ENRICHMENT AND RESTRICTION MAPPING OF RABBIT GLOBIN GENES

N.J. Hanscomb

Thesis submitted for the Degree of Doctor of Philosophy
University of Edinburgh
1978
ABSTRACT

(a) I have made DNA columns using the procedure of Gilham (1971) (Methods in Enzymology XXI 194) to bind oligo dT or globin cDNA to cellulose or Sephadex, and the procedure of Noyes and Stark (1975) (Cell 5 301) to bind oligo dT, poly dT or cDNA to m-diazobenzoyl cellulose and p-diazobenzyl polystyrene. I have used oligo dT bound in all these ways to these column beads and poly dT bound to m-diazobenzoyl cellulose or p-diazobenzyl polystyrene as primers for cDNA synthesis from globin mRNA with AMV reverse transcriptase. This priming reaction worked well with oligo dT bound to m-diazobenzoyl cellulose, less well with poly dT bound to the same beads and poorly with all other combinations of primer and beads that I tested. All the beads to which I have tried to bind DNA covalently bind DNA tightly even when they have not been activated chemically and most of the DNA bound tightly to chemically activated m-diazobenzoyl cellulose or p-diazobenzyl polystyrene can be removed by washing the columns with non-homologous RNA.

(b) I have used the procedures of Southern (1975) (J. Mol. Biol. 98 503) for transferring DNA from agarose gels to nitrocellulose filters and hybridising the DNA bound to these filters with radioactive DNA or RNA probes as a means to study the organisation of DNA sequences around the rabbit globin genes. I have used cDNA made from globin cDNA and also cRNA or DNA probes from plasmids carrying globin cDNA inserts. I have
made two new cDNA plasmids one carrying the 5' 400 base pairs of \( \beta \) globin cDNA and the other the 3' 200 base pairs. I found that all the globin probes I used hybridised to many sequences within rabbit DNA restriction digests and the ribosomal genes hybridised particularly strongly. This anomalous hybridisation is not caused by contamination of globin probes with ribosomal probe. Much of this anomalous hybridisation can be eliminated by washing the hybridised nitrocellulose sheets at a higher temperature, for example 2 x SSC at 80°C as against 2 x SSC at 65°C. I found that there are probably 2 \( \beta \) globin genes in rabbit in Hind 3 fragments of 15 and 18 Kb. nearer the genes, on the 5' side there is probably an Hl site about 2000 base pairs from the Hl site within the genes and on the 3' side there is probably an Hl site 900 base pairs from the Hl site in the genes.
ACKNOWLEDGEMENTS

I thank particularly Maureen Walsh for typing a draft of the manuscript free and in her spare time. Mary Taggart not only helped enormously with DNA preparations but was always ready to make up solutions and get chemicals at a moment's notice. Barbara Smith made some excellent enzyme preparations. Peter Walker helped with my arguments with the MRC and GKAC. He also read the manuscript as did Ed. Southern. Much of this thesis is founded upon the work of Ed. Southern and many of the ideas are his. Many people have helped me through this thesis but the warmth of Deborah Keith, Saveria Campo and Ken and Anne Gummerson come particularly to my mind. The Salisbury Centre has been a spiritual and emotional guide to me.

I hate and despise the MRC for refusing to give me financial support for this work.
ABBREVIATIONS

AMV : Avian myeloblastosis virus

SSC : Standard saline citrate (0.15 M NaCl, 15 mM Na citrate)
CONTENTS

INTRODUCTION
The study of rare DNA sequences in eukaryotic DNA 1
   Detection of rare sequences 3
   Sequences associated with genes 4
   RNA transcripts 6

MATERIALS AND METHODS
Animal DNA 10
Bacterial DNA 10
Bacterial plasmid DNA 11
Animal RNA 11
cDNA preparation 12
poly dT synthesis 13
cRNA (cRNA from globin cDNA) 13
cRNA from plasmids 14
Formamide acrylamide gels 14
Preparation of fragments of rabbit DNA 15
Kinase labelling 15
Chemical labelling of oligo dT 16
Binding of DNA fragments or oligo dT to beads 16
Synthesis of diazobenzyl polystyrene 17
Nick translations 18
Restriction enzymes 18
Agarose gel electrophoresis 18
Transfer of DNA from agarose gels to nitrocellulose filters 19
Elution of DNA from agarose gels 20

RESULTS AND DISCUSSION: PART 1
(a) INTRODUCTION
   Enrichment of rare sequences from the DNA of a eukaryot 22
CONTENTS (continued)

Approaches to the synthesis of cDNA columns 24

(b) EXPERIMENTS

Preparation of globin cDNA columns: Binding DNA to beads 27
Adventitiously bound DNA 30
Binding of oligo dT 31
Use of oligo dT columns as primers for cDNA synthesis 32

RESULTS AND DISCUSSION: PART 2

(a) INTRODUCTION

Restriction analysis of rare sequences in total eukaryotic DNA 40

(b) EXPERIMENTS

Restriction mapping of globin genes 43
Probe excess hybridisation 44
Choice of probe 45
Hybridisation conditions 46
Signal strength 48
DNA track background 50
DNA falls off transfers 54
Globin cDNA plasmids:
   (a) cRNA probes from plasmids 56
   (b) Preparation of 2 new globin cDNA plasmids 59
   (c) cRNA probes from the PMB9 200 and PMB9 400 inserts 61
Nitrocellulose background 63
Globin cDNA plasmids (continued):
   (d) Nick translated plasmid probes 64
   (e) PMB9 200 and PMB9 400 cRNA probes 68
RNA probes are better than DNA probes 70
Ribosomal genes 70
Globin bands 76
CONTENTS (continued)

CONCLUSIONS: PART 1

cDNA columns

Other column approaches to gene enrichment

CONCLUSIONS: PART 2—

Restriction mapping of single copy genes within mammalian genomes

REFERENCES
INTRODUCTION

The study of rare DNA sequences in eukaryotic DNA

Although breeding experiments have been able to provide a great deal of information about organisation and regulation of genes within prokaryotic genomes such experiments have told us less about the eukaryotic genome and less still about the mammalian genome. Even in the most studied eukaryotic genetic system, namely Drosophila, we know little about regulation of genes or their organisation. There is no sign at present that breeding experiments can ever be powerful enough to elucidate the mammalian genome in the same detail as we understand the prokaryotic genome without an enormous amount of labour. There are three main reasons for this; eukaryotic genomes are larger than prokaryotic, breeding rates are slower for eukaryots than for prokaryots and it is very difficult to handle the very large numbers of large organisms required to detect mutations in chosen regions of a genome. The work of Chovnick et al. (1977) shows just how much labour is required to study even a relatively simple locus in Drosophila using genetic techniques. Similar experiments in mammals would surely be much harder.

Over the last few years molecular approaches to the eukaryotic genome have completely outstripped conventional genetic approaches in providing information about gene organisation, though so far it has only been possible to study in detail sequence organisation of eukaryotic DNA as a whole or sequences within the DNA that are present in many copies per
genome such as ribosomal genes, histone genes and satellite DNAs. There are sufficient copies of any of these sequences within a cell and the sequences are sufficiently different from the bulk of the DNA that density centrifugation can purify the sequences enough that they can then be detected, after digestion with restriction enzymes and gel electrophoresis, or in the electron microscope, as discrete size classes of DNA.

These studies have done much to explain the nature of repetitive DNA in eukaryots but it has been known for some time that most of the mammalian genome consists of sequences of DNA present in one or a few copies per cell (see Lewin (1974) and Southern (1974) for reviews) and that most mRNA species hybridise to this single copy part of the mammalian genome (Gelderman et al. (1969) and Ielli et al. (1971)). I wish to understand the regulation and organisation of mammalian genes in the same detail as we now understand the prokaryotic genome. Since we know that all genes are not expressed at the same time and are not all transcribed with the same frequency in mammals it follows that it is impossible to study regulation or organisation of any gene in any detail through molecular genetics by studying the bulk DNA, and since most sequences of mammalian DNA are present in only one or a few copies per genome it is unlikely that sequences of DNA present as multiple copies per genome or separable from bulk DNA by density centrifugation have a sequence organisation or gene regulation typical of the majority of genes. A sequence of DNA containing
a gene present in only one or a few copies per cell is more likely to be typical of the bulk genome and, even if it is not, it might well show an organisation and regulation different from either multiple copy eukaryotic genes or single copy prokaryotic genes. Indeed results on the organisation of the Drosophila genome from the work of Hogness et al (unpublished) endorse this: clones from the $87BC$ heat shock site show at least 2 genes each repeated several times and with spacers between the genes that are different for each repeat unit. This is still a repeated gene system but it shows that there are several different ways to organise genes.

Detection of rare sequences

Rare sequences within eukaryotic DNA are very difficult to study and even detect because they are hidden within a $10^5 \text{ to } 10^7$ fold excess of other sequences. Hybridisation with radioactive probes is the only technique available which offers sufficient specificity or sensitivity for identifying one single copy sequence against such a background and even to do this it is necessary to have a fairly pure radioactive probe for the sequence of interest. In practice probes are prepared from cytoplasmic RNA species and at present only some cytoplasmic mRNAs can be purified sufficiently to be useful as probes. Thus the only DNA sequences available for sequence analysis or even measurement at present are those that code for the limited range of RNA species available as probes. Most single copy mammalian DNA probably does not code for cytoplasmic mRNAs (Lewin 1974; Southern 1974). Globin mRNA is one type of RNA that can be purified enough to be useful as a probe and hybridisation of probes prepared from
globin mRNA back to the cell genome has shown that globin genes are present in one or a few copies per genome (Harrison et al, 1972; Old et al, 1976). This is a rough estimate but it agrees with the genetic evidence in humans (Haemoglobins, Comparative Molecular Biology Models for the study of disease (1974), Gandini et al, 1977). Because there are only a few globin genes the haemoglobins also show that it is unnecessary to have multiple copies of a gene in order to have a cell make nearly all its protein from that gene during differentiation.

**Sequences associated with genes**

Studies in which radioactive probes are hybridised to unfractionated total DNA do not provide information about the sequences associated with genes. Such sequences could be important because they probably contain the control signals for transcription and processing of RNA from rare genes. There is some genetic evidence for this in Drosophila (Chovnick et al, 1977) and of course a vast literature on the subject from prokaryots. Also work from Hogness' group (unpublished) shows that the Drosophila histone genes are transcribed off different strands and in pairs; H3 and H4 are transcribed off different strands and away from a site between them and so are H2A and H2B. This hints at a bidirectional promoter sequence between each of these pairs of genes. The existence of a globin RNA precursor of defined length (Kwan et al (1977) and Bastos and Aviv (1977)) in mouse cells shows that there must be at least two important processing points for this RNA: where the precursor is cleaved to generate the mRNA and where either the initiation of transcription of the
precursor begins or where the precursor is cleaved from a pre-
precursor. By looking to see which sequences are often found
adjacent to different genes from the same organism or the same
genes from different organisms it should be possible to tell which
sequences are important for regulation and expression of genes
because sequences that occur near genes more often than would
be expected by chance or improbably symmetrical sequences are
probably being conserved by natural selection and thus may serve
some function. Improbable sequences are found in the sea urchin
histone gene repeat unit (Birnstiel et al, unpublished) and also
around the replication origin in SV40 (Berg's group and Fiers' group).

Bishop and Freeman (1973) made an attempt at studying the
sequences associated with globin genes. They fractionated duck
DNA by denaturing it then reassociating it taking different Cot
classes. By hybridising these fractions with duck globin cDNA
they were able to provide evidence for repeated sequence DNA at
the 3' ends of the duck globin genes. These experiments are
open to the criticism that it is not really clear that repeated
sequence DNA adjacent to the globin genes is causing globin genes
to appear amongst DNA hybridising at low Cot values, any sort
of mismatched random hybridisation will produce the same
results. This is a type of problem that will be discussed in
detail later in this thesis. Very recently Flavell, Jeffreys and
Grosveld (1977) have reached the same conclusion as Bishop and
Freeman using rabbit DNA and a similar experimental approach to
that taken by Bishop and Freeman (1973).

RNA transcripts

Another approach to sequences associated with single copy genes is to look at the primary RNA transcripts. The advantage is that there is both selection in the cell for which sequences are transcribed and amplification of the transcribed sequences with respect to the original gene. Both of these points make it easier to enrich the individual sequence of interest and thus free it sufficiently from other different sequences to make detailed sequencing possible or to make the RNA useable as a probe. However there are several disadvantages to this approach. The RNA is no use for studying control of transcription from a gene and by the very fact of being a transcript it is unlikely to contain all the signals for stopping or starting transcription. RNA is unlikely to be any use for studying DNA binding proteins involved in gene regulation and the binding of cell proteins to pieces of DNA has proved valuable in understanding gene regulation in prokaryotes and may well prove even more valuable in eukaryotes where a firm basis for studying regulation will not be available through breeding experiments. Recently a number of groups have found that RNAs from viruses can be spliced together after they have been transcribed from widely different parts of the virus genomes. This means that RNAs isolated from eukaryotic cells may not represent the true sequence organisation in the genome. The inserts that David Glover finds in some of the 28S Drosophila ribosomal genes also suggest this (Glover and Hogness, 1977).
The genome DNA is then a better material to use to study the sequences associated with eukaryotic genes but at the moment only a few RNA species are available anything like pure enough to use as probes for sequences within total eukaryotic DNA and so very few such DNA sequences can be studied. This situation has been improved recently by the cloning into bacterial plasmids of cDNAs from mRNAs and this technique should make available many more pure probes for eukaryotic genes. This in turn opens up the possibility of cloning DNA sequences that contain genes and associated sequences from eukaryotic genomes. Although cloning allows study of rare DNA sequences pure and in large quantities it is quite difficult for rare eukaryotic genes and so any techniques that allow study of rare DNA sequences within eukaryotic DNA without recourse to cloning are still useful, not only a priori but also as a standard against which to compare clones to check that the clone contains a true copy of the genome sequence. Furthermore, while clones will clearly be useful for studying transcription of eukaryotic genes (Mertz and Gurdon, 1977) it will still be necessary to show that the clone is transcribed in the same way as the gene within the genome and is subject to the same regulatory steps. Thus clones of eukaryotic genes will not obviate the need to develop techniques to study eukaryotic genes within the genome, where chromosomal conformation might be important in regulating gene expression. There is already
evidence that chromosomal conformation is important (Levy and Dixon (1977), Weintraub and Groudine (1976), Garel and Axel (1976)).

In this thesis I shall discuss approaches to enriching rare sequences associated with genes relative to total organismal DNA and the application of Southern's gel techniques (Southern, 1975) and the cDNA plasmid technology to the study of rare sequences associated with genes in DNA that has not been enriched for the sequence of interest. I have chosen to study the rabbit globin system. There are several reasons for choosing the globin genes for study:

1. There is more than one globin gene and the different genes are likely to be regulated differently. The \( \alpha \) genes always being expressed in erythropoietic cells whereas the \( \beta \)-like genes will be expressed differently in foetus' and adults.

2. In humans and probably rabbits there are several \( \beta \)-like genes and these may show differential transcriptional control. They are likely to provide an ideal system for studying DNA sequence organisation between genes because the genes are genetically linked and probes will become available for the different genes of the complex (the human \( \beta, \gamma \) and \( \delta \) genes for example). This system is therefore an ideal choice of system with which to tie together mammalian genetic and molecular map distances. (See "Haemoglobins comparative molecular biology models for study of disease (1974)" for the genetic data).

3. Once the technology is established for studying globin genes it is fairly easy to move from working with rabbits to working with
humans or mice. The globin genetics in humans and perhaps mice is the best characterised of all mammalian genetic systems. In humans the thalassemias (Weatherall and Clegg, 1972) provide a ready made set of mutations including deletion and possible insertion mutants ideal for studying in vivo regulation of the globin genes in relation to their DNA biology. Mice have the advantages that they breed quickly, can be used for experimental genetics and by mammalian standards their overall genetics is well characterised.

4. A technique for detecting defects in globin DNA could be applied through amniocentesis to reduce the incidence of thalassemia as a disease.

Rabbit is a good animal to use to develop the technology for studying globin DNA because (a) rabbit globin mRNA is better studied than any other mRNA (b) it is available more pure than any other mRNA and in large quantities (c) one individual rabbit will provide sufficient DNA or mRNA for very detailed analysis thus making it possible to allow for DNA heterogeneity between individuals. (d) Rabbit globin cDNA plasmids were the first available.
MATERIALS AND METHODS

Animal DNA

Tissues were homogenised with 4 x 20 sec. bursts in a Silverson heavy duty mixer with the medium head in 0.32 M Sucrose, 10 mM Tris pH 8, 10 mM MgCl₂, strained through 4 - 6 layers of cheesecloth and spun 5,000 r.p.m. 20 minutes in a Sorvall RC5, to pellet nuclei. Nuclei were then resuspended in 0.1 M EDTA 0.15 M NaCl pH 10.5, 10% SLS was added to 1% and pronase (Calbiochem) added to 200 micrograms/ml. After 3 hours at 60°C the DNA was deproteinised by repeated phenol extraction then chloroform extraction then spooled from 70% ethanol. Once redissolved in water the DNA was treated with 20 micrograms/ml. RNase A (Sigma) for 1 hour at 37°C then 20 micrograms/ml. pronase for 30 minutes, taken to 0.3 M in Na Acetate and phenol extracted, chloroform extracted then spooled again from 70% ethanol. The DNA was then dissolved in water.

Bacterial DNA (Barbara Smith did these DNA preparations)

Cultures were grown to stationary phase. L broth was used as growth medium if unlabelled DNA was required. For ³²P labelled DNA the growth medium contained:

1.5 g/litre KCl
5.0 g/litre NaCl
1.0 g/litre NH₄Cl
12.0 g/litre Tris
1 mM MgCl₂
14 g/litre glucose
1% bactopeptone
4 mCi ³²P orthophosphate (Amersham)
Cells were pelleted from medium in a Sorvall RC5, washed in 10 mM Tris 1 mM EDTA then lysed with 100 mg. lysozyme (Sigma) per litre of original culture in 10 mM Tris pH 7.5 1 mM EDTA. SLS was added to 0.5% and the solution shaken for 6 hours. After that the DNA was deproteinised with phenol and purified further as for animal DNA.

Bacterial plasmid DNA (Colicin E1 derivatives PCR1, PMB9 and globin recombinants made from them) for 1 litre of cell culture:

Cells were grown to an O.D. of 1.0 in L broth then 150 micrograms/ml. chloramphenicol added and the culture shaken at 37°C for 12 hours. Cells were pelleted from solution in a Sorvall RC5 then resuspended in 500 ml. 10 ml. Tris pH 8.0 1 mM EDTA and pelleted again. Cells were then suspended in 15 ml. 25% Sucrose 0.05 M Tris pH 8 at 4°C and after 5 minutes 100 mg. lysozyme was added. After 5 minutes 3 ml. of 0.5 M EDTA pH 8.5 was added and after a further 5 minutes 24 ml. of 0.1% Triton X-100, 62.5 mM EDTA, 50 mM Tris pH 8 was added. The lysed cells were then spun at 20,000 r.p.m. in an MSE 8 x 50 rotor for 30 minutes to remove debris, 0.95 g/ml. CsCl and ethidium bromide to 500 micrograms/ml. were added to the supernatant and the plasmids separated from bulk DNA by density centrifugation for 72 hours. The lower plasmid band was removed, extracted 3 times with CsCl saturated aqueous isopropanol, once with phenol, then dialysed against water. Sometimes the DNA was then concentrated by ethanol precipitation.

Animal RNA

Tissue was homogenised in 20 mM Tris pH 7.4, 1% NaCl, 2% TNS
(Eastman), 1% SLS, then phenol extracted until no interface remained, chloroform extracted twice then precipitated with ethanol. The pellet was redissolved in 10 mM Tris 10 mM MgCl₂ and treated with 20 μg./ml. DNAse I (Sigma) for 2 hours at 37°C then with 10 μg./ml. pronase for 30 minutes at 37°C in 0.02% SLS then extracted once with phenol, once with chloroform then precipitated with ethanol.

Rabbit globin mRNA was bought from Searle.

cDNA preparation

I used dATP, dTTP, dCTP, dGTP, at 200 μM each. One or more labelled with ³²P or ³H (Amersham), 100 mM Tris pH 8.0, 40 mM KCl, 200 μg./ml. BSA, 2 mM dithiothreitol, 4 μg./ml. oligo dT (pT₁₀ PL Biochemicals), 7 mM MgCl₂, 20 units/ml. Searle ribonuclease inhibitor, 40 μg./ml. Searle α + β globin mRNA, about 350 units/ml. reverse transcriptase (a gift from J.W. Beard). For single strand cDNA I added 500 μg./ml. Actinomycin D (Bohringer). The reaction was incubated for 5 hours at 37°C, then extracted once with phenol, twice with chloroform then taken to 0.3 M in NaOH and incubated at room temperature overnight. If double strand cDNA was to be used for making ccRNA (see later) then the solution was neutralised with HCl and used directly in the ccRNA reaction mixture. If single strand cDNA had been made then this was neutralised with HCl, mixed with 100 μg. of E. coli sonicated single strand DNA and passed through a Sephadex SP50 column in 0.3 M NaCl. If required the DNA was then precipitated with ethanol. When actinomycin D was used in the reaction mixture the cDNA at the end was about 1% resistant to S1 nuclease.
Under the conditions described about 100 - 200 μg./ml. of double 
strand cDNA could be made from 40 μg./ml. of mRNA. Much of this is 
approximately full length double strand globin cDNA as judged by 
agarose gel electrophoresis. Single strand cDNA made under these 
conditions has about 50% of the molecules full length.

Searle ribonuclease inhibitor contains no priming activity 
for AMV reverse transcriptase.

Poly dT synthesis

As for globin cDNA except that poly ra (Miles) was used as 
RNA template, only 3H dTTP was present as nucleotide and oligo dT 
was present at a weight ratio of 1:4 with respect to poly ra to 
make poly dT about 40 bases long.

ccRNA (cRNA from globin cDNA)

One or two 32P labelled nucleotides usually about 1 n mole 
of each at 100 Ci/mM to transcribe 1-5 μg. of cDNA. Other un-
labelled nucleotides at 1 mM. 10 mM NaCl2, 20 mM Tris pH 8.0, 
40 mM KCl, 10 units/ml. Searle ribonuclease inhibitor, 2 mM 
dithiothreitol,100 μg./ml. BSA, 10 μg. of ribotrinucleotide AUG, 
4 units of RNA Polymerase (Bohringer) all in a final volume of 
200 μl. incubated for 2 hours at 37°C then 1-5 μg. of DNase I added and 
the reaction incubated for 45 minutes at 37°C, after which 40 μg. 
E. coli tRNA was added and 10 μg. poly ra. The reaction was 
extracted twice with phenol then passed through Sephadex SP50. 
AUG increases the efficiency of transcription by about 30% presumably 
by acting as a primer. Searle ribonuclease inhibitor does not contain 
anything that acts as a primer or template for RNA polymerase. 
The salt concentrations used were optima for transcribing cDNA.
FIGURE 1

Size of ccRNA

The histograms are profiles of $^{32}$P globin ccRNA c.p.m. from 0.5 cm. slices of 10\% acrylamide 99\% formamide gels. I used 0.25 \( \mu \)g. of 9S globin RNA, 0.5 \( \mu \)g. rabbit ribosomal RNA and 3 \( \mu \)g. of \textit{E. coli} tRNA as markers on the left hand gel (gel A) and 3 \( \mu \)g. of \textit{E. coli} tRNA on the right hand gel (gel B). Gel B was electrophoresed at 2 mA for 5 hours, gel A at 2 mA for 17 hours. Markers were detected by scanning the gels in a U.V. scanner then the gels were sliced and measured for radioactivity by Čerenkov counting the slices.
$^{32}\text{P cpm}$

18S 9S

$^{32}\text{P cpm}$

4S

5S

4S
RNA was usually synthesised under these reaction conditions to a weight ratio of RNA to DNA of 1-4%. After purification over Sephadex SP50 the RNA was about 90% TCA precipitable and 90% sensitive to RNAse A. Ken Gummerson fingerprinted oligonucleotides from ccRNA and got a pattern of about the correct complexity for α + β globin mRNA and with major oligonucleotides apparently identical to those published for ccRNA (Poon et al, 1974).

RNA prepared in the absence of ribonuclease inhibitor was about 100 bases long whereas when prepared in the presence of inhibitor much of it was probably as long as globin mRNA (see figure 1). The gels in figure 1 are formamide acrylamide gels run at room temperature and some of the radioactive RNA comigrates with the marker RNAs suggesting that these gels are not completely denaturing at room temperature in agreement with Spohr et al (1976).

**ccRNA from plasmids**

One or two 32P labelled nucleotides at 100 Ci/mM or more and using about 1 nmole per μg. of plasmid to be transcribed. Other nucleotides at 1 mM, 15% glycerol, 30 mM Tris pH 8, 10 mM mercaptoethanol, 180 mM KCl, 10 mM MgCl₂, 10 units/ml. Searle ribonuclease inhibitor, 4 units of RNA polymerase. Otherwise as for ccRNA.

**Formamide acrylamide gels**

Prepared in 10 cm. x 0.5 cm. tubes. 99% formamide buffered to a pH meter value of 9 with 0.4% barbituric acid. Electrophoresis in 10 - 20 mM NaCl at 2 mA/gel. Samples loaded in 100% formamide and heated to 60°C before loading. Otherwise as in Staynov et al (1972).
FIGURE 2

Size of rabbit DNA fragments

These are profiles of ethidium bromide fluorescence from adjacent gel tracks of a 2% agarose gel. Electrophoresis was from right to left. The smooth profile with a lot of low molecular weight DNA is the profile for the DNAse I digested rabbit DNA. The profile showing bands is a Hae III digest of human DNA. The band sizes are taken from Cooke (1976), though I have converted the sizes from daltons to base pairs.
Preparation of fragments of rabbit DNA

5 mg. rabbit DNA in 50 mM Tris pH 8,10 mM MgCl₂ with 1 μg. alkaline phosphatase and 5 μg. DNase I in 2 mls. Reaction incubated at 37°C for 12.5 minutes then extracted twice with phenol and 3 times with chloroform then precipitated by addition of 50% TCA to a concentration of 10%. The pellet was washed x 3 in ethanol/ether and dissolved in 500 μl. H₂O. 67% of the DNA remained acid precipitable. On a 2% agarose gel the DNA showed a smear with most of the DNA smaller than 200 base pair double strand DNA so probably with a single strand length less than 400 bases (figure 2). The quantity of alkaline phosphatase used was selected so as to remove 5 - 10% of the 5' phosphate groups on the DNA.

Kinase labelling

Polynucleotide kinase was a gift from Ken Murray.

(a) DNA fragments

3.35 mg. of fragments from rabbit DNA in 500 μl. of 5 mM dithiothreitol, 10 mM MgCl₂, 100 mM Tris pH 7.4, 25 μCi γ³²P ATP (Amersham) at 3 Ci/mM and 10 units polynucleotide kinase, 20 hours 37°C. Solution extracted once with phenol, once with chloroform then DNA precipitated with 10% TCA, washed in ethanol/ether and redissolved in H₂O. Final specific activity 3000 c.p.m./μg.

(b) Oligo dT (pT₁₀)

Removal of 5' phosphate:

30 μg. oligo dT in 250 μl. 100 mM Tris pH 7.4, 10 mM MgCl₂,
5 mM dithiothreitol, 10 μg. alkaline phosphatase (Sigma) 37°C 16 hours. Heated at 100°C for 5 minutes then 1 μg. pronase added and the reaction incubated for 3 hours at 37°C then extracted twice with phenol and twice with chloroform then passed over Sephadex G10 in H₂O.

Addition of 5'32P phosphate:

3.3 μg. of T₁₀, 5 μCi 32P ATP, 100 mM Tris pH 7.4, 10 mM MgCl₂, 5 mM dithiothreitol, 0.5 units polynucleotide kinase 3 hours 37°C. Extracted once with phenol then passed through Sephadex G25 in water.

Chemical labelling of oligo dT (T₁₀)

Dephosphorylated T₁₀ (0.5 μg.) labelled by the trichlor-acetonitrile procedure of Symons (1972), 2.5μCi of 32P orthophosphate was incorporated into oligo dT.

Binding of DNA fragments or oligo dT to beads

Sephadex G10 and cellulose Cellex N1 (Biorad) were washed in 30% ethanol 1% HCl at room temperature for a week before use. This helps remove carboxyl groups.

(a) aqueous binding to Sephadex or cellulose with carbodiimide:

5 mg. of bead in a small siliconised glass tube. 20 μl. of a solution of N-cyclohexyl-N'-(2-(4-morpholinyl)-ethyl carbodiimide (MNTS) (Fluka) 100 mg./ml. in 0.1 M 2-N morpholino ethane sulphonate buffer (MES) (Fluka) pH 6.0 and 10 or 20 μg. of 32P DNA fragments or 0.1 μg. oligo dT incubated 5 days 37°C in sealed tubes. The DNA was dried down onto the beads before adding the reaction solvent in this and subsequent binding procedures.
(b) **binding to Sephadex or cellulose in pyridine using carbodiimide:**

5 mg. of bead in 20 µl. of dry freshly distilled pyridine containing 100 mg./ml. of dicyclohexyl carbodiimide and 10 - 20 µg. DNA fragments or 0.1 µg. oligo dT incubated 5 days 37°C in sealed tubes. Dicyclohexyl carbodiimide is more reactive than the carbodiimide in (a) and so the reaction might be expected to work better in pyridine. However, the DNA fragments were not soluble in pyridine and this may counteract the reactivity advantage.

(c) **attachment to chloromethyl polystyrene:**

chloromethyl polystyrene beads SX1(Cl) (Biorad) or P(Cl) (a gift from Zerolit Ltd.) were incubated with 10 µg. of DNA fragments or 0.1 µg. of oligo dT in water or in pyridine or in the absence of solvent for 5 days at 37°C.

(d) **binding to diazobenzyl cellulose or diazobenzyl polystyrene:**

as described in Noyes and Stark (1975) except that in later experiments I used 100% formamide instead of 80% DMSO. 5 mg. cellulose per assay.

**Synthesis of diazobenzyl polystyrene**

Polystyrene beads SX1 (Biorad) were brominated on the benzene rings by treatment with Br₂ with I₂ catalyst for 3 days at 25°C, conditions that should introduce one Br atom per ring (Merrifield, 1964).

Brominated polystyrene was then treated with a saturated solution of NaNH₂ in liquid NH₃ at -50°C for 6 weeks. The resulting beads were used to make diazobenzyl polystyrene by the procedure of Noyes and Stark (1975).
Nick translations

Performed roughly according to the nick translation procedure of Maniatis et al (1975). I use 40 μM unlabelled nucleotides and about 10 μM of one or two 32P labelled ones. 50 mM Tris pH 8.0, 5 mM MgCl2, 10 mM mercaptoethanol, 50 μg/ml BSA, 1.5 x 10^-9 g/litre DNase I, 1.5 pl. Böhringer DNA polymerase I and 0.5 μg DNA in a final volume of 50 pl. After 2 hours incubation at 15°C I extract the solution once with phenol after adding NaCl to 0.3 M and 50 μg. E. coli sonicated single strand DNA then pass the solution through Sephadex SP50 in 0.3 M NaCl.

Restriction enzymes

All were gifts from Barbara Smith except R2 which was prepared by Ken Cummerson and myself, HaeIII which was prepared by H.J. Cooke and Hha, originally from Cold Spring Harbor Laboratories, which was a gift of S.A. Endow. Pst, Hae III, HhaI, HpaI, BglII were used in 10 mM Tris pH 7.4 10 mM mercaptoethanol, 10 mM MgCl2.

R2 and Hind 3 were used in the same buffer supplemented with 50 mM NaCl.

Bam was used in 10 mM Tris pH 8, 10 mM MgCl2, 10 mM mercaptoethanol, Rl in 100 mM Tris pH 7.5, 5 mM MgCl2, 10 mM mercaptoethanol. All were made by a standard protocol developed by Barbara Smith and Howard Cooke (this laboratory).

Agarose gel electrophoresis

Slab gels for running restriction digests of eukaryotic DNA subsequently to be used for blotting (see later) were
19 cm. x 19 cm. x 0.6 cm. with slots 0.85 cm. or 0.5 cm. wide and 0.6 cm. thick.

Other slab gels were 0.3 cm. thick but the same otherwise. Two types of cylindrical gels were used, small ones 24.5 cm. x 0.8 cm. diameter and large ones 20 cm. x 2.5 cm. diameter. Gels were run in E buffer (Loening, 1967) at 20 - 100 volts for 3 - 20 hours until the orange G tracking dye loaded with the DNA reached the bottom of the gel. 0.6 cm. slab gel slots were loaded with 15 - 30 µg. of restricted eukaryotic DNA per slot, 0.3 cm. slab gels with 1 - 5 µg. of restricted eukaryotic DNA per slot, small cylindrical gels with 30 µg. and large cylindrical gels 200 - 400 µg. 2 - 5% formamide was added to the DNA samples to aid loading onto the gels by increasing the solution density without increasing the viscosity. Formamide may also help disrupt hybridisation of sticky ends in restricted DNA.

Transfer of DNA from agarose gels to nitrocellulose filters

Millipore HAWP was always used as nitrocellulose; Sartorius or Schleicher and Schüll nitrocelluloses seem no better (Southern, unpublished). Slab gels were transferred as described by Southern (1975) except that 0.6 cm. thick gels were soaked in 0.5 M NaOH 1.5 M NaCl for 1.5 hours then neutralised in 3 M NaCl, 0.4 M Tris pH 7.5, 0.1 M acetic acid for 3 hours before transfer in 20 x SSC. DNA was transferred from large cylindrical gels using an apparatus built by Ed. Southern: after electrophoresis the gels were stained in 0.5 µg./ml. of ethidium bromide in water for 6 hours, photographed under U.V. light then the DNA denatured
in situ for 6 hours using 0.1 M NaOH. The gels were then neutralised by soaking for 16 hours in 0.3 M NH$_4$ acetate pH 5.0 in 5 litres/6 gels with one change of buffer. Gels were then placed on their sides on strips of nitrocellulose which in turn lay upon a large slab of 2% agarose in 0.3 M NH$_4$ acetate. 25 - 50 volts was applied across the gels at right angles to the original direction of gel electrophoresis through a large filter paper wick so that a replica of the original gel DNA profile was reproduced on the nitrocellulose. Complete transfer of DNA took 1 or 2 days.

DNA was transferred from small cylindrical gels and slices from slab gels using the electrophoretic procedure described in Arnheim and Southern(1977).

I shall refer to the transfer procedure where the DNA is washed out of the gel by flow of buffer as blotting and the electrophoretic transfers I shall call transfers.

Elution of DNA from agarose gels

After electrophoresis bands detected by U.V. fluorescence with ethidium bromide were cut out of the gels (large cylindrical gels 20 cm. x 2.5 cm.) put back into the original electrophoresis tube and the DNA eluted into a dialysis bag by short electrophoresis (30 volts, 30 minutes). At the end of the elution the polarity was reversed for 30 seconds to stop DNA sticking to the dialysis bag (Southern, unpublished) then the solution in the bag taken to 0.3 M in NaCl, extracted with phenol until no interface was left between the phenol and water phases,
then the phenol removed from the aqueous phase with ether and the DNA precipitated with ethanol. Recovery 10 - 50% of theoretical.
RESULTS AND DISCUSSION: PART 1

(a) Introduction

Enrichment of rare sequences from the DNA of a eukaryot

There are two approaches to the enrichment of a rare DNA sequence. Either the DNA of interest can be enriched as native double stranded DNA or as single stranded DNA. For complete purification of a single copy sequence the only practicable method at present is to clone it in a bacterial plasmid because no physical method exists capable of enriching a DNA sequence $10^6$ fold with respect to other DNA sequences and vast quantities of starting DNA would be required in order to finish with a reasonable yield of pure single sequence anyway. Double stranded DNA is probably required for cloning and so any enrichment technique that maintains the DNA double stranded is more useful.

By partially enriching a sequence of interest from total DNA it may be possible to examine the sequence using techniques that would give too high a non-specific background with total DNA. The use of density centrifugation to enrich repeated sequence DNAs is an example. If the DNA sequences associated with a single copy gene are to be studied by hybridisation or by enzymatic extension of a DNA primer on the gene template then there is no disadvantage to denaturing the DNA and once denatured it is possible to use hybridisation to enrich a sequence and this is much the most powerful way to do so. I chose to try making globin cDNA columns for enriching globin DNA sequences in total rabbit DNA by hybridisation. The advantage of columns is that
at each biochemical step in a purification only the beads of
the column need be handled and this is easier quicker and more
efficient than trying the same steps in solution with very small
quantities of DNA.

Many people have tried to use columns with covalently bound
nucleic acid to purify DNA or RNA (for review see Gilham, 1974).
Probably the earliest really successful application was the use
of oligo dT cellulose to purify mRNA (Edmonds and Caramela, 1969).
Shih and Martin (1973, 1974) used SV40 RNA bound to cellulose
using Gilham's procedures (Methods in Enzymology XXI 194) to
purify SV40 DNA fragments and Hoyes and Stark (1975) used a
procedure of their own to bind SV40 DNA to cellulose and then
purify SV40 RNA from SV40 transformed cells.

There are three main difficulties with these techniques:
non-specific binding of nucleic acid to the columns (Kitos et al,
1972) poor yields of hybrid (Shih and Martin, 1973) and lack of
stability of the bonding of the biochemical supposed to be bound
covalently to the column (Kolb et al, 1975).

To tackle the first two of these problems I chose to
compare cellulose and polystyrene as column materials. Cellulose
has been used for most nucleic acid column work but for peptide,
chemistry in solid phase polystyrene beads have been used.
Polystyrene might give lower non-specific binding of DNA than
does cellulose because polystyrene has less, small pores than
cellulose being a tight hydrophobic bead and therefore provides
a smaller surface area for random non-covalent binding of DNA.
while providing better access of large molecules to small pieces of nucleic acid bound to the polystyrene. It has been suggested that lignin is responsible for the background binding of DNA to cellulose (Delarco and Guroff, 1975). If this is true then polystyrene might give lower background binding than cellulose in that polystyrene does not contain lignin though it does have the disadvantage that being hydrophobic it might form hydrophobic bonds with DNA bases.

Polystyrene may give better yields of hybrids between column bound DNA and free DNA and better accessibility of bound DNA to enzymes because it will have a large pore size in water whereas cellulose, Sephadex and Sepharose will all form expanded gel matrices in water with small pores. DNA bound to polystyrene may then be much less hindered by other bits of polystyrene nearby than DNA bound to cellulose, Sephadex or Sepharose. I hoped that by using highly reactive chemical groups in the preparation of all the columns firm binding of the DNA to the column could be ensured.

**Approaches to the synthesis of cDNA columns**

There are two approaches to making a cDNA column, either bind oligo dT covalently to the column and use the oligo dT as a primer against mRNA to make cDNA or alternatively bind the cDNA directly to the column. The first of these approaches has the advantage that a lot of oligo dT can be used in the reaction used to bind it to the column whereas cDNA is usually available in small quantities only. In addition oligo dT is a much smaller
molecule than cDNA being 10 bases long compared to 500 or more for cDNA and in a procedure for linking DNA to cellulose such as Gilham's procedure (Gilham, 1964) where the 5' phosphate group on the DNA is used these are 50 times as frequent in oligo dT as in cDNA.

Attachment of a primer to beads then extension of the bound primer was used by Jovin and Kornberg (1968) to make RNA or DNA homopolymers attached to columns, by Panet and Khorana (1974) to make pieces of a tRNA gene, by Venetianer and Leder (1974) to make globin cDNA cellulose and by Anderson and Schimke (1976) partially to purify ovalbumin genes.

Once cDNA has been hybridised to a larger piece of DNA it is theoretically possible to use the cDNA as a primer against the longer piece of DNA and extend the cDNA enzymatically. In this way radio-labelled DNA from the sequences at the 5' end of that gene can be prepared. If this is to work with column immobilised cDNA then the reaction used to attach the cDNA to the column must leave free and unmodified the 3' hydroxyl group on the cDNA so that only techniques that use either the 5' end or bases within the DNA sequences are of any use for linking DNA to columns if the DNA is subsequently to be used as a primer. I chose to try the procedure of Gilham (Methods in Enzymology XX1 194) with the modification of using commercial oligo dT 10 bases long as the nucleic acid for linking to cellulose. In this procedure carbodiimide is used to condense the 5' phosphate groups on DNA with hydroxyl groups on cellulose to form phosphodiester bonds:
cellulose – OH + P - DNA  \[\text{carbodiimide}\]  \rightarrow\] cellulose –(P)– DNA + H₂O

For both cellulose and polystyrene I used a modification of the Noyes and Stark (1975) procedure where DNA bases react with aromatic diazo compounds to link cDNA directly to the columns:

\[\begin{align*}
\text{N₂} & \rightarrow \text{DNA} \\
\text{DNA} & \rightarrow \text{DNA} + \text{N₂}
\end{align*}\]

For polystyrene alone I tried direct reaction of DNA with chloromethyl polystyrene:

\[\begin{align*}
\text{CH₂Cl} & + \text{DNA} \\
\text{DNA} & \rightarrow \text{DNA} + \text{Cl}⁻
\end{align*}\]
RESULTS AND DISCUSSION: PART 1

(b) Experiments

Preparation of globin cDNA columns

Binding of DNA to beads

I had available a range of bead materials to which to bind DNA. Cellulose Cellex Nl is a non-ionic cellulose of high purity which was used in the detailed studies of Panet and Khorana (1974) on DNA binding to and behaviour on cellulose. Sephadex G10 was also used by Panet and Khorana. These two differ chemically — one is cellulose, the other Sephadex, physically, Cellex Nl is a fine powder but G10 is a large tight bead, and ionically, Cellex Nl is non-ionic whereas Sephadex G10 probably has some carboxyl groups (manufacturers data). Thus they may have different adsorption behaviour towards DNA. I used these mainly to study the carbodiimide linking of DNA to cellulose, which many people have used successfully (see Gilham, 1974). To study the procedure of Noyes and Stark (1975) I had the Kiles m-aminobenzylxoxymethyl cellulose. The major advantages of this material and the procedure of Noyes and Stark in general are that the cellulose is used as very small beads giving large surface areas in small amounts of cellulose, leaving bound DNA floating free on the surface of the bead rather than sterically hindered within a gel matrix (Noyes and Stark, 1975) and the activation step for binding DNA uses a very reactive chemical intermediate namely the diazobenzyl group. These features might lead to high efficiencies of covalent DNA binding, low non-covalent binding of DNA and high efficiencies
for enzyme reactions on bound DNA. The disadvantage is that
the cellulose is too fine to use in columns and dissolves in
some buffers, in particular Tris above about pH 8.5.

For polystyrene I chose to use chloromethyl polystyrene
because it was a readily available form of polystyrene bead and
this form of polystyrene activation has been used in solid phase
peptide work as the reactive group for binding the peptide or
amino acid to the column (Merrifield, 1964). I obtained SX1(Cl)
a 1% cross-linked 200 - 400 mesh bead sold by Biorad and P(Cl)
a 0.5 mm diameter bead as a gift from Zerolit Ltd. These latter
beads are precursors used in the manufacture of standard ion
exchange beads. I determined the chlorine content of these beads
by fusing a sample with sodium metal then dissolving the NaCl in
water and precipitating the chloride ion with radioactive
$^{110m}$Ag$^+$. In this way I obtained a value of $4.5 \pm 0.6 \mu$moles of
chlorine per milligram of P(Cl) as against $1.3 \pm 0.1 \mu$moles/mg.
for SX1(Cl) the latter agreeing well with manufacturers value
of $1.34 \mu$moles/mg. Because the Noyes and Stark procedure for
linking DNA to cellulose uses such an easily synthesised and
reactive chemical intermediate I tried to adapt it to polystyrene.
I tried to make p-amino benzyl polystyrene from SX1 beads (these
beads are the same as SX1(Cl) except they have not been chloro-
methylated) (see methods). I was unable to assay the synthesis
of these aminated beads because the I.R. spectroscopy equipment was
not sensitive enough and the polystyrene beads were too hard
to grind into a smooth mull to produce good spectra anyway.
Mass spectroscopy equipment was not available to me.
TABLE 1

LEGEND

1 µg. of E. coli DNA was used per assay. Specific activity was 233,500 c.p.m./µg. and it was a gift from Barbara Smith. It was denatured by boiling before use. ³H FLA DNA was a gift from Phoebe Mounts. FLA is a derivative of HeLa cells. 1 µg. of this was used in the assay, denatured before use. About 1 ng. of ³H globin cDNA was used per assay. DNA fragments were prepared as described in methods. Outline biochemical steps for this experiment are also described in methods. m-diazobenzoyl linking is the procedure of Noyes and Stark (1975) but note the use of formamide as solvent in some assays. The cellulose used in the DMSO experiments is a different batch from that used in the formamide experiments. After the chemical reaction was terminated beads were washed in 0.3 M NaCl 1% Triton, then soaked overnight in 10 mls. 99% formamide 0.1% SLS at 60°C. Beads were then washed in water on glass fibre filters, dried at 60°C and radioactivity measured by scintillation counting in a toluene based scintillation fluid. Control column is the column material without any chemical activation.
<table>
<thead>
<tr>
<th>Material</th>
<th>Solvent</th>
<th>DNA</th>
<th>% of DNA bound to control column</th>
<th>% of DNA bound to activated column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>H₂O</td>
<td>10 µg. ³²P fragments</td>
<td>5 ± 2</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Cellex N1 (NI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G10 (G10)</td>
<td>H₂O</td>
<td></td>
<td>18 ± 11</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>CHLOROMETHYL LINKING</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zerolit</td>
<td>H₂O</td>
<td>10 µg.</td>
<td>28 ± 5</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>Chloromethyl-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzyl-</td>
<td>No solvent</td>
<td></td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Polystyrene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(Cl)</td>
<td>H₂O</td>
<td>20 µg.</td>
<td>1 ± 0.5</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>Bio-Beads SX1 (Cl)</td>
<td>H₂O</td>
<td>10 µg.</td>
<td>6 ± 1</td>
<td>35 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-DIAZO BENZOYL LINKING</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>80% DMSO</td>
<td>³H FLA DNA</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td></td>
<td></td>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td></td>
<td>³H globin cDNA</td>
<td>35</td>
<td>63</td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>100% formaldehyde</td>
<td>³²P coli DNA</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td></td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>100% formaldehyde</td>
<td>³²P coli DNA</td>
<td>44 ± 8</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td></td>
<td></td>
<td>30 ± 30</td>
<td>74 ± 4</td>
</tr>
</tbody>
</table>
Table 1 shows the results of binding a range of DNAs to these beads under a range of conditions. Binding to column materials that have not been chemically activated is in most cases an appreciable fraction of binding to activated materials. Where fairly long single strand DNA has been used (Globin cDNA, $^{3}$H FLA DNA, $^{32}$P coli DNA) non covalent binding to Sephadex, cellulose or polystyrene is sometimes very high and this agrees with a common observation that it is very difficult to purify cDNA over Sephadex columns in the absence of carrier DNA because the cDNA sticks to the Sephadex. This background was not reduced by washing beads in a range of buffers spanning a wide range of salt concentrations nor was 1 M potassium thiocyanate (a powerful chaotropic agent) effective nor was 1% starch solution able to remove the DNA.

The results from this experiment were very erratic as evidenced by the large standard errors. The efficiency of the chemical reaction between the DNA and the beads in each case will depend on the actual concentration of DNA in the reacting solution. To maximise the concentration of DNA I used very small volumes of solution, just enough to wet the beads, and thus small absolute differences in the amount of solution that evaporates during the reaction can give rather large differences in DNA concentration.

Noyes and Stark (1975) used 80% DMSO at 4°C for 2 days as their standard reaction conditions in their procedure. Table 1 does seem to show that longer times and using formamide rather than DMSO both increase the efficiency of the reaction. 100% formamide is probably a much better denaturing solution than 80%
<table>
<thead>
<tr>
<th>Material</th>
<th>Means of binding DNA fragments to material</th>
<th>% DNAse Insensitive 32P on control material</th>
<th>% DNAse Insensitive 32P on activated material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>Aqueous Carbodiimide</td>
<td>28 ± 1</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>Cellex Nl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G10</td>
<td>&quot;</td>
<td>35 ± 5</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>Biobeads Sx1 (Cl)</td>
<td>Aqueous Solution</td>
<td>31 ± 1</td>
<td>62 ± 15</td>
</tr>
</tbody>
</table>

Beads from the experiments of table 1 were washed free of toluene scintillant with ethanol then washed in 3 M NaAc pH 7.2 then in distilled water then digested with 40 μg/ml of DNAse II in 400 μl. NaAc pH 4.5 + 1 mM MgCl₂. At the end of the experiment beads were washed in water then in 10 mls. 99% formamide 0.1% SLS overnight, in water again then dried and scintillation counted in a toluene based scintillant.
DMSO and thus may inhibit reassociation or aggregation of single strand DNA. Since single strand DNA reacts better than double strand with diazobenzyl groups (Noyes and Stark, 1975) maintenance of the DNA single stranded may explain the improvement in DNA binding when formamide is used. Since formamide and DMSO are very similar chemically the change from DMSO to formamide is highly unlikely to affect any chemical aspect of the reaction. Because the binding of DNA to unactivated cellulose is also affected by the change from DMSO to formamide it is possible that much of the difference is simply in adsorption of DNA to the beads (see later).

I am surprised to see that DNA binds so reliably to SX1(Cl) beads in the absence of solvent yet so little to SX1 beads. Although this result seems to me at least plausible chemically in that no solvent is really necessary in the reaction except to carry away the Cl⁻ leaving group another explanation may be that SX1(Cl) and SX1 beads do have different adsorption properties for DNA and this would invalidate SX1 beads as controls against which to assess chemical binding of DNA to SX1(Cl) beads.

Adventitiously bound DNA

The adventitiously bound DNA is very tightly bound to all the column beads in Table 1 as judged by its ability to survive quite stringent washes. To study this further I took some of the materials from the experiments of Table 1 which had bound to them DNA labelled with ³²P only in the 5' phosphate group and treated them with 40 μg/ml. DNAse II at 37°C overnight (Table 2).
Since DNase II cleaves DNA leaving 3' phosphate groups then 5' phosphate groups bound covalently to beads should be resistant to DNase II. 30% of the radioactivity that bound to columns that had not been activated and thus had no covalently bound DNA was resistant to DNase II and only 60% of the radioactivity bound to activated columns was resistant to DNase II. Thus a lot of DNA that does bind to activated columns (> 40%) is sensitive to DNase II and therefore is not covalently attached through its 5' phosphate group (5' covalent attachment is supposed to occur using the carbodiimide technique) and since 30% of radioactivity bound to non-activated columns is resistant to DNase II 30% of DNA that is not covalently bound is still resistant to DNase II. This experiment shows that a lot of the DNA that appears to be bound covalently to beads using either carbodiimide linking or chloromethyl linking, as measured by washing the beads after the chemical reactions, is probably not covalently bound at all and is certainly not covalently bound by the 5' phosphate group on the DNA. The experiment also shows that DNA that is not covalently bound to beads can still be very tightly bound and can be inaccessible or poorly accessible to enzymes. This seems true for cellulose, Sephadex and polystyrene.

**Binding of oligo dT**

A shorter piece of DNA might be less prone to adventitious binding to columns because it can form less, weak non-covalent bonds and since the reason for doing these experiments was as a model system for binding oligo dT to columns I repeated some of
TABLE 3
BINDING OF $^{32}$P OLIGO dT ($^{32}$pT$_{10}$) TO COLUMNS

<table>
<thead>
<tr>
<th>Material</th>
<th>Means of binding Oligo dT to material</th>
<th>% Oligo dT (0.1 µg.) bound to control material</th>
<th>% Oligo dT (0.1 µg.) bound to activated material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Beads SX1 (Cl)</td>
<td>No solvent</td>
<td>1.3 ± 0.2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Bio-Beads SX1 (Cl)</td>
<td>DMSO</td>
<td>1.6 ± 0.04</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Bio-Beads SX1 (Cl)</td>
<td>H$_2$O</td>
<td>0.9 ± 0.4</td>
<td>4 ± 0.6</td>
</tr>
<tr>
<td>Zerolit P(Cl)</td>
<td>No solvent</td>
<td>- - -</td>
<td>6 ± 1.5</td>
</tr>
<tr>
<td>Zerolit P(Cl)</td>
<td>DMSO</td>
<td>- - -</td>
<td>5 ± 1.5</td>
</tr>
<tr>
<td>Zerolit P(Cl)</td>
<td>H$_2$O</td>
<td>- - -</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>Zerolit P(Cl)</td>
<td>Pyridine</td>
<td>- - -</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Cellex N1</td>
<td>Aqueous Carbodiimide</td>
<td>3 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Sephadex G10</td>
<td>&quot;</td>
<td>4 ± 2</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

$^{32}$P Oligo dT was prepared as described under chemical labelling of oligo dT in methods. It was used with carrier unlabelled oligo dT. Beads washed in 10 mls. water overnight at end of reaction, filtered onto GFA filters, washed in water and ethanol/ether and scintillation counted.
### TABLE 4
ACTIVITY OF REVERSE TRANSCRIPTASE (AMV) ON OLIGO dT COLUMNS

<table>
<thead>
<tr>
<th>Material</th>
<th>Means of binding Oligo dT</th>
<th>% efficiency of reverse transcriptase on Oligo dT adsorbed to control column</th>
<th>% efficiency of reverse transcriptase on bound Oligo dT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio–Beads SX1 (Cl)</td>
<td>No solvent</td>
<td>$1 \pm 0.1$</td>
<td>$1.7 \pm 0.3$</td>
</tr>
<tr>
<td>Bio–Beads SX1 (Cl)</td>
<td>DMSO</td>
<td>$1 \pm 0.3$</td>
<td>$2 \pm 0.4$</td>
</tr>
<tr>
<td>Bio–Beads SX1 (Cl)</td>
<td>H$_2$O</td>
<td>$0.7 \pm 0.2$</td>
<td>$1.2 \pm 0.2$</td>
</tr>
<tr>
<td>Zerolit P (Cl)</td>
<td>No solvent</td>
<td>--</td>
<td>$3 \pm 1$</td>
</tr>
<tr>
<td>Zerolit P (Cl)</td>
<td>DMSO</td>
<td>--</td>
<td>$1.4 \pm 0.4$</td>
</tr>
<tr>
<td>Zerolit P (Cl)</td>
<td>H$_2$O</td>
<td>--</td>
<td>$4 \pm 2$</td>
</tr>
<tr>
<td>Zerolit P (Cl)</td>
<td>Pyridine</td>
<td>--</td>
<td>$7 \pm 4$</td>
</tr>
<tr>
<td>Cellex</td>
<td>Aqueous Carbodiimide</td>
<td>$2.4 \pm 0.01$</td>
<td>$3 \pm 1$</td>
</tr>
<tr>
<td>Sephadex G10</td>
<td>&quot;</td>
<td>$1 \pm 0.1$</td>
<td>$4 \pm 1$</td>
</tr>
</tbody>
</table>

Beads were washed before the enzyme reaction in ethanol then water.

My standard reverse transcriptase reaction mixture was used with $^3$H dCTP as label, 20 \( \mu \)l of reaction mix per 5 mg of beads and 0.1 \( \mu \)g of globin mRNA 37°C 15 hours. After the reaction the beads were washed overnight in 10 mls. 1% Triton then filtered onto GFA filters and counted in toluene based scintillant. Efficiency is expressed relative to transcription of 0.1 \( \mu \)g of mRNA in the same reaction cocktail but with free oligo dT as primer. Incorporation into DNA determined in this case by TCA precipitation.
these binding procedures with oligo dT and in fact I used the techniques that are supposed to bind DNA by its 5' phosphate group in the hope that then enough of each primer molecule would be free and unhindered away from the beads actually to function as a primer. Oligo dT also contains only one of the 4 DNA bases and T may not be the base most prone to adventitious binding to beads. Table 3 reveals that oligo dT is less prone to adventitious binding but also seems to bind rather poorly to activated material and adventitious binding is still a high proportion of total binding. The poor efficiency of the binding reaction need not be important because only a ratio of about 1:65 weight for weight of oligo dT is required to prime transcription of globin mRNA with reverse transcriptase in liquid phase so there is no need to have very much oligo dT bound to the columns. Globin mRNA is 65 times longer than oligo dT.

**Use of oligo dT columns as primers for cDNA synthesis**

I went on and used the materials from Table 3 as primers for reverse transcriptase against a template of globin mRNA (Table 4) to test the accessibility of the column bound primers to reverse transcriptase and globin mRNA. Efficiency of the reaction is judged relative to the efficiency of the reverse transcription of the same amount of mRNA in solution with free oligo dT as primer. The columns serve as very poor primers and this might be expected from the previous experiment which showed that DNA bound to these beads is poorly accessible to DNase II. This is in fair agreement with Anderson and Schimke (1976) who found
### Table 5

<table>
<thead>
<tr>
<th>Material</th>
<th>% inhibition of reverse transcriptase</th>
<th>% adsorption of cDNA to solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zerolit P(Cl)</td>
<td>- - -</td>
<td>95</td>
</tr>
<tr>
<td>p-amino benzyl polystyrene</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>m-amino benzoyl cellulose (dirty)</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>Cellex N1</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>Sephadex G10</td>
<td>10</td>
<td>70</td>
</tr>
</tbody>
</table>

11 tubes were prepared with a standard reverse transcriptase reaction cocktail with 0.1 µg. of globin mRNA and 20 ng. of oligo dT. 5 of these tubes contained 5 mg. per tube of one of the types of bead. 6 tubes did not contain these. After 2 hours at 37°C beads were added to 5 more tubes and all 11 tubes were left at 37°C for a further 2 hours. At the end of this incubation beads were washed in 1 ml. water, this water was taken to 10% in TCA and filtered through GFA glass fibre filters, the filters were washed in 10% TCA then ethanol/ether, dried and measured for radioactivity by scintillation counting. Results are expressed relative to the amount of TCA precipitable radioactivity in the tube that contained no beads at all.
cellulose columns with oligo dT to be about 5% efficient as primers. I found also that commercial oligo dT cellulose (Collaborative Research) had no priming activity at all. These columns are not poor primers in these experiments merely because there was not enough oligo dT present to prime 0.1 μg. of globin mRNA. About 1.5 ng. of oligo dT would have been enough in theory and all except one of the beads tested (Biobeads SXI(C1) control Table 3) had this much or very nearly so.

Another explanation is that the column material itself inhibits reverse transcriptase. To test this I mixed the reaction mixture used to test the priming efficiency of beads in the experiments of Table 4 with 20 ng. of free oligo dT in the presence or the absence of column materials for 2 hours. At 2 hours I added column beads to those tubes that did not already contain beads then I left all the tubes for a further 2 hours. This tests both the adsorption of cDNA to the bead materials (those tubes where the beads were added only after most of the cDNA synthesis was over) and inhibition of transcription of mRNA by the presence of beads (the tubes where the beads were there from the beginning and the knowledge from the other tubes of how much cDNA adsorbs to the beads). The results are presented in Table 5 and it is clear both that cDNA adsorbs strongly to the beads and also that reverse transcription is appreciably inhibited by the presence of the beads. With the exception of dirty m-amino benzoyl cellulose it seems very unlikely that this inhibition could account for the very poor priming efficiency of the beads with oligo dT bound to them, the inhibition is not
large enough. The dirty m-amino benzoyl cellulose is dirty because it was taken straight from the bottle that it was sold in and in this state it is pale yellow presumably due to degradation of the aromatic groups attached to the cellulose. Since the other celluloses do not inhibit reverse transcriptase so severely it may be that these aromatic groups inhibit reverse transcriptase. When this cellulose is purified according to Noyes and Stark (1975) it becomes white and so the dirty material is probably a poor control for inhibition of reverse transcriptase by m-amino benzoyl cellulose.

Another explanation for the poor priming efficiency of oligo dT columns could be that the oligo dT is too small a molecule and that either hybridisation of the oligo dT to the mRNA or binding of reverse transcriptase to the complex is inhibited by the material of the beads. Oligo dT is probably only about 30–40 Angstroms long and this is about the diameter of a protein molecule. To test whether oligo dT is too short to serve as an efficient primer I lengthened oligo dT to make poly dT about 40 bases long (see methods) and bound this and also oligo dT to polystyrene or cellulose by the procedure of Noyes and Stark (1975). This procedure seemed to me a more efficient way to bind a large molecule to a bead than the carbodiimide or chloromethyl approaches because it uses bases within the DNA sequence rather than just the 5' terminal base and also because the finely divided cellulose may give less steric hindrance anyway. I then used the resulting beads to prime cDNA synthesis from globin mRNA. Results are again expressed relative to the
### Table 6

<table>
<thead>
<tr>
<th>Column Material</th>
<th>% Efficiency of cDNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>oligo dT</td>
<td></td>
</tr>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>poly dT</td>
<td></td>
</tr>
<tr>
<td>p-amino benzyl polystyrene</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>oligo dT</td>
<td></td>
</tr>
<tr>
<td>p-amino benzyl polystyrene</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>poly dT</td>
<td></td>
</tr>
<tr>
<td>m-amino benzoyl cellulose</td>
<td>4, 14</td>
</tr>
<tr>
<td>oligo dT</td>
<td></td>
</tr>
<tr>
<td>m-amino benzoyl cellulose</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>poly dT</td>
<td></td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>oligo dT</td>
<td></td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>poly dT</td>
<td></td>
</tr>
</tbody>
</table>

Oligo dT and poly dT celluloses and polystyrenes were prepared according to the Noyes and Stark (1975) procedure using 1 μg. of oligo dT or poly dT per 5 mg. of bead and using 100% formamide rather than 80% DMSO. Diazo cellulose or polystyrene is the chemically active bead, amino cellulose or polystyrene the control bead. Reverse transcription as described in the legend to Table 4. After the reaction beads were washed in water, 99% formamide + 1% Triton then 1 hour at 60°C in 99% formamide 1% Triton then water then ethanol/ether then dried at 60°C. In the formamide wash at 60°C no more c.p.m. were removed by washing for longer than 1 hour.
**TABLE 7**

**EFFECT OF WASHING DNA COLUMNS WITH 1.6 mg./ml. RABBIT RIBOSOMAL RNA**

<table>
<thead>
<tr>
<th>Column Material</th>
<th>DNA</th>
<th>% DNA retained on column without RNA wash</th>
<th>% DNA retained with RNA wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-amino benzyl polystyrene</td>
<td>$^{32}P$ coli</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>p-diazo benzyl polystyrene</td>
<td>$^{32}P$ coli</td>
<td>3.5</td>
<td>0.8</td>
</tr>
<tr>
<td>m-amino benzoyl cellulose</td>
<td>$^{32}P$ coli</td>
<td>63</td>
<td>6</td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>$^{32}P$ coli</td>
<td>87</td>
<td>6</td>
</tr>
<tr>
<td>m-diazo benzoyl cellulose</td>
<td>Globin cDNA</td>
<td>---</td>
<td>10</td>
</tr>
</tbody>
</table>

DNA was bound to cellulose or polystyrene by the procedure of Noyes and Stark (1975) though using 100% formamide as solvent. Washing of bound DNA initially as in Noyes and Stark (1975). The RNA wash was for 1 hour at room temperature with 1.6 mg./ml. rabbit liver RNA depleted for mRNA by passage through a column of oligo dT cellulose. This RNA contained no DNAse activity.
efficiency of transcription in solution using oligo dT as primer. The poly dT was about 35% as efficient a primer as oligo dT in solution. Table 6 shows the results and it is clear that poly dT is not a more efficient primer than oligo dT when bound to cellulose or polystyrene. In these experiments the beads carried a 5 - 10 fold excess of primer relative to the amount required to transcribe all the mRNA.

The length of the primer does not then seem very important but evidently the nature of the bead is important, oligo dT cellulose prepared according to Noyes and Stark (1975) seems a very efficient primer for cDNA synthesis and this cellulose seems to bind DNA very efficiently also. Since the major difference between this bead material and any of the other beads I have used whether cellulose, Sephadex or polystyrene is that the beads are very small I think that much of the oligo dT will be free on the bead surface and thus more readily available to mRNA and reverse transcriptase than in the larger types of bead. Noyes and Stark (1975) discuss this aspect of their material. However, I have found with DNA bound to beads using carbodiimide or chloromethyl techniques that merely washing beads in buffers or in formamide will not remove adventitiously bound DNA so I wished to test how tightly DNAs were bound to the beads in the Noyes and Stark procedure. I washed some beads with bound DNA in a large excess of non-homologous RNA to try and compete this DNA from the cellulose or polystyrene should it not be bound covalently. Table 7 shows that the RNA did indeed remove much of the bound single strand DNA from cellulose and polystyrene. Note however that
globin cDNA had been bound to m-diazobenzoyl cellulose as cDNA and I have not tested cellulose where oligo dT was bound and then used as a primer to make cDNA. In so far as the same chemical groups are available in cDNA as in oligo dT with the addition of many more in cDNA able to react by the Noyes and Stark procedure I would be surprised if oligo dT proved to be more reliably bound than cDNA.

Because RNA can elute much of the bound DNA from this DNA cellulose either the DNA was never bound covalently at all or the covalent linkage is very unstable. This raises the possibility that I simply failed to use the Noyes and Stark (1975) protocol correctly.

Noyes and Stark (1975) made SV40 DNA cellulose and used it to bind in vitro prepared SV40 cRNA and also to extract SV40 specific RNA from the total RNA of cells transformed by SV40. Liz Rogers and I repeated some of these experiments to check that I had indeed made the DNA cellulose properly. We prepared SV40 DNA cellulose according to Noyes and Stark (1975) and showed that 1 mg. of it carrying 8 µg. of SV40 DNA bound 50% of 1000 c.p.m. of 32P SV40 cRNA in the presence of 100 µg. of unlabelled E. coli RNA. 5 mg. of Human DNA cellulose prepared in the same way bound 4% of the SV40 cRNA. We also showed that SV40 DNA cellulose extracted 0.02% of RNA from cells transformed by SV40 just as did Noyes and Stark though we did not characterise the RNA so extracted. Thus I have been able to repeat the results of Noyes and Stark (1975) in showing that m-diazobenzoyl cellulose does bind DNA efficiently and that when SV40 DNA is bound to this
cellulose it can then bind SV40 cRNA. In addition I have found that this supposedly covalently bound DNA is mostly not bound covalently to the cellulose or at least can be eluted with non-homologous RNA.

I conclude from these experiments that it is possible to made cDNA columns efficiently using the Noyes and Stark (1975) procedure but the DNA bound is not stably attached to the cellulose. I find that cellulose Cellex N1, Sephadex G10 and polystyrene will all bind DNA using a variety of chemical reactions but again a lot of the DNA that binds is not stably attached, and, whereas with cellulose prepared according to Noyes and Stark, bound oligo dT will serve as a primer for efficient cDNA synthesis this is not the case with the other beads. This disagrees with Jovin and Kornberg (1968) and Venetianer and Leder (1974) both of whom have reported reliable synthesis of stable columns, though it is in reasonable agreement with the more thorough studies of Panet and Khorana (1974) who found some difficulty in binding poly dT to cellulose or Sephadex and more difficulty in using it as an enzyme primer. Through all my experiments I have always observed that polystyrene or cellulose that has not been activated chemically behaves much as does activated material in binding DNA and acting as an enzyme primer though it does so less well. Unless beads are washed very thoroughly before use as primers for DNA synthesis and after the primer has been bound to the bead then there may be enough primer leached off the bead during the enzyme reaction to prime efficient synthesis of DNA. This DNA can then adsorb
readily to the beads as I have shown (Table 5). This DNA then behaves as though it has been primed from covalently bound primer. This may explain the results of Jovin and Kornberg (1968) and Venetianer and Leder (1974).

It is clear that DNA cellulose columns can be used to enrich rare DNA sequences from eukaryotic DNA (Anderson and Schimke (1976)) but if the columns are as unstable or inefficient as they appear to be from my experiments then they are a poor basis for studying rare sequences because the DNA bound to the column will leach into the solution passed through the column and losses of hybrid formed from DNA supposed to be covalently bound to the beads and DNA hybridised to that DNA may be very severe. This type of problem is already known in the case of insulin-sepharose where all the activity of the insulin-sepharose columns has been accounted for by insulin leaching off the columns (Kolb et al, 1975).

In the experiments where I made oligo dT beads using Gilham's procedures to bind the oligo dT to the beads I think the beads served as poor primers for cDNA synthesis because the oligo dT is trapped within a gel matrix. Polystyrene may be a poor support material for a variety of reasons. The primer may be sterically hindered and the hydrophobic nature of the substance may repel, bind or simply inhibit enzymes. Polystyrene seems to bind DNA adventitiously just as badly as does cellulose, and this observation may support the view of Delarco and Guroff (1975) that lignin causes the background binding of DNA to cellulose in that both cellulose and polystyrene bind DNA and the only
component of cellulose that is remotely like polystyrene is the lignin contaminant in that they both are polymers containing aromatic groups. If this is correct then celluloses from which the lignin has been removed (Delarco and Guroff, 1975) will probably be very good column beads from the point of view of background binding anyway. Though in my experiments Sephadex gives backgrounds as bad as those with cellulose, and Sephadex presumably contains no lignin at all.

Although I find the attachment of DNA to cellulose using the Noyes and Stark (1975) procedure to be unstable I have nevertheless found that SV40 DNA columns made in this way will bind SV40 cRNA. In my experiments and in the equivalent experiments of Noyes and Stark there is always a 10 fold or more excess of DNA bound to the cellulose over RNA to be hybridised so that if 90% of the DNA falls off the cellulose there is enough still attached to the cellulose to hybridise the RNA. The free DNA fragments will hybridise to the RNA but will leave RNA tails or provide DNA tails that will bind much of the RNA to the DNA cellulose. I note within the paper of Noyes and Stark (1975) that the maximum ratio of RNA they could hybridise to cellulose bound DNA was 10% and that is about the figure I find for DNA that is stably bound to the cellulose.

From this series of experiments it was clear to me that cDNA columns were not a reliable approach to studying rare genes within eukaryotic DNA. I therefore turned to restriction mapping and cloning as a better approach to rare genes. The next sections of this thesis deal with experiments in restriction mapping and cloning.
RESULTS AND DISCUSSIONS

PART 2

(a) INTRODUCTION

Restriction analysis of rare sequences in total eukaryotic DNA

Electrophoresis in agarose gels separates DNA fragments according to their molecular weight. If total DNA from a eukaryot is digested with a restriction enzyme and the digest run in an agarose gel then any sequence will have a characteristic mobility in the gel. The size of fragment carrying a given sequence depends upon which restriction enzyme is used. By using a range of enzymes individually and in combination it is possible to construct a map of the sites for restriction enzymes around a sequence if that sequence can be detected in the gel. If it were possible to do this for a single copy gene sequence then a very large amount of information could be obtained about gene organisation in eukaryots without recourse to cloning every single sequence of interest. There are already examples of such analyses in the case of integrated virus sequences in transformed cell DNA (Botchan et al 1976; Ketner & Kelly, 1976). This approach would provide background information for cloning genes and tell how many copies there are of a rare gene. In situations where a gene is present in many copies per genome restriction mapping of sequences near the gene will tell whether the same sequences are associated with each copy of the gene. Restriction mapping will tell how closely linked genes map and how large deletions are in known regions (e.g. the thalassemias) as long as a restriction enzyme is available that does not cut the DNA between the genes or within the deletion.
It is impossible to see one particular single copy sequence in a total eukaryotic DNA restriction digest in an agarose gel by fluorescence. The signal is swamped by the signal from thousands of other sequences of similar size and non-specific dyes will not pick out an individual single copy sequence anyway. Radioactive probes for hybridisation might be specific enough and in theory can be made radioactive enough to detect single copy sequences in fairly small quantities of DNA but until recently there has been no way for rendering the DNA within an agarose gel readily accessible for efficient hybridisation. Botchan et al (1973) extracted mouse DNA from gel slices by homogenising the slices in buffer then bound the DNA to nitrocellulose filters and hybridised the filters with SV40 cRNA to look for SV40 DNA sequences integrated into the mouse genome. Nusum and Tonegawa (1976) dissolved gel slices of mouse DNA in sodium perchlorate, removed the DNA over hydroxylapatite columns and hybridised it with $^{125}$I labelled mouse K chain mRNA in solution. In this way they were able to provide evidence for somatic rearrangement of immunoglobulin genes. Since the K chain mRNA is impure the only acceptable way to screen an agarose gel for restriction fragments homologous to the K chain mRNA is by hybridising in unlabelled DNA excess. It is a general advantage of the slicing and DNA elution approach to hybridisation across agarose gels that impure probes can be used. The major disadvantages to this approach are that the resolution is poor being limited by the size of slice chosen and the procedure is extremely laborious. Also, because the hybridisation is performed in DNA excess, very different quantities of hybridisable DNA in
different gel slices will give the same hybridisation signal, further reducing resolution. Two other approaches have been developed for screening agarose gels with radioactive probes. Shinnick et al (1975) dried agarose gels after denaturing the DNA in situ with potassium hydroxide and then hybridised the dried gel with an excess of radioactive RNA probe. The efficiency of hybridisation using this procedure is quite low presumably because most of the DNA is trapped within the gel and is poorly available for hybridisation. A better approach has been developed by Southern (1975): after denaturing DNA in the gel the gel is placed in contact with filter paper soaked in 20 x SSC and in contact with a bath of 20 x SSC. A sheet of nitrocellulose is placed on top of the gel and paper towels on top of that. The flow of 20 x SSC through the gel washes the DNA onto the nitrocellulose. In this way a nitrocellulose replica of the gel is created and the replica may then be hybridised with radioactive RNA or DNA. As a technique for studying rare sequences the major limitation is the caprices of hybridisation to nitrocellulose (Ivanov and Markov (1979); Haas et al (1972); Flavell et al (1974)) though Rotchan et al (1976) and Ketner and Kelly (1976) used the technique to analyse the SV40 DNA integrated into the genomic DNA of transformed rat or mouse cells. In this thesis I shall describe attempts to use the technique to analyse globin genes in rabbit DNA.
RESULTS AND DISCUSSION

PART 2

(b) EXPERIMENTS:

Restriction mapping of globin genes

I needed to adapt the techniques of Southern (1975) for detecting sequences within agarose gels to the detection of single-copy genes within restriction enzyme digests of mammalian DNA. The gel system must be able to resolve enough DNA to give a hybridisation signal despite any losses that may occur during transfer of the DNA to the filter and during annealing of radioactive probe to the DNA on the nitrocellulose. Southern (1975) has discussed the detection of radioactivity on such strips of nitrocellulose and has concluded in favour of autoradiography. Using a $^{32}$P labelled radioactive probe 1000 c.p.m. in a band can easily be detected in an overnight exposure of the nitrocellulose to the film. For a sequence 500 base pairs long within a genome of $3 \times 10^9$ base pairs and hybridising with a probe of specific activity $10^8$ c.p.m./µg. then about $50 - 100$ µg. of total DNA will contain enough of the single copy sequence to give a signal of 1000 c.p.m. if the hybridisation goes to completion. $50 - 100$ µg. of mammalian DNA when restricted and applied to an agarose gel requires a gel area of 1-2 cm$^2$. if the DNA is to spread through the gel during electrophoresis with high resolution, so that quite large gels are needed to take this much DNA. Initially I used gels 2.5 cm. in diameter and 20 cm. long with a capacity of about 250 µg. of restricted mammalian DNA. With gels of this size the blotting procedure (see methods) is very inefficient so Southern developed an electrophoretic transfer
procedure which allows complete transfer of DNA even from large gels onto nitrocellulose (see methods).

**Probe excess hybridisation**

Hybridisations to nitrocellulose strips in the Southern (1975) procedure are performed in probe excess in the sense that the total hybridisation solution contains more of the sequence to be studied than there is that sequence attached to the nitrocellulose. For example in 200 μg. of rabbit DIA there will be about $10^{-4}$ μg. of globin DNA whereas $4 \times 10^{-2}$ μg. of radioactive probe would be used in the hybridisation in a typical experiment. Probe excess has the advantage that in theory all the hybridisable DNA on the strip will be hybridised with probe and therefore signal strengths will be maximised. If non-duplexed probe tails are removed then the amount of probe hybridised at any point will be proportional to the amount of complementary sequence at that point. For reasons already discussed this method also allows better resolution than does hybridisation with limiting amounts of probe.

Because in theory quantitation of hybridisation is possible with the probe excess approach if the unhybridised tails are removed from hybridised probe molecules and because RNase will do this to RNA probes while leaving filter bound DNA intact there is a clear advantage to using RNA rather than DNA probes. However probe excess means that small amounts of contaminant within the probe can give disproportionately large signals if the contaminant is homologous to a repeated sequence in the total DNA because even the contaminant can be present in high enough concentration to hybridise strongly to the repeated sequence DNA. This could
also be a problem if a pure probe contains a sequence with cross homology with repeated sequence DNA. Pure probes are thus required if there is to be much chance that the major sequence within the probe is the only sequence detected in the DNA.

**Choice of probe**

Starting from globin mRNA there are really three approaches to making high specific radioactivity probes, one is to iodinate the RNA, the second is to make labelled cDNA and the third is to make unlabelled cDNA then prepare labelled RNA from that. I chose to make cDNA then make cDNA from that with *E. coli* RNA polymerase. The main disadvantage of iodination is that mRNA is always contaminated with ribosomal RNA and the radiolabelled RNA will hybridise at least as strongly as will the globin RNA when hybridising in globin probe excess.

I have already discussed the potential advantages of using RNA rather than DNA probes and for those reasons I preferred cDNA to cDNA.

I checked that Avian Myeloblastosis Virus (AMV) reverse transcriptase copies rRNA very poorly and showed that it makes cDNA copies of rRNA $10^{-5}$ times as efficiently as it copies globin mRNA. Even this low use of the rRNA template may result from mRNA contamination of rRNA. Whatever the sequence copied in the rRNA experiment cDNA from rRNA will be $< 10^{-6}$ of the DNA when globin mRNA is copied with reverse transcriptase assuming the mRNA is 10% contaminated with rRNA. Manufacturers' (Scarle) acrylamide gels show the mRNA to be about 2% contaminated with intact rRNA.
I used globin cDNA as a template for *E. coli* RNA polymerase to make ccRNA (ccRNA, see methods) of specific activity $\geq 10^8$ c.p.m./µg. using $^{32}$P labelled ribonucleotide triphosphates. This probe should be $< 10^{-6}$ rRNA and thus when hybridised in even $10^4$ fold excess to DNA on nitrocellulose filters should give a hundred fold stronger signal from globin hybridisation than from ribosomal hybridisation because under these conditions 0.01% of the globin probe should hybridise to globin gene on the nitrocellulose and this is still 100 times more hybridised signal than there is ribosomal probe in the entire hybridisation mixture.

The ccRNA probe will be contaminated with ccRNA from mRNA other than globin. Bishop *et al* (1975) provide evidence that 90% of duck reticulocyte mRNA is globin and 10% is a population of 200-300 other mRNAs. If this data is applicable to rabbit reticulocytes then each contaminant mRNA is about 0.1% of the globin probe. When nitrocellulose bound DNA is hybridised in $< 10^3$ fold excess of globin probe the globin hybridisation should be stronger than the contaminant hybridisation.

Ken Gummerson fingerprinted ccRNA and found no evidence for any oligonucleotides other than those that could be derived from globin RNA (Gummerson unpublished).

**Hybridisation conditions:**

Flavell *et al* (1974) have studied the kinetics of hybridisation in probe excess to nitrocellulose bound DNA and found that under the sorts of conditions I wished to use (see legend to figure 3 for typical hybridisation conditions) the rate of saturation of filter bound DNA with probe was proportional to the concentration of
FIGURE 3

HYBRIDISATION OF GLOBIN ccRNA TO RESTRICTION DIGESTS OF MAMMALIAN DNA

This is a 7 day autoradiograph on Kodak BB54 X-ray film of nitrocellulose strips carrying 200 μg./strip of restricted DNA from agarose gels. The strips have been hybridised in dialysis bags with 8 strips per bag and 10 x 10^6 c.p.m. of globin ccRNA at 150 x 10^6 c.p.m./μg. in 2 x SSC 0.1% SE, with 40 μg. E. coli tRNA and 10 μg. Poly rA. Hybridisation was for 40 hours at 60°C in a total volume per dialysis bag of about 5 mls. After hybridisation I washed each set of 8 strips briefly in 20 mls. 2 x SSC 0.1% SE then in 500 mls. 2 x SSC for 30 minutes, then in 500 mls. 2 x SSC for 30 minutes all at 60°C then in 50 μg./ml. RNase A in 2 x SSC at 37°C for 30 minutes (strip 16 did not receive this RNase treatment and strip 15 only had 5 minutes). Finally all were washed in 5 litres 2 x SSC for 60 minutes at 60°C, strip 16 separate from the rest in 500 mls., then the strips were dried at 60°C under vacuum. The rabbit R1 band sizes on these strips are 13.5 Kb, 10.2 Kb, 7.7 Kb. scored against λ R1 or λ Hind 3 markers (see Figure 4).
Ethidium bromide fluorescence profiles of restricted rabbit DNA in large agarose gels after electrophoresis. The big gels contain: (1) - (4) rabbit R1, (5) Rabbit Hind 3 (6) Rabbit Hind 3/Bam. About 200 μg. of DNA per gel. The small marker gel contains 0.2 μg. of λ R1.
A microdensitometer scan of one of the rabbit R1 tracks from figure 3. The 3 major bands are clearly visible and some of the minor bands. I have marked the positions and sizes of the 3 major bands on the figure. Electrophoresis was from right to left.
probe, independent of the amount of DNA on the filter and about
10 times slower than the reaction rate of the same reaction with
both components in solution. Using this data and assuming a globin
probe concentration of 10 ng./ml. then about 140 hours of
hybridisation will be required to saturate the globin genes
on a filter when the hybridisation is performed in 2 x SSC at 65°C.
In figure 3 I show an autoradiograph of an experiment performed
in this way using 32P-rRNA as radioactive probe at 60 ng./ml. and
using nitrocellulose strips carrying 200 μg. of restricted DNA
electrophoresed in large agarose tube gels then transferred
electrophoretically to the nitrocellulose. There are several
major bands and many minor bands on each track though with this
and all such figures the bands are very difficult to see. The
original autoradiographs show the bands more clearly than do the
prints. I have marked the positions of some bands on the
photographs with pen marks at the sides. Tracks where DNA has
been digested with the same enzyme show the same pattern, whereas
those digested with different enzymes show a different pattern.
The technique is at least reproducible that far. These bands rise
above a very high background of radioactivity that seems to map
out the fluorescence profile of the DNA originally in the agarose
gels (figure 4) and this radioactivity is not very sensitive to
RNase (figure 3). Figure 5 shows a Joyce-Loebl microdensitometer
scan of one of the tracks from figure 3 and it is clear that even
the major bands are a small fraction of the hybridisation. It is
also clear that had the nitrocellulose been assayed by scintillation
counting slices rather than autoradiography the bands could not
have been seen with any certainty, the stochastic counting error being comparable to the strength of the bands above the background. Bands on the nitrocellulose are not as sharp as those on the stained gel, they spread during transfer.

**SIGNAL STRENGTH:**

The band signal strength is at least a factor of 10 lower than calculated. This could either mean that DNA is falling off the filters or that the probe is not saturating the hybridizable DNA on the filters, or that most of the DNA in the gel fails to attach to the nitrocellulose during the transfer. The probe may fail to saturate the DNA either because I did not hybridise for long enough or because, although the probe is present in excess overall, in the region of a band the hybridisation may occur in filter DNA excess if the mixing of the probe in the hybridisation is inefficient. Flavell *et al* (1974) present data that in experiments similar to mine the probe saturates the DNA on the filters and so this cannot explain the low signal strength in my experiments and either most of the DNA fails to reach the nitrocellulose during transfer or falls off the nitrocellulose during hybridisation. Although I have evidence that DNA falls off filters during hybridisation (figure 8 and 9 and relevant text) this phenomenon will only explain band signal strengths down to about 10% of the expected value. Since I sometimes observed that the band signal was 1% of the expected value I conclude that as much as 90% of DNA originally in the agarose gel fails to attach to the nitrocellulose in the Southern (1975) procedure. Botchan *et al* (1976) find that the efficiency of transfer of adenovirus or λ DNA to nitrocellulose is
dependent on the size of the DNA fragment and upon the agarose concentration in the gel. For 1.4% gels they find a range of efficiencies from 20% - 90%. My transfer efficiencies may be lower because I denature and neutralise my gels for longer and also, in some experiments, because I have used the electrophoretic transfer procedures. Jeffreys and Flavell (1977) show clearer and stronger globin probe hybridisation than I observe. The major differences between their protocol and mine is that they run their agarose gels with DNA that has been denatured prior to application to the gel. I interpret this difference between their result and mine as indicating that denaturation within agarose is inefficient either because large DNA strands fail to separate or because they reassociate while the gel is being neutralised before and during transfer. See the section headed "Nick translated plasmid probes" for further comments on saturation of nitrocellulose bound sequences.

Because bands are not appearing on transfers with the quantity of c.p.m. predicted from the amount of DNA loaded onto the original gels and because the background hybridisation to the DNA track is rather insensitive to RNAase, it is not possible to draw any quantitative conclusions from the autoradiograph. There is one other explanation for the low signal strengths I see. Globin genes might be present in a whole range of sizes of restriction fragment. It seems to me completely unnecessary to invoke this to explain my data. My reasons for saying that the DNA track background is not globin genes will appear later in the thesis in the sections on DNA Track background and on Ribosomal genes.
Since the background hybridisation was preventing me from seeing the bands clearly I tried to find conditions that would remove it. Cage and Manning (1976) found a high background when hybridising $^{125}$I silk fibroin mRNA across CsCl gradients of DNA. At 50 ng/ml RNA probe much of the mRNA hybridisation measured was hybridisation to main band DNA which did not contain the bulk of the silk fibroin DNA. These anomalous hybrids were poorly matched. They found this effect only with DNA from the same organism as the RNA probe. I examined the background I found in figure 3 by loading rabbit DNA onto one set of nitrocellulose discs and Echinus esculentus DNA onto another set. I hybridised the DNA on these filters with globin cDNA then melted the hybrids on the filters through a range of temperatures. Under the hybridisation conditions used, namely those used in figure 3, most of the hybridisation should be background and I would be unlikely to detect much true globin hybridisation so that the melting profile I see should be the melting profile of the background hybridisation. However, I have evidence that DNA is lost from filters when filters are heated through this range of temperatures (figure 8). This complicates the interpretation of the melts but because loss of DNA is likely to be the same for the rabbit and the Echinus DNAs it does not invalidate comparison of the melting profiles for the hybrids formed by globin RNA with the two types of DNA. Use of tritiated DNA would have eliminated this problem because loss of DNA from the filters could
1.25 cm. diameter nitrocellulose discs were loaded with high molecular weight DNA. Rabbit and Echinus esculentus DNAs were denatured by boiling in water then taken to about 4 x SSC and 25 µg. of one or the other type of DNA loaded onto each nitrocellulose disc in 1 ml. of solution under weak suction. The filters were washed briefly in 0.1 x SSC then the DNA was baked onto the filters by 2 hours at 80°C under vacuum. The filters were hybridised with 2 x 10⁶ c.p.m. of globin cRNA at 140 x 10⁶ c.p.m./µg. in 1 ml. of 2 x SSC 0.2% SLS at 60°C with 30 µg. of rabbit ribosomal RNA carrier. This hybridisation was done in a dialysis bag. After 7 days the filters were washed in 2 litres 2 x SSC through a range of temperatures starting at 60°C. At various temperatures the heating was held so that the solution temperature was constant for about 5 minutes then one or two filters were removed and put at 4°C and the rest were heated further. When all the filters had been removed the whole set were treated with 20 µg./ml. of RNase A in 2 x SSC at 37°C for 1 hour then washed for 1 hour in 2 litres 2 x SSC at 60°C, dried and scintillation counted. Filters with no DNA had about 40 c.p.m. when washed at 60°C or 90°C. The upper curve is the Echinus filters and the lower curve the rabbit ones.
The graph shows the change in $^{32}\text{P cpm}$ with temperature in degrees Celsius. As the temperature increases from 60°C to 100°C, the $^{32}\text{P cpm}$ decreases significantly, indicating a decrease in the activity of a particular process with increasing temperature.
then have been measured. Similar considerations apply to the experiments of figures 7 and 9.

Figure 6 shows the melting profiles for the hybrids on both sets of filters. The hybrids on both sets of filters melt with a T_m in the range 70 - 72°C in 2 x SSC, and these hybrids remain even with RNAse treatment. Hybridisation to a transfer carrying a restriction digest of Echinus DNA using exactly the same probe RNA as for figure 3 though supplemented with 120 μg. of unlabelled mouse RNA shows a background pattern that maps out the DNA profile as with rabbit DNA restriction digests in figure 3 only rather weaker. No bands are visible though. These experiments disagree with Gage and Manning in that I do see hybridisation to DNA that is from a different organism from my probe RNA. This cross-hybridisation, the low T_m of the hybrids and the T_m being the same with rabbit and Echinus DNAs suggests that I am seeing poorly matched or short hybrids with little homology to my probe molecule. The observation that the hybridisation to restriction digests marks out the original gel fluorescence profile indicates that the sequences within the DNA are probably randomly distributed through the genomes. These hybrids seem rather insensitive to RNAse (figure 3 and 6) perhaps suggesting they are short well matched hybrids rather than long poorly matched ones.

Gage and Manning found that when they hybridised their RNA to DNA on nitrocellulose filters at lower RNA concentration the hybridisation gave a much truer picture of the location of the silk fibroin genes. I hybridised a series of rabbit DNA nitrocellulose discs at a range of cRNA concentrations then washed them
Disc filters of rabbit DNA were prepared as for figure 6, prehybridised in 2 x SSC, 0.1% SLS at 60°C with 250 μg/ml of rabbit liver RNA for 30 minutes then hybridised in glass vials with various concentrations of 32P rabbit globin cRNA at 130 x 106 c.p.m./μg and with 250 μg/ml of rabbit liver RNA as carrier. After 4 days all the filters were washed briefly in 50 mls. of 2 x SSC at 50°C then in 80C mls. 2 x SSC at 65°C for 30 minutes. They were then treated with 50 μg/ml. RNase A in 2 x SSC for 30 minutes at 37°C then washed in 2 x SSC at 65°C for another 30 minutes. Filters were then Čerenkov counted in 2 x SSC, washed for 15 minutes at 75°C, counted again, washed for 15 minutes at 80°C and counted again.
successively at 65°C, 75°C and 80°C in 2 x SSC measuring them for radioactivity at each step. If the low T\textsubscript{m} globin hybridisation was strongly dependent upon the ccRNA concentration in the hybridisation and these hybrids were not formed at low ccRNA concentration whereas true hybrids were, then by melting the filters through the T\textsubscript{m} of the background I should see a much more marked percentage drop in measured c.p.m. at high ccRNA concentrations than at low. Figure 7 shows that this does not happen much if at all, and so my background hybridisation seems to be of a different type from that seen by Gage and Manning. Also, in support of this conclusion, hybridising with lower probe concentrations to transfers reduces the overall radioactivity and does not enhance any bands relative to the background in my experiments. The background seen by Gage and Manning might be silk fibroin like sequences that really do band on CsCl gradients with the main band DNA. Higher probe concentrations would increase hybridisation to these sequences particularly if these sequences only represent a small subset of the silk fibroin sequence. At low probe concentration there will be enough probe to hybridise appreciably to the silk fibroin genes but not enough of the subset of silk fibroin sequence to hybridise much to the main band. At high probe concentration there will be enough to saturate the silk fibroin genes and the main band sequences. The argument is the same in principle as the one I have already presented over contamination of globin probes.

A more sensitive way to test the differences in hybridisation fidelity of the bands in transfers relative to the background is to do the melts actually with the transfers and measure the loss
Nitrocellulose strips of rabbit DNA were prepared as for those in figure 3. They were hybridised with 20 x 10^6 c.p.m. of ccRNA at 265 x 10^6 c.p.m./µg. in about 5 mls. of 0.3 M NaCl, 0.2% SLS at 60°C. The dialysis bag also contained 2.5 mg. of rabbit liver RNA and about 10 µg. of poly rA and 30 µg. of proteinase K. Filters were hybridised for 4 days, then washed for 15 minutes in 0.3 M NaCl 0.2% SLS at 60°C then in 250 mls. 2 x SSC 0.1% SLS at 60°C (removed 10^6 Čerenkov c.p.m.) then in 1 litre 2 x SSC at 60°C for 30 minutes (removed 60,000 Čerenkov c.p.m.) then in 50 mls. RNAse A at 50 µg./ml., 2 x SSC, 37°C, (removed 5,000 c.p.m.) then in 1 litre of 2 x SSC at 65°C for 45 minutes (removed 3750 c.p.m.) then in 1 litre of 2 x SSC at 60°C for 30 minutes (removed 1000 c.p.m.). Filters were then dried under vacuum at 60°C and then autoradiographed under BB54 X-ray film for a week. After that the filters were washed in 2 x SSC at 80°C for 15 minutes, dried under vacuum and autoradiographed at -70°C with RP Royal Xomat X-ray film sensitised by flashing (Bonner and Laskey, 1974) and in contact with an Ilford fast tungstate X-ray intensifier screen. The autoradiograph was then exposed for 4 weeks. The left hand strip is rabbit Hind 3 and the right hand one rabbit Hind 3/Rfl. The major Hind 3 band sizes are 15 Kb., 13 Kb., 7.8 Kb. and the major Hind 3/Rfl band is 2 Kb. measured against λ markers.
of background relative to the bands. In figure 8 I show 2 strips that were hybridised as in figure 3, autoradiographed after being washed at 65°C, then washed again at 80°C and autoradiographed again with an X-ray intensifier screen as signal amplifier (Laskey unpublished). This procedure shows clearly both that the background and also some of the bands have been selected against strongly by washing at 80°C. The disadvantage to this procedure is that most of the hybridisation to the retained bands is lost as well. I estimate from comparing the 65°C and 80°C washes from figure 8 that 50 - 80% of the band hybridisation has been lost. This means that to do these experiments requires starting with 200 μg per gel of restricted DNA and autoradiographing the final hybridised nitrocellulose for 4 weeks with an intensifier screen in order to see the bands. This is a cumbersome and slow procedure.

I conclude from this section on the DNA track background that this background results from hybridisation of short stretches of probe molecule to short stretches of homology randomly distributed within the DNA of any organism. This phenomenon can be overcome by washing the hybridised nitrocellulose at a higher temperature. The experiments described in figures 10, 11, 14, 15, 16, 17, 18, 19, 20 all endorse these conclusions. The text associated with these figures contains comments on the implications of these later experiments for the DNA track background.
Nitrocellulose discs were loaded with 25 μg. of rabbit or Echinus esculentus DNA as for figure 6 except that I used 1.5 M NH₄Ac for loading and washed the filters in 1.5 M NH₄Ac before baking. Southern has shown that DNA binds more reliably to nitrocellulose in NH₄Ac than in 4 x SSC (unpublished). I hybridised the discs with 30 ng./ml. cRNA at 120 x 10⁶ c.p.m./μg. in 2 mls. 2 x SSC, 0.1% SLS and with 4 μg. poly rA, 10 μg. E. coli tRNA and 150 μg. rabbit liver RNA as carriers. Discs were hybridised in a glass vial under paraffin. At various times a disc or two was removed and stored at -20°C. Finally I washed all the discs in 1 litre 2 x SSC for 1 hour at 60°C, then RNAsed them (37°C, 2 x SSC, 50 μg./ml. RNase A, 30 minutes) then washed them for another hour in 2 x SSC at 60°C, and dried and scintillation counted them. Upper curve is the rabbit DNA, lower curve the Echinus.
DNA falls off transfers

I have already suggested that part of the low signal strength seen in figure 3 results from loss of DNA from the nitrocellulose during the hybridisation and washing. I did an experiment to study the kinetics of hybridisation of globin RNA to DNA bound to nitrocellulose that bears upon the loss of band hybridisation at 80°C in the experiment of figure 5 and upon the low signal strength in hybridisation generally. I made a set of small disc filters and bound 25 μg. of total rabbit DNA to each of them then hybridised the set with 30 ng./ml. ccRNA for various times. The time course of hybridisation is completely different from that predicted by solution hybridisation kinetics or the kinetics found by Flavell et al (1974) for hybridisation of RNA to DNA bound to nitrocellulose. The radioactivity bound to the discs rises to a peak within 7 hours then falls away slowly (figure 9). Hybrids once formed are likely to be stable at 65°C and RNA was not degraded at all during the hybridisation as judged by TCA precipitation so the simplest interpretation of the experiment in figure 9 is that the hybridised DNA is falling off the filters. This is in agreement with Haas et al (1972) and Ivanov and Markov (1975) who both observed severe loss of DNA from nitrocellulose filters during hybridisation. Loss of DNA can help explain why signals were so low in the experiments of figures 3 and 8, and will explain why washing transfers at 80°C removes most of the true well matched hybrids from the filters as well as removing poorly matched hybrids if high wash temperatures increase
the rate of loss of DNA. Loss of DNA during hybridisation poses the serious problem that there is little point in trying to hybridise the DNA on transfers to saturation if that takes about 140 hours and 90% of the DNA has been lost from the filters during that time. Much the same signal strength will be seen by hybridising for 20 hours and having 80 – 90% of the hybrids retained on the filter (see figure 9). Alternatively probe concentrations of 70 ng./ml. will be required to saturate DNA on the nitrocellulose within 20 hours. I think that the reason my time course of hybridisation differs from that seen by Flavell et al (1974) is simply that I hybridise for longer times giving the filter bound DNA more chance to fall off.
Globin cDNA plasmids

(a) cRNA probes from plasmids

All the experiments I have described so far have used $\alpha + \beta$ globin cRNA probe. Bernard Mach gave me some of the rabbit $\alpha$ and $\beta$ globin cDNA plasmids constructed in his laboratory (Rougeon et al. 1975; Rougeon and Mach 1977) in particular the $\beta$ globin gene containing plasmids, PCR1 $\beta$G7 and PCR1 $\beta$G19, and the $\alpha$ globin gene containing plasmid PCR1 $\alpha$G59. I shall refer to these as $\beta$G7, $\beta$G19 and $\alpha$G59. They have been prepared from double strand globin cDNA and attached to the plasmid PCR1 at the R1 site of that plasmid by 2 blocks of poly G/poly C. They have been characterised as $\alpha$ and $\beta$ rabbit globin plasmids by hybridisation (Rougeon and Mach, 1977) and by restriction mapping (Southern, unpublished). These plasmids are better probes than globin cRNA because the insert cDNA sequence is pure in the sense that it does not contain any other rabbit DNA sequences than $\alpha$ or $\beta$ globin. The plasmids are also better because using them it is possible to distinguish $\alpha$ genes from $\beta$ genes. These plasmids can also help decide whether the anomalous hybridisation seen with cRNA is just a property of cRNA, of $\alpha$ or of $\beta$ probes or of all globin probes. I made cRNA from these plasmids and hybridised the RNA to transfers of rabbit DNA restriction digests using the same hybridisation protocols as I have been using for cRNA. The gels I used were slab gels with 20 $\mu$g. of DNA/track and the DNA was transferred from the gels to the nitrocellulose electrophoretically (see methods). I used less DNA in these experiments because I had higher specific radioactivity probes at about $4 \times 10^8$ c.p.m./$\mu$g.
Filter strips carrying restricted DNA were prepared by electrophoretic transfer of DNA from slices of slab gels (see methods). These strips were washed in 0.33 M NaCl 0.1% SLS then hybridised for 60 minutes with 50 μg/ml rabbit liver RNA at 60°C in the same solution. They were then hybridised with 35 x 10^6 c.p.m. of cRNA from the Hha fragment of PCR1 β c7 at 410 x 10^6 c.p.m./μg with 40 μg E. coli tRNA, 8 μg poly rA and 200 μg rabbit liver RNA as carriers, 130 x 10^6 c.p.m. of α G59 cRNA was used for the α filters (not shown). The hybridisation was performed in a dialysis bag attached to an electric motor so that the bag was rotated throughout the hybridisation in a 5 litre beaker of 0.33 M NaCl 0.2% SLS. The bag contained 10 mls. of solution and the hybridisation was for 30 hours at 58°C. At the end of the hybridisation I washed the filters in 250 mls. 0.33 M NaCl 0.2% SLS for 30 minutes at 60°C then in 2.5 litres 0.33 M NaCl 0.2% SLS for 30 minutes at 60°C then in 2.5 litres 0.33 M NaCl for 1 hour at 63°C then in 50 mls. 0.33 M NaCl at 65°C with 50 μg/ml. RNAse A for 30 minutes then for 2 hours in 0.33 M NaCl at 65°C. Filters were dried in air overnight at room temperature.

Strips are Rabbit Hind 3, Rabbit Rl, Rabbit Rl, Rabbit Hind 3/Rl, Rabbit Hind 3/Rl, λ Rl. Strip 3 was not treated with RNAse. The prints are from overnight autoradiographs at -70°C with XH-1 film sensitised by flashing from 20 cm. with a flash gun as described by Bonner and Laskey (1974), and using a fast tungstate screen as amplifier. The track comes from a different
gel from the other tracks. The 4 most prominent large bands in track 3 size at 15 Kb., 10.5 Kb., 8.3 Kb., 7.5 Kb. (arrowed), In order to see the bands on the print I have exposed that track longer than the other tracks so that intensities are not comparable.
and X-ray intensifier screens amplifying the autoradiographic signal by a factor of about 8. For \( \alpha \) G59 I made cRNA from the entire plasmid, for \( \beta \) G7 I used an Hha I fragment 1800 base pairs long carrying the entire \( \beta \) globin insert and purified away from the rest of the plasmid by agarose gel electrophoresis. This fragment was prepared by Ed. Southern. It is theoretically more useful than the whole plasmid because cRNA from the fragment should be 25% globin whereas the whole plasmid is only 5% globin. Thus 5 times less cRNA from the fragment than from the plasmid should contain as much globin cRNA. This reduces the amount of probe RNA needed to provide any selected concentration of globin probe sequence in the hybridisation and consequently should reduce the amount of background radioactivity that binds to the nitrocellulose relative to that which hybridises to DNA. cRNA from the \( \beta \) G7 Hha I fragment did indeed prove more successful than cRNA from the entire \( \alpha \) G59. The \( \alpha \) probe gave higher nitrocellulose backgrounds than the \( \beta \). It was impossible to see any convincing bands on any of the transfers hybridised with the \( \alpha \) probe. I show some of the \( \beta \) probe gel tracks in figure 10. Some of the bands seem the same size as those with cCRNA (compare the R1 band sizes in the legends to figures 3 and 10) though it is very difficult to tell whether the bands that look the same really are the same, sizing against the phage \( \lambda \) markers (gifts from Barbara Smith) used on all gels is not accurate enough to say that the patterns are more than similar and hence in subsequent figures I have not always included the sizes of all bands. The phage \( \lambda \) track shown in figure 10 has been hybridised with cRNA from the Hha I fragment from \( \beta \) G7
Nitrocellulose strips carrying restriction digest were prepared by electrochoretic transfer of slices from slab gels. They were hybridised with either $5 \times 10^6$ c.p.m. of cRNA from the Hha fragment of $\alpha G7$ or with $15 \times 10^6$ c.p.m. of cRNA from $\alpha G59$. Both probes were at $100 \times 10^6$ c.p.m./μg and with 100 μg rabbit liver RNA, 3 μg poly rA and 100 μg E. coli tRNA as carriers. Hybridisation was at 60°C for 38 hours as for figure 10. Filters were washed as for figure 10 except that strips 2 and 3 were not treated with RNAse. Strips are rabbit Hae III (α probe) rabbit Hae III (α probe) rabbit Hae III (β probe). The prints are from 8 day autoradiographs as for figure 10. The 330 base pair band in the β track is marked on the picture. I scored bands on the autoradiograph then sized them by reference back to the fluorescence profile of the mouse satellite M2 step ladder on the gel (not shown).
THE GLOBIN INSERT IN PBR122 IS NOT TRANSCRIBED IN VIVO

I grew a 500 mls. culture of \( \beta \) G19 in E. coli HB101 to an O.D. of 1.2 in a low phosphate medium without \(^{32}\)p then added chloramphenicol to 150 \( \mu \)g./ml. (see methods). After 30 minutes at 37°C I added 3 mCi \(^{32}\)P orthophosphate and after a further 16 hours lysed the cells and separated the cell DNA, plasmid DNA and cell + plasmid RNA on CsCl/ethidium bromide gradients (see methods). I dialysed the RNA pellets from the bottoms of the gradients into water at 4°C after extracting most of the ethidium bromide with 3 isopropanol/CsCl extractions (see methods) then precipitated the RNA with ethanol. I DNAsed the RNA (90 minutes, 37°C, 100 ml. Tris pH 8, 10 mM KCl, 20 \( \mu \)g./ml. DNase I) and checked on a gel that this removed all contaminating plasmid or cell DNA. I then treated the RNA with 20 \( \mu \)g./ml. pronase + 0.03% SLS for 30 minutes at 60°C then extracted the RNA once with phenol, twice with chloroform then precipitated the RNA with ethanol from 0.3 M NaAc. I recovered about 4.5 mgs. of RNA at 50,000 c.p.m./pg. and hybridised this to a blot of various plasmids: I put the blot in a 100 ml. measuring cylinder and added the \(^{32}\)P RNA in 4 mls. of 0.33 M NaCl, 0.1% SLS and rotated the cylinder at about 10 r.p.m. on an electric motor at 60°C for 38 hours. At the end of this time I washed each half of the blot for 45 minutes in 2 litres 2 x SSC then for 1 hour in fresh 2 x SSC at 60°C then RNAsed tracks 1 - 7 at 60°C for 30 minutes with 50 \( \mu \)g./ml. RNase A, then washed all tracks for 90 minutes in 2 x SSC (2 x 2 litres) tracks 11-15 separately from 1-7. Tracks 8-10
contain $^{32}$P $\beta$ G19 DNAs and these were not hybridised at all with $^{32}$P RNA. Tracks 8 and 10 show severe nuclease degradation. They are R1 digests of $\beta$ G19 and the degradation is probably caused by a nuclease in the plasmid DNA. I have seen this type of degradation several times. Phenol extracting plasmids off CsCl gradients or centrifuging a second time on CsCl gradients reduces the severity of the contaminating nuclease. The unlabelled plasmids on this gel were centrifuged twice on CsCl/ethidium bromide gradients and show no degradation when digested with R1. The $^{32}$P $\beta$ G19 R1 globin bands are still visible on both the fluorescence picture (left) of the gel and the autoradiograph (right) of the blot. The 600 base pair dimer of the globin bands is also visible.

**TRACKS**

1. $\alpha$ G59 (R1 digest) 2. $\alpha$ G59 3. $\alpha$ G59 (R1 digest)
4. $\beta$ G19 (R1 digest) 5. $\beta$ G19 6. $\beta$ G19 (R1 digest)
7. Double strand globin cDNA 8. $^{32}$P $\beta$ G19 (R1 digest)
9. $^{32}$P $\beta$ G19 10. $^{32}$P $\beta$ G19 (R1 digest) 11. $\beta$ G19
12. $\beta$ G19 + PMB9 (R1 digest of both) 13. PMB9
14. PMB9 (R1 digest) 15. $\lambda$ Hind 3 (weak)

0.1-1 $\mu$g DNA per track.

The right hand picture is an 8 day amplified autoradiograph (figure 10) and I can see no hybridisation to the globin bands from $\beta$ G19 or the double strand cDNA. A longer exposure still shows no hybridisation to these DNAs, but on this the other bands are over-exposed.
A restriction map of \( \beta \text{G19} \) is shown in figure 13. Since \textit{in vivo} labelled RNA from an \textit{E. coli} carrying \( \beta \text{G19} \) does not hybridise to the two \( \beta \) globin fragments from \( \beta \text{G19} \) but does hybridise to both the PCR1 piece of \( \beta \text{G19} \) and to PMB9 (which contains a piece of DNA in common with PCR1) I conclude that \textit{in vivo} RNA polymerase transcribes the globin insert from \( \beta \text{G19} \) poorly if at all.
indicating how readily globin plasmid cRNA will bind to non-homologous DNA. The \( \lambda \) bands are arrowed at the right of the photograph. Jeffreys and Flavell (1977) find no hybridisation of nick translated P\( \Phi \)Cl DNA to phage \( \lambda \). P\( \Phi \)Cl contains the same piece of plasmid DNA as is present in the H\( \Phi \)a I fragment I used as probe. Thus the hybridisation I observe is probably not indicative of a long sequence homology between plasmid and phage DNAs. This hybridisation of globin plasmid RNA to non-homologous DNA supports my interpretation of the DNA track background as being hybridisation of random short stretches of chance homology between probe and nitrocellulose bound DNA. The plasmid cRNAs do both give hybridisation all the way down the DNA tracks as did cRNA indicating that this anomalous hybridisation is not caused by impurities in cRNA nor by either \( \alpha \) or \( \beta \) globin RNA alone. (See section "DNA track background").

These cRNA pictures are very poor and since the banding pattern is not obviously identical to the pictures with cRNA I tried to confirm that some globin hybridisation was visible. Maniatis et al. (1976) showed a detailed restriction map for the rabbit \( \beta \) globin plasmid they made. In particular they showed that a 330 base pair piece is excised from the \( \beta \) globin gene within the coding sequence with Hae III. I digested rabbit organismal DNA with Hae III, prepared transfers from the digest and hybridised them with cRNA from the H\( \Phi \)a I fragment from \( \Phi \)C7. If the cRNA really hybridises to \( \beta \) globin genes then a band should be visible in rabbit DNA at 330 base pairs. One is just visible (figure 11). The experiment is very difficult because 330 base pair pieces of DNA bind poorly to nitrocellulose (Southern 1975, will probably
fall off when hybridised (Haas et al, 1972) and will give a very small signal anyway because 330 base pairs is very little DNA. This is weak evidence that some of the hybridisation to transfers of rabbit organismal DNA is globin. However, many other bands are visible on these transfer tracks and some look the same size with α as with β probes suggesting that something other than globin genes is also hybridising to the probes. (See discussion under "Ribosomal genes").

(b) Preparation of 2 new globin cDNA plasmids

cRNA from α G59, β G7, β G19 and the Rha I fragments from these are poor probes. The insert is walled off from the rest of the plasmid by blocks of poly G/poly C and this may inhibit transcription of the insert by RNA polymerase, at least in vivo. RNA polymerase fails to transcribe globin inserts in β G19 at all (figure 12, and Kourilsky et al, 1977). Furthermore there is really no guarantee that any band seen in transfers hybridised with these probes is globin DNA. Ed. Southern pointed out that the 5' and 3' halves of the globin inserts in these plasmids can be used separately by cleaving the plasmids with R1 at a site in the middle of both the rabbit α and β globin genes (Salser et al, 1976). If the halves are hybridised to transfers where the restriction enzyme used to prepare the transfer is not R1 and does not cleave the genes then both halves must give the same banding pattern for globin genes (for example Hind 3). If R1 is used or R1 plus an enzyme that does not cleave the genes then different patterns can be expected with the two halves of the plasmids. If another enzyme is used that does cut the gene then one half of the plasmid will hybridise to both ends of the gene in the DNA and the other half to only one end, Bam is an example for
This should be a stringent way of showing that hybridisation is to globin DNA within rabbit organismal DNA. There are other advantages to this approach. The random hybridisation to the DNA track in transfers might be caused by one specific sequence within the globin probes, for example the poly A/poly T tail at the 3' end of the cDNAs, using the two halves separately can test this. For \( \alpha \)G59 Southern cleaved the plasmid with Hind 3 and Rl and purified the two bands by gel electrophoresis. For \( \beta \)G19 there is a neater way to purify the two ends of the globin gene. In building this plasmid Rooseon and I each were able to introduce 2 additional Rl sites between the plasmid and the poly C/poly C flanking the globin insert. I cleaved the plasmid with Rl to generate 3 fragments, one being PCR1, one the 5' 400 base pairs of the \( \beta \)globin insert and one the 3' 20C base pairs of the \( \beta \)globin insert. Polarity here refers to globin mRNA. I purified these small globin bands by agarose gel electrophoresis then cloned them in the plasmid PHB9 (figure 13 for schema). I made 2 clones carrying the 200 base pair 3' end of the \( \beta \)globin gene and 3 clones carrying the 400 base pair 5' end of the gene. These plasmids are potentially excellent probes for \( \beta \)globin genes. Not only can they help decide which bands in transfers are \( \beta \)globin genes but also they can distinguish 5' gene fragments from 3' and thus allow construction of an unambiguous restriction map around the \( \beta \)globin genes. Both plasmids contain PHB9 and poly C/poly C so that in rabbit Rl digests on transfers any bands due to PHB9 or poly C/poly C hybridisation will be the same with both plasmids. In \( \beta \)G19 the globin insert is walled off from the rest of the plasmid.
SYNTHESIS OF 2 NEW GLOBIN cDNA PLASMIDS

This is a schema for the synthesis of PMB9 plasmids carrying either the 5' or the 3' end of the β globin cDNA from rabbit.

I digested 90 μg. of PCRI β G19 to completion with Rl then purified the globin insert bands on an agarose gel (see methods). I mixed 10-20 ng. of each insert separately with 10-20 ng. of Rl cleaved PMB9 in 6 μl. of 0.2 M Tris pH 8.0, left them overnight at 0°C then took the solution to 50 mM Tris pH 7.5, 0.8 mM EDTA, 10 mM MgCl₂, 2 mM ATP, 10 mM DTT, and 0.2 μl T4 ligase, all in 50 μl. then incubated the mix for 6 hours at 12°C then for 3 days at 0°C.

I transfected each of these DNAs into HB101 as follows:

1. Grew HB101 to O.D. 1.25
2. Harvested and suspended cells in ½ original volume of 50 mM CaCl₂
3. Stood cells for 15 minutes at 0°C
4. Harvested and resuspended them in 1/10 original volume 50 mM CaCl₂
5. 0.3 ml. cells added to 0.2 ml. DNA in 10 mM Tris pH 8, 10 mM MgCl₂, 10 mM CaCl₂
6. 0°C, 25 minutes
8. Room temp. 10 min.
9. 1 ml. L broth added, shaken 37°C, 40 min.
10. 10 mls. 0.7% soft agar at 45°C added
11. Plated on 4 L broth plates containing 25 μg./ml. tetracycline.
FIGURE 13 (continued)

After growing the colonies overnight at 37°C I picked some colonies onto a master plate and also onto millipore sheets upon plates. I screened some plates simply by replica plating the original plate onto a millipore plate and growing the millipore plate colonies overnight. I then hybridised the millipore sheets with globin cDNA (2 x 10^6 c.p.m. per sheet at 120 x 10^6 c.p.m./μg) essentially according to Grunstein and Hogness (1975). In this way I screened about 10,000 colonies from 2 plates and recovered 5 globin containing colonies, 2 from the 200 fragment transfection and 3 from the 400 fragment transfection. I cloned the cells by growing small liquid cultures and replating them, screened picked colonies again according to Grunstein and Hogness (1975), grew preparative cultures, prepared DNA and purified the globin fragments from the plasmids as described in methods. I shall call these PHB9 plasmids 200 (1), 200 (2), 400 (1), 400 (2), 400 (3).
FIGURE 14

HYBRIDISATION OF cRNA OFF THE EXCISED INSERTS FROM PMB9 200 and PMB9 400 TO RABBIT DNA RESTRICTION DIGESTS

These are blots of slab gels of rabbit DNA. The left hand gels have been hybridised with cRNA from either the 200 or the 400 globin fragments and the right hand gel with cRNA off PCR1.


The blobs of radioactivity between tracks 6 and 7 and 15 and 16 are Hind 3 and R1 markers. On the original gels I marked the positions of some of these bands with India ink then on the blot marked the band positions with ball point pen ink. Finally after hybridising the blots I marked the bands with $^{32}$P ink or $^{14}$C ink. I have done this with all subsequent gels too.

Tracks 1-6 were hybridised with 200 cRNA, tracks 7-12 with 400 cRNA, 13-15 with 200 cRNA, 16-18 with 400 cRNA, 19-24 with PCR1 cRNA.

Tracks 1-12 were prehybridised for 10 hours with 65 µg./ml. rabbit liver RNA, 50 µg./ml. Poly rA, 1.3 µg./ml. poly dC at 60°C, then washed in 2 litres 2 x SSC for 30 minutes. I used 22 x $10^6$ c.p.m. of 200 cRNA, 27 x $10^6$ c.p.m. of 400 cRNA and 28 x $10^6$ c.p.m. of PCR1 cRNA all at 400 x $10^6$ c.p.m./µg. with 80 µg. E. coli RNA, 20 µg. poly rA and 5 µg. poly dC. I denatured the RNAs by taking them to 100°C for 3 minutes in water then I hybridised the probes to the blots at 62°C in:
FIGURE 14 (continued)

2.5 cm. diameter cylinders rotated at 10 r.p.m. with 15 - 20 ml.
of 0.33 M NaCl, 0.2% SLS for 20 hours. After pouring off the
radioactive solution at the end of the hybridisation I washed the
filters in 500 ml. of 2 x SSC at 50°C for 30 minutes in their
hybridisation tubes then washed them all together in 2.5 litres
of 2 x SSC for 30 minutes then in 5 litres 2 x SSC for 60 minutes.
The filters shown in the figures were not treated with RNAse.
Those that were so treated from this experiment got 50 µg./ml.
RNAse A, 37°C, 30 minutes in 2 x SSC. RNAse only changed the
quantity of radioactivity on the blot and neither enhanced nor
selected against any bands. The prehybridisation I used seemed
to make no difference to anything as judged against other filters
hybridised in this experiment but not shown in the figure.

The pictures are from 6 week amplified autoradiographs
(figure 10).
by poly G/poly C at each end. In the new plasmids (which I shall call PMB9 200 and PMB9 400) only one end is walled off so that they should be better templates for cRNA transcription.

(c) cRNA probes from the PMB9 200 and PMB9 400 inserts

The inserts in both PMB9 200 and PMB9 400 can be excised from the PMB9 with R1 and purified away from the PMB9 by agarose gel electrophoresis. After elution from the gels 10 - 50 μg quantities of pure insert DNA can readily be prepared so that very large quantities (0.5 - 1 μg.) of pure globin probe can be used in hybridisations without undue difficulty or expense. I purified the inserts from these plasmids and made cRNA from the inserts in order to get as pure a globin probe as possible at high specific activity and sensitive to RNAse. I also made cRNA from the plasmids PMB9 and PCR1 then hybridised all these RNAs to Southern blots of rabbit DNA. The result is completely unexpected. On rabbit DNA tracks where the DNA has been digested with the same enzyme but hybridised in one case with the 200 globin probe and in another case with the 40C globin probe then I often see the same bands with both probes. The pattern of these bands is different for each of the restriction enzymes used to digest the DNA (figure 14). While this is the result expected for globin bands in Hind 3 digests I see the same phenomenon in R1 and Hind 3/R1 digests where I know that bands common to both PMB9 200 and PMB9 400 cannot be globin bands because of the way in which PMB9 200 and PMB9 400 are constructed. The 3 major R1 bands seen with both PMB9 200 and PMB9 400 probes in figure 14 are the same sizes as the three major R1 bands seen with cRNA in figure 3.
The cRNAs from PMB9 and PCR1 give a set of bands that are the same sizes as the major bands in R1 and Hind 3/R1 tracks hybridised with 200 and 400 probes and the same sizes as two bands in Hind 3 digests that appear with 200 and 400 probes. I have drawn the bands that I am referring to at the right of the figure 14 photograph. None of these bands can be globin genes. They appear with non-globin probes and do not show the expected pattern of hybridisation with globin probes. All the probes used in this experiment show track background hybridisation and this supports my earlier conclusion (see section on DNA track background) that this hybridisation is just random short stretches of chance homology between probe and filter bound DNA. It also indicates that anomalous bands and track background hybridisation occur together (figure 14) and vanish together (figure 8) suggesting they are related phenomena. I shall discuss the nature of the anomalous bands later (see Ribosomal genes section). There are bands within the tracks of figure 14 that appear only with PMB9 200 namely the small R1 and Hind 3/R1 bands (arrowed on left of figure 14) and there are bands that are the same size as the 2 large bands in the Hind 3 digest in the 80°C picture from figure 8 (arrowed at left of figure 14, the large bands in the 2 left hand PMB9 200 Hind 3 tracks). These R1, Hind 3/R1 and Hind 3 bands are good candidates for globin genes.

Prehybridisation of the nitrocellulose filters with rabbit liver RNA and treatment of the filters after hybridisation with RNAse A reduced the overall levels of radioactivity but did not
change the pictures qualitatively for the filters hybridised with globin probes. These extra treatments almost eliminated hybridisation with the plasmid cRNAs. This difference between the globin and plasmid probes may just be quantitative. The globin probes hybridised more strongly anyway.

**Nitrocellulose background**

With RNA probes the binding of probe to the nitrocellulose has usually been a less serious background problem than the hybridisation of probe down the DNA track. In figure 14 the strip hybridised with the 400 probe shows quite a high overall background. The clearest influence on overall background is drying the filter out in the presence of excess radioactivity, this always sticks radioactivity to the filters and often ruins them. The background on filters that have not dried out in the presence of probe is more or less proportional to the amount of radioactive probe in the hybridising solution and it is not usually serious below $2.5 \times 10^6$ c.p.m./ml. (my data and also P. Rigby (unpublished)) though very radioactive blobs are often found on the filters regardless of the hybridisation conditions. (See section on "Nick translated plasmid probes" for further comments on nitrocellulose background). The transfers shown in figure 14 and all subsequent ones were prepared using the standard blotting procedure rather than the electrophoretic procedures (see methods). The standard blotting procedure gives better resolution than does the electrophoretic procedure.
FIGURE 1

HYBRIDISATION OF NICK TRANSLATED GLOBIN DNA PROBES TO
RESTRICTED RABBIT DNA

I nick translated PMB9, PMB9 200 (2) and PMB9 400 (1) to
50-60 x 10^6 c.p.m./µg. with 32P and hybridised 0.5 µg. of each of
these in Denhardt's solution to blots of rabbit DNA. I denatured
the probes by boiling for 10 minutes in 0.15 M NaCl, added 125 µg.
E. coli single strand sonicated DNA as carrier then hybridised
(for 20 hours) at 65°C in 2.5 cm. diameter cylinders rotated at
10 r.p.m. I pretreated the blots in Denhardt's solution at 65°C
for 10 hours before hybridising them in Denhardt's solution.
Hybridisation volumes were 15-20 ml. After hybridisation I washed
the filters separately, first in 500 ml. of 2 x SSC, 0.5% SLS at
70°C for 30 minutes then in 1 litre of 2 x SSC, 0.5% SLS at 70°C
for 1 hour then for another hour in 2 x SSC, 0.5% SLS then for
another 1.5 hours under the same conditions. After this I dried the
filters at room temperature, autoradiographed them briefly and
discovered the high background on the PMB9 400 filter, washed that
filter for 16 hours in 2 x SSC, 0.5% SLS at 70°C, baked it dry
then autoradiographed all three filters for 4 weeks under XH-1
film flashed and with a screen as signal amplifier (figure 10).
Tracks are: First block 1. Hind 3, 2. Hind 3/R1 3. R1
5. Hind 3/R1 6. R1
Third block: Same track order as the previous blocks.
Markers: Hind 3, R1, Hind 3, Hind 3, Mus R2 Satellite
bands. The first block is from a different gel from block 2 and block 3. The markers on block one are λ Hind 3 (left) λ Rl (right) and the λ Rl is next to λ Hind 3 on the left hand edge of block 2. Block 1 was hybridised with PMB9, block 2 with PMB9 200 and block 3 with PMB9 400. The position of the 2000 base pair Rl and Hind 3/Rl band in the PMB9 400 block is marked on the photo as is the position of the 900 base pair Rl and Hind 3/Rl band in the PMB9 200 block. Note also the large bands in PMB9 200 Hind 3 and perhaps in PMB9 400 Hind 3. These have about the same size as the large two Hind 3 bands in figure 8. Notice also the minor Rl, Hind 3/Rl large bands in the PMB9 200 block. These are like the major bands seen with previous probes.
(d) **Nick translated plasmid probes**

Kelly *et al* (1970) and Maniatis *et al* (1975) have described a procedure that they call nick translation that allows the radioactive labelling to high specific activity of double stranded unlabelled DNA. Denhardt (1966) developed a medium in which labelled DNA probes could be hybridised to nitrocellulose filters carrying unlabelled DNA without the radioactive DNA binding to the free nitrocellulose and without inhibiting more than 50% the hybridisation of the probe DNA to the nitrocellulose bound DNA. I labelled PMB9, PNB9 200 and PNB9 400 using this procedure (see methods) and hybridised these probes to blots of rabbit DNA in Denhardt's medium. This type of procedure was pioneered by Botchan *et al* (1976) for viral DNAs. Since Botchan *et al* got clean specific hybridisation to SV40 DNA on blots using SV40 DNA probe, and since RNase A did not remove anomalous hybrids when using RNA probes in my experiments (figure 14) one major potential advantage of RNA probes seems invalid, namely that RNA/DNA duplicates that are not globin could be destroyed with RNase. RNA still has the advantage that it can be labelled to a higher specific activity than can nick translated DNA. Indeed it is very difficult to label the plasmid DNAs to a high enough specific radioactivity for this experiment. The signal seen is very weak. I show the result in figure 15. PMB9 gives very little track hybridisation, PMB9 200 a lot more track hybridisation with two large and strong Hind 3 bands (arrowed in figure 15). These two Hind 3 bands are the same size as the large Hind 3 bands in figures 3, 8 and 14 namely 15 and 18 Kb. PNB9 200
FIGURE 16

HYBRIDISATION OF \( \alpha \) GLOBIN DNA TO RABBIT DNA RESTRICTION DIGESTS

I prewashed blots of rabbit DNA in Denhardt's solution for 11 hours at 65°C then hybridised them with 0.5 \( \mu g \) of either \( \alpha_A \) or \( \alpha_B \) at 70 x 10^6 c.p.m./\( \mu g \) with 50 \( \mu g \) of E. coli single strand sonicated DNA as carrier. Hybridisations were for 18 hours at 68°C in rotating tubes with 17 ml. total volume of Denhardt's solution. I denatured the probes in 500 ml of 2 x SSC 0.5 M SLS at 63°C for 1 hour then in 4 litres of 2 x SSC 0.5 M SLS at 65°C for 7 hours. I dried the filters at room temperature then autoradiographed them for 4 weeks with an amplifier (figure 10).

Tracks: All DNA is rabbit unless otherwise stated and derives from the liver of one rabbit.

1. \( \lambda \) Hind 3/R1 (marker track)
2. Bgl 2
3. Bgl 2
4. R1 (note bands)
5. Hind 3 (note bands)
6. Hind 3/R1 (note bands)
7. R1 (partial)
8. \( \lambda \) R1 (marker track)
9. Bgl 2
10. Bgl 2/R1
11. R1 (partial)
12. Hind 3
13. Hind 3/R1
14. R1
15. Mus R2 (Satellite bands marked)
16. \( \lambda \) Hind 3/R1 (marker track)
17. Pst
18. Pst/R1
19. R1 (partial)
20. Bam (note bands)
21. Bam/R1
22. R1 (partial)
23. \( \lambda \) R1 (marker track)
24. Pst
25. Pst/R1
26. R1 (Partial)
27. Bam (note bands)
28. Bam/R1 (note bands)
29. R1
30. Mus R2 (satellite bands marked)
Tracks 1-8 and 16-23 were hybridised with $\alpha B$, tracks 9-15 and 24-30 with $\alpha A$. The Hind 3/R1 and Hind 3 bands in tracks 5 and 6 size at $5.5 \times 10^6$ daltons (8.3 Kb.).
also gives a 900 base pair Hind 3/Rl and Rl band (arrowed in figure 15) and PMB9 400 gives a very high overall background but with a 2400 base pair Hind 3/Rl and Rl band (arrowed in figure 15). PMB9 200 also gives some very faint bands that are the same sizes as the strong marked anomalous bands in figure 14. Note that the PMB9 200 seems to give more hybridisation to the entire DNA track for each enzyme digest than does PMB9. This might just be random variation between blots though. It is clear also that this track hybridisation is a property of DNA probes as well as RNA (see discussion under DNA track background). The huge nitrocellulose background binding seen with PMB9 400 is very common when using DNA probes to DNA bound to nitrocellulose and about 50% of filters are useless as a result. On good filters the overall nitrocellulose background is lower than with RNA probes (see the section on nitrocellulose background). The band and track background signal strengths are very much lower.

I nick translated the two halves of α G59, the 5' fragment αB and the 3' fragment αA (Southern unpublished) and hybridised these to blots. The background and the signal strength are again very low (figure 16) but some bands are visible, a band at about 5.5 x 10^6 daltons in the Hind 3 and Hind 3/Rl tracks (arrowed on left of figure 16), two bands in the Rl track (arrowed on right).

The Rl bands, as measured against the λ markers on the gel, are the same size as the larger two bands seen in Rl tracks when β globin or plasmid probes are used (figures 3, 14) and it is already clear that these are not globin bands. It will be surprising if the 5.5 x 10^6 dalton Hind 3/Rl and Hind 3 band really contains an α
globin gene because rabbit α-globin genes are expected to contain an R1 site (Salser et al, 1976).

Because these experiments using DNA plasmid probes to blots gave either no signal or very weak signals and gave background binding of probe to the nitrocellulose comparable to the signal I nick translated some of the agarose gel purified 200 and 400 globin inserts and hybridised these to blots using 0.5 μg. of insert in each experiment compared to 0.5 μg. of plasmid in previous experiments. This gives about a 25 fold increase in actual globin concentration and thus a 25 fold kinetic improvement in the experiment, and this may increase the signal strength relative to the background if globin sequences in DNA are not being saturated with DNA probe. I would expect the hybridisation with nick translated plasmid plus insert to be far from saturation. Actual globin probe concentration is only about 1 ng./ml. (figures 15 and 16). In the experiments of figures 15 and 16 I see ≈ 0.1% the theoretical maximum of c.p.m. in bands under conditions where only about 20% of the DNA will have fallen off the filters (figure 9). There are two disadvantages to using the excised inserts though. With the whole plasmids as probes there is the chance of concatemer formation by solution hybridisation of plasmid fragments. This may generate a probe hybridising to the DNA bound to the nitrocellulose that is much bigger than the globin gene. Flavell et al (1974) say that this concatemer hybridisation only accounts for about 20% of filter hybridisation under similar conditions to mine, but still this is lost by nick translating globin inserts and using them as probes. The second disadvantage is that the probe molecules will self
reassociate in solution and this will probably render most of the probe unavailable for hybridisation to filter bound DNA when probe molecules as short as the globin inserts are being used. When I did the experiment the 200 fragment gave no hybridisation at all. The 400 fragment gave a band at 1900 base pairs in RI digests of DNAs from several different tissues of one rabbit that was about 3 times more intense than the band of similar size seen using the whole PMB9 400 plasmid as probe. This experiment does show that there is little or no difference between rabbit tissues in this probable 5' globin fragment out to 2000 base pairs. The background binding of probe DNA to nitrocellulose was still high relative to the signal strength with both the 200 and 400 probes. In this experiment I used the wash procedure of Botchan et al (1976) using 2 x SSC at pH 8.5. This removed the DNA background hybridisation to filter bound DNA very effectively but with blots where whole PMB9 200 or PMB9 400 plasmid or 200 insert has been used as probe this wash removes all the hybridisation as well. Even allowing for the effect of the 2 x SSC pH 8.5 wash the failure of the nick translated insert probes to improve much upon nick translated whole plasmid probes suggests that failure to saturate globin DNA on nitrocellulose is not the main reason for the low signal strengths seen. This conclusion agrees with that I drew in the section "Signal strengths", namely that inefficient transfer and loss of DNA from filters are the main reasons for low signal strengths in hybridisation.
I prewashed these blots in Denhardt's solution at 65°C for 10 hours, then hybridised them with 60 x 10^6 c.p.m. of PMB9 400 (3) cRNA at 525 x 10^6 c.p.m./µg. in 20 mls. Denhardt's solution + 0.2% SLS, at 62°C for 17.5 hours. After the hybridisation I washed the filters in 500 ml. 2 x SSC, 0.5% SLS at 58°C for 1 hour then in 4 litres 2 x SSC, 0.5% SLS at 64°C for 4.5 hours then in 4 litres 0.33 M NaCl, 0.5% SLS at 64°C for 1.5 hours. I dried the filters at room temperature overnight then autoradiographed them for 10 days with an amplifier screen (figure 10).

Tracks: DNA is rabbit
1. Rl
2. Hind 3/Rl
3. Hind 3
4. Rl
5. Hind 3/Rl
6. Hind 3
7. Hind 3/Rl
8. Rl
9. Hind 3

Tracks 1-6 contain DNA derived from pooled brain and testis of 10 rabbits, tracks 7-9 contain DNA from the liver of one rabbit. Note the extra low molecular weight Hind 3/Rl band in tracks 2 and 5 as compared to 7 (arrowed). Others are visible on the original autoradiograph.
FIGURE 18

HYBRIDISATION OF PMB9 200 AND PMB9 400 cRNA S TO BLOTS OF RABBIT RL DIGESTS

This blot had the same treatment as did those of figure 17 except that the right hand half of this one was hybridised with PMB9 200 (1) cRNA rather than PMB9 400 (3) and this filter was washed an extra 24 hours at 67°C in 2 x SSC 0.5% SLS because it had a much higher background on the first autoradiograph.

The DNA derives from a series of individual rabbits, each DNA deriving from one tissue of one rabbit.

Tracks:
1. ⌧ Hind 3 (marker)
2. Rabbit A Testis Rl
3. Rabbit 4 Liver Rl
4. Rabbit 5 Testis Rl
5. Rabbit 1 Liver Rl
6. Portent strain rabbit kidney Rl
7. Rabbit 2 Liver Rl
8. ⌧ Rl (marker)
Tracks 9-14 repeat 2-7 and track 15 is ⌧ Hind 3/Rl (marker).

All rabbits were New Zealand white as have been all rabbits used in this thesis. The 2 tracks of Portent are the only exceptions to this.

1-8 were hybridised with 400 cRNA 9-15 with 200.

Note the strong anomalous 4.5 Kb band in tracks 2 and 9 (arrowed) and a weaker counterpart in track 6. Note also the set of big bands in most 400 tracks and in tracks 11, 12, 13. These size at 13, 10, 6.5 Kb. Note also the 1.5 - 2 Kb set of bands in most 400 tracks.

The ⌧ Hind 3 bands marked are 22, 9, 6, 3.9 Kb approx.
(e) **PMB9 200 and PMB9 400 cRNA probes**

One problem that I have not found with RNA probes but have always found with DNA probes is that of getting enough radioactivity hybridised to the DNA on the nitrocellulose to see anything at all on the autoradiograph. Since both ends of the globin genes and the plasmids show track hybridisation and non globin bands that are insensitive to RNase there is no longer such an advantage to using the globin inserts as templates for cRNA synthesis as compared to using the whole plasmids with inserts. Furthermore, since cRNA from the plasmids with inserts will probably be longer than cRNA from the inserts themselves this increase in probe size might well compensate for the lower globin probe concentration that is practicable using cRNA from plasmids with inserts rather than cRNA from the inserts. For these reasons I made cRNA off PMB9 200 and PMB9 400 and hybridised these to some blots in Denhardt's solution.

I used Denhardt's solution in the hope that it would reduce RNA binding to nitrocellulose just as it reduces DNA binding relative to the binding in 3 x SSC (Denhardt (1966)). Background binding of probe to nitrocellulose is lower than in previous RNA experiments suggesting that Denhardt's solution does help and the band and track background signal strengths are higher (figure 17). The high signal strength suggests that increase in probe size does compensate for lower probe concentration. The autoradiographs show probe hybridisation marking out the DNA tracks on the blots but many bands rising above that (figure 17). The Hind 3/Rl and Rl band patterns look similar to and have bands the same size as bands seen in Hind 3/Rl and Rl digests from previous experiments. The
Hind 3 pattern looks different from the pattern seen in previous experiments. In all digestes more bands are visible than ever before and this may explain why the Hind 3 pattern looks different. Note also that the rabbit DNA digestes used in this experiment differ between the filters. The DNA used on the left hand 6 tracks was made by pooling tissues from 10 rabbits. The DNA used on the right hand 3 tracks was from the liver of an individual rabbit. There are more low molecular weight bands in the Hind 3/R1 and R1 digestes using DNA from pooled rabbits than using DNA from one rabbit (the major extra band is arrowed with a large arrow in figure 17) suggesting DNA sequence differences between individual rabbits. The resolution and signal strength in this experiment are better than in the experiments of figures 8, 14 and 15 and this may explain why I see the extra bands. I also hybridised PMB9 200 and PMB9 400 cRNAs to a blot carrying R1 digestes from several different individual rabbits (figure 18). The result is surprising in that there are clear differences in the PMB9 400 hybridisation patterns between different digestes. Many bands are the same size as those I have seen many times before but some are not and these differ between individuals. Most surprising of all is the strong extra band in the far left hand track that shows up with PMB9 400 and PMB9 200 (arrowed in figure 18). Note also the faint large PMB9 200 bands in tracks 11, 12 and 13 which correspond to strong bands in the PMB9 400 tracks (arrowed on right of figure 18) and which are the same size as the 3 major R1 bands seen in previous experiments. This is further evidence that these bands are not globin as they appear with both PMB9 200 and PMB9 400 probes.
FIGURE 19

HYBRIDISATION OF PCD4 RIBOSOMAL PROBE TO BLOTS THAT HAVE ALREADY BEEN USED FOR GLOBIN HYBRIDISATIONS

I nick translated PCD4 to $220 \times 10^6 \text{ c.p.m./} \mu\text{g.}$ and hybridised it to blots that I have already used for globin probe hybridisation. I prewashed the blots for 10 hours in Denhardt's solution then hybridised them all in a 2.5 cm. tube with $50 \times 10^6 \text{ c.p.m.}$ of PCD4 denatured by boiling. The tube contained a total of about 40 ml. of Denhardt's solution and 50 $\mu$g. of E. coli single strand sonicated carrier DNA. After 18 hours hybridisation at $68^\circ \text{C}$ I washed the filters in 500 ml. 2 x SSC 0.5% SLS at $68^\circ \text{C}$ for 1.5 hours then in 5 litres 2 x SSC 0.5% SLS at $68^\circ \text{C}$ then in another 5 litres 2 x SSC 0.5% SLS at $68^\circ \text{C}$ then finally for 10 minutes at $75^\circ \text{C}$ in the same buffer. I dried the filters at room temperature overnight then autoradiographed them with amplification for 8 days. The 2 x SSC used in this experiment was at pH 8.5 whereas previously I have used it at pH 7.0 - 7.5.

In the figure I show 6 gels. Top left is a gel from the experiment described in figure 14. It is not actually one of the ones shown in figure 14 but is essentially the same. Tracks are 1. Hind 3, 2. Hind 3, 3. Hind 3/R1, 4. Hind 3/R1, 5. R1, 6. R1, 7. Hind 3 (marker) then 8-13 repeat 1-6.

On the right of this gel are the right hand two blocks from figure 15.

Beneath these two gels are the two gels from figure 16. The left hand gel on the next photo is the gel from figure 17. On the right of this are 3 gel tracks from figure 3, tracks 5, 6, 9.
The ribosomal bands shown in Figure 19 line up perfectly with nearly all the bands of similar size in the globin hybridisations, the autoradiographs superimpose. The only exceptions to this are the Hind 3 and Hind 3/Rl bands at 8.3 Kb in figure 16. These bands look like ribosomal bands but do not line up with bands in figure 19. — Note the bands in the Rl track of rabbit DNA track 12 of the figure 16 gels rehybridised in figure 19. There is a band at the same size as the strong band in track 2 of figure 18 suggesting that this band is ribosomal.

Notice also the big blob of radioactivity on the left hand gel from the second photo and compare it with figure 17 which shows it also. Two probes are then picking out the same bit of the blot for high non specific binding. The result is not merely residual radioactivity from the first hybridisation reappearing on the second autoradiograph.
Figure 14 experiment

Figure 15 experiment

Figure 16 experiment

Figure 18 experiment

Figure 23 experiment
RNA probes are better than DNA probes:

From the experiments of figures 10, 11, 14, 15, 16, 17, 18, which compare nick translated DNA probes with cRNA probes from plasmids, I am left with the strong impression that the RNA probes are better. The major reason for this is simply that with DNA probes I was unable to get a strong enough signal. The DNA probes probably reassociate in solution and render most of the probe unavailable for hybridisation to filter bound DNA. They are always at lower specific radioactivity than the RNA probes too. It is not clear from my experiments whether excising the globin inserts to make cRNA as opposed to making cRNA from the whole plasmid is worthwhile.

Ribosomal genes

The major high molecular weight bands I have seen in all blots with all enzymes to digest the DNAs and with all probes have looked just like the rabbit ribosomal gene restriction patterns. Adrian Bird gave me some of the plasmid PCD4, a PSC101 plasmid carrying the entire Xenopus laevis ribosomal gene repeat unit inserted into PSC101 with Rl. I nick translated this and hybridised it to blots that I have already used for experiments with globin probes. I show the results in figure 19. There is very little background radioactivity or track hybridisation on these pictures. Botchan et al (1976) also found low backgrounds when they used SV40 DNA probes. This lowering of backgrounds is probably caused by the rather more stringent 2 x SSC pH 8.5 wash that I used in this experiment and that Botchan et al used in theirs. It is quite clear that a set of bands in all blots whether hybridised with RNA or DNA
HYBRIDISATION OF GLOBIN RNA TO PCD4

On the left is a picture of ethidium bromide fluorescence of a gel. In the middle is a blot of this gel that has been hybridised with cRNA from either the 400 β-globin fragment (left half of blot) or the 200 fragment (right half of blot). On the right of this blot is a picture of the same blot hybridised with nick translated PCD4. Hybridisation protocols are essentially those of figures 14 and 19.

Tracks:
1. PCR1 β G19 R1
2. PCD4 Hind 3/R1
3. PCD4 R1
4. RNA polymerase + R1/Hind 3
5. RNA polymerase + R1
6. " " + R1/Hind 3
7. " " + R1
8. 1 μg. poly dC, 10 μg. poly rA, 80 μg. E. coli tRNA, + R1
9. 0.2 μ moles UTP, CTP, GTP, ATP, 7 m moles tris, 50 m moles KCl, 2 m moles Mercaptoethanol, 2 m moles DTT, 1.5 m moles MgCl₂, R1.

Then a repeat of these tracks from 10-18.

Tracks showing hybridisation are 1, 2, 3, 8, 10, 11, 12, 17.

Where the blot has been hybridised with globin RNA most of the tracks have not been treated with RNase but the β G19 tracks have been.

The blot hybridised with globin probes was autoradiographed for 14 days with an amplifier screen. The PCD4 hybridisation was autoradiographed for 8 days with a screen.
probes is indeed the ribosomal genes (the ribosomal band sizes are shown in figure 14). This set of bands seen with globin probes on autoradiographs superimposed upon the bands seen on the autoradiographs when the same filters were rehybridised with PCD4. It is also clear that some bands are not ribosomal genes for example the small Hind 3/Rl and Rl bands and the large Hind 3 bands I see with globin probes. Most interesting of all the extra band seen on the left hand PNB9 400 and PNB9 200 tracks of figure 18 hybridises more strongly with the globin probes than with the ribosomal probe (left hand track of right hand photograph in figure 19) and yet is highly unlikely to be globin because it is in an Rl digest yet hybridises to both PNB9 200 and PNB9 400. If it is a ribosomal fragment it must be part of a spacer region or a very infrequent piece of a repeat unit because it hybridises so poorly with PCD4. One explanation for the globin probes hybridising to ribosomal genes is that something used to make probes for these experiments is contaminated with a ribosomal plasmid. I checked this by running a gel carrying all my buffers and unlabelled RNA or DNA carriers, RNA polymerase, and all my unlabelled nucleotides, and treated this gel as a normal gel for blotting. I hybridised the blot with cRNA from the 400 and 200 \( B \) globin gene fragments (figure 20). These RNAs hybridise to Rl fragments from \( B \) c19. The 200 fragment RNA hybridises to the 200 and 400 fragment DNAs (track 10) but the 400 RNA does not hybridise to the 200 DNA (track 1). They both also hybridise to the PSC101 plasmid (top) fragment from PCD4, to the ribosomal RNA sequence
fragment (bottom) and weakly to the ribosomal spacer fragment (middle) (tracks 2, 3, 11, 12). They do not hybridise to the tracks carrying buffers etc. They hybridise to poly dC as well (tracks 8, 17), suggesting that the reason the 200 fragment hybridises to the 400 is that it contains a higher proportion of poly G/poly C and the RNA polymerase transcribes a higher proportion of homopolymers from the 200 than from the 400 fragment. These globin RNAs probably hybridise to PSC101 because the globin DNA fragments are contaminated with PNB9 which shares sequence homology with PSC101. This will not explain their hybridisation to the X. laevis ribosomal fragments though and so the globin probes really do seem to hybridise to ribosomal DNA.

I rehybridised this blot with nick translated PCD4. At the specific activity of this probe, $2 \times 10^8$ c.p.m./µg., I would be able to detect about 1 pg. of contaminant ribosomal DNA in the tracks on the blot that contained buffers etc. None can be detected (figure 20). Nick translated PCD4 does not seem to hybridise to the globin fragments either. This experiment shows that none of my buffers are contaminated with ribosomal DNA and since several DNA preparations of several types have been used to make probes, some very highly purified (the β globin inserts for example) these also are highly unlikely to be contaminated. Furthermore I obtained ribosomal gene patterns with cRNA before this laboratory had any ribosomal plasmids (figures 3 and 19).

This experiment of figure 20 shows directly that cRNA from the globin plasmid inserts will indeed hybridise to ribosomal DNA at at least two places. It seems then that my radioactive
probe molecules are unlikely to be contaminated with ribosomal probe and that globin ccRNA, PCR1 or PNB9 cRNA, 5' or 3' globin cRNA, PNB9 400 and PNB9 200 cRNA, nick translated 5' and 3' α-globin DNA and nick translated PNB9 200 can all hybridise weakly to several parts of the ribosomal DNA in rabbit or Xenopus, the nick translated DNAs less so than the RNAs. There is not a lot in common between these probes let alone between the probes and the DNAs they recognise in blots. Only the plasmids carrying globin inserts and the inserts themselves have poly G/poly C sequences. Only ccRNA and the 3' ends of the globin cDNA plasmid inserts carry poly A/poly T sequences. Globin inserts excised from plasmids and purified on gels contain very little contaminating plasmid DNA yet give stronger binding to ribosomal DNA on blots than the plasmids alone. Although I have used rabbit ribosomal RNA as carrier in many, though not all, RNA experiments I have not used it in experiments with nick translated DNA where I have used sonicated single stranded E. coli DNA. Poly rA as carrier neither prevents nor causes this anomalous hybridisation and nor does poly dC. The simplest explanation is that under low stringency hybridisation conditions any probe will bind to any high G/C DNA. There is considerable evidence that stretches of RNA/DNA hybrids as short as 10 bases long are stable at 60 - 70°C in 2 x SSC particularly if they are rich in G/C base pairs (see Walker, 1969 for discussion). There is some support for this within my experiments. The only RNA experiment in which I do not see ribosomal genes is that of figure 8 after washing the filters at 80°C and these are the conditions that greatly reduce the
random track hybridisation too. At 60 - 65°C with RNA probes I get the most binding to ribosomal DNA whereas with nick translated DNA probes hybridised at 70°C and washed at 70 - 75°C I get less ribosomal hybridisation.

Filters hybridised with nick translated globin DNA then washed at pH 8.5 at 70°C show no DNA track hybridisation or ribosomal hybridisation and very few bands. These DNA hybridisation and washing conditions are probably rather more stringent than the standard RNA hybridisation conditions. This is unlikely to be a complete explanation because all probes do not always hybridise as strongly to the same ribosomal DNA bands on blots so that there must be some sequence selection between probes, some binding more strongly to one stretch of ribosomal DNA, some more strongly to another stretch. In support of my conclusion Ken Gross probably sees ribosomal genes when hybridising histone DNA plasmids to organismal DNA from several organisms including rabbit when he uses hybridisation conditions comparable to mine.

Another possible explanation for the binding of my probes to ribosomal genes is that there really is some biologically important homology between parts of the ribosomal gene repeat unit and my probes. I do not think this is very likely because I find it hard to see what PMB9, PC1, both ends of the β globin gene and perhaps the Echinus sea urchin genes are likely to have in common with several parts of the rabbit ribosomal genes. The β globin fragments are unlikely to contain biologically important promoters for RNA polymerase, nor are they both likely to carry
ribosomal RNA homology of biological importance in recognition of mRNA by ribosomal RNA because only one of the fragments carries the 5' end of the globin gene. Furthermore the probes not only recognise the R1 or Hind III/R1 fragment in the *Xenopus laevis* ribosomal repeat unit that contains the bulk of the ribosomal RNA sequences but they also recognise the fragment that contains mostly spacer sequences as well.

In R1 digests of rabbit DNA my various probes recognise 3 or 4 ribosomal fragments indicating that each probe has at least 3 recognition sites within the ribosomal repeat unit and there are not likely to be 3 biologically important homologies in widely different parts of the ribosomal repeat unit.

Whether the homology is biologically important or not, not all probes recognise the same bands of ribosomal DNA with the same frequency suggesting that either there are different recognition sites for each probe or that there are a limited number of sites with different affinities for the probes. Either of these explanations is possible but the second of the two leaves open the possibility of mapping the sites of hybridisation within the rabbit ribosomal repeat unit and in any case further mapping experiments should be able to tell how many sites there are. If there really are a few sites that all probes recognise then the sites might be biologically important. However this anomalous hybridisation is strikingly like the sorts of hybridisation found when using homopolymers to separate the strands of phage DNAs (Szybalski et al, Methods in Enzymology XXI 394) where homopolymers that are not truly complementary to the DNA form RNase
resistant hybrids with several (30–70) sites on the DNAs and this sort of random short hybridisation seems a sufficient explanation for the hybridisation of my probes to ribosomal genes.

**Globin bands**

Some bands in some blots obey the rules that must be obeyed when using PMB9 400 or PMB9 200 probes if the bands are β globin genes. From these bands I can construct a crude restriction map for rabbit globin genes. There are probably 2 genes in Hind 3 bands of 15 and 18 Kb (figures 8, 15). Nearer the genes there is an Rl site on the 5' side at about 1500–2500 bases (this may be real variation between rabbits) and on the 3' side at 900 bases (figures 8, 14, 15, 17, 18).

Jeffreys and Flavell (1977) find one β gene with Rl sites 2.6 Kb to the 5' end of the Rl site within the gene and 0.9 Kb to the 3' end. They do not detect the 5' variation that I observe though they do find extra bands that they suggest are diverged globin genes. My data is obtained using lower stringency hybridisation and wash conditions so I may be detecting even greater divergence and there may be differences in these diverged sequences between rabbits. I find 2 non-ribosomal Hind 3 fragments that hybridise to β globin probes. These fragments are bigger than any restriction fragments observed by Jeffreys and Flavell who used different restriction enzymes so that there may be 2 genes with flanking sequences that only differ at large distances from the genes. Jeffreys and Flavell mention this possibility themselves.
CONCLUSIONS: PART 1

cDNA columns

I have used the procedures of Gilham (1964, 1971), Noyes and Stark (1975) and a procedure using chloromethyl polystyrene to bind DNA to Sephadex, cellulose or polystyrene. Gilham's procedures use carbodiimide to activate the 5' phosphate group of DNA so that the DNA will bind to hydroxyl groups on cellulose or Sephadex. Noyes and Stark made diazobenzoyl cellulose and used this to bind DNA by attack of bases within single strand DNA on the diazobenzoyl group attached to the cellulose. I have found that each of these procedures will bind DNA to beads of Sephadex, cellulose or polystyrene but DNA bound by Gilham's procedures to Sephadex or cellulose or by any procedure to polystyrene is then poorly accessible to enzymes. DNA bound to cellulose by the Noyes and Stark procedure is readily accessible to enzymes and this is more likely to be a property of the cellulose than the binding procedure, the cellulose in the Noyes and Stark procedure is very finely divided so very little DNA can become trapped within the cellulose matrix. All the column materials I have used inhibit AMV reverse transcriptase, Sephadex doing this least, and all bind DNA even without chemical activation. Much of the DNA that binds to all the beads even without chemical activation behaves as though it were covalently bound in that it is tightly bound as defined by washing in buffers and formamide and, if oligo dT has been bound, serves as primer for AMV reverse transcriptase. Using the Noyes and Stark procedure I have found that much of the DNA bound to beads that have not been activated chemically can be removed from the beads.
by washing in an RNA solution and most of the DNA bound to chemically activated beads can also be washed off with RNA suggesting that very little DNA that binds to beads in the Noyes and Stark procedure is actually covalently bound. Despite this I have found all my materials bind DNA, behave as primers and hybridise to RNA in ways similar to those observed by previous authors suggesting that I have not simply failed to use the materials properly. I have not found polystyrene to have any advantages over cellulose or Sephadex. The poor enzyme accessibility of the DNA on some of these columns and the lack of stability of the attachment of the DNA to all of them indicate to me that they are not the method of choice for partial purification of rare DNA sequences from mammalian DNA.

Other approaches

Flavell has developed a better column procedure where cDNA is hybridised to DNA or RNA in solution then the hybrids are specifically removed from the hybridisation reaction by chromatography on homopolymer columns. This approach has given many thousand fold purifications of globin genes from rabbit DNA. The main advantages when compared to my approach are that very large quantities of homopolymer can be attached to the columns so that the poor accessibility of bound DNA is less serious. There is still a vast excess of accessible DNA bound to the column and the cDNA/rabbit DNA hybrids need only be passed through the homopolymer columns or hybridised to them briefly so that less of the column bound DNA is likely to fall off during hybridisation to
the cDNA/rabbit DNA hybrids. Problems are that yields of purified rabbit globin DNA are still only about 10% of theoretical values and it is not very easy to remove all the cDNA that is an inevitable and large scale contaminant of the purified globin DNA when this approach is used (Jeffreys and Flavell, unpublished). Flavell has used cDNA lengthened with a stretch of poly C so that the cDNA will bind to a poly I column. This means that the cDNA cannot be used as a primer on a genome DNA template to obtain radio-labelled sequences at the 5' end of the genes.

Now that we have cDNA plasmids there are several approaches that will probably be better than either mine or Flavell's. I mention three of these. DNA from plasmids carrying cDNA could be hybridised to genome DNA then the hybrids purified by passage through a column carrying bound DNA from the same plasmid but without the cDNA insert. This would work best with strand separated plasmid DNAs. cDNA plasmids could be cleaved within the cDNA with a restriction enzyme and the single strand pieces that carry free 3' hydroxyl groups that fall within the cDNA purified and hybridised in solution to genome DNA. These pieces of plasmids and cDNA can then serve as primers against the genome DNA to radiolabel, with DNA polymerase, sequences at the ends of genes. The hybrids can be purified either over plasmid DNA columns, over homopolymer columns exploiting the homopolymer sequences that are used to link cDNAs into plasmids, or over sulphhydryl columns if some of the DNA is mercurated. Finally Thomas et al (1976) have developed a technique called r-looping whereby RNA/DNA
hybridisation can be used to purify double strand DNA. Since double strand DNA is easier to use for cloning and hybridisation is the best way to purify DNA this technique may be the best use of hybridisation and column techniques. R-looping itself is a hybridisation technique but when mercurated RNA is used to form the r-loop the hybrids can specifically be purified over sulphhydryl columns. The disadvantages are that the technique is difficult, optimal conditions have to be worked out for every single piece of DNA you want to purify, and it may not work at all if the regions around a gene have a lower $T_m$ than the gene itself.
CONCLUSIONS PART 2

Restriction mapping of single copy genes within mammalian genomes

Although I have found very great difficulty in using the Southern (1975) transfer to look at globin genes my experiments do allow me to describe conditions where the procedure will probably work reliably. Starting from whole organismal DNA without any prior fractionation, about 200 μg of DNA will be required per gel track. RNA probes of 5 x 10^8 c.p.m. per μg. will be required and hybridisations will have to be at 65°C or above in Denhardt's medium for 20 hours with the nitrocellulose washed after hybridisation for about 10 hours at 65°C in 2 x SSC then for 30 minutes or so at 80°C or more. Nick translated DNA probes are unlikely to hybridise strongly enough but if used at all the same hybridisation and washing protocols as for RNA will suffice. Nick translated DNA probes may be less effective either because the two strands of the probe renature during the hybridisation so reducing greatly the effective probe concentration or because they are always at a lower specific activity than the RNA probes. The difference between the signal strengths with RNA and with DNA probes is so great though that differences in specific activity may not explain the difference in hybridisation, compare say figures 10 and 17 with figures 15 and 16.

To saturate globin bands on filters in under 20 hours will require about 70 ng/ml. of globin probe if the rate of saturation is really 10 fold slower for filter bound DNA than for DNA in solution (Flavell et al, 1974). It is not really practicable to
use such high probe concentrations because even with a probe that is all globin and carries no plasmid RNA 70 ng./ml. at $5 \times 10^8$
c.p.m./pg. in $35 \times 10^6$ c.p.m./ml. and such a high concentration of radioactivity will give high background binding of probe to the nitrocellulose. $10 \times 10^6$ c.p.m./ml. is probably about the limit.

Shaken sealed plastic bags or rotating cylinders can be used for hybridisations. Rotating cylinders have the advantage that smaller volumes of liquid can be used because the bulk of the liquid is only in touch with a small area of the nitrocellulose at any instant. The background streaking of radioactivity over the nitrocellulose is often very serious with both techniques and almost impossible to remove. Nitrocellulose filters can be stacked together in either hybridisation vessel, moving liquid over the filters tends to even up the background binding but has little effect on band intensity.

Another approach that will probably work is partially to purify a restriction fragment containing a gene either by preparative gel electrophoresis or r-looping (Thomas et al., 1976) then restriction map this fragment by restriction with other enzymes, gel electrophoresis and blotting. Because the DNA used for blotting is now partially purified much more gene can be loaded onto any gel slot than is possible using organismal DNA. With 50 fold purified preparative gel purified DNA about twice as much gene can be loaded onto a gel track even without re-restricting that DNA and after re-restricting 50 - 100 times as much depending on the original degree of purification. With re-restricted partially
purified DNA not only can more gene be loaded onto a gel track but also, because the DNA is now enriched for the gene, the non-specific hybridisation observed when blots are hybridised at low stringency will be much weaker relative to the hybridisation to the gene sequence. Thus low stringency hybridisation conditions can be used and this will probably increase the signal from the gene sequence even further mainly by reducing loss of DNA from the filters. Restriction mapping by comparison to the restriction map of the probe cDNA will show whether the DNA fraction really contains the gene for that cDNA or just a sequence that is similar to the cDNA sequence. Purified DNA excess hybridisation over the radiolabelled probe will probably do this too.

Jeffreys and Flavell (1977) have shown recently that if rabbit DNA is denatured before gel electrophoresis then blotted from the gel onto nitrocellulose normally and the DNA on the nitrocellulose hybridised with a globin DNA or RNA probe then the signal from globin hybridisation is larger than when the usual Southern procedure is used to make the blots. This probably indicates that denaturation within agarose is inefficient and helps explain the low efficiency of hybridisation to transfers that I have found throughout this thesis. For example, in the experiment shown in figure 3 loss of DNA from the filters during hybridisation will explain why band signal strength should be down a factor of 2-3 from the expected value but not why it is down a factor of 10 or more. The remainder of the deficit is probably
explained by DNA that renatures during transfer and is unavailable for hybridisation or DNA that never denatured in the gel. Running gels with DNA that is already denatured is clearly going to be the approach of choice for looking at single copy genes on blots of total organismal DNA though the DNA used does need to have less nicks than is permissible for the usual Southern procedure and so it may be difficult to detect large bands on blots when running the DNA denatured.

My observations of several probes hybridising not only to the entire DNA track in Southern blots but also to specific sequences within the DNA track that are not truly homologous to the probe must stand as a criticism of doing hybridisations at low stringency. It seems as though in order to detect specific hybrids within genome DNA using globin probes hybridisation must be done at fairly high stringency and the hybrids melted to within 5°C or so of the expected T_m of true hybrids. This is particularly true for experiments where probe DNA or RNA from one organism is hybridised to genome DNA from another organism at low stringency to look for related sequences. My experiments suggest that these experiments are uninterpretable without further criteria for recognising the sequences detected.
REFERENCES


Cell 8 163.
U.S.A. 72 1184.
MELLER M., WHITFIELD C., RAS K.V., RICHARDSON N. and BISHOP J.O.
MERRIFIELD R.B. (1964) Biochem. 3 1385.
1502.
OLD J., CLEGG J.B., WEATHERALL D.J., OTTOLENGHI S., COMI P., GIGLIONI
13.
POOH R., PADDOCK G.V., HEINDELL H., WHITCOME P., SALSER W., KACIAN
ROUCHEON F., KOURILSKY P., and NACH B. (1975) Nucleic acid Res. 2
2365.
SALSER W., BOCHEN S., BROWN B., ADLI F.E., FEDOROFF N., FRY K.,
HEINDELL H., PADDOCK G., POOR R., WALLACE B. and WHITCOME P.
WEATHERALL D.J. and CLEGG J.B. (1972) "The Thalassemia Syndromes" (Blackwell scientific publications).