the same fraction of the sequences of the family would again reassociate, such is the nature of the second order reaction. Thus it is extremely difficult to remove the last traces of homologously reassociated DNA in the reaction. It is for this reason that low inputs of DNA and short incubation times are used.

The reassociated component was removed as far as possible by concentrating the DNA taken into the second cycle to ten times that of the first cycle reaction and incubating for at least twice as long as in the first reaction. A preliminary experiment showed that after this time no further reaction was seen. In the case of the cytoplasmic RNA/DNA reaction the homologous reassociated
component made up a considerable fraction of the total duplexed DNA seen as 30% reassociation on homo-incubation. It is understood however, that not all of the first cycle reassociated material is removed.

The close similarity of the homologous reassociation rates of the complementary strands from the hybrids could mean that these strands belong to one family or that they belong to different families with similar numbers of sequences. To decide this complementary strands from one hybridization were reannealed with heterologous RNA, using the homologous RNA as the control reaction. Complementary sequences to N RNA were recovered after a one hour incubation of 1μg/ml mb DNA with 2μg/ml N RNA at 65° in 0.12 M-PB. Similarly, strands complementary to cytoplasmic RNA were obtained from a one hour incubation of 15μg/ml cytoplasmic RNA with 1μg/ml mb-DNA. The complementary strands of the DNA were recovered as described previously. In a second cycle set of reactions using these isolated single strands each was incubated without RNA, with its homologous RNA, and with heterologous RNA. Thus strands complementary to N RNA were incubated in the second cycle again with that RNA, also with cytoplasmic RNA, and without RNA. In a separate experiment second cycle N RNA complementary strands were reacted with N RNA which had been treated with alkali as in the procedure for the recovery of DNA strands from the hybrid. No reaction in this case, or in a control with 10μg/ml E.coli RNA, was seen.

The results of second-cycle homologous and cross-reactions are shown in Fig. 8a. These results again reflect the large differences in the effective concentration of reacting sequences between the RNA preparations. It is seen that the N RNA reacts faster and to a greater extent with DNA first fractionated on N RNA than with that prepared from cytoplasmic RNA. Cytoplasmic RNA on the
Fig. 8A

Second cycle homologous and heterologous hybridization reactions.

Filled circles; homologous NRNA reaction.
Open circles; heterologous NRNA reaction.
Filled triangles; heterologous cytoplasmic RNA reaction.
Open triangles; homologous cytoplasmic RNA reaction.
Filled squares; the reassociation reaction of complementary DNA strands from a first cycle reaction with NRNA.

Fig. 8B

Second cycle homologous and heterologous hybridization reactions with DNA strand concentration adjustments.
Symbols as for Fig. 8A.
fig 8
other hand reacts faster and to a greater extent with DNA from N RNA prepared strands than with its homologous counterpart. This effect can be explained by again considering the effective concentration of complementary strands in the first cycle reaction. Virtually all of the sequences that can react with N RNA are removed in the first cycle incubation. This is not so with the cytoplasmic RNA where the concentration of reacting sequences is so small that after most of the sequences have hybridized, the effective concentration becomes such that much larger amounts of complementary RNA sequences or much longer incubation times would be required to complete the reaction. Similarly, strands which are complementary to cytoplasmic RNA may occur within families which are partly reacted under the conditions employed and thus be unavailable for reaction with RNA. The concentration of DNA used in this experiment is low enough to render the latter possibility less likely, and it is more probable that the effective concentration of the complementary RNA sequences is not sufficient to raise the complementary sequence concentration to high enough Cot values. This effect is again seen in the second cycle reaction where the cytoplasmic RNA reaction proceeds more slowly and in neither is completed. The second cycle reaction between cytoplasmic RNA and N RNA complementary strands largely bears out the earlier interpretation of the rate and extent of the cytoplasmic reaction. It is predictable that the second cycle reactions should be twice as fast in the case of the N RNA complementary strands from the first cycle than those derived from the cytoplasmic RNA reaction. This is because there should be at least twice as many strands present in the incubation, a carry over from the extents of first cycle reactions. The concentration of N RNA was too high in the reaction shown in Fig. 6a for rate differences to be
seen, the cytoplasmic RNA for the most part bears out this prediction. Fig.8b shows second cycle reactions in which strand concentration is the same. This was achieved by pooling complementary strands from first cycle cytoplasmic RNA hybridization reactions.

In each of the second cycle reactions, the reaction rate is slower than on the first cycle. Theoretically, since the sequence concentrations are the same in both first and second cycles, the rates should be the same. This certainly should be the case for N RNA complementary strands derived from a reaction which is complete. This effect is difficult to account for unless specificity requirements change on re-cycling. If this is the case, the melting temperature (Tm) should also be different. Melting profiles of first and second cycle hybrids and reassociated molecules are shown in Fig.9(a and b). These profiles show increasing spread and decreasing Tm on re-cycling.

It is difficult to see why specificity requirements should change on re-cycling. Clearly whatever the effect it must reside in the DNA component of the reaction, since in both cycles the conditions of the incubation were identical. Therefore the effect of re-cycling reassociated DNA was investigated.
Fig. 9A

Thermal chromatography of 1st and 2nd cycle hybrids on HAP with 0.12MPB.

Open circles NRNA hybrid
Filled circles (right hand curve) cytoplasmic RNA hybrid.
Filled circles (left hand curve) second-cycle hybrids.

Heterologous and homologous second cycle hybrids were indistinguishable.
Fig. 28

Thermal chromatography of reassociated mb DNA strands on HAP with 0.12M PB.

Filled circles; sheared native mb DNA.

Open circles; first cycle reassocaited mb DNA.

Filled triangles; second cycle reassociated mb DNA.

Open triangles; third cycle reassociated mb DNA.
The Effect of Reannealing DNA.

To study the effect on the DNA of re-cycling, the DNA-DNA reaction was studied in the following way. 10ug/ml mb DNA was incubated for 2 hours at 65°C in 0.12 M-PB. After this time 20% of the DNA had reassociated. Since only very small changes in extent of the reaction after 2 hours at this concentration were seen, the reaction was considered complete. The effective concentration of the sequences which had reassociated was therefore 20% of the initial concentration (2ug/ml mb DNA). The reassociated fraction was readjusted to 20% of the concentration of the initial reaction, denatured and reannealed for the same time and under the same conditions as for the first cycle reaction. If all the sequences of the families involved in the first reaction had reacted, then complete reassociation should be seen on the second cycle. The results (Table 3) show that this did not occur. The reaction observed after 2 hours incubation was 70% ± 2 in an average of three experiments, and the overall rate of the reaction was slower by a factor of almost 2. Reassociated DNA from the first reaction, undenatured on the second, re-chromatographed to a value of 92% after 2 hours incubation at 65°C.

The discrepancy between the theoretical and observed extent of the reaction on the second cycle could be accounted for by assuming that the first cycle reaction had not gone to completion. The change in the rate however, cannot be accounted for in this way. Both the change in rate and the discrepancy in extent of the reaction can be accounted for by assuming a certain amount of breakdown in the molecule. The rate of the reaction is piece length dependent (Britten and Kohne, 1966, Wetsm and Davidson, 1968) and if under the conditions used in the reaction certain of the sequences had become reduced to a size less than the minimum stable duplex piece length
complete reassociation would not occur. These two alternatives were investigated in the following way:

Cot values of I were attained in two ways. a) mb DNA was incubated for 1 hour at a concentration of 0.1mg/ml., and, b) mb DNA was incubated for 10 hours at a concentration of 0.01mg/ml. The reassociated fraction was recovered from both incubations and, as previously, reannealed at a concentration of 2 ug/ml. in a second cycle reaction for 2 hours. These results are shown in Table 3, and Fig. 9b shows melting profiles.

Although the difference is small between the rates and extent of reactions of the reassociated DNA's from the different reactions, the results are indicative of breakdown of DNA in the annealing process. The fact that neither fraction approaches the theoretical expected value of reassociation on a second cycle, implies that some breakdown occurs on HAP chromatography. This conclusion was substantiated and a rather interesting effect of piece length seen when the second cycle reaction was driven to completion with unlabelled large excesses of total DNA. In this experiment the sequence concentration effect was examined. Large excesses of DNA (1mg/ml. unlabelled total DNA) were included in the second cycle reassociation reaction; these results are also shown in Table 3. Unlabelled DNA was reassociated in order to increase the concentration of complementary strands five-fold over unreassociated unlabelled DNA. The latter DNA however, raised the reaction to values higher than the pre-fractionated DNA. This result suggests that the longer piece lengths of the unfractionated DNA have helped stabilise the shorter pieces of the re-cycled material.

The change in rate can similarly be accounted for by piece length of the
TABLE 3

THE EFFECT OF REANNEALING DNA ON THE RATE AND EXTENT OF THE REACTIONS

REACTIONS CARRIED OUT IN 0.12M PB. at 65°C.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10  20  40  60  80  100  120</td>
</tr>
</tbody>
</table>

1st cycle reassociation
1x10^{-2} mg/ml ab DNA

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8  12  16  19  19.2  19.4  19.4</td>
</tr>
</tbody>
</table>

2nd Cycle reassociation

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20  28  42  55  65  68  70</td>
</tr>
</tbody>
</table>

2nd cycle undenatured reassociated control

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-  -  -  92.5  -  -  92</td>
</tr>
</tbody>
</table>

2nd cycle reassociation A

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22  30  42  57  67  70  72</td>
</tr>
</tbody>
</table>

2nd cycle reassociation B

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18  25  38  51  64  66  68</td>
</tr>
</tbody>
</table>

2nd cycle homologous reassociation

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-  -  -  72  -  -  76</td>
</tr>
</tbody>
</table>

2nd cycle homologous reassociation

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reassociation after incubation time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-  -  -  80  -  -  82</td>
</tr>
</tbody>
</table>

A * reassociation reaction using DNA from 1st cycle reaction with 0.1mg/ml DNA for 1 hr.

B * reassociation reaction using DNA from 1st cycle reaction with 0.01mg/ml DNA for 10hrs.

1* homologous reaction with enriched re-iterated DNA.

2* homologous reaction with unreassociated DNA.
the DNA. Tm decrease must to some extent reflect the overall decrease in
the numbers of base pairs formed per sequence length. If an initial
nucleation between two strands of the DNA reduces the specificity
requirements for further nucleations between the strands, then, when the
sequence length is reduced the possibility of forming more base paired
regions is reduced.

The rate decrease of the second cycle reaction will, to some extent,
affect the extent of the reaction. If the rate was halved on re-cycling
then the time of the reaction to completion must be doubled. Extending
the time course of the second cycle reaction does not produce this result,
probably because the rate of reduction of piece length in the DNA matches
the rate of nucleation in the reaction.

e) Sequences Common to Liver and Kidney NRNA's.

Exactly the same approach used to study sequences common to nuclear and
cytoplasmic RNA's was used to estimate the sequences common to liver and kidney
RNA's.
Kidney nuclear RNA was found to have an identical saturation effect in the hybrid reaction to liver mRNA, although the effective concentration of sequences was higher by a factor of two in the kidney nucleus than in the liver. The complementary DNA was of a reiterative class similar to that of the liver DNA and of a similar proportion in the genome. These results are shown in Fig.10a.

Complementary DNA was isolated as described previously for nuclear and cytoplasmic RNA's. For the kidney first cycle reaction 1mg/ml. NRNA was reacted with 1ug/ml pre-fractionated mBDNA. In the liver first cycle reaction 2mg/ml. liver NRNA was used. The same amount of complementary DNA from each tissue was taken into the second cycle. The second cycle reactions are shown in Fig.10b. The same amount of each RNA was used in both cycles thus maintaining the same effective concentration of sequences in the RNA. The complementary sequence DNA concentration was also the same in each case, no adjustment being required as both RNA preparations gave the same saturating effect at the same value. The second cycle homologous reassociation reactions are shown in Fig.11a, for the complementary strands derived from both tissue RNA's. Although the differences are small it is seen that the complementary sequences have proportional membership of sequence families of differing reiterative values. The kidney derived complementary sequences are proportionally representative of more highly reiterated families than are those derived from the liver. This is reflected in the rates of the reactions. The liver complementary sequences are more equally representative of all of the reiterated families.

In the second cycle heterologous reactions the differences in the reaction rate again reflect the complementary strand bias between families. The extent
Fig. 10A

Reaction of mouse liver and kidney NRNA's with $5 \times 10^{-4}$ mg/ml mb DNA.

Filled crossed circles; the reassociation reaction of $5 \times 10^{-4}$ mg/ml mb DNA.

Filled circles and continuous line; the reassociation reaction of $5 \times 10^{-3}$ mg/ml mb DNA.

Filled squares; hybrid reaction with $5 \times 10^{-2}$ mg/ml liver NRNA.

Open squares; hybrid reaction with $2.5 \times 10^{-2}$ mg/ml kidney NRNA;

Filled and open triangles; hybrid reactions with $1 \times 10^{-1}$ mg/ml liver NRNA and $5 \times 10^{-2}$ mg/ml kidney NRNA respectively.

Fig. 10B

Second cycle hybridization reactions.

Filled and open circles; second cycle homologous reactions of liver NRNA (2mg/ml) and kidney NRNA (1mg/ml) respectively.

Filled and open squares; second cycle homologous reactions of liver NRNA ($1 \times 10^{-1}$mg/ml) and kidney NRNA ($5 \times 10^{-2}$mg/ml) respectively.

Filled triangles; second cycle heterologous reaction of kidney NRNA ($5 \times 10^{-2}$mg/ml).

Open triangles; second cycle heterologous reaction of liver NRNA ($1 \times 10^{-1}$ mg/ml).
fig. 10
Fig. 11A
Second cycle homologous reassociation reactions.
Filled circles; reassociation reaction of $1 \times 10^{-2}$ mg/ml mb DNA (Left axis).
Open circles; the homologous reassociation reaction of complementary DNA strands from a first cycle reaction with kidney mRNA, with $1 \times 10^{-2}$ mg/ml mb DNA (Right axis).
Filled squares; the homologous reassociation reaction of complementary DNA strands from a first cycle reaction with liver mRNA, with $1 \times 10^{-2}$ mg/ml mb DNA (Right axis).
Crossed filled circles; reassociation reaction of complementary strands from a first cycle reaction with kidney mRNA.

Fig. 11B
Second cycle homologous reassociation reactions.
Filled circles; reassociation reaction of $5 \times 10^{-3}$ mg/ml mb DNA (Left axis).
Open circles and squares; as for Fig. 11A, reaction of liver and kidney complementary strands with $5 \times 10^{-3}$ mg/ml mb DNA.
fig. II
of the reaction in each case is noteworthy. Liver NRNA completes the reaction with heterologous complementary DNA less well than does kidney NRNA in the same situation. This must to some extent be the product of the effect of sequence concentration and breakdown in the DNA. The homologous reaction in both cases is more extensive than the heterologous, which means that some families are not common to both RNA's, although it may reflect the differences in sequence concentration between the RNA's. These conclusions are substantiated by the results shown in Fig.11b. In this case the first cycle reactions were stopped when only half completed, and the complementary DNA homologously reassociated in a second cycle reaction. The bias of the kidney complementary strands to the more highly reiterated sequences is seen more clearly in this situation.
A Model System For Hybridization and Reassociation in Solution.

Since the conclusions drawn from the reactions between RNA and DNA are based on the interpretations of the reactions shown in Fig.3 namely, that the reaction of RNA in a reassociation system both accelerates the overall reaction rate and depresses the overall reaction extent, a model was constructed to substantiate this interpretation.

The hybridization reaction has been formally demonstrated to be sequence concentration dependent and therefore second order in reaction rate (Bishop, 1969). With excess complementary sequences the reaction will become pseudo first order, the effect of this is seen in plateauing in the RNA/DNA reactions.

As in the DNA-DNA reactions (Wetmur and Davidson, 1968) the rate limiting event must be the nucleation event, that is, the formation of one or a few correct base pairs at some in-register point along the two strands. In the ideal case of reassociation and hybridization reactions occurring simultaneously in solution, at each nucleation site on the DNA either of the two reactions can occur. The type of reaction that occurs will depend upon the relative concentration of complementary sequences in the DNA and RNA. The reactions may be written thus:

\[
\begin{align*}
\text{ss DNA} + \text{ss DNA} & \xrightarrow{K} \text{DNA/DNA} \quad (1) \\
\text{ssDNA} + \text{RNA} & \xrightarrow{K} \text{RNA/DNA} \quad (2)
\end{align*}
\]

These reactions represent the model situation where all of the RNA is complementary to one single strand of DNA (ss DNA). Since the complementary strand can enter into both reactions its rate of depletion will be determined by the rates of both reactions.

Assuming both reactions have the same overall rate constants, let \( a \) the
single stranded initial concentration, and \(b\) = the initial concentration of complementary RNA. If, after time \(t\), \(x\) amount of reassociation has occurred whilst \(y\) amount of hybridization has occurred; reactions 1 and 2 may be represented thus:
\[
\begin{align*}
(a-x-y) + (a-x) & \xrightarrow{K} x \\
(a-x-y) + (b-y) & \xrightarrow{K} y
\end{align*}
\]

The rate of formation of \(x\) and \(y\) is therefore:
\[
\frac{dx}{dt} = k(a-x-y)(a-x)
\]
and:
\[
\frac{dy}{dt} = k(a-x-y)(b-y)
\]

Solving these simultaneous equations for \(y\) gives
\[
t = \frac{1}{kb} \log \left[ \frac{\frac{a^2 - ax}{a^2 - a^2x - abx}}{\frac{a^2 - ax^2}{a^2 - a^2x - abx}} \right]
\]

The derivation of this equation is shown in appendix 2.

A computer was programmed to solve for \(x\) with varying values of \(b\), \(k\) and \(t\) with \(a\) maintained as unity. The programme scanned \(k\) values from 0.1 to 1 at intervals of 0.1. At each stage \(b\) values from 0 and 0.1 to 0.5 the value of \(a\) in geometric progression were substituted at \(t\) intervals of between 1 and 10.

A further important restriction was placed on the system to mimic the performance of an isotopically labelled reaction in which the DNA is labelled and the RNA is unlabelled. In this situation the product is made up of double stranded DNA with both strands labelled and the hybrid of which only one strand, the DNA, is labelled. In the model case, the proportion of RNA/DNA to DNA/DNA will be the same as the input ratio of the RNA and DNA. Thus the proportion of hybrid \((y)\) to reassociated molecules \((x)\) can be represented:
\[ y = \frac{b \times x}{a} \]

where only one strand of the hybrid is labelled, 'y' has one half of this value.

For every value of x calculated at increasing values of b and k, the computer was asked to calculate y and finally to summate x and y.

The results of this programme are shown in Fig.12 (a, b and c). One rather striking feature is the effect made by even fractional inputs of the complementary strand. The crossing over feature is also interesting especially in relation to the increasing values of 'k'. Clearly k reflects complexity; the lower the 'k' value the longer the reaction time.
Fig. 12

Computer simulation of hybridization reaction. For details see text.
A

B

duplex

duplex

t

t

C

D

duplex

duplex

t

t

E

F

duplex

duplex

t

t

K = 0.2
DISCUSSION

In viral and bacterial genomes, Britten and Kohne (1966) showed a linear relationship between 'k' (the second order rate constant) and genome size in terms of numbers of base pairs. This relationship does not hold for eukaryote genomes because of the occurrence of base sequences sufficiently related that they can reassociate. An, as yet, undetermined number of such families exist with different numbers of members. Clearly the numbers and sizes of families will be determined by the stringency of the conditions employed in measurement; this is seen in the dependence of the reassociation reaction rate and extent on the conditions of incubation (for a review see Walker, 1969).

At a given incubation condition the population of sequences within a family will react at a rate proportional to the family size. Thus each family of sequences in the mammalian genome will have its own second order rate constant. Therefore the overall rate constant for mammalian DNA is the sum of a number of rate constants. The number being determined by the number of different family classes of sequences in the DNA.

Considering the second order rate constant (Glasstone, 1951):

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}$$

where 'a' and 'b' are the initial concentration of the reactants and x is the decrease of each after time 't'.

The overall rate constant for 'n' families will be:

$$k = k_1 + k_2 + k_3 + \ldots + k_{n-1} + k_n.$$
When $k_n$ is the $n$th family of sequences with an effective concentration of $a_n$ reacting with complementary sequences at a concentration of $b_n$ showing a decrease of $x_n$ after time $'t'$. 

The hybridization reaction in the mammalian genome is extremely complex when viewed in this way. Families may show very large differences in size of membership and may, in hybridization reactions, show widely differing values of complementarity to sequences in the RNA. From the second order equation it is clear that when $'b'$ becomes large with respect to $'a'$ the reaction becomes kinetically first order. In the hybridization reaction certain families may show extensive complementarity to sequences in the RNA, making the reaction one of pseudo-first order. This situation may exist together with the situation in other families where the extent of complementarity is less and the family membership is small, a situation in which little or no reaction would take place. It is possible that certain sequences could be equally represented in the RNA (the same number of transcriptory products) but because of differences in the family size of their complementary sequences in the DNA, widely differing extents of hybridization could occur between these sequences.

There is one other important aspect of the occurrence of families of sequences and the second order reaction. It is best seen from the model curves (Fig.12) that the time course of the reaction is determined by the value of $k$. Though even at high $k$ values the second order reaction is essentially never complete, the reaction of certain families will be more complete than others after any given time ($t$). At any given value of $'t'$ the difference between the hybridisation and reassociation levels of a family of sequences is determined by the effective concentration of
complementary sequences in the RNA. The extent of the hybridization reaction will be determined by the concentration of the different RNA's present in the incubation solution. The results of differences in effective concentration will be greatest when small families of DNA sequences have only small amounts of complementary sequences in the RNA. If the overall effective concentration of reacting sequences is low, less frequently represented sequences may not react during the time of the experiment.

The results show that the hybridization reaction is restricted to the reiterated sequences of the genome. Repeated attempts using RNA's from many different sources and obtained by different preparation procedures failed to show any reaction with the uniquely occurring sequences. Attempts in which the sequences of the slow fraction were concentrated in the presence of RNA sequences and incubated over extended periods failed to yield a DNA strand which on recovery from the hybrid would homologously reassociate with slow fraction DNA. Because of the restriction of hybridization to the reiterated sequences it is impossible to quantify the amount of the genome transcribed. It is possible that the unique sequences of DNA do not have a transcriptory product, although some evidence for hybridization occurring with unique sequences has been presented (Drake and Britten; 1968; Davidson and Hough, 1969). It is by no means certain, however, that these reactions were with unique sequences. The difficulty of taking a second order reaction to virtual completion means that the last reiterated sequences cannot be removed. In hybridization experiments designed to anneal unique sequences, the reaction with RNA of any reiterated sequences which remain will mask the formation of any DNA-RNA unique sequence hybrids. It is quite probable then, that the above mentioned workers have only observed the reaction of reiterated
sequences. In results obtained from systems using immobilised DNA, since the reaction rate is reduced by a factor of about 10 (Britten; 1969) compared to the liquid phase reaction, only reiterated sequences have been measured.

It is a common misconception to think that if a non-reiterated sequence is transcribed many times its hybridization will be observed. If a Cot value of 10,000 is required before a unique sequence will react, then a very high proportion of the RNA must be complementary to one unique sequence before hybridization occurs. To react one unique sequence it would be necessary to employ a system sufficiently sensitive to detect 1x10^{-14}% hybridization of the DNA (taking a unique sequence length as 1,000 base pairs). Clearly only small multiples of this value could be hybridized with any given RNA because of the difficulty in obtaining sufficiently high Cot values with many sequences. If there are unique sequences amplified in transcription the total number of sequences transcribed must be reduced, hence the overall complementarity (% hybridization) must be reduced. Thus, if the RNA concentration is left constant and the average copy number of sequences in the RNA is raised, the rate of reaction will be increased but the overall extent of the reaction will be reduced. The interpretation of plateau values or extent of the reaction is difficult because any one value can be attained in a variety of ways depending upon the number of copies of the complementary sequences in the RNA and their relative proportions to the reiterated sequence families in the DNA.

From the rate of the reactions observed there must exist many RNA copies of the reiterated DNA sequences. These copies may in fact be the transcrip
tory product of only a very few sequences, but hybridization could occur
with all the family members. There may exist several such families in which one or two members are transcribed but all of which hybridize. This is likely to be the case for most, if not all, of the reiterated sequences seen to hybridize with nuclear RNA.

About 20% of the major band fraction of mouse DNA will reassociate at Cot values about 10, after this further reaction is virtually unmeasurable. Since some 6% of the total mb DNA will hybridize with NRNA this means that 12% of the total mb DNA is complementary to this RNA. This is about half of the amount of reiterated DNA in the major band fraction. From the homologous reassociation experiments it is seen that complementarity extends through almost the entire spectrum of reiterated sequences. Since the hybridization reaction is essentially complete the 6% of the genome complemented can be considered an absolute value. Yet, considering the distribution of the hybridized DNA sequences throughout the reiterated families of sequences it is difficult to understand why the hybridization value is so low; why in fact, the hybridization value is not one half of the total reiterated sequence value at completion. Obviously the simplest explanation of this is that two or more families exist which are reiterated to the same extent. Also, families may differ in their relative proportions within the reiterated fraction of the genome. In the first case RNA homology with one family out of two or more of similar reiterated value would in the homologous reassociation reaction show complementarity to that reiterated class and reveal nothing of inter-class family differences. The second explanation is equally feasible. In this case, if one considers the possibility of there being quite considerable differences in family reiterated values together with there being differences in proportion of families, then it is easy to see that a perfectly smooth
second order reaction would be seen on reassociation. On hybridization, two or three highly reiterated families amounting to 12% of the mb DNA could explain the observed results.

There is however, one further possible explanation of the limited extent of the hybridization reaction. If one considers the possibility that what constitutes a sequence family in terms of reassociation may be different from that which constitutes a family in terms of hybridization, then the observed extent of the hybridization reaction might be the ceiling value of that reaction. Put another way, in terms of the reassociation reaction the reiterated sequences constitute some 20% of the mb DNA, but in terms of the hybridization reaction they may only constitute 12%. If this were the case it would mean that a stable hybrid molecule would require more extensive complementarity in base pairing than the reassociated molecule. Hybrid molecules on thermal chromatography show a much greater range of stabilities and a lower Tm than does its reassociated counterpart. Since the maximum values for duplex formation occur some 20-25°C below the Tm it follows that under the experimental conditions employed, more severe demands have been made of the hybrid than of the reassociated molecule in terms of base sequence matching. This in itself means that sequence family size has been more restricted in the hybridization reaction. The extent of second-cycle reactions also support this explanation. Given the fact that it is DNA breakdown which prevents the reaction approaching the theoretically expected 100% value, the differences between the extents of second cycle reassociation and hybridization reactions must reflect the stability requirements. The hybridization value, since it is more reduced, reflects the greater sequence length complementarity requirements.
It is known that the stability of the duplex is affected both by the base composition (Marmur and Doty, 1961) and by the sequences of the bases along the individual strands, in particular in the mixed ribose–deoxyribose polymers (Chamberlin et al., 1963; Chamberlin and Patterson, 1965; Riley et al., 1966). From this work it is seen that U-containing polymers are less stable than their T analogs so that poly rA-poly rU melts 12°C lower than poly dA-poly dT in 0.1 M Na+. Poly dA-poly rU has a Tm at least 19°C lower than poly rA-poly dT, whilst poly dG-poly rC on the other hand is more stable than poly dC-poly dG. Work on artificial polymers by Lipsett et al. (1961) and by Michelson and Monny (1967) shows clear relationship between oligomer chain length and increase in Tm of the duplex. Considering these points it is clear that more extensive base pairing will be required to form a stable hybrid duplex with an RNA of high AU value (more DNA like) than one with a high GC value. Since, in the main, the hybrid molecules observed are made up of non-ribosomal RNA the extent of base pairing in these molecules must be greater than that in their reassociated counterparts. Again, this means that the conditions for hybrid formation are more stringent and, as in the case of reassociation, the more stringent the conditions, the smaller the reiterated class size. Thus the overall extent of the hybridization reaction may be less than the reassociation reaction due to the differences in the stability requirements for the reactions and therefore the differences in effective concentrations of the sequences involved. The effective concentration of the RNA sequences that will hybridize in the cytoplasm is at least 20 times less than that of the nucleus in liver cells. Considering the differences in amounts of RNA in the nucleus and cytoplasm of a liver cell there could in fact
be the same number of complementary sequences occurring to the same extent in terms of number of molecules in each; the effective concentration of the former will be much higher than the latter simply because of the large differences in amounts of the total RNA. It is not surprising then, that cytoplasmic RNA fails to compete as effectively or as extensively as does NRNA in an immobilised DNA hybridization scheme with labelled total cell RNA. (Shearer and McCarthy, 1967). These authors interpreted their results as demonstrating the existence of a fraction of RNA sequences restricted to the nucleus. There is other evidence which suggests the existence of an RNA fraction confined to the nucleus (for a review see Harris, 1964).

The results presented here might be interpreted to show the occurrence of all cytoplasmic RNA's within the nuclear RNA fraction but the absence of certain NRNA's from the cytoplasmic fraction. There are, however, two points worth consideration: 1) The overall similarity in the family representation of the DNA complementary to both RNA classes. 2) The incompleteness of both first and second cycle reactions with cytoplasmic RNA.

It is possible to argue from these points that the observed effects, both in competition experiments and in re-cycling isolated complementary strands, are due to the effective concentration of the RNA sequences involved. If a family of sequences in the DNA is half reacted with the complementary sequence in a NRNA preparation then at least 20 times longer incubations or 20 times higher concentrations of cytoplasmic RNA would be required to produce the same result with the same sequences. The results show that competition does not reflect the absence of certain molecular species of RNA in the cytoplasm but their lesser abundance. Of the total NRNA sequences in liver nuclei about 20%
appear to be of the class which hybridizes with the reiterated DNA. In kidney nuclear RNA preparations this fraction appears to make up 40% of the total sequences. The overall extent of the reaction is the same in both cases although from the rates of the homologous reassociation reactions kidney complementary DNA appears to be made up of a higher proportion of more highly reiterated sequences. From the second cycle homologous and heterologous hybridization reactions it is however, clear that a considerable overlap exists between the sequences complementary to the two different NRNA's. A similar result was shown by Hoyer and McCarthy (1964) employing an immobilised DNA hybridization procedure with total RNA.

The second cycle heterologous reaction using kidney RNA is interesting and difficult to interpret for it is odd that the heterologous should be almost as extensive as the homologous reaction. Although the differences are small the rate of reaction is greater in the heterologous case. This effect can only in part be due to concentration of RNA sequences. The results could possibly be explained in terms of stability requirements if kidney NRNA had less stringent requirements than liver NRNA in the formation of a stable hybrid duplex. Relaxed conditions for duplex formation would result in higher sequence reiterative values although it need not necessarily mean that the reiterated fraction constitutes a greater proportion of the total. All reactions with NRNA from kidney should then proceed faster and more extensively than those with liver NRNA because the kidney complementary sequences in the DNA should be of higher reiterative values. The performance of kidney NRNA in the second cycle heterologous reaction could thus be explained in terms of the liver sequences being raised in reiterative value relative to that in the
homologous reaction. A knowledge of the base constitution of the non-ribosomal NRNA's from liver and kidney would be of considerable value in interpreting the results seen. If indeed it were known that kidney NRNA was higher in G C content than liver NRNA it would help substantiate this interpretation.

Nuclear RNA is known to be both heterogeneous in size and in its rate of turnover in the nucleus (Brown and Gurdon, 1964; Nemer and Infante, 1965; Gazarjan, 1966; Samarina, 1964; Ingle et al, 1965). Sedimentation coefficients vary between 15 and 60S, with a main peak of about 18S and more or less heterogeneous material in the region of 20-30S. Rapidly labelled RNA is seen first in the higher coefficient values of the gradient and spreads throughout on longer times of labelling. Initially the label in RNA, associated with the chromosomes. This RNA fraction sediments principally over the range 25-30S although a considerable fraction shows greater size heterogeneity at still higher values (Samarina et al, 1965; Georgiev et al, 1963). The existence of a rapidly labelled high molecular weight RNA fraction confined to the nucleus has been established beyond reasonable doubt, but its fate and relation to mRNA's and mRNA's are controversial. Two basic interpretations can be considered:

I. The high molecular weight RNA molecules are marked by an exceedingly high turnover rate with a half-life of about 2-20 minutes (Warner et al, 1966; Attardi et al, 1966; Gazarjan, 1966). They are made and degraded inside the nucleus (Harris, 1963, 1964; Warner et al, 1966; Attardi et al, 1966). However, although rapid labelling of this RNA is consistently observed, unequivocal proof of rapid and complete degradation has not been presented.
Experiments with actinomycin D indicate that either there is not a preferential degradation of rapidly labelled RNA (Attardi et al, 1966) or it is more stable than rRNA precursor (Kemp and Mandel, 1966). On the other hand some investigators point out that at least a fraction of newly synthesised RNA is unstable and degrades upon transfer of the cells to non-radioactive medium or in the presence of actinomycin (Scherrer et al, 1963; Grosdev and Tichnov, 1964). Under these conditions the heaviest fractions of the rapidly labelled RNA are shown to disappear completely from the gradient (Gazarjan, 1966).

Despite the controversies concerning the rate and conditions of breakdown it appears to be generally agreed that RNA is made and broken down in the nucleus without ever having left it, this may suggest an as yet obscure function for the short lived fraction of mRNA.

2. The high molecular weight RNA is a precursor of cytoplasmic mRNA and because of its large size may represent a polycistronic mRNA (Scherrer, 1965; Scherrer et al, 1966). Tracer kinetic data must however, rule out a direct precursor-product relationship (Attardi et al, 1966). In addition the operation of special mechanisms selecting only the mRNA segments needed for cytoplasmic translation must be envisaged (Scherrer et al, 1966).

Since neither view alone embraces all of the facts, the existence of two parallel pathways of high molecular weight RNA turnover in the nucleus has been considered (Soeire et al, 1966).

A precursor-product relationship has been argued by Hadjiolov (1967) on the basis of RNA synthesis rate. Goldstein et al (1965) presented data which allows the calculation of the estimated time of completion of precursor RNA of about 80-160 minutes. Although very little data is available on this
topic Hadjiolov concludes that the completion time of one RNA molecule in animal cells could perhaps be reckoned in hours. Greenburg and Penman (1966) estimated a lower limit of two to three minutes for the completion of 45S rRNA in HeLa cells. On the other hand a kinetic study showed that the degradation time is within one or two minutes (Gazarjan, 1966). These results are contradictory and they show the difficulty of arriving at sensible conclusions regarding nuclear RNA. The discrepancy must be even larger in non-growing cells where the overall rate of RNA synthesis is ten to one hundredfold slower.

Certainly it is a plausible solution to this paradox to accept that high molecular weight NRNA is a precursor of other RNA molecules. Since it cannot be the immediate precursor of any type of cytoplasmic RNA (Harris, 1964) conversion into stable NRNA's has been suggested (Hadjiolov, 1967). Transformation of high molecular weight RNA into stable 18S RNA has been put forward by several workers (Georgiev, 1964; Floyd, 1966; Brown and Gurdon, 1964; Steele and Busch, 1966). It is interesting that the maximum template activity of RNA in protein synthesis resides in the 18S region of sucrose gradients (Brawerman et al, 1963; Di Giralamo et al, 1964). Formation of stable 18S RNA is, however, rather a slow process, which is not compatible with the high rate of turnover of high molecular weight early labelled RNA (Georgiev, 1966).

On the basis of tracer kinetic and hybridization data Georgiev has concluded that the RNA of the nucleus can be divided into two classes. Ribosomal RNA, derived from the 45S precursor, and heavy rapidly labelled DNA-like RNA. This latter class was subdivided into (a) chromosomal 18S RNA which is ultimately found as mRNA in the cytoplasm, and (b) an RNA fraction
which functions and is broken down in the nucleus (for review see Georgiev, 1967). At best, tracer kinetic data is inconclusive. The validity of the argument of an RNA restricted to the nucleus appears to lie in hybridization results.

Samarina et al (1965) studied hybridizability between DNA and different RNA fractions prepared from nuclei by the hot phenol method. It was shown that the hybridizability of non-ribosomal RNA is lower by a factor of 10 to 25 than that of the rapidly labelled high molecular weight RNA. An increase in the DNA/RNA ratio leads to a proportional increase in the percent of hybridization until saturation occurs and a further increase in DNA raises the percent of hybridization only slightly. These results were obtained using an immobilized DNA hybridization system. It is important to note that the same amounts of RNA from different extractions of RNA prepared at different temperatures was used. No account was taken of the proportions of the relative non-rRNA sequence concentrations in each. This result merely reflects the heterogeneity of NRNA with regard to phenol extraction temperature.

A further series of experiments, performed in this case by Arman and Georgiev (1967) and reviewed by Georgiev (1967), studied the relative complementarity of labelled NRNA from different zones of a sucrose gradient. Nuclear RNA appears on a sucrose gradient with a main peak position about 18S, with a considerable amount of heterogeneous material to values up to 50 or even 60S (Samarina et al, 1965; Georgiev et al, 1963; Ingle et al, 1965; Yoshikawa et al, 1964; Gasarjan et al, 1966). Newly formed RNA, detected by the incorporation of pulsed labels appears first in the heavier zones of
the gradient and only at longer times does it begin to follow the UV
distribution.

Hybridization experiments with RNA derived from gradients after different
times of labelling showed that rapidly labelled heavy RNA hybridized better
than the total NRNA and also better than 18S. It was inferred from this
that the heavy early labelled fraction contained replicas from a larger number
of cistrons than did the 16S component. Several other interpretations are
open to these results particularly considering the conditions of the experi-
ment which employed high ratios of DNA/RNA. In that the same amounts of RNA
were used from each fraction of the gradient, the effective concentration of
sequences in that fraction about the 16S region will be lower since most of
this fraction will be made up of ribosomal RNA sequences.

Arian and Georgiev performed further studies by re-cycling RNA,
uncomplexed after first annealing to DNA, with new portions of DNA. It was
found that after the first transfer the hybridization percent falls rapidly,
but after the third or fourth transfer it stops at a relatively low but
significantly constant level, at both long and short labelling times. Brown
and Gurdon (1966) observed a similar effect in labelled RNA derived from the
anucleolate mutant Xenopus laevis where no rRNA can be labelled. It was
concluded from these results that the bulk of the NRNA is represented by
replicas from a relatively limited number of cistrons. In both of these
experiments high ratios of DNA/RNA were used, and in both cases 10 to 20%
of the RNA was hybridized. With only small changes in the percent bound
after the first cycle. It has been suggested by Church and McCarthy (1968)
that these results reflect the transcription of non-reiterated sequences in
the DNA although insufficiently high Cot values were employed to react all but the most reiterated DNA. The difference in the results seen between RNA's after different labelling times in the Arion and Georgiev experiments requires more careful consideration. RNA recovered from one hour labelling of the cells showed some 20% hybridization to the DNA on the first cycle. RNA labelled for 20 hours gave less than half of this value. Each gave similar values on a second annealing and each showed constant background values on subsequent hybridizations with new samples of RNA, the values being higher with the long-labelled RNA. This result shows that only 20% of these RNA preparations was effective in their reactions with DNA. The hybridization reaction with longer labelled RNA is obscured by the presence of non-reacting labelled species of RNA at the concentrations employed.

The final experiments from Arion and Georgiev involved competition reactions between labelled rRNA and unlabelled cytoplasmic competing RNA. The heavy early labelled fraction was shown to be reduced by 20% of its uncompetet hybridization value whilst the 16S fraction showed over 50% reduction. These values are of the order expected on a sequence concentration basis. Highest values could be expected for the heavy early labelled fraction because of the relative absence of rRNA sequences; in competition with cytoplasmic RNA the observed effect should be less than for the 16S fraction which should have a lower effective concentration because of the presence of rRNA sequences. These results, like those of Shearer and McCarthy (1967) are inconclusive in their demonstration of RNA sequences restricted to the nucleus.
Considering the relative concentration of non-ribosomal RNA sequence between cytoplasm and nucleus, it is perhaps a little surprising that cytoplasmic RNA competes to the extent observed. This might, in part, be explained from the lower specificity of the reaction of cytoplasmic RNA revealed in the lower Tm on melting the hybrid (Results section c.).

Certainly, if the requirements for a stable duplex were relaxed for one particular fraction of RNA relative to another the complementary family size would be larger and the reaction would be faster and more extensive. A similar effect has been shown for the cytoplasmic RNA in competition experiments (Birnboim et al 1967). In this case competition specificity was reduced when mammalian cytoplasmic RNA was used as the competitor. This effect may be due to the base constitution of the effective cytoplasmic sequences.

The most convincing evidence for the restriction of an RNA fraction to the nucleus comes from Harris and his associates. In this work the heterokaryon cell was employed to considerable advantage. Methods were devised for recovering and separating nuclei from heterokaryons between HeLa and hen erythrocytes (Fisher and Harris, 1962). The hen erythrocyte nucleus, when incorporated into HeLa cell cytoplasm enlarges and produces increasing amounts of RNA as it gets bigger. Analysis of the RNA made in the two types of nuclei showed that normal 28 and 18S RNA as well as the rapidly labelled high molecular weight RNA were being made in the HeLa nucleus, whilst only the rapidly labelled high molecular weight RNA was synthesised in the hen nucleus. The presence of hen protein synthesis in the cytoplasm was investigated by immunological methods, and could not be detected. An even
more sensitive method failed to detect hen specific surface antigens (Watkins and Grace, 1967). Thus the HeLa cell nucleus appears to dominate protein synthesis in the heterokaryon, even in the case where only one HeLa nucleus shares a cytoplasm with several hen nuclei.

The question arises whether the RNA being turned over in the hen nuclei passes to the cytoplasm but fails to programme HeLa ribosomes, or whether it remains restricted to the nucleus. Sidebottom and Harris (1968) showed that by irradiating the HeLa nuclei in the heterokaryon they could completely cancel HeLa rRNA synthesis. Subsequent to irradiation the heterokaryon was incubated with labelled RNA precursors, it was found that the erythrocyte nuclei continued to synthesise RNA, but this labelled RNA did not appear to be transferred to the cytoplasm. Since the hen nucleus contains only inactive vestigial nucleoli no rRNA was being synthesised, thus the RNA synthesis observed must be of the heterogenous polydisperse class.

The macrophage, like the hen nucleus in the heterokaryon, is without nucleoli. Watts and Harris (1959, and Harris 1959) showed that virtually all of the rapidly labelled polydisperse RNA synthesised in the macrophage nucleus could not be detected in the cytoplasm. Actinomycin D produces gross structural changes in the nucleolus (Schoefl, 1964) at concentrations which are too low to inhibit the transcription of RNA elsewhere in the nucleus (Perry, 1963). When cells which have been incubated with radioactive RNA precursors are exposed to actinomycin D the labelled RNA in the cell nucleus undergoes degradation, and again very little of it is transferred to the cytoplasm (Paul and Struthers, 1963). Clearly, the nucleus does not transfer genetic information to the cytoplasm unless it has a functional nucleolus; and it is
therefore reasonable to conclude that this structure and thus rRNA is involved in some fundamental way in the process of information transfer.

Intranuclear RNA turnover is much more pronounced in stationary or slowly growing cells, or in cells transferred from a rich to a poor medium, than it is in cells growing exponentially (Attardi et al., 1966; Haussais et al., 1966; Bellamy, 1966). In this situation, and in the macrophage nuclei, it is certain that RNA is made and totally broken without leaving the nucleus. There is some evidence that 28S exists in a pool in the nucleus though 16S does not and is observed, consequently, to turnover faster (Perry, 1966). Harris (1968) has suggested that the rapid turnover of the 16S region of the gradient may in fact reflect the turnover of the rapidly labelled polydisperse component. This, Harris claims, has the same average polynucleotide chain length as the 16S RNA detected by ultraviolet absorption, the difference in sedimentation reflecting a difference in shape and not size although this view is not supported by other techniques for determining RNA size (Scherrer, 1967; Loening, 1969).

Apart from the observation that synthesis of polydisperse 16S occurs all over the cell nucleus, the 16S fraction also contains several species which are selected out on MAK chromatography (Bramwell and Harris, 1967). Furthermore the specific activity of labelled ribosomes in the cytoplasm shows that 28S and 16S components are not closely coupled in their synthesis. Under certain conditions of labelling the 16S fraction may be labelled whilst the 28S is not (Perry, 1966). Also the cytoplasm does not appear to contain any ribosomal subunits which have additional RNA attached to them (Perry and Kelley, 1966). Despite differences in the relative rates of turnover of the
two ribosomal RNA components, it is clear that they are derived from one precursor molecule (Birnstiel et al. 1969).

Though it is not surprising that a fraction of RNA purporting to be 'messenger' should be made all over the nucleus, it is somewhat astonishing that it should be made in such amounts. In other words far more RNA is made than the fraction that becomes functional. Furthermore, if the generally held values of molecular weight are accepted (Scherrer and Marcaud, 1965) then a large fraction of the non-ribosomal RNA produced exceeds by about 50 fold the expected and observed molecular weights of messenger molecules (Keuchler and Rich, 1969). Yet, the rate of formation, base composition and the ability to stimulate protein synthesis in a cell free system (Scherrer et al., 1963), supports 'messenger' designation for the polydisperse fraction of RNA.

If this fraction is indeed a polycistronic precursor molecule, then the question remains whether all of the RNA sequences found in the nucleus are found in the cytoplasm? Given that certain sequences are broken down in the nucleus, are these sequences the same or different from those sequences translated in the cytoplasm?

Due to the lack of specificity in the mammalian nucleic acids hybridization reaction this technique cannot give a definite answer to this question. The results may show that there are many copies of only a few sequences, or alternatively, that there are relatively few copies of many sequences. In that the sequences hybridized are members of extensive families of sequences in the DNA the alternatives cannot be resolved. The answer to this question is critical to an understanding, not only of the control of transcription and ultimately of protein synthesis, but also to an understanding of the organization
of the sequences in eukaryote DNA.

Britten and Davidson (1969) and Georgiev (1969) have postulated regulatory roles for the reiterated sequences of the DNA. In each case the models derived have RNA restricted to the nucleus fundamental to their framework. In the Britten and Davidson model the reiterated sequences of DNA are seen as regulatory in function with mediation through sequence specific complexes, with nucleus restricted RNA's. The type of complex suggested is between double stranded DNA and single stranded RNA. Bekhov et al (1969 a and b), have presented evidence suggesting that sequence specific binding between chromosomal RNA and genomic DNA determines the sites at which the transcription-inhibiting chromatin proteins bind to the DNA. The model also allows for mediation through proteins produced by the NRNA. Although experiments have shown that protein synthesis may occur in the nucleus, and in one case totally in the nucleus (Hommel & Bessman 1964), they are largely unconfirmed. Frenster (1965) suggested that RNA might act effectively as a derepressor by annealing with the nonsense strand of the DNA thus leaving the sense strand open for transcription. This mechanism certainly would account for repetitious sequences, for the complementary strand at the derepressor RNA's transcription site must be identical to the sense strand being de-repressed. In either case, from the rate of production of NRNA its role must be a short lived one and must also (if they are up to 60S in size) cover up to 20,000 bases of the DNA.

The Georgiev model is altogether more precise and has more experimental data to substantiate it. Based on the operon model of Jacob and Monod (1961) it consists of two main parts: promotor-proximal acceptor (or non-informative) zone, and promotor distal structural (or informative) zone. The non-informative
zone contains acceptor loci which do not carry any information but specifically interact with regulatory proteins. The informative zone contains structural and regulatory genes carrying information regarding structural proteins, enzymes, and regulatory proteins. Different operons may contain identical or similar acceptor loci, the most multiple acceptor loci being localised in the proximal part. On the other hand, one operon contains a number of different acceptor loci, reacting with different regulatory proteins.

It is suggested that the whole operon is transcribed and consequently a large RNA molecule is produced. The replica from the non-informative part of the operon is then degraded and the structural zone, now a 'messenger' molecule is transferred into the cytoplasm.

Certainly this model provides a plausible explanation of known facts such as the formation and turnover of large RNA molecules, and the presence of closely related sequences and their scrambling with non-reiterated sequences within the genome (Britten and Kohne, 1965). It has been shown that the bulk, if not all, of the high molecular weight RNA is combined with protein particles (Samarina et al., 1965; Perry and Kelley, 1968). These proteins are aggregates of high molecular weight consisting of a number of subunits (Samarina et al., 1968; Kricherskaya and Georgiev, 1968). On the basis of this model it is possible that these protein particles are involved in regulating breakdown of the large RNA molecules to 'messenger' size. This model is substantiated by the large body of evidence in support of the scheme of breakdown of the 45S rRNA precursor molecule to produce the ribosomal RNA subunits. It was demonstrated in actinomycin chase experiments that the giant 45S rRNA breaks down to produce the 26 and 18S rRNA's of the cytoplasm.
(Scherrer et al., 1963; Perry et al., 1964). Also it has been demonstrated that the process of breakdown is non-conservative, almost 50% of the 45S molecule being broken down completely (Lerman et al., 1965; Jeanteur et al., 1968; Willems et al., 1968; Quagliariello and Ritossa, 1968). Strong support of this model also comes from the observations of Hell (1964) and Wilt (1965) who demonstrated that initiation of haemoglobin formation in explants of chick embryos is resistant to actinomycin after the seven somite stage but can be inhibited by puromycin. These results can best be explained by assuming that a pre-existing 'stable' messenger is made to function by controls after transcription of the haemoglobin gene, but before the onset of translation.

The experiments of Paul and Gilmour (1966 and 1968), Ananieva and Koslov (1966) and Ananieva et al. (1968) might be interpreted to provide indirect evidence for involvement of the reiterated sequences in the regulation of transcription. These workers obtained in vitro synthesised RNA from chromatin, de-histoned chromatin and de-proteinised chromatin. Annealing each of these preparations with DNA from the same source as the chromatin and at the same concentrations, showed that the RNA made on the de-proteinised DNA hybridized about five times better than that from whole chromatin and twice as much as that from de-histoned chromatin. Furthermore, tissue specific differences could be demonstrated between the RNA made from chromatin derived from different tissue. At the concentrations used, hybridization must have been restricted to the repeated sequences.

It is easily seen that any model for the mammalian genome based on the juxtaposition of control and informational sequences will immediately experience quantitative problems when translocation is considered. Clearly,
with each translocation the control sequence requirement must be doubled for it is known that as closely related characters as the polypeptide chains of an enzyme molecule may be situated in two distinct chromosome loci and on two different chromosomes (Horovitz and Metsenberger, 1965). It is difficult to understand why the reiterated sequences or the polydisperse RNA of the nucleus should be thought of as regulatory at all, other than the obvious fact that there is at this date no other easily assignable role for them.

Fairly large differences in the hybridization of the reiterated sequences have been detected between differentiated systems, both in hormonal response (O'Malley et al, 1968) and in embryonic development (Glisin et al, 1966; Church and McCarthy, 1967). Differences amounting to 100% were observed between the measured RNA's in the successive stages of the embryogenesis of Xenopus (Davidson et al, 1968). If all of the RNA hybridized in these situations was 'control' RNA one would have a situation in which the regulatory molecules were almost as varied as the genes themselves. Clearly the regulatory molecules cannot be as varied as the genes, nor can they even remotely approach such a situation; if it were so, then the informational potentiality of the genome would be consumed in self-regulation.

When the stability requirements of a reassociated molecule are considered it is surprising that related sequences do not constitute a greater proportion of the genome. Walker (1969) concluded that the stability requirements may be met by 20 base pairs and possibly even fewer. On this basis sequences that are seen as reiterated might well be expected to be expressed as proteins for it is reasonable to suggest that many proteins will share sequences of
seven amino acids. This hypothesis does not at first sight gain much support from complementarity studies. Hybridization values as high as 10% of the genome have been observed from the RNA of a differentiated cell (Paul and Gilmour, 1968) and differences as large as 80% between different tissues (Church and McCarthy, 1968). Considering the number of differentiated cell types there is insufficient DNA in the genome to cover the requirements if the sequences hybridized were 'informational'. The same argument, however, has been used against the sequences being 'control'. It is doubtful whether this paradox could be settled assuming that both 'informational' and 'control' sequences were hybridized when taking into account the values involved. It is possible that different RNA's from different sources may have different stability requirements and thus account for the observed hybridization values. The results of tissue comparisons presented here (results section E.) differ considerably from the results of tissue comparisons obtained from the immobilised DNA technique employing competition hybridization (Church and McCarthy, 1968). Since the salt concentrations and temperatures employed were roughly the same it is difficult to see why the differences should be so great unless one assumes that the immobilised DNA hybridization procedure is more demanding in its stability requirement and is thus more sensitive, consequently revealing greater differences. However, as the overall amount of the genome bound is roughly the same in both cases it is reasonable to conclude that the two procedures are of equal sensitivity.

The scheme of work presented for studying sequence homology between the nucleic acid species has many appealing aspects. The extent and rate of the hybridization reaction can be measured and from this the effective concentration
of sequences in the RNA. The re-cycle homologous reassociation reaction shows the proportional distribution of family sequences complementary to the different RNA preparations. The recovery of the DNA strand from a hybridization reaction and its further reaction can provide a great deal of information. This is most clearly seen in tissue comparisons (Results section E.).

From a comparison of the second cycle reaction it is clear that the distribution of RNA's are distinctly different in the liver and kidney. A much higher proportion of RNA sequences in the kidney are complementary to more highly reiterated families of sequences in the DNA than in the liver RNA. The difference is however, one of proportion, for liver RNA also has a fraction complementary to these sequences in the DNA. The liver RNA would appear to have a greater range of complementarity to sequences in the DNA from the extent and rate of the homologous reassociation reaction. Using complementary strands of DNA derived from an incomplete first cycle reaction, the differences between tissues is even more marked in the second cycle homologous reassociation reaction. It may be concluded from this result that a greater number of different family sequences have hybridized with liver NRNA than with kidney NRNA on the first cycle. This is seen in the rate and extent of the second cycle homologous reassociation reactions. Kidney complementary DNA reacts with sequence families that are proportionally more highly reiterated and thus the reaction progresses faster than does the liver complementary DNA reaction. The liver RNA is quite different in its complementarity distribution. Although it has membership within the more highly reiterated families it has a more even distribution between the families.
The second cycle hybridization results show that different families of DNA sequences have hybridised with the RNA's from the two tissues; this is seen in the differences between the second cycle heterologous hybrid reactions. This result considered with the second cycle homologous reassociation reaction and the first cycle hybrid reaction presents a paradox. Both first cycle reactions attained similar hybridization values and, in the second cycle reassociation reactions although the rates were different, the overall extent of the reactions were the same. Yet, the second cycle heterologous hybridization reactions show differences: This can only be explained by different families occurring with about the same reiterative values. One such family set must have its complement more frequently represented in liver NRNA and the other its complement more frequently represented in kidney. The emphasis should be on the word 'frequent' for the differences can only reflect the effective concentration of the RNA species themselves.

Although most proteins may be expected to fall into families having a common sequence of seven amino acids there must occur exceptions. The informational sequences in the DNA for these proteins will fall into the 'unique' category. Since it is difficult and perhaps even impossible, with the available techniques, to hybridise these cistrons, the differences that are observed in hybridization experiments must be minimum estimates.

Therefore studies in homology with the mammalian genome, either in reassociation or hybridization will continue to be limiting because of the complexity of the genome itself. The relaxed conditions of reaction specificity relative to the simpler genomes makes interpretation of the results.
difficult, and the conclusion drawn can only be tentative.

Further resolution may possibly be achieved by firstly resolving the karyotype. If an organism contained 40 chromosomes then the isolation and recovery of one of these should reduce the complexity of the DNA by one fortieth of the total. Similarly, the effective concentration of the slow sequences would be increased by a factor of forty. This is arrived at by assuming that firstly, all of the chromosomes are of the same size, and secondly, that there is bias in the distribution of sequence families between chromosomes. If members of every family of sequences were represented on every chromosome then the DNA for one chromosome would be as complex as the total and only the effective concentration of the unique sequences would be increased. Chromosomes are, however, not of uniform size and in the cases of very small chromosomes, some of which can be no larger than the E.coli genome, proportional representation of family sequences would result in a unique copy situation. If this is unlikely, since it is generally held that increase in genome size resulted from the tandem duplication and subsequent divergence of already existing sequences, then the small chromosomes can only be made up of one, or a very small number of families. Reducing the larger chromosomes in size should present a similar situation.

It is likely that the polytene chromosomes of insects will be most amenable to this type of approach. Expressly from the point of view of RNA/DNA relationship, the separation of a single insect chromosome with only one puff should provide the ideal material to resolve many of the problems that emerge due to the complexity of the DNA and the heterogeneity of the RNA species.
Appendix I

The Isolation of RNA/DNA Hybrid Molecules by Buoyant-Density Centrifugation.

Introduction.

Because of the difference in density between DNA and RNA equilibrium density centrifugation was an obvious choice of technique for the separation and recovery of hybrid molecules. That this technique has not enjoyed wide application can probably be accounted for by the fact that, in general, only very small amounts of hybrid have to be resolved from the very large amounts of non-hybridized material. But there are other limiting factors, principle among which has been the extent of separation of the hybrid component. In the case of the heavy ribosomal cistrons of Xenopus laevis, which not only make up a proportionally larger fraction of the genome than that generally seen, but which are also in themselves distinctly separated from the major component of the DNA, resolution of the rDNA/rRNA molecules was clearly demonstrated (Wallace and Birnstiel 1966). Hybrid molecules formed with DNA from within the major fraction of the genome present a more difficult problem.

Since the equilibrium position of a molecule in a density gradient is determined by the density of the molecule it must be dependent, in the case of a hybrid, upon the ratio of RNA: DNA in the molecule. Thus the greater the proportion of RNA making up the hybrid the greater the distance of separation from non-hybridized DNA in a density gradient. Hybrid molecules formed between RNA and sheared DNA should on this basis be more easily resolved from the non-hybridized material than those formed with high-molecular weight
DNA, since the density of the molecule should be greater due to the proportionally greater amount of RNA making up the molecule.

The quantitative limitations of the technique already mentioned, could to some extent be relieved by prior fractionation of the DNA, or by prior enrichment of the hybrid component of the total nucleic acid placed on the gradient. Removal of the slowly renaturing sequences of the DNA would in this respect, be to an advantage as would the recovery of only duplexed material from the reaction mixture prior to centrifugation.

In this work HAP was used with some success for enriching the amount of hybrid in the total amount of material on the gradient. Though HAP chromatography is limited to some extent in its usefulness, being restricted to sheared DNA, and also because of the further reduction of molecular weight during the chromatographic procedure, these factors as already discussed can actually be to an advantage in the subsequent resolution of hybrid molecules.

Results

The practical procedures were as described in the section 'Materials and Methods'. Unlabelled DNA was prepared from mouse livers, $^{32}P$ labelled RNA was prepared by the hot phenol method from L cells and from nuclei derived by the citric acid procedure from L cells.

Reiterated sequences in the DNA preparation were enriched in certain of the experiments described by prior renaturation to a Cot value of 10 and subsequent recovery from HAP. In other experiments the hybrid component on the gradient was enriched by recovery from HAP of the total duplexed material from the reaction. In both cases recovery was by salt chromatography.
all cases the gradients were made up in 0.12 MPB. Centrifugation was performed in the MSE 8 x 25 angle rotor, the conditions, the fractionation procedure and the measurement of radioactivity and optical density were as described in the 'Methods' section.

In the first result shown (Fig. 13a) the hybridization reaction was performed using total unlabelled DNA (1x10^{-2} mg/ml) and total cellular^{32}P RNA (5x10^{-2} mg/ml) specific activity 1.12 x 10^6 cpm/mg) in 0.12 MPB for 4 hours at 60°C, a total incubation volume of 125 ml was used. After incubation for 4 hours the total duplexed material was recovered from HAP in two fractions, that eluting between 0.12 M and 0.2 MPB and that between 0.2 and 0.3 MBP. The former showed no detectable retention of counts on nitro-cellulose filters subsequent to fractionation of the gradient, whilst the latter showed the fractionation pattern shown (Fig.13a). About 20% of the OD was recovered in the 0.2M to 0.3MPB salt fraction of the chromatogram and some 10% of the radioactivity. The total amount of duplex material recovered over the 0.2M to 0.3MPB region was split into four centrifuge tubes, each tube containing about 4 OD units at a density of 1.70 gms. cm^{-3}CsCl. By rough calculation, at least 75% of the input OD on the gradient should be made up by RNA. After fractionation corresponding fractions from the four tubes were pooled and the OD determined. RNA/DNA duplexes were then trapped on nitro-cellulose filter discs and the radioactivity measured. About 2.5% of the radioactivity put onto the gradient was recovered on the discs. Slightly less than 25% of the input OD was recovered from the gradient.

The poor resolution seen on fractionation (Fig.13a) was not unexpected. Wallace and Birnstiel (1966) showed that, unlike the ribosomal hybrid, non-
ribosomal RNA hybrid molecules were not significantly displaced in the density gradient from denatured DNA. There may be several reasons for this. It may, for instance, be the case that the increase in density due to the collapse of the secondary structure of the molecule, and in the case of the renatured component incomplete base pairing in the molecule produces the observed overlap with the hybrid densities. Alternatively, the poor resolution may be due to the relative contribution of RNA and DNA in the hybrid molecule. The latter is probably the most likely explanation.

An explanation concerning the relative amounts of RNA and DNA in the hybrid molecule cannot, however, completely explain the result shown (Fig.13a). The short pieces of DNA used (about 200-250 base pairs) are at least two orders of magnitude shorter than the 18s rRNA molecule. Thus, unless the RNA of the hybrid was considerably reduced in size during the procedure of isolation, an explanation concerning relative amounts cannot hold. In order to investigate the effects of HAP on the RNA of the hybrid molecule the experiment described above was repeated. In this case, however, repetitious DNA was previously enriched by HAP chromatography and the hybrid was not chromatographed.

A hybridization reaction was performed with $2 \times 10^{-3}$ mg/ml HAP enriched repetitious DNA and $1 \times 10^{-2}$ mg/ml $^{32}$P total cellular RNA in 0.12M PB at 60°C for 4 hours. Using a starting density of 1.70 g cm$^{-3}$ CsCl no radioactivity could be detected on the filters subsequent to centrifugation. At a starting density of 1.78 gms. CsCl the result shown in Fig.14a was obtained. The peak of radioactivity occurs at a density of 1.81 gms cm$^{-3}$, determined by pooling these fractions and measuring the refractive index of an aliquot. The difference between this result and that shown in Fig.13a can only be explained in terms of the breakdown of the RNA component of the hybrid.
molecule on HAP chromatography.

The effect of HAP on the size of RNA was further investigated with respect to temperature. $^{32}$P labelled nuclear RNA ($1.0 \times 10^6$ cpm/mg RNA) was absorbed onto HAP in 0.05 MFB at room temperature (about 23°C) and recovered in 0.3MFB. This material was then used in a hybridization reaction with HAP enriched repetitive DNA. The experiment performed was exactly as described above, CsCl gradients being formed from a starting density of 1.78 gm. cm$^{-3}$ CsCl. The result of this experiment is shown in Fig.13b. This experiment was repeated using the same RNA which, in this case, had not been previously absorbed onto HAP. In two separate experiments, the hybridization reaction was performed with RNA at a concentration of $1 \times 10^{-2}$ mg/ml in each case, whilst the incubation time was 4 hours in one experiment and 8 hours in the other. The results of these reactions are shown in Fig.14b together with the result of a hybridization reaction using the same RNA previously incubated with $5 \times 10^{-2}$ mg/ml RNAase for 1 hour at 37°C.

Interpretation of Figs. 13 and 14b are difficult. The two peaks seen in the first few fractions of Fig.13b may be the result of partial breakdown of the RNA by the treatment with HAP. The peak seen in the middle region of this gradient occurs at a mean density lower than that of the corresponding peaks seen in Fig.14b which supports the breakdown interpretation. The actual densities involved differ only by 0.002 gm. cm$^{-3}$ though the differences are clearly seen when corresponding fraction numbers are compared.

The attributing of peaks to particular RNA classes is also difficult from the results available. Possibly the best evidence for the high density peak being made up of rRNA comes from the melting curves shown in Fig.15. Melting
experiments were performed by separately pooling filters 1 to 5 and 13 to 19 derived from the experiment described above using $2 \times 10^{-3}$ mg/ml HAP enriched repetitious DNA and $1 \times 10^{-2}$ mg/ml $^{32}$P MRNA incubated for 4 hours. Filters were first washed in 10 ml 0.12 MPB at 60°C and at 5°C intervals in the same buffer by transferring the filters between scintillator tubes containing the buffer and heated to the appropriate temperature. The tube contents were subsequently dried and counted.

From Fig. 15 it can be seen that the high density peak melts at temperatures considerably higher than the low density peak and from this evidence alone it is reasonable to assign this peak to rRNA and its DNA complement. The consistent occurrence of this peak irrespective of the source, concentration, or incubation time suggests that this RNA species is in great excess in the reaction, a feature which also supports its interpretation as rRNA.

In the absence of double-label data the melting profiles shown are, together with the adherence of the molecules to nitro-cellulose, good evidence for the banding patterns observed being due to the formation of hybrid molecules.

**Discussion**

Though the centrifugation experiments described in this appendix were undertaken to obtain hybrid molecules for the purpose of studying the place of elution of hybrid molecules on salt chromatograms of HAP, the results in themselves are interesting, though they perhaps reveal some discouraging aspects of the effect on RNA of HAP chromatography. Indeed, if the breakdown
interpretation is correct then the usefulness of the HAP technique, with respect to the isolation of specific RNA molecules and their DNA complement, is limited.

Hybrid molecules on CsCl gradients appear to band over two density ranges, the smaller with a mean density of 1.78 gm. cm\(^{-3}\). These density positions are considerably higher than even the Xenopus ribosomal hybrids at saturation which showed a mean value of 1.77 gm. cm\(^{-3}\). (Wallace and Birnstiel: 1966). The high densities observed may be interpreted in terms of the RNA/DNA ratio in the hybrid and also the base constitution. When filters from each peak region were pooled and subjected to digestion with RNAase a six-fold decrease in radioactivity was observed from the smaller and more dense peak, whilst a 2.4 decrease was seen in the larger and lighter peak. This substantiates the deduction that the RNA:DNA ratio largely determines the high density banding positions. In the absence of data from experiments in which both nucleic acid species in the hybrid are differently labelled this cannot be conclusive.

Considering the possible range of matched sequences in mammalian hybrid molecules it is somewhat surprising to see almost symmetrical bands in the density gradient. Band symmetry suggests homogeneity which makes the assignment of the larger and lower density band difficult. From the experiments presented it is clear that this RNA fraction is enriched in the nuclear preparation and that at the concentrations and incubation times used the hybridization reaction has not been completed. Using higher concentrations of RNA was found to be impracticable for, even in the results shown, the gradients were greatly overloaded. Overloading the gradient, particularly with the RNA component being in the greater excess results not only in RNA
pellet formation on the bottom of the tube but also the danger of CsCl coming out of the solution, in which case the gradient is destroyed. Also in this respect the rotor temperature is critical. At the high initial densities of CsCl involved in these experiments even a small drop in temperature below 25 °C resulted in the CsCl coming out of solution and pelleting on the bottom of the tube.

Further work with higher specific activity RNA and labelled DNA should prove interesting, especially regarding the mRNA low density hybrid. Considering the molecular species concentrations and reaction times involved in these experiments it can be concluded that the mRNA component which has its complement in all but the most reiterated DNA is also homogeneous with regard to size and possibly with regard to base constitution.
**Fig. 13A**

Buoyant density distribution of hybrid and reassociated molecules recovered from HAP. Reaction carried out with $1 \times 10^{-2}$ mg/ml mb DNA, and $5 \times 10^{-2}$ mg/ml $^{32}$P total cellular RNA (specific activity $1.12 \times 10^6$ cpm) in 0.12MPB at 60°C for 4 hrs. Starting density 1.70 gms cm$^{-3}$ CsCl.

Open circles; optical density.

Filled circles; radioactivity.

**Fig. 13B**

Buoyant density distribution of hybrid molecules using RNA recovered from HAP $^{32}$P mRNA (specific activity $1 \times 10^6$ cpm) recovered from HAP (for details see text) reacted at a concentration of $1 \times 10^{-2}$ mg/ml with $1 \times 10^{-2}$ mg/ml mb DNA in 0.12 MPB at 60°C. Starting density 1.78 gms cm$^{-3}$ CsCl.

Open circles; optical density.

Filled circles; radioactivity.
Fig. 14A

Buoyant density distribution of hybrid molecules using DNA recovered from HAP.

Reaction carried out with $2 \times 10^{-3}$ mg/ml DNA recovered from HAP (for details see text) and $1 \times 10^{-2}$ mg/ml $^{32}$P total cellular RNA (specific activity $1.12 \times 10^6$ cpm) in 0.12 MPB at $60^\circ$C for 4 hrs.

Starting density $1.7$ gms cm$^{-3}$ CsCl.

Open circles; optical density.

Closed circles; radioactivity.

Fig. 14B

Buoyant density distribution of hybrid molecules.

Hybridization reactions carried out using $^{32}$P RNA (specific activity $1 \times 10^6$ cpm) at a concentration of $1 \times 10^2$ mg/ml with $1 \times 10^{-2}$ mg/ml DNA, in 0.12 MPB at $60^\circ$C for 4 hrs. and 8 hrs.

Filled circles; reaction time - 4 hrs.

Filled triangles; reaction time - 8 hrs.

Filled squares; RNAased RNA, reaction time 8 hrs.
fig. 14
Fig. 15

Thermal chromatography of hybrid molecules recovered from CsCl gradients.

Right hand curve: high density hybrid.

Left hand curve: low density hybrid.

Thermal chromatography with 0.12 MPB.
Appendix 2.

Derivation of the equation

\[ t = \frac{1}{kb} \log \left[ \frac{a^3 - ax}{a^3 - a^2 x - abx} \right] \]

from the reaction rate equations

\[ \frac{dx}{dt} = k(a-x-y)(a-x) \quad (1) \]

and

\[ \frac{dy}{dt} = k(a-x-y)(b-y) \quad (2) \]

From (1) and (2)

\[ \frac{dy}{dx} = \frac{b-y}{a-x} \]

\[ \therefore \quad \frac{dy}{b-y} = \frac{dx}{a-x} \]

\[ -\log(b-y) = -\log(a-x) + \log c \quad (3) \]

when \( t = 0, x = 0, y = 0 \)

\[ -\log b = -\log a + \log c \]

\[ \therefore \quad \log c = -\log \frac{b}{a} = \log \frac{a}{b} \]

(3) gives

\[ -\log b \left( \frac{b-y}{a-x} \right) = \log c = \log \frac{a}{b} \quad (4) \]

\[ \therefore \quad \log \left( \frac{a-x}{b-y} \right) = \log \frac{a}{b} \]

\[ \therefore \quad b(a-x) = a(b-y) \]

\[ -bx = -ay \]

\[ \therefore \quad \frac{y}{x} = \frac{b}{a} \quad \text{and from (4)} \quad \frac{b}{a} = c. \]
In (1) \[
\frac{dx}{dt} = k \left[ a - x - cx \right] (a-x) \\
= k \left[ a - (1+c)x \right] (a-x) \\
= k(1+c) \left( \frac{a}{1+c} - x \right) (a-x)
\]

let \( k' = k(1+c) \) and \( d = \frac{a}{1+c} \)

then \[
\frac{dx}{dt} = k' (d-x) (a-x)
\]

and \[
\frac{dx}{(d-x)(a-x)} = k' dt
\]

Integrating \( x \) and \( t \).

\[
\frac{1}{a-d} \int_0^x \left[ \frac{1}{d-x} - \frac{1}{a-x} \right] dx = k' \int_0^t dt
\]

\[
\frac{1}{a-d} \left[ -\log (d-x) + \log (a-x) \right]_0^x = k' t
\]

\[
\frac{1}{a-d} \log \frac{a-x}{d-x} \int_0^x = k' t
\]

\[
\therefore \quad k' t = \frac{1}{a-d} \left[ \log \left( \frac{a-x}{d-x} \right) - \log \frac{a}{d} \right]
\]

\[
k' t = \frac{1}{a-d} \log \frac{d(a-x)}{a(d-x)}
\]

\[
\therefore \quad t = \frac{1}{k' (a-d)} \log \frac{d(a-x)}{a(d-x)} \quad (5)
\]

By substitution in (5) for \( k' = k(1+c) \)

\[
d = \frac{a}{1+c}
\]

and \( c = \frac{b}{a} \)

gives the equation

\[
t = \frac{1}{kb} \log \left[ \frac{a^3 - a^2 x}{a^3 - a^2 x - abx} \right]
\]
Appendix 3

Work-flow for First and Second Cycle Reactions:

This text should be read in conjunction with the work-flow diagram included. The sequence of events starts at the top left corner (Box T). The principle path of events is shown in heavy lines.

1 volume of mb DNA in 0.12MPB, made up from 0.1 vol. mb DNA in alkali (Box T) and 0.9 volumes 0.12MPB (Box 2) at the desired incubation temperature, and is passed through HAP. The eluate is retained.

The eluate (Box 3) is brought to the same concentration and volume in alkali as the starting solution in Box I. Box 3 is used in two reactions. In the first cycle control reaction the DNA is made up to 1 volume by the addition of 0.9 volumes of 0.12MPB from Box 2. In the first cycle hybridization reaction (Box A, principle pathway) the DNA is made up to 1 volume with 0.9 vol. RNA in 0.12PB from Box 4. The incubation time for both reactions is the same.

After incubation the total duplexed material is recovered from HAP and after the digestion of the RNA in alkali the DNA is again concentrated and made up to either 0.05 or 0.1 volumes in alkali (Box B).

Box B is the starting point of second cycle reactions. In some instances a second cycle cleaning reaction was performed in the same way as the first reaction event. DNA in 0.05 volumes alkali is made up to 0.1 volumes with 0.05 volumes 0.24 MPB from Box 6, the whole is passed through HAP and, as in the first event, the eluant is concentrated and used in the hybridization reactions.
WORK-FLOW DIAGRAM FOR
1st. & 2nd. CYCLE REACTIONS
The second cycle control reaction is performed using 0.1 volume from Box B and 0.9 volume 0.12 MPB from Box 2. The second cycle hybridization reactions are of two types. Homologous hybridization is that using the same RNA as in the first cycle reaction (Box 4). Heterologous hybridization uses different RNA (Box 4). In both cases the reaction is the same, 0.1 volume second cycle DNA in alkali from Box B is incubated with 0.9 volume RNA in 0.12 MPB from Boxes 4 and 4, giving Boxes C and D.

An homologous reassociation reaction is performed using second cycle DNA from Box B together with unlabelled mb DNA from Box 5 (both 0.05 volume) and making up to 1 volume with 0.9 volumes MPB from Box 2.
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