THE CLONING OF POLYADENYLATED RIBONUCLEIC ACID SEQUENCES FROM DROSOPHILA

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DECLARATION:

I declare that this thesis has been composed by myself, and that all the work described herein is my own.

Philip Mason
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SUMMARY

It had been found previously that a major component of poly(A) containing RNA from Drosophila larvae was the 14S large mitochondrial ribosomal RNA. In order to develop a specific screening system for this RNA it was decided to isolate mitochondrial rDNA by molecular cloning. Mitochondrial DNA from Drosophila melanogaster embryos was fragmented with restriction enzymes, and the fragments cloned in bacterial plasmids. Some of the fragments were not recovered in the recombinant plasmids, however a plasmid containing the entire large mitochondrial ribosomal DNA sequence was identified. DNA from this plasmid was found to be complementary to about 30% of cDNA made against Drosophila poly(A) containing RNA.

Fat bodies from Drosophila melanogaster third instar larvae were isolated in bulk, and poly(A) containing RNA was purified from them. This was translated in a cell free system derived from rabbit reticulocytes. Alcohol dehydrogenase was shown to be a major product of translation, by immunoprecipitation with sera made against the purified protein. In addition a number of polypeptides with masses of about 80,000 daltons were seen. These have previously been identified as chains of the major proteins of larval serum.

Fat body poly(A) containing RNA was used as a template for the enzyme reverse transcriptase, and cDNA was synthesized, made double stranded and inserted into the plasmid pCM2. A number of recombinant plasmids were isolated. Thirteen of these were analysed in some detail; twelve were found to contain mitochondrial rRNA sequences and one appeared to contain a sequence complementary to the mRNA for one of the 80,000 dalton polypeptides by a hybrid arrested translation assay.

INTRODUCTION

The Control of Gene Expression in Prokaryotes

In the 1950s, after the elucidation of the structure of DNA in 1953 (Watson and Crick, 1953) with its well known implications about the mechanism of DNA replication, many biologists addressed themselves to the question of how gene expression is controlled. In 1961, in a now classical review paper, Jacob and Monod (1961) presented a model for the control of the lactose operon of *E. coli*. The essential features of this model were found, in the 1960s, to apply to gene expression in prokaryotic cells in general and the biochemical mechanisms involved were established (Reviewed by Lewin, 1974a, and Goldberger *et al.*, 1976).

Expression of bacterial operons is controlled by the interaction of proteins with DNA at sites near the beginning of transcription. For many enzymes involved in catabolism the operon is repressed in the absence of the inducer - usually the substrate or one of its early metabolic products - by a repressor molecule which binds to an operator sequence near to the starting end of the structural genes and prevents RNA polymerase from transcribing the operon. If the inducer is present it binds to the repressor protein and greatly reduces its affinity for the operator sequence. RNA polymerase can now move from its binding site, distal to the operator, through the operator and can then transcribe the structural genes. In addition transcription is sensitive to the level of cyclic AMP present in the cell. When cAMP levels are high, a condition which prevails when there is a lack of a 'good' carbon source such as glucose, it binds to a protein, the cAMP receptor protein, and the resulting complex binds at a site close to the RNA polymerase binding site of operons coding for catabolic

enzymes. The binding of this complex is required for RNA polymerase binding to operons coding for catabolic enzymes which degrade poorer carbon sources such as lactose. By this mechanism the best available carbon sources are used, since in the presence of glucose, for example, cAMP levels are low, the complex does not form, and RNA polymerase binding to the lac promoter will be repressed.

For many operons involved in anabolism transcription is modulated by a repressor molecule which binds to the operator and prevents transcription only when complexed with a corepressor molecule, often the product of the anabolic pathway. In still other cases, binding of regulatory proteins at sites near to the beginning of transcription is required to stimulate transcription.

Bacterial operons respond quickly to changes in the environment, and can be 'switched off' almost as quickly as they are 'switched on'; this is possible because bacterial mRNAs are short lived templates; that transcribed from the lac operon, for example, has a half life of about fifty seconds.

The success of this work with bacteria has encouraged attempts to understand gene expression in eukaryotes in similar detail. However, the situation in eukaryotes may be very different. The development of a single cell into a plant or animal containing a myriad of differentiated cell and tissue types is likely to require a higher order of control mechanisms. I shall consider two aspects of eukaryote molecular biology which are very different from prokaryote molecular biology and which may have a bearing on the control of gene expression in the increased complexity of eukaryote development. These are the organisation of eukaryote DNA and the transcription of DNA and processing of the RNA transcripts.

DNA Organisation in Eukaryotes

Because of the complexity of eukaryotes the techniques used by Jacob and Monod and others cannot be applied to them.

The most striking difference between the genomes of higher organisms and bacteria is that whilst bacteria contain about the right amount of DNA we expect they will need to code for a reasonable number of proteins the genomes of higher organisms consist of many times that amount. Considering only "unique" DNA sequences (see below), there is enough in the mammalian genome to code for ten times the number of proteins a mammal could reasonably be expected to produce (Lewin, 1974b).

Much of what was known of DNA organisation and gene expression in eukaryotes up until the mid-1970s came from studying the nucleic acids of higher organisms directly. A particularly powerful technique in these early studies was nucleic acid reassociation or hybridization (reviewed by Lewin, 1974b). In essence, denatured nucleic acids will form double stranded structures, in solution under standard conditions, with a rate that is proportional to the concentration of particular sequences in the nucleic acid population; the formation of double stranded nucleic acids can be easily monitored by chromatographic, biochemical or spectrophotometric methods. When nuclear DNA of higher organisms is sheared, denatured, and allowed to reassociate, at least three complexity classes are observed (Britten and Kohne, 1968). Highly repeated DNA (rapidly renaturing) consists of short repeated sequences lying in tandem arrays (Southern, 1970). Moderately repeated DNA consists of sequences repeated about 20 - 1000 times per haploid genome and unique DNA consists of sequences present once, or only a few times, per haploid genome. In contrast, if *E. coli* DNA is analysed in the same way nearly all the DNA reassociates in the

unique complexity class.

The established laws of genetics and mutagenesis are consistent with most protein encoding genes being present only once per haploid genome. This was confirmed by hybridization studies for globin (Bishop et al., 1972; Packman et al., 1972) chick ovalbumin (Sullivan et al., 1973) and silk fibroin (Suzuki et al., 1972). It would seem, therefore, that most protein coding genes are in the unique DNA complexity class.

Davidson et al. (1973) performed reassociation experiments using Xenopus laevis DNA of varying lengths with an excess of unlabelled 450 base long DNA. Reassociation was stopped when moderately repeated but not single copy DNA would have renatured. It was found that as the length of the labelled DNA increased the proportion of labelled DNA containing duplexes increased. The conclusion from this is that moderately repeated sequences are interspersed among unique sequences in the genome. The data from these and similar experiments on different organisms show that in organisms as diverse as insects and man the organisation of DNA sequences in the genome is basically the same with about half the genome being made up of short ($\sim$ 300 base pairs) lengths of moderately repeated DNA interspersed with longer ($\sim$ 1000 base pairs) lengths of single copy DNA (e.g. Graham et al., 1974; Goldberg et al., 1975).

Bishop and Freeman (1974), using labelled complementary DNA (cDNA) made by copying duck globin mRNA with the enzyme reverse transcriptase, showed that the duck globin gene is contiguous to repetitive sequences. Davidson et al. (1975) were able to isolate the single copy DNA which was interspersed with repetitive DNA in the genome of sea urchin embryos. By hybridization of this DNA with

mRNA from sea urchin embryos they showed that most of the mRNA sequences were coded for by this DNA fraction.

It seems, therefore, that structural genes in higher organisms are organised in an ordered fashion, interspersed with short, moderately repeated sequences, and that this organisation has been conserved through evolution. These observations have lead to the proposition of models of the control of gene expression in eukaryotes. According to these models (Britten and Davidson, 1969; Davidson and Britten, 1973; Georgiev et al., 1974) batteries of genes, which need not be close together in the genome, are under the control of the contiguous repetitive elements. Such batteries of genes may be those responding to a hormonal signal, or those expressed in the same tissue at a particular stage of development.

Transcription and RNA Processing

The mRNAs of bacteria are short lived molecules so that the rate of production of a particular protein depends, to a large extent, on the rate of transcription of the corresponding gene. In contrast, gene expression in eukaryotes may be modulated at a number of distinct levels; these include transcription of DNA into the primary RNA transcript, processing of this nuclear transcript, export of mRNA to the cytoplasm, and translation of the relatively stable cytoplasmic mRNA. The relative importance of these types of control in development has not been established.

Nuclear RNA is rapidly turned over within the nucleus and, until very recently, accurate measurements of its sequence content have been difficult. A small part of this nuclear RNA is processed into cytoplasmic mRNA, processing usually involving the addition of a poly(A) tail to the 3' end of the RNA (Darnell et al., 1973) and of a 5'

methylated 'cap' structure (Rottman et al., 1974). For a given cell, nuclear RNA is about ten times more complex than cytoplasmic RNA (reviewed by Lewin 1975a and b). This means that non-protein coding DNA sequences are transcribed; it may be that these non-protein coding nuclear RNAs have a regulatory function or that post-transcriptional control, selection of which RNAs are processed and exported to the cytoplasm, plays an important role. Macnaughton et al. (1974) showed that nuclear RNA molecules containing globin encoding sequences in duck red blood cells were about three times as large as cytoplasmic globin mRNA. The significance of RNA processing and rapidly turned over nuclear RNA remains unclear.

Cloning Eukaryotic Genes

In the early 1970s, techniques were developed which will enable a more detailed and accurate analysis to be made of DNA organisation and gene expression in eukaryotes. These techniques allowed the preparation of large quantities of specific fragments of eukaryotic DNA by incorporating DNA fragments into a bacterial plasmid, infecting bacterial cells with such plasmids, cloning the bacterial cells, and preparing plasmid DNA from a culture of bacterial cells derived from a single colony (Morrow et al., 1974; Wensink et al., 1974; Glover et al., 1975). If a pure mRNA is available the cloned DNA coding for that mRNA can be identified amongst a mixture of cloned DNAs by nucleic acid hybridization. Kedes et al. (1975) were able to isolate fragments of sea urchin DNA containing the histone genes in this way. Shortly afterwards it was demonstrated that DNA copies of mRNA molecules could be made double stranded, incorporated into plasmids, and cloned in the same way (Rougeon et al., 1975; Rabbitts, 1976; Maniatis et al., 1976).

Using these techniques, if fairly pure mRNA preparations are available, plasmid DNAs containing all or part of the mRNA sequence can be isolated. These can be used to pick out, by hybridization, a recombinant plasmid containing the corresponding genomic DNA sequence from a library of plasmids containing random fragments of genomic DNA (Grunstein and Hogness, 1975). Hence it is possible to isolate large (milligrams) quantities of genomic DNA which includes the structural gene coding for a particular polypeptide. Using cloned genomic DNA, questions about the structure of genes and the nature of their flanking sequences can be asked directly. Labelled probes can be prepared from the cloned DNA sequences and can be used to study the transcription and processing of one particular RNA species.

These techniques have been used to isolate structural genes from higher organisms. For technical reasons, the first genes to be studied in this way were those which code for a mRNA, or structural RNA, which is present in a cell or tissue at high concentration. These early studies revealed a new phenomenon concerning gene structure in eukaryotes. When the genomic DNA sequences were compared with the corresponding mRNA, cDNA, or tRNA sequences the two were found not to be exactly complementary but the genomic DNA contained, within the RNA coding sequence, one or more lengths of sequence which were not represented in the cytoplasmic transcript. This has been found to apply to most genes which have so far been isolated, although it should be noted that these are not "typical" genes in that they are all required to direct the synthesis of large amounts of RNA. Some genes shown to contain intervening sequences are rabbit β-globin (Jeffreys and Flavell, 1977), mouse β-globin (Tiemeier et al., 1978; Tilghman et al.,

1978), chick ovalbumin (Breathnach et al., 1977), mouse immunoglobulin light chains (Brack and Tonegawa, 1977) and yeast tRNA (Goodman et al., 1977). The presence of these intervening sequences introduces the novel possibility of controlling sequences situated within structural genes.

In some cases the transcription of these genes has been studied and it appears that the primary transcripts contain the intervening non-coding sequences which must therefore be spliced out of the RNA molecules during the production of the mature mRNA (Tilghman et al., 1978; Roop et al., 1978). This phenomenon of RNA splicing was first shown to occur in studies on animal viruses, where several different mRNA molecules have the same 5' leader sequence which maps at only one site in the genome (Berget et al., 1977; Chow et al., 1977; Klessig, 1977). The nuclear precursor to these RNAs is a long RNA molecule reminiscent of heterogenous nuclear RNA (Bachenheimer and Darnell, 1975). Mature mRNAs must arise therefore by a number of specific splicing events. Several observations point to the possible universality and importance of RNA splicing in eukaryotes (reviewed by Abelson, 1979). The sequences around the coding/intervening sequence junctions are similar in mouse globin,immunoglobulin and chick ovalbumin genes (Tilghman et al., 1978; Tonegawa et al., 1978; Catterall et al., 1978). De Robertis and Olson (1979) showed that oocyte nuclei of Xenopus would transcribe a yeast tRNA gene which contained an intervening sequence and that the RNA trascript was processed accurately.

The Use of Drosophila melanogaster in Molecular Biology.

A lot of effort is currently being put into investigating the molecular biology of the dipteran Drosophila melanogaster. This species

is particularly suitable for this type of study for a variety of reasons. Its genome size is small, an order of magnitude or so simpler than that of a mammal and its life cycle is short, taking only two weeks to develop from a fertilized egg into a flying imago. Perhaps its main advantage is that there is already a wealth of genetical and biochemical information available on the organism since it has been a major laboratory animal for over 50 years. Another advantage of Drosophila is that, once DNA sequences are isolated, their distribution in the genome is easily determined by applying the technique of *in situ* hybridization to the giant salivary gland chromosomes (Pardue *et al.*, 1973; Wensink *et al.*, 1974).

The interspersion pattern of repetitive DNA in Drosophila is somewhat different from that found in most other eukaryotes in that repetitive sequences of lengths 0.5 - 13Kb are interspersed with single copy sequences of lengths greater than 13Kb (Manning *et al.*, 1975; Crain *et al.*, 1976). Apart from this difference Drosophila possesses all the features of typical eukaryote molecular biology.

The giant salivary gland chromosomes of Drosophila larvae are organised as a series of bands which can be visualized in the light microscope. Since the discovery of this organization the bands have been thought to represent individual genes. Judd *et al.*, (1972) studied a small region of the X-chromosome using both genetical and cytological techniques and found that for this region one functional group could be identified for each band plus interband present. However, a typical band plus interband represents enough non-repetitive DNA to code for about twenty average sized polypeptides. The complexity of Drosophila nuclear RNA (Turner and Laird, 1973; Levy *et al.*,

1976) requires that a substantial amount of this 'non-coding' DNA be transcribed. The functional significance of this intergenic DNA, and its transcripts, may be clarified by examination of the structure and expression of specific Drosophila genes using the molecular cloning techniques discussed above.

Drosophila genes which have been studied by these techniques so far include histone genes (Lifton et al., 1978), ribosomal RNA genes (White and Hogness, 1977; Glover and Hogness, 1977; Wellauer and Dawid, 1977; Pellegrini et al., 1977) and the group of genes induced in Drosophila tissue culture cells and living tissues by "heat shock" (reviewed by Ashburner and Bonner, 1979).

The heat shock loci are particularly interesting in that they comprise a set of genes for proteins whose expression appears to be under co-ordinate control. After a brief heat shock ($37^{o}C$) is administered to Drosophila tissue culture cells or living animals, nine loci are activated, and can be seen to 'puff' in salivary gland polytene chromosomes (Ritossa, 1962; Ashburner, 1970). The heat shock induces the transcription of a new set of mRNAs and the appearance of eight new polypeptides whilst the synthesis of mRNAs and proteins made prior to heat shock is depressed (Tissières et al., 1974; McKenzie et al., 1975; Spradling et al., 1977; Moran et al., 1978). Most of the heat shock genes have now been isolated by molecular cloning (Schedl et al., 1978; Lis et al., 1978; A. Tissières, personal communication). Two of the loci, 87A and 87C have so far been studied in detail. Both these loci code for the most abundant, 70,000 dalton heat shock protein (Ish-Horowicz et al., 1977; Moran et al., 1979; Artavanis-Tsakonas et al., 1979). The 87A locus appears to

code for only the mRNA for this protein but transcription at 87C involves the production of other RNAs of unknown function which probably do not code for protein and may be restricted to the nucleus (Henikoff and Meselson, 1977; Livak et al., 1978; Lis et al., 1978). It is tempting to speculate that these RNAs have a controlling function. Further study of the heat shock system may reveal fundamental aspects of gene organisation and the control of expression. However, the physiological significance of the heat shock response is not known and it is certainly not a typical example of gene action; this set of genes may be specially adapted to respond to stress and may not behave like developmentally regulated genes.

The genome of Drosophila contains several families of dispersed, repeated genes (Rubin et al., 1976; Finnegan et al., 1978; Potter et al., 1979). These families of repeated genes appear to code for abundant cytoplasmic polyadenylated RNAs of unknown function and are flanked by short direct repeated sequences. Potter et al. (1979) and Strobel et al. (1979) showed that these sequences were mobile within the genome and underwent significant rearrangements in fly populations or in tissue culture cell lines. The significance of these observations is not clear; there are, however, some indications that similar phenomena exist in other eukaryotes. Repeated elements with the same properties have been found in yeast (Cameron et al., 1977) and movable elements have been shown to occur in the genomes of Xenopus and other animals (Perlman et al., 1976; Phillips, 1979). In addition, transposable genetic elements in maize (McClintock, 1956) and Drosophila (Green, 1977) have been demonstrated by purely genetical studies.

The Aims of This Work

As a first step in trying to answer some of the questions and problems posed above, I decided to study a set of Drosophila genes which are expressed, in the normal life cycle of Drosophila, in a single tissue and at a particular stage of development. I chose the third instar larval fat body because it can be isolated in bulk and is therefore amenable to biochemical analysis.

The long term aims of this project, beyond the time scale of the present work, will be to isolate a set of genes which are expressed, at various levels, in third instar larval fat body. The organisation of these tissue specific genes in the genome can then be studied by *in situ* hybridization, and the sequences flanking, or inserted into the structural genes could be compared for any homology, or for the presence of repeated gene families, thus testing models of developmental regulation of the Britten and Davidson type. Such cloned genes will also be interesting in themselves to examine the organization of Drosophila structural genes and to compare this organization with that found for other genes isolated elsewhere, e.g. heat shock genes, in order to build up a picture of a "typical" Drosophila gene. Probes can be prepared from in and around the isolated structural genes and used to analyse the sequence content of nuclear RNA molecules from a known region of the genome which has a known function.

The Fat Body of Drosophila Third Instar Larvae.

The fat bodies of third instar larvae of Drosophila are in the form of long whitish sheets of tissue which run along most of the length of the animal. They are associated with the salivary gland and the genital discs and play an active role in intermediary metabolism, storage of reserves, and synthesis of serum proteins. At the

end of larval life the fat bodies are histolysed and imaginal fat bodies develop anew within the pupa. Third instar larval fat bodies grow by increasing their cell size, not their cell number and their nuclei contain polytene chromosomes which can be visualized in the same way as those in larval salivary glands. As well as the possibility of studying tissue specific gene expression the application of molecular cloning techniques to fat body mRNAs may lead to the isolation of the sequences of the mRNAs, and ultimately genes, of several interesting proteins known to be synthesized in larval fat body. These are alcohol dehydrogenase and the larval serum proteins.

Alcohol Dehydrogenase

The enzyme, alcohol dehydrogenase, of Drosophila melanogaster, has been well studied biochemically, the entire amino acid sequence is known (D. Thatcher, personal communication) and the locus and its surrounding loci have been the subject of intensive genetic analysis in the laboratories of W. Sofer, M. Ashburner and B. Clarke. A number of different electrophoretic forms exist (Grell et al., 1965) an observation that has made ADH one of the favourite battlegrounds in the neutralist v. selectionist argument. ADH is synthesized in fairly large amounts, accounting for about 2% of newly synthesized protein, in the active feeding stages of Drosophila - the larvae and the imago (Dunn et al., 1968; W. Sofer, personal communication). Selection systems for ADH null mutants and ADH positive flies have been developed (O'Donnell et al., 1975; Vigue and Sofer, 1976) and many mutations in and around the structural gene have been isolated (Schwartz and Sofer, 1976; O'Donnell et al., 1977; Woodruff and

Ashburner, 1979a,b). This surfeit of biochemical and genetical information makes the locus an ideal one for molecular biological studies. Should any"regulatory" mutations be turned up, their changes at the DNA level will be of great interest.

Most of the ADH activity in third instar larvae is present in fat body or in tissues associated with fat body (Ursprung et al., 1970). It is likely, therefore, that ADH mRNA is a reasonably abundant, say, greater than 2%, component of larval fat body mRNA.

The Larval Serum Proteins

The major proteins of Calliphora and Drosophila third instar larval serum are synthesized in the fat body (Munn et al., 1969; Sekeris and Scheller, 1977; Sekeris et al., 1977; Kemp et al., 1978; Roberts et al., 1977; D. Roberts, personal communication). The synthesis of these proteins is specific to the larval stage and they may function as storage proteins or precursors of the adult cuticle. They are further discussed in the Results section entitled "Larval Serum Proteins Are a Major Product of Fat Body RNA Translation".

Scheme of Work

The first part of the "Results" section describes the cloning of Drosophila mitochondrial ribosomal DNA. The rationale behind this work is explained in the introduction to that section. The next step was to analyse the variety of proteins encoded by third instar larval fat body mRNA by cell free translation and to identify ADH and the larval serum proteins amongst the translation products. The next step was to construct a library of cDNA sequences, cloned into bacterial plasmids and representing, hopefully, the mRNA sequences present

in the tissue. These cloned cDNAs could then be used as specific probes to identify cloned genomic DNA segments which carried genes expressed in third instar larval fat body. This thesis describes these cell free translation studies and the construction and preliminary analysis of a cDNA clone library.

ABBREVIATIONS

DNA	deoxyribonucleic acid
rDNA	ribosomal DNA
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
poly(A)	polyadenylic acid
poly(A) RNA	polyadenylated RNA
DNAase	deoxyribonuclease
pol.1	DNA polymerase 1 from E. coli
reverse transcriptase	RNA dependent DNA polymerase from avian myeloblastosis virus.
S1	Single strand specific nuclease from Aspergillus oryzae.
terminal transferase	terminal deoxynucleotidyl transferase from calf thymus.
cAMP	adenosine 3'5' cyclic monophosphoric acid
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
TTP	thymidine 5'-triphosphate
ATP	adenosine 5'-triphosphate
Amp	ampicillin
Cam	chloramphenicol
Tet	tetracycline
Tet^S	sensitive
Tet^R	resistant
ADH	alcohol dehydrogenase
bis-acrylamide	N,N'-methylenebisacrylamide
BSA	bovine serum albumin
cpm	counts per minute
d	daltons
Md	megadaltons (10^6d)
DEAE	diethylaminoethyl

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-amino ethyl ether) N,N'-tetraacetic acid.
eIF2	eukaryotic initiation factor 2
GFC	glass fibre filter
HAP	hydroxylapatite
K	10^3 revolutions per minute
LSP	larval serum proteins
MDL	mRNA dependent lysate
NP40	nonidet-P40
PBS	phosphate buffered saline
PPO	2-5-diphenyloxozole
POPOP	1-4-bis-2-(4-methyl-5-phenyloxozole)-benzene
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Trishydroxymethylaminomethane
SDS	sodium dodecyl sulphate
Kb	10^3 base pairs.
ds DNA	double stranded DNA.

MATERIALS AND METHODS

Culture of Drosophila

Flies of the Drosophila melanogaster strain, Oregon R, were reared in population cages at 25°C. Each cage contained about 30g of imagos. The flies were fed daily on yeast paste containing 1% propionic acid which was spread on 3% agar in "Ribena Medium". To keep the population going eggs and embryos were brushed off the agar trays each day in 0.7% NaCl, collected on nylon mesh, dechorionated by immersion in 1.2% sodium hypochlorite for 3 minutes, washed well with sterile 0.7% NaCl and pipetted onto culture medium in small milk bottles. After growth at 25°C for 12 - 14 days flies from the bottles were transferred to a new cage.

For rearing larvae for RNA extraction the washed, dechorionated embryos were pipetted onto autoclaved yeast paste in plastic ventilated boxes, and grown at 25°C. After 4 days most of the animals in the box were 3rd instar larvae and these were used for extraction of RNA from whole larvae, or from their fat bodies.

To obtain embryos for DNA extraction or for mitochondrial DNA preparation a food tray was removed from the cage after 20 hours and the embryos brushed off after another hour.

The timing of the harvesting of embryos and larvae was based on the following schedule of development at 25°C (from Bodenstein, 1950).

	Hours	Development
	0	Laying of egg
	0 - 22	Embryonic development
1st instar larva	22	Hatching
2nd instar larva	47	1st moult
3rd instar larva	70	2nd moult
Pupa	118 - 123	Formation of pupa
Imago	214	Emergence of imago

RNA Preparations

When working with RNA all glassware was sterilized by baking at 200°C for 12 hours, all solutions and other apparatus were sterilized by autoclaving or by treatment with diethylpyrocarbonate.

(a) Preparation of Cytoplasmic RNA from 3rd Instar Larvae

This method is based on that of Kirby (1965) and Parish and Kirby (1966). 3rd instar larvae were harvested by washing them off the autoclaved yeast with tap water and collecting them in a tea strainer. They were washed well with sterile water and transferred to a glass dounce. They were then homogenised in 5 - 10 times their volume of ice cold lysis medium (30mM Tris (pH 7.5), 0.1M NaCl, 10mM $MgCl_2$, 25 µg/ml polyvinyl sulphate, 25 µg/ml spermine) on ice using a loose fitting motor driven teflon pestle. Triton X-100 was added to 0.5% and the mixture homogenised again briefly. The homogenate was filtered through 200 mesh nylon gauze and centrifuged for 5' at 10K at 0°C in the Sorval HB4 rotor to pellet nuclei. The supernatant was then mixed with an equal volume of ice cold Kirby salts (1% sodium triisopropylnaphthalenesulphonate, 6% sodium-4-amino-salicylate, 1% NaCl, 6% butan-1-ol, 0.5% SDS). An equal volume of Kirby phenol

(550ml water saturated phenol : 70g m-cresol : 0.5g 8-hydroxyquinoline) was added and the mixture shaken for 10 minutes at room temperature. The phases were separated by centrifugation for 5 minutes at 10K. The phenol phase was re-extracted with a half volume of sterile water and the pooled aqueous phases were re-extracted with a half volume of Kirby phenol. Two volumes of ethanol : m-cresol 9 : 1 were added to the final aqueous phase and this was left at $4^{o}C$ overnight to precipitate RNA. The precipitate was collected by centrifugation (15' 12K), re-suspended in sterile water, adjusted to 0.2M sodium acetate (pH 5) and RNA precipitated by the addition of 2.5 volumes of 95% ethanol. After standing overnight at $-20^{o}C$ the precipitate was collected again and the ethanol precipitation repeated.

Oligo-dT cellulose chromatography

The dried pellets of RNA were dissolved in 2 - 5ml of binding buffer (0.4M NaCl, 10mM Tris (pH 7.5), 1mM EDTA, 0.1% SDS) and passed slowly through a small (500mg) column of Oligo-dT cellulose.(Aviv and Leder, 1972). The flow-through solution was passed through the column twice more. The column was then washed by passing binding buffer through it until the absorbance of the flow-through solution was less than 0.03 at a wavelength of 260nm. RNA bound to the column under these conditions was eluted with 10mM Tris (pH 7.5) 1mM EDTA, 0.1% SDS. Fractions (usually the first 1ml fraction) containing significant amounts of RNA were made 0.4M in NaCl and the binding and eluting was repeated. The bound fractions from the second elution were pooled, made 0.2M in CH_3COONa (pH 5) and RNA precipitated by the addition of 2.5 volumes of ethanol and stored at $-20^{o}C$ for at least 12 hours.

Pellets of Poly(A) containing RNA were washed with 3M CH_3COONa (pH 6) exactly as described by Palmiter (1973).

(b) Preparation of Total RNA from 3rd Instar Larvae

This method is a modification of that described by Strohman et al. (1977) and is based on that of Cox (1968).

Larvae were collected and washed as above. About 10g of larvae were weighed into a flask and sufficient freshly made 7M guanidinium chloride at -20°C was added to make the final solution 6M. (7M guanidinium chloride was prepared each day by neutralizing solid guanidinium carbonate with concentrated HCl, decolourizing with activated charcoal and filtering through Whatmann No.1 filter paper (Cox, 1968)). CH_3COOK (pH 5) was added to 0.1M. The mixture was homogenized well in a glass dounce with a tight fitting motor driven teflon pestle with intermittent cooling in an ice:ethanol bath. The homogenate was filtered through 200-mesh nylon gauze and debris were removed by 2 successive centrifugations (10' at 5K). A half volume of 95% ethanol at -20°C was added to the final supernatant. This was left at -20°C overnight.

RNA was reprecipitated, extracted with chloroform:butanol, precipitated from 3M CH_3COONa (pH 6) and then reprecipitated with ethanol exactly as described by Strohman et al. (1977).

Poly(A) containing RNA was purified from total RNA by oligo-dT-cellulose chromatography as described above except that the eluate from the first cycle of binding was heated to 70°C for 2 minutes before NaCl was added to 0.4M for the second cycle of binding.

(c) Preparation of Polysomal RNA from Drosophila Tissue Culture Cells

Cells of Schneider Line 3 were incubated at 25°C, resuspended, and collected by centrifugation. The cells were lysed and the polysomes obtained from the cleared lysates by sucrose density gradient centrifugation exactly as described by McKenzie et al. (1975). The fractions containing polysomes were pooled, made 15mM in EDTA (pH 7.0) and RNA extracted by the Kirby phenol procedure described above.

(d) Bulk Isolation of Fat Bodies and Preparation of Fat Body RNA

3rd instar larvae were collected and washed as before. About 50g of larvae were loaded into a metal tissue grinder (Gallenkamp) and ground into a plastic beaker sitting on ice. An equal volume of ice cold Drosophila Ringers Solution (0.75% NaCl, 0.035% KCl, 0.021% $CaCl_2$, 5mM Tris (pH 7.5) (Ephrussi and Beadle, 1936))was added and the mixture swirled briefly and decanted into 50ml polypropylene centrifuge tubes. After a brief centrifugation (2', 3K at 0°C), a thick layer of fat containing tissue had formed on the top of the homogenate. This was withdrawn using a siliconised 10ml pipette with a wide orifice and pipetted straight into the phenol/lysis mixture of Kemp et al. (1978). RNA was purified by phenol and chloroform: isoamyl alcohol extractions as described by Kemp et al. (1978). Poly(A) containing RNA was purified using oligo-dT cellulose as described.

(e) Preparation of Rabbit Globin mRNA

Globin mRNA was prepared from rabbit reticulocyte polysomes which were purified by a modification of the method described by Labrie (1969).

Rabbits were made anaemic and reticulocytes prepared from them and washed as described below under "Preparation of the Reticulocyte

Lysate". Reticulocytes were lysed by rapidly mixing them with twice their volume of ice cold 2mM $MgCl_2$, 1mM DTT. Cell debris were removed by centrifugation.(15', 7K, 0°C). The supernatant was layered on top of 5ml of 40% sucrose in 10mM KCl, 1.5mM $MgCl_2$, 10mM Tris (pH 7.4) 10mM DTT in 25ml tubes and centrifugation carried out for 3 hours at 28K and 2°C in the MSE 8 x 25 rotor. The polysome pellet was resuspended in 2.5 ml of 50mM Tris (pH 7.5), 30mM KCl, made 0.5% in SDS and 400µg/ml in proteinase K. After 2 hours at room temperature the solution was made 0.5M in NaCl and loaded onto a column of oligo-dT cellulose. Oligo-dT cellulose chromatography was carried out as described above except that the binding buffer was 0.5% SDS and the eluting buffer contained no SDS. The bound RNA was precipitated with ethanol. The 9S globin mRNA was further purified by sucrose density gradient centrifugation.

(f) Preparation of Guinea Pig Liver Transfer RNA

Livers from two guinea pigs were pooled, cut into small pieces and homogenized in 2ml per gram of ice cold 0.25M sucrose, 125mM KCl, 10mM NaCl, 10mM Tris (pH 7.5), 0.5mM spermidine, 1mM $MgCl_2$, 0.5mM DTT, in a Dounce homogeniser with a tight fitting motor driven teflon pestle. The homogenate was centrifuged for 15' at 15K and 2°C. The supernatant was decanted off and centrifuged for 2 hours at 40K in a Beckmann Angle Type 40 rotor at 2°C. The supernatant was decanted and extracted with an equal volume of water saturated phenol. The phenol phase was re-extracted with a small amount of buffer and then the pooled aqueous phase was re-extracted twice with an equal volume of fresh phenol. The final aqueous phase was precipitated with ethanol and stood at -20°C overnight. The RNA was pelleted, dried, dissolved in water to a final concentration of 10mg/ml and stored at -20°C.

Scintillation Counting

The radioactivity in macromolecules bound to glass fibre or nitrocellulose discs was measured by immersing the discs in PPO/POPOP toluene counting fluid and counting in a Packard scintillation counter. Liquid samples were counted in NE260 (Nuclear Enterprise). P^{32}-labelled nucleic acids in solution were counted directly.

Sucrose Density Gradient Centrifugation

(a) For Analysis and Purification of RNA

RNA was dissolved at about 200µg/ml in 5mM Tris (pH 7.5), 100mM NaCl, 1mM EDTA, 0.5% SDS. 0.5ml or less was layered on top of a 13ml 15% - 30% sucrose gradient in the same buffer. Centrifugation was for 16 hours at 25K and 25°C in a Beckmann SW 40 rotor. The gradients were analysed and fractionated by pumping them through an ISCO continuous flow cell absorbance monitor. For preparation of 9S globin mRNA and for most other gradients the RNA solution was heated at 70°C for 3' and cooled before loading. RNA was recovered from gradient fractions by ethanol precipitation.

(b) For Size Fractionation of Double Stranded DNA

DNA was dissolved in 5mM Tris (pH 7.5), 5mM EDTA, 50mM NaCl and loaded onto a 13ml gradient of 5% - 20% sucrose in the same buffer. Centrifugation was for 7½ hours at 35K in the Beckmann SW 40 rotor at 20°C. The gradient was fractionated by pumping out ½ml fractions from the bottom and counting the radioactivity in each fraction.

Binding RNA to Nitrocellulose Filters

Cytoplasmic poly(A) containing RNA from third instar larvae was bound to "Millipore" filters essentially as described by Brawerman et al. (1972).

RNA was dissolved at about 20µg/ml in 0.5M KCl, 1mM $MgCl_2$, 10mM Tris (pH 7.6). The solution was passed slowly, under gravity, through a Millipore filter which had been presoaked overnight and washed thoroughly in the same buffer. The RNA in the flow-through fraction was precipitated with ethanol. The RNA bound to the filter was eluted by shaking the filter for 1 hour in a few mls of water. The solution was adjusted to 0.2M CH_3COONa (pH 6) and RNA precipitated with ethanol. Both RNA fractions were analysed by sucrose density gradient centrifugation.

Preparation of Mitochondrial DNA

DNA from highly purified mitochondria was prepared by a modification of the methods of Bultmann and Laird (1973) and Klukas and Dawid (1976).

Embryos were harvested, dechorionated and washed as before. Highly purified mitochondria were obtained after homogenization, filtration, preparation of a crude mitochondrial pellet, and centrifugation of the resuspended pellet on a sucrose step gradient exactly as described by Bultmann and Laird (1973). The pellet of pure mitochondria was resuspended in 8ml of ice cold 0.15M NaCl, 0.1M EDTA, 0.1M Tris (pH 8.5) and 2ml of 10% SDS were added. The resulting lysate was slowly warmed to room temperature and then gently homogenised in a Dounce homogeniser with a loose fitting glass pestle. The solution was centrifuged at 30K for 30' in a Beckmann SW 50 rotor at 25°C. The supernatant was made 0.1% in ethidium bromide and 1M in CsCl, chilled by standing on ice and then centrifuged for 10' at 10K and 2°C in the Sorvall centrifuge. The volume of the supernatant was then measured and ethidium bromide was added again to 0.1%. Enough

CsCl was added to make the final density of the solution 1.55 g/ml, taking into account the caesium added before and that removed as caesium dodecyl sulphate in the last centrifugation. The solution was centrifuged for 60 hours at 36K and 25°C in the Beckmann A 40 rotor after which the tubes were viewed in ultraviolet light. The lower of the two fluorescent bands was collected, diluted with 0.1% ethidium bromide and the solution brought to a final density of 1.55 g/ml by addition of CsCl. The centrifugation was repeated. The lower band from this second isopycnic centrifugation step was collected and ethidium bromide removed by shaking 3 or 4 times with an equal volume of CsCl-saturated-water-saturated-isopropanol, whereby the ethidium bromide is removed into the upper organic phase. The aqueous phase was dialysed for 48 hours against 3 x 1 litre of 1mM Tris (pH 7.5) and the mitochondrial DNA concentrated by freeze drying.

Preparation of Embryonic Nuclear DNA

Embryos were harvested and dechorionated as described above. They were washed well with 0.7% NaCl, 0.01% Triton X-100. They were then homogenized in 2 volumes of ice cold 0.2M sucrose, 30mM Tris (pH 7.5), 10mM EDTA, 2.5mM $CaCl_2$ and the homogenate filtered through 200 mesh nylon gauze. The filtrate was centrifuged at 10K for 5 minutes and the pellet resuspended in 1 volume of 0.1M NaCl, 30mM Tris (pH 7.5), 10mM EDTA. Proteinase K was added to 400µg/ml and SDS was added to 1%. The mixture was incubated for 1 hour at 37°C. An equal volume of Kirby salts was then added and the solutions mixed. The solution was then extracted with an equal volume of Kirby phenol by gentle shaking for 1 hour at room temperature. The aqueous phase and the interphase from the first extraction were re-extracted with Kirby phenol. The two phenol phases and the interphase from the second

extraction were extracted for half an hour with a half volume of Kirby salts. The aqueous phases were pooled and extracted twice with chloroform. The final aqueous phase was dialysed overnight against 10mM Tris (pH 7.5) and then brought to a density of 1.55 g/ml by addition of solid CsCl and made 0.1% in ethidium bromide. After centrifugation for 3 days at 35K in a Beckmann A 40 rotor the fluorescent DNA band was collected and the DNA repurified by another CsCl-ethidium bromide density gradient centrifugation step. After collecting the DNA from this gradient ethidium bromide was removed with iso-propanol and CsCl removed by dialysis as before. The DNA was concentrated by precipitation with ethanol.

The Preparation and Use of Restriction Endonucleases

The restriction endonuclease BamI from Bacillus amyloliqufaciens H was prepared by a modification of the method of Wilson and Young (1975) kindly provided by D. Ward and K. Murray. About 24g of cells (grown by S. Hughes) were suspended in twice their volume of 10mM Tris (pH 7.5) 10mM β-mercaptoethanol and sonicated with one minute bursts until the absorbance of the solution at 280nm reached a maximum. The solution was then centrifuged for 2 hours at 35K and 4°C in the M.S.E. 8 x 50 rotor. The supernatant was then made 1M in NaCl and applied to a large (300ml) column of Biogel A 0.5M equilibrated with 1M NaCl, 10mM Tris (pH 7.5), 10mM β-mercaptoethanol. The column was run in the cold room at a flow rate of 20ml/hr. The fractions containing most of the protein (A_{260}/A_{280} ratio 1.5-1.8) were pooled and dialysed against 10mM K PO_4 (pH 7.4), 5mM β-mercaptoethanol, 0.1mM EDTA, 10% glycerol. Further purification by chromatography on DEAE cellulose and phosphocellulose was exactly as described by Wilson and Young (1975).

Other restriction endonucleases were obtained as follows. Eco.RI from J. Bishop and J. Davies. HindIII from J. Bishop. HaeIII from J. Bower. PstI from J. Jenkins. XbaI and KpnI from Miles Research Ltd.

For restriction of DNA the reaction volume was usually 10 - 50 µl and contained DNA at about 100µg/ml, 10mM Tris (pH 7.5), 10mM $MgCl_2$, 10mM β-mercaptoethanol and 100mM NaCl and sufficient restriction enzyme to give complete digestion. Incubation was at 37°C for 1 - 3 hours after which the reaction was stopped by adding EDTA to 20mM or by heating at 70°C for 3 minutes. The buffer for XbaI was 0.1M NaCl, 6mM Tris (pH 7.5), 6mM $MgCl_2$ whilst that for KpnI was 6mM each of NaCl, Tris (pH 7.5), $MgCl_2$ and β-mercaptoethanol.

Agarose gel electrophoresis

(a) Agarose was dissolved in tris borate buffer (90mM Tris, 90mM boric acid, 3mM EDTA (pH 8.3)) to final concentrations of 0.5% - 2% by boiling under reflux for 15'. After cooling to 55°C slab gels 16 x 0.8 cm were cast. 0.2 - 1µg of DNA in a solution containing 3.5% Ficoll and 0.005% bromophenol blue were loaded per slot. Electrophoresis was carried out for about 18 hours at 30V at room temperature after which time the slab was stained by immersion for 1 hour in tris borate buffer containing 1µg/ml ethidium bromide. Bands were visualized under ultraviolet light and photographed with a Polaroid camera fitted with a red filter.

For rapid analysis of DNA samples small tube gels (10 x 0.5cm) were cast in tris borate buffer containing 5µg/ml ethidium bromide. These were run for 2 hours at 50V after which the DNA bands could be visualized immediately under U.V. light.

The molecular weight of DNA fragments was estimated by comparison with Eco.RI digested λDNA or BamI and Eco.RI digested pCM2 DNA run on the same gel.

(b) For determining the size of plasmids in crude bacterial lysates a modification of the method of Barnes (1977) was used. A loopful of bacteria from a single colony was resuspended in 100µl of tris borate and 20µl of 5% SDS, 100mM EDTA were added. The mixture was vortexed, incubated at 70°C for 10' and then vortexed again, made 3.5% in Ficoll and 0.005% in bromophenol blue and 100µl were loaded into a gel slot and electrophoresis and staining carried out as before.

(c) For purifying a single restriction fragment by agarose gel electrophoresis a modification of the method described by Tabak and Flavell (1978) was used. 20µg of pMD417 DNA, mixed with a trace of pMD417 DNA which had been labelled to a very high specific activity with P^{32} by nick translation, was digested to completion with Eco.RI and electrophoresed in two wide (2cm) slots in a 0.8% slab gel as described. After electrophoresis the gel was stained and the part of the gel containing the two 0.5×10^6 dalton DNA bands was cut out and placed between two electrodes in a plastic sandwich box in such a way that the direction of electrophoresis would be at right angles to that of the first electrophoresis. The electrode compartments were then sealed off with fresh agarose gel and filled with buffer. Cubes of agarose were cut out of the gels immediately to the anodal side of the two bands and these were filled with a suspension of hydroxylapatite in tris borate. Electrophoresis was now performed for about 30 minutes at 50V during which time the fluorescent bands could be seen moving into the HAP filled wells. The HAP was then withdrawn from the wells with a spatula and resuspended in 1ml of 0.4M sodium phosphate (pH 7.0). The suspension was poured into a small water-jacketed column which was maintained at 70°C and was left for 15 minutes with occasional resuspensions. The buffer was then drained off and the elution repeated

with 1ml of fresh sodium phosphate buffer. The eluted fractions were counted by Cerenkov counting and the peak fractions (the first two) were pooled and applied to a large (30ml) Sephadex G50 column equilibrated with column buffer (0.3M NaCl, 50mM Tris pH 7.5). 1ml fractions were collected, counted, and the peak fractions pooled. The DNA was concentrated by ethanol precipitation. The resulting purified DNA fragment proved to be an excellent substrate for nick translation without any further treatment.

The Plasmid Vectors

The plasmid cloning vectors used in this study were pBR322 (Bolivar et al., 1977) and pCM2 (Bishop, 1979). Maps of these plasmids, showing their important restriction enzyme target sites and their antibiotic resistance determinants are shown in Fig. 1.

Production of Recombinant DNA Molecules

(a) By Ligation

Plasmid DNA, linearized by treatment with HindIII, was mixed with HindIII generated fragments of mitochondrial DNA in a 5 - 10 times molar excess of mitochondrial DNA. The plasmid DNA was at a final concentration of about 0.5pmoles/ml. The final volume of the reaction mixture was 50µl and it contained 66mM Tris (pH 7.6), 10mM $MgCl_2$, 1mM EDTA, 40mM NaCl, 10mM DTT, 125µg/ml BSA, 0.1mM ATP and 0.015U of T4 DNA ligase. Incubation was for 2 hours at 10°C and the reaction was stopped by the addition of 0.5ml of ice cold TMC (10mM Tris (pH 7.5), 10mM $MgCl_2$, 10mM $CaCl_2$).

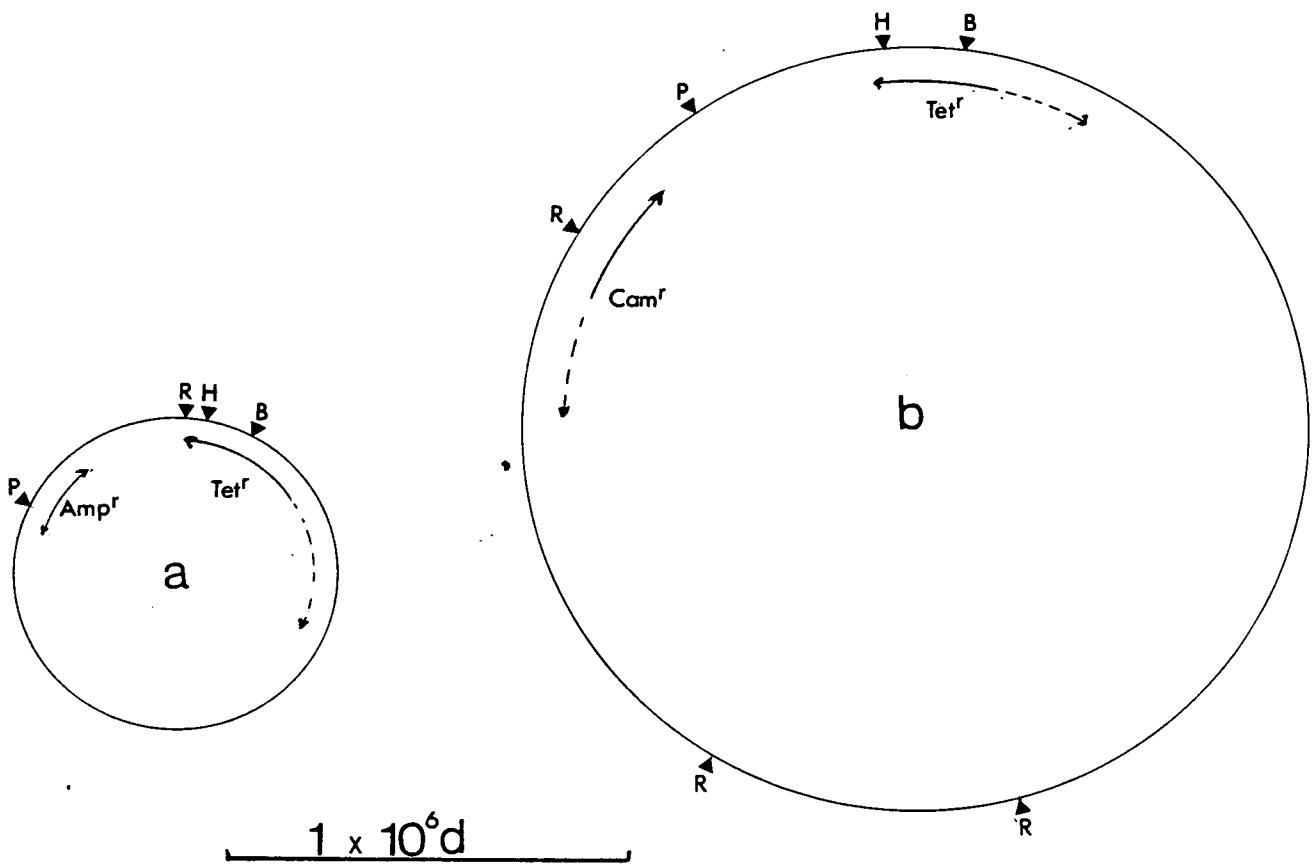


Figure 1.

Maps of the two plasmid cloning vectors used in this study, showing the important restriction enzyme target sites and antibiotic resistant determinants. (a) is pBR322 (Bolivar et al., 1977). (b) is pCM2 (Bishop, 1979). R = EcoR1, P = PstI, H = HindIII, B = BamH1. Amp = ampicillin. Tet = Tetracycline, Cam = chloramphenicol, r = resistance determinant.

pBR322 is 2.6×10^6 daltons.

pCM2 is 6.3×10^6 daltons.

(b) By annealing of Complementary Polynucleotide tails

Plasmid DNA which had been linearized by restriction and extended with a polynucleotide tail by the use of terminal transferase was mixed in approximately equal molar proportions with mitochondrial DNA fragments, or with *in vitro* synthesised double-stranded DNA, which had been tailed with the complementary nucleotide (Lobban and Kaiser, 1973). The final concentration of plasmid DNA was about 0.1 pmole/ml. The final volume of the annealing mixture was 50µl and it contained 100mM NaCl, 10mM Tris (pH 8.3) and 1mM EDTA. The solution was heated at 65°C for 10 minutes, cooled slowly to 45°C, maintained at 45°C for 2 hours and cooled slowly to room temperature, whereupon 0.2mls of ice cold TMC were added.

Transfection

Transformation of *E. coli* cells with plasmid DNA was performed by a modification of the method of Mandel and Higa (1970). *E. coli* cells of strain Hb101 were grown in 25 ml of L-broth at 37°C to an optical density of 0.5 at 650nm. After cooling on ice for 15 minutes the cells were collected by centrifugation (10K, 8 minutes) and the pellet resuspended in 12.5ml of 0.5M $CaCl_2$. After 15 minutes on ice the cells were collected again and resuspended in 2.5ml of 0.5M $CaCl_2$. 0.1ml of this solution of "competent" cells was then mixed with 0.05ml of recombinant plasmid DNA solution prepared as described above. The mixture was left on ice for 15 minutes and then heated at 37°C for 2 minutes before 1ml of L-broth was added and incubation continued at 37°C for 30 minutes. Finally, 2.5ml of BBL top agar, containing the appropriate antibiotic to select for transformed cells, was added and the mixture poured onto LB agar plates containing antibiotic. The

antibiotic concentrations were: chloramphenicol 50µg/ml in LB bottom, 25µg/ml in BBL top, tetracycline 25µg/ml and 12.5µg/ml and ampicillin 100µg/ml and 80µg/ml. The plates were incubated at 37°C for 36 hours. Screening for possible recombinant plasmids was carried out by serially plating single colonies from the transfection plates onto 2 LB plates containing different antibiotics.

Preparation of Plasmid DNA

Plasmid DNA was prepared from bacteria essentially as described by Wensink et al. (1974).

25ml of L-broth containing the appropriate selecting antibiotic (tetracycline at 25µg/ml or chloramphenicol at 50µg/ml or ampicillin at 100µg/ml) was inoculated with bacteria from a single colony. This was shaken overnight at 37°C. The culture was then decanted into 200ml L-broth supplemented with the antibiotic and 1% glucose. This was shaken at 37°C until the optical density at 650nm was 1 after which an appropriate blocking agent was added (100µg/ml Cm for Cm^S cells, 100µg/ml Tet for $Cam^r Tet^S$ cells, 200µg/ml rifampicin for Tet^r Cam^r cells) and shaking continued at 37°C for 16 hours to permit amplification of the plasmids. The bacteria were then collected by centrifugation, washed by resuspension in 30ml of 10mM Tris (pH 7.4) 1mM EDTA and collected again. The pellet was resuspended with a glass Dounce homogeniser in 4ml of ice cold 25% sucrose, 50mM Tris (pH 8.1), 40mM EDTA. 1.2ml of 10mg/ml lysozyme in the same sucrose solution was added and the mixture stood on ice for 5 minutes with occasional swirling. 1.2ml of 0.5M EDTA(pH 8.1) were then added. After a further 5 minutes on ice 10.8ml of 0.1% Triton X-100, 62.5mM EDTA, 50mM Tris (pH 8.1) was added and the mixture shaken and left on ice for 10

minutes or until lysis appeared to be complete. The solution was centrifuged for 50 minutes at 25K and 2°C in the M.S.E. 6 x 16 swing out rotor to remove bacterial DNA. The supernatant was made 1.55g/ml by the addition of solid CsCl and 0.1% in ethidium bromide and centrifugation carried out for 3 days at 35K in a Beckmann A40 rotor. The tubes were viewed under U.V. light and the lower band, containing covalently closed circular plasmid DNA was collected and the DNA repurified by another CsCl-ethidium bromide centrifugation. Ethidium bromide and CsCl were then removed as before. Plasmid DNA was dissolved in 10mM Tris (pH 7.5) and stored at -20°C.

Handling of Recombinant DNA

All procedures involving bacteria harbouring eukaryotic-prokaryotic recombinant DNA were carried out in a C2 containment facility observing the precautions recommended by the Genetic Manipulation Advisory Group.

Preparation and Use of a Cell Free Translation System from Rabbit Reticulocytes

(a) Preparation of the Reticulocyte Lysate

The method used is essentially that of Hunt and Jackson (1973). 5-7 lb rabbits were made anaemic by subcutaneous injections of 1.25% acetylphenylhydrazine on 4 successive days. The rabbits were given 3ml each on day 1, 2.5ml on days 2 and 3, and 2ml on day 4. On day 8 the rabbits were injected in their ear vein with 2ml Nembutal and 2,000 units of Heparin. When the injection had taken effect blood was withdrawn by cardiac puncture into a large syringe containing 20ml of NKM (0.13M NaCl, 7.5mM $MgCl_2$, 5mM KCl) containing 0.1% heparin. The blood was kept on ice until all blood had been collected. It was

then filtered through 5 or 6 layers of new muslin and centrifuged at 6K for 10 minutes. The supernatant was poured off and the red cells were mixed well with ice cold NKM and the centrifugation repeated. The cells were washed twice more with NKM. The volume of the washed red cells was measured using a blunt ended 10ml pipette and 1.5 volumes of ice cold water were added to lyse the cells and the mixture quickly shaken. The lysate was centrifuged at 30,000g (18K in Sorvall GSA rotor) for 15 minutes and the supernatant divided into 1ml aliquots and stored in liquid nitrogen.

At this stage the protein synthesising capacity of the lysate was checked. 1ml of lysate was thawed, and 5µl of creatine kinase (5mg/ml in 50% glycerol) were added. An aliquot of this was removed (-haem) and the rest was made 20µM in haemin (+haem). Both aliquots were then made 0.1M KCl, 0.5mM $MgCl_2$, 10mM creatine phosphate, 50µM in all 20 amino acids and 50µCi/ml in a mixture of 5 tritiated amino acid (>100 Ci/mmole). The final volume of the incubation mixture was 25µl. Incubation was carried out at 30°C and samples were withdrawn at time intervals and decolourized and the radioactivity counted exactly as described by Pelham and Jackson (1976). Figure 2 shows one such experiment. In this experiment the radioactive label is incorporated into proteins made by translation of endogenous mRNAs, chiefly globin mRNA. The absence of added haemin does not affect the initial rate of protein synthesis but after 5 minutes the rate decreases and protein synthesis has almost stopped by 10 minutes. This phenomenon is well documented (for Review see Revel and Groner, 1978) and is due to the accumulation of a translational repressor which phosphorylates the initiation factor eIF2 thus preventing reinitiation of translation.

The extent of variation between batches of lysate was considerable, especially with respect to the linearity of amino acid incorporation. The example shown in Figure 2 is the most linear seen. Other batches

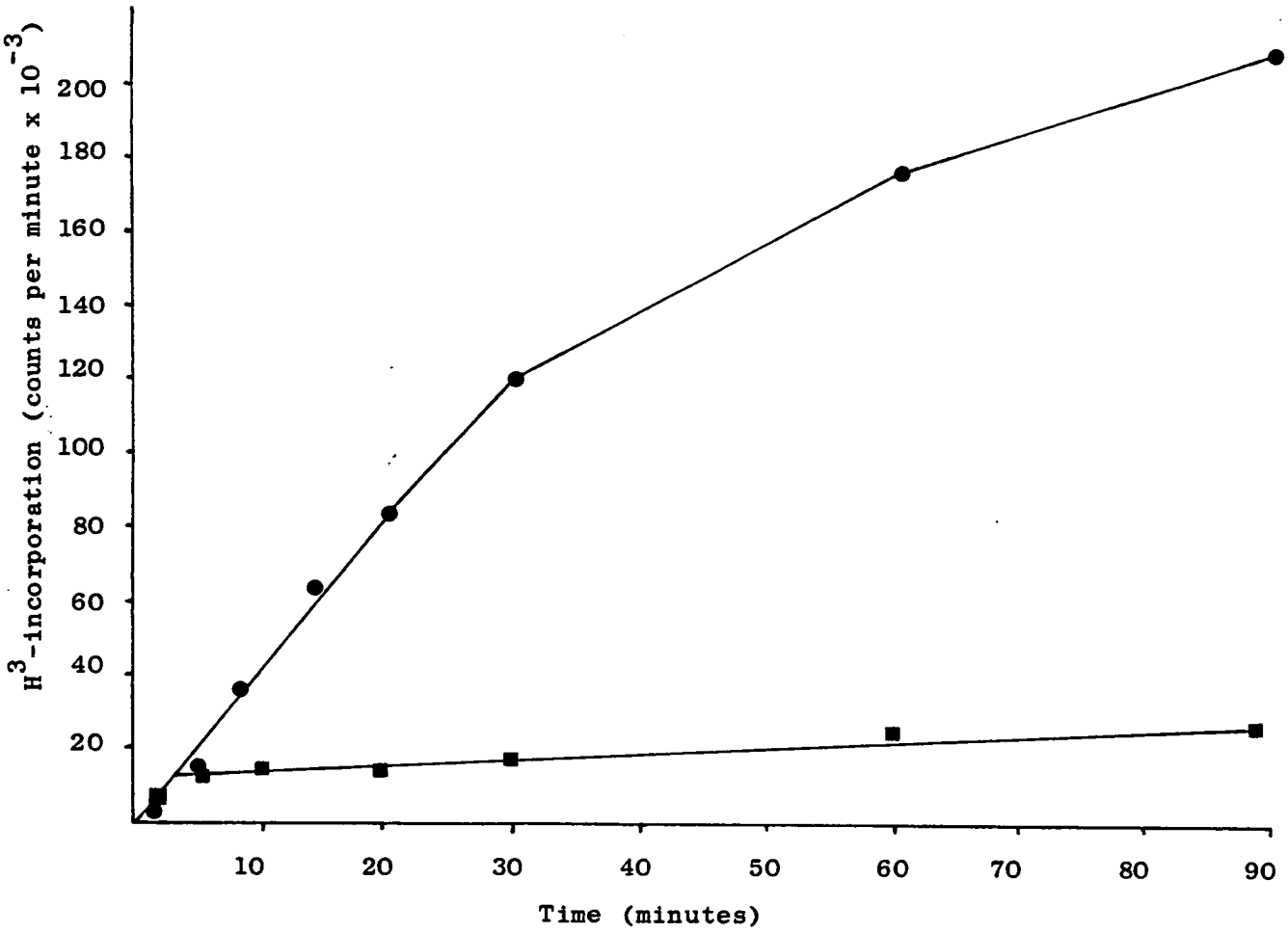


Figure 2.

Endogenous protein synthesis activity of the reticulocyte lysate. Reticulocyte lysates were prepared as described. A sample of the lysate was made 50µg/ml in creatine kinase, 20mM Tris (pH 8.2), 0.1M KCl, 10mM creatine phosphate, 0.5mM $MgCl_2$, 50µM each of 20 amino acids and 50µCi/ml of high specific activity H^3-amino acid mixture. Two 50µl aliquots were taken one was supplemented with 20µM haemin.● Both were incubated at 30°C. Amino acid incorporation was monitored by taking samples at intervals and measuring TCA precipitable radioactivity as described. The graph shows the incorporation of radioactivity into the 50µl samples of lysate.

showed considerable slowing down after 45 minutes. When incubation was continued for longer than 2 hours, incorporation into protein stopped and sometimes decreased.

(b) Preparing a mRNA Dependent Lysate (MDL)

The lysate was treated with micrococcal nuclease in the presence of $CaCl_2$ and the calcium ions chelated with EGTA exactly as described by Pelham and Jackson (1976). Amino acids were not added at this stage but were added to MDL aliquots just before use.

In order to reduce endogenous protein synthesis as much as possible the nuclease was first assayed by monitoring its ability to reduce the template activity of the endogenous message. An aliquot of lysate, supplemented with haem, salts, amino acids, etc. as above was made 1mM in $CaCl_2$ and 20 - 40µg/ml in micrococcal nuclease (Boehringer). The mixture was incubated at 20°C and aliquots removed at intervals, made 2mM in EGTA and incubated at 30°C in the presence of 50µCi/ml of tritiated amino acids. Radioactivity incorporated was measured after 30 minutes. Figure 3 shows the results of such an experiment. The conditions shown to be optimum here, 40µg/ml nuclease for 15' at 20°C were generally found to be satisfactory. Incubation for a shorter time at 25°C or for a longer time at 20°C with less nuclease both gave a lysate with less protein synthesising activity. The nuclease was assayed in this way each time a batch of lysate was to be nuclease treated. The activity of the nuclease, as measured by this assay, fell by less than a half in 2 years when dissolved in water and stored at -20°C.

Batches of about 6ml of lysate at a time were converted to MDL. To check the response of the MDL to added mRNA I incubated an aliquot of MDL with 20µg/ml rabbit globin mRNA and 50µCi/ml tritiated amino acids as above. Figure 4 shows the results of such an experiment.

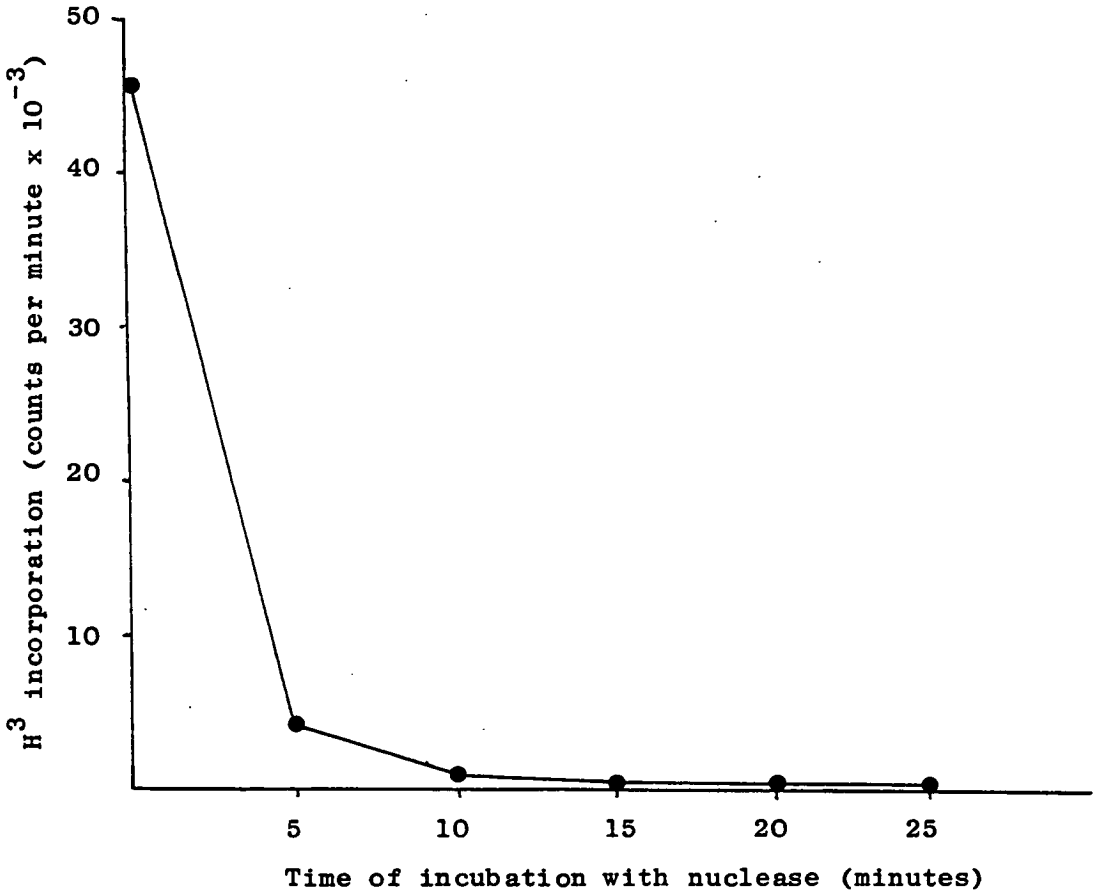


Figure 3.

Assay of micrococcal nuclease activity. Reticulocyte lysates were supplemented with haemin and all constituents of the final protein synthesis mixture except the radioactive amino acid. 1ml of lysate was made 1mM in $CaCl_2$ and 40µg/ml in micrococcal nuclease. This mixture was incubated at 20°C. At time intervals as shown, samples were removed, made 2mM in EGTA and, in this experiment, 40µCi/ml in H^3-leucine. The samples were incubated at 30°C and the radioactivity incorporated after 30' was measured.
N.B. When leucine was the labelled amino acid, the concentration of cold amino acids was 50µM except for leucine, which was 30µM.

Consistently 65 - 75% of the activity in the reticulocyte lysate was present after nuclease treatment and addition of globin mRNA. The activity of the MDL without added mRNA was reduced to less than 0.5% of its level before nuclease treatment.

(c) The Use of the MDL

MDL prepared as above was stored in 150µl aliquots in liquid nitrogen. For routine checking of mRNA preparations an aliquot was removed, made 50µM in all 20 amino acids and 50µCi/ml in a tritiated amino acid mixture or tritiated leucine (in which case the cold leucine concentration was 20µM). For translation of Drosophila mRNA the MDL was made 7mM in cAMP. To 25µl of this 1 - 2µl of Drosophila mRNA solution (1mg/ml) was added and incorporation of radioactivity monitored as above. For analysis of translation products by acrylamide gel electrophoresis the MDL was made up in the same way but the amino acid to be used as the radioactive label was omitted. 50 - 100 µCi of S^{35}-methionine (>1000Ci/mmol), H^3-leucine (>100Ci/mmol), or S^{35}-cysteine (~500Ci/mmol) were dried down and 0.5µl of Drosophila mRNA (1mg/ml) and 10µl of MDL were added. Incubation was for 45 minutes at 30°C after which 1 - 2µl of the MDL was subjected to electrophoresis as described below.

Hybrid Arrested Translation

Plasmids containing putative Drosophila cDNA sequences were restricted with HaeIII. The restricted DNA solution was made up to a final volume of 150µl with 0.1M NaCl and extracted with 75µl water saturated phenol for 10 minutes at 37°C, and then with 75µl of chloroform. The aqueous phase was extracted with 150µl of chloroform. The phenol/chloroform and chloroform phases were back extracted with 100µl

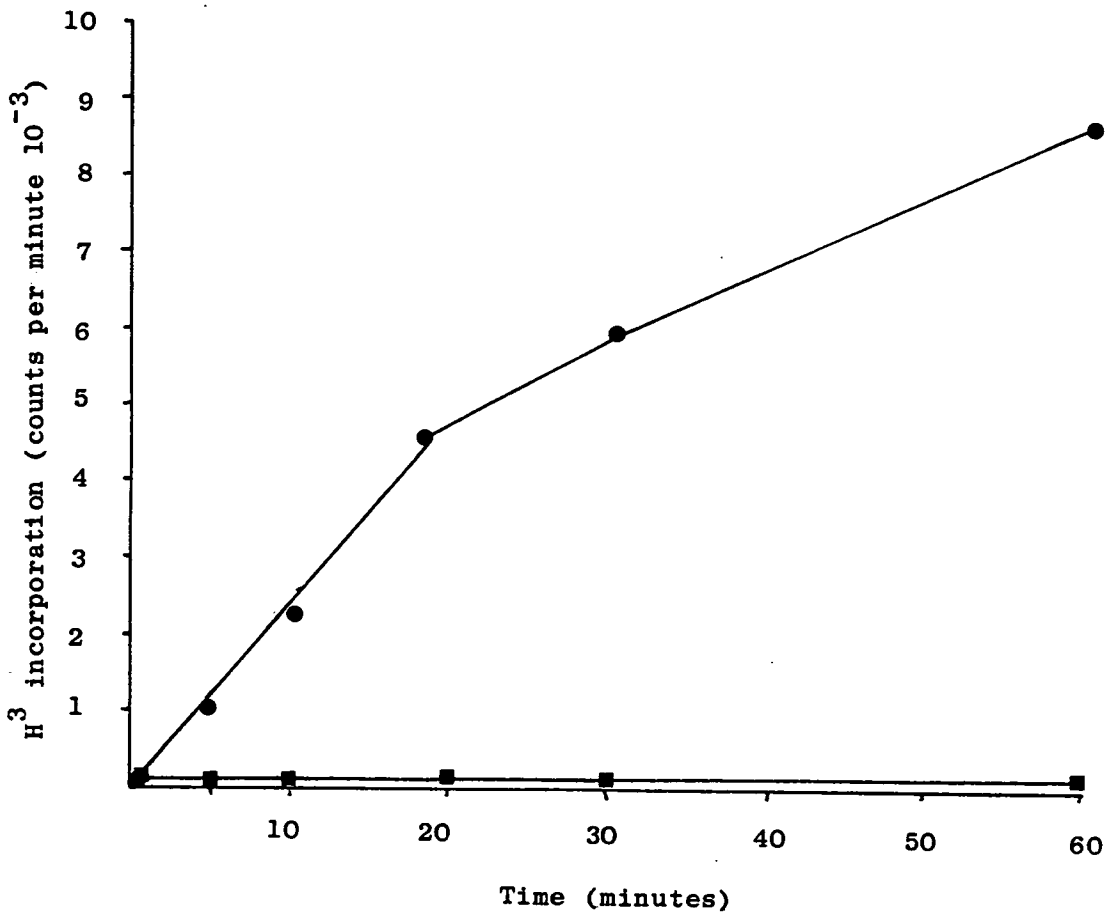


Figure 4.

Protein synthesis in the MDL. A batch of reticulocyte lysate was converted to message dependent lysate as described. Aliquots were then made 50µM in all 20 amino acids and 50µCi/ml in a mixture of H^3 amino acids. Samples were incubated at 30°C and 5µl aliquots taken at the time intervals shown and the incorporated radioactivity measured as described.

■ MDL without added mRNA

● MDL + 20µg/ml rabbit globin RNA.

of 0.1M Tris(pH 7.5). The aqueous phases were pooled and DNA precipitated with ethanol. Thereafter the conditions for hybridization of the restricted plasmid with mRNA and translation of hybridized and melted forms were exactly as described by Paterson et al. (1977). 5µg of total poly(A) RNA from fat body was used per hybridization and the amount of plasmid DNA used was calculated to give a 4 times excess of complementary sequence taking into account the length of the inserted Drosophila DNA and assuming each mRNA comprises 5% of the poly(A) RNA. The final 'hybrid' and 'melted' fractions were taken up in 5µl of 10mM Tris (pH 7.5) and stored in liquid nitrogen. 1µl of this solution was added to 10µl of MDL and 50µCi of a radioactively labelled amino acid as described above.

Polyacrylamide gel Electrophoresis

(a) Electrophoresis of cDNA

The constituents of the gel mixture were 7.5% acrylamide, 0.2% bis acrylamide, 1 x Tris Borate buffer (pH 8.3), 0.075% ammonium persulphate, 0.0375% TEMED. Slab gels 15cm x 15cm x 1.5mm were cast. Gels were run in Tris-borate buffer in a vertical gel apparatus. Samples of P^{32}-labelled double stranded DNA were loaded in 1 x Tris Borate buffer, 6% Ficoll, 0.005% bromophenol blue. Included on each gel was a sample of nick translated and HaeIII restricted pMB9 DNA. Electrophoresis was for 2 hours at 150V after which the blue dye had travelled half-way down the gel. Gels were dried at 80°C for 3 hours under vacuum and exposed to preflashed X-ray film using calcium tungstate intensifying screens (Laskey and Mills, 1977) at -70°C for about 1 day for every 1000cpm/track. X-ray films were developed by standard methods. The developed X-ray film was scanned in a Joyce-Loebl

densitometer and the number average molecular weight of double stranded cDNA calculated from a comparison of the scans of the cDNA and the molecular weight markers.

(b) SDS-Polyacrylamide Gel Electrophoresis of Proteins

The discontinuous buffer system of Laemmli (1970) was used. Proteins were stacked by electrophoresis through 2cm of a 3% or 5% stacking gel and resolved by electrophoresis through a 13%, 15%, or 13 - 20% gradient gel. The dimensions of the resolving gel were 12cm x 15cm x 1.5mm. Electrophoresis was for 4 - 6 hours at a constant current of 100V. Gels were fixed by immersion in 50% TCA overnight. Gels were stained by immersion in 0.2% Coomassie brilliant blue in 50% TCA for 1 hour at 37°C and destained at 37°C in 7% acetic acid containing a few beads of Dowex-1 ion exchange resin. When gels were subjected to fluorography they were immersed in 7% acetic acid for 1 hour after fixing or taken from the destaining mixture and impregnated with the scintillant, PPO, exactly as described by Bonner and Laskey (1974). Gels were dried as before and exposed to preflashed X-ray film at -70°C (Laskey and Mills, 1975). When more than 100,000cpm of S^{35} was loaded per sample, gels were dried directly after staining, or directly after fixing and immersion in 7% acetic acid, and exposed to X-ray film at room temperature.

Samples of labelled proteins or tissue extracts for electrophoresis contained in a final volume of 5 - 20µl 0.0625M Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue, (Laemmli, 1970) and were heated at 100°C for 2 minutes just before loading. For analysis of cell free translation products only 1 - 2µl of MDL were loaded per track. If more than this was used the gels were

overloaded for some of the higher molecular weight reticulocyte proteins and the pattern of translation products was consequently distorted. Gels were always overloaded with respect to globin. Most gels included one track of S^{35}-labelled "heat shock" proteins from Drosophila melanogaster as molecular weight markers.

Preparation and Incubation of Drosophila Tissues

For labelling the newly synthesised proteins of whole larvae with tritiated amino acids 50µCi of the high specific activity tritiated amino acid mixture were dried down in a small plastic tube. Two 3rd instar larvae were gently ripped open in 0.03M sodium phosphate buffer (pH 6.8), 0.04M KCl, 0.011M NaCl, 0.003M $CaCl_2$ 0.021M $MgCl_2$ (Ashburner, 1970) and transfered to the tubes. They were left at 23°C for 30 minutes after which the protein was extracted exactly as described by Tissières et al. (1974).

For labelling of newly synthesised larval fat body proteins 30µCi of S^{35}-methionine was dried down. Fat bodies from 5 or 6 animals were dissected out in the same buffer as before and introduced into the tube. The liquid carried over with the tissues was sufficient to dissolve the labelled methionine. Incubation and protein extraction was as above.

To prepare a sample of larval serum, 3rd instar larvae were punctured with a sharp needle and the serum squeezed out. Serum from 2 or 3 animals was pooled and mixed with electrophoresis sample buffer.

S^{35}-methionine labelled "heat shock" proteins from tissue culture cells were prepared exactly as described by McKenzie et al. (1975).

Immunological Methods

Purified alcohol dehydrogenase from Drosophila melanogaster ADH^{11} (Schwartz and Sofer, 1976) was kindly provided by Dr David Thatcher. The protein was dissolved in PBS (20mM sodium phosphate buffer pH 7.4, 150mM NaCl) at 200µg/ml. 0.5ml aliquots of this solution were mixed with 0.5ml of Freunds adjuvant (Difco) and the mixtures stored in liquid nitrogen. On day 1 of the immunization schedule about 2ml of blood was taken from each of 3 rabbits (control sera). Aliquots of antigen-adjuvant mixture were made into an emulsion by repeated squirting through a syringe needle. Rabbits were injected with 1ml of the emulsion (i.e. 100µg of antigen) per rabbit by injecting about 0.25ml at each of 4 different subcutaneous sites. This injection was repeated on days 17, 33 and 53. On days 24, 41 and 61 about 2ml of blood was taken from each rabbit by bleeding from the ear vein. The serum was prepared and tested for the presence of antibodies by a modification of the procedure of Ouchterlony (1958).

Agarose was dissolved to 1.5% in PBS and the solution made 0.5% in phenol and poured onto clean microscope slides (2.5ml per slide). After the gel had set 2 groups of 7 circular holes (3mm in diameter) were cut in each slide such that in each group a central hole was surrounded by 6 others, all of which were 6mm from the central hole and equidistant from one another. The central holes were filled with serum, at various dilutions in PBS and the outer holes with a crude homogenate of Drosophila larvae at different dilutions in PBS. The slides were left in a humid chamber at room temperature for 20 hours and then examined for the presence of precipitin lines between the inner and outer holes. Precipitin lines were seen best with a 1:16 wt:volume Drosophila extract. At day 61 serum antibodies were

detected with serum concentrations of 1/64 for 2 out of the 3 rabbits. Ouchterlony slides were stained with Thiazine Red and dried exactly as described by Crowle (1958).

Immunoprecipitation of ADH from the Cell Free Translation System

The method used is a modification of that of Kessler (1975) and was given to me by Dr Chris Leaver who also kindly provided the formaldehyde treated Staphylococcus aureus cells.

100µg of mRNA were translated in 200µl of MDL containing 150µCi of H^3-leucine or S^{35}-methionine, 80µg/ml tRNA and 7mM cAMP as described. Incubation was for 40 minutes at 30°C after which an aliquot was decolourized, TCA precipitated and counted as before. 100µl of PBS containing 1% Triton X-100 and 0.5% sodium deoxycholate were then added and the mixture centrifuged for 1 hour at 35K and 4°C in a Beckmann A40 rotor. The supernatant was divided into 25µl aliquots, each of which was mixed with 5µl of serum at various dilutions in PBS and 20µl of PBS + Triton X-100 and sodium deoxycholate. The mixture was incubated at 37°C for 1 hour and then at 4°C overnight. The next morning a 10% suspension of formaldehyde treated Staphylococcus aureus cells (Kessler, 1975) were centrifuged (3 minutes in a microfuge) and resuspended to 10% in 0.5% NP 40, 150mM NaCl, 5mM EDTA, 50mM Tris (pH 7.4) 0.02% sodium azide. This centrifugation and resuspension was repeated twice, after which 15µl of the suspension was added to each tube of lysate/serum mixture. The tubes were incubated at 27°C for 1 hour with occasional shaking. The cells were centrifuged out of the suspension and washed three times with 0.5ml of PBS containing 1% Triton X-100 and 0.1% SDS. Finally, the cells were resuspended in electrophoresis sample buffer, heated at 100°C for 3 minutes, centrifuged

again and an aliquot of the supernatant TCA precipitated and counted as before. Sufficient counts per minute of this supernatant were analysed by electrophoresis.

cDNA-RNA hybridization

cDNA-RNA hybridization in RNA excess was carried out essentially as described by Bishop et al. (1974). Tritiated cDNA and an excess of its poly(A) RNA template were mixed in 10mM Tris (pH 7.5) in a final volume of 90µl. The solution was heated at 100°C for 3 minutes and a 10µl aliquot was removed and diluted into 0.19ml of ice-cold water. The reaction tube was then placed in a 70°C bath and after 30 seconds the solution was made 0.36M in NaCl and 30mM in Tris (pH 7.5). At intervals thereafter, 10µl aliquots were taken and diluted into 0.19ml of ice-cold water. For each aliquot taken, the amount of cDNA which had hybridized with the RNA was estimated by digestion with the nuclease S1 which digests single-stranded but not hybridized DNA (Sutton, 1971).

The 0.2ml samples were made 70mM in NaCl, 30mM in sodium acetate (pH 4.5), 0.1mM in $ZnSO_4$ and 50µg/ml in denatured carrier DNA. Two 100µl aliquots were taken, one was treated with sufficient S1 nuclease to digest all single-stranded DNA and leave double-stranded nucleic acids intact (predetermined by assay) and both were incubated at 50°C for 40 minutes. TCA precipitable counts per minute in both samples were measured as usual. The radioactivity in the S1 treated sample was expressed as a percentage of that in the undigested sample. This percentage was plotted against the product of the original RNA concentration (from the absorbance at 260nm of the original RNA solution) and the time (Bishop et al., 1974). Several such reactions, with RNA

concentrations differing by as much as 1000 were performed in constructing the final curve.

Theory of cDNA-RNA Hybridization and Treatment of Results

DNA-RNA hybridization follows the kinetics of a second order reaction

$$\text{DNA} + \text{RNA} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \text{DNA-RNA}$$

If Do and Ro are the initial reactant concentrations and DR is the concentration of the duplex

$$\frac{dDR}{dt} = K_1(Do - DR)(Ro - DR) - K_2DR$$

In the hybridization experiments described here the RNA is in such excess over the cDNA that the concentration of RNA in the hybrid form is negligible compared with the initial RNA concentration, and $R_o - DR = Ro$. Furthermore, $K_2DR \simeq 0$. Thus

$$\frac{dDR}{dt} = K_1R_o(Do - DR)$$

Integrating with the condition DR = 0 at t = 0

$$\frac{DR}{Do} = 1 - \frac{1}{e^{K_1Rot}}$$

In homogenous RNA populations, the complexity of the RNA is directly proportional to the $R_o t_{\frac{1}{2}}$ (product of the initial RNA concentration and the time at which the hybridization is half complete). It follows that the reaction rate and the abundance of the homogenous RNA are both inversely proportional to the $R_o t_{\frac{1}{2}}$ (Bishop et al., 1975)

$$C \alpha R_o t_{\frac{1}{2}}$$

$$A \alpha \frac{1}{R_o t_{\frac{1}{2}}}$$

In a heterogenous population where not all the RNA families contain the same number of members discrete abundance classes can be observed. In these cases the equation for the hybridization curve is given by

$$\frac{DR}{Do} = \Sigma P_n - \Sigma_e \frac{Pn}{KnRotPn}$$ Bishop et al. (1974)

where P is the proportion of hybridized cDNA molecules in each class, n is the abundance class and Kn is the rate constant for each class, given by $K = 0.69 \times \frac{1}{R_o t_{\frac{1}{2}}}$ (Bishop, 1972).

The complexity in each class is thus proportional to the product of the $R_o t_{\frac{1}{2}}$ and P

$$C \alpha R_o t_{\frac{1}{2}} \times P$$

The data from the hybridization experiments were listed as a series of P values (proportion of cDNA which is S1 resistant) and their corresponding $R_o t$ values. These were processed by a computer, programmed according to the above theory, which draws the best fitting curve to the points (the one with the lowest sum of squares of deviations). The complexity of each class is then calculated from the $R_o t_{\frac{1}{2}}$ and P values read off from the curve, using the fact that a mixture of rabbit α and β globin mRNAs of complexity 4×10^5 daltons, react with their complementary DNAs with a $R_o t_{\frac{1}{2}}$ of 6×10^{-4} mol.sec.l^{-1} (Bishop et al., 1974).

Dividing the complexity by the number average molecular weight of RNA, taken as 6×10^5 daltons for Drosophila (from Izquierdo, 1976), we get the approximate number of different mRNA sequences in each abundance class.

Reverse Transcription of mRNA

(a) Tritiated cDNA for use in hybridization experiments was prepared essentially according to the method of Verma et al. (1972) and Kacian et al. (1972). The final reaction volume was 100µl and contained

reverse transcriptase (supplied by Dr J.W. Beard) 60μCi of H^3 dCTP (25.5 Ci/mmol) 2mM each of dGTP, TTP and dATP, 1.5μg/ml of oligo thymidylic acid ($(pT)_{10}$), 50mM Tris (pH 8.3), 20mM DTT, 6mM $MgCl_2$, 60mM NaCl, 0.8mg/ml of actinomycin D and 2μg of mRNA. The mixture was incubated at 37°C for 90 minutes and the reaction stopped by adding EDTA to 10mM 40μl of 1M NaOH were then added and the mixture incubated at 37°C for 1 hour. 80μl of NaH_2PO_4 were then added and the mixture extracted with phenol and chloroform as described above. The final aqueous phase was passed through a column of Sephadex G-50 in 0.3M NaCl 50mM Tris (pH 7.5) and the radioactivity in each fraction monitored by counting an aliquot in N.E. 260. The peak fractions were pooled and the cDNA precipitated with ethanol.

(b) For preparation of cDNA as the first step in in vitro synthesis of double-stranded cDNA for use in cloning the procedure was exactly as described above except that the final reaction volume was 500μl and contained 50μg of mRNA, 30μCi of P^{32} dCTP (350Ci/mmole) and was 0.05mM in cold dCTP. Incubation was for 2 hours at 37°C.

Synthesis of the Second DNA Strand

The P^{32}-labelled cDNA was treated with DNA polymerase I large fragment (Jovin et al., 1969) in the presence of 66mM Tris (pH 7.4), 6mM $MgCl_2$, 5mM DTT, 0.03mM dGTP, TTP, dCTP and dATP and in a final volume of 300μl contained 60μCi of H^3 dCTP (25.5Ci/mmol). After incubation for 3 hours at 30°C phenol/chloroform extraction, Sephadex G-50 chromatography, and ethanol precipitation were carried out as before.

The product of the DNA polymerase I reaction was treated with S1 nuclease in the presence of 50mM sodium acetate (pH 5.0) and 1mM $ZnCl_2$

at 37°C for 30 minutes. The DNA was recovered by phenol chloroform extraction and chromatography as before.

Addition of 3' oligoadenylic acid tails

Homopolymer blocks of adenosine residues were added to the 3' termini of the S1 treated double-stranded DNA by using the enzyme terminal transferase (Lobban and Kaiser, 1973) kindly supplied by Irving Johnston. The reaction volume was 200µl and contained 100mM Hepes (pH 7.0), 1mM DTT, 4mM $MgCl_2$, 20µCi of P^{32}-labelled dATP, and 0.125mM dATP in addition to the DNA and the enzyme. Incubation was for 5 minutes at 37°C after which the "tailed" DNA was purified by phenol/chloroform extraction, chromatography and ethanol precipitation.

Nick Translation

Plasmid DNAs, or the purified Eco.RI generated fragment of pMD417, were labelled *in vitro* to a high specific activity essentially as described by Rigby *et al.* (1977). Plasmid DNAs were nicked by treatment with highly purified DNAase. The final reaction volume of 250µl contained 66mM Tris (pH 7.5), 6mM $MgCl_2$ DNA at 50µg/ml and 10ng/ml of DNAase. Incubation was for 7 minutes at 20°C after which phenol/chloroform extraction was performed and the DNA ethanol precipitated.

Nick translation was carried out in a volume of 10µl containing 0.3µg of DNA, 66mM Tris (pH 7.5), 6mM $MgCl_2$, 5mM DTT, 50µCi of P^{32}-labelled dCTP (350 Ci/mmole), 0.033 mM each of dATP, dGTP, and TTP and DNA polymerase I. Incubation was at 30°C for 3 hours after which nick translated DNA was purified by pheno/chloroform extraction, chromatography on Sephadex G-50 and ethanol precipitation.

Annealing Experiments

(a) Annealing Plasmids with cDNA

Putative mitochondrial rDNA containing plasmids were annealed with 14S cDNA, plasmid pMD417 and putative cDNA containing plasmids were annealed with fat body cDNA using essentially the same procedure. The "14S cDNA" used in these experiments was made as described above using as the template for reverse transcriptase, mRNA from Drosophila embryos, sedimenting at 14S, and was a gift from Dr John Bishop.

Plasmids were first linearized either by sonication (4 x 30 second bursts in 0.3M NaCl, 10mM CH_3COONa (pH 5), followed by passage through a small (1ml) chelex column equilibrated with the same buffer) or by treatment with restriction enzyme HaeIII (followed by phenol/ chloroform extraction). In either case the DNA was then concentrated by ethanol precipitation.

The plasmid DNA was mixed with about 10^5cpm of tritiated cDNA in a large excess of plasmid DNA in 10mM Tris (pH 7.5). The solution was heated at 100°C for 3 minutes, transferred to a 70°C bath and made 0.36M NaCl, 30mM Tris (pH 7.5). Incubation at 70°C was carried out until the $C_o t$ value (original DNA concentration X time) was about 40 times that required for the reassociation of half of the plasmid DNA. The reaction products were then analysed in either of two ways:

(1) The percentage of cDNA in hybrid was determined using nuclease S1 exactly as described under "cDNA-RNA hybridization".

(2) The percentage of cDNA in hybrid was determined using hydroxylapatite (HAP) chromatography (Bernardi, 1962). A portion of the reaction mixture was diluted into 0.012M sodium phosphate buffer (pH 6.8) and mixed with 100mg of HAP in a water-jacketed column at 68°C. The HAP was resuspended in the solution several times over 15 minutes.

The buffer was then drained off and replaced with 1ml of 0.12M phosphate buffer at 68°C. The HAP was resuspended in this buffer several times over 5 minutes. The buffer was then drained off and the elution with 1ml of 0.12M phosphate buffer repeated a further six times. The elution was then repeated in the same way with 3 x 1ml portions of 0.4M phosphate buffer. All the eluted fractions were then precipitated with TCA, collected on GFC filters and counted in the usual way. The proportion of cDNA complementary to plasmid DNA was calculated as the counts eluted by 0.4M phosphate divided by the total counts eluted.

(b) Experiments with Filter-bound Plasmid DNA

Plasmid DNA was loaded onto nitrocellulose filters as described by Kindle and Firtel (1978).

0.5µg of each plasmid DNA was incubated at 55°C for 15 minutes in 50µl of a solution containing 0.2M CH_3COONa (pH 4.2). The solution was then diluted to 0.5ml with water and 0.5ml of 1N NaOH were added. After 15 minutes at room temperature 2ml of neutralizing solution were added (1 part 1N HCl:1 part 1M Tris (pH 8.0):2 parts 3M NaCl). The solution was then passed slowly (0.2ml per minute) through a 3cm diameter nitrocellulose filter (Millipore) which had been soaked and washed in 2 x SSC (SSC is 0.15M NaCl, 0.015M sodium citrate). The discs were then washed with 10ml of 2 x SSC and baked in a vacuum oven for 2 hrs at 80°C.

Three smaller discs, 13mm in diameter, were cut from each larger one. Pretreatment of these discs before hybridization was exactly as described by Maniatis et al. (1978) based on the procedure first described by Denhardt (1966). Finally the discs were stacked in small

vials and incubated overnight at 70°C in hybridization buffer. This contained a final volume of about 0.5ml 4 x SET (SET is 0.15M NaCl, 0.03M Tris (pH 8), 2mM EDTA), 0.1% SDS, 0.1% sodium pyrophosphate, 10µg/ml poly(A), 50µg/ml denatured salmon sperm DNA, 10µg/ml *E. coli* DNA, and about 10^5 counts per minute of either denatured P^{32}-labelled 0.5Md EcoRI fragment of pMD417 or H^3-labelled fat body cDNA.

After hybridization the filters were washed exactly as described by Maniatis *et al.* (1978) and counted in the usual way.

Southern "blotting" experiments

Restricted Drosophila embryonic DNA (3µg/track) was electrophoresed in 0.75% agarose gel as described. After electrophoresis the gels were stained, viewed under ultraviolet light and the DNA transferred to strips of nitrocellulose exactly as described by Southern (1975). The filters, after baking, were pretreated, hybridized, and washed exactly as described by Maniatis *et al.* (1978). The probes used were denatured, nick-translated plasmid DNAs (about 10^6cpm of P^{32}/ filter). The washed and dried filters were exposed to pre-flashed X-ray film, against a tungstate intensifying screen at -70°C (Laskey and Mills, 1977).

Solutions and media not detailed in the text:-

"Angelas" larval culture medium.
150g maize meal
22g flaked yeast
20g agar
130g treacle
3 pints of water
5mls of propionic acid

"Ribena" medium.
Bacto agar 29.7g
Dextrose 78.8g
Sucrose 19.9g
Flaked yeast 23.6g
Blackcurrant juice 160ml
H_2O 1160ml
1.25N NaOH 34ml

These ingredients are mixed and autoclaved. After cooling 14ml of 1.25N NaOH and 13ml of 40% propionic acid, 4% phosphoric acid are added. The solution is mixed and poured into plastic trays to set.

L-broth
Difco Tryptone 10g
Difco yeast extract 5g
NaCl 10g
H_2O 1 litre

LB-bottom as for L-broth, plus 15g Difco agar.

BBL top
BBL Trypticase 10g
NaCl 5g
Difco agar 6.5g
H_2O 1 litre.

Suppliers of Reagents

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England.

Oligo-dT-cellulose was purchased from Collaborative Research, U.S.A.

PPO and POPOP were from Packard Ltd.

NE260 was bought from Nuclear Enterprise, Scotland.

Nitrocellulose filters were from Millipore.

GFC and 3MM paper was from Whatman, as were DEAE cellulose (DE52) and phosphocellulose

Biogel, hydroxyapatite and Dowex-1 were obtained from Bio-rad.

Proteinase K was bought from Merk.

Sephadex was sent from Pharmacia, Uppsala, Sweden.

Micrococcal nuclease, creatine phosphate and creatine kinase were obtained from Boehringer.

X-ray film, cassettes and intensifying screens were supplied by Kodak.

Ampicillin was sent by Beecham Research Laboratories.

Actinomycin D was purchased from Calbiochem.

Unless stated otherwise in the text, all other biochemicals were supplied by Sigma.

RESULTS

The Isolation of Mitochondrial Ribosomal DNA by Molecular Cloning

Introduction

Figure 5 shows the methodology for cloning and identification of specific mRNA sequences from a heterogenous population of mRNAs. I was interested in using this methodology in order to isolate sequences present in the fat body of third instar larvae of Drosophila melanogaster. The eventual aim of this work is to isolate a set of genes which are expressed in the same tissue at the same stage of development. Some of the abundant fat body proteins are of special interest since they are well characterized biochemically and genetically, and have well known developmental profiles. These are the larval serum proteins, at present under intense investigation in the laboratories of Dr D.B. Roberts and Dr D.M. Glover, and alcohol dehydrogenase (Ursprung et al., 1970; O'Donnell et al., 1977).

One might expect that the most common mRNA sequences in fat body mRNA are the ones which code for these abundant proteins and that a major proportion of the clones obtained after following the procedure outlined in Figure 5 would contain sequences complementary to these mRNAs. Previous work in this, and other, laboratories has shown that this may not be the case for between 50% and 80% of polyadenylated RNA from Drosophila larvae, or their fat bodies, consists of a non-messenger sequence.

Larval Poly(A) containing RNA contains a high proportion of 14S Large Mitochondrial Ribosomal RNA

When polyadenylated RNA from Drosophila larvae is subjected to sucrose density gradient centrifugation a large peak is found with a sedimentation coefficient of 14S (Izquierdo, 1976). Izquierdo showed

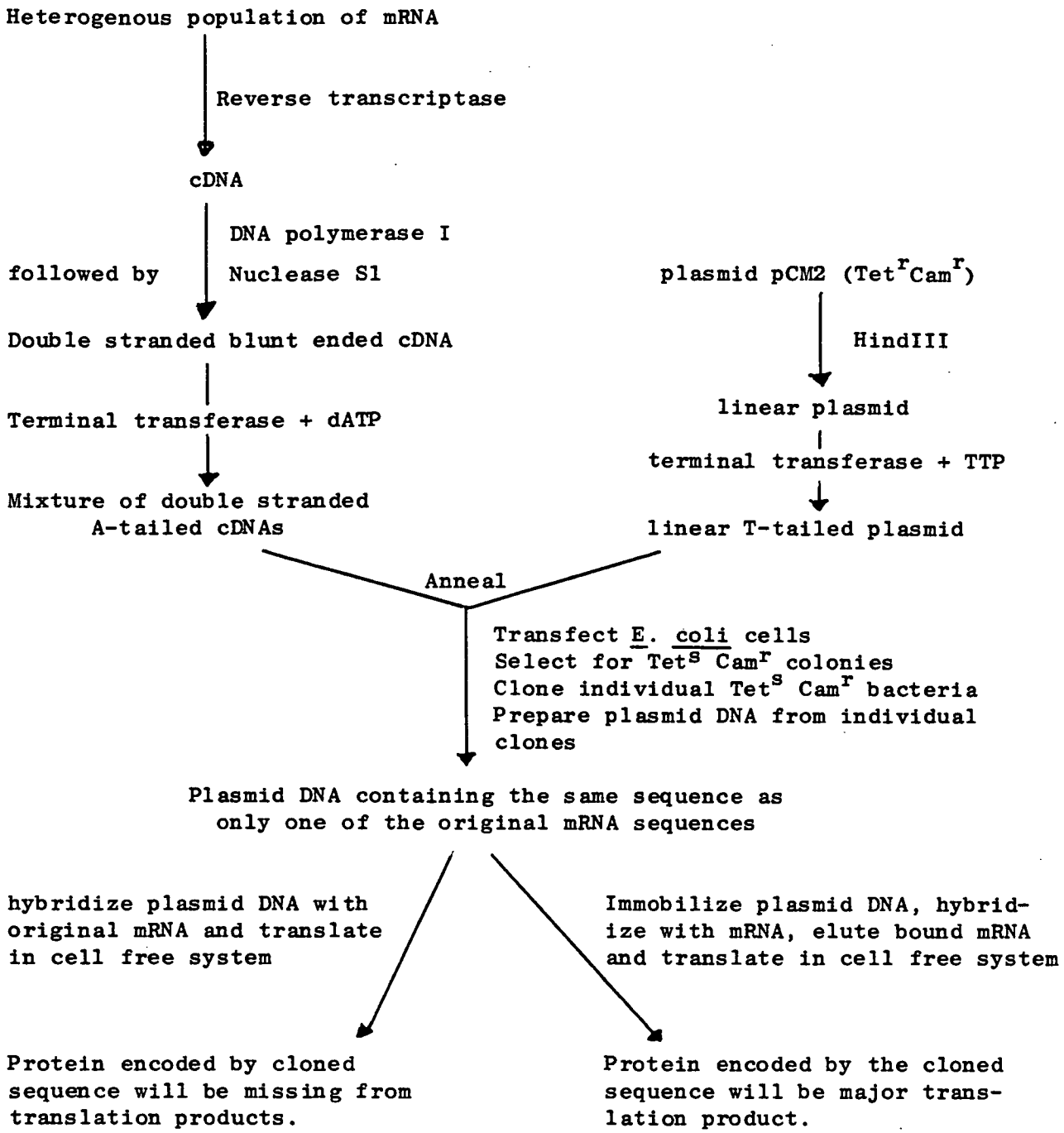


Figure 5.

A summary of the procedure used for cloning and identification of specific mRNA sequences from a heterogenous population of mRNAs.

that this 14S RNA consisted largely of a single species. Kinetic analysis of larval or imaginal poly(A) RNA also revealed the presence of a single, abundant RNA species, accounting for 70% - 80% of the RNA. Izquierdo and Bishop (1979) showed that this RNA was complementary to mitochondrial DNA. Several other workers have reported the presence of an abundant 13 - 14S poly(A) RNA of mitochondrial origin (Spradling et al., 1976; Alwine et al., 1977).

Klukas and Dawid (1976) showed that the large mitochondrial rRNA in Drosophila melanogaster is A-rich and has a sedimentation coefficient, under their conditions, of 13S. The same workers showed that the mitochondrial DNA coding for this large rRNA is located on a HindIII restriction fragment of length 5.2 Md. Izquierdo and Bishop (1979) showed that 14S RNA is transcribed from a HindIII fragment of total Drosophila DNA of length 5.2 Md. The kinetic analysis carried out by Izquierdo (1976) depends upon the copying of the RNA into complementary DNA (cDNA) by the enzyme, reverse transcriptase, which is primed at a poly(A) region of the template RNA. Since this analysis showed the presence of the abundant 14S RNA, this RNA species must act as an efficient template for the enzyme. It is likely, therefore, that if RNA from Drosophila larvae, or their fat bodies, was used in the procedure shown in Figure 5, more than half of the clones obtained would contain mitochondrial DNA sequences. Unless these clones could be easily identified their presence would complicate attempts to pick out clones which contained real abundant mRNA sequences. Therefore, it was decided to develop either a method of removing 14S RNA from mRNA preparations or a system of screening for mitochondrial rDNA sequences in recombinant plasmids.

Binding Drosophila mRNA to Millipore Filters

Early attempts to remove 14S RNA from preparations of cytoplasmic poly(A) RNA from third instar larvae were based on the observations of Gorski et al. (1974). These workers found that only 30% of mouse globin mRNA, purified by oligo dT-cellulose chromatography, bound to Millipore filters (HAWP 25mm) under conditions where other mRNAs bind. Analysis of the bound and unbound fractions revealed that the unbound RNA contained shorter poly(A) regions (35-45 adenine residues) than the bound fractions. The poly(A) tails in real mRNA, are added to the RNA molecules post-transcriptionally, in the nucleus (Darnell et al., 1971; Perry et al., 1974). The poly(A) region or regions in 14S RNA presumably reflect the base sequence of mitochondrial rDNA. Since the two poly(A) regions arise by unrelated events it was thought that they may well be of different sizes and therefore that 14S RNA and mRNA may bind differentially to Millipore filters.

Poly(A) containing RNA from Drosophila third instar larvae was passed through a Millipore filter in high salt (Brawerman et al., 1972). RNA that had bound was eluted with sterile water. Bound and unbound fractions were analysed by sucrose density gradient centrifugation. Repeated attempts to remove 14S RNA using this approach failed. Most of the RNA (90% - 95%) was in the unbound fraction and both fractions contained a prominent 14S peak.

It was decided, therefore, to prepare large quantities of mitochondrial rDNA by molecular cloning of restriction fragments (Morrow et al., 1974; Chang et al., 1975). This DNA could then be coupled to finely divided cellulose (Noyes and Stark, 1975) and used to hybridize 14S RNA out of mRNA preparations. Alternatively cloned mitochondrial rDNA could be labelled in vitro and used as a probe to identify and screen out any cDNA clones containing mitochondrial sequences.

Preparation and Characterization of Drosophila Mitochondrial DNA

DNA was extracted from highly purified mitochondria of Drosophila embryos (Bultmann and Laird, 1973) according to the method of Klukas and Dawid (1976). Closed circular mitochondrial DNA was purified by twice banding in caesium chloride-ethidium bromide gradients. Figure 6 shows DNA from a second gradient after agarose gel electrophoresis. The three bands seen are closed circular DNA, linear molecules and open circular molecules in order of decreasing mobility. The molecular weight of the linear DNA from this gel appears to be about 12Mdal in comparison with the molecular weight markers. This is in good agreement with published values (Bultmann et al., 1976).

Klukas and Dawid (1976) showed that treatment of the circular mitochondrial DNA with the restriction endonuclease HindIII split the DNA into four fragments of molecular weights 5.3, 3.6, 2.9 and 0.3Md. They were able to show that the large mitochondrial rRNA was transcribed entirely from the largest HindIII fragment. To characterize the genome further, I located the four target sites for the restriction enzyme Eco.RI on this map. Mitochondrial DNA was digested to completion with HindIII, Eco.RI, PstI or a mixture of two of these enzymes, and the resulting fragments were separated by agarose gel electrophoresis. Figure 7 shows an example of such a gel. The enzyme PstI appears to cleave a small proportion of mitochondrial DNA molecules at a unique site so that in double digests involving PstI extra weak bands are seen but no bands generated by Eco.RI or HindIII alone disappear.

Figure 8 shows the map derived from deducing a unique order for the restriction fragments and considering the data of Klukas and Dawid (1976). The sizes of the fragments shown in Figure 8 differ slightly from those derived from the electron microscope observations of Klukas

Figure 6.

Mitochondrial DNA from Drosophila melanogaster. Mitochondrial DNA was prepared from embryos and purified on ethidium bromide caesium chloride gradients. The DNA was electrophoresed in a 0.5% agarose gel in Tris Borate buffer, the gel stained with ethidium bromide and the bands visualized under ultra violet light. (a) DNA from the upper band of the isopycnic gradient. (b) an Eco.RI digest of λ DNA. The bands correspond to molecular weights 15.8, 13.7, 4.7, 3.7, 3.5, 3.0, 2.1Md. (c) DNA from the lower band of the isopycnic gradient. The three bands are the closed circular, the linear, and the open circular forms of mitochondrial DNA in order of decreasing mobility.

Figure 7.

A 0.5% agarose gel of mitochondrial DNA after digestion with various restriction enzymes.

(a)(e)(i) An Eco.RI digest of phage λ DNA.
(b) mitochondrial DNA linear and open circular forms
(c) mitochondrial DNA digested with Eco.RI and HindIII.
(d) mitochondrial DNA digested with Eco.RI.
(f) mitochondrial DNA digested with PstI.
(g) mitochondrial DNA digested with PstI and Eco.RI.
(h) mitochondrial DNA digested with PstI and HindIII.

The molecular weights at the right of the photograph refer to the masses in Md of the mitochondrial DNA fragments in tracks c, d, g and h and were calculated relative to the λ-Eco.RI fragments and, in other gels, relative to Eco.RI and BamI fragments of plasmid pCM2 DNA. The molecular weights of the weak PstI generated bands are not shown. The conditions for restriction and electrophoresis are described under Materials and Methods.

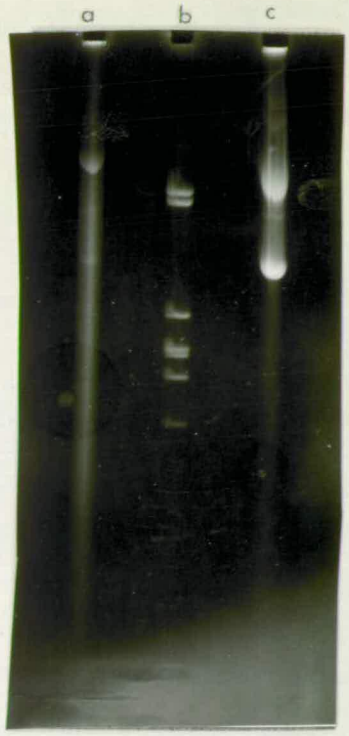


Fig.6

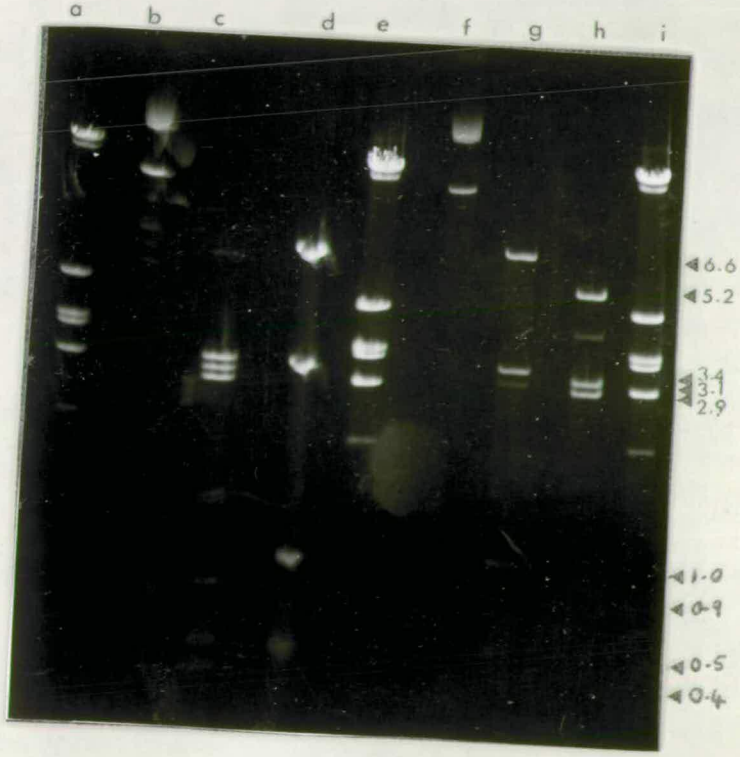


Fig.7

Figure 8.

A restriction map of mitochondrial DNA derived from that of Klukas and Dawid (1976) and my own restriction mapping.

△ Eco.RI target site ▲ HindIII target site

— HaeIII target site

The dotted lines represent ribosomal RNA genes.

S - small ribosomal RNA

L - large ribosomal RNA.

The molecular weights as calculated from my electrophoresis data in Md are:

Eco.RI	A 0.5	B 3.4	C 1.0	D 6.6
HindIII	A 5.2	B 0.3	C 2.9	D 3.1

The molecular weights of the Eco.RI HindIII double digestion fragments are, working clockwise from the HindIII site at 12 o'clock 0.4, 0.5, 3.4, 0.9, 0.1, 0.2, 2.9, 3.1Md.

The 0.1 and 0.2Md fragments are assumed to have run off the gel shown in Figure 7 track c, and the 0.3Md fragment cannot be seen in Figure 7 track h.

The total molecular weight from addition of these fragments is 11.5Md, 0.5Md smaller than the size of the DNA derived from electron microscope studies. This is probably because this AT rich DNA is relatively light per unit length.

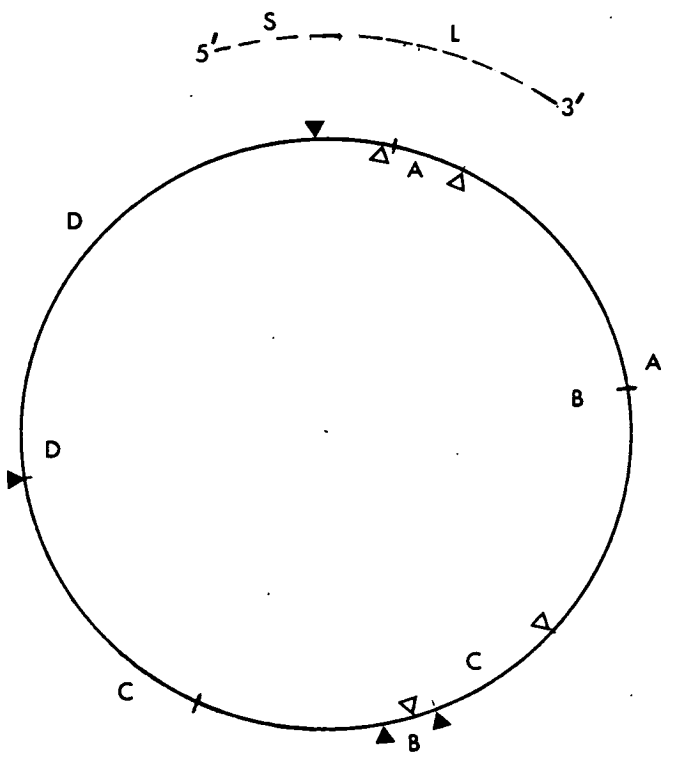


Fig.8

and Dawid. This is most likely because DNA of high A+T composition, like Drosophila mitochondrial DNA, is relatively light per unit length. The fragments will be referred to by their masses as derived from my studies and shown in Figure 8. In addition I found that Drosophila mitochondrial DNA has no site for the enzyme BamI.

Attempts to clone mitochondrial ribosomal DNA

Attempts were made to isolate the large ribosomal RNA gene by molecular cloning. The strategy used is illustrated in Figure 9. Briefly, HindIII restricted DNA of the plasmid cloning vector pCM2 (Bishop, 1979) was mixed with HindIII restricted mitochondrial DNA in a 4 - 5 fold molar excess of mitochondrial DNA. The mixture was then placed at 10°C for 2 hours in the presence of T4 DNA ligase. During this time we expect the HindIII generated sticky ends to anneal together and the ligase to covalently join 2 DNA molecules, or to form covalently closed DNA circles from one original type of molecule. Some of these annealing and ligation events will result (should ?) in the insertion of the mitochondrial DNA fragments into the HindIII site of pCM2. The ligated DNA was then used to transform E. coli cells of the strain HB101. Chloramphenicol resistant colonies from this transformation were replica plated on plates containing tetracycline (Tet) and plates containing chloramphenicol (Cam). Since foreign DNA is inserted into the gene conferring Tet resistance any Tet sensitive, Cam resistant (Tet^s Cam^r) colonies are likely to harbour pCM2 molecules which contain inserted DNA. The combined results of two such experiments are shown.

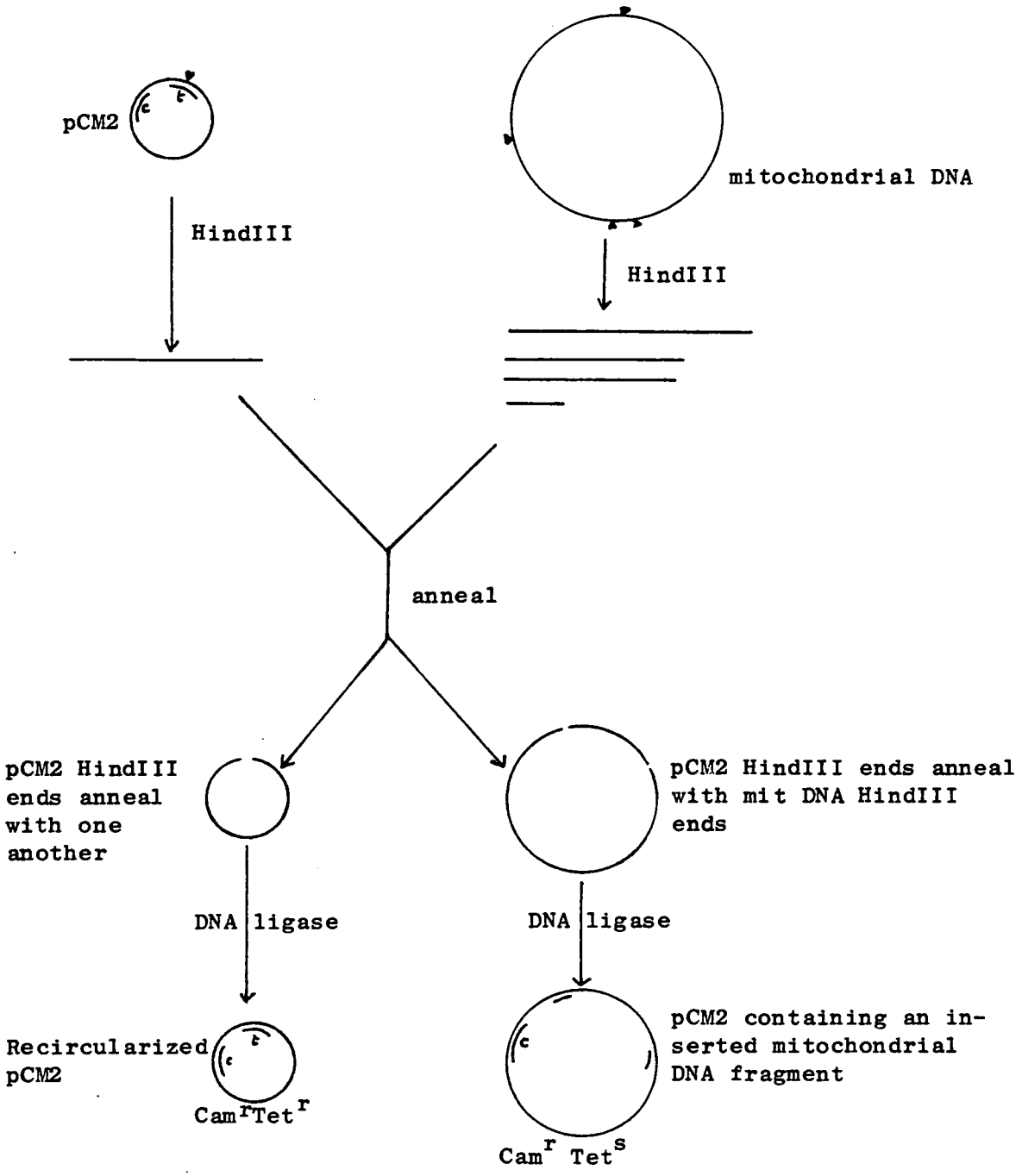


Figure 9.

Strategy for cloning mitochondrial DNA HindIII fragments.

▲ is a HindIII target site. The inner lines represent the antibiotic resistance determinants of pCM2. c is chloramphenicol resistance, t is tetracycline resistance.

DNA in ligation	Cam^r colonies	$Cam^r Tet^s$ colonies
-	0	-
5nmole pCM2 HindIII	6240	0/45
5nmole pCM2 HindIII + 25nmole mit-DNA HindIII	3920	52/924

A number of the $Cam^r Tet^s$ colonies were streak plated, single colonies picked, grown in L-broth and the plasmid DNA from each colony extracted and purified in caesium chloride-ethidium bromide gradients. The plasmid DNAs were treated with HindIII and electrophoresed in agarose gel in order to identify any plasmids containing a 5.2Mdal fragment inserted into the pCM2 DNA. Figure 10 shows the results of the analysis of 24 plasmids from two separate experiments.

The plasmids fall into three classes. 12 plasmids contain an inserted fragment which migrates slightly faster than the 3.0Md fragment of phage λ DNA (Class I). 5 plasmids are restricted to show a single band of the same mobility as linear pCM2 DNA (Class II). 6 molecules show 2 strong bands and a weak band, the weak band having a mobility faster than that of linear pCM2 DNA. Plasmid pMD211 does not fall into these classes. It runs slightly slower than pCM2 and appears to contain only one site for the enzyme HindIII.

Figure 11 shows further analysis by Eco.RI and HindIII restriction of 2 representatives from each of classes I and III. The total mass of DNA from class I molecules is about 2.9Md greater than that of pCM2. Class III molecules have a mass some 1.1Md less than pCM2. The likely explanation is that class I molecules contain the 2.9Md HindIII generated mitochondrial DNA fragment and class III molecules have a segment of pCM2 deleted from them, including the HindIII site

Figure 10. Restriction analysis of putative mitochondrial DNA clones.

Plasmid DNAs were prepared as described, restricted with HindIII and electrophoresed in 0.5% agarose gels.

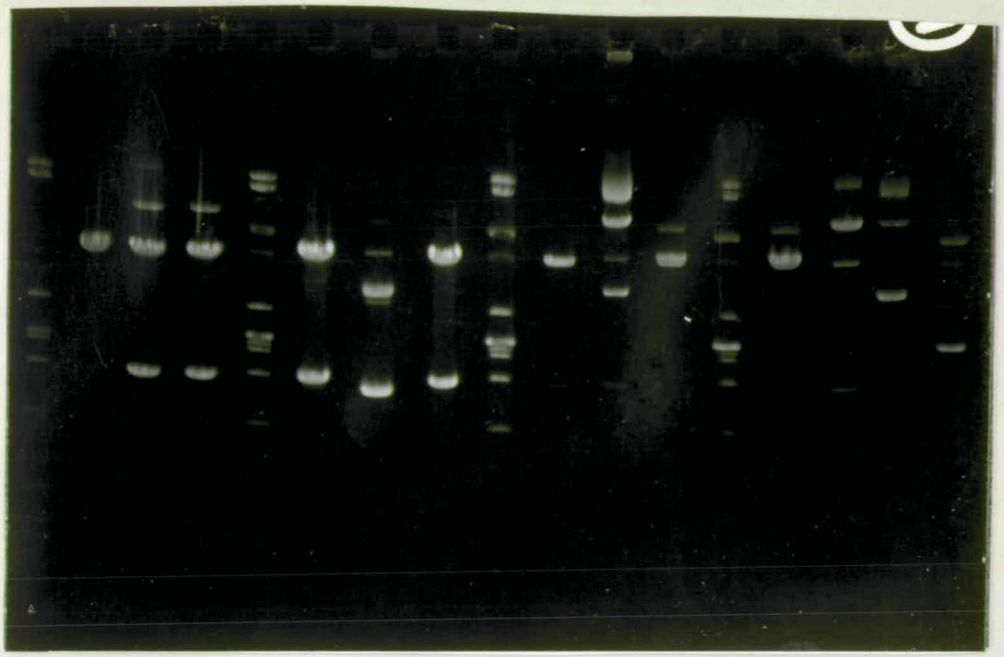
Tracks are

A.(right to left). pCM2 unrestricted, pMD101-103, pCM2+ Eco.RI treated λ DNA, pMD104-106, pCM2+Eco.RI treated λ DNA, pMD107-109, pCM2+Eco.RI treated λ DNA, pMD110-112, Eco.RI treated λ DNA.

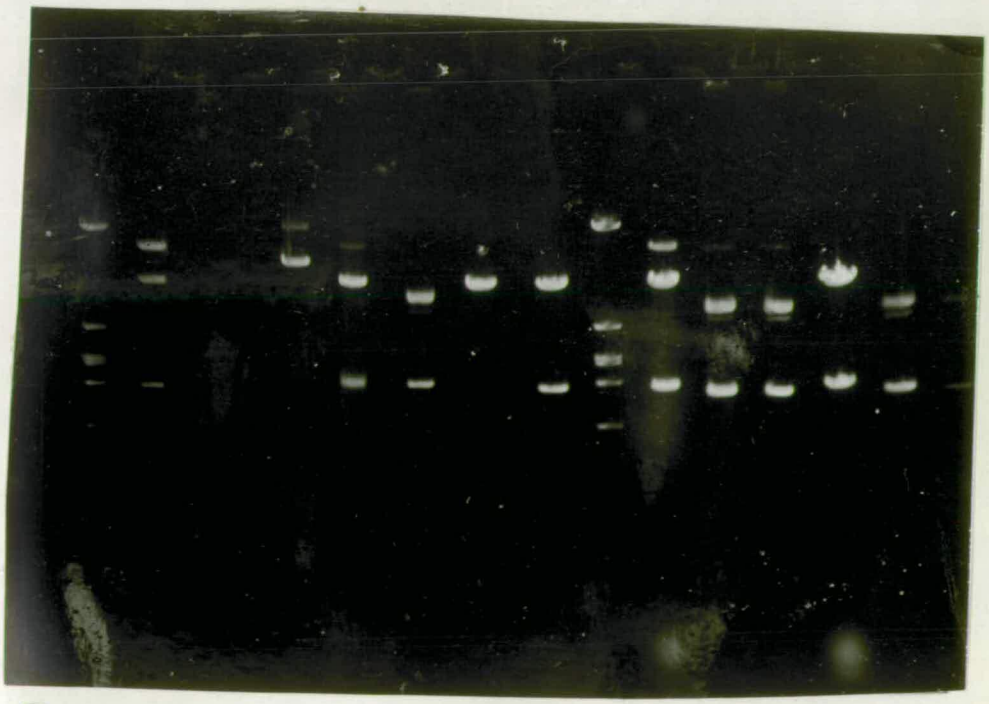
B.(right to left). pMD201-206, Eco.RI treated λ DNA, pMD207-212, Eco.RI treated λ DNA.

The bands in pCM2 are closed circular, linear and open circular molecules in order of decreasing mobility.

The bands in Eco.RI treated λ DNA are 2.1, 3.0, 3.5, 3.7, 4.7, 13.7, 15.8Md in order of decreasing mobility.



A



B

Fig.10

and rendering them Tet^S. The three bands seen in the HindIII digests of these plasmids are the supercoiled DNA, linear molecules and open circular molecules.

Figure 12 shows the restriction analysis of pMD112, one of the class II plasmids. A small 0.3Md HindIII fragment is cleaved out of this plasmid. Class II molecules, therefore, contain the smallest HindIII generated mitochondrial DNA fragment inserted into pCM2. Figure 12 shows that this HindIII fragment contains a target site for Eco.RI as expected from the map shown in Figure 8.

The interesting and unexpected result of these initial cloning experiments is that only 2 out of a possible 4 HindIII fragments are successfully inserted into pCM2 to yield plasmids which can replicate and function in *E. coli*. In addition a further 24 colonies from these experiments were examined by the colony gel procedure described below, and none contained a plasmid large enough to include a 5.2Md insert. Of the many possible reasons for this, the first one that I tested was that parts of the sequence of these fragments were not compatible in some way with the plasmid pCM2, perhaps inducing deletions or blocking replication. Therefore the experiment was repeated using the plasmid pBR322 (Bolivar *et al.*, 1977), again trying to insert the HindIII fragments into a HindIII site. This plasmid contains the same Tet^r conferring sequence as pCM2 and also contains a sequence conferring resistance to ampicillin (Amp).

Several Amp^r Tet^S colonies were isolated as described above and these were screened for the presence of a 5.2Mdal insert by a more rapid method (Barnes, 1977). A loopful of bacteria from a single colony was lysed and loaded directly onto an agarose gel. After electrophoresis and staining the closed circular DNA can be visualized

Figure 11.

Restriction enzyme analysis of plasmid DNA. DNAs were restricted and the fragments subject to electrophoresis in a 1% agarose gel. The tracks are

(a) pMD203 x HindIII. (b) pMD203 x HindIII x Eco.RI.
(c) pMD202 x HindIII. (d) pMD202 x HindIII x Eco.RI
(e)(j)(m) λ DNA x Eco.RI. (f) pMD108 x HindIII.
(g) pMD108 x HindIII x Eco.RI. (h) pMD107 x HindIII.
(i) pMD107 x HindIII x Eco.RI. (k) pCM2 x HindIII.
(l) pCM2 x HindIII x Eco.RI.

Plasmids pMD107 and pMD203 are referred to in the text as Class I molecules. pMD108 and pMD202 are Class III molecules. The Class I molecules contain a 2.9Md insert, which is cleaved out by HindIII (a,h) and does not contain any EcoRI sites (b,i). The Class III molecules are not restricted by HindIII (c,f) and contain less DNA than pCM2 (d,g). The figures at the right of the photograph refer to the sizes of Eco.RI x HindIII generated pCM2 DNA fragments. The sizes of λ Eco.RI fragments are given in the legend to Figure 10.

Figure 12. Analysis of pMD112.

Plasmid DNA was restricted and subjected to electrophoresis in a 2% agarose gel. The tracks are

(a) pMD112 treated with Eco.RI.
(b) pMD112 treated with HindIII.
(c) pMD112 treated with Eco.RI and HindIII.
(d) mouse satellite DNA digested with Eco.RII (a gift from Dr Crispin Phillips). The fastest and second fastest running bands are 240 and 480 base pairs long respectively (approximately 0.16 and 0.32Md).

The gel shows the pMD112 contains a fragment of about 0.3 Md which is cleaved out by HindIII and which contains a Eco.RI target site. pMD112 is an example of a Class II molecule as referred to in the text.

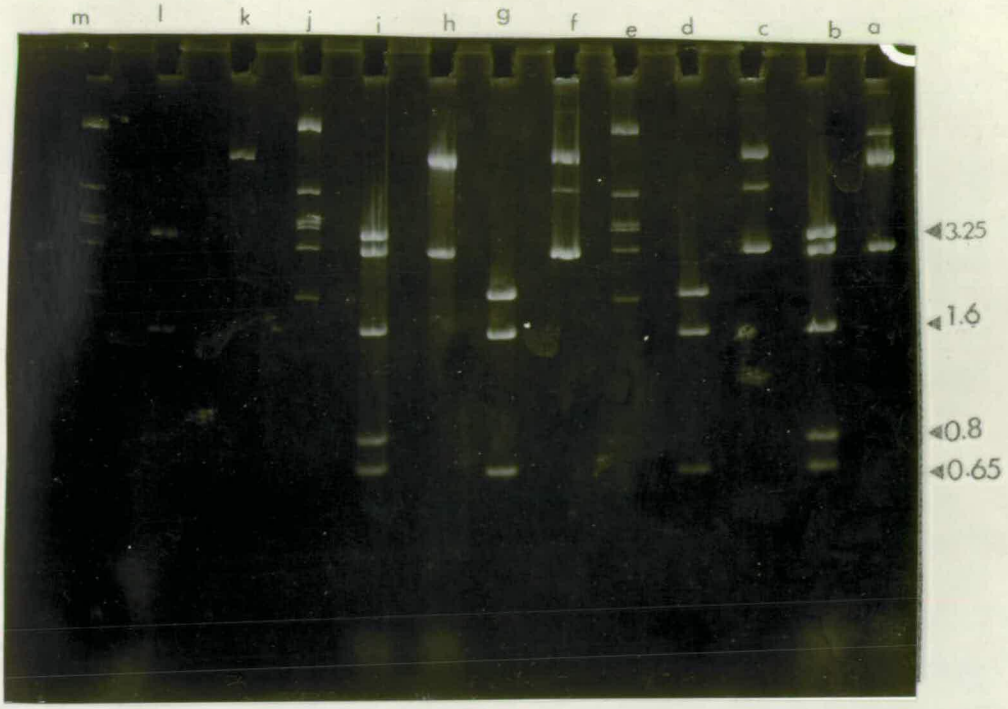


Fig.11

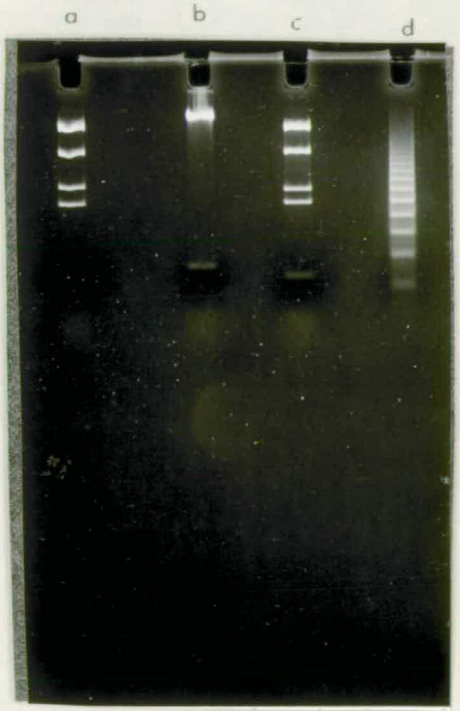


Fig.12

in the gel. Closed circular DNAs of known sizes were run on these gels as size markers. From this experiment no plasmids big enough to contain the 5.2Md mitochondrial DNA fragment were seen (16 were looked at).

It could be that the 5.2Md fragment will not enter the HindIII site of these related plasmids to give viable plasmids which render their host cells Tet^s. Consequently, an attempt was made to insert the HindIII fragments of mitochondrial DNA into the single PstI site of pBR322 which is located in the Amp gene. The procedure is illustrated in Figure 13.

HindIII cleaved mitochondrial DNA was tailed with cytidine residues and PstI cleaved pBR322 was tailed with guanidine residues using the enzyme terminal transferase. These DNAs were annealed in equimolar proportions and used to transform E. coli cells. Any Amp^s Tet^r colonies may harbour plasmids containing mitochondrial DNA inserted at the PstI site of pBR322. The tailing in this experiment was performed by Mr John Jenkins.

Out of 127 Tet^r colonies obtained from this experiment, 21 were Amp^s. These were analysed as before by the colony gel procedure. Most of the plasmids were about the same size, or very slightly bigger, than pBR322. 2 of the plasmids were about the right size to contain an insert of about 3.0Md. A further 2 plasmids were about 0.5 - 1.0Md smaller than these. These latter two were of interest since they do not have the molecular weight expected of pBR322 containing any one of the mitochondrial DNA HindIII fragments. Plasmid DNA from these two colonies was prepared, and analysed by restriction with HindIII and Eco.RI. Figure 14 shows the restriction patterns. This gel shows that plasmid pMD417 contains an insert which contains two

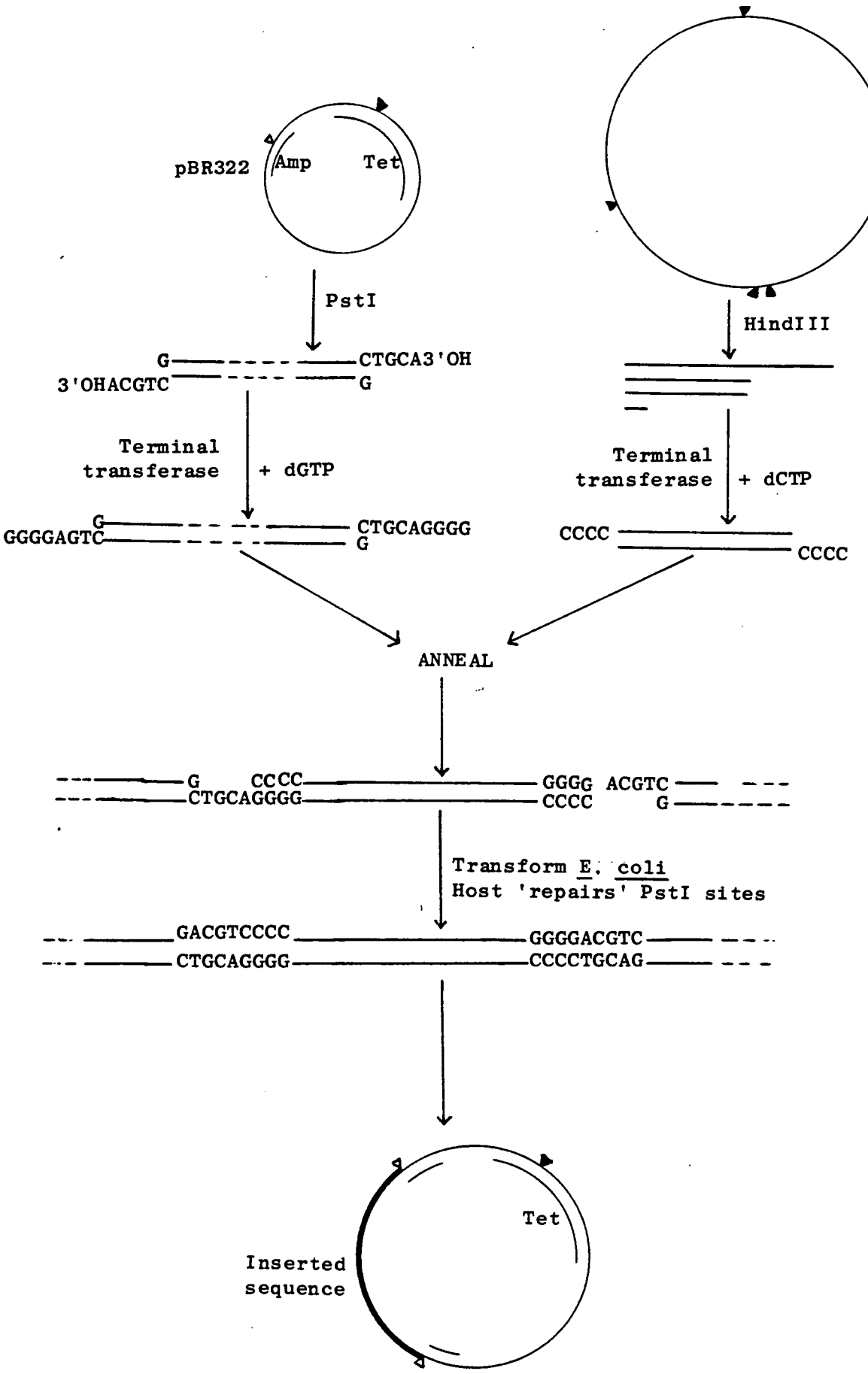


Figure 13.

The strategy for cloning mitochondrial DNA into the PstI site of pBR322. △ is a PstI target site. ▲ is a HindIII target site.

Eco.RI sites, separated by 0.5Md. Consideration of the map of the Eco.RI sites in mitochondrial DNA shown in Figure 8 would indicate that the portion of the genome containing these Eco.RI sites is that of the gene for the large mitochondrial rRNA.

To test this possibility plasmid DNA from pMD417 and from other plasmids was sonicated to a length of about 400 base pairs, denatured, and annealed in excess with cDNA prepared from the 14S peak from a Drosophila embryo poly(A) RNA preparation (this cDNA was a gift from J. Bishop). After annealing, the mixtures were treated with S1 nuclease, which digests single stranded but not double stranded nucleic acids, to determine how much of the cDNA was complementary to a sequence in the plasmids.

Plasmid	Percentage of cDNA resistant to S1
pBR322	5%
pMD412	6%
pMD417	51%

Similar results were obtained in three such experiments. It seems, therefore, that a sequence represented in pMD417 is complementary to an abundant 14S poly(A) RNA.

Figure 15 shows an example of an agarose gel used to derive a map of restriction sites in the plasmid pMD417. Figure 16 shows the restriction map of pMD417 derived from analysis using restriction enzymes HaeIII, Eco.RI, PstI, HindIII and from the known restriction map of pBR322 (Bolivar et al., 1977). It seems that the inserted fragment extends from a HindIII site in mitochondrial DNA to a site

Figure 14. Eco.RI sites in plasmids pMD412 and pMD417.

Plasmid DNAs were restricted and subjected to electrophoresis in a 0.5% agarose gel as described.

(a) and (f) Eco.RI treated phage λ DNA.
(b) pMD417 DNA treated with Eco.RI and BamI.
(c) pMD417 DNA treated with Eco.RI.
(d) pMD412 DNA treated with Eco.RI and BamI.
(e) pMD412 DNA treated with Eco.RI.

The gel shows that pMD417 contains an insert having two Eco.RI sites. The sizes in Md of the Eco.RI fragments of pMD417 were calculated from this and other gels.

Figure 15. Restriction enzyme analysis of plasmid pMD417.

Plasmid DNAs were restricted and subjected to electrophoresis in a 1% agarose gel as described.

(a)(k) Eco.RI treated phage λ DNA.
(b) pMD417 DNA treated with PstI and HindIII.
(c) pBR322 DNA treated with PstI and HindIII.
(d) pMD417 DNA treated with Eco.RI and HaeIII.
(e) pMD417 DNA treated with HaeIII.
(f) pBR322 DNA treated with HaeIII.
(g) pMD417 DNA treated with Eco.RI and PstI.
(h) pBR322 DNA treated with Eco.RI and PstI.
(i) pMD417 DNA treated with Eco.RI.
(j) pBR322 DNA treated with Eco.RI.

The molecular weights shown at the right of the photograph refer to tracks g and i and were calculated from this gel and other gels. Those shown at the left refer to track b.

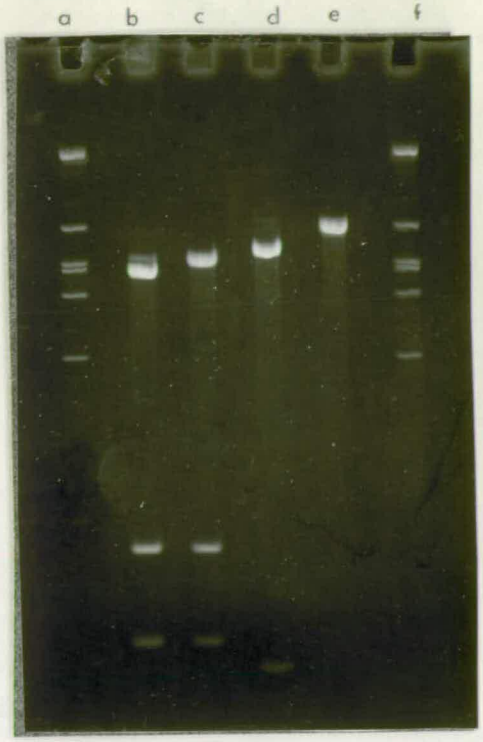


Fig.14

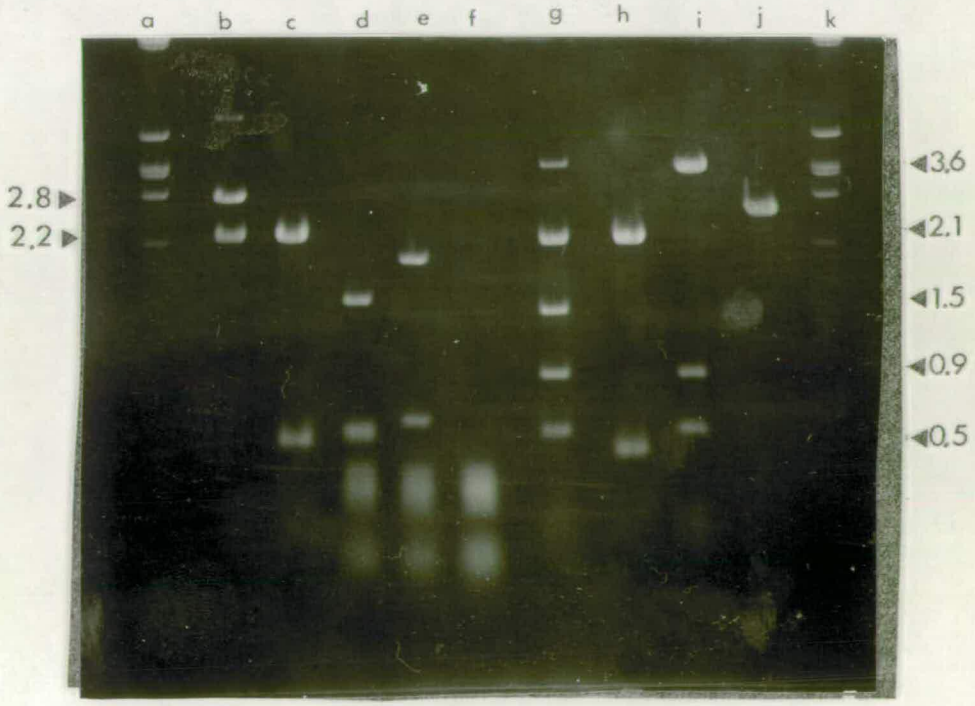


Fig.15

2.4Md along the 5.2Md HindIII fragment and will, therefore, contain the entire gene for the large mitochondrial ribosomal RNA. DNA inserted into the PstI site of pBR322 by the procedure shown in Figure 13 should contain a PstI site at either side of the inserted fragment. pMD417 contains only one PstI site which is located midway along the 5.2Md HindIII fragment. The likely explanation is that C tails have been added onto the mitochondrial DNA at this point, possibly by terminal transferase initiating at a nick or a breakpoint in the DNA. The absence of a PstI site at the other pBR322-mitochondrial DNA boundary is more difficult to explain.

pMD417 therefore, is a plasmid which contains the entire gene coding for the large mitochondrial ribosomal RNA. It could be useful in specifically hybridizing out 14S RNA from Drosophila poly(A) RNA preparations or in preparing labelled probes to identify mitochondrial sequences in cDNA clones.

Figure 16.

(a) A restriction map of mitochondrial DNA derived as described in the legend to Figure 8.

(b) A restriction map of plasmid pMD417 derived from the gel shown in Figure 15 and other gels and from the restriction map of pBR322 (Bolivar et al., 1977). The thin line represents the mitochondrial DNA insert. The thick line represents pBR322 DNA.

The dotted line represents the large mitochondrial ribosomal RNA.

H HindIII Target site

R Eco.RI target site

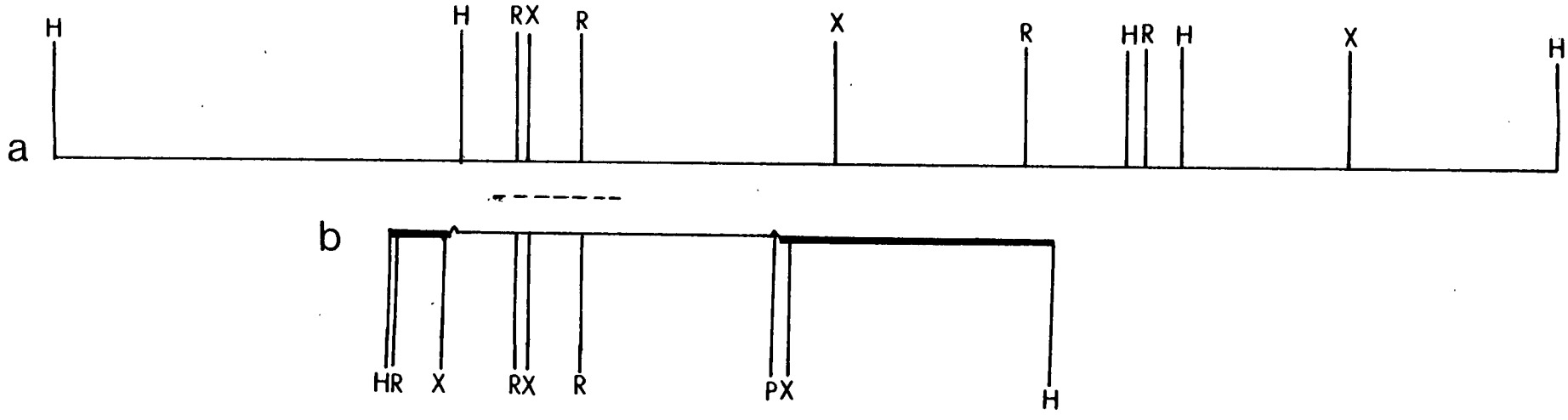
X HaeIII target site

P PstI target site

Λ mitochondrial DNA/plasmid DNA boundary

In b the only HaeIII target sites shown are those flanking the PstI site of pBR322 and the one in mitochondrial DNA.

The size of pMD417 is 5.0Md. The sizes of various fragments of pMD417 are shown in Figure 15.



1Mdal

Fig.16

Translation of Messenger RNA from Drosophila in a Message Dependent Cell Free Translation System from Rabbit Reticulocytes

Introduction

The proteins encoded by mRNA populations, or partially purified mRNAs can be analysed by translating the mRNAs *in vitro* and resolving the proteins or polypeptides synthesised. This technique can be used to assay the complexity of mRNA populations, since an abundant mRNA will show an abundance of the polypeptide for which it codes. The technique can also be used to determine the polypeptide encoded by cloned cDNA or genomic DNA sequences. A cloned DNA sequence may hybridize to, and block the translation of a mRNA containing a complementary sequence (Paterson *et al.*, 1977). Alternatively, cloned DNAs can be used to purify their complementary DNAs by hybridization. Translation of the mRNA purified in this way shows which protein the cloned DNA codes for (Lewis *et al.*, 1975; Kindle and Firtel, 1978). Of the many cell free translation systems available the nuclease treated reticulocyte lysate of Pelham and Jackson (1976) translates added mRNA the most efficiently and has the lowest endogenous mRNA activity.

Translation of Drosophila mRNA in the MDL

I was interested, primarily, in examining the translation products of mRNA from a single tissue, Drosophila third instar larval fat body. However, since only limited quantities of this mRNA can be routinely prepared, I decided to use total larval mRNA to calibrate the translation system.

Initially, I tried to translate cytoplasmic poly(A) containing RNA from Drosophila larvae in the MDL. This RNA was prepared by homogenising fresh larvae in the presence of RNAase inhibitors

(polyvinyl sulphate and spermine), spinning out the nuclei and extracting the supernatent by the Kirby-phenol method (Kirby, 1965; Parish and Kirby, 1966). This was the method of choice since large yields of undegraded RNA can be obtained routinely. Figure 17 shows the sucrose density gradient profile of RNA made by this method. 30mg of RNA yielding 3-400µg of poly(A) RNA can be prepared from 8g of larvae. When this poly(A) RNA was used as a template in the MDL little, if any, protein synthesis was detected.

When purified globin mRNA was translated in the MDL in the presence of tritiated amino acids it stimulated incorporation by about 100-fold (Fig. 18). However when globin and Drosophila cytoplasmic mRNA were added to the lysate together a 10-fold reduction in amino acid incorporation into globin was observed. It seems from this result that Drosophila RNA, or a constituent of the cytoplasmic RNA preparation is inhibiting protein synthesis in the reticulocyte lysate. Cytoplasmic poly(A) RNA was further purified by

(1) Sucrose density gradient centrifugation

(2) Further cycles of oligo(dT) cellulose chromatography

(3) Washing with 3M CH_3COONa (pH 6) (Palmiter, 1973)

but none of these treatments removed its inhibitory effect on the reticulocyte lysate.

It was important to determine whether Drosophila poly(A) RNA was inhibiting the lysate or whether a protein synthesis inhibitor was introduced during the RNA preparation. To this end I prepared polysomes from Drosophila tissue culture cells growing at 25°C by the method described by McKenzie *et al.* (1975). The polysome peaks from a sucrose gradient were pooled and RNA prepared from them by Kirby-phenol extraction. I obtained insufficient material to purify

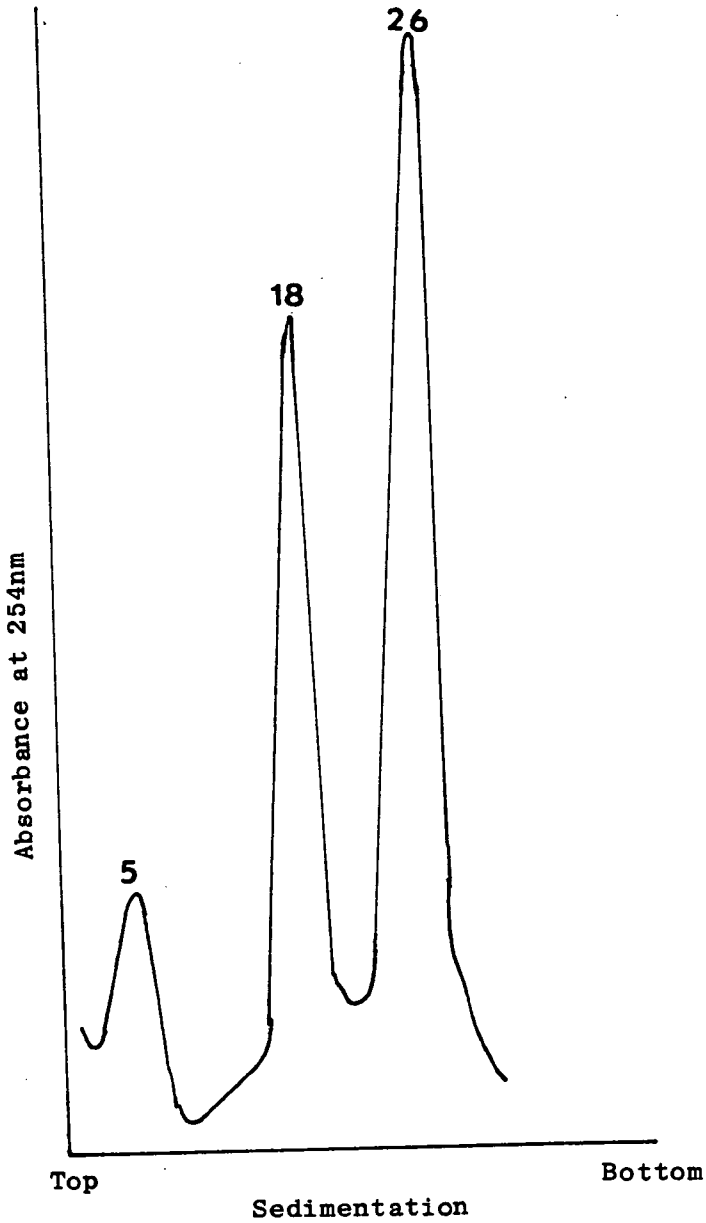


Figure 17.

Sedimentation profile of cytoplasmic RNA from Drosophila third instar larvae. RNA was prepared from the cytoplasm of Drosophila third instar larvae by the Kirby phenol extraction procedure. 100µg of RNA in 0.1M NaCl, 1mM EDTA, 5mM Tris (pH 7.5), 0.5% SDS (NETS) were loaded onto a 13ml gradient of 15% - 30% sucrose in NETS. Centrifugation was at 25K r.p.m. for 16 hours in a Beckmann SW40 rotor. Absorbance at 254nm was monitored by pumping the gradients through a continuous flow cell spectrophotometer (ISCO).

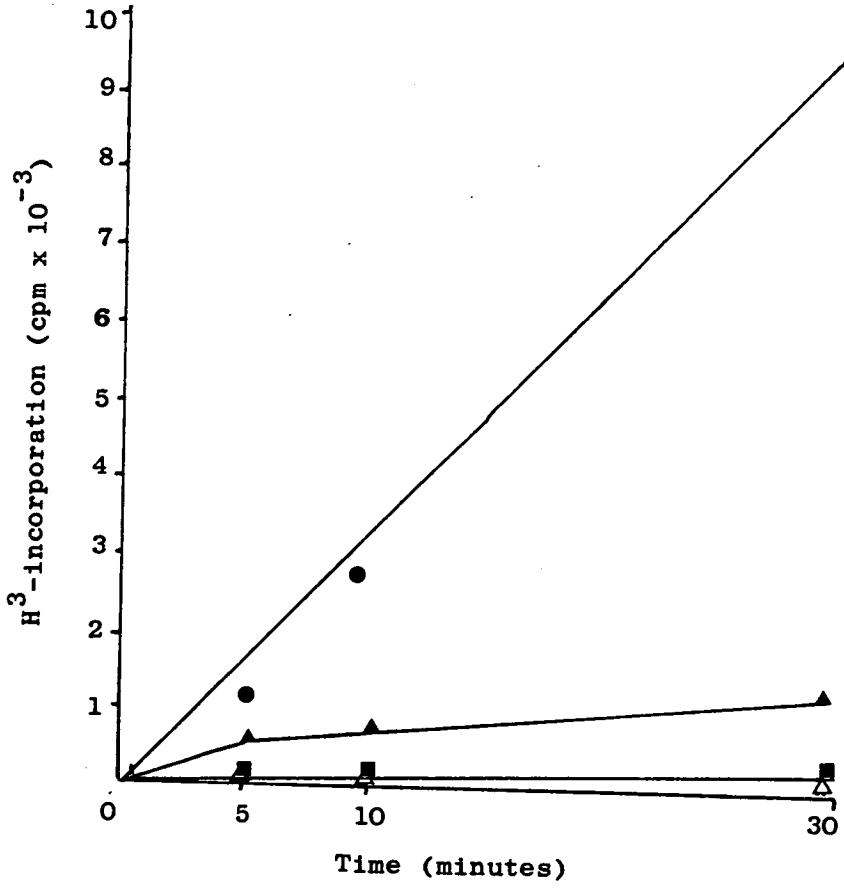


Figure 18.

Translation of Drosophila cytoplasmic poly(A) RNA in the MDL. Aliquots of MDL were made 50μM in all 20 amino acids and 50μCi/ml in a H^3 amino acid mixture and RNA added as follows

△ no RNA added

● 20μg/ml rabbit globin mRNA

■ 40μg/ml Drosophila cytoplasmic poly(A) RNA

▲ 20μg/ml globin RNA and 40μg/ml Drosophila cytoplasmic poly(A) RNA

Incubation was at 30°C. The graph shows the counts per minute incorporated in 5μl of MDL.

the poly(A) RNA. Total polysomal RNA was washed with 3M CH_3COONa (pH 6) and translated in the MDL in the presence of 40µCi/ml of a H^3-amino acid mixture.

RNA	c.p.m. in 5µl lysate after time t @ 30°C		
	t = 0'	t = 10'	t = 30'
-	159	146	170
20µg/ml globin RNA	146	2743	7409
500µg/ml polysomal RNA (unwashed)	139	147	146
250µg/ml polysomal RNA (washed)	131	323	580
500µg/ml polysomal RNA (washed)	112	353	706
250µg/ml polysomal RNA (washed) + m 20µg/ml globin RNA	125	2072	4455

The results show that polysomal RNA stimulates protein synthesis in the lysate which is almost linear for 30'. The presence of Drosophila polysomal RNA does not greatly inhibit translation of globin mRNA. The lack of protein synthesis in the aliquot containing unwashed RNA is due to the presence of heparin, used in the polysome preparation as a RNAase inhibitor, which is soluble in 3M CH_3COONa (pH 6) (Palmiter, 1973). It seems, therefore, that the inhibition observed with Drosophila cytoplasmic RNA is neither a property of Drosophila mRNA, nor of the Kirby phenol extraction procedure.

I decided, therefore, to prepare Drosophila RNA by a method known

to yield translatable RNA from other sources. The method I used was essentially that described by Strohman et al. (1977) which is based on that of Cox (1968). Fresh larvae were homogenised in 6M guanidinium chloride and total RNA was purified by two ethanol precipitations, a chloroform butanol extraction and a high salt precipitation. Poly(A) RNA was obtained by oligo(dT) cellulose chromatography. The yield of poly(A) RNA by this method was very low compared with other methods. 8g of larvae gave about 3mg of RNA which yielded about 80µg of poly(A) RNA after two successive purifications by oligo(dT) cellulose chromatography.

This poly(A) RNA was translated in the MDL (Fig. 19). The RNA is translated efficiently at first but after 10 minutes very little more protein is synthesized. The RNA has the same effect on the translation of added globin RNA. It seems that this RNA contains a component which inhibits protein synthesis in the MDL. The kinetics of this inhibition are characteristic of inhibition involving the haem controlled repressor (Legon et al., 1974). Such inhibition can result from lack of haemin (Zucker and Schulman, 1968) or the presence of double stranded RNA (Ehrenfeld and Hunt, 1971) or oxidized glutathione (Kosower et al., 1972) and involves the accumulation of a repressor which phosphorylates the initiation factor eIF2 and prevents reinitiation of translation. Protein synthesis is therefore normal at first but is almost completely inhibited after 10'. Legon et al. (1974) showed that such inhibition can be overcome by including in the lysate unphysiologically high levels of 3'5' cAMP. Figure 20 shows the effect of cAMP on the translation of poly(A) RNA from Drosophila larvae prepared by the guanidinium chloride method. The results show that at cAMP concentrations above 5mM protein synthesis is almost linear for

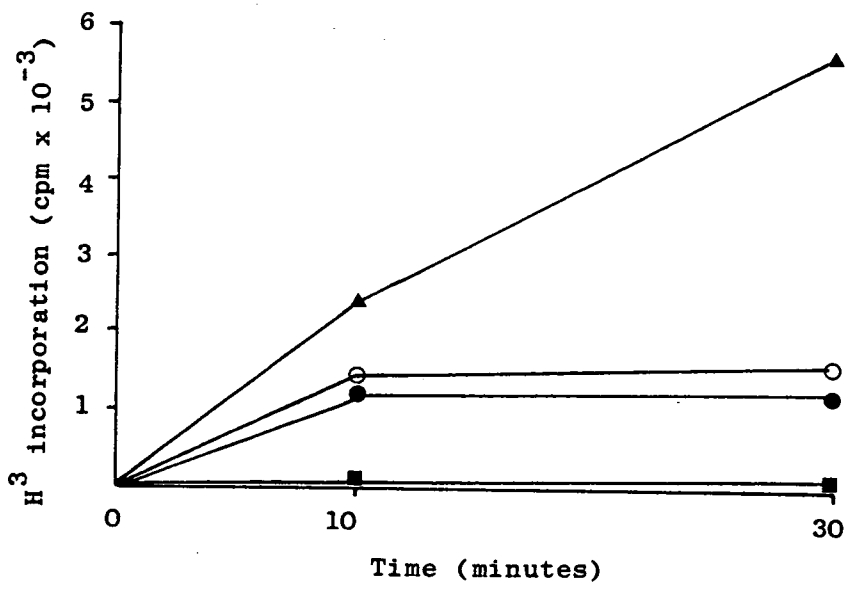


Figure 19.

The translation of larval mRNA prepared using 6M guanidinium chloride.

Aliquots of MDL were made 50µM in all 20 amino acids and 50µCi/ml in a H^3-amino acid mixture and RNA added as follows

■ no RNA added

▲ 20µg/ml rabbit globin mRNA

● 40µg/ml Drosophila poly(A) RNA

○ 20µg/ml globin mRNA + 40µg/ml Drosophila poly(A) RNA

Incubation was at 30°C. The graph shows the counts per minute incorporated in 5µl of MDL.

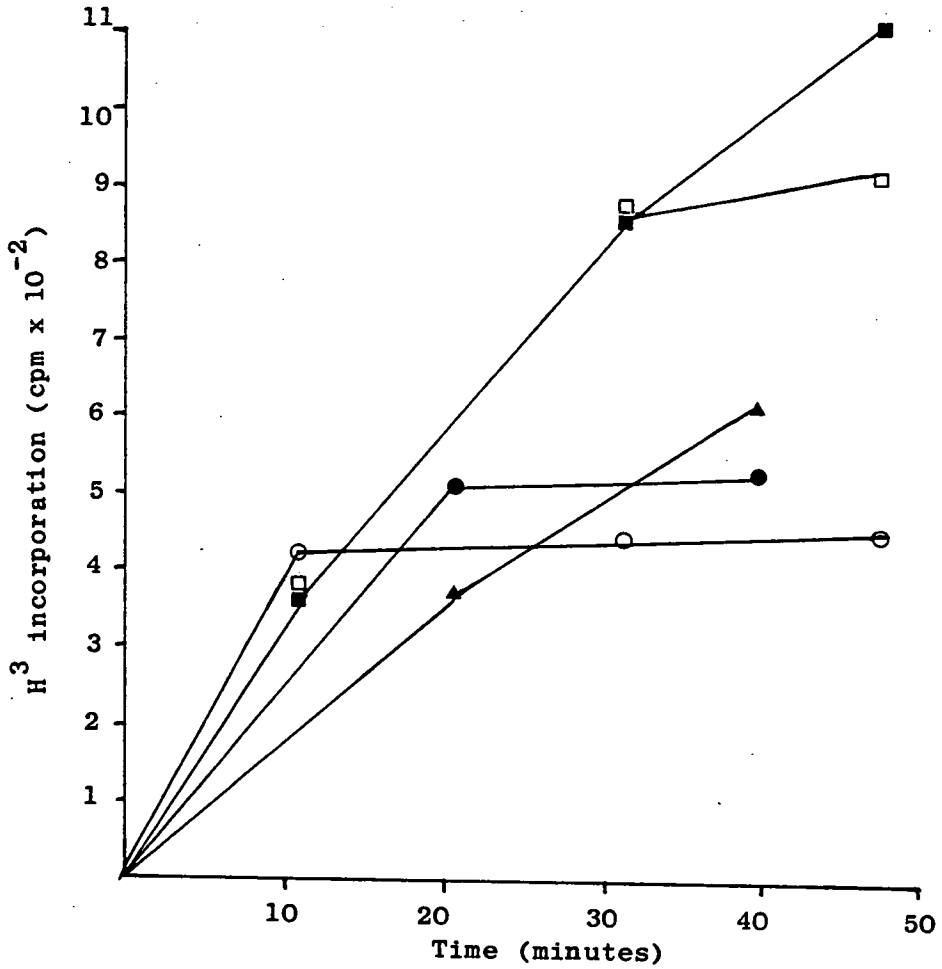


Figure 20.

The effect of 3'5' cAMP on the translation of Drosophila RNA in the MDL.

MDL was made 50μM in all 20 amino acids, 50μCi/ml in H^3 amino acid mixture, and 40μg/ml in Drosophila poly(A) RNA prepared using 6M guanidinium chloride and purified by chromatography using oligo(dT) cellulose. In addition aliquots were supplemented with

- ○ no cAMP
- ● 1.7mM cAMP
- □ 5mM cAMP
- ■ 10mM cAMP
- ▲ 15mM cAMP

Incubation was at 30°C and 5μl aliquots were taken for measurement of radioactivity incorporated.

N.B. Two separate experiments are shown combined here.

about 30'. Above 10mM cAMP incorporation is lower, but remains linear for longer. Accordingly, 7 - 8mM cAMP was included in lysates for the translation of Drosophila RNA.

I also assayed the effect of tRNA on protein synthesis using Drosophila mRNA. A range of concentrations of guinea pig liver tRNA were added to the lysate and incubation carried out as usual. The results (Figure 21) show that tRNA at a concentration of 80µg/ml increases incorporation about 1.5 fold and that at concentrations as high as 400µg/ml it is not inhibitory. 80µg/ml tRNA was included in some of the later translations.

Using RNA made by the guanidinium chloride method, incorporation of radioactive amino acids into protein in the MDL was about one third of that using globin mRNA when H^3-leucine or the H^3 amino acid mixture was used and about two thirds of that using globin mRNA when S^{35}-methionine was the label.

Translation of mRNA from larval Fat Body

Fat bodies were isolated from third instar larvae by grinding the larvae and collecting the material which floats on top of Drosophila Ringers solution after a short low speed centrifugation. Attempts were made to isolate RNA from this material using 6M guanidinium chloride as described above. The low yields which I found associated with this method, however, meant that the amount of poly(A) RNA obtained from 50g of larvae was of the order of a few micrograms.

Therefore, I adopted the method described by Kemp et al. (1978). Fat bodies were collected and pipetted straight into a phenol/lysis mixture and extracted with phenol and chloroform:isoamyl alcohol. The total nucleic acids were then precipitated with ethanol and poly(A)

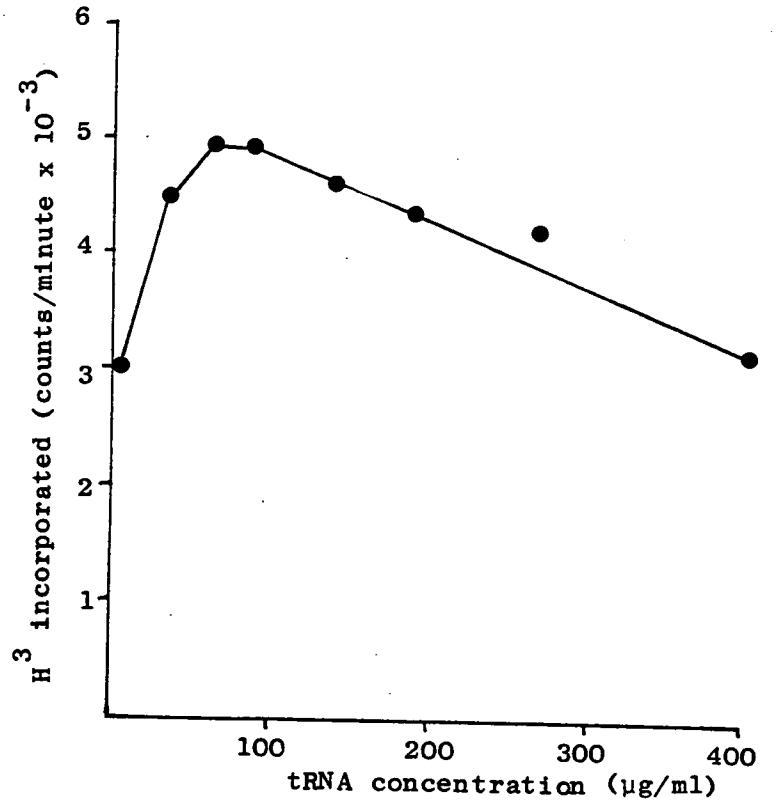


Figure 21.

The effect of tRNA on the translation of Drosophila RNA in the MDL.

MDL was made 40µg/ml in Drosophila poly(A) RNA prepared using guanidinium chloride, 50µM in all 20 amino acids, 50µCi/ml in H^3 amino acid mixture and 8mM in cAMP. Aliquots were supplemented with different amounts of guinea pig liver tRNA and incubation carried out for 30' at 30°C. The graph shows the counts per minute incorporated in 5µl of lysate.

RNA purified by two cycles of oligo(dT) cellulose chromatography. This material again showed dependence on cAMP for efficient translation. The efficiency of translation of this fat body mRNA was less than half that of RNA prepared by the guanidinium chloride method but the yield was considerably better. 30µg of fat body poly(A) RNA were obtained from 50g of third instar larvae.

Analysis of poly(A) containing RNA from Drosophila Third Instar Larval Fat Body

The procedure for cloning and identifying specific mRNA sequences, shown in Figure 5 necessitates the use of cell free translation to identify the proteins encoded by the cloned sequences. My first step, therefore, was to analyse fat body mRNA translation products to determine the variety of proteins coded by the mRNA and also to verify that alcohol dehydrogenase and larval serum protein mRNAs are major components of fat body mRNA, as expected from previous studies (Ursprung et al., 1970; Sekeris and Scheller, 1977; Sekeris et al., 1977; Kemp et al., 1978; Roberts et al., 1977).

Preparation of RNA from Isolated Fat Bodies

Methods for isolating organs in bulk, from insect larvae, have usually been relatively long processes involving grinding, filtration and isopycnic centrifugation steps (Fristrom and Mitchell, 1965; Zweidler and Cohen, 1971; Kemp et al., 1978). Because of the high endogenous nuclease content of third instar larval fat body I wanted to reduce the time taken for tissue isolation to a minimum. The method adopted was to grind about 50g of larvae in a tissue grinder, suspend the ground material in cold Drosophila Ringers solution, and take as fat body tissue the material which floats on the Ringers solution after a short, low speed, centrifugation. This material, when viewed under a microscope, appears to consist mainly of fat body tissue, contaminated with a few Malphigian tubules and pieces of gut.

RNA was extracted from this material by phenol extraction as described by Kemp et al. (1978) and poly(A) RNA was purified by two

Figure 22. Sucrose density gradient centrifugation of Fat Body RNA.

(a) 100µg of RNA extracted from the fat body of third instar larval fat body were dissolved in 0.5ml 0.1M NaCl, 1mM EDTA, 5mM Tris (pH 7.5), 0.5% SDS (NETS) and layered on top of a 13ml gradient of 15% - 30% sucrose in NETS. Centrifugation was at 25K r.p.m. for 16 hours in a Beckmann SW40 rotor. Absorbance at 254nm was monitored by pumping the gradients through a continuous flow cell spectrophotometer (ISCO).

The profile shows a few differences from that of cytoplasmic RNA (Fig. 17). The total RNA shown here will contain substantial amounts of nuclear RNA and DNA.

(b) 100µg of poly(A) containing RNA, prepared by oligo(dT) cellulose chromatography from the RNA preparation shown in (a), was subjected to sucrose density gradient centrifugation in the same rotor as that in (a).

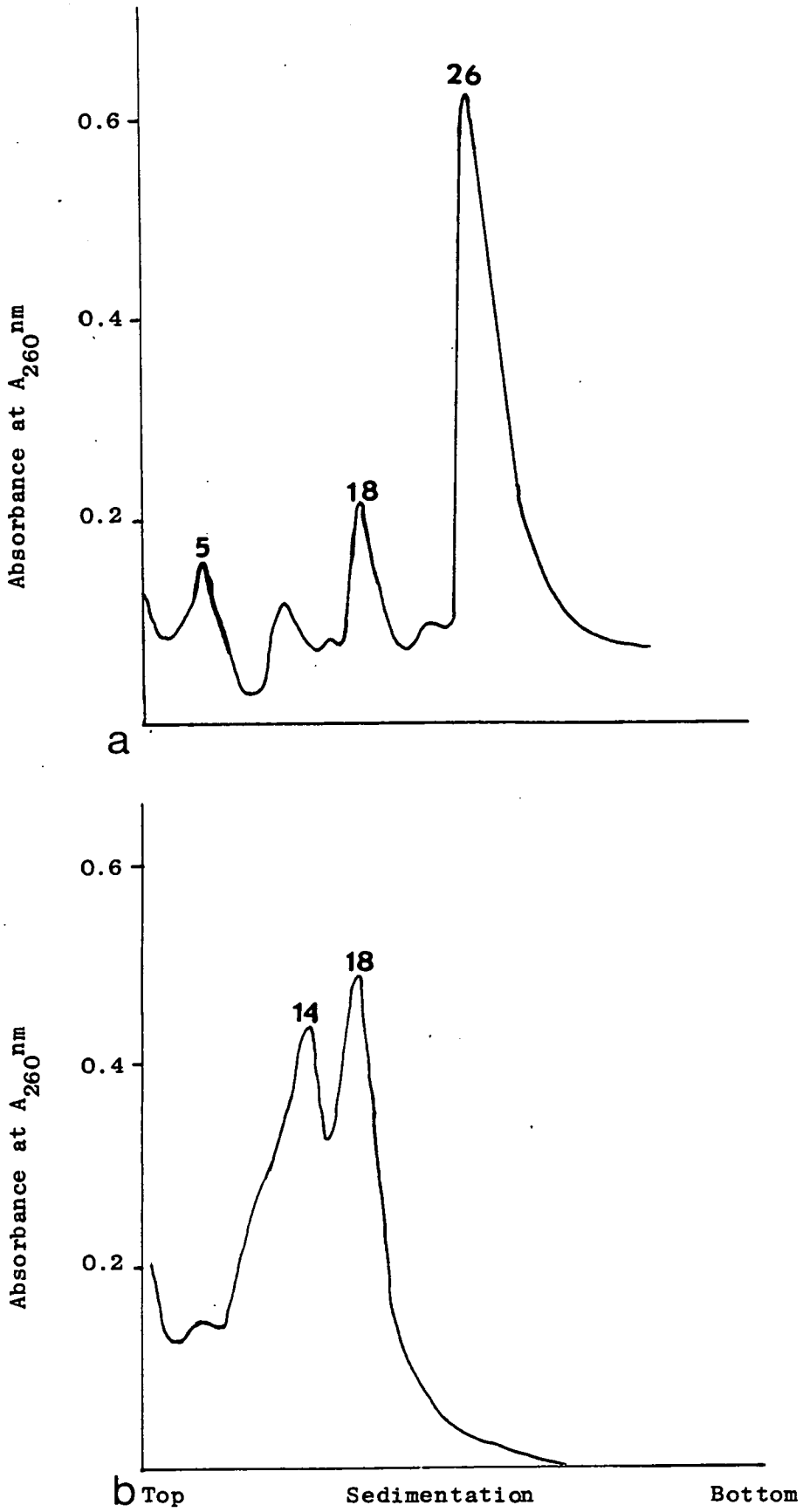

26
0.6
0.4
18
0.2
5
Absorbance at A_{260}^{nm}
a
0.6
18
14
0.4
0.2
Absorbance at A_{260}^{nm}
b Top
Sedimentation
Bottom

Fig.22

cycles of oligo(dT) cellulose chromatography. Figure 22 shows the sucrose density gradient profile of RNA bound and unbound to oligo(dT) cellulose. The bound fraction shows large peaks at 14S and 18S indicating that the poly(A) RNA contains a large proportion of 14S large mitochondrial rRNA, and is still substantially contaminated with 18S rRNA.

The poly(A) RNA was translated in the MDL using S^{35}-methionine as the labelled amino acid. The products of the translation were electrophoresed in a discontinuous SDS polyacrylamide gel (Laemmli, 1970) and the gel dried and subjected to autoradiography. Figure 23 shows the results of this experiment. The most prominent feature of the translation products is a very heavily labelled polypeptide with a molecular weight of about 27,000d with respect to the molecular weight markers used. In addition a group of prominent bands are seen with molecular weights of about 80,000d and five or six other prominent bands are present.

Figure 24 shows the result of an experiment in which fat bodies were hand dissected out of third instar larvae and incubated in the presence of S^{35}-methionine. The patterns of the proteins synthesized *in vivo* and those made by *in vitro* translation are very similar. This shows that the fat body material isolated in bulk consists largely of genuine fat bodies. The main difference is that the larger proteins are not seen in the *in vitro* translation track. This is most likely due to the fact that translation of this fat body RNA in the MDL continues for only 40 minutes which is insufficient time for the completion of long polypeptide chains.

In other gels which are not shown here the products of translation of total larval RNA were analysed and found to contain a more complex

Figure 23.

The translation products of poly(A) RNA from Drosophila third instar larvae. All four tracks are from the same 13% polyacrylamide gel (Laemmli, 1970).

(a) 20,000cpm of S^{35} labelled "heat shock" proteins.

(b) 130,000cpm of S^{35} methionine labelled translation products of fat body poly(A) RNA in the MDL.

(c) 10µg of purified ADH from Drosophila melanogaster.

(d) Serum from one third instar Drosophila larvae.

The conditions for electrophoresis, staining, destaining and autoradiography are described in Methods. (a) and (b) are photographs of X-ray film, developed after being in contact with the dried gel for 4 days. (c) and (d) are photographs of the Coomassie blue stained bands in the dried gel.

The figures at the left of the photograph refer to the molecular weights of the "heat shock" proteins in track (a) in thousands of daltons (from Moran et al., 1978).

Figure 24.

Protein synthesis in fat bodies. The samples were electrophoresed in a 13% polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel was impregnated with PPO (Bonner and Laskey, 1974) and placed next to pre-flashed X-ray film (Laskey and Mills, 1975) and developed after two days at -70°C.

(a) 20,000cpm of S^{35} labelled translation products of fat body poly(A) RNA in the MDL.

(b) 20,000cpm of S^{35} labelled proteins extracted from dissected fat bodies after incubation for 30' at 23°C in 0.03M sodium phosphate buffer (pH 6.8), 0.04M KCl, 0.011M NaCl, 0.003M $CaCl_2$, 0.021M $MgCl_2$ containing 30µCi of S^{35}-methionine.

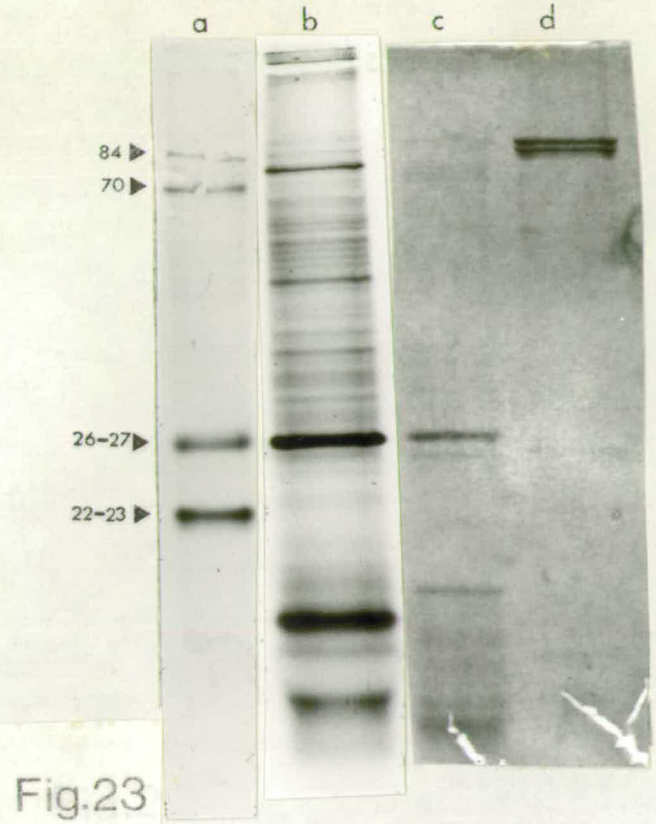


Fig.23

Fig.24

pattern of labelled polypeptides. This indicates that a substantial purification of fat body specific mRNA sequences has been achieved.

Larval Serum Proteins are a Major Product of Fat Body RNA Translation

The major protein of third instar larvae of the dipterans Calliphora and Drosophila is a large protein consisting of six or seven subunits of approximate molecular weight 80,000d (Munn et al., 1971; Roberts et al., 1977). In both species the protein is synthesized in the fat body (Munn et al., 1969; Roberts et al., 1977). Sekeris et al. (1977) and Kemp et al. (1978) showed that poly(A) RNA from fat bodies of Calliphora larvae could be translated into this major protein, calliphorin, in a cell free translation system. Sekeris et al. (1977) were also able to show that poly(A) RNA from Drosophila larvae was translated to give a protein which could be precipitated by antibodies to calliphorin.

Work in the laboratory of Dr David Roberts has shown that two abundant proteins, larval serum proteins (LSPs) 1 and 2 are synthesized in the fat body of Drosophila third instar larvae and secreted into the haemolymph. LSP1 is the more abundant protein and is analogous to calliphorin; it gives three bands in SDS gels of approximate molecular weights 72 - 80,000d. LSP2 gives a band of molecular weight 75000d(Roberts et al.,1977). I was interested in identifying these polypeptides amongst the products of translation of fat body poly(A) RNA. Larval serum was withdrawn from three or four third instar larvae, boiled in SDS sample buffer, and run on a gel alongside the translation products of fat body poly(A) RNA. The result is shown in Figure 23. A number of the major bands of the translation products co-migrate with the major Coomassie blue stained bands found in the larval serum. The molecular weights of these bands, compared with

the markers used is 75 - 80,000d. These bands, therefore, are tentatively identified as polypeptide chains of LSP1 and LSP2. The resolution of bands in this region of the gels varied, in some gels, e.g. Figure 26, two heavily labelled bands are resolved from the one seen in Figure 23 (track b).

Alcohol Dehydrogenase is a Major Product of Fat Body RNA Translation

Ursprung et al. (1970) showed that most of the alcohol dehydrogenase (ADH) activity in Drosophila larvae is present in the fat body or in tissues associated with fat body. It seems likely, therefore, that ADH is synthesized in fat body. Drosophila ADH is a hexamer of subunits of molecular weight about 26,000d which contains no methionine (D. Thatcher personal communication). This introduced two problems; firstly S^{35}-methionine could not be used to label ADH - the only practical alternatives are tritiated amino acids and tritium is about 10 times less efficient in fluorography than S^{35}; secondly, 26,000d is very close to the molecular weight of the most heavily labelled polypeptide seen in translations of total larval RNA, fat body RNA, or after in vivo incubation of fat bodies, when S^{35}-methionine is used as the label (Figs. 23 and 24). Therefore, I initially asked if ADH could be resolved in Laemmli gels when amino acids other than methionine were used to label the proteins.

Third instar larvae were gently opened and about 20μCi of a mixture of tritiated amino acids in Drosophila Ringers solution were introduced. Two larvae at a time were bathed in this solution and then the proteins were extracted and subjected to electrophoresis and fluorography. The experiment was performed on wild type larvae of the strain used in this study and on larvae of a strain homozygous

for the mutation ADH n^2 (Schwartz and Sofer, 1976). ADH n^2 larvae have been shown to contain no protein which cross reacts with antibodies made against Drosophila ADH. The result, shown in Figure 25, shows that a band running slightly slower than the 27,000d "heat shock" band is present in wild type larval proteins but not in proteins from ADH n^2 larvae. This suggests that ADH is made in relatively large quantities by third instar larvae; secondly, the protein that is most heavily labelled with S^{35}-methionine is much less conspicuous when other amino acids are used as the label.

In another experiment fat body RNA was translated using H^3-leucine as the labelled amino acid and the products were electrophoresed alongside S^{35}-methionine labelled products. Figure 26 shows that at the molecular weight of about 27,000d the leucine labelled band, though prominent is not so heavily labelled as the band at the position labelled with methionine. This suggests that the 27,000d protein seen as the major translation product when methionine is the label is of moderate abundance but is very rich in methionine residues. It leaves open the possibility that a significant part of the 27,000d band labelled with leucine consists of ADH.

To confirm the presence of ADH in fat body RNA translation products the protein was immunoprecipitated from the MDL after translation.

Highly purified ADH from Drosophila melanogaster ADH n^{11} flies (Schwartz and Sofer, 1976) was a gift from David Thatcher. Antisera to this protein were raised in rabbits.

Fat body RNA was translated in a large volume of MDL using H^3-leucine as the radioactive label. After ribosomes had been removed by centrifugation, aliquots of the MDL were incubated with various

Figure 25.

The newly synthesized proteins of wild type and $ADHn^2$ larvae of Drosophila melanogaster.

The photograph shows the fluorograph of a 13% polyacrylamide SDS gel (Laemmli, 1970). The tracks are

(a) 5,000cpm of S^{35}-methionine labelled Drosophila "heat shock proteins.

(b) 30,000cpm of H^3-amino acid labelled newly synthesized proteins of $ADHn^2$ third instar larvae.

(c) 30,000cpm of H^3-amino acid labelled newly synthesized proteins of wild type third instar larvae. Exposure was for one week at $-70^{o}C$.

The preparation of the samples is described in Methods. The arrow to the right of the photograph indicates the position of a band in track (c) which is absent in track (b). The figures at the left indicate the molecular weights of the bands in track (a) in thousands of daltons.

Figure 26.

S^{35}-methionine and H^3-leucine labelled translation products of third instar larval fat body poly(A) RNA.

The photograph shows the fluorograph of a 13 - 20% gradient polyacrylamide SDS gel (Laemmli, 1970). The tracks are

(a) 5,000cpm of S^{35}-methionine labelled translation products of fat body poly(A) RNA.

(b) 30,000cpm of H^3-leucine labelled translation products of fat body poly(A) RNA.

(c) 3,000cpm of S^{35}-methionine labelled "heat shock" proteins.

The preparation of the samples and the conditions for electrophoresis and fluorography are described in Methods.

The figures at the right indicate the molecular weights of the bands in Track (c) in thousands of daltons.

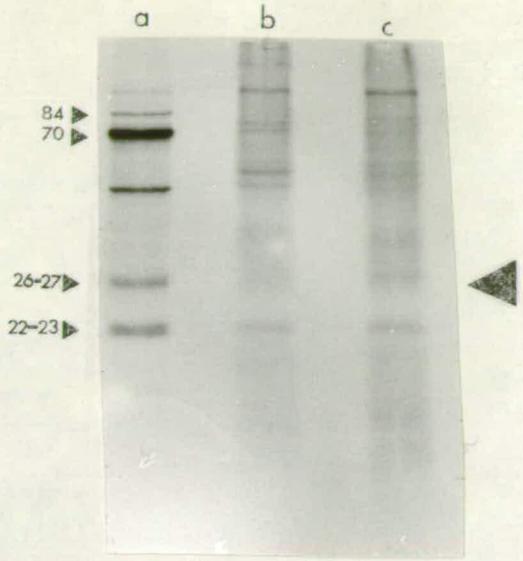


Fig.25

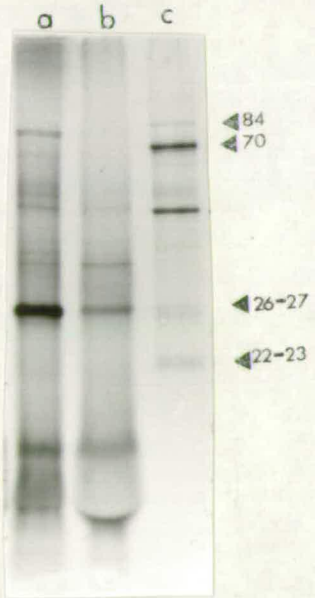


Fig.26

concentrations of antiserum and the antibody/antigen complexes were collected using formaldehyde treated Staphylococcus aureus cells essentially as described by Kessler (1975).

A total of 4.0×10^6 cpm of H^3 were incorporated into protein in this experiment. 3.2×10^6 cpm remained in the supernatant after ribosomes had been removed. One twelfth aliquots (2.6×10^5) cpm were then incubated with serum, and treated with Staphylococcus aureus cells as described in Methods. The final, washed, Staphylococcus aureus cells were bolied in 50µl of SDS electrophoresis sample.buffer and centrifuged in a microcentrifuge and 5µl of the supernatant was TCA precipitated and the precipitated radioactivity measured as described.

a Serum concentration	b cpm immuno-precipitated	c Percentage immuno-precipitated = $= \frac{(b-245) \times 10}{2.6 \times 10^5} \times 10^2$
0	245	0%
neat	1066	3.2%
½	1059	3.1%
¼	1285	4.0%
1/16	770	2.0%
1/64	556	0.8%

The amount of radioactivity sedimenting with the S. aureus cells depended upon the concentration of antiserum used and was, at the optimum antiserum concentration, 4% of the total radioactivity incorporated. The immunoprecipitate was electrophoresed and the gel

Figure 27.

Immunoprecipitation of H^3-leucine labelled translation products of fat body mRNA with anti-ADH sera.

The preparation of antisera and the conditions for immunoprecipitation, electrophoresis and fluorography are described in 'Materials and Methods'. The gel was a 15% polyacrylamide SDS gel. The tracks are

(a) and (e) 1,000cpm of S^{35}-methionine labelled "heat shock proteins.

(b) 5,000cpm of H^3-leucine labelled translation products precipitated with anti-ADH serum.

(c) 40,000cpm of total translation products labelled with H^3-leucine.

(d) 1,000cpm of translation products labelled with H^3-leucine and precipitated with normal rabbit serum.

The gel was impregnated with PPO as described and placed next to pre-flashed X-ray film at -70°C for one week (Laskey and Mills, 1975).

Figure 28.

Immunoprecipitation of H^3-leucine and S^{35}-methionine labelled translation products of fat body mRNA with anti-ADH sera. Samples were

(a) and (e) 33,000cpm of S^{35}-methionine labelled translation products.

(b) 6,000cpm of H^3-leucine labelled translation products immunoprecipitated with anti-ADH sera.

(c) 40,000cpm of H^3-leucine labelled translation products.

(d) 7,000cpm of S^{35}-methionine labelled heat shock "proteins".

(f) 4,000cpm of S^{35}-methionine labelled translation products immunoprecipitated with the same anti-ADH serum as that used for sample (b).

(g) 3,000cpm of S^{35}-methionine labelled translation products immunoprecipitated with anti-ADH serum from a second rabbit.

Samples were electrophoresed in a 13% - 20% gradient polyacrylamide SDS gel which was impregnated with PPO and exposed to pre-exposed X-ray film at -70°C for one day (Laskey and Mills, 1975).

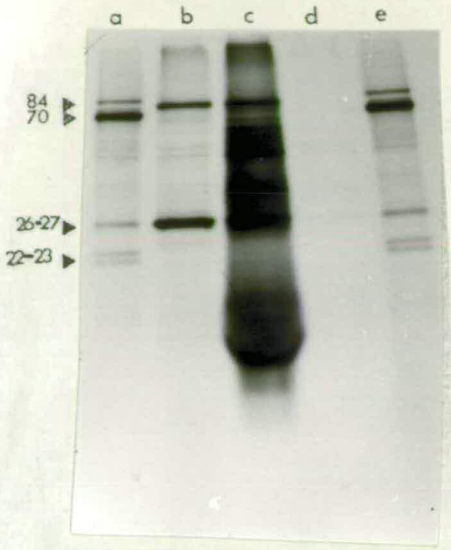


Fig.27

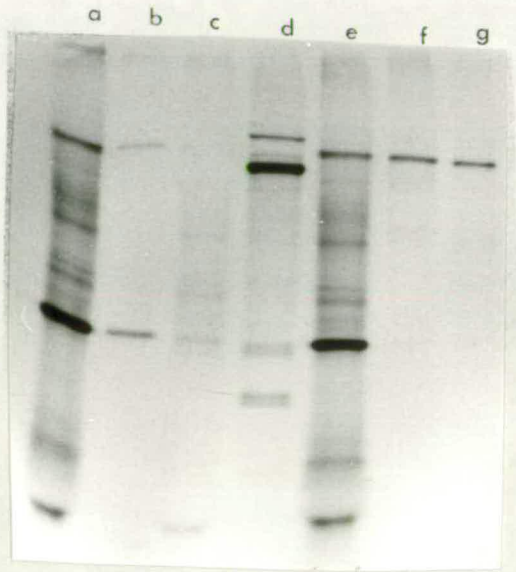


Fig.28

subjected to fluorography. The result is shown in Figure 27. Two bands can be seen in the immunoprecipitate of approximate molecular weights 27,000d and 80,000d. The experiment was repeated using S^{35}-methionine as the radioactive label. In this experiment, at the optimum antiserum concentration, 2.5% of the total counts incorporated sedimented with the Staphylococcus aureus cells. The electrophoresis of this immunoprecipitate is shown in Figure 28. The 80,000d band is labelled by methionine whereas the 27,000d band is not. The presence of a 27,000d polypeptide, specifically precipitated by antiserum to ADH and containing no methionine is good evidence that this band represents the ADH subunit. The band at 80,000d may be a protein which is a minor contaminant in the ADH preparation but which is a relatively abundant product of fat body mRNA translation.

From Figures 27 and 28 it can be seen that most of the leucine which is precipitated by ADH antisera is in the 27,000d polypeptide. However I do not know if all the ADH in the MDL was precipitated out or only a proportion of it, nor have the losses during the washing of the S. aureus cells been measured. We can conclude, therefore, that ADH is a major translation product of fat body mRNA, accounting for at least 3% of leucine incorporation.

Analysis of Fat Body Poly(A) RNA by Nucleic Acid Hybridization

Cell free translation studies suggest, therefore, that fat body poly(A) RNA codes for about 10 abundant polypeptides. Five of these have been identified; four, tentatively as constituents of the larval serum proteins and one as alcohol dehydrogenase. That the probability of obtaining cDNA clones containing the sequences coding for these polypeptides will be relatively high depends upon the

assumption that proteins made in abundance are encoded by mRNAs present in the cell in relatively high concentrations (and also upon the fact that the *in vitro* gene synthesis and cloning procedures do not favour certain sequences - this will be discussed later).

Correlation between abundant proteins and abundant mRNAs seems to be a general phenomenon (e.g. Paterson and Bishop, 1977; Hastie and Held, 1978). The complexity of poly(A) RNA populations can be estimated by mRNA-cDNA hybridization in mRNA excess (Bishop *et al.*, 1974).

Poly(A) RNA from fat body tissue of third instar larvae was used as the template for the enzyme reverse transcriptase and tritiated complementary DNA (cDNA) was isolated. This was then hybridized with an excess of the same RNA which was used as the template. The extent of hybridization after various times was measured by assay with S1 nuclease. Figure 29 shows the result of the experiment and the mRNA complexity calculated from the hybridization data are shown below.

Abundance class	Percentage of mRNA *1	Corrected $R_o t_{\frac{1}{2}}$ *2	No. of 6×10^5 dalton sequences
1	40	0.72×10^{-3}	1
2	60	1.6×10^{-1}	200

*1 Observed percentage S1 resistance normalized to 100% hybridization.

*2 Observed $R_o t_{\frac{1}{2}}$ values corrected for the dilution resulting from the presence of sequences in the other abundance class.

The theory underlying mRNA-cDNA hybridization kinetics is outlined in 'Materials and Methods'. Briefly, the curve obtained is a computer

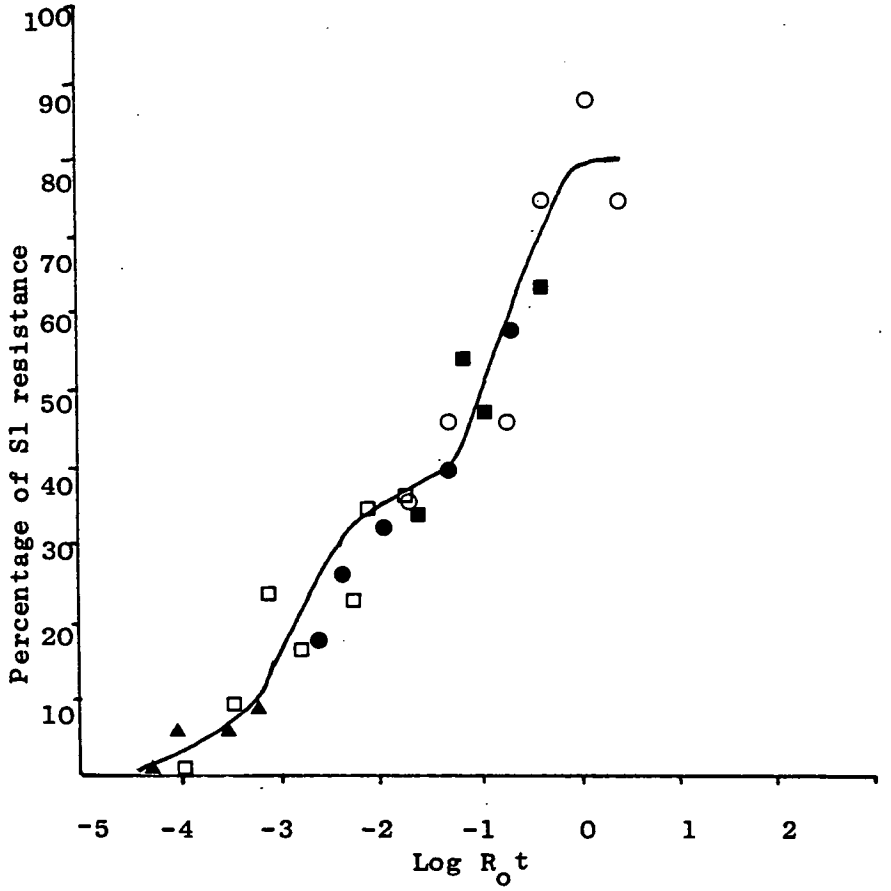


Figure 29.

Hybridization kinetic analysis of fat body poly(A) RNA. Poly(A) RNA from third instar larval fat body was used as a template for the enzyme reverse transcriptase and tritiated cDNA isolated. A trace of labelled cDNA was hybridized with a vast excess of the same RNA used as the template. Aliquots were removed after various times of annealing and the percentage of cDNA in hybrid determined with the use of single stranded specific nuclease S1.

$R_o t$ = original RNA concentration x time.

The curve was constructed using data from five separate hybridization experiments.

fit to the actual data points, based on the assumption that the data can be fit by a small number of ideal first order reactions. The computer selects the number of kinetic components which gives the lowest sum of squares of deviations (ssd) of the data points from the calculated curve. Many mRNA populations in eukaryotes are adequately described as consisting of two or three such kinetic components, or abundance classes (Bishop et al., 1974; Hereford and Rosbash, 1977; Axel et al., 1976).

The first transition in Figure 29 represents a single RNA species accounting for 40% of the mass of cDNA (and, by inference, of RNA, see Bishop et al., 1974). This is almost certainly due to 14S mitochondrial rRNA which has been found to be the major component of poly(A) RNA from Drosophila cells and whole animals (Izquierdo, 1976; Alwine et al., 1977; Spradling et al., 1977).

No evidence for a class of about 10 abundant mRNAs can be obtained from this hybridization data. Hybridization occurs over five logs of $R_o t$ which indicates that mRNA sequences at a number of different concentrations are present. The curve shown, containing two kinetic components is the best fit to the data but the ssd value at 0.08 is large and implies that the actual data deviate considerably from the curve drawn. This may mean that, apart from 14S RNA, the mRNAs are not organised into discrete abundance classes but exhibit a more continuous distribution.

The Proportion of 14S specific cDNA in Fat Body Poly(A) RNA cDNA

The $R_o t$ analysis described above gives a figure of 40% for the proportion of cDNA made against fat body poly(A) RNA which is 14S RNA specific. This figure is important in that it gives an estimate

of the probability of any cDNA clones containing mitochondrial DNA sequences after following the procedure shown in Figure 5. In addition, this proportion was measured by hybridization of fat body cDNA with an excess of plasmid pMD417 DNA. This plasmid contains the entire coding sequence for the 14S large mitochondrial rRNA.

Plasmid DNA from pBR322 and pMD417 was sonicated to a length of about 400 base pairs, denatured and hybridized with a trace amount of tritiated fat body cDNA. The amount of cDNA in hybrid after annealing for an appropriate time (until plasmid DNA was totally re-annealed) was determined by assay with S1 nuclease or by hydroxylapapatite(HAP)chromatography.

Plasmid	% S1 resistance	% eluting from HAP in 0.4M phosphate buffer
pBR322	5%	2%
pMD417	35%	27%

The value obtained from both assays is that about 30% of fat body cDNA forms double stranded structures with pMD417 DNA. This figure is likely to be an underestimate because small regions of hybrid, e.g. with cDNAs of less than 50 base pairs, may be dissociated under the 65°C, 0.12M phosphate buffer conditions used to elute single stranded DNA from HAP. This is especially likely since large mitochondrial rDNA has a high A-T (82%) composition (Klukas and Dawid, 1976). Similarly, hybrid regions of high A+T composition form relatively unstable duplexes and may be digested with S1 nuclease. The $R_o t$ data partially, but not completely, overcome this problem because the value obtained is the mass of cDNA in the first transition as a

percentage of the mass of cDNA which finally becomes hybridized. Thus any loss of 14S specific duplex is compensated for to some extent. We can conclude that a large proportion, probably greater than 40%, of cDNA made against fat body poly(A) RNA contains sequences of mitochondrial rDNA.

Cloning Fat Body cDNA sequences

The procedure for producing large (milligram) quantities of faithful DNA copies of sequences represented in mRNA has been described (Efstratiadis et al., 1976; Maniatis et al., 1976; Monahan et al., 1976; Rougeon and Mach, 1976). The procedure involves a series of enzymatic steps wherein mRNA is copied into complementary DNA, this is made double stranded and then inserted into a bacterial plasmid. Such plasmids are used to transform laboratory strains of the common enteric bacterium E. coli. The transformed E. coli cells can be cloned and milligrams of plasmid DNA extracted from them. In this way it is possible to purify a large quantity of a DNA sequence complementary to a single mRNA species (Fig. 5).

I used this procedure to prepare a number of such cloned recombinant plasmids, using Drosophila melanogaster third instar larval fat body poly(A) containing RNA as the initial template. The exact conditions used for each step are described in 'Materials and Methods'.

Preparation of cDNA

The enzyme, reverse transcriptase, from avian myeloblastosis virus will synthesize DNA molecules complementary to a RNA template in the presence of a DNA primer (Verma et al., 1972; Kacian et al., 1972; Ross et al., 1972). For copying poly(A) RNA, oligo(dT) is used as a primer; it binds to the poly(A) sequence at the 3' end of the RNA and the cDNA is synthesized in a 5' to 3' direction.

The template I used was the fat body poly(A) RNA, some of whose properties were described above. The reaction mixture contained high levels of all four deoxyribonucleotides, a condition which has been shown to favour the synthesis of longer transcripts (Efstratiadis

et al., 1975). Actinomycin-D was included in the reaction - this inhibits the formation of a second new DNA strand by the enzyme using the first cDNA strand as a template (Ruprecht et al., 1973).

Synthesis of cDNA was monitored by including in the reaction P^{32}-labelled dCTP and measuring the incorporation of P^{32} into TCA precipitable material. From 50µg of poly(A) RNA in the reaction, 5µg of cDNA were synthesized. Since the poly(A) RNA has been shown to contain a substantial amount of 18S ribosomal RNA, which will not serve as a template for reverse transcriptase, these figures lead to an underestimate of the efficiency of the reaction.

The reaction mixture was treated with sodium hydroxide to hydrolyse the RNA, extracted with phenol to remove the enzyme and chromatographed on Sephadex G-50 to remove the unincorporated nucleotide triphosphates.

The length of the cDNA was determined after it had been made double stranded.

Synthesis of the Second DNA Strand

The enzyme, DNA polymerase I from E. coli (pol I) will catalyse the addition of mononucleotide units to the 3' hydroxyl terminus of a primer DNA chain. There is an absolute requirement for a DNA template (Kornberg, 1969). It has been found (Efstratiadis et al., 1976; Rougeon and Mach, 1976) that the cDNA product of the reverse transcriptase reaction will serve as a template for pol I without the need for any exogenous primer. This is thought to be due to the fact that the cDNA strand hooks over at its 3' end. When the first reaction is carried out in the absence of Actinomycin D short hairpins of double stranded DNA are found in this region (Efstratiadis et al., 1976). In the presence of Actinomycin D, presumably the

region of double stranded DNA is only one or two bases long, but sufficient to prime second strand synthesis.

I used pol I to make a second DNA strand complementary to the cDNA. The reaction was followed by using tritiated dCTP as one of the deoxyribonucleotide triphosphates. About 30% of the cDNA made in the first reaction was made double stranded in the pol I reaction.

The product of this reaction, then, is a length of double stranded DNA, the two strands of which are covalently joined at one end. The molecules will also contain projecting 5' ends - representing regions of cDNA which were not copied by pol I. Treatment with the single strand specific nuclease S1 should break this hook and remove the projecting 5' ends.

The product of the pol I reaction was extracted with phenol, chromatographed on Sephadex G-50, and then digested with S1 nuclease. As expected more than half of the P^{32} counts (first strand) were digested by S1 whilst most (80%)of the H^{3} counts remained TCA precipitable.

The Size of In Vitro Synthesized Double Stranded DNA

In order to select the larger double stranded DNA molecules for cloning, half of the S1 treated DNA was run on a native 5% - 20% sucrose gradient, and the gradient fractionated. Fractionation of the DNA by this method was not very good, the gradient consisted of a single peak near the top of the tube. The fractions were pooled in two groups representing the larger and smaller parts of this peak. Samples of these two fractions were then subjected to polyacrylamide gel electrophoresis and autoradiography. Figure 30 shows the photograph of a gel containing the size fractionated DNA and molecular weight markers. The film was scanned with a densitometer and the

number average size of each DNA fraction was calculated from the trace. These were 880 base pairs for the larger DNA and 750 base pairs for the smaller.

The outstanding feature of the gel shown in Figure 30 is that a discrete band representing DNA of length about 1200 base pairs is present in the pattern of the larger DNA fraction. Discrete size classes of cDNA, representing partial copies of rabbit globin mRNA, have been shown to be generated by reverse transcriptase, and presumably represent preferential sites for termination by the enzyme (Efstratiadis et al., 1975). Presumably the 1200 base pair molecule seen here arose by this mechanism and is a copy of an abundant poly(A) containing species - probably the 14S large mitochondrial ribosomal RNA. The double stranded DNA used in subsequently described experiments is part of the same batch described above, but was not fractionated on sucrose gradients, nor sized on gels. It is assumed to have a number average length of 800 base pairs.

Addition of Polyadenylic Acid Tails to Double Stranded cDNA

The strategy used to incorporate double stranded DNA molecules into bacterial plasmids by the dA dT joining method has been described (Jackson, Symons and Berg, 1972; Lobban and Kaiser, 1973; Wensink et al., 1974). The enzyme terminal transferase from calf thymus is used to add a string of deoxyadenosine residues onto the 3' ends of the DNA to be inserted. The plasmid vector DNA is linearized by the use of a restriction enzyme and is similarly tailed, but with thymidine residues. If the two molecules are annealed together they will form circular DNA molecules containing the entire complement of DNA from the plasmid and the 'insert' DNA. Such hybrid molecules can be used to transform E. coli cells which will grow and reproduce.

Figure 30.

The size of *in vitro* synthesized Double Stranded DNA. P^{32}-labelled double stranded DNA was prepared using poly(A) RNA from fat body tissue as the original template. The DNA was centrifuged in a 5% - 20% sucrose gradient as described and two size fractions collected. DNA from these fractions was electrophoresed in a 7.5% polyacrylamide gel for 2 hours at 150V after which the gel was dried and placed next to preflashed X-ray film using calcium tungstate intensifying screens. Exposure was for 3 days.

The tracks were

(a) and (d) 500cpm of P^{32}-labelled HaeIII restricted pMB9 DNA.

(b) 500cpm of the larger fraction of double stranded cDNA.

(c) 300cpm of the smaller fraction of double stranded cDNA.

The figures to the left of the photograph refer to the sizes of DNA in the bands in tracks (a) and (d) in base pairs.

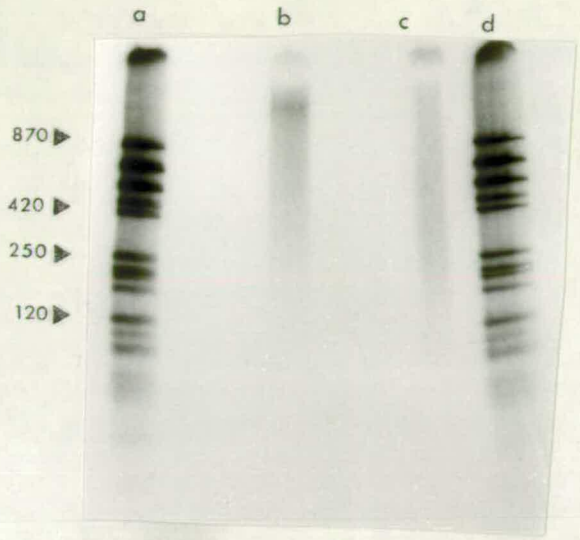


Fig.30

Half of the double stranded DNA prepared as above was incubated with terminal transferase in the presence of P^{32}-labelled dATP. The average number of residues added per DNA 'end' is calculated from the increase in TCA precipitable counts of P^{32}. Approximately 90 residues per end were added in this experiment.

The double stranded, tailed, cDNA was then purified by phenol extraction and Sephadex G-50 column chromatography, and concentrated by ethanol precipitation. About 30ng of this material was recovered at the end of this series of reactions. Since half of the material was put aside at two separate stages of the process, this 30ng of tailed DNA was prepared from a quarter, or about 12µg of the original template RNA. The theoretical maximum yield is therefore 24µg so the overall efficiency of the process is 0.125%. As well as the losses resulting from the low efficiencies of the enzymes reverse transcriptase and DNA polymerase I, material was lost each time phenol extraction and Sephadex G-50 chromatography were performed.

Assuming a number average length of 800 base pairs for the double stranded DNA 30 ng is equivalent to 60fmoles.

Preparation of Recombinant DNA Molecules

The next step was to anneal the dA tailed ds DNA molecules with linear dT tailed molecules from a suitable plasmid vector. I used the plasmid pCM2 (Bishop, 1979) which was cleaved with the restriction enzyme HindIII and tailed with thymidine residues (50 residues/tail) by Melville Richardson. Some of the properties of pCM2 have been discussed earlier and by Bishop and Davies (1979).

The two DNAs were mixed together in annealing buffer in approximately 1:1 molar ratios. After annealing the solutions were used to transform competent *E. coli* cells of the strain Hb101. The cells

were then plated out on plates containing the antibiotic chloramphenicol. Only Hb101 cells containing a viable pCM2 plasmid will grow. The results of one such transfection were

DNA in Annealing Mixture	No.of Cam^r colonies obtained
-	0
5fmol of pCM2 dT + 2.5fmol λ HaeIII dA	110
5fmol pCM2 dT + 1.5fmol fat body cDNA dA	71
5fmol pCM2 dT + 3fmol fat body cDNA dA	34
5fmol pCM2 dT + 6fmol fat body cDNA dA	41
5fmol of pCM2 dT	2

The background of growth with pCM2 dT alone is very low (0.5 x 10^3 colonies/pmol). The transfection efficiency in the presence of HaeIII restricted dA tailed phage λ DNA is 6 x 10^4 colonies/pmol vector whilst that in the presence of dA-tailed fat body cDNA is about 2 x 10^4/pmol vector.

Each colony from the experimental plates was then picked and tested for its growth on plates containing the antibiotic tetracycline. Bacteria harbouring plasmids in which the Tet gene is interrupted by foreign DNA should not grow on these plates. In one experiment 139 out of 146 Cam^r colonies were Tet^s. These were named pFB101-239. In a second identical experiment another 135 Cam^r Tet^s colonies were produced. These were named pFB301-435.

These recombinant plasmids should contain sequences represented in the mRNA population of Drosophila third instar larval fat bodies. The next task is to identify which proteins these sequences partially encode.

Screening and Analysis of Cloned cDNA Sequences

Selection for Larger Cloned cDNA

Initially, the plasmids containing the largest inserts were selected for further study. To identify these, I used the "colony gel" procedure described by Barnes (1977). Briefly, a loopful of bacteria from a plate were lysed and loaded directly onto an agarose gel. After electrophoresis and staining the supercoiled plasmid DNA can be visualized in the gel. Figure 31 shows an example of such a gel. From the 139 Cam^r Tet^s colonies from the first transformation experiment 17 were selected as containing plasmids with larger inserts. Plasmid DNA from these plasmids was purified by caesium chloride-ethidium bromide banding of bacterial lysates as described in the Methods section.

Restriction Analysis of Recombinant Plasmids

After large (approximately 100 µg) quantities of DNA of each recombinant plasmid had been prepared a more accurate measurement of the size of the inserted cDNA fragments could be obtained by restriction enzyme analysis. Plasmid DNAs were digested with the enzymes BamI and Eco.R1 and the resulting fragments were separated in agarose gels and their molecular weights determined with reference to the known molecular weights of the BamI-Eco.R1 generated fragments of the plasmid vector pCM2. Figure 32 shows an example of such a gel and the results of this and other gels are summarized below.

A striking feature of these results is the number of plasmids which contain a cDNA sequence with a target site for the enzyme Eco.R1. A sequence of 500 base pairs, in random order, would have only a one in eight chance of containing the six base pair Eco.R1 target sequence. The map of mitochondrial DNA shown in Figure 8 shows that the sequence

Plasmid No.	Restriction Enzyme Target Site in insert	Size of insert in base pairs
pFB 101	1 Eco.R1	120
pFB 105	1 Eco.R1	500
pFB 122	-	450
pFB 132	1 Eco.R1	370
pFB 141	-	220
pFB 147	-	370
pFB 151	1 Eco.R1	450
pFB 153	-	600
pFB 154	1 Eco.R1	300
pFB 161	1 Eco.R1	750
pFB 163	1 Eco.R1	450
pFB 168	-	220
pFB 172	-	500
pFB 174	1 Eco.R1	600
pFB 177	1 Eco.R1 + 1 BamI	1000
pFB 181	1 Eco.R1	600
pFB 186	-	650

of the gene for the 14S large mitochondrial ribosomal RNA contains two Eco.R1 sites. It is therefore very likely that the cloned cDNA sequences which contain Eco.R1 sites are '14S' sequences.

Consideration of the stochiometry of the bands seen in digests of plasmid pFB 177 suggests that this DNA is a mixture of two plasmids, one containing on Eco.R1 site in the insert and the other a BamI site. Two separate colonies from a plate of pFB 177 containing

Figure 31.

Tet^s Cam^r colonies from the transformation of Hb101 with pCM2/cDNA hybrid molecules were lysed as described and loaded directly onto a 0.7% agarose gel. After electrophoresis and staining the closed circular plasmid DNA can be visualized in the gel (Barnes, 1977). The tracks are, left to right, a pCM2 containing colony, eleven different Tet^s Cam^r colonies, another pCM2 containing colony.

Figure 32.

Plasmid DNAs were treated with restriction enzymes Eco.R1 and BamI and the resulting fragments separated in a 0.75% agarose gel.

The tracks are
(a) and (n) pCM2, (b) pFB154, (c) pFB141, (d) pFB147, (e) pFB174, (f) pFB177, (g) pFB186, (h) pFB153, (i) pFB161, (j) pFB163, (k) pFB168, (l) pFB172, (m) pFB181.

The figures at the right of the photograph refer to the molecular weights in megadaltons of the fragments in tracks (a) and (n).

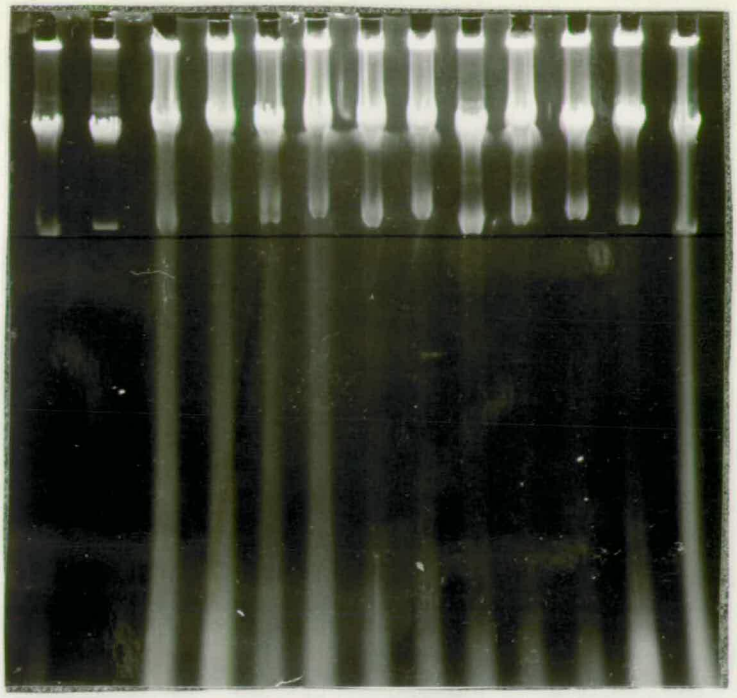


Fig.31

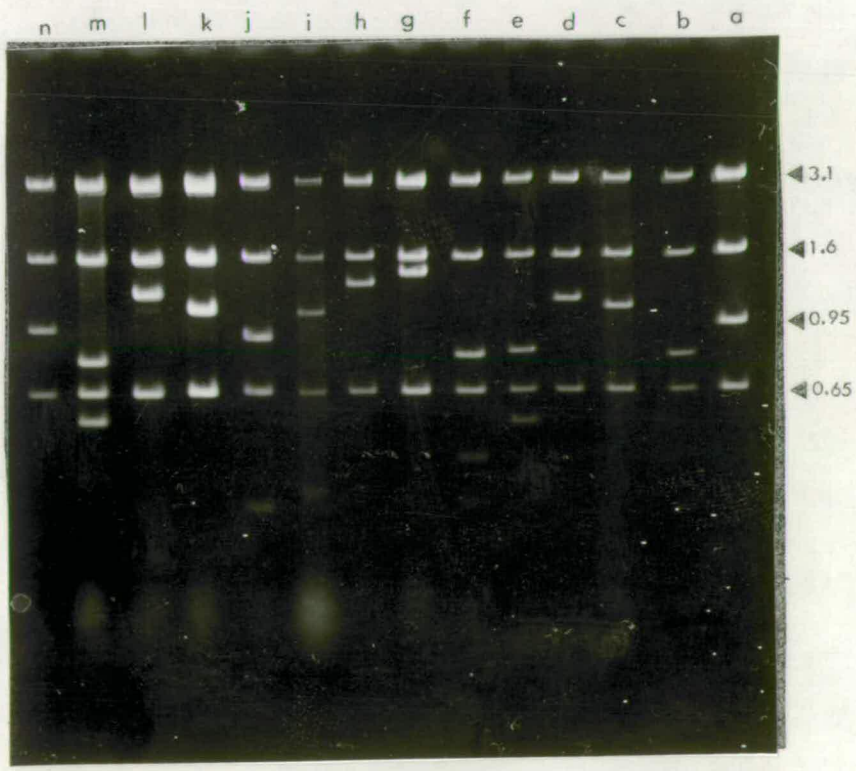


Fig.32

bacteria were grown up and plasmid DNA isolated from each one. A plasmid with a cDNA insert of 500 base pairs containing a BamI site and no Eco.R1 sites was isolated from both colonies. Figure 33 shows restriction enzyme analysis of this plasmid. In the hybrid arrested translation experiment described below, pFB 177 DNA containing the mixture of two plasmids was used whereas in the Southern blotting experiments the purified pFB 177 DNA was used.

Screening Recombinant Plasmids for '14S' sequences

Figure 16 shows the restriction map of plasmid pMD 417 in relation to the 14S mitochondrial ribosomal RNA. The 0.5Md (750bp) Eco.R1 fragment of this plasmid contains a sequence from the middle of the 14S gene. It was thought that the probability of 14S cDNA sequences containing part of this sequence was high. Only if reverse transcription began at the 3' end of 14S RNA, or 400 bases or so from the 5' end, would large 14S cDNA sequences be cloned which did not show complementarity to this fragment. The presence of Eco.R1 sites in so many of the cloned cDNA sequences seemed to support this idea.

It was decided therefore to purify this fragment, label it with P^{32} and use it as a hybridization probe to pick out the plasmids containing 14S RNA sequences. The fragment was purified by agarose gel electrophoresis of Eco.R1 restricted pMD 417 DNA followed by electrophoresis of the 0.5Md band into HAP (Tabak and Flavell, 1978). The resulting DNA was labelled to high specific activity (10^8 cpm/µg) with P^{32} dCTP by nick translation. The other probe used in this experiment was H^3-labelled fat body cDNA.

Plasmid DNA (about 0.5µg of each plasmid) was partially depurinated, denatured, neutralized and loaded onto 13cm diameter nitrocellulose

filters and the filters baked. Small filters (13mm diameter) were cut from the larger ones and one set of filters were hybridized with 10^5 cpm of P^{32}-labelled "14S probe", another with 10^5 cpm of H^3-labelled fat body cDNA. After hybridization, which took place in small vials in about 0.5mls of solution for 16 or so filters, the filters were washed and counted. The results of this experiment are shown on the next page. Although the results are presented in terms of counts per minute the experiment is not an accurate quantitative one since several factors were not controlled.

The amount of DNA per small filter may vary from filter to filter depending upon the physical properties of the various filtering apparati used in loading the DNA. The accessibility of filters to the hybridization solution may have varied between filters since it is impossible to stack them in such a small volume with equal gaps between all filters. The conclusions from the experiment are therefore not strictly quantitative - in effect the experiment yields the same kind of information as the Grunstein-Hogness colony hybridization technique (Grunstein and Hogness, 1975) where the counts per minute reported here are proportional to the degrees of blackening seen on Grunstein-Hogness filters.

The plasmids which hybridized a lot of '14S probe' were assumed to contain 14S cDNA. It is not surprising that these plasmids also hybridize a lot of fat body cDNA since 14S cDNA has been shown to be the major component of fat body cDNA. Therefore plasmids pFBs 151, 168, 172, 181 and from another similar experiment pFBs 105, 161, 174 were assigned as containing 14S sequences and were not studied further. These all contain Eco.R1 sites in the cDNA inserts with

Experiment No.	Plasmid DNA	cpm P^{32} 14S probe hybridized	cpm H^{3} cDNA hybridized
1	-	26	44
1	-	29	45
1	pCM 2	25	38
1	pCM 2	42	39
1	pMD 417	10850	3960
1	pFB 122	432	215
1	pFB 151	1480	870
1	pFB 153	435	1050
1	pFB 163	488	1285
1	pFB 168	624	2717
1	pFB 172	8248	1355
1	pFB 177*[1]	2401	1723
1	pFB 181	3558	4450
1	pFB 186	89	220
2	-	25	25
2	-	25	30
2	pCM 2	105	55
2	pCM 2	141	59
2	pMD 417	6480	2350
2	pFB 172	10288	4316
2	pFB 147	309	879
2	pFB 177*[2]	130	209
2	pFB 177*[2]	158	560

*[1] pFB 177 contaminated with another plasmid.

*[2] Recloned pFB 177 containing an insert of 500 bp which has a target site for BamI, but not one for Eco.Rl.

the exception of pFB 172 which shows strong hybridization to '14S probe' and to fat body cDNA. The likely explanation is that this cDNA was transcribed from within the part of 14S RNA complementary to the 0.5Md fragment of mitochondrial DNA.

The remaining plasmids, which show low levels of hybridization with 14S probe, fall, roughly, into two classes; those which hybridize small amounts of fat body cDNA (pFB 122, pFB 177, pFB 186) and those which hybridize relatively large amounts of fat body cDNA (pFB 147, pFB 153, pFB 163). pFB 177 DNA before purification of the single plasmid behaves as a 14S cDNA containing plasmid; after recloning of the single pFB 177 plasmid it behaves as a plasmid containing no 14S sequences. We conclude that the plasmid pFB 177, before further purification, was contaminated with a 14S cDNA containing plasmid.

Now the highest level of hybridization with fat body cDNA shown by 14S cDNA containing plasmids is about 4000 cpm (pMD 417, pFB 172, pFB 181) and 14S cDNA has been shown to represent at least 40% of fat body cDNA. The figure of about 1000 cpm of cDNA hybridizing to pFBs 153, 163 and 147 would therefore mean that these cDNAs represent about 10% of the total cDNA or about 20% of the real mRNA sequences. No evidence for such abundant mRNA was found from $R_o t$ analysis or from cell free translation studies. It is possible, therefore, that these plasmids contain cDNAs complementary to 14S RNA, but not to any, or to very little, of the 0.5Md mitochondrial DNA fragment used as the 14S probe. This unfortunate conclusion is strengthened by the observation that the insert in pFB 163 contains a site for Eco.Rl.

All recombinant plasmids show hybridization to both probes which is above that shown by the vector pCM 2. This may be explained by the presence of the poly dA-dT sequences in all the recombinant plasmids which will hybridize to the A + T rich 14S probe and the poly-T containing radioactive cDNA.

Hybrid Arrested Translation

Some of the plasmids which showed low levels of hybridization to 14S probe were further studied by hybrid arrested translation (Paterson et al., 1977). Plasmid DNAs were linearized by restriction with HaeIII and hybridized in excess with fat body mRNA under conditions which favour the formation of RNA-DNA hybrids. After hybridization half of each reaction mixture was heated to 100°C to denature the nucleic acids and the other half was left in the hybrid form. The 'hybrid' and 'melted' RNAs were precipitated with ethanol and translated in the messenger RNA dependent reticulocyte lysate. Comparison of the pattern of polypeptides synthesized by the two fractions may show which polypeptide is partially encoded by the cloned sequence since the cloned DNA should hybridize to and block the translation of, its complementary mRNA. The polypeptide should therefore be missing in the products of the 'hybrid' RNA but present in the 'melted' RNA translation products.

Figure 34 shows the results of this analysis for plasmids pCM 2, pFB 153 and pFB 177. No differences are seen between the hybrid and melted fractions for pCM2 or pFB 153. mRNA in hybrid with pFB 177 DNA, however, appears to stimulate the synthesis of only one major methionine containing polypeptide of molecular weight approximately 80,000 d whereas after melting, two polypeptides are resolved

Figure 33.

Two separate colonies from a plate of colonies harbouring plasmid pFB177 were picked, DNA purified from them, restricted and the resulting fragments separated in a 0.7% agarose gel.

The tracks are

(a) pCM2 DNA digested with BamI and Eco.R1.

(b) and (c) Two separately isolated pFB177 DNAs digested with Eco.R1.

(d) and (e) pFB177 DNAs digested with BamI.

(f) and (g) pFB177 DNAs digested with BamI and Eco.R1.

The figures at the left of the photograph refer to the molecular weights in megadaltons of the fragments in track (a).

Figure 34.

Hybrid arrested translation. Plasmid DNA was fragmented, denatured, and hybridized with mRNA from larval fat body as described. Half of each hybridization solution was heated to 100°C to denature the nucleic acids (melted) and half was left in hybrid (hybrid). The RNA in each fraction was recovered and translated in the MDL in the presence of S^{35}-methionine (Paterson et al., 1977). The MDL was electrophoresed in a 13%-20% polyacrylamide gel which was impregnated with PPO and exposed to preflashed X-ray film (Laskey and Mills, 1975). Exposure was for 5 days at -70°C. The tracks are

(a) and (i) S^{35}-labelled 'heat shock' proteins (6000 cpm)
(b) pCM2 'hybrid' fraction (15000 cpm)
(c) pCM2 'melt' (17000 cpm)
(d) pFB153 'hybrid' (15000 cpm)
(e) pFB153 'melt' (17000 cpm)
(f) pFB177 hybrid (6000 cpm)
(g) pFB177 melt (12000 cpm)
(h) pFB177 hybrid (12000 cpm)

The figures at the right of the photograph refer to the molecular weights of the bands in tracks (a) and (i) in thousands of daltons.

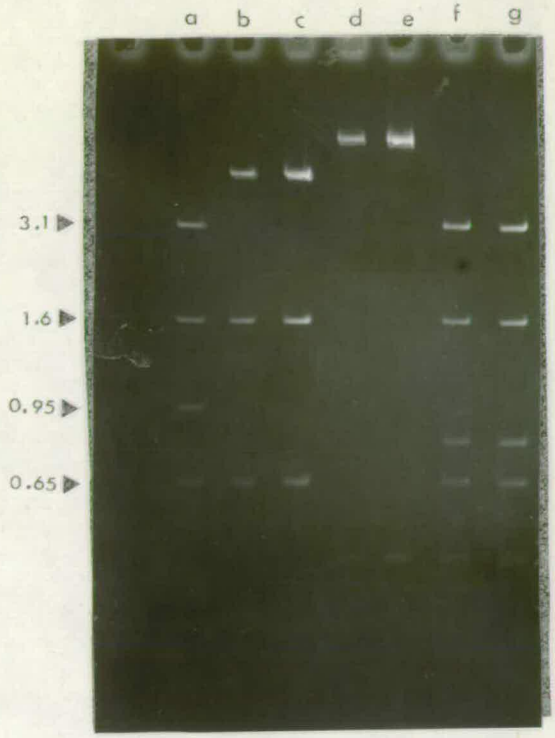


Fig.33

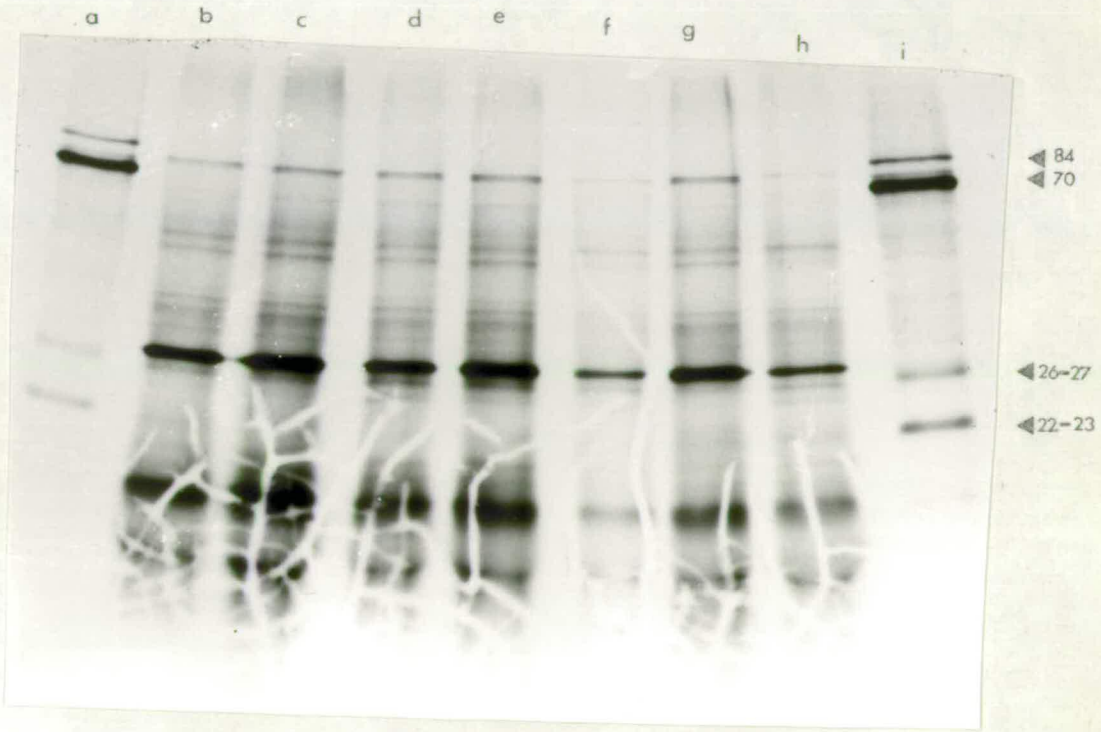


Fig.34

at this position. RNA hybridized with pCM 2 or pFB 153 stimulates the synthesis of two polypeptides which migrate to this position. This result indicates that plasmid pFB 177 contains part of the sequence for a major methionine containing 80,000d polypeptide. Arguments presented earlier suggested that the methionine containing bands in this region represented chains of the larval serum proteins.

It should be remembered that the pFB 177 DNA used in this experiment is contaminated with a 14S cDNA containing plasmid. There seems to be no intrinsic reason why 14S cDNA should specifically block the translation of one mRNA species but this possibility cannot be overlooked.

In a second HART experiment using plasmids pFB 153, pFB 163 and pFB 147, no differences in 'hybrid' and 'melted' translation products were found when S^{35}-methionine, S^{35}-cysteine, or H^{3}-leucine were used as the labelled amino acid.

Southern Blotting Experiments

The genomic DNA sequences complementary to cDNAs cloned in plasmids pFB 122, pFB 153, pFB 163, pFB 177 and pFB 186 were investigated by applying the Southern Blotting technique (Southern, 1975).

Nuclear DNA from Drosophila embryos was restricted with various restriction enzymes, electrophoresed in agarose gels, denatured, transferred to nitrocellulose filters, and the filters hybridized with P^{32}-labelled plasmid DNAs. The filters were then washed and exposed to X-ray film. In this way we expect to determine the sizes of restriction fragments of genomic DNA which contain sequences cloned in the plasmids.

Figure 35 shows the result of this analysis for plasmids pCM 2

and pFB 186. Nick translated pCM 2 DNA does not hybridize to any Drosophila DNA restricted by Eco.Rl or BamI, but it does show bands when hybridized to DNA restricted with Kpn 1 and Xba 1. This result implies that the enzymes have been contaminated with plasmid DNA. pFB 186 DNA hybridizes with an Eco.Rl fragment of 3.4Md, a Xba 1 fragment of 6.7Md and to BamI and Kpn 1 fragments which only just enter the gel, as well as to the Kpn 1 and Xba 1 fragments resulting from contamination of these enzymes. In the same experiment pFB 122 showed exactly the same pattern as pFB 186.

Figure 36 shows the result of analysis using pFB 153, pFB 177 and pMD 417 (pMD 417 DNA contains the entire sequence coding for the large mitochondrial rDNA). In this experiment 5 µg of Drosophila DNA were loaded per track and the mass of restricted pCM 2 DNA used as molecular weight markers was calculated so that the 0.95Md fragment of pCM 2 would hybridize with the same amount of radioactively labelled plasmid DNA as would a sequence of Drosophila DNA present once per haploid genome, and complementary to 500 base pairs of the plasmid.

The results show that pMD 417 contains DNA complementary to Eco.Rl fragments of Drosophila DNA of molecular weights approximately 6.6, 3.4 and 0.5Md, representing the Eco.Rl fragments of mitochondrial DNA (Fig. 16). It hybridizes to a BamI fragment which only just enters the gel and to one which is a little faster.

pFB 153 oontains DNA complementary to a BamI fragment which hardly enters the gel and to a Eco.Rl fragment of about 6.6Md. (Although this fragment runs slightly faster than the 6.3Md pCM 2 band under these conditions it travels the same distance, in the same

Figure 35.

Restricted DNA was electrophoresed in 0.75% agarose gel and transferred to nitrocellulose filters (Southern, 1975). The tracks are

(a) 2ng pCM2 DNA digested with Eco.R1 and BamI.

The other tracks contain 2µg of Drosophila embryonic nuclear DNA digested with:

(b)(f) Eco.R1, (c)(g) BamI, (d)(h) KpnI, (e)(i) XbaI.

The filters were prehybridized, hybridized with P^{32}-labelled probes, and washed as described by Maniatis et al., (1978), dried and exposed to preflashed X-ray film using tungstate intensifying screens (Laskey and Mills, 1977). The probes were

(A) 10^6 cpm nick translated pCM2 DNA (10^7 cpm/µg).

(B) 3.5×10^6 cpm nick translated pFB186 DNA (3×10^7 cpm/µg).

Exposure times were (A) 1 week, (B) 18 hours.

The figures to the left of (A) refer to the molecular weight in megadaltons of the bands in track (a).

The figures to the right of (B) refer to the molecular weights in megadaltons of pCM2 DNA digested with BamI, and Eco.R1 + BamI which were run on the same gel but are not shown.

Figure 36.

Electrophoresis, transfer, hybridization and exposure were as in the legend to Figure 35.

The tracks were

(c)(d)(g) 200pg pCM2 DNA digested with Eco.R1 + BamI, plus 100pg pCM2 DNA digested with BamI.

(b)(e)(h) 5µg Drosophila embryonic nuclear DNA digested with Eco.R1

(a)(f)(i) 5µg Drosophila embryonic nuclear DNA digested with BamI.

The probes were

(A) 4×10^6 cpm nick translated pMD417 DNA (5×10^7 cpm/µg).

(B) 3×10^6 cpm nick translated pFB153 DNA (5×10^7 cpm/µg).

(C) 3×10^6 cpm nick translated pFB177 DNA (5×10^7 cpm/µg).

Exposure times were (A) 5 days, (B) and (C) 3weeks.

The figures to the right refer to the molecular weights in megadaltons of the bands in tracks (d) and (g). Those to the left refer to the Eco.R1 fragments of mitochondrial DNA in track (b). All tracks shown were run on the same 0.75% agarose gel.

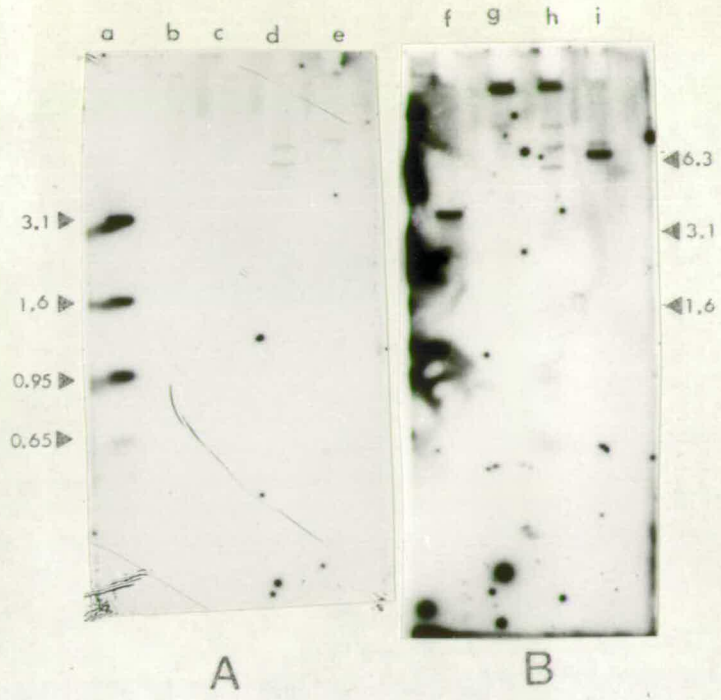

a b c d e
f g h i
3.1
1.6
0.95
0.65
6.3
3.1
1.6
A
B

Fig.35

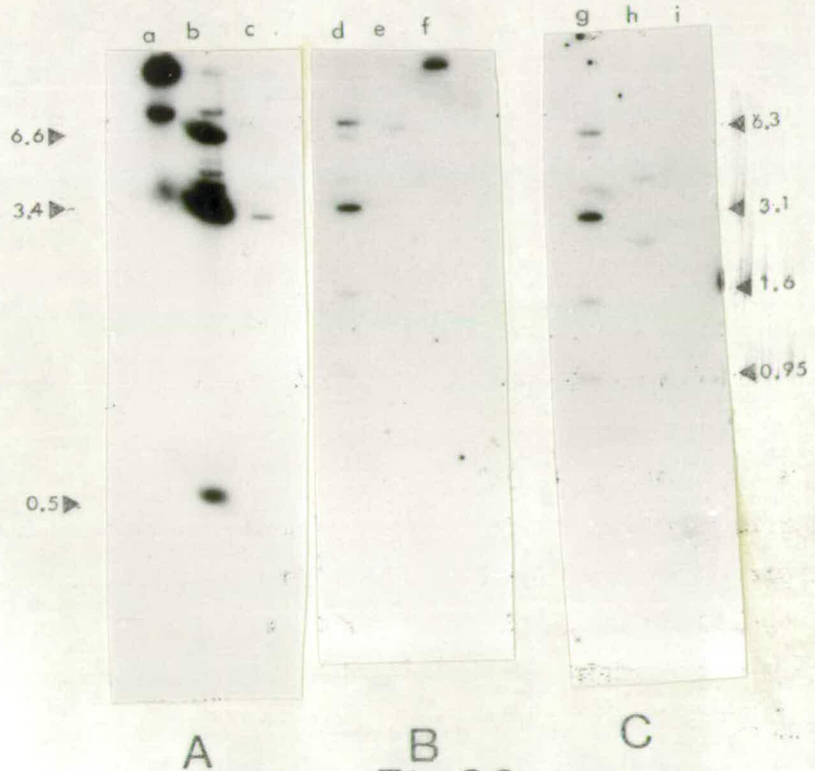

a b c
d e f
g h i
6.6
3.4
0.5
6.3
3.1
1.6
0.95
A
B
C

Fig.36

gel, as the 6.6Md mitochondrial DNA band complementary to pMD 417 DNA. It is therefore assumed to be this band). The extent of hybridization with the BamI fragment indicates that this sequence is present more than once per genome. In the same experiment (data not shown) pFB 186 DNA was shown to hybridize to fragments present many times per haploid genome and pFB 163 showed exactly the same pattern as pFB 153.

pFB 177 DNA hybridizes to DNA fragments of molecular weights 2.6Md and 4.4Md which are present once or a few times per haploid genome. No clear hybridization with BamI generated fragments is seen.

The results are summarized below.

Plasmid	Eco.R1 fragments (Md)	BamI fragments	No.of copies Per Haploid genome
pMD 417	6.6, 3.4, 0.5	2 large fragments	many
pFB 153 and pFB 163	6.6	large fragment	many
pFB 122 and pFB 186	3.4	2 large fragments	many
pFB 177	4.4, 2.6	none visible	one or a few

Considering the maps of mitochondrial DNA, 14S rRNA and pMD 417 (Figs. 8 and 16) these results are consistant with the notion that plasmids pFB 122 and pFB 186 contain cDNA sequences which were transcribed from the part of 14S RNA between the 3'end and the first Eco.R1 site. Hence they do not hybridize with the '14S probe' but do hybridize with the 3.4Md Eco.R1 fragment of mitochondrial DNA. Mitochondrial DNA is present in the nuclear DNA preparation as a contaminant in quantities

of several copies per genome as is shown by the Southern transfer result using labelled pMD 417 DNA. The large BamI 'fragments' hybridizing to pMD417 and pFBs 186, 122, 153 and 163 presumably represent open circular mitochondrial DNA and the slightly faster one, linear mitochondrial DNA. It was shown earlier that mitochondrial DNA contains no site for BamI.

pFB 153 (and pFB 163) behave as if they contain cDNA sequences which were transcribed from about 400 bases from the 5' end of 14S RNA and almost reach the end. Therefore they show little complementarity with the '14S probe' and hybridize to the 6.6Md Eco.R1 fragment of mitochondrial DNA.

Further support for these interpretations comes from the fact that pFB 122 and pFB 186, and pFB 153 and pFB 163 were in the same classes with respect to their hybridization with '14S probe' and fat body cDNA. Plasmid pFB 163 contains a Eco.R1 site in the insert; in order to comply with the above interpretations this must be very close to the origin of reverse transcription.

pFB 177 seems to contain a DNA sequence which was transcribed from a real mRNA and which is present once or only a few times, per haploid genome. It is difficult, from Figure 36, to be exact about the copy number. The degree of blackening of the X-ray film over the two Eco.R1 fragments is about that expected of a single copy sequence, but the control band (the 0.95Md pCM 2 band) is from a lower molecular weight region of the gel. As discussed by Southern (1975) lower molecular weight DNA does not bind to nitrocellulose filters so well - this would lead to an overestimate of copy number. In the higher molecular weight part of the gel DNA does not transfer

so efficiently from the gel to the filter - this would lead to an underestimate of copy number. The two bands each show significantly less blackening than the 3.1Md marker band which represents about 3 copies per genome and is in the same region of the gel. We can say therefore that each Eco.R1 band contains a half, or one (or possibly two) copy of the sequence cloned in pFB 177. The absence of BamI fragments complementary to pFB 177 is not altogether surprising since the DNA insert in this plasmid contains a BamI site. This would mean that (a) less nick translated probe is available to hybridize to each BamI fragment and (b) the possibility of the BamI fragments being so small that they are not seen in this analysis is increased.

In fact two very faint BamI bands of approximately 4.4 and 5.3Md can be seen by studying the X-ray film from which the photograph shown in Figure 36 was taken but these are so faint one cannot be sure they are there at all.

The presence of two Eco.R1 fragments of Drosophila genomic DNA complementary to pFB 177 DNA (which does not contain an Eco.R1 site in the insert) may be explained in three ways:

(i) the Drosophila DNA is not completely digested by Eco.R1.

(ii) the gene coding for the pFB 177 insert sequence is present in two copies, each of which is contained on a Eco.R1 fragment of a different size.

(iii) the gene is present in only one copy but contains an inserted non-coding sequence which contains an Eco.R1 site.

There are several reasons for believing that possibility (i) is not the case. Firstly the two bands are of approximately equal intensity which would only arise by chance if the larger band was a partial digestion product. However, when the same digested DNA was

hybridized with pMD 417 (Fig. 36), partial digestion products are seen, but they are one or two orders of magnitude less intense than the complete digestion products. Secondly, a portion of the Eco.R1 digestion mixture was mixed with phage λ DNA during digestion of the Drosophila DNA and the λ DNA was completely digested as far as could be determined by viewing the stained bands in an agarose gel.

For possibility (iii) to be the case, because the two bands are of approximately equal intensities the non-coding sequence would have to be inserted at a point near to the middle of the sequence cloned in pFB 177. Again this may only occur by chance. We cannot really distinguish between possibilities (ii) and (iii) from the data shown here.

pFB 177, then, appears to be the only plasmid containing a real mRNA sequence from the thirteen examined from the first transformation experiment. That it contains a sequence with the properties of a real mRNA sequence is supported by data from two independent experiments, hybrid arrested translation and Southern blotting. All the other twelve plasmids examined appear to contain sequences transcribed from 14S mitochondrial ribosomal RNA.

DISCUSSION

Mapping Mitochondrial DNA

Klukas and Dawid (1976) located the target sites for the restriction enzymes HindIII and HaeIII in the circular mitochondrial DNA of Drosophila melanogaster. They also mapped the ribosomal RNA genes and an A + T rich 'early melting region' in relation to these restriction sites. Wolstenholme and Fauron (1976) mapped four Eco.R1 sites and determined the position of the A + T rich region in relation to these sites. I mapped the 4 Eco.R1 sites in relation to the 4 HindIII sites (Fig. 8). Since this work was done two groups have published restriction enzyme maps of D. melanogaster mitochondrial DNA which include the sites for Eco.R1 and HindIII and the results of both are in fairly good agreement with those reported here (Bonner et al., 1978; Shah and Langley, 1979). Bonner et al. (1978) report that the smallest HindIII generated fragment does not contain a site for Eco.R1 but that such a site lies just outside this fragment. My mapping experiments led to the conclusion that the small 0.3Md HindIII fragment contains an Eco.R1 site and the cloned 0.3Md fragment (in pMD112) is cleaved by Eco.R1. Shah and Langley (1979) find an Eco.R1 site in the small HindIII fragment.

Bonner et al. (1978) report the presence of a site for the restriction enzyme PstI. This site maps within the 3.4Md Eco.R1 generated fragment, close to an Eco.R1 site. I found that a PstI site, which mapped within the 3.4Md Eco.R1 fragment was present, but only in a small proportion of the molecules making up the mitochondrial DNA preparation which I used (Fig. 7). This result could be explained by assuming that the PstI did not completely digest the mitochondrial DNA. No experiments were performed to specifically exclude this

possibility but there are reasons for believing it unlikely. The phenomenon was observed several times, in different experiments, using PstI alone and in combination with either HindIII or Eco.Rl. In the double digestion experiments the proportion of PstI sensitive molecules, as judged by the intensities of the PstI generated faint bands was always about the same. The PstI was used under conditions which normally gave complete digestion of other DNAs at the time and mitochondrial DNA was completely digested with other restriction enzymes using standard conditions for these enzymes.

The most likely interpretation, therefore, is that there is some sequence heterogeneity of mitochondrial DNA molecules within the fly population which I was using. Intraspecific heterogeneity of mitochondrial DNA sequences detected by restriction enzyme analysis has been reported before. Potter et al. (1975) studied the restriction patterns of mitochondrial DNA from various mammals using the enzyme HaeIII. They found that the mitochondrial DNA of any individual animal appeared to be completely homogenous. However, mitochondrial DNAs from very closely related species such as donkey and horse were found to have only about half of their HaeIII sensitive sites in common and several intraspecific differences were found (in human and horse mitochondrial DNAs). Buzzo et al. (1978) studied mitochondrial DNA from individual rats from the same colony using Eco.Rl. Again intraspecific differences were found but the mitochondrial DNA of any one individual was homogenous. Avise et al. (1979) using several type II restriction enzymes detected intraspecific heterogeneity in mitochondrial DNAs of several Peromyscus species.

Shah and Langley (1979) studied the restriction enzyme site distribution in the mitochondrial DNAs of several Drosophila species and

found that many of the sites were not conserved within this genus. These workers did not detect any intraspecific heterogeneity.

Molecular Cloning of Mitochondrial DNA

In view of its small size and ease of isolation animal mitochondrial DNA will possibly be the first eukaryotic 'genome' to be completely sequenced. This work could lead to an understanding of the structure and control of mitochondrial genes (reviewed by Tzagoloff et al., 1979) and may shed some light on the evolutionary origin of mitochondria. If mitochondrial DNAs could be incorporated into bacterial plasmids biochemical study of their structure and function would be simplified since large yields of the DNA could be obtained using only microbiological techniques. The mitochondrial genome of the rat has been cloned in this way (Chang et al., 1975).

Here, I tried to clone the mitochondrial genome of D. melaongaster by cloning its four HindIII generated fragments. Only the 2.9Md and the 0.3Md fragments were successfully cloned in the HindIII site of pCM2 or pBR322. The reason for the failure of the other two fragments to form a recombinant plasmids which will successfully replicate in viable E. coli cells can only be speculated upon. Parts of the mitochondrial genome may be expressed and interfere with the physiology of the host or the plasmid mediated antibiotic resistance or parts of the mitochondrial DNA may block plasmid replication. Parts of the mitochondrial genome of Paramecium have been found to be 'unclonable' in the same way in this laboratory (P. Kelly, A. Tait and J. Bishop, unpublished) and many workers have found the same phenomenon with Drosophila mitochondrial DNA.

Using a second cloning strategy 2.5Md of the 5.2Md HindIII generated fragment were cloned into pBR322. The resulting plasmid,

pMD417, was shown to contain the entire gene for the large mitochondrial rRNA and was found to be perfectly stable. Therefore, about one half of the D. melanogaster mitochondrial genome has been cloned into bacterial plasmids. The 3.1Md HindIII fragment, none of which has been cloned, consists almost entirely of a region of very high A + T content (Klukas and Dawid, 1976). Bultmann et al. (1976) and Shah and Langley (1979) showed that the length of this A + T rich region varied widely within the genus Drosophila whereas the rest of the genome was much more evolutionarily conserved. It is likely therefore that this region does not have an important biological function. Therefore the cloning reported here has resulted in the incorporation of about two thirds of the functional mitochondrial DNA of D. melanogaster into bacterial plasmids. Although I was mainly interested in the isolation of the large mitochondrial rRNA gene, the plasmids may be used in a detailed study of the organisation and expression of the D. melanogaster mitochondrial genome.

The Translation of Drosophila mRNA in a Cell Free System from Rabbit Reticulocytes

The first method of extracting RNA from Drosophila larvae which I used was to homogenize fresh larvae in the presence of ribonuclease inhibitors, centrifuge out the nuclei, and extract RNA from the cytoplasmic fraction using Kirby salts and phenol (Kirby, 1965; Parish and Kirby, 1966). The inhibitors used were spermine and polyvinylsulphate (PVS). This method has been in use in this laboratory for a number of years and has been found to give a reproducibly high yield of undegraded RNA. I found, however, that the poly(A) containing RNA purified from this RNA inhibited protein synthesis in the reticulocyte lysate. Since spermine is often added to reticulocyte cell free

systems, and since RNA extracted from polysomes by the Kirby phenol procedure did not inhibit the lysate we conclude that the polyanion PVS is responsible for the inhibition and that it cannot be removed from RNA by repeated sucrose density gradients, high salt washes or oligo-dT-cellulose chromatography.

The guanidium chloride method of RNA extraction based on the method of Cox (1968) yielded poly(A) containing RNA from larvae which proved to be a good stimulator of protein synthesis in the reticulocyte lysate. However, in my hands the yields obtained from this procedure were so small that its use in the isolation of fat body RNA would be impractical. The method finally adopted, that of Kemp et al. (1978) gave good yields of RNA which stimulated the lysate about half as well as that made using guanidinium chloride.

Using all these methods the yield of poly(A) containing RNA was about 1% of total RNA. Considering that about half of this, from larvae, is 14S mitochondrial rRNA, the yield of mRNA is very small. Recent results (J. Manning, unpublished) suggest that many Drosophila larval mRNAs have very small or no 3'poly(A) tracts. These would not bind to oligo-dT-cellulose and would not be present in the analysis described here.

Poly(A) containing RNA purified either from total larval RNA made using guanidinium chloride, or from fat body RNA prepared by phenol extraction, showed dependence on unphysiologically high levels of 3'-5' cAMP for efficient translation in the reticulocyte lysate. This is reminiscent of inhibition involving the haem controlled repressor and can result from lack of haemin (Zucker and Schulman, 1968) or the presence of double stranded RNA (Ehrenfeld and Hunt (1971) or oxidized glutathione (Kosower et al., 1972). Legon et al. (1974)

showed that all three inhibitions can be prevented by inclusion in the lysate of high levels (3-10mM) of 3'5'cAMP (reviewed by Revel and Groner, 1978). Since in this case the inhibition is brought about by the addition of a preparation of RNA we assume that the inhibitory substance is double stranded RNA. Farrel et al. (1977) showed that very low levels (50ng/ml) of double stranded RNA, such as some viral RNAs, activate a translational inhibitor, and an eIF2 kinase. The translational inhibitor is a different molecule from the haem controlled repressor. The phosphorylation of eIF2 is responsible for inhibition of protein synthesis. 3'5'cAMP probably acts by inhibiting the eIF2 kinase but its precise mode of action is unknown.

The cDNA Clone Bank

The proportion of 14S mitochondrial rRNA sequences in third instar larval fat body was estimated in two ways. Hybridization of cDNA with an excess of plasmid pMD417 DNA indicated that at least 30% of the cDNA was transcribed from 14S RNA. Hybridization of fat body cDNA with an excess of its RNA template showed that 40% - 50% of the cDNA behaved as if it had been transcribed from a single RNA species, and that a significant amount (at least 40%) of the cDNA behaved as a separate kinetic component representing about 200 other different RNA species. Fat body cDNA was made double-stranded and inserted in the plasmid cloning vector pCM2 by the poly dA-dT joining method. Thirteen of the resulting recombinant plasmids containing larger inserted fragments were studied in some detail. Twelve of these were found to contain sequences present in mitochondrial rRNA and one behaved as the sequence of a real mRNA. It would seem that at one or more stages during the production of the clone bank, 14S specific sequences are selected for.

This selection may have occurred at the stage at which DNA polymerase I is used to make the single stranded cDNA double stranded. The reverse transcriptase reaction was carried out in the presence of Actinomycin D which inhibits the formation of a second new strand of DNA by the enzyme using the first cDNA strand as a template (Ruprecht et al., 1973). Inclusion of Actinomycin D in the reaction has been found, in this laboratory, to allow the synthesis of longer cDNA transcripts. The second enzyme used in the series of reactions, DNA polymerase I from E. coli, will synthesize a second DNA strand complementary to the cDNA in the presence of a DNA primer, hydrogen bonded to the first strand. In the case of cDNAs synthesized in the absence of Actinomycin D the primer is the short piece of second strand formed at the 3' end of the cDNA by the DNA dependent DNA polymerase activity of the reverse transcriptase. Rougeon and Mach (1976), studying the synthesis of a second DNA strand complementary to rabbit globin cDNA, found that the cDNA could serve as an efficient template for DNA polymerase I without any exogenous primer, when Actinomycin D was included in the first reaction. They compared the efficiency of this 'self-primed' synthesis using full length cDNA and shorter partial cDNA copies and found that second strand synthesis was much more efficient when full length cDNAs were used. The product of self-primed second strand synthesis was found to be a molecule which would snap back after denaturation, but which lost this property after treatment with S1 nuclease. The authors conclude that the cDNA product loops over near its 3' end forming a well-matched region of duplex which acts to prime second strand synthesis. The fact that shorter cDNAs were inefficient primer-templates suggests that such loop structures are characteristic of the 5' end of the globin mRNA sequence.

It would seem then, that if self priming is used, as it is here, to make the cDNA double stranded, we are selecting for cDNA sequences which have sufficient secondary structure to form well matched 3' terminal loops. It is a matter for speculation how many cDNA sequences will be capable of forming these loops. It is likely that cDNA transcribed from 14S RNA will contain considerable secondary structure, reflecting that of its ribosomal RNA template, and will consequently be able to form the double stranded DNA regions necessary to prime the synthesis of the second DNA strand. This may have been the point at which 14S specific sequences were selected. Sequences incapable of forming loops will remain single stranded and will be digested by S1 nuclease in the next enzymatic step of the process.

The second stage at which selection for specific sequences may have occurred was in the selection of only the longer cloned cDNAs for further study. Longer cloned cDNAs are preferred for several reasons. Very short cDNAs may be copies of only the 3' non-coding region of the mRNA in which case they would not block the translation of the mRNA in a hybrid arrested translation experiment. Also the amount of radioactive label which can be incorporated into a cloned DNA segment is directly proportional to its length and for very short cDNAs it may be impossible to incorporate sufficient label to give a positive result in Southern blotting experiments or in the screening of a genomic DNA library. Efstratiadis et al. (1975) studied the cDNAs synthesised by reverse transcription of rabbit globin mRNA and silkmoth chorion mRNAs. They found that as well as full length copies of the mRNAs the reaction generated discrete sized, partial cDNA copies. This result implies that there are, within the mRNA sequences, preferential sites for the termination of reverse transcription. These sites are presumably

related to the nucleotide sequences around the points of termination. For some mRNAs, these preferential sites may be less than about 300 bases from the 3' end of the molecule. Here, such cloned cDNAs will be specifically underrepresented in the larger cloned cDNAs which were selected, by the colony gel procedure, for more detailed study.

The Sequence in pFB177

The sequence cloned in pFB177 was shown to block the appearance of a major methionine containing polypeptide of 80,000d in a hybrid arrested translation experiment using fat body mRNA. The major methionine containing polypeptides of this molecular weight, encoded by larval fat body mRNA, are the chains of the larval serum proteins LSP1 and LSP2. It is not possible to say, from the present results, which polypeptide chain of these proteins is partially encoded by the sequence. The pFB177 DNA which gave this result was contaminated with a plasmid containing part of the 14S RNA sequence. There seems to be, however, no *a priori* reason why this mitochondrial sequence should block the translation of a specific mRNA. In the same experiment pFB153, which was later shown to contain part of the 14S sequence, gave a negative result.

Southern blotting analysis of the sequence cloned in pFB177 led to the conclusion that the sequence was present once or twice per haploid genome. The cloned sequence, which does not contain an Eco.RI sensitive site hybridized to two Eco.RI generated fragments of Drosophila genomic DNA. Evidence was presented that this was not due to partial digestion of the DNA. There remain three other possible explanations.

(i) There exists, in the fly culture which I used, a polymorphism for one of the Eco.RI sites nearest to the gene, which may be present in one copy.

(ii) The genomic sequence contains a non-coding intervening sequence within the part of the sequence cloned in pFB177.

(iii) The sequence is present twice per haploid genome on Eco.RI generated fragments of different sizes.

We cannot distinguish between these possibilities from the present results. The two bands seen in the Southern blotting experiment, however, were of roughly equal intensities. This is a requirement of interpretation (iii) but would only arise by chance if (i) or (ii) were correct. Isolation of genomic DNA containing the sequence will provide an answer to this problem.

Recent Developments

Recent technological developments have superceded the technical approach to the isolation of structural genes described here, at least as far as genes coding for fairly abundant mRNAs are concerned. These developments have concerned the use of phage lambda as a molecular cloning vector. A rapid *in situ* plaque hybridization procedure has been developed (Benton and Davis, 1977). Safe and easy to use lambda cloning vectors have been constructed (Leder, Tiemeir and Enquist, 1977; Blattner *et al.*, 1977) and *in vitro* packaging systems have been developed which greatly increase the efficiency of introducing lambda DNA into bacterial cells compared with transfection with phage DNA (Hohn and Murray, 1977; Sternberg *et al.*, 1977). Using these techniques it is possible to screen a collection of phage containing an entire animal genome and to grow up milligrams of the positive sequences in a matter of weeks (Maniatis *et al.*, 1978). For example, to achieve the preliminary aims of the work described here, namely the isolation of several genes expressed at a high level in the third instar larval fat body of Drosophila we could screen a library of

Drosophila DNA packaged into phage lambda with labelled fat body cDNA and with labelled 14S probe. The plaques which give a positive signal with the cDNA but not with the probe could be further purified and DNA isolated from them. The DNAs, cloned in phage lambda could then be further characterized by hybrid arrested translation or positive mRNA selection (Kindle and Firtel, 1978). In fact this sort of approach has already led to the successful isolation of several of the LSP genes (D.M. Glover, personal communication) and the gene coding for alcohol dehydrogenase (M. Goldberg, unpublished).

For mRNAs which cannot be isolated as fairly abundant species, however, cDNA cloning remains a necessary first step in order to be able to prepare a highly labelled, specific probe with which to screen a genomic library.

Future Work

Future work on this project will consist of the isolation of several genomic sequences which code for abundant fat body mRNAs by the phage library screening method. Sequences coding for less abundant mRNA will be isolated by cDNA cloning followed by screening of the phage library. In this way a comprehensive selection of fat body specific genes could be built up as a basis for a study of gene organisation in Drosophila and tissue specific gene expression.

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