STUDIES ON CLONED HUMAN DNA

by

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ABBREVIATIONS

The abbreviations used in this work are as laid down in the Biochemical Journal "Instruction to Authors" (Biochem. J. (1978), 169, 1-27), with the following additions:

Amp.	: ampicillin
bisacrylamide	: NN^1 - methylene bisacrylamide
bp	: base pairs
BSS	: balanced salt solution
cDNA	: complementary DNA
DMS	: dimethyl sulphate
DNase	: deoxyribonuclease
ds	: double stranded
EDTA	: ethylene diamine tetra acetate
hsp	: heat shock protein
HZ	: hydrazine
IPTG	: isopropyl- β-D-thiogalactopyranoside
kb	: kilo base pairs
LDB	: loading buffer
mA	: milli amps
MOPS	: 3 (N-morpholino) propanesulphonic acid
mRNA	: messenger RNA
mV	: milli volts
NE	: 50mM sodium chloride, 0.5mM EDTA pH 7
ON-R	: oligodeoxyribonucleotide-R
PAGE	: polyacrylamide gel electrophoresis
p.f.u.	: plaque forming units
RNase	: ribonuclease
r.p.m.	: revolutions per minute
R.S.B.	: reticulocyte standard buffer

SET	: 0.15M sodium chloride, 30mM Tris-HCl pH 8,1mM EDTA
SDS	: sodium dodecyl sulphate
SM	: storage medium
SSC	: 0.15M sodium chloride, 0.015M sodium citrate pH 7
STET	: 8% (w/v) sucrose, 50mM Tris-HCl pH 8, 50mM EDTA, 5% (v/v) triton X-100
TBE	: 100mM Tris-HCl pH 8.3, 80mM boric acid, 1mM EDTA
TBS	: Tris buffered saline
TE	: 10mM Tris pH 8, 1mM EDTA
TEA	: 40mM Tris pH 7.4, 5mM sodium acetate, 1mM EDTA
TEMED	: N,N^1, N^1,N tetramethylethylenediamine
TET	: tetracycline
Tris	: tris (hydroxymethyl) aminomethane
tRNA	: transfer RNA
µg	: microgram
X.-gal.	: 5-bromo-4-chloro-3-indolyl-β-galactoside

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SUMMARY

This thesis describes recombinant DNA studies on certain families of repeated DNA in the human genome.

The initial objective was to isolate and characterise the gene(s) for human heat-shock protein 70 (hsp 70) using a complementary DNA (cDNA) clone (pHS2) prepared previously by others and reported to hybridise to mRNA for hsp 70. The insert from this clone was used to screen two recombinant bacteriophage libraries containing human genomic DNA. After certain technical problems had been overcome, a number of corresponding genomic clones were isolated. Restriction maps were constructed for the four genomic clones which possessed the greatest homology to pHS2, as indicated by Southern blotting at high stringency. These maps suggested that three of the clones (λHr02, λHr03 and λHr06) were overlapping clones derived from the same region of the genome, whereas the remaining clone (λHr05) was derived from a completely distinct region.

The nucleotide sequence of the insert of pHS2 was determined as a preliminary to sequencing the genomic clones. Analysis of the sequence indicated that it was a GC-tailed cDNA copy of a fragment of ribosomal RNA (rRNA), rather than a heat-shock sequence. The rDNA fragment encompassed a region extending over nucleotide 3600-3923 of human 28S rDNA with only one mis-match. Nevertheless it was clear from genomic blots, comparisons of restriction maps and the frequency of genomic clones isolated that the genomic clones did not contain members of the rDNA tandem repeat. They were therefore analysed further.

A portion of λHr02 containing the region hybridising to pHS2 was subcloned and the nucleotide sequence of a 823 base-pair region determined. This showed that the region in λHr02 recognised by pHS2 was a rDNA pseudogene. It was related to a 451 base-pair segment of human

28S rDNA, extending over nucleotide 3627-4105. This rDNA pseudogene has suffered a number of mutations and is not immediately flanked by other rDNA sequences. Regions further upstream and downstream of this pseudogene were analysed by Southern blotting, using two fragments of the human ribosomal clone pA4 (which contain areas not recognised by pHS2) as probes. No sequences corresponding to these regions were detected in the genomic clone, λHr02. This indicated that this rDNA pseudogene (designated H28S-01) was a dispersed member of the tandem rDNA repeat, an 'orphon', and is the first ribosomal orphon to be characterised in detail. Additional Southern blotting evidence suggests that there are some repeated DNA sequences surrounding this pseudogene. The sequence immediately 3' to this pseudogene was shown to possess homology to a region extending over nucleotide 146-170 of the genomic 1.9 kb Hind III repeat, a portion of the human long interspersed middle repetitive sequence, L1Hs.

Southern blot and restriction map analyses indicate that clone λHr05 also contains a dispersed rDNA pseudogene. Clone λHr04 may contain an even more diverged rDNA pseudogene as it possesses even less homology to pHS2 than the other genomic clones, as determined from Southern blotting at high stringency.

Two mechanisms are considered for the generation of H28S-01. One possibility is that it was generated by a DNA-mediated mechanism, as proposed for other orphons. Unequal crossing-over caused by misaligned tandem repeats might have resulted in a looped out segment being excised and reintegrated at a different site in the genome. A second possibility is that this rDNA pseudogene was generated through an RNA-mediated mechanism. An rRNA fragment with suitable secondary structure may have primed its own reverse transcription, the resulting complementary DNA being subsequently integrated non-specifically into the genome.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

In 1968, using the technique of DNA reassociation, Britten and Kohne first reported that DNA sequences could be divided into three main categories : highly-repetitive DNA, intermediate-repetitive DNA and unique DNA. Since that time advances in techniques for the manipulation of DNA have allowed a greater understanding of some members of these groups and their organisation. The members of two of these classes of DNA are quite well characterised. The most highly-repetitive DNA sequences of the eukaryotic genome are those of DNA satellites, which are comprised of very short nucleotide sequences (of variable base composition) repeated many times in tandem (Southern, 1975; Manuelidis, 1978). The unique DNA fraction is made up principally of single-copy genes, coding for various proteins. Intermediate-repetitive DNA, however, encompasses a broad range of 'lower' - copy-number families of repeated DNA (which collectively comprise 30-40% of the DNA of most eukaryotic genomes), and it is this latter class which will be dealt with in more detail in this Introduction.

Both protein-coding and non-protein coding repeated DNA families are represented in this intermediate-repetitive group, with members of these families organised as clusters or lying dispersed, often on different chromosomes (although combinations of both these are found). Clusters may be small, comprising a few members (e.g. human globin genes (Orkin, 1978; Fritsch *et. al.*, 1980; Kaufman *et. al.*, 1980; Lauer *et. al.*, 1980; Proudfoot and Maniatis, 1980)) or alternatively they may be large, with extensive repetition of members which are tandemly repeated (e.g. sea urchin histone genes (Kedes, 1979) and human rRNA genes (Wilson *et. al.*, 1982)). Examples of dispersed DNA families include the *Alu* (Houck *et. al.*, 1979; Rubin *et. al.*, 1980) and L1 families (Singer and Skowronski, 1985; Loeb *et. al.*, 1986). In

higher mammals most members of the intermediate-repetitive group appear to have arisen through one of two main processes : that of tandem duplication and/or retroviral-like transposition.

1.1 Clustered duplicated gene families

1.1.1 Occurrence and mechanism of generation

Some gene families are organised as small clusters in the genome. Examples of such families include the globin genes (Section 1.1.3) and the heat shock protein 70 genes (Section 1.1.4). Members of these clusters are thought to have arisen by a process in which an ancestral gene was duplicated (often more than once) to produce a small array of genes lying in close proximity, in tandem.

The best understood mechanism by which these tandem gene copies are thought to have been generated is by that of homologous reciprocal recombination (at meiosis), with unequal crossing-over. This is depicted in Figure 1.1. This mechanism is most easily envisaged in a situation where two similar tandem copies of a gene already exist. If the two chromatids (copies of a chromosome produced by replication) containing these genes align properly, then no effect on gene number will be observed upon recombination (Figure 1.1.a). However should these chromatids be misaligned, recombination will result in the generation of two different chromosomes; one in which a gene has been deleted and the other in which a gene has been duplicated (Figure 1.1.b). The formation of a hybrid gene (indicated as A/A' in Figure 1.1) is also a consequence of unequal crossing-over. Such duplication is important in evolutionary terms as it provides the potential for one copy of a gene to diverge while the other maintains its function.

This model for duplication cannot however be applied to the duplication of a single gene (e.g. the ancestral gene from which others are derived). However should this gene become flanked by

LEGENDS

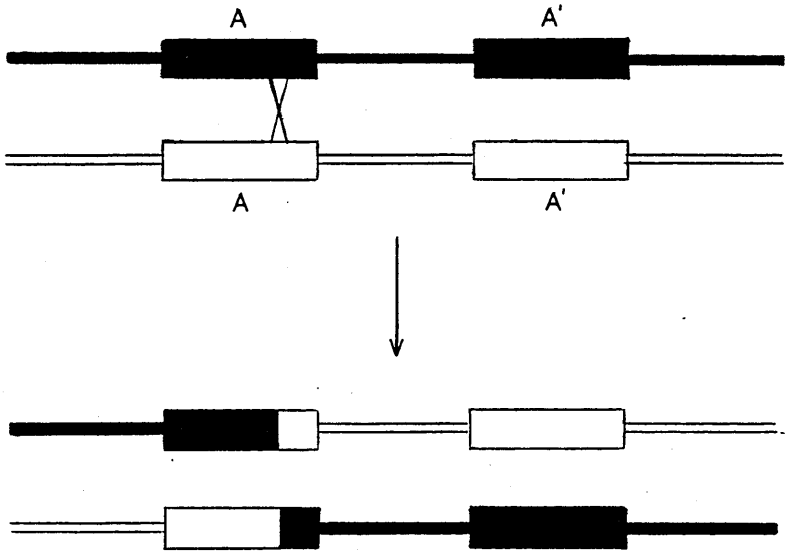
Figure 1.1

Increase in gene number by homologous recombination involving unequal crossing-over

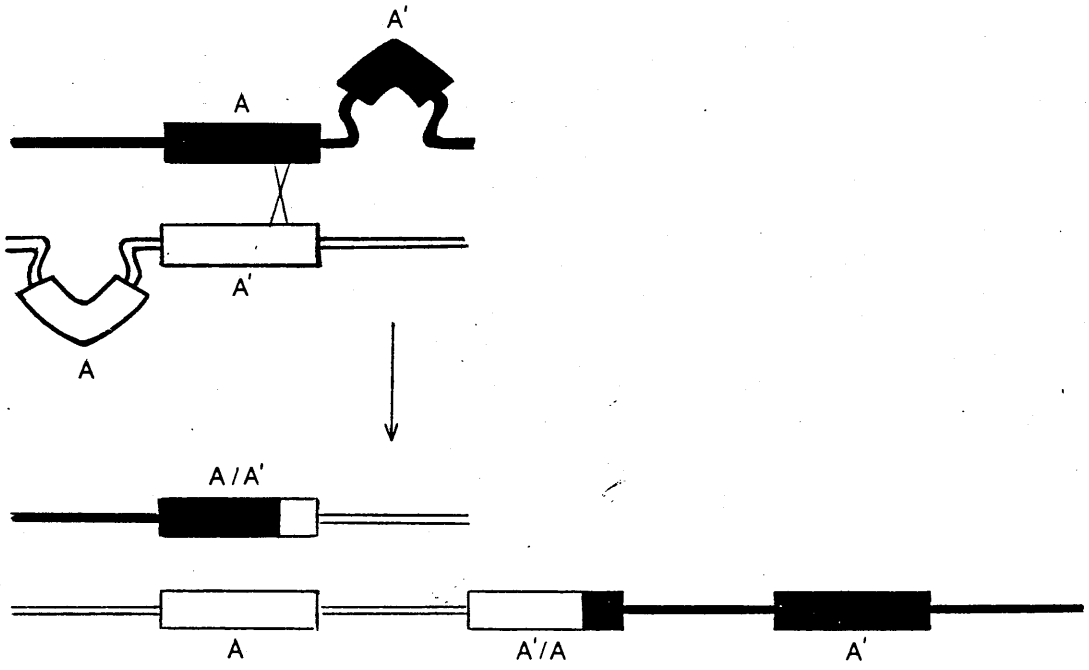
This diagram shows a representation of two tandem copies, A and A′, of a gene (or region of DNA) on two recombining non-sister chromatids (distinguished by light and dark shading) at meiosis. Reciprocal crossing-over on copy A of properly aligned chromatids (a) does not affect gene number, whereas unequal crossing-over between A and A′ on misaligned chromatids (b) generates different products containing one and three copies of the gene, respectively.

Taken from Adams, R.L.P., Knowler, J.T. and Leader, D.P. "The Biochemistry of the Nucleic Acids" 10th edition, 1986.

Figure 1.1



a



b

similar copies of repeated DNA it may undergo 'incidental' duplication as the repeated sequences are subjected to recombination with unequal crossing-over.

1.1.2. Pseudogenes

Although it is clear that certain mutations may lead to new and useful functions in a duplicated gene, others can be so deleterious that they can render such a gene functionally useless. Such genes are called pseudogenes. The first pseudogene to be reported was that of a 5S ribosomal RNA gene of *Xenopus laevis* by Jacq *et. al.*, (1977). This pseudogene lies downstream of the functional 5S RNA gene, is 20 nucleotides shorter at its 3' end than its functional counterpart (101 instead of 121 nucleotides) and in the remaining portion differs from the functional gene by nine base changes (Miller *et. al.*, 1978). No RNA corresponding to this pseudogene has been found *in vivo* and thus it appears to lie inert in the genome. Since the discovery of the 5S RNA pseudogene many other pseudogenes have been identified and characterised. As some of these pseudogenes have arisen by DNA-mediated duplication of functional genes they consequently show some (if not all) of the major structural features of expressed genes e.g. promotors, exons, introns etc. These pseudogenes often lie silent within their respective active gene clusters. The most likely way in which genes have been silenced is by base substitution or deletion. Another type of pseudogene (processed pseudogenes) also exists and these lie dispersed in the genome. These will be dealt with, however, in Section 1.3.4.

1.1.3 Globin genes

Perhaps the best characterised multigene family, the members of which exist in small clusters and appear to have arisen by tandem gene duplication, is that of the globin gene family. The globin genes are thought to have evolved from a single ancestral gene through a series

of events involving duplication, transposition and mutation. It is presumed that an initial duplication gave rise to the prototypes of the two globin gene families: the α-globin and β-globin genes. The common origin of these two families is indicated by the fact that in amphibians they are still linked on the same chromosome (Jeffreys et. al., 1980). In many species these two families are now on separate chromosomes, the result, it is presumed of a translocation event. In man, these two globin gene families lie on different chromosomes: the α-gene cluster on chromosome 16 (Deisseroth et. al., 1977) and the β-gene cluster on chromosome 11 (Gusella et. al., 1979). Genetic, restriction mapping and sequencing studies indicate that, in man, the α-cluster is composed of three functional genes (one zeta (embryonic gene) and two alpha (adult genes)) and two pseudogenes (one zeta and one alpha)(Lauer et. al., 1980; Proudfoot and Maniatis, 1980) and extends over a 28 kb region of the genome (Figure 1.2). The β-cluster is larger, stretching over a 50 kb region, and contains five functional genes (one epsilon (embryonic gene), two gamma (foetal genes), one delta and one beta (adult genes)) and one (beta) pseudogene (Fritsch et. al., 1980 ; Kaufman et. al., 1980). The organisation of the globin gene clusters in other vertebrates is similar, but the types and numbers may vary in detail. In rabbit, for example, four beta-like genes have been identified: three functional genes and one pseudogene (Hardison et. al., 1979; Lacy et. al., 1979), while in goat there are seven beta-like genes: five functional and two pseudogenes (Cleary et. al., 1981; Townes et. al., 1984). In most cases the functional genes are arranged 5' to 3' in the same order in which they are expressed during development (embryonic → foetal → adult). An exception, however, is the β_c-cluster of chicken where the two adult genes are bounded by two embryonic genes. In all cases, the genes within each cluster show close homology. Mapping and sequencing analyses indicate

Figure 1.2

Organisation of the human α-like and β-like globin gene families

a. Map of human α-globin gene cluster, taken from Proudfoot and Maniatis, (1980).

b. Map of human β-globin gene cluster, taken from Fritsch et.al., (1980).

Neither map is drawn to scale.

Key:

α : alpha globin gene

β : beta globin gene

γ : gamma globin gene

δ : delta globin gene

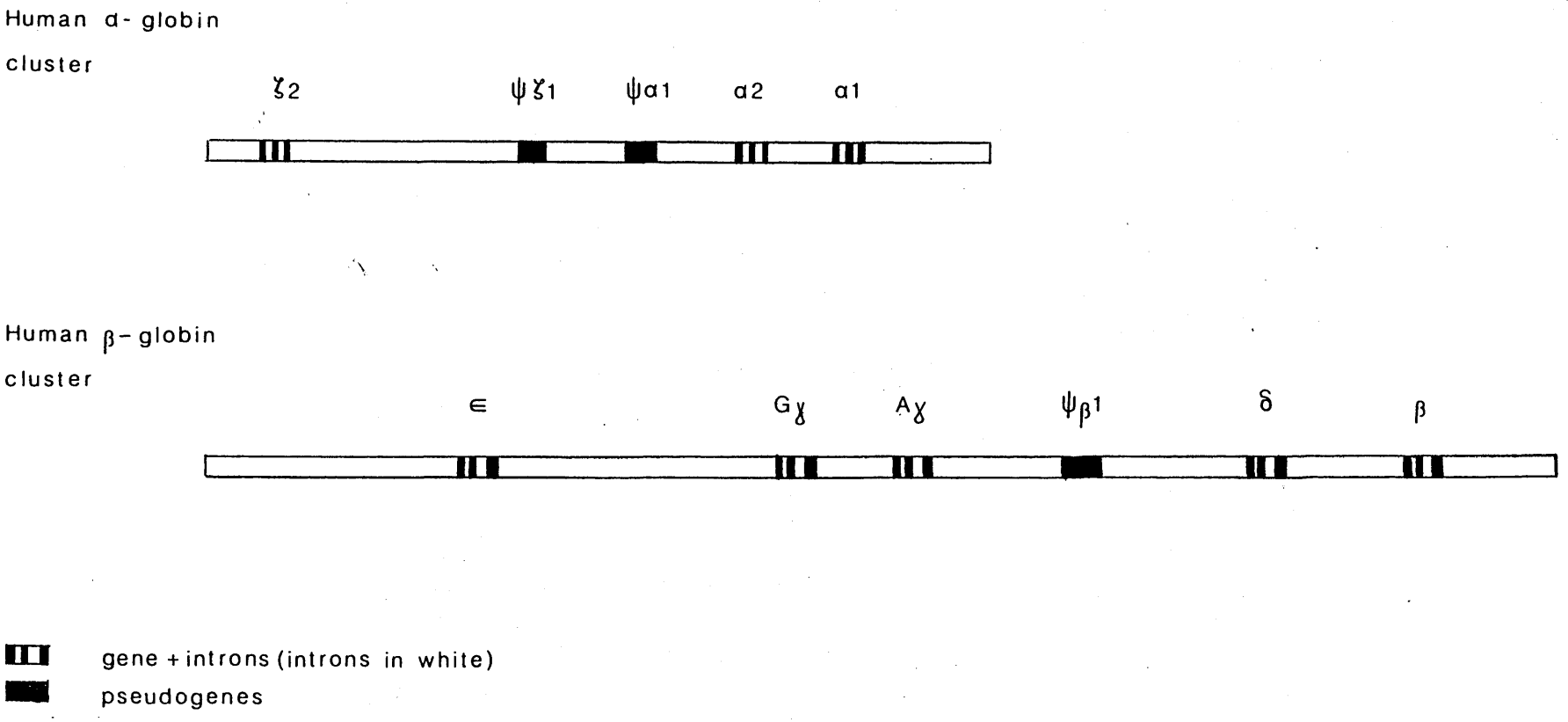
ζ : zeta globin gene

G_γ : gamma gene which produces a gamma globin protein with glycine at position 136

A_γ : gamma gene which produces a gamma globin protein with alanine at position 136

ψ : pseudogene

Figure 1.2


Human α- globin
cluster
ζ2
ψζ1
ψα1
α2
α1
Human β- globin
cluster
∈
Gγ
Aγ
ψβ1
δ
β
gene + introns (introns in white)
pseudogenes

that the globin pseudogenes are commonly located between the adult and foetal or embryonic genes. These pseudogenes are also products of globin gene duplication, but their sequences have since diverged so that they no longer code for a functional globin gene. Further evidence which suggests tandem gene duplication with unequal crossing-over has occurred during the evolution of the globin gene families comes from studies of certain haemoglobinopathies (Lang and Lorkin, 1976). In haemoglobin anti-Lepore an extra gene has arisen between the δ- and β-genes. This gene has a β/δ fusion structure and is consistent with generation by the mechanism of unequal crossing-over shown in Figure 1.1. Evidence of contraction of gene number, the other consequence of unequal crossing-over is illustrated in haemoglobin Lepore where the β- and δ-genes have been replaced by a δ/β fusion gene.

1.1.4 Heat shock protein 70 genes

Another family of genes the members of which are thought to have arisen by duplication is that of the heat shock protein 70 (hsp 70) genes of *Drosophila*.

The first indication that multiple hsp 70 genes existed emerged when it was shown that RNA encoding hsp 70 hybridised to two cytological loci in *Drosophila melanogaster*; 87A7 and 87C1 (Henikoff and Meselson, 1977; Spradling *et. al.*, 1977). Mapping and sequencing studies on *Drosphila melanogaster* (Livak *et. al.*, 1978; Schedl *et. al.*, 1978; Artavanis-Tsakonas *et. al.*, 1979; Craig *et. al.*, 1979; Moran *et. al.*, 1979) and other *Drosophila* species (Leigh Brown and Ish-Horowicz, 1981) have revealed a similar organisation of hsp 70 genes at the two hsp 70 loci found in each species: two hsp 70 genes in opposite orientation (Figure 1.3). Since most species studied so far show such an arrangement (at both their respective hsp 70 loci) it is suggested that these two bands arose originally by duplication of a single band. The arrangement at 87C1 of *Drosophila melanogaster*,

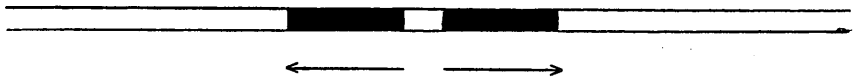
Figure 1.3

Organisation of hsp 70 genes at loci 87A and 87C of different species of Drosophila

Shaded regions correspond to hsp 70 genes. Arrows indicate orientation of these genes. Maps are not drawn to scale.

Figure 1.3

87A

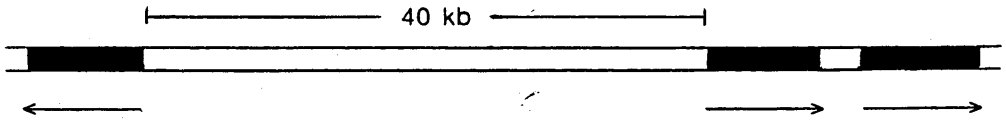


D. melanogaster
D. simulans
D. mauritiana

87C

D. simulans
D. mauritiana

87C



D. melanogaster

however, is slightly different. Three copies of the hsp 70 gene are present. One of these genes (which is in inverted orientation relative to the others) is located approximately 40 kb upstream from the remaining pair which lie in tandem, approximately 0.8 kb apart (Ish-Horowicz and Pinchin, 1980)(Figure 1.3). This arrangement is thought to have arisen through DNA insertion between the two diverging hsp 70 genes of the ancestral locus with tandem duplication of one of these copies. This third copy at 87C1 seems to be the result of a later duplication event since it is not present in several related species.

Another heat-inducible gene in Drosophila, hsp 68 (which resides at cytological locus 95D), seems to be related to hsp 70 (Holmgren et. al., 1979). Melting studies of duplexes formed between isolated hsp 70 and hsp 68 genes indicate a 15% DNA sequence divergance between the genes (Holmgren et. al., 1979).

The hsc 70 (heat shock cognate) genes of Drosophila, located at cytological loci 70C, 87D and 88E, also appear to be related to hsp 70 (Ingolia and Craig, 1982; Craig et. al., 1983). These cognate genes are 75% identical in DNA sequence in the protein coding region to each other and to the hsp 70 and hsp 68 genes. Transcripts of these genes are normally present in non heat-shocked cells.

In yeast there are 8 related hsp 70 genes, though their organisation in the genome is not fully determined (Ingolia et. al., 1982).

Although studies are still in an early stage, there also appears to be a family of hsp 70 genes in mammals. These genes code for three main hsp 70-like proteins: hsp 70, hsc 70 and grp 78 (present in secretory cells). Primate cells also have an additional member, that of hsx 70 (which is heat-inducible, E1A inducible and cell cycle regulated)(Pelham, 1986). The precise number and organisation of these hsp 70 genes is not yet known.

1.2 Tandem duplicated gene families

1.2.1 Occurrence and mechanism of generation

Other multigene families of the intermediate-repetitive group are present in large tandem arrays in the genome. Examples of such families include the rRNA genes and the histone genes. As for the smaller clusters of genes described in Section 1.1, these multigene families are thought to have arisen by homologous reciprocal recombination with unequal crossing-over. In this case, such an event has occurred many times, producing an extensive repetition of gene family members, in tandem. Such large tandem arrays differ from the smaller ones described above in one other characteristic. This is that members have a much closer resemblance to one another (see Section 1.5, below).

1.2.2 5S rRNA genes

The 5S rRNA genes have been analysed in most detail in *Xenopus laevis*. In most chromosomes of *X.laevis* there is a cluster of 5S rRNA genes located at or near the telomere (end of the chromosome). There are two distinct 5S rRNA gene families comprising separate tandem arrays. These families encode slightly different 5S rRNA sequences, one expressed during oogenesis and the other throughout the developmental cycle (excluding embryogenesis)(Brown and Sugimoto, 1973; Fedoroff, 1979). About 20,000 genes code for oocyte 5S RNA. Each repeating unit contains a variable AT-rich spacer, a single gene, a spacer of about 73 bp and a pseudogene (Jacq *et. al.*, 1977; Fedoroff and Brown, 1978). Different repeating units can frequently differ in their spacer length. The other 'somatic' 5S RNA genes are less abundant (Fedoroff, 1979). In *Xenopus borealis* the somatic 5S RNA unit is 850 bp long and consists of a GC-rich spacer and a gene copy (Fedoroff, 1979).

1.2.3 Large ribosomal RNA genes

The 18S and 28S rRNA genes of higher eukaryotes, together with the 5.8S rRNA genes are organised in a basic unit (Figure 1.4), and it is this unit which is repeated many times in tandem. These tandem arrays are found in nucleolar organiser regions (particular chromosomal regions found associated with the nucleolus where rRNA synthesis occurs). In X.laevis there is one nucleolar organiser region (comprising 400-500 tandemly-arranged rDNA copies) while in primates there are several (Hsu et. al., 1967).

1.2.4 Histone genes

Also occurring in multiple copies are the genes for the histones: H1, H2A, H2B, H3 and H4. The most well-characterised histone gene cluster is that of sea urchin. In several sea urchin species there are as many as 300-600 copies of each histone gene (Old and Woodland, 1984). All five early histone gene species are present in a basic unit which is repeated many times in tandem (Figure 1.5)(Schaffner et. al., 1978; Kedes, 1979). There appear to be no intervening sequences within the mRNA coding regions. In sea urchin, at least, all histone genes share the same polarity. The main difference between species lies in the non-transcribed spacers. These differ not only in length but in sequence. The spacer regions between the genes are smallest in Psammechinus miliaris and largest in Lytechinus pictus (Kedes, 1979)(Figure 1.5).

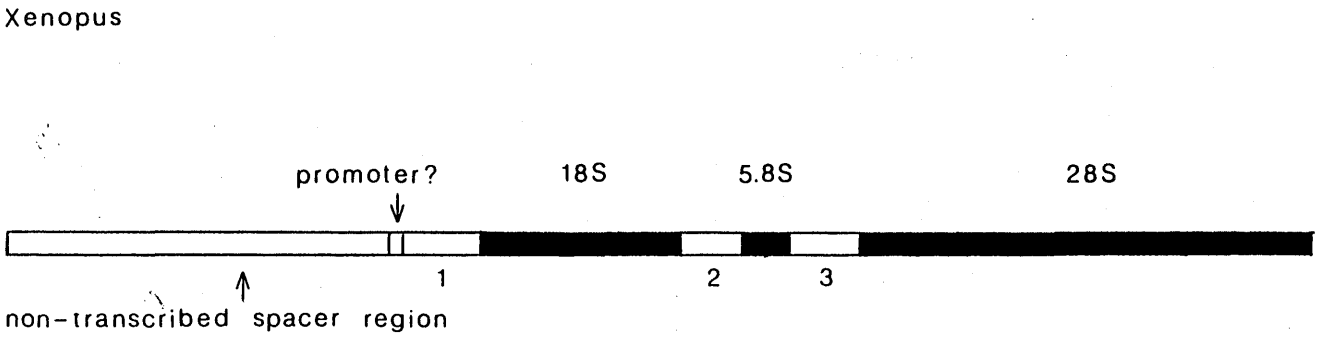
The above quintet pattern of organisation has also been found in Drosophila (Figure 1.6), however the polarity of the genes is different. In Drosophila there are 100 copies of this basic repeating unit. These repeat units can be divided into two classes, which differ by an insertion of 240 bp of spacer. The two classes are arranged in tandem, shorter ones sometimes adjacent to the longer type. Drosphila histone genes, however, are not all transcribed from the same strand, as in

Figure 1.4

Repeat unit of ribosomal DNA of Xenopus

Schematic illustration of a repeat unit of ribosomal DNA in Xenopus. Shaded regions correspond to ribosomal genes. Map not drawn to scale.

Figure 1.4



1,2 and 3 : transcribed spacer regions

Figure 1.5

Organisation of the early histone genes of different sea urchin species

Schematic diagram of the early histone repeat units from three sea urchin species: Lytechinus pictus (L. pictus); Strongylocentrotus purpuratus (S. purpuratus); Psammechinus miliaris (P. miliaris). Shaded regions indicate regions encoding proteins. Arrows show orientation of the genes. The numbers indicate the lengths of the spacer regions (in base pairs). Map not drawn to scale.

Figure 1.5

L. pictus	820	1350	610	790	1100
S. purpuratus	690	1140	610	580	830
P. miliaris	420	880	420	510	675

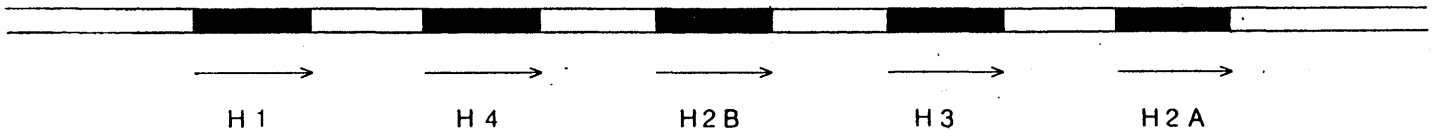
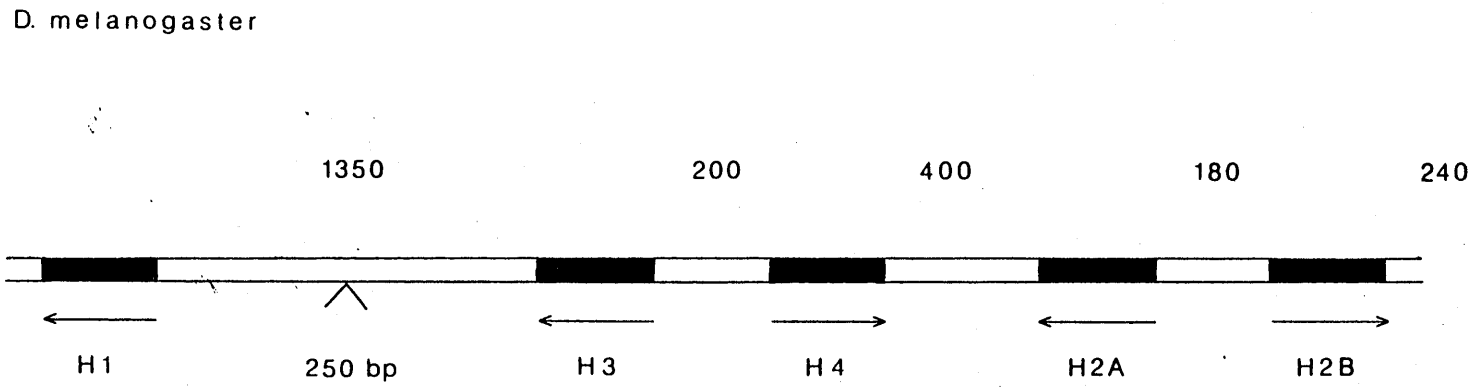


Figure 1.6

Organisation of the histone genes of Drosophila melanogaster

Schematic diagram of the histone gene repeat unit of Drosophila melanogaster. Shaded regions indicate regions encoding proteins. Arrows show orientation of the genes. The numbers indicate the lengths of the spacer regions (in base pairs). Map not drawn to scale.

Figure 1.6



sea urchin. H4 and H2B are transcribed from one strand whereas H1, H3 and H2A are transcribed from the other (Lifton et. al., 1977; Kedes, 1979).

1.3 Dispersed gene families

1.3.1 Occurrence and mechanism of generation

Not all families of repeated DNA in the intermediate-repetitive class have arisen through the mechanism of tandem duplication. Another mechanism which involves retroviral-like transposition (RNA-mediated mechanism) is thought to be responsible for the generation of certain other families. Families, the members of which have been generated by such a mechanism, are collectively known as retroposons. Two interspersed families of the intermediate-repetitive group, the members of which have arisen by retrotransposition are the short and long interspersed repetitive elements (SINES and LINES respectively). The SINES are typified by the Alu - family in man (Schmid and Jelinek, 1982; Sharp, 1983) while the LINES are typified by the L1 elements: L1Hs in man (Singer, 1982) and L1Md in mouse (Loeb et. al., 1986). They differ from retroviruses and transposons,such as Ty (Eibel et. al., 1980) and Copia (Levis et. al., 1980) in that they lack long terminal repeats and in the case of Alu also appear to lack the ability to code for the machinery for their own transposition. Members of such families have been generated by reverse transcription of the respective RNA species followed by the subsequent non-specific integration of the DNA copy into the genome by a transposition mechanism (characterised by flanking target-site direct repeats). As the DNA copy is inserted non-specifically into the genome, members of retroposon families lie dispersed in the genome.

1.3.2 SINES

The Alu - family, so called since most of its members contain Alu I restriction sites, is the most dominant interspersed family in man

(Houck et. al., 1979). There are about 500,000 copies of Alu in human DNA. Several Alu - members have been characterised by nucleotide sequence analysis (Jelinek et. al., 1980). Alu - family members usually consist of a head to tail tandem arrangement of two related sequences about 130 bp long, each terminating in an A-rich segment at what corresponds to the 3' end of the transcript (Figure 1.7). The 5' half contains a 31 bp insert which is usually, but not always, absent in the 3' half (Duncan et. al., 1981). In the genome these sequences can be flanked by direct repeats 5-20 bp long (Bell et. al., 1980; Baralle et. al., 1980; Duncan et. al., 1981). Alu - elements possess significant sequence homology to the small cytoplasmic 7S RNA. About 100 nucleotides at the 5' - end and 50 nucleotides at the 3'-end of the 300 nucleotides 7S RNA have about 80% identity to the consensus Alu - sequence (Ullu et. al., 1982). The central portion of the 7S RNA primary sequence however is not represented in the Alu - repeat (Ullu and Tschudi, 1984)(Figure 1.7). Some (but not all) Alu - elements are capable of being transcribed by RNA polymerase III (Jagadeeswaran et. al., 1981); the transcript includes RNA complementary to the whole Alu - element and terminates a little way beyond it (possibly at an oligo (dT) stretch in flanking 3' DNA). It is suggested that this 3' region hybridises to the A-rich region and then acts as a priming site for reverse transcription, the reverse DNA copies then being reinserted into the genome at random locations (Jagadeeswaran et. al., 1981). These Alu - sequences retain their RNA polymerase III internal promotors and thus have the potential for further transposition. However the accumulation of mutations may in some cases inactivate these promotors and deprive the Alu - sequence of the capacity for further transposition.

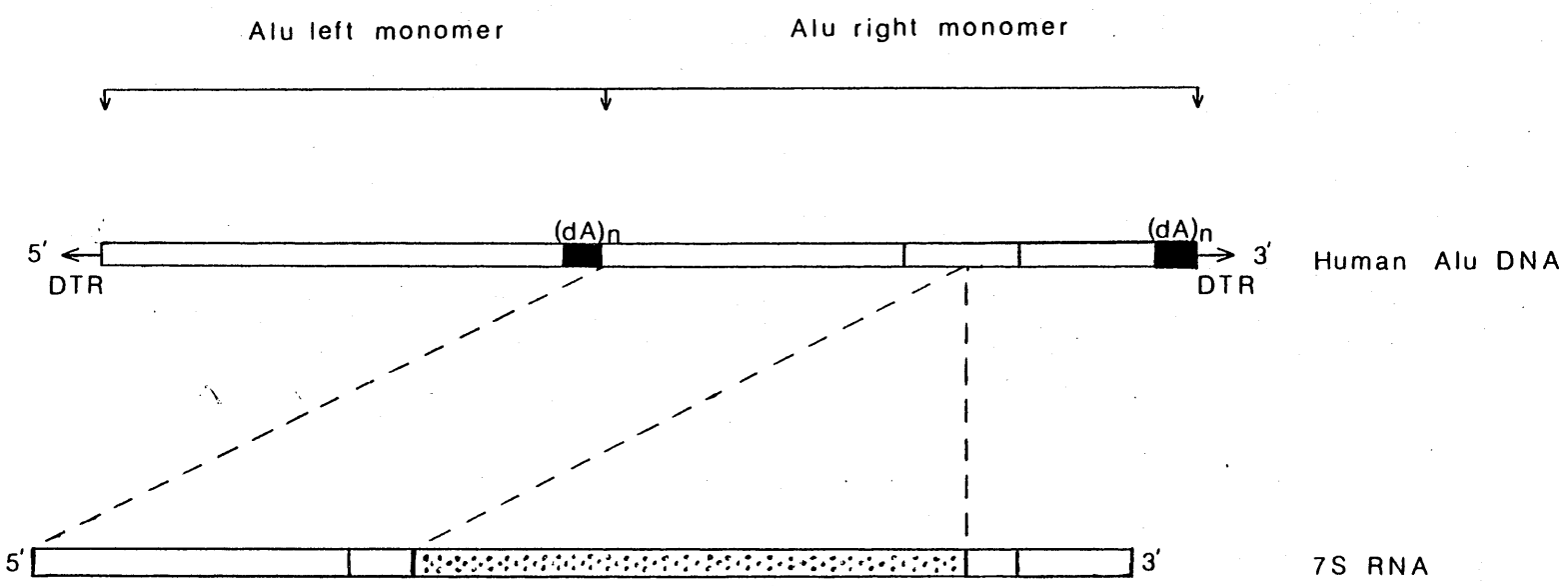
Alu - equivalents have been reported in other primates, e.g. monkey (Grimaldi et. al., 1981), as well as in rodents, e.g. mouse

Figure 1.7

Structural features of Alu-DNA and relationship to 7S RNA

Schematic diagram of Alu-DNA and 7S RNA. Dotted region represents the central sequences of 7S RNA absent from either Alu-repeat. Maps not drawn to scale.

Figure 1.7



DTR : direct repeat

(Krayev et. al., 1980) and chinese hamster (Haynes et. al., 1981). A comparison of the structure of human and rodent Alu-like DNAs is shown in Figure 1.8. The best homology between human and rodent Alu-like DNAs is found with the 3' 130 bp sequence of Alu (Haynes et. al., 1981)(Figure 1.8). Rodent Alu-like DNA also contains a 32 bp insert which is different in nucleotide sequence from the 31 bp insert present in the human Alu - repeat. Excluding the insert regions, the human and rodent Alu - DNA sequences have approximately 80% identity.

1.3.3 LINES

L1 elements are believed to be the only major family of long interspersed repeated elements in primate DNA. There are about 10^4-10^5 copies in the human genome (designated L1Hs), and these account for 2-3% of the total DNA. L1Hs family members are heterogeneous in length. The largest are 6-7 kb in size, but shorter truncated versions predominate, usually missing sequences from the end designated 5', but generally containing the 3' end (Figure 1.9)(Kole et. al., 1983; Grimaldi et. al., 1984). The 3' sequence terminates in an A-rich region of variable length (Grimaldi et. al., 1984). Individual L1Hs elements are flanked by small direct repeats, which are consistent with a mechanism which generates staggered DNA strand breaks at the point of their insertion (Grimaldi et. al., 1984). These characteristics, as in the case of Alu - repeats, suggest L1Hs elements may have been dispersed through an RNA-mediated mechanism, the truncation in some cases being consistent with their generation by varying extents of reverse transcription.

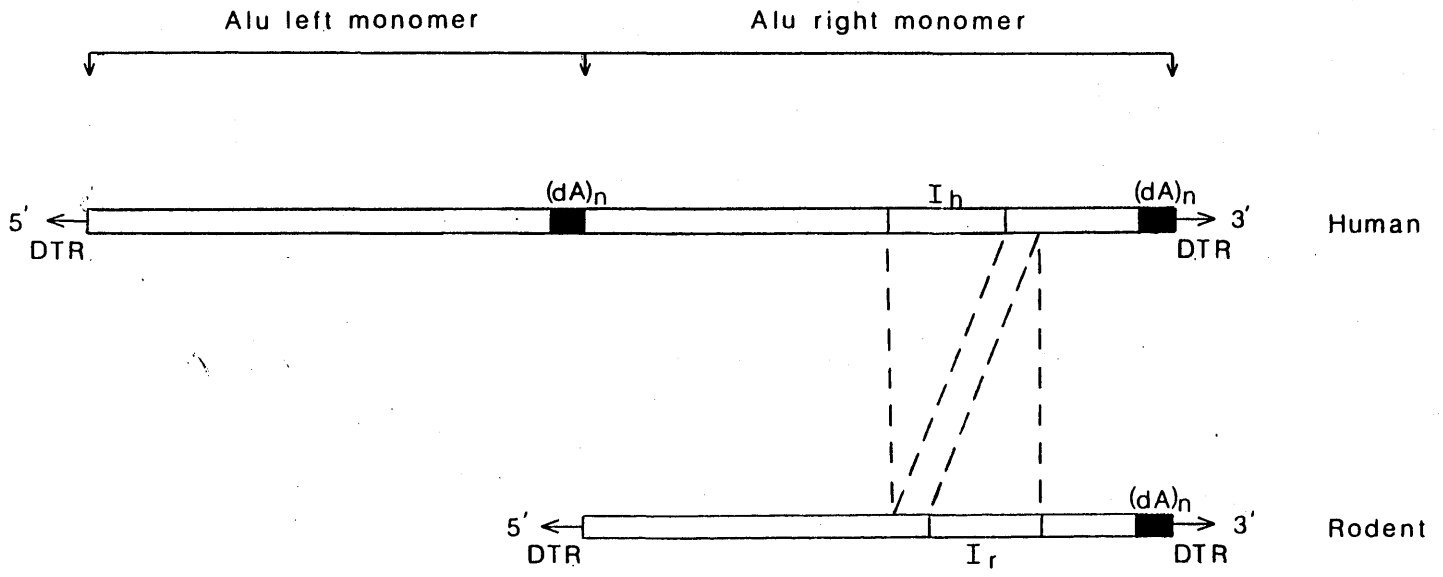
Similar sequences are also present in the genomes of mice (L1Md) (Figure 1.9) and other rodents (Fanning, 1983; Voliva et. al., 1983). They too are most often truncated at their 5'-ends, possess an A-rich tail at their 3'-ends and are flanked by small (less than 15 bp) direct repeats, suggesting dispersal through an RNA intermediate (Gebhard et.

Figure 1.8

Structural features of rodent Alu-like DNA and relationship to human Alu DNA

Schematic diagram of human and rodent Alu-DNA. I_h corresponds to a 31 base pair insert found only in human Alu-DNA. I_r corresponds to a 32 base pair region found only in rodent Alu-DNA. Maps not drawn to scale.

Figure 1.8



DTR:direct repeat

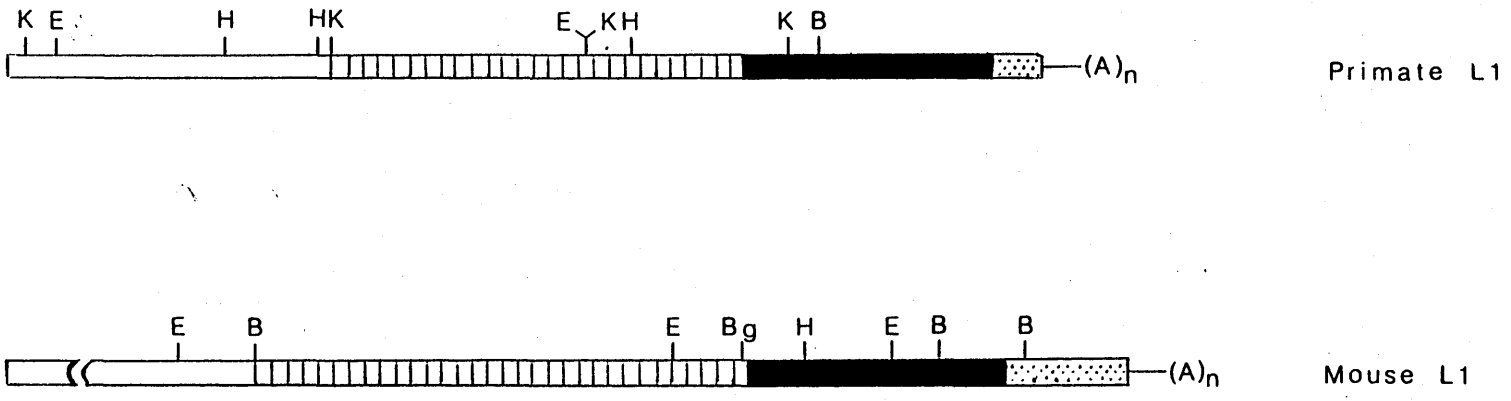
Figure 1.9

Structural features of primate and mouse LINE 1 elements, L1Hs and L1Md

The restriction endonuclease sites indicated (B - Bam HI; Bg - Bgl II; E - Eco RI; H - Hind III and K - Kpn I) are found in many but not all family members. Regions where sequence comparison shows the two L1s to be homologous are shaded. Regions where cross-hybridisation shows homology are hatched. The dotted segments at the 3′ end are not homologous.

Taken from Singer and Skowronski, (1985).

Figure 1.9



al., 1982; Fanning, 1983; Voliva et. al., 1983; Wilson and Storb, 1983; Voliva et. al., 1984).

Restriction endonuclease analyses of L1 elements indicate that the longer L1Hs and L1Md elements can give rise to several different characteristic restriction endonuclease fragments which were previously considered to be generated from separate repeated families. These were the 1.9 kb Hind III family (Manuelidis, 1982), 1.2 kb, 1.5 kb and 1.8 kb primate Kpn I families (Grimaldi et. al., 1984) and the mouse 0.5 kb and 4 kb Bam HI and 1.3 Eco RI families (Brown and Dover, 1981; Soriano et. al., 1983). Sequence data and Southern blot hybridisation studies indicate that these fragments are contiguous in L1. Neither primate nor rodent L1 elements appear to have terminal repeats (Rogers, 1985). DNA sequence comparison suggests that the longer L1 elements of rodents and primates have 60% identity in a 1.5 kb region near the 3'-end but the similarity ends 200 bp from the 3'-end of the primate sequence and 750 bp from the 3'-end of the mouse sequence (Singer et. al., 1983).

L1 elements also possess open reading frames (Figure 1.10). There are thought to be four open reading frames in primate L1 elements (Singer and Skowronski, 1985) but analysis of a complete L1Md member suggests only two open reading frames in mouse (Loeb et. al., 1986). It is not yet known whether these encode proteins required for L1 transposition, although the predicted product from one of these open reading frames bears some similarity to reverse transcriptase (Loeb et. al., 1986).

1.3.4 Processed pseudogenes

Not all pseudogenes have been generated by tandem duplication, as described in Section 1.1.2. For some there is evidence to suggest that an RNA template is involved in their generation, as for Alu - repeats. These pseudogenes generally resemble mutated DNA copies of

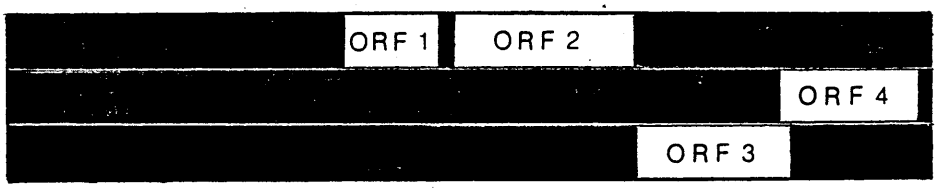
Figure 1.10

Apparent open reading frames of L1 elements

a. Apparent open reading frames (ORFs) in primate L1 elements (Singer and Skowronski, 1985)

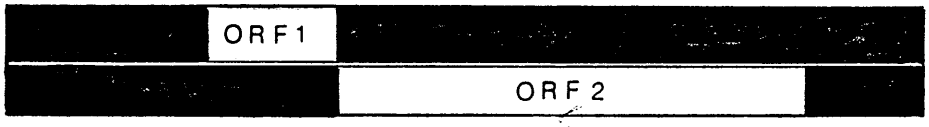
b. Apparent open reading frames of one L1Md element (Loeb et.al., 1986).

Figure 1.10



primate L1

a



L1Md

b

mature RNA. Thus in most cases they possess a poly(A) tail (which is absent from the functional gene) at the 3'-end of the pseudogene in a position equivalent to the poly(A) on mRNA; lack all introns present in the functional gene; and are flanked by direct repeat sequences immediately preceding the transcriptional start and immediately following the poly(A) tail. They reside at chromosomal locations distant from functional genes. These pseudogenes are referred to as processed pseudogenes (Sharp, 1983).

The above characteristics indicate that these pseudogenes have arisen by the reverse transcription of mature RNA to produce a complementary DNA which was subsequently inserted into the host chromosome at a staggered break (Figure 1.11). The exact source of reverse transcriptase and nature of the primer for reverse transcription are not yet known, although a suggestion is that retroviruses may provide the reverse transcriptase. One model which has been proposed for priming reverse transcription rests on the observation that the target sites of transposition frequently have a dA-rich segment at their 5'-ends (Moos and Gallwitz, 1983; Vanin, 1984). A staggered break at such a point would provide an oligo (dT) attachment site for the poly(A) tail of the mRNA, and this oligo(dT) segment would act as a primer for reverse transcription (Figure 1.11).

In general most processed pseudogenes lie inert in the genome, unlikely to be transcribed as they lack 5' promotor sequences. One exception is the processed calmodulin gene of chicken (Stein et. al., 1983). It is not known yet what promotor sequences are responsible for the expression of this gene.

Processed pseudogenes are abundant for 'house-keeping' genes such as β-tubulin (Wilde et. al., 1982) and β-actin (Moos and Gallwitz, 1982 and 1983), which are expressed in the germ line. There are a few

Figure 1.11

Proposed mechanism for processed pseudogene formation

The following steps are proposed for processed pseudogene formation:

a. staggered nucleolytic cut at A/T-rich target site.

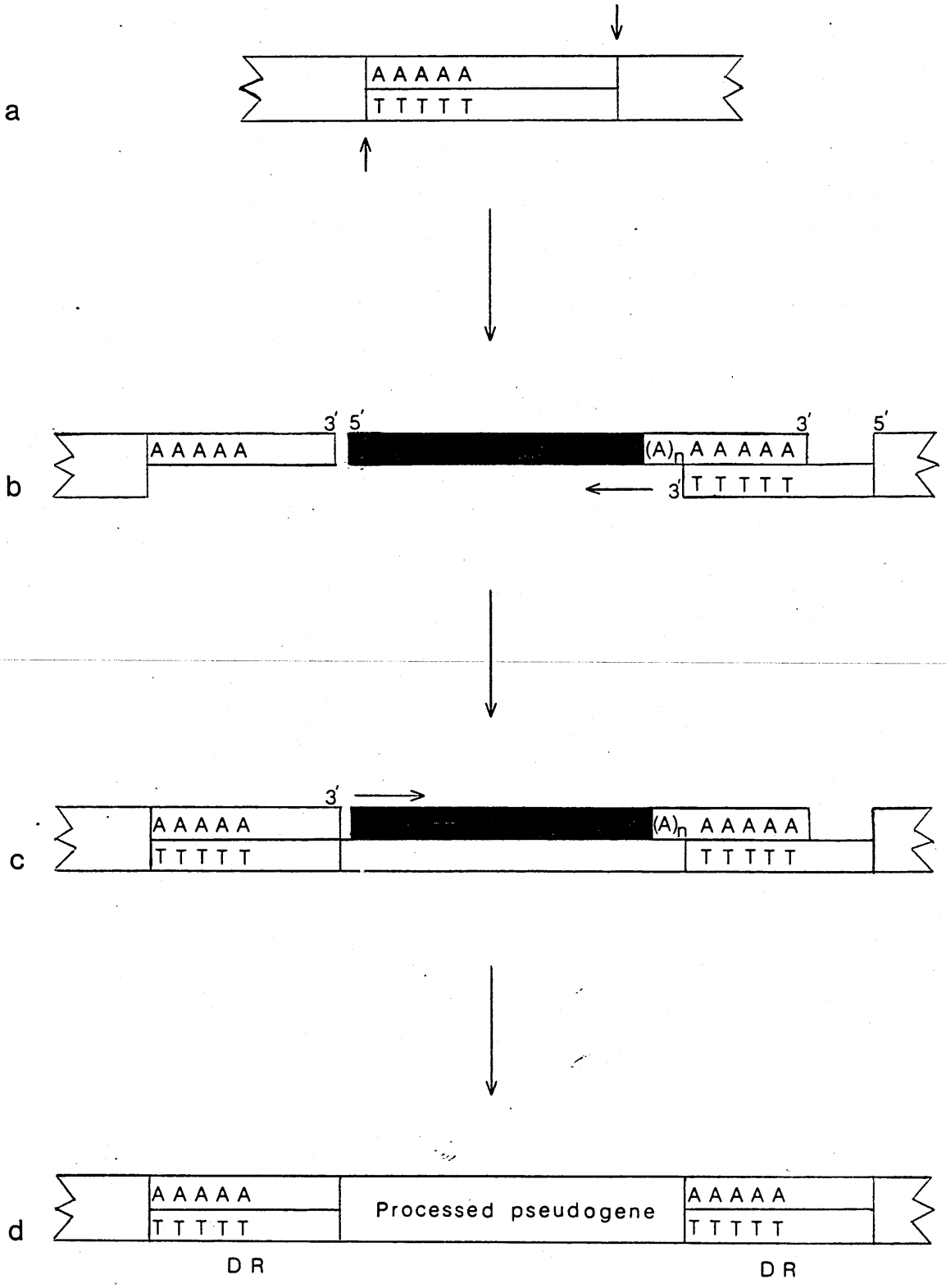
b. annealing of mRNA (solid shading) to 3′ protruding oligo (dT) end. This serves as a priming site for reverse transcription.

c. RNA is displaced or degraded for the synthesis of the second DNA strand.

d. processed pseudogene flanked by target site direct repeats (DR).

Taken from Adams, R.L.P., Knowler, J.T. and Leader D.P. "The Biochemistry of the Nucleic Acids" 10th edition, 1986.

Figure 1.11


a
A A A A A
T T T T T
b
3′
5′
A A A A A
(A)n A A A A A
3′
5′
3′
T T T T T
c
3′
A A A A A
T T T T T
(A)n A A A A A
T T T T T
d
A A A A A
T T T T T
Processed pseudogene
A A A A A
T T T T T
D R
D R

examples of processed pseudogenes corresponding to proteins only found in differentiated cells e.g. α- globin (Nishioka et. al., 1980), and the manner in which these must have been integrated into the germ-line is unclear.

1.3.5 Small nuclear RNA pseudogenes

Another type of RNA, other than mRNA, which can generate pseudogenes by mechanisms which involve reverse transcription is small nuclear RNA. Small nuclear RNAs (snRNAs), U_1 to U_6, are a family of abundant discrete RNAs found associated with proteins in ribonucleoprotein particles in the nuclei of eukaryotes. U_1, U_2, U_4 and U_6 RNAs are nucleoplasmic and are thought to be involved in splicing and processing of mRNA precursors, while U_3 RNA is restricted to the nucleolus and has been implicated in the processing of rRNA (Busch et. al., 1982). Hybridisation experiments indicate that each snRNA species is encoded by approximately 100-2000 genes which lie dispersed in the genome (Hayashi, 1981; Manser and Gesteland, 1981; Westin et. al., 1981; Lund and Dahlberg, 1984). These snRNAs give rise to two structural classes of pseudogene. The first encode full-length snRNAs and contain scattered base substitutions and insertions (Manser and Gesteland, 1981; Westin et. al., 1981). They additionally have short 3' A-rich segments at their ends or preceding a 3' direct repeat sequence (Van Arsdell et. al., 1981; Denison and Weiner, 1982). Since poly(A) is not normally present on snRNAs, such pseudogenes must have been derived from aberrantly polyadenylated molecules. The second class contains pseudogenes that are truncated to various degrees at their 3' ends (Bernstein et. al., 1983). The U_3 pseudogenes, in particular, are severely truncated at their 3' end and at nearly identical positions. U3 snRNA has been found to act as a self priming template in vitro for AMV reverse transcriptase, generating a cDNA that is of closely similar length to the U3 pseudogenes (Bernstein et. al., 1983). It is suggested

that these truncated pseudogenes might have been generated by the integration of similar self-primed cDNAs formed *in vivo*.

1.4 Orphons

A class of repeated DNA having some features of members of both categories 1.2 and 1.3 above, has been termed 'orphon.' This designation was initially coined for dispersed members of the tandem multigene families that encode histones and ribosomal RNAs in yeast, *D. melanogaster* and sea urchin (Childs *et. al.*, 1981). It is likely that orphons encompass disparate DNA species, perhaps including functional genes as well as inactive genes of different types. Some orphons have been cloned and studied in more detail. One such orphon, a histone H3 orphon from the sea urchin *L. pictus*, retains greater than 98% identity in both its coding and 5' and 3' flanking region with its counterpart in the parent gene cluster (Childs *et. al.*, 1981) and is thought to have arisen through a DNA-mediated mechanism. Childs *et. al.*, (1981) proposed that during unequal crossing-over events, misaligned tandem repeats might form a looped out segment which would be a potential target for excision during repair processes. The excised DNA segment would then reintegrate into the genome and create such an orphon. The structure of the early histone 2B orphon from sea urchin *Strongylocentrotus purpuratus* (Liebermann *et. al.*, 1983) suggest however that it was more likely to be derived through an RNA-mediated process. The *S. purpuratus* orphon has a 32 bp long poly(A) tract at its 3'-end (although histone mRNAs are not normally polyadenylated) and is flanked by six-nucleotide direct repeats, indicative of its integration at a staggered chromosomal break. Although rRNA orphons were noted in Southern blotting studies (Childs *et. al.*, 1981), no individual members were studied.

1.5 Maintenance of similarity among different members of a gene family

Although it might be expected that a pair of DNA sequences would diverge independently after tandem duplication, there is clear evidence that in some repeated DNA families the copies have remained in a remarkably similar state. Corrective mechanisms must therefore exist to rectify such divergances as they occur and maintain sequence homogeneity. Two processes in particular have been postulated. One proposal is that a series of unequal homologous crossing-over events of the type shown in Figure 1.1 can lead to homogenisation of the structure of the members of tandemly-arranged genes (Petes, 1980; Szostak and Wu, 1980). The other proposed process is that of gene conversion where one copy of a gene is replaced by another (in part or whole) in a non-reciprocal process (Baltimore, 1981)(Figure 1.12).

Studies by Petes, (1980) and Szostak and Wu, (1980) have shown that tandemly-arranged genes such as rDNA genes appear to be maintained by unequal-crossing-over. By inserting a (marker) LEU 2 gene into the rDNA gene cluster of yeast (strain leu^-) they have been able to show, by restriction analysis, that deletions and duplications due to unequal crossing-over do occur during mitotic growth.

While unequal crossing-over events can take place only among tandemly-arranged genes of a family, gene conversion can take place between either tandem copies of a gene on the same chromosome, tandem genes interacting from sister chromatids, or a pair of related genes located anywhere in the chromosomes of the organism (Baltimore, 1981). In gene conversion (Figure 1.12), gene A interacts with gene A' in such a way that part or all of the nucleotide sequence of A becomes identical to that of A'. In the interaction, gene A and gene A' retain their integrity and their physical locations but a non-reciprocal alteration in the structure of one gene occurs. This

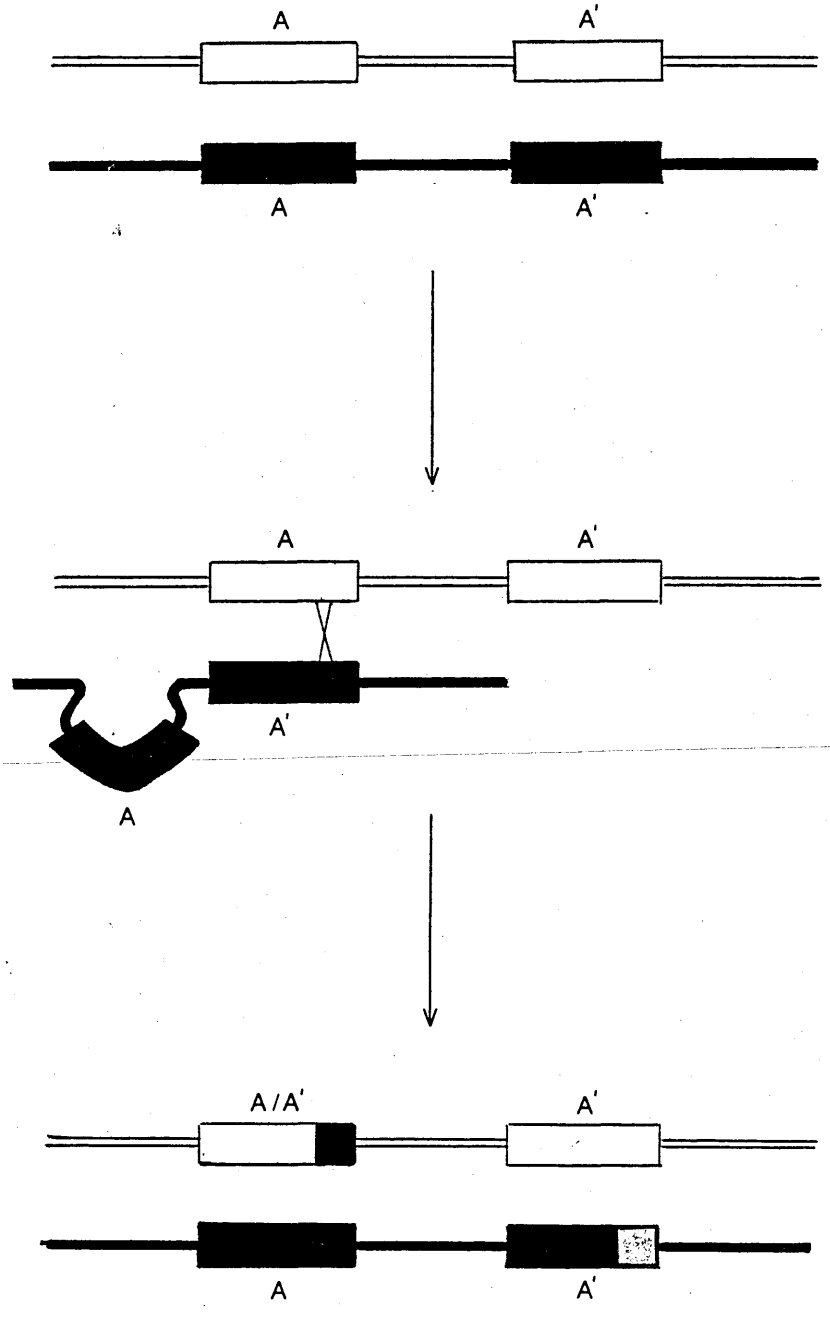
Figure 1.12

Gene conversion

Schematic diagram of proposed process of gene conversion.

This diagram shows a representation of two tandem copies, A and A′, of a gene (or region of DNA) on two recombining chromatids (distinguished by light and dark shading). Non-reciprocal crossing-over on copy A of mis-aligned chromatid generates e.g. gene A with a portion homologous to gene A′ while gene A′ remains unaffected.

Figure 1.12



process -- avoids many of the consequences of unequal crossing-over, such as gene loss and rearrangement. Families in the intermediate-repetitive group, the members of which have been maintained by gene conversion include the globin genes, hsp 70 genes and the immuno-globulin genes.

Slightom et. al., (1980) were the first to provide evidence that gene conversion may occur in β-globin genes. They found regions of homology between adjacent β-like globin genes on one chromosome that were not reflected on the other. Similarily, evidence has been presented for the involvement of gene conversion in the maintenance of the human α-globin genes in man (Liebhaber et. al., 1981) and for the segmental homology between a mouse α-globin pseudogene and functional mouse α-globin gene (Miyata and Yasunga, 1981).

As the process of unequal crossing-over cannot act on inverted repeats it has been suggested by Leigh Brown and Ish-Horowicz, (1981) that the hsp 70 genes are also subject to gene conversion.

Additional homology among the subregions of a pair of immuno-globulin γ-genes (Miyata et. al., 1980) indicates that gene con-version occurs in immunoglobulin genes too.

OBJECTIVES

The initial objective of this work was to study one of the multigene families described above; that of the heat-shock proteins. The human cDNA clone, pHS2, was used for this purpose as it had been reported to correspond to the γ-polypeptide of the major 72,000-74,000 group of HeLa cell heat-shock proteins (Cato et. al., 1981). In the course of this work it emerged that clone pHS2 does not contain heat-shock DNA sequences. Nevertheless the genomic clones isolated using this probe did contain other members of the multigene families described in this Introduction, and it is a consideration of these that constitutes the bulk of the work described in this thesis.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS

Part A : Materials

2.1 Suppliers

The names and addresses of the suppliers used are given below.

Aldrich, Gillingham, Dorset, U.K.

Amersham International plc., Amersham, Bucks., U.K.

Anglian Biotechnology Ltd., Colchester, Essex, U.K.

A. & J. Beveridge Ltd., Edinburgh, U.K.

BBL Microbiology Systems, Becton Dickinson & Co., Cockeysville,U.S.A.

B.D.H. Chemicals, Poole, Dorset, U.K.

Beckman Ltd., High Wycombe, Bucks., U.K.

Bio-rad Laboratories Ltd.,Watford, Herts., U.K.

Boehringer Mannheim, Lewes, East Sussex, U.K.

B.R.L./Gibco Ltd., Paisley, Renfrewshire, U.K.

Difco Laboratories, East Molesey, Surrey, U.K.

Flurochem Ltd., Derbyshire, U.K.

Hannimex X-rays, London, U.K.

Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Pharmacia Fine Chemicals AB, Uppsala, Sweden.

P.L. Biochemicals Inc., Wisconsin, U.S.A.

Schleicher & Schüll, Dassel, W. Germany.

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Uniscience Ltd., Cambridge, U.K.

Whatman, Cambridge, U.K.

2.2 Radiochemicals

All of the radiochemicals used were supplied by Amersham International plc.

α [^{32}P] dATP,dCTP,dGTP,dTTP : specific activity 3000 Ci/mmol

γ [^{32}P] ATP : specific activity 3000 Ci/mmol

2.3 Chemicals and enzymes

Most of the reagents used were of Analar grade, supplied by B.D.H. Chemicals Ltd. with the exception of the following:

DMS	: Aldrich
restriction enzymes	: Anglian Biotechnology Ltd.
tripticase agar base	: BBL Microbiology Systems
Biogel - P60	: Bio-rad Laboratories Ltd.
ATP	: Boehringer Mannheim
calf intestinal alkaline phosphatase	: "
caesium chloride	: "
dATP	: "
dCTP	: "
dGTP	: "
dTTP	: "
DNA polymerase I	: "
DNA polymerase I Klenow fragment	: "
proteinase K	: "
restriction enzymes	: "
T4 DNA ligase	: "
Tris	: "
IPTG	: BRL/Gibco Ltd.
lambda DNA	: "
phenol	: "
restriction enzymes	: "
urea	: "
X-gal.	: "
bactoagar	: Difco Laboratories
bactotryptone	: "
trypsin	: "

yeast extract	: Difco Laboratories
formamide	: Flurochem Ltd.
polyvinylpyrrolidine	: "
piperidine	: Koch-light Laboratories
dithiothreitol	: "
hydrazine	: Kodak
ficoll	: Pharmacia Fine Chemicals
polyethyleneimine cellulose	: "
polynucleotide kinase	: P.L. Biochemicals Inc.
polyethylene glycol 6000	: Serva, A. & J. Beveridge Ltd.
nitrocellulose	: Schleicher & Schüll
agarose (type : medium EEO)	: Sigma Chemical Co. Ltd.
ampicillin	: "
bovine serum albumin	: "
chloramphenicol	: "
DNase I	: "
ethidium bromide	: "
lysozyme	: "
MOPS	: "
N-lauryl sarcosine	: "
RNase (types I-A & XII-A)	" "
Salmon testes DNA	: "
spermidine	: "
tetracycline	: "
yeast tRNA	: "

2.4 Media and antibiotics

Details of the media used in this study are given below.

2.4.1	L-broth	: 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride pH 7.4. Sterilised by autoclaving at 15 p.s.i. for 15 minutes.
2.4.2	L-bottom agar	: L-broth plus 0.7% (w/v) bactoagar. Sterilised as above.
2.4.3	BBL-bottom agar	: 1.1% (w/v) tripticase agar base, 0.5% (w/v) sodium chloride, 0.8% (w/v) bactoagar. Sterilised as above.
2.4.4	BBL-top agarose	: 1.1% (w/v) tripticase agar base, 0.5% (w/v) sodium chloride, 0.7% (w/v) agarose, 10mM magnesium sulphate. Sterilised as above.
2.4.5	L-bottom agar plus ampicillin	: Ampicillin added to a final concentration of 30-50µg/ml in L-bottom agar for amp. plates. Stock ampicillin stored at 25mg/ml in water at -20°C. Sterilised by filtration.
2.4.6	L-bottom agar plus tetracycline	: Tetracycline added to a final concentration of 12.5-15µg/ml in L-bottom agar for tet. plates. Stock tetracycline stored at 12.5mg/ml in ethanol/water (50% (v/v)) at -20°C. Sterilised by filtration.
2.4.7	L-bottom agar plus ampicillin, X-gal.and IPTG	: Onto each L-bottom agar plus ampicillin plate spread X-gal. (30µl)(stored as 2% (w/v) in dimethyl formamide at -20°C), IPTG (30µl)(stored as 2% (w/v) in sterile water at 4°C) and water (60µl) before use.

2.5 Solutions and buffers

Details of the solutions and buffers used in this study are given below.

2.5.1 For screening genomic DNA libraries and phage DNA isolation

2.5.1.a	lambda diluent	: 10mM Tris-HCl pH 7.5, 10mM magnesium sulphate, 1mM EDTA.
2.5.1.b	SET (1X)	: 0.15M sodium chloride, 30mM Tris-HCl pH 8, 1mM EDTA.

2.5.1.c	Denhardt's solution (50X)	: 1% (w/v) bovine serum albumin, 1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidine. Sterilised by filtration.
2.5.1.d	Phage hybridisation buffer	: 4X SET, 10X Denhardt's solution, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 50µg/ml denatured sonicated salmon testes DNA, 5µg/ml poly A, U, I and C. Store at -20°C.
2.5.1.e	SM	: 0.1M sodium chloride, 8mM magnesium sulphate, 5mM Tris-HCl pH 7.5, 0.01% (w/v) gelatin.
2.5.1.f	TE	: 10mM Tris-HCl pH 8, 1mM EDTA.

2.5.2 For agarose gel electrophoresis of DNA

2.5.2.a	Loening phosphate electrophoresis buffer	: 36mM Tris pH 7.7, 30mM sodium dihydrogen phosphate, 1mM EDTA.
2.5.2.b	sample loading buffer	: 50% (w/v) glycerol, 50% (v/v) appropriate electrophoresis buffer containing 0.025% (w/v) bromophenol blue.

2.5.3 For 5' end-labelling of oligodeoxynucleotide, ON-R, with polynucleotide kinase

2.5.3.a	kinase buffer, pH 7.6	: 70mM Tris-HCl pH 7.6, 10mM magnesium chloride, 5mM dithiothreitol.

2.5.4 For electro-elution of DNA from agarose gels

2.5.4.a	TEA electrophoresis buffer	: 40mM Tris pH 7.4, 5mM sodium acetate, 1mM EDTA.

2.5.5 For nick-translation of DNA

2.5.5.a	NE	: 50mM sodium chloride, 0.5mM EDTA pH 7.

2.5.6 For hybridisation of Southern-blotted DNA

2.5.6.a	SSC (1X)	: 0.15M sodium chloride, 0.015M sodium citrate pH 7.
2.5.6.b	prehybridisation buffer (65°C)	: 4X SET, 5X Denhardt's solution, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 50µg/ml denatured sonicated salmon testes DNA, 5µg/ml poly A, U, I and C.

2.5.6.c	hybridisation buffer ($65^{o}C$)	: 5X SET, 1X Denhardt's solution, 0.5% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 50ug/ml denatured sonicated salmon testes DNA, 5µg/ml poly A, U, I and C.
2.5.6.d	prehybridisation and hybridisation buffer ($42^{o}C$)	: 4X SET, 1X Denhardt's solution, 0.1% (w/v) SDS, 50% (v/v) formamide 0.1% (w/v) sodium pyrophosphate, 50µg/ml denatured sonicated salmon testes DNA, 5µg/ml poly A, U, I and C.

2.5.7 For Northern blotting of RNA

2.5.7.a	LDB	: formamide (500µl), 10X RB (100µl), formaldehyde (500µl).
2.5.7.b	RB electrophoresis buffer (1X)	: 20mM Mops, 5mM sodium acetate pH 7, 1mM EDTA.

2.5.8 For construction of subclones

2.5.8.a	alkaline phosphatase buffer	: 50mM Tris-HCl pH 9.5, 1mM spermidine, 0.1mM EDTA.
2.5.8.b	ligase buffer	: 40mM Tris-HCl pH 7.6, 10mM magnesium chloride, 1mM dithiothreitol.

2.5.9 For small scale plasmid DNA preparation

2.5.9.a	STET	: 8% (w/v) sucrose, 50mM Tris-HCl pH 8, 50mM EDTA, 5% (v/v) triton X-100.

2.5.10 For end-labelling DNA restriction fragments

2.5.10.a	kinase buffer, pH 8	: 50mM Tris-HCl pH 8, 10mM magnesium chloride.

2.5.11 For preparative polyacrylamide gel electrophoresis

2.5.11.a	TBE	: 100mM Tris-HCl pH 8.3, 80mM boric acid, 1mM EDTA.
2.5.11.b	sample loading buffer	: 50% (v/v) glycerol, 50% (v/v) TBE containing 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue.
2.5.11.c	20% (w/v) acrylamide solution	: 19% (w/v) acrylamide, 1% (w/v) bisacrylamide.
2.5.11.d	4% (w/v) acrylamide gel solution	: 20% (w/v) acrylamide solution (10mls), 10X TBE (5mls), glycerol (5mls), water (30mls),

10% (w/v) ammonium persulphate (0.4mls).

acrylamide plug: 5mls above solution plus 40µl TEMED.

main gel : rest of above solution plus 30µl TEMED.

2.5.12 For elution of DNA from acrylamide gels

2.5.12.a	elution buffer	: 0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% (w/v) SDS.

2.5.13 For Maxam and Gilbert sequencing

2.5.13.a	pyridine formate	: 4% (v/v) formic acid pH 2 (with pyridine). Stored at 4°C.
2.5.13.b	carrier DNA	: 1mg/ml salmon testes DNA.
2.5.13.c.	DMS buffer	: 50mM sodium cacodylate pH 8, 10mM magnesium chloride, 0.1mM EDTA. Stored at 4°C.
2.5.13.d	DMS stop	: 1.5M sodium acetate pH 7, 1M mercaptoethanol, 100µg/ml yeast tRNA.
2.5.13.e	HZ stop	: 0.3M sodium acetate, 0.1mM EDTA, 50µg/ml yeast tRNA.
2.5.13.f	saturated NaCl	: sodium chloride (31.7g), water (88.1g).

2.5.14 For sequencing gels

2.5.14.a	6% (w/v) sequencing acrylamide gel solution	: 20% (w/v) acrylamide solution (15mls), 10X TBE (5mls), water (13.4mls), urea (21g), 10% (w/v) ammonium persulphate (0.38mls), TEMED (20µl).
2.5.14.b	sample loading buffer	: 99% (v/v) formamide, 0.05% (w/v) xylene cyanol.

2.5.15 For subculture of HeLa cells

2.5.15.a	trypsin	: 0.25% (w/v) trypsin, 10.5mM sodium chloride, 1mM sodium citrate, 0.002% (w/v) phenol red.
2.5.15.b	EDTA solution	: 0.6mM EDTA, 0.17M sodium chloride, 3.4mM potassium chloride, 10mM disodium hydrogen

phosphate, 2.4mM potassium dihydrogen phosphate.

2.5.16 For isolation of high molecular weight DNA from HeLa cells

2.5.16.a TBS : 0.136M sodium chloride, 5mM potassium chloride, 25mM Tris pH 7.4, 0.0015 % (w/v) phenol red.

2.5.17 For preparation of cytoplasmic RNA

2.5.17.a BSS : 0.116M sodium chloride, 5.4mM potassium chloride, 1mM magnesium sulphate, 1mM sodium dihyrogen phosphate, 1.8mM calcium chloride, 0.002% (w/v) phenol red, 5.6% (w/v) sodium bi-carbonate to pH 7.

2.5.17.b RSB : 10mM sodium chloride, 1.5mM magnesium chloride, 10mM Tris-HCl pH 7.4.

Part B : Methods

2.6 E.coli host strains

The E.coli host strains used in this study were HB101 (F^-, hsdS20 ($r_B^- m_B^-$), recA 13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r) xyl-5, mtl-1, supE44, λ^-)(Boyer and Roulland-Dussoix, 1969) JM109 (recA1, endA1, gyr A96, thi, hsdR17, supE44, relA1, λ^-, Δ(lac-proAB),(F', traD36 proAB, $lacI^q Z$ M15))(Yanisch-Perron et.al., 1985) and NM526 (sup F, rec A^+)(Frischauf et.al., 1983).

The strains were stored as 50 : 50 mixtures of stationary phase culture : glycerol at -20°C and on appropriate agar plates at 4°C.

2.7 Human genomic DNA libraries in bacteriophage lambda

A human genomic DNA library, constructed by the procedure of Frischauf et. al., (1983), was a kind gift of Dr. A. M. Frischauf, EMBL, Heidelberg. The genomic library was a partial Sau 3AI digest of human lymphocyte DNA which had been cloned into the phage lambda vector EMBL 3 (Figure 2.1). It had a titre of 9×10^9 p.f.u./ml and was stored in caesium chloride/lambda diluent solution (Section 2.5.1.a)at 4°C. The general strategy for its construction is outlined in Figure 2.2.

A second human genomic DNA library, constructed by the procedure of T. Maniatis et. al., (1978) was a kind gift of T. Maniatis. The genomic library was a partial Hae III digest of human foetal liver DNA which had been cloned into the phage vector Charon 4A using synthetic Eco RI linkers. It had a titre of 1.5×10^7 p.f.u./ml.

2.8 Screening genomic DNA libraries

Recombinant phage were screened for complementarity towards the cDNA probe, pHS2 (see Introduction), using the plaque filter/ hybridisation procedure of Benton and Davies, (1977). The principle of the method is as follows. The plaques to be screened are first

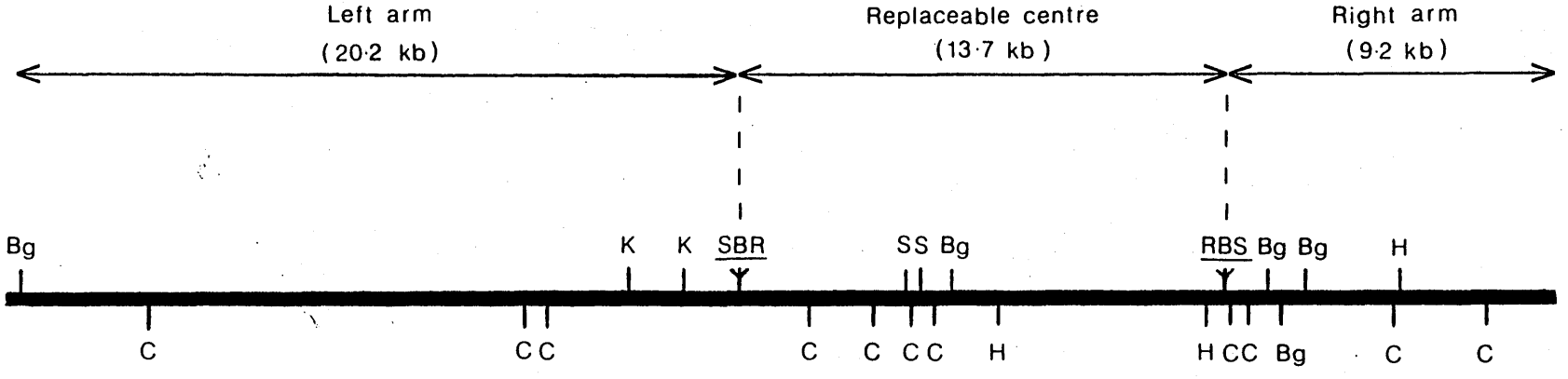
Figure 2.1

Restriction endonuclease map of vector EMBL3 DNA

This restriction map of EMBL3 DNA is taken from Frischauf et.al., (1983).

Figure 2.1

EMBL 3



B = Bam HI H = Hind III

Bg = Bgl II K = Kpn I S = Sal I

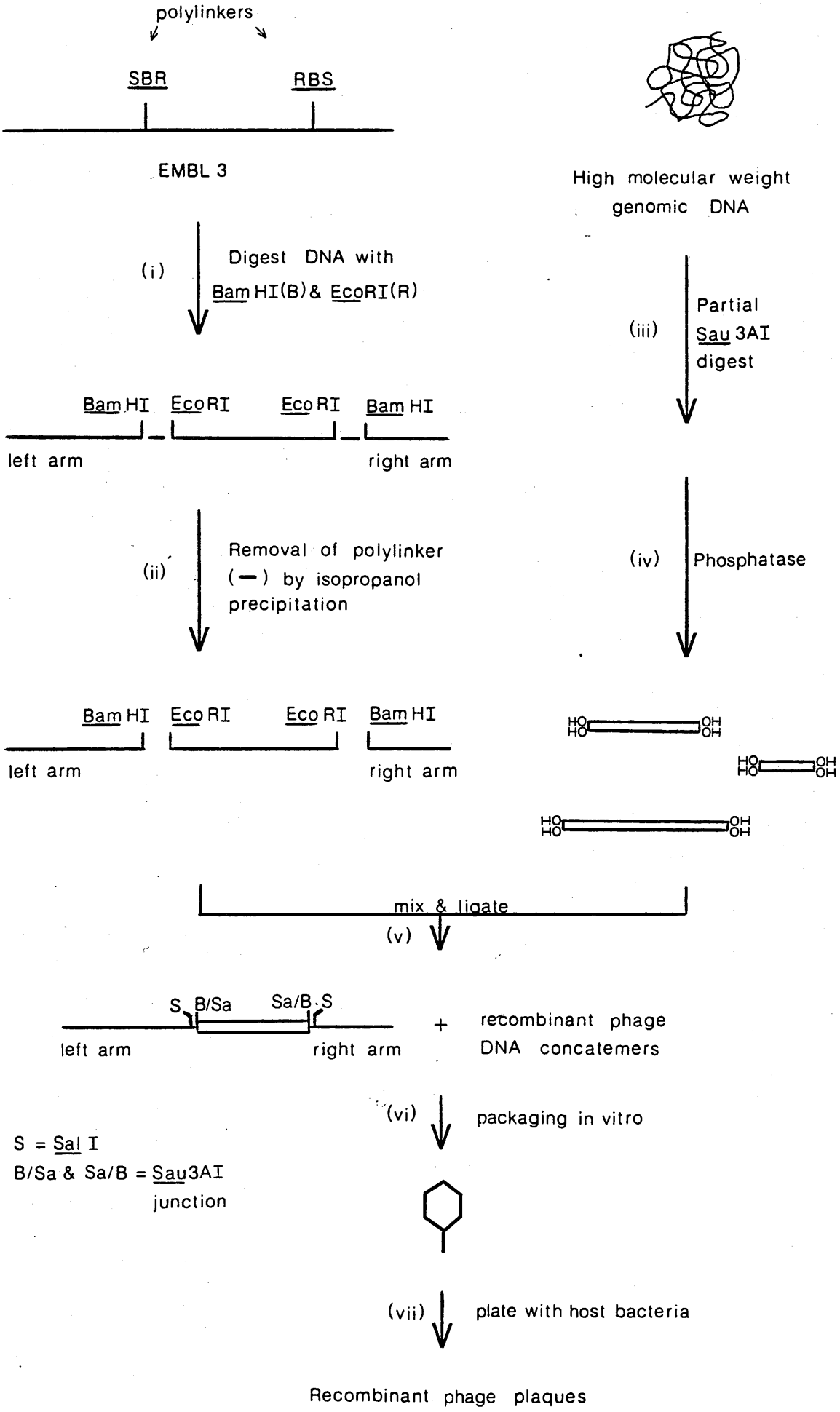
C = Cla I R = Eco RI SBR & RBS = Sal I / Bam HI / Eco RI polylinker

Figure 2.2

Construction of a genomic DNA library using the phage lambda vector EMBL3

This diagram illustrates the procedure described in Frischauf et.al., (1983). The EMBL3 vector is digested with Bam HI and Eco RI which cut within the polylinker sites (i). The tiny Bam HI/Eco RI polylinker fragments is discarded in the isopropanol precipitation (ii). High molecular weight genomic DNA is partially digested with Sau 3AI (iii). The fragments are treated with alkaline phosphatase to remove their 5' - phosphate groups (iv). The vector arms are ligated to the partially digested genomic DNA (v). The recombinant phage are packaged in vitro (vi) and plated onto agar plates with host bacteria (NM526) (vii).

Figure 2.2


polylinkers
SBR
RBS
EMBL 3
High molecular weight genomic DNA
(i)
Digest DNA with Bam HI(B) & EcoRI(R)
(iii)
Partial Sau 3AI digest
Bam HI
EcoRI
EcoRI
Bam HI
left arm
right arm
(ii)
Removal of polylinker (—) by isopropanol precipitation
(iv)
Phosphatase
Bam HI
EcoRI
EcoRI
Bam HI
left arm
right arm
mix & ligate
(v)
S
B/Sa
Sa/B
S
left arm
right arm
+
recombinant phage DNA concatemers
(vi)
packaging in vitro
S = Sal I
B/Sa & Sa/B = Sau3AI junction
(vii)
plate with host bacteria
Recombinant phage plaques

replica plated. This is achieved by placing a nitrocelluose filter disc onto the surface of the agar plate (Figure 2.3). Phage become absorbed onto the filter from the plaques and DNA is thus transferred from the plaques to the filter. It is denatured by alkali treatment of the filter and, after neutralisation, immobilised by baking. The filter is then incubated with a radioactively-labelled probe for the DNA sequence of interest. The position of hybridisation of the probe is located by autoradiography and so the corresponding plaque can be picked from the master plate. This procedure permits the screening of several thousands of plaques simultaneously.

2.8.1 Plating recombinant phage

Using a platinum loop, 100mls of L-broth (Section 2.4.1) were inoculated with a single colony of E.coli host strain NM526 and incubated overnight at 37°C in an orbital shaker (180 cycles/minute). The bacteria were sedimented using a bench-top centrifuge (Beckman model TJ-6) at 2,500 r.p.m. for 15 minutes at 4°C. The bacterial pellet was resuspended in 0.2 volumes of 10mM magnesium sulphate. The resuspended bacteria (200µl) were mixed with enough phage to give approximately 5,000 plaques and incubated at 37°C for 15-20 minutes. BBL top-layer agarose (4mls)(Section 2.4.4), held at 50°C, was added to the bacteria/phage mixture and poured evenly over a 9cm diameter petri dish containing BBL bottom-layer agar (Section 2.4.3). Once the top layer had set the plate was incubated upside down at 37°C. Agarose rather than agar is necessary in the top layer to prevent this tearing when taking filter replicas.

2.8.2 Taking filter replicas

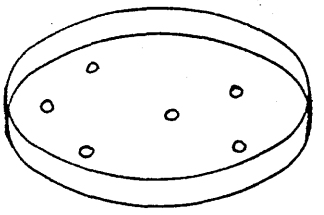
The following morning the phage plates were cooled at 4°C for at least 1 hour. Using sterile (ethanol flamed) blunt-ended forceps, a 9cm diameter nitrocellulose filter was lain onto the agarose surface

Figure 2.3

Procedure used for screening a genomic library

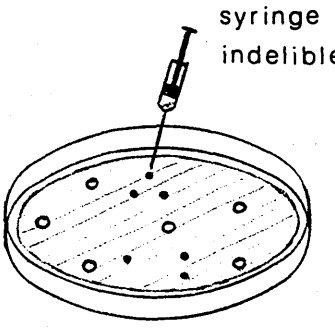
Diagrammatic representation of this procedure, as outlined in Section 2.8.

Figure 2.3



Master plate of recombinant phage

Place nitrocellulose filter on plate



Mark filter and plate with an asymmetric code

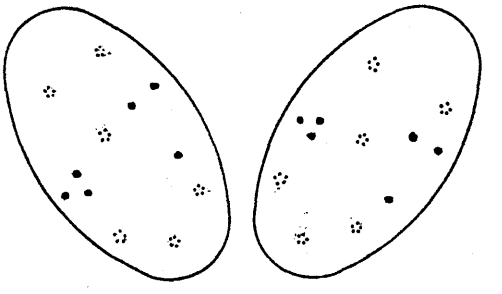
Transfer filters to:

1 0·2 M NaOH;1·5 M NaCL

2 0·2 M Tris-HCL pH 7·6;1·5 M NaCL

3 2 X SET. Air dry filters

4 Bake filters in vacuum oven

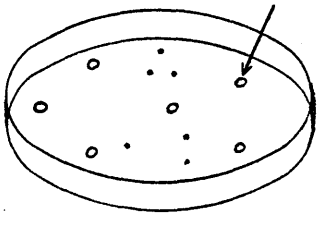


Remove replica filters

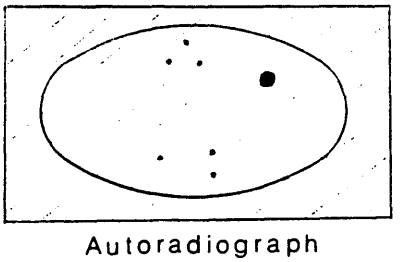
1 Hybridise to ^{32}P labelled probe

2 Wash and air dry filters

3 Autoradiograph



Master plate



Autoradiograph

and the plates returned to 4°C for 20 minutes. The plate and filter were marked in three places with an asymmetric code using a syringe with the finest possible needle and indelible ink. The filters were carefully removed and transferred to a. 0.2M sodium hydroxide, 1.5M sodium chloride for 20 seconds - 5 minutes, b. 0.2M Tris-HCl pH 7.6, 1.5M sodium chloride for 1 minute and c. 2X SET (Section 2.5.1.b) until ready for next stage. The filters were allowed to air dry on Whatman 3MM paper for 1 hour and then baked for 2 hours in a vacuum oven at 80°C.

2.8.3 Prehybridisation and hybridisation of the filters

The filters were pre-wet in 4X SET (Section 2.5.1.b) at room temperature for 10 minutes, floating and sinking each filter separately. They were then soaked at 65°C in 4X SET, 10X Denhardt's solution (Denhardt, 1966)(Section 2.5.1.c), 0.1% (w/v) SDS for 1 hour (using 10mls per filter). The filters were then stacked together in a 1 litre wide-necked screw-top plastic bottle of suitable diameter and just enough phage hybridisation buffer (Section 2.5.1.d) added to cover the stack (approximately 1ml buffer per filter). They were pre-hybridised for 3 hours at 65°C, with shaking. The ^{32}P-labelled nick-translated DNA probe (Section 2.15) was denatured with 0.1 volume 1M sodium hydroxide for 10 minutes, then 0.1 volume 0.1M Tris-HCl pH 7.6 followed by 0.1 volume 1M HCl. An aliquot (1µl) of the probe was removed and the ^{32}P radioactivity estimated from the Cherenkov radiation using the ^{3}H channel of a scintillation spectrometer. Enough probe was added to the filters to give approximately 10^6 Cherenkov c.p.m. per filter. The filters were hybridised at 65°C for 18 hours with shaking.

2.8.4 Washing the filters

The probe solution was poured off and the following washes carried out at 65°C with 250mls of solution: a. 2 x 20 minutes in

4X SET, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate; b. 4 x 15 minutes in 2X SET, 0.1% (w/v) SDS; c. 1 x 15 minutes in 0.2X SET, 0.1% (w/v) SDS; and d. 1 hour at room temperature in 3mM Tris base (unbuffered). The filters were allowed to dry at room temperature on Whatman 3MM paper for approximately 30 minutes.

2.8.5 Autoradiography

The dried filters were attached to a sheet of Whatman 3MM paper by their edges and covered with cling film. They were exposed to Fuji X-ray film (NIF RX 100), using a Dupont intensifying screen, at -70°C for 18-48 hours, as indicated.

2.8.6 Plaque purification

Well separated positive plaques were chosen and excised together with the underlying agar using the narrow end of a sterile pasteur pipette. The fragments of agar were washed out into 1ml SM (Section 2.5.1.e) containing one drop of chloroform and left at room temperature for 1-2 hours to allow the phage to diffuse out of the agar. An average plaque yielded 10^6 - 10^7 infectious phage particles which were stored at 4°C in SM/chloroform. Ten-fold serial dilutions of the phage stock were prepared. NM526 bacteria in 10mM magnesium sulphate (Section 2.8.1)(100µl) were added to each dilution (100µl) and plated as outlined in Section 2.8.1. These plates were screened as previously described and positive plaques again isolated. The above plaque purification process was repeated until a plate was obtained in which all of the plaques gave positive hybridisation to the probe. This usually required 2-3 rescreenings.

2.9 Preparation of phage DNA

Phage DNA was prepared according to the procedure of Yamomoto et. al., (1970)with modification.

2.9.1 Preparation of concentrated phage stock

A plate of the required phage clone was prepared on which there

were 10-100 plaques. Using a sterile pasteur pipette a single well-separated plaque was removed and added to 200µl of a freshly saturated overnight culture of NM526 bacteria. This was left at room temperature for 20 minutes before being transferred to a 100ml conical flask containing 20mls L-broth which included 5mM magnesium sulphate. The flask was shaken at 37°C in an orbital shaker (180 cycles/minute) until lysis occurred (usually between 4-6 hours). The culture becomes cloudy as the bacteria grow and then clears upon lysis. Chloroform (1ml) was added and the flask shaken for a further 5 minutes. The culture medium was decanted off (leaving the chloroform behind) and the broken bacteria were sedimented by centrifugation for 20 minutes at 2,000 r.p.m. and 4°C in a Beckman bench-top centrifuge. The supernatant was transferred to a fresh tube and stored at 0°C until the next stage. The average yield of phage was approximately 10^{11} p.f.u./ml.

2.9.2 Large scale amplification and concentration of phage

To 1 litre of L-broth containing 5mM magnesium sulphate in a 2 litre flask, was inoculated a portion of saturated overnight culture of NM526 bacteria (7mls). This was grown until it gave an A_{650} of 0.3 (approximately 3 hours). Phage (5×10^5 p.f.u.) were added and shaking continued at 37°C until lysis occurred (approximately 3-4 hours). Chloroform (10mls) was then added and the flask shaken for a further 5 minutes. The culture medium was decanted off, avoiding the chloroform, and the broken cells sedimented at 4,000 r.p.m. for 20 minutes at 4°C, in the 6 x 500ml rotor of a Hi-Spin 21 M.S.E. centrifuge. The supernatant was decanted and the sediment discarded. To the supernatant DNase I was added to 10µg/ml and RNase A (type I-A) to 10µg/ml and this then incubated at room temperature for 30 minutes. Solid sodium chloride was then added to 2% (w/v) and poly-

ethylene glycol 6000 to 8% (w/v) at room temperature, and the phage left to precipitate at 4°C overnight. They were then sedimented by centrifugation at 6,000 r.p.m. for 30 minutes at 4°C in a 6 x 500ml M.S.E. rotor. The phage were resuspended in lambda diluent (20mls) (Section 2.5.1.a), and 0.71g of caesium chloride per ml added to give a density of 1.5. The solution was clarified by centrifugation for 30 minutes at 1,500 r.p.m. in a Beckman bench-top centrifuge and the phage solution transferred to heat-sealable VTi50 tubes (Beckman No. 34405 3.5" x 1") topping up with a solution of lambda diluent containing 0.71g caesium chloride/ml. This was then subjected to centrifugation at 50,000 r.p.m. and 25°C in a VTi50 rotor of a Beckman ultracentrifuge for 18 hours. The blue/white phage band was viewed by illuminating the tube with white light and the side of the tube pierced with a 21 gauge needle just below the phage band which was removed using a 5ml syringe. The phage band was dialysed against 4 x 500ml changes of 10mM Tris-HCl pH 7.5, 1mM EDTA, 10mM magnesium sulphate to remove the caesium chloride (3-4 hours).

2.9.3 Recovery of phage DNA

The dialysate was extracted three times with an equal volume of phenol saturated with lambda diluent (Section 2.5.1.a). The, upper, aqueous phase was then extracted twice with an equal volume of ether. To the resulting, lower, aqueous phase 2.5 volumes of ethanol was added and the DNA left to precipitate at -70°C for 20 minutes. It was then sedimented by centrifugation at 10,000 r.p.m. for 10 minutes in an HB-4 rotor of a Sorval centrifuge at 0°C, resuspended in 200-400µl of TE (Section 2.5.1.f) and stored at 0°C - 4°C.

2.10 Digestion of DNA with restriction endonucleases

The site specificities and conditions of incubation of the restriction endonucleases used in this study are shown in Table 2.1.

2.10.1 Single digests

A typical restriction enzyme digestion mixture contained the following: DNA (1μg), restriction endonuclease (1-5 units) in the appropriate buffer (Table 2.1) and in a total volume of 15μl with incubation at 37°C for 1-2 hours.

2.10.2 Double digests

Digestions involving two restriction enzymes were performed sequentially, with adjustment of the buffer composition (where necessary) after the first digestion.

Visualisation of the digestion products was carried out by gel electrophoresis (Sections 2.11 and 2.22.3).

2.11 Agarose gel electrophoresis of DNA

Agarose gels were prepared by the method of Southern, (1975).

Horizontal 0.5% (w/v), 0.7% (w/v) and 1% (w/v) agarose gels were used. Electrophoresis in Loening phosphate buffer (Loening, 1967) (Section 2.5.2.a) was performed at 40-60V until the marker dye had migrated two-thirds of the distance to the end of the gel. Samples were applied with 0.4 volumes of loading buffer (Section 2.5.2.b). DNA was visualised using a U.V. illuminator, after staining in a solution of ethidium bromide (final concentration 0.5μg/ml)/electrophoresis buffer, and photographed.

2.12 DNA size markers for gel electrophoresis

In order to determine the size of DNA fragments produced from a restriction digest, the digest was subjected to agarose gel electrophoresis and a DNA size-marker (a mixture of fragments of DNA of known size) subjected to electrophoresis in parallel. Some of the size-markers used in this work are shown in Table 2.2. The distance of migration of the known DNA fragments from the well of the gel was plotted against their size on semi-logarithmic graph paper (an example of this is shown in Figure 2.4). The standard curve obtained

Table 2.1

Conditions for restriction endonuclease digestion

The site specificities and conditions of incubation of the restriction endonucleases used in this study are shown.

Table 2.1

Restriction enzyme	Digestion buffer	Incubation temperature	DNA recognition sequence
Acc I	Medium salt	37°C	GT↓[AG/CT]AC
Asp 718	Medium salt	37°C	G↓GTACC
Bam HI	Medium salt	37°C	G↓GATCC
Bgl II	Low salt pH9.5	37°C	A↓GATCT
Cla I	Low salt	37°C	AT↓CGAT
Dra II	Low salt	37°C	PuG↓GNCCPy
Eco RI	High salt	37°C	G↓AATTC
Hae III	Medium salt	37°C	GG↓CC
Hind III	Medium salt	37°C	A↓AGCTT
Hinf I	Medium salt	37°C	G↓ANTC
Kpn I	Low salt	37°C	GGTAC↓C
Nco I	Medium salt	37°C	C↓CATGG
Pst I	Medium salt	21-37°C	CTGCA↓G
Sal I	High salt	37°C	G↓TCGAC
Sma I	Sma I buffer	37°C	CCC↓GGG
Sph I	Medium salt	37°C	GCATG↓C
Sst I	Low salt	37°C	GAGCT↓C
Sty I	Medium salt	37°C	C↓C[AA/TT]GG
Xba I	High salt	37°C	T↓CTAGA

Buffer	NaCl	KCl	Tris	$MgCl_2$	DTT	pH
Low salt	0mM	0mM	10mM	10mM	1mM	7.5
Medium salt	50mM	0mM	10mM	10mM	1mM	7.5
High salt	100mM	0mM	50mM	10mM	1mM	7.5
Sma I buffer	0mM	20mM	10mM	10mM	1mM	8.0

Table 2.2

Bacteriophage lambda size markers

The size of fragments generated by *Hind* III and *Hind* III/*Eco* RI digests of bacteriophage lambda DNA are shown.

Table 2.2

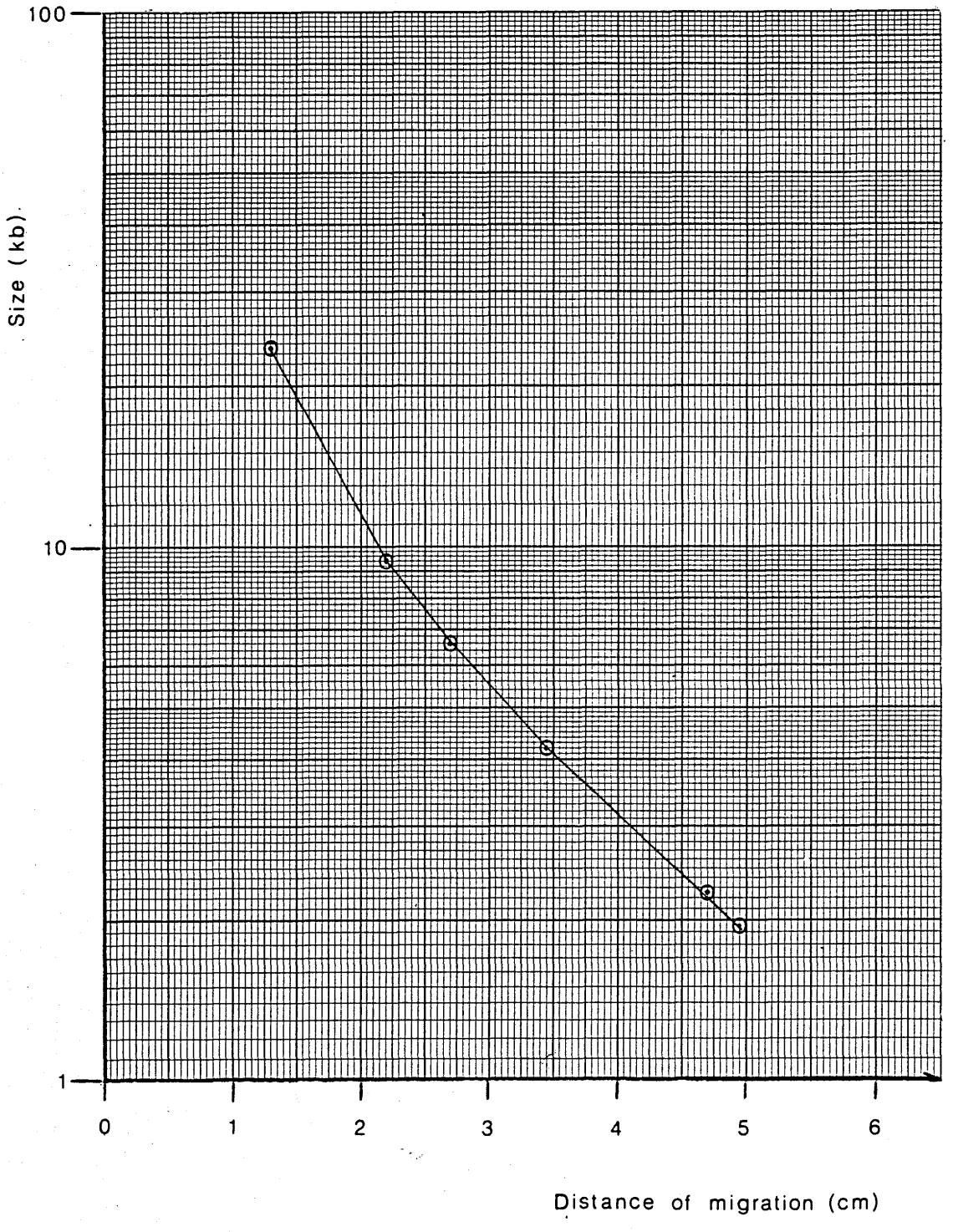
HindIII digest of lambda DNA (kb)	HindIII / EcoRI digest of lambda DNA (kb)
23.7	23.7
9.46	5.24
6.61	5.05
4.26	4.21
2.26	3.41
1.98	1.98
0.58	1.90
	1.57
	1.32
	0.93
	0.84
	0.58

Figure 2.4

Example of standard curve relating the size of DNA fragments to their electrophoretic mobility in agarose gel

This standard curve is for fragments of bacteriophage lambda DNA generated by digestion with Hind III, and outlined in Section 2.12

Figure 2.4



was then used to calculate the size of the unknown DNA fragments from their distance of migration.

2.13 Restriction mapping of recombinant lambda clones by partial digest/oligodeoxynucleotide hybridisation

The mapping procedure of Rackwitz et. al., (1984) was employed, with modification to reaction times and volumes. The exact procedure used in this work is described below and a general outline of the procedure is depicted in Figure 2.5.

2.13.1 5'end-labelling of oligodeoxynucleotide, ON-R, with polynucleotide kinase

The synthetic oligodeoxynucleotide ON-R (5'- GGG CGG CGA CCT -3') complementary to the right cohesive end of lambda DNA was used in this study.

The following reaction mixture was assembled in an Eppendorf tube: ON-R (a gift of H. Lehrach, EMBL, Heidelberg)($10^{-3}A_{260}$ units), γ [^{32} P] ATP (20µCi), polynucleotide kinase (10 units) in kinase buffer pH 7.6 (Section 2.5.3.a) and in a total volume of 10µl. Incubation was at 37°C for 1 hour. The extent of the reaction was checked by applying a small aliquot of this mixture to a thin layer of polyethyleneimine cellulose and subjecting to chromatography with 0.7M potassium phosphate pH 3.5. The thin layer plate was dried with a hairdryer and subjected to autoradiography (Section 2.8.5). An incorporation of $\geq$50% of the γ [^{32} P] ATP was deemed satisfactory. This is shown in Figure 2.6. The reaction was stopped by heating for 1 minute at 100°C and the sample then diluted to 1ml with TE (Section 2.5.1.f) and stored at -20°C.

2.13.2 Partial digestion of recombinant lambda DNA

To test partial digestion conditions, recombinant phage DNA (2µg) was digested with the required restriction enzyme (1.5 - 2 units) under the appropriate conditions (Table 2.1) in a total reaction volume of 50µl. Aliquots (10µl) were withdrawn at suitable

Figure 2.5

Procedure used for restriction mapping of recombinant lambda clones by partial digest/oligodeoxynucleotide hybridisation

Diagrammatic representation of the procedure of Rackwitz et.al., (1984), as outlined in Section 2.13.

Figure 2.5

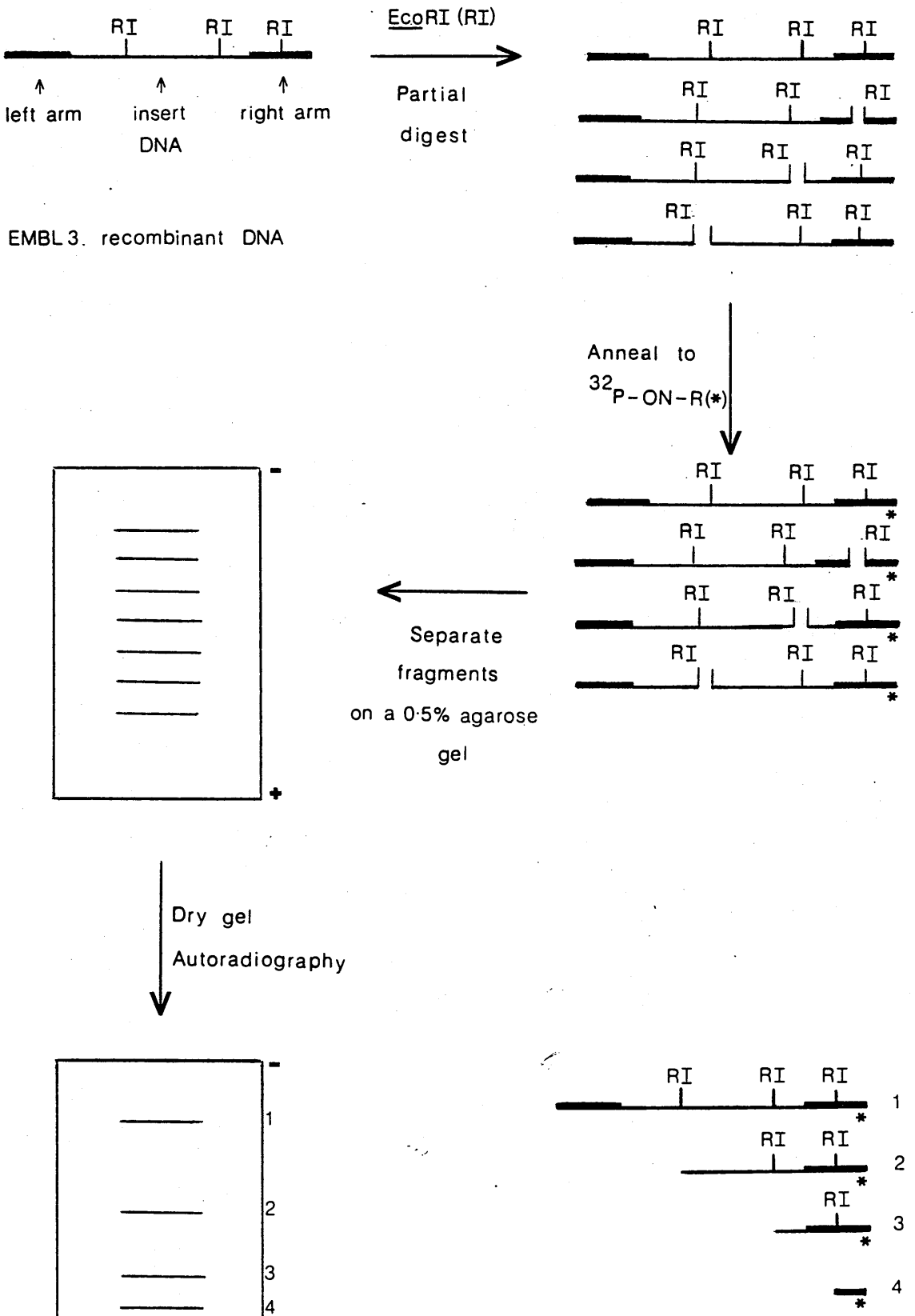
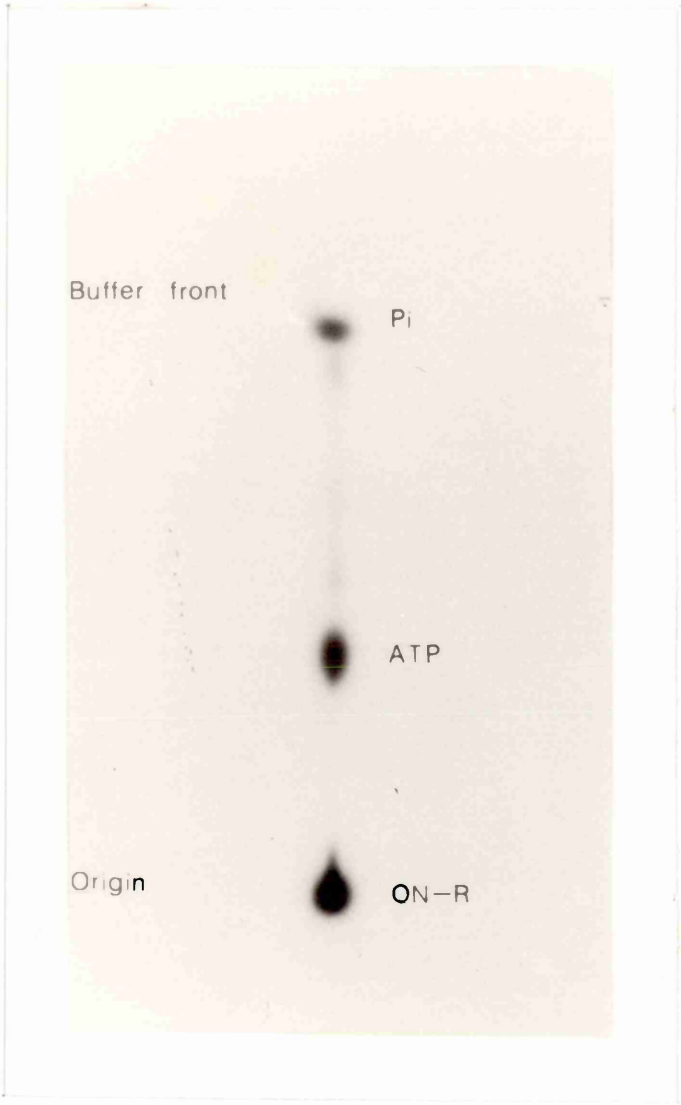

RI
RI
RI
left arm
insert DNA
right arm
EMBL 3. recombinant DNA
EcoRI (RI)
Partial digest
Anneal to
^{32}P-ON-R(*)
Separate fragments on a 0·5% agarose gel
Dry gel
Autoradiography
1
2
3
4
Autoradiograph
Bands on the autoradiograph correspond to fragments 1-4 above.

Figure 2.6

5′ end-labelling of oligodeoxynucleotide ON-R

This autoradiograph shows the extent of 5′ end-labelling of ON-R as determined by chromatography on polyethyleneimine cellulose. For further details see Section 2.13.1.

Figure 2.6



time points between 0-60 minutes and the reaction stopped by the addition of EDTA to 20mM. Samples (5µl) from each time point were analysed on 0.7% (w/v) agarose gels. Electrophoresis was performed at 40V for 3.5 - 4 hours in Loening phosphate buffer (Section 2.5.2.a). Visualisation of the DNA was as previously described (Section 2.11). A typical digestion pattern is shown in Figure 2.7. The time points used were altered if a range of digestion products was not observed. In general, digestion for 1, 2, 4, 8 and 16 minutes generated the required range of digestion products, which were pooled, as appropriate, and stored at -20°C until required.

2.13.3 Hybridisation of the recombinant lambda DNA partial digests with oligodeoxynucleotide probe ON-R and gel electrophoresis

An aliquot (0.75µg) of pooled partial digested recombinant DNA was mixed with ON-R (200,000 Cherenkov c.p.m.) and incubated at 37°C for 2 hours. Samples for each digest were subjected to electrophoresis on 0.5% (w/v) agarose gels at 40V for 24 hours in Loening phosphate buffer. The gel was dried onto Whatman DE-81 cellulose paper, under vacuum (no heat on gel-dryer), and then subjected to autoradiography.

2.14 Electroelution of DNA from agarose gels

This procedure is based on the method of Yang et. al., (1979) with modification.

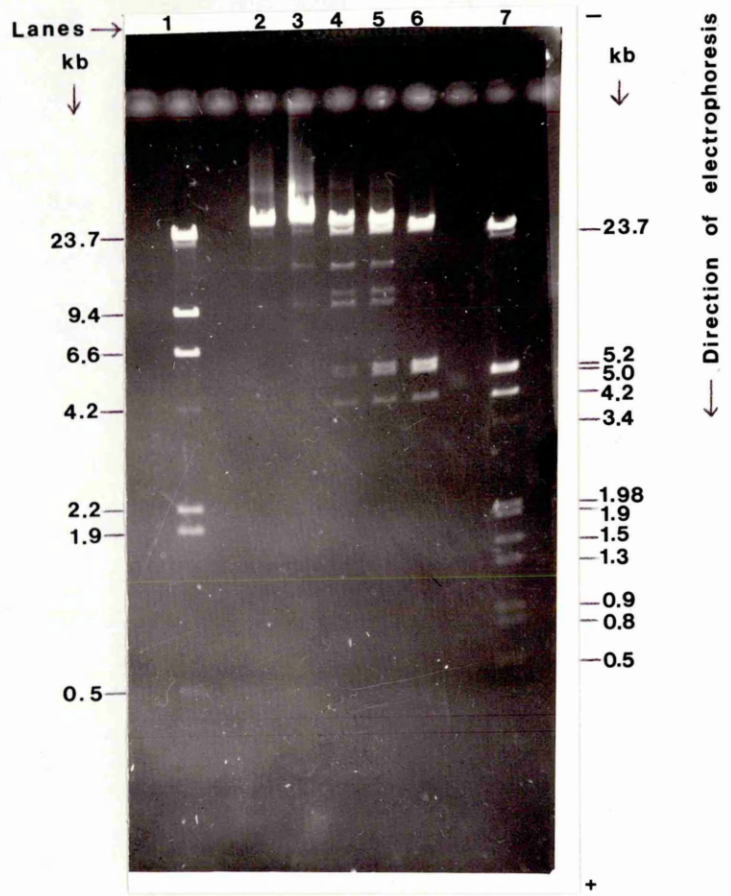
DNA (20µg) was cleaved with the required restriction endonuclease (50 units) under the appropriate conditions (Table 2.1) in a total volume of 50µl at 37°C. After the addition of 0.4 volumes of loading buffer (Section 2.5.2.b) the sample was subjected to electrophoresis on a 1% (w/v) agarose gel at 60V for 45 minutes in TEA electrophoresis buffer (Section 2.5.4.a). The DNA was then visualised (Section 2.11), the required band(s) located and cut out using a scalpel. The gel slice was then placed in a small dialysis

Figure 2.7

Agarose gel electrophoretic separation of DNA fragments generated by partial endonucleolytic digestion

EMBL3 DNA was partially digested (Section 2.13.2) with Hind III for 1 minute (lane 2) 2 minutes (lane 3), 4 minutes (lane 4), 8 minutes (lane 5), 16 minutes (lane 6), and subjected to electrophoresis, in parallel with a Hind III digest of bacteriophage lambda DNA (lane 1) and a Hind III/Eco RI digest of bacteriophage lambda DNA (lane 7) on a 0.7% (w/v) agarose gel (Loening phosphate buffer : Sections 2.5.2.a and 2.11). The photograph is of the gel stained with ethidium bromide.

Figure 2.7


Lanes →
1
2
3
4
5
6
7
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
kb
23.7
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
0.9
0.8
0.5
Direction of electrophoresis
−
+

bag with a minimal volume of TEA (usually 200µl) and subjected to electrophoresis for a further 1-2 hours followed by back-electrophoresis for 1-2 minutes. The DNA solution was then transferred to an Eppendorf tube and subjected to centrifugation in a M.S.E. microfuge for 5 minutes to sediment any agarose debris. The DNA was then precipitated in the presence of 0.1 volume of 3M sodium acetate pH 6 and 2.5 volumes of ethanol, in a fresh Eppendorf tube, at -20°C overnight. The DNA was recovered by centrifugation at 4°C for 15 minutes in the M.S.E. microfuge. The DNA was then washed with ethanol (300µl) and subjected to centrifugation for a further 10 minutes. The DNA was then dried under vacuum and an appropriate volume of TE added.

2.15 Nick translation of DNA

For labelling DNA molecules or restriction fragments for hybridisation experiments the method of nick translation (Rigby et. al., 1977) was used.

A typical reaction contained the following: DNA (0.5 - 2µg), α [^{32}P] dATP (50µCi), dCTP (5µM), dTTP (5µM), dGTP (5µM), DNase I (10^{-4} µg), E.coli DNA polymerase I (5 units) in medium buffer (Table 2.1) and in a total volume of 12µl. Incubation was at 15°C for 3-4 hours. NE (100µl)(Section 2.5.5.a) was then added and the sample applied to a column of Biogel P-60 in a 1.5ml Eppendorf tip (which had been packed at the narrow end with siliconised glass wool). Portions of NE (12 x 100µl) were applied to the column, collecting each eluted fraction separately. The fractions were then checked with a mini-monitor and the peak fractions collected (usually 3 fractions centred on tube 5 or 6) and pooled. An aliquot (1µl) was removed and the ^{32}P radioactivity estimated from the Cherenkov radiation using the ^{3}H channel of a scintillation spectrometer. The

sample was stored at -20°C until required.

2.16 Determination of radioactivity

An aliquot (1µl) of ^{32}P-labelled sample was placed in a scintillation vial and the ^{32}P radioactivity estimated from the Cherenkov radiation using the ^{3}H channel of a scintillation spectrometer. This procedure is 30% efficient for ^{32}P estimation.

2.17 Southern blotting and hybridisation of blotted DNA

2.17.1 Southern blotting

The transfer of DNA from agarose gels to nitrocellulose paper was achieved by Southern blotting (Southern, 1975).

An agarose gel was prepared for Southern blotting as follows. The gel was immersed in 1. 0.5M sodium hydroxide, 1.5M sodium chloride (500mls) for 30 minutes; 2. distilled water (500mls) for 1-2 minutes; 3. 0.5M Tris-HCl pH 7.6, 1.5M sodium chloride (500mls) for 30 minutes and 4. distilled water (500mls) for 1-2 minutes. The gel was then transferred to the blot apparatus which was assembled as shown in Figure 2.8. Both the nitrocellulose paper and Whatman 3MM paper were soaked in 2X SSC (Section 2.5.6.a) before laying onto the gel. Once assembled the blot was left for at least 18 hours at room temperature. The nitrocellulose filter was then carefully removed from the apparatus, marking the well slots of the gel with a pen, and transferred to 2X SSC for 5-10 minutes. The filter was then allowed to air dry on Whatman 3MM paper for 30 minutes and then baked for 2 hours in a vacuum oven at 80°C.

2.17.2 Hybridisation of blotted DNA

2.17.2.a Hybridisation at 65°C

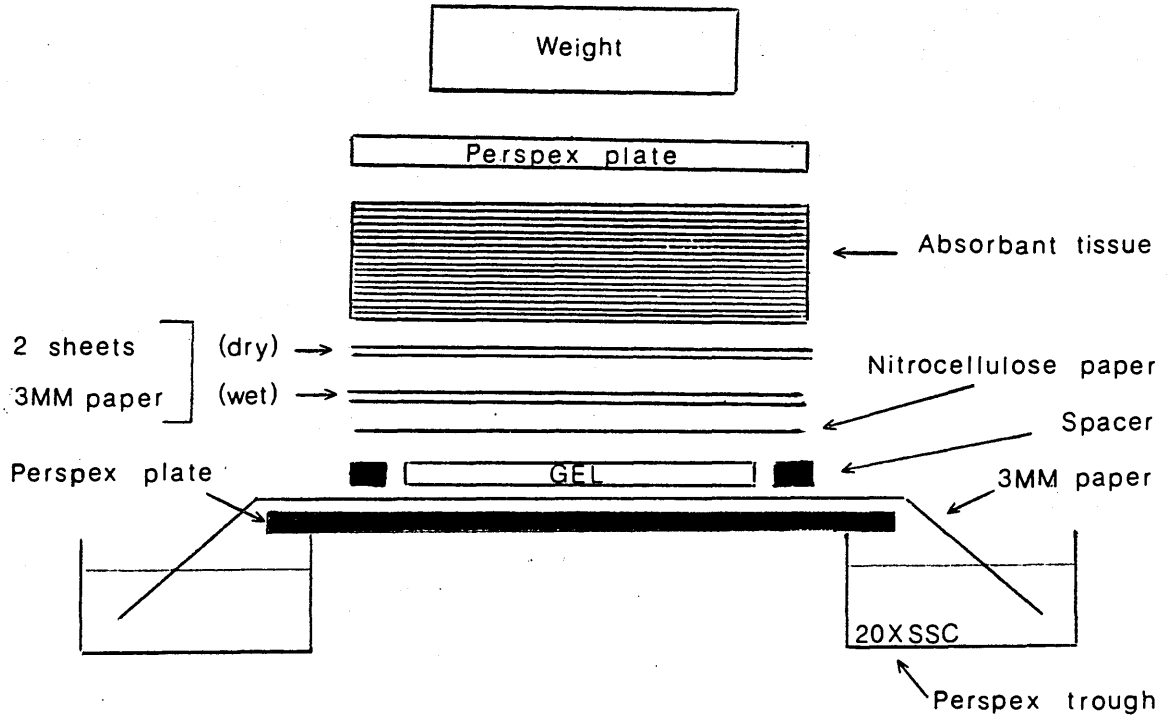
The Southern blotted nitrocellulose filter to be hybridised was placed in a polythene bag with prehybridisation buffer (Section 2.5.6.b)(25-30mls). After the exclusion of all air bubbles, the open end of the bag was sealed with a heat sealer and the filter

Figure 2.8

Apparatus for Southern transfer

Details of Southern blotting are outlined in Section 2.17.1.

Figure 2.8


Weight
Perspex plate
Absorbant tissue
2 sheets
3MM paper
(dry)
(wet)
Nitrocellulose paper
Spacer
Perspex plate
GEL
3MM paper
20XSSC
Perspex trough

then incubated at 65°C in a shaking waterbath for 2 hours. The bag was then opened, the fluid decanted, and hybridisation buffer (Section 2.5.6.c)(50µl/cm^2 paper) and 10^6 Cherenkov c.p.m. denatured ^{32}P-labelled probe (Section 2.15)(for a 11cm x 15cm filter) added to the bag. Once resealed, the bag was returned to the waterbath and the filter hybridised for 16-24 hours at 65°C. The probe solution was then poured off and the filter subjected to the following washes at 65°C with 250mls of solution: a. 3 x 30 minutes in 2X SET, 0.4% (w/v) SDS and b. 1 x 30 minutes in 2X SET, 0.1% (w/v) SDS. The filter was allowed to air dry for approximately 30 minutes before being subjected to autoradiography.

2.17.2.b Hybridisation at 42°C with formamide

The Southern blotted nitrocellulose filter to be hybridised was placed in a polythene bag with hybridisation buffer containing formamide (Section 2.5.6.d)(25-30mls) and incubated at 42°C in a shaking waterbath for 2 hours. Hybridisation was then performed, with fresh hybridisation buffer (50µl/cm^2 filter) and 10^6 Cherenkov c.p.m. denatured ^{32}P-labelled probe, at 42°C in a shaking waterbath for 16-24 hours. The filter was then washed with 250mls of solution as follows: a. 5 x 10 minutes in 2X SET, 0.1% (w/v) SDS at room temperature and b. 2 x 30 minutes at 45°C in 0.1X SET, 0.1% (w/v) SDS. The filter was allowed to air dry for approximately 30 minutes before being subjected to autoradiography. The advantage of this method is that filters can be washed at a higher temperature with 0.1X SET, 0.1% (w/v) SDS if 'background' hybridisation is observed.

2.18 Northern blotting and hybridisation of blotted RNA

Northern blotting was based on the method of Thomas, (1980) with modification.

RNA samples (1µg or less) were heated for 5 minutes at 60°C in the presence of LDB (Section 2.5.7.a)(7.5µl/sample) and water

(2.5µl/sample) before being applied to a 1% (w/v) agarose/2.2M formaldehyde gel, with 0.5 volumes of sample loading buffer (Section 2.5.2.b). They were then subjected to electrophoresis at 60V in RB/2.2M formaldehyde buffer (Section 2.5.7.b) until the dye had migrated three-quarters of the distance to the end of the gel. The section of the gel possessing DNA size markers was cut out and stained in RB/10% glycerol/0.5µg/ml ethidium bromide and photographed. The part of the gel to be blotted was then soaked for 30 minutes in 1-2 volumes 20X SSC before being transferred to the blot apparatus (as for Southern blotting. Figure 2.8). The nitrocellulose was pre-wet with 20X SSC before lain onto the gel. After 18 hours the nitrocellulose filter was removed and treated as described for a Southern blot except using 20X SSC to rinse. Hybridisation of blotted RNA was carried out at 42°C in the presence of formamide (Section 2.5.6.d).

2.19 Construction of subclones

In order to analyse further regions of interest in the recombinant lambda clones, these were subcloned into the vector pUC18 (Yanisch-Perron et. al., 1985). The plasmid pUC18 consists of a pBR322 derived ampicillinase gene and origin of DNA replication ligated to the 5' portion of the lac z gene of E.coli. A DNA insert (polylinker) containing an array of unique restriction enzyme sites has been introduced into the lac region of this plasmid (Figures 2.9 and 2.10). When introduced into a suitable strain of E.coli carrying only the complementary 3' part of the lac z gene the plasmids give rise to blue colonies on indicator plates containing X-gal, a chromogenic substitute for β-galactosidase. Cloning DNA fragments into any of the polylinker restriction sites usually inactivates the lac z gene giving rise to white colonies (Figure 2.11). The

Figure 2.9

Restriction endonuclease map of vector pUC18 DNA

This map was constructed on the basis of information presented by Yanisch-Perron et.al., (1985). The nucleotide position is indicated beside each restriction enzyme.

Key

Amp^r : ampicillin resistance gene

ori : origin of replication

lac fragment: lac gene fragment

Figure 2.9

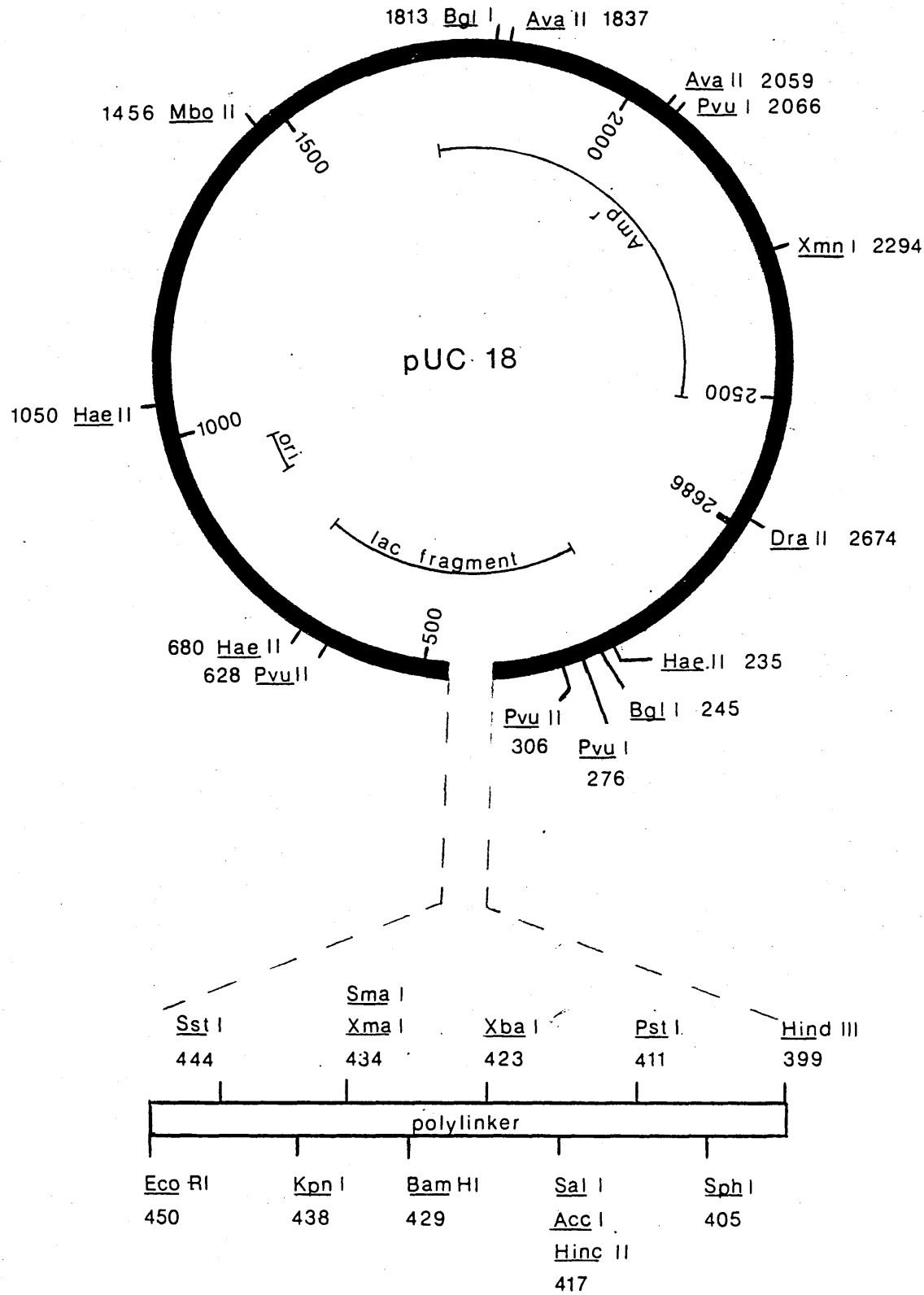

1813 Bgl I
Ava II 1837
Ava II 2059
Pvu I 2066
1456 Mbo II
1500
2000
Amp r
Xmn I 2294
pUC 18
2500
1050 Hae II
1000
ori
2686
Dra II 2674
lac fragment
500
680 Hae II
628 Pvu II
Hae II 235
Pvu II
306
Bgl I 245
Pvu I
276
Sma I
Xma I
Sst I
444
434
Xba I
423
Pst I
411
Hind III
399
polylinker
Eco RI
450
Kpn I
438
Bam HI
429
Sal I
Acc I
Hinc II
417
Sph I
405

Figure 2.10

Multiple cloning polylinker of pUC18

Taken from Yanisch-Perron et.al., (1985).

Figure 2.10

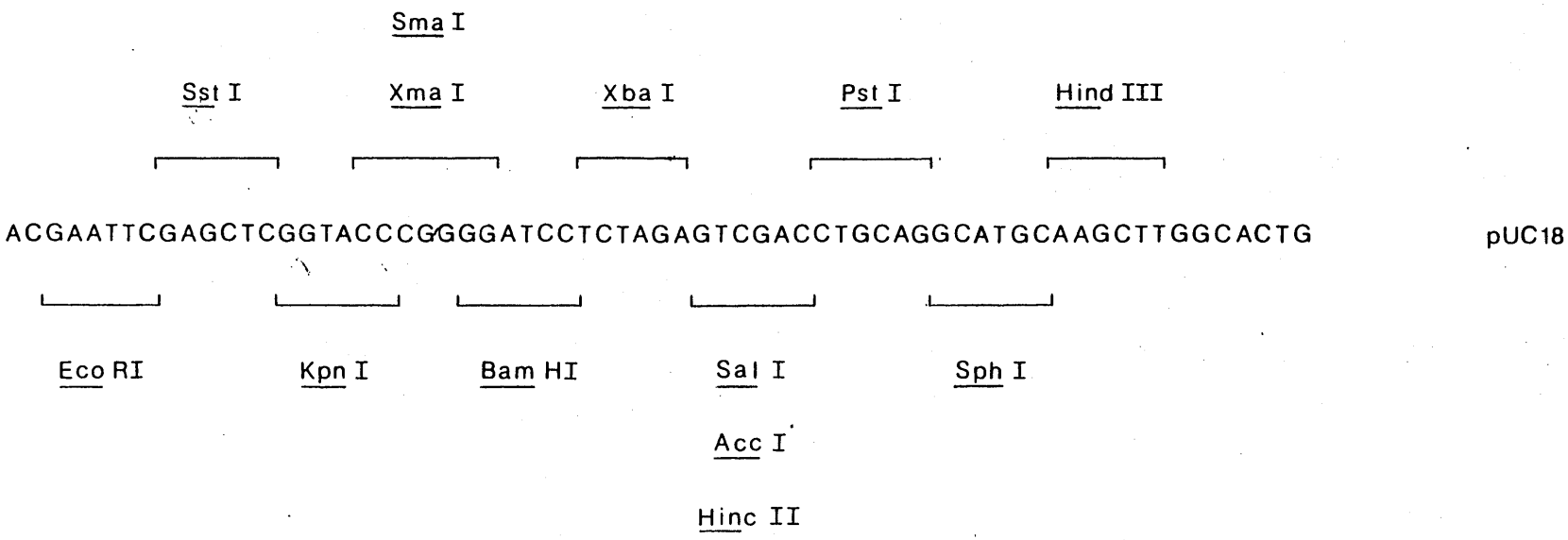
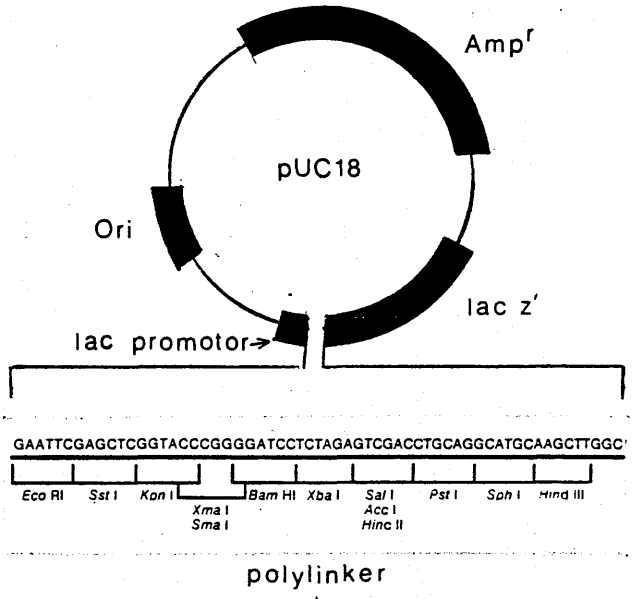

Sma I
Sst I
Xma I
Xba I
Pst I
Hind III
ACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTG
pUC18
Eco RI
Kpn I
Bam HI
Sal I
Sph I
Acc I
Hinc II

Figure 2.11

Construction of subclones

Diagrammatic representation of this procedure, as outlined in Section 2.19.

Figure 2.11



FOREIGN DNA

(restriction fragment

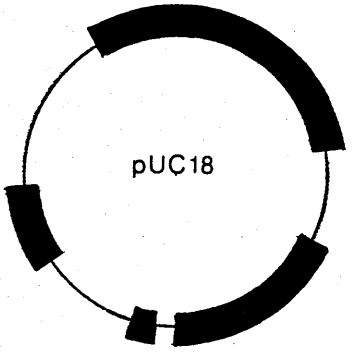
from parent clone)

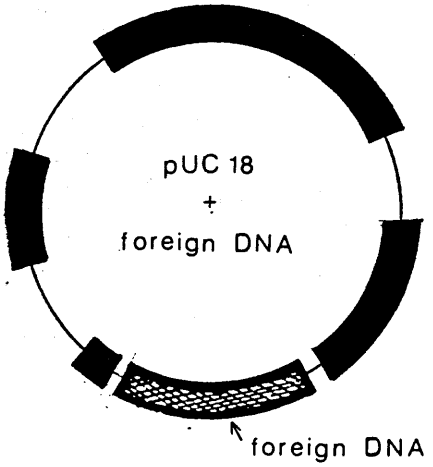
digest with eg BamHI

digest with eg BamHI

Anneal with

T4 DNA Ligase





Transformants form blue colonies on Amp / X-gal / IPTG plates

Transformants form white colonies on Amp / X-gal / IPTG plates

following section describes the procedures for subcloning.

2.19.1 Restriction digestion of vector and DNA of interest

Vector pUC18 DNA (5-10μg) and DNA to be subcloned (5μg) were digested with the appropriate enzyme(s). The digests were then heated for 10 minutes at 70°C before being extracted three times with an equal volume of phenol saturated with water. To the, upper, aqueous phase 0.1 volume 3M sodium acetate pH 6 and 2.5 volumes ethanol was added and the DNA left to precipitate on dry ice for 15 minutes. DNA was recovered by centrifugation in a M.S.E. microfuge for 15 minutes at 4°C. The DNA samples were then dried under vacuum.

2.19.2 Alkaline phosphatase treatment of pUC18 DNA

pUC18 DNA was treated with alkaline phosphatase as follows. Vector DNA (5-10μg), calf intestinal alkaline phosphatase (35mU) in alkaline phosphatase buffer (Section 2.5.8.a) and in a total volume of 20μl was incubated at 37°C for 30 minutes. The DNA was then extracted three times with phenol saturated with water and twice with ether. The DNA was then precipitated and recovered as described in Section 2.19.1 above. TE was then added to the DNA to give 0.3μg/ml.

2.19.3 Ligation reaction

A 5-10 molar excess of 'insert' over vector DNA was used. The following were assembled in an Eppendorf tube: digested lambda DNA, pUC18 DNA, ATP (0.5mM) and DNA ligase (1 unit) in ligase buffer (Section 2.5.8.b) and in a total volume of 30μl. Incubation was at 15°C for 18 hours. Control ligations of digested pUC18 self-ligation and digested 'phosphatased' pUC18 self-ligation were included.

2.19.4 Transformation of subcloned DNA

Half and one-tenth of the ligation mixtures were used to trans-

form 100µl of competent JM109 cells as described in Section 2.20. Control transformations of 1. undigested vector, 2. unligated vector, and 3. unligated digested phosphatased vector were included. Transformation mixtures were spread onto amp./X-gal./IPTG plates (Section 2.4.7) and incubated at 37°C.

2.20 Transformation of E.coli with plasmid DNA

This procedure is based on the method of Lederberg and Cohen, (1974).

2.20.1 Preparation of cells competent for transformation

To 500mls of L-broth in a 1 litre flask were inoculated 2.5mls of saturated overnight culture of host cells. Cells were grown to an A_{600} of 0.2. The cells were harvested by centrifugation at 5,000 r.p.m. for 15 minutes at 4°C in a 6 x 500ml M.S.E. rotor of a M.S.E. Hi-Spin 21 centrifuge. The supernatant was decanted and the cells resuspended in cold 100mM calcium chloride (250mls). They were then incubated on ice for 20 minutes. The cells were then subjected to centrifugation at 6,000 r.p.m. for 10 minutes in the M.S.E. 6 x 500ml rotor. The cells were resuspended in 100mM calcium chloride (5mls) and sterile glycerol (0.5mls) added. Portions (1ml) were aliquoted, shock frozen in liquid nitrogen, and stored at -70°C.

2.20.2 Transformation procedure

Frozen competent cells were allowed to thaw in an ice/water mixture for 30 minutes. Plasmid DNA (0.2 - 100ng) was then added to the cells (100µl) and incubated on ice for 30 minutes. After 'heat-shocking' for 2 minutes at 37°C the transformation mixtures were plated directly onto tetracycline, ampicillin, or amp./X-gal./IPTG plates (Section 2.4) (depending on the plasmid used) using a sterile (ethanol-flamed) glass spreader, and incubated at 37°C.

2.20.3 Growth and storage of E.coli transformed with plasmid

L-broth (10mls) in a 50ml flask was inoculated with 0.1ml of saturated overnight culture of transformed bacteria (from a single colony) and cells grown to an A_{600} of 0.5. An aliquot (2mls) of the growing culture was added to sterile glycerol (3mls). This culture was then kept at -70°C. When required for use an aliquot of this mixture was plated on an appropriate agar plate and incubated at 37°C.

2.21 Preparation of plasmid DNA

2.21.1 Small scale plasmid DNA preparation

This procedure is based on the method of Holmes and Quigley, (1981).

A single colony of transformed bacteria was picked and spread thoroughly over half of a L-agar plate (containing the appropriate antibiotic(s)). The plate was then incubated at 37°C. The following morning the bacteria were scraped off the plate with a platinum loop (taking care not to rip the agar). The bacteria were resuspended in STET (1ml)(Section 2.5.9.a) in an Eppendorf tube (1.5ml). Lysozyme (10µl of a 20mg/ml stock) was then added and the contents of the tube thoroughly mixed before being transferred to a waterbath at 95°C for 7 minutes. Centrifugation was then performed in a M.S.E. microfuge for 15 minutes at room temperature. The supernatant was transferred to a fresh Eppendorf tube (2.5ml) and RNase A (type XII-A)(2µl of 1mg/ml stock) added. The tube was then incubated at 37°C for 15 minutes. Diethylpyrocarbonate (1µl) was added and the tube incubated for a further 10 minutes at 65°C. 5M ammonium acetate (0.4ml) and isopropanol (0.9ml) was then added and the tube placed on dry ice for 5 minutes. Centrifugation was then performed for 10 minutes at room temperature in a M.S.E. microfuge. The sediment was washed with 0.3M

ammonium acetate/70% isopropanol and subjected to centrifugation for a further 10 minutes. The DNA was dried under vacuum, TE (20µl) added and stored at -20°C.

2.21.2 Large scale plasmid DNA preparation

This method is based on the procedure of Birnboim and Doly, (1979) with modification.

L-broth (100mls) containing the appropriate antibiotic (tetracycline 20µg/ml or ampicillin 40µg/ml) was inoculated with a single colony of transformed bacteria and incubated for 18 hours at 37°C in an orbital shaker (180 cycles/minute). Portions of this culture (2 x 5ml) were then inoculated into 2 x 2 litre flasks containing 2 x 1 litre of L-broth and the bacteria grown to an A_{600} of 0.8 at 37°C. Chloramphenicol was added to a concentration of 165µg/ml and the flasks shaken for a further 20 hours at 37°C. The bacteria were then harvested by centrifugation at 5,000 r.p.m. for 10 minutes at 4°C in a 6 x 500ml M.S.E. rotor of a M.S.E. Hi-Spin 21 centrifuge. The supernatant was decanted and resuspended in 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8 (9.5mls per 2 litre culture). The suspension was transferred to rigid-walled Ti60 tubes and lysozyme (0.5mls per 2 litre culture)(40mg/ml in above buffer) added. After mixing the tubes were incubated on ice for 30 minutes. To the tubes was then added 0.2M sodium hydroxide, 0.1% (w/v) SDS (20mls per 2 litre culture). The tubes were then left on ice for a further 5 minutes. After this time elapsed 3M sodium acetate pH 4.8 (15mls per 2 litre culture) was then added and incubation on ice continued for a further 60 minutes. Centrifugation was then performed in a Beckman Ti60 rotor for 30 minutes at 30,000 r.p.m. in a Beckman ultracentrifuge. The supernatant which contains the plasmid DNA was retained, the volume measured and 0.6 volumes of isopropanol added. The supernatant was then left at room temperature, in 30ml corex

tubes, for 10 minutes to precipitate the DNA. The DNA was sedimented by centrifugation in a Sorval centrifuge at 8,000 r.p.m. for 15 minutes at room temperature in a Sorval SS-34 rotor. The DNA was dissolved in TE (total volume 30mls) and caesium chloride (28.9g) and ethidium bromide (1.8mls of a 10mg/ml stock) added to a density of 1.59. After clarification of the solution at 1,500 r.p.m. in a Beckman bench-top centrifuge at room temperature, the solution was transferred to VTi50 heat-sealable tubes. Centrifugation was then carried out at 50,000 r.p.m. in a VTi50 rotor of a Beckman ultra-centrifuge for 16 hours and 20°C. After centrifugation the DNA bands were visualised by U.V. illumination. The lower closed circular DNA band was removed by side puncture of the tube, using a 21 gauge needle and 5ml syringe to withdraw the band. Further purification of the DNA was achieved by a second centrifugation in a VTi65 rotor, in VTi65 heat-sealable tubes (Beckman No. 344057 2" x 0.5"), at 65,000 r.p.m. and 20°C. The lower band was again collected by side puncture of the tube. The DNA solution was extracted four times with an equal volume of isopropanol saturated with water and caesium chloride to remove the ethidium bromide. The lower, aqueous, phase was collected, measured and 4 volumes TE added. The DNA was then precipitated in the presence of 2 volumes of ethanol at -20°C for 18 hours. The DNA was then sedimented by centrifugation at 10,000 r.p.m. for 30 minutes at 4°C in a Sorval HB-4 rotor. The DNA was resuspended in TE (100µl) and reprecipitated in an Eppendorf tube in the presence of 0.1 volume 3M sodium acetate and 2.5 volumes of ethanol on dry ice for 15 minutes. Recovery of the DNA was performed by centrifugation in a M.S.E. microfuge for 15 minutes at 4°C. The DNA was washed with ethanol (300µl) and subjected to centrifugation for a further 10 minutes. The DNA was dried under

vacuum, TE (100µl) added and stored at -20°C.

2.22 Analysis of nucleotide sequences of DNA

The sequence of DNA was determined by the chemical method of Maxam and Gilbert, (1980). The principle of this method is as follows. The DNA fragment to be sequenced is purified and labelled at one end only. This is achieved by first labelling both ends of the DNA fragment (Section 2.22.1) cleaving the DNA fragment with a restriction enzyme and then purifying the products electrophoretically (Sections 2.22.2 and 2.22.3). The purified DNA fragment, labelled at a single end is then subjected to a set of four separate chemical treatments, the end result of which is cleavage of the DNA backbone at one, or in some cases two, of the four bases. Each procedure involves three steps: 1. modification of a base; 2. removal of the modified base from its sugar, and 3. DNA strand scission at that sugar. Conditions are chosen such that only partial modification is obtained giving a family of DNA molecules of different lengths with the same labelled ends (Section 2.22.4). These molecules are separated and analysed according to size on a high resolution denaturing polyacrylamide gel (Section 2.22.5). By subjecting a set of samples obtained with the four different series of chemical reactions to electrophoresis in parallel on the same gel it is possible to determine the nucleotide sequence (Figure 2.12).

2.22.1 End-labelling DNA restriction fragments

2.22.1.a 3' end-labelling with E.coli DNA polymerase I Klenow fragment

The following protocol is based on the method of Drouin, (1980).

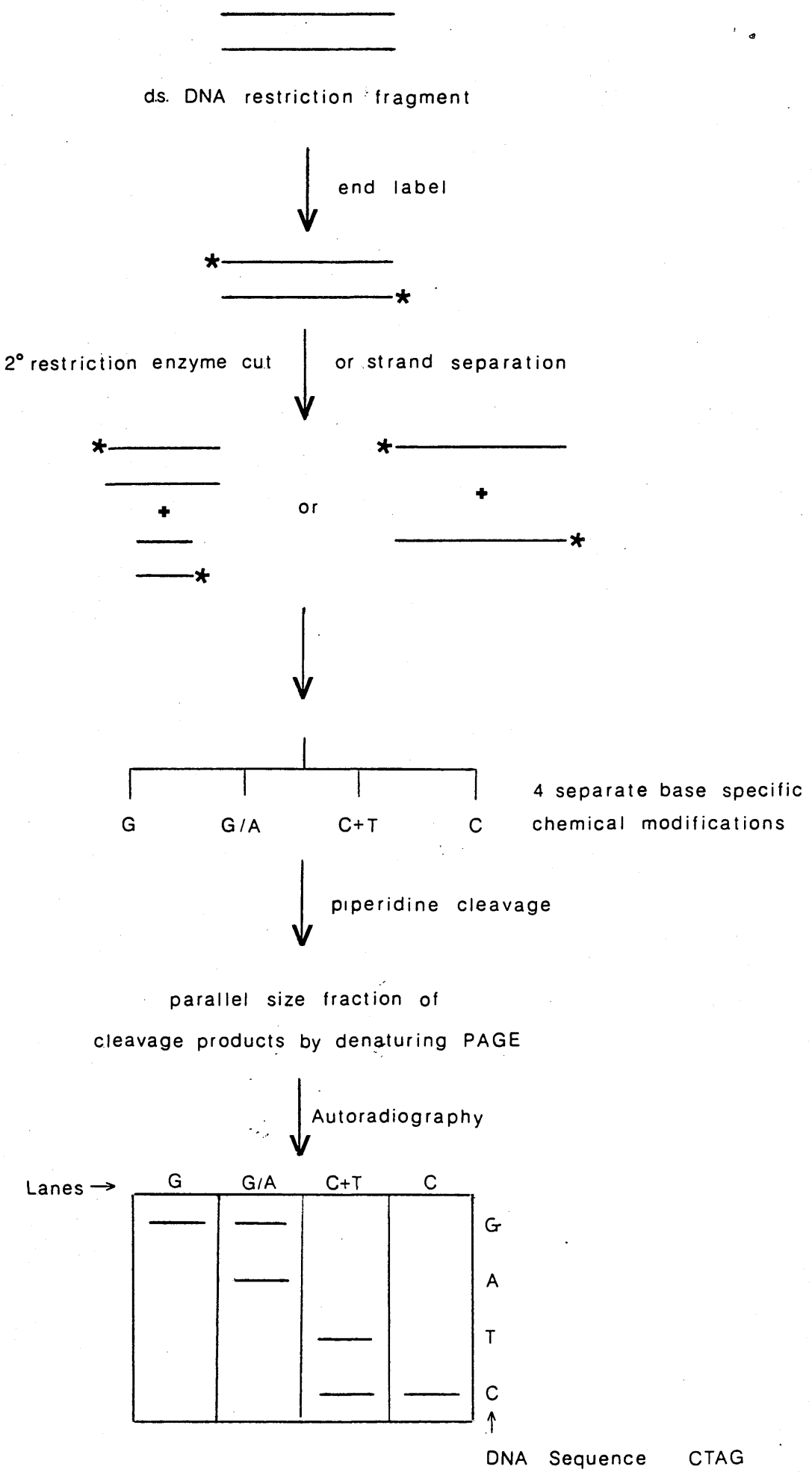
DNA (5µg) was digested with a restriction enzyme which generated 5' protruding ends. After the addition of EDTA to 10mM the digest was incubated at 65°C for 10 minutes and the DNA ethanol precipitated

Figure 2.12

General strategy used in the nucleotide sequencing procedure of Maxam and Gilbert

Details of this procedure are outlined in Section 2.22.

Figure 2.12


ds. DNA restriction fragment
end label
2° restriction enzyme cut
or strand separation
or
G
G/A
C+T
C
4 separate base specific chemical modifications
piperidine cleavage
parallel size fraction of cleavage products by denaturing PAGE
Autoradiography
Lanes →
G
G/A
C+T
C
G
A
T
C
DNA Sequence CTAG

and dried (Section 2.21.2). The following reaction was assembled in an Eppendorf tube: DNA (5μg), αI^{32} P I dNTP (30μCi), the three remaining dNTP's (5μM each), Klenow fragment of DNA polymerase I (1 unit) in medium buffer (Table 2.1) and in a total volume of 14.25μl. Incubation was at 20°C for 30 minutes. DNA was then precipitated on dry ice for 5 minutes in the presence of 2.5M ammonium acetate (90μl) and ethanol (360μl). The DNA was sedimented by centrifugation for 5 minutes at 4°C in a M.S.E. microfuge. The supernatant was discarded to radioactive waste and the DNA resuspended and reprecipitated in the presence of 0.3M sodium acetate pH 6 (100μl) and ethanol (300μl) on dry ice for 15 minutes. The DNA was recovered by centrifugation for 5 minutes and then dried under vacuum.

2.22.1.b 5' end-labelling with T4 polynucleotide kinase

This procedure is based on the method of Chaconas and Van de Sande, (1980).

2.22.1.b.1 Phosphatase reaction

To remove 5' phosphate groups from a restriction fragment, the DNA (5-10μg) was incubated with calf intestinal alkaline phosphatase (35mU) in TE (100μl) at 37°C for 60-75 minutes. The DNA solution was then extracted two times with an equal volume of phenol saturated with TE. The upper, aqueous, phase was collected and DNA precipitated in the presence of 0.1 volume 3M sodium acetate pH 6 and 3 volumes ethanol for 15 minutes on dry ice. After centrifugation for 5 minutes at 4°C the DNA was resuspended in 0.3M sodium acetate pH 6 (100μl) and ethanol (300μl) and reprecipitated on dry ice. After a further 5 minute centrifugation the DNA was dried under vacuum.

2.22.1.b.2 Polynucleotide kinase reaction

The following reaction was assembled in an Eppendorf tube: phosphatased DNA (5-10μg), dithiothreitol (4.5μM), αI^{32} P I ATP

(60μCi), polynucleotide kinase (5 units) in kinase buffer pH 8 (Section 2.5.10.a) and in a total volume of 11μl. Incubation was at 37°C for 30 minutes. The DNA was then precipitated on dry ice for 15 minutes in the presence of 2.5M ammonium acetate (40μl) and ethanol (160μl). Centrifugation was at 4°C for 5 minutes in a M.S.E. microfuge. The supernatant was then discarded to radioactive waste. The DNA was then resuspended in 0.3M sodium acetate pH 6 and ethanol, reprecipitated, sedimented and dried as described above (Section 2.22.1.a).

The end-labelled DNA fragment was then digested with a second restriction enzyme to liberate two fragments labelled at one end only.

2.22.2 Preparative polyacrylamide gel electrophoresis

The end-labelled fragments were separated by polyacrylamide gel electrophoresis.

Vertical 160 x 160 x 1.5mm 4% (w/v) polyacrylamide gels (Section 2.5.11.d) were used. Electrophoresis in TBE electrophoresis buffer (Section 2.5.11.a) was carried out at 200V for 2-3 hours or until the dyes had migrated the required distance to the end of the gel. Samples were applied to the gel with 0.4 volumes sample loading buffer (Section 2.5.11.b). In 4% (w/v) acrylamide gels, xylene cyanol migrates with fragments approximately 350 base pairs in size and bromophenol blue with fragments approximately 70 base pairs in size. DNA was visualised as previously described (Section 2.11).

2.22.3 Elution of DNA from acrylamide gels

The following procedure was based on the method of Maxam and Gilbert, (1980).

The required band(s) were located on the gel and cut out using a scalpel. The gel slice was then placed in a 1.5ml Eppendorf tip (which had been packed at the narrow end with siliconised glass wool

and the tip heat-sealed). The gel fragment was then crushed with a glass rod, elution buffer (Section 2.5.12.a)(600µl) added and the open end covered with parafilm. The tip was then placed in a 15ml corex tube and incubated at 37°C for 18 hours. The sealed end of the tip was then cut off and the buffer/DNA solution allowed to drain into the 15ml corex tube. The tip was rinsed with aliquots (4 x 200µl) of elution buffer. The DNA was then precipitated in the presence of 2.5 volumes of ethanol at -70°C for 30 minutes. The DNA was recovered by centrifugation at 3,500 r.p.m. and -10°C for 30 minutes. The DNA was resuspended in 0.3M sodium acetate pH 6 (400µl) and transferred to an Eppendorf tube. Centrifugation was carried out for 3 minutes to sediment any pieces of acrylamide before transferring the supernatant to a fresh Eppendorf tube. The DNA was re-precipitated in the presence of ethanol (1ml) at -70°C for 30 minutes and recovered by centrifugation in a M.S.E. microfuge for 15 minutes. The DNA was then dried under vacuum.

2.22.4 Chemical cleavages for Maxam and Gilbert sequencing

The reagents and solutions used during Maxam and Gilbert, (1980) sequencing are indicated in Section 2.5.13.

The reactions used in this work were specific for guanine, guanine and adenine, cytosine and thymine, and cytosine. The reactions were performed in 1.5ml siliconised Eppendorf tubes. The end-labelled fragment (at least 200,000 Cherenkov c.p.m.) was dissolved in water (11µl) and carrier DNA (4µl). Aliquots (3.5µl) were then dispensed into 4 Eppendorf tubes and the steps outlined in Table 2.3 carried out simultaneously for all four reaction mixtures. The DNA samples from all four reactions were then dissolved in 1M piperidine. They were incubated at 90°C for 30 minutes and then frozen on dry ice. The samples were then dried under vacuum. Once

Table 2.3

Chemical cleavage procedure used for nucleotide sequence determination according to Maxam and Gilbert

Taken from Maxam and Gilbert, (1980).

Table 2.3

To lyophilised ^{32}P DNA add 11µl water and 4µl carrier DNA (1µg/µl)

Then aliquot :	G	A/G	T+C	C
DNA sample	3.5µl	3.5µl	3.5µl	3.5µl
DMS buffer	98 µl	—	—	—
Saturated NaCl	—	—	—	8 µl
Water	—	11 µl	6 µl	—

Then add :

Start first:

A
1. 2.5µl Pyridine Formate 2. 30°C for 70 minutes.

Then :

G	T	C
1. 0.5µl DMS	1. 15µl HZ	1. 15µl HZ
2. 20°C for 5 minutes.	2. 20°C for 8 minutes.	2. 20°C for 10 minutes.
3. 24µl DMS-Stop	3. 60µl HZ Stop	3. 60µl HZ Stop
4. 400µl EtOH	4. 250µl EtOH	4. 250µl EtOH
5. -70°C for 15 minutes.	5. -70°C for 15 minutes.	5. -70°C for 15 minutes.

3,4 -70°C then Lyophilise

5. Add 10µl water

6,7 -70°C then Lyophilise.

For G , T , C .

6. Centrifuge for 5 minutes . Discard supernatant to radioactive sink .

7. Add 60µl 0.3M NaAc. Vortex .

8. Add 200µl EtOH . Vortex .

9. -70°C for 15 minutes . Centrifuge for 5 minutes . Discard supernatant.

10. Wash with 200µl 70% EtOH .-70°C for 5 minutes . Centrifuge for 5 minutes

11. Discard supernatant and lyophilise pellet .

dry they were dissolved in water (2 x 20µl), freezing the samples on dry ice and drying down the DNA after each addition of water. The samples were then dissolved in a suitable volume of sample loading buffer (Section 2.5.14.b) to give 10,000 Cherenkov c.p.m. per µl.

2.22.5 Sequencing gels

Sequencing gels were prepared by the method of Sanger and Coulson, (1978).

Vertical 480 x 220 x 0.1mm 6% (w/v) sequencing polyacrylamide gels (Section 2.5.14.a) were used. Electrophoresis in TBE was carried out at 25mA in a safety cabinet. Samples were boiled for 2 minutes before each loading. Three successive loadings were made on each gel. The volume of each sample applied in loadings 1, 2 and 3 was 1.5µl, 1µl and 1µl respectively. The dye was allowed to migrate 20cm before the second loading, 15cm before the third and 13cm before the gel was finally removed from the gel apparatus. The gel was then covered in cling film and subjected to autoradiography.

2.23 Computer analysis of DNA sequences

Sequences were stored on a PDP 11/34 computer. Routine manipulation of sequences employed the following programs of Staden, (1979): SEQLST, SEQEDT, CUTSIT, TRNTRP. Sequences were aligned to known related sequences using the program PALIGN (P. Taylor, Institute of Virology, University of Glasgow, unpublished) to identify the main regions of homology, followed by subjective matching of other regions by eye, where necessary. Unknown sequences were transferred to the VAX cluster at EMBL, Heidelberg, and compared with sequences in the Genbank database using the program, WORDSEARCH, of the UWGCG sequence analysis software package (Devreux et. al., 1984).

2.24 Cell culture

2.24.1 Maintenance of HeLa cells

HeLa cells (Gey et. al., 1952) were maintained as monolayers in 80oz. glass Winchester bottles. The growth medium was Eagle's minimal essential medium (Eagle et. al., 1959) supplemented with 10% (v/v) calf serum, penicillin (100 units/ml) and streptomycin (100µgs/ml). Bottles were seeded with 2 x 10^7 cells in 180mls growth medium and grown in an atmosphere of 5% (v/v) carbon dioxide at 37°C. The monolayer was confluent after 2-3 days.

2.24.2 Subculture of HeLa cells

Cells were subcultured by removal from the glass with a solution of trypsin and EDTA. The cell monolayer was washed with a 1:4 solution of trypsin: EDTA (10mls)(Sections 2.5.15.a and 2.5.15.b). The monolayer was then treated with a further portion of trypsin: EDTA solution (10mls) until opaque, at which point the solution was poured off, leaving approximately 1ml of solution on the monolayer. As soon as the cells began to detach from the glass, growth medium (10mls) was added, and the cells were shaken into suspension. The cell density of the suspension was measured and used to subculture further bottles.

2.25 Isolation of high molecular weight eukaryotic DNA from HeLa cells

This protocol is based on the method of Blin and Stafford, (1976).

Cell monolayers were washed twice with TBS (Section 2.5.16.a). Using a cell scraper, the monolayer was transferred into TBS (5-10mls). The cell suspension was subjected to centrifugation at 2,000 r.p.m. for 10 minutes at 4°C in a Beckman bench-top centrifuge. The cells were resuspended at a concentration of 10^8 cells/ml in ice-cold TE. Ten volumes of 0.5M EDTA pH 8, 100µg/ml proteinase K,

0.5% (w/v) N-lauroyl sarcosine was then added. The suspension of lysed cells was incubated at 50°C for 3 hours. The suspension was extracted three times with an equal volume of phenol saturated with TE. The lower, aqueous, phase was then dialysed against 4 litres of 50mM Tris-HCl pH 8, 10mM EDTA, 10mM sodium chloride, with several changes, until the A_{270} of the dialysate was less than 0.05. The sample was then treated with 100µg/ml RNase A (type XII-A)(DNase-free) at 37°C for 3 hours. The sample was extracted twice with an equal volume of phenol saturated with TE and twice with an equal volume of chloroform. The upper, aqueous, phase was extensively dialysed against TE. The DNA sample was then concentrated by ethanol precipitation (Section 2.21.2) and stored at 0°C-4°C.

2.26 Preparation of cytoplasmic RNA

This procedure was based on the method of Penman, (1966 and 1969).

Cell monolayers were washed twice with ice-cold BSS (50mls) (Section 2.5.17.a). Using a cell scraper, the cells were transferred into BSS (10mls) and sedimented by centrifugation at 2,000 r.p.m. for 3 minutes at 4°C in a Beckman bench-top centrifuge. The cells were washed twice by resuspending in BSS (10mls) followed by centrifugation as described above. The cells were then resuspended in RSB buffer (Section 2.5.17.b) at a density of 5×10^7 cells/ml. This suspension was left on ice for 10 minutes. The cells were then disrupted in a stainless steel Dounce homogeniser (10 strokes). The nuclei were sedimented by centrifugation at 2,000 r.p.m. for 10 minutes at 4°C. To the supernatant SDS was added to 0.5% (w/v) and EDTA with respect to 10mM. RNA was isolated by extracting four times with an equal volume of phenol/chloroform and twice with an equal volume of chloroform. RNA was precipitated from the upper, aqueous,

phase by the addition of 0.1 volume 2M sodium chloride plus 2 volumes ethanol. After 20 hours at -20°C RNAs were collected by centrifugation at 10,000 r.p.m. for 20 minutes and -10°C.

CHAPTER THREE

RESULTS

3. RESULTS

3.1 Isolation of genomic clones related to clone pHS2

3.1.1 Optimisation of screening conditions

Initially a number of unsuccessful attempts were made to screen libraries of recombinant human genomic DNA in bacteriophage lambda using the nick-translated insert of clone pHS2. The problems encountered were high background and inability to detect any positive plaques (Figure 3.1.a). It was considered that one or more of the following could have been the cause of the problems: the library, the probe, the method of screening. As the genomic library initially employed (originally prepared by T. Maniatis from foetal liver DNA in bacteriophage lambda vector Charon 4A) had undergone a number of amplifications it was decided to procure a more recently-constructed library, and a human lymphocyte DNA library in lambda vector EMBL3 was obtained. Screening was still unsuccessful however (Figure 3.1.b). In order to determine whether either probe or screening method was responsible the library was screened in addition with a second probe an actin cDNA clone, p749 (Shani et. al., 1981), which had been successfully used to isolate genomic clones from a lambda library (Leader et. al., 1985). Furthermore such actin genomic clones were available as 'positive controls' of the new genomic library. One such clone, λmA36, was used instead of the library in initial 'trouble-shooting' experiments.

Attempts to rescreen a lawn of λmA36 with the ^{32}P-labelled insert of p749 again met with difficulties, the eventual resolution of which identified two factors responsible. A combination of the use of autoclaved filters and of whole nick-translated plasmid pHS2 had been reducing the chance of detecting positive plaques (Figures 3.1.c , 3.1.d and 3.1.e). Autoclaved filters appeared to 'wet'

Figure 3.1

Optimisation of conditions for screening genomic libraries in bacteriophage lambda

a. Plaque filter replicas of recombinant phage from the Charon 4A human foetal DNA library were prepared as described in Section 2.8 using autoclaved nitrocellulose filters. Hybridisation was performed at 65°C, using nick-translated pHS2 (Cato et. al., 1981) as probe (Section 2.8.3). After 18 hours hybridisation, the filters were washed and subjected to autoradiography at -70°C (Sections 2.8.4 and 2.8.5). One such autoradiograph is shown.

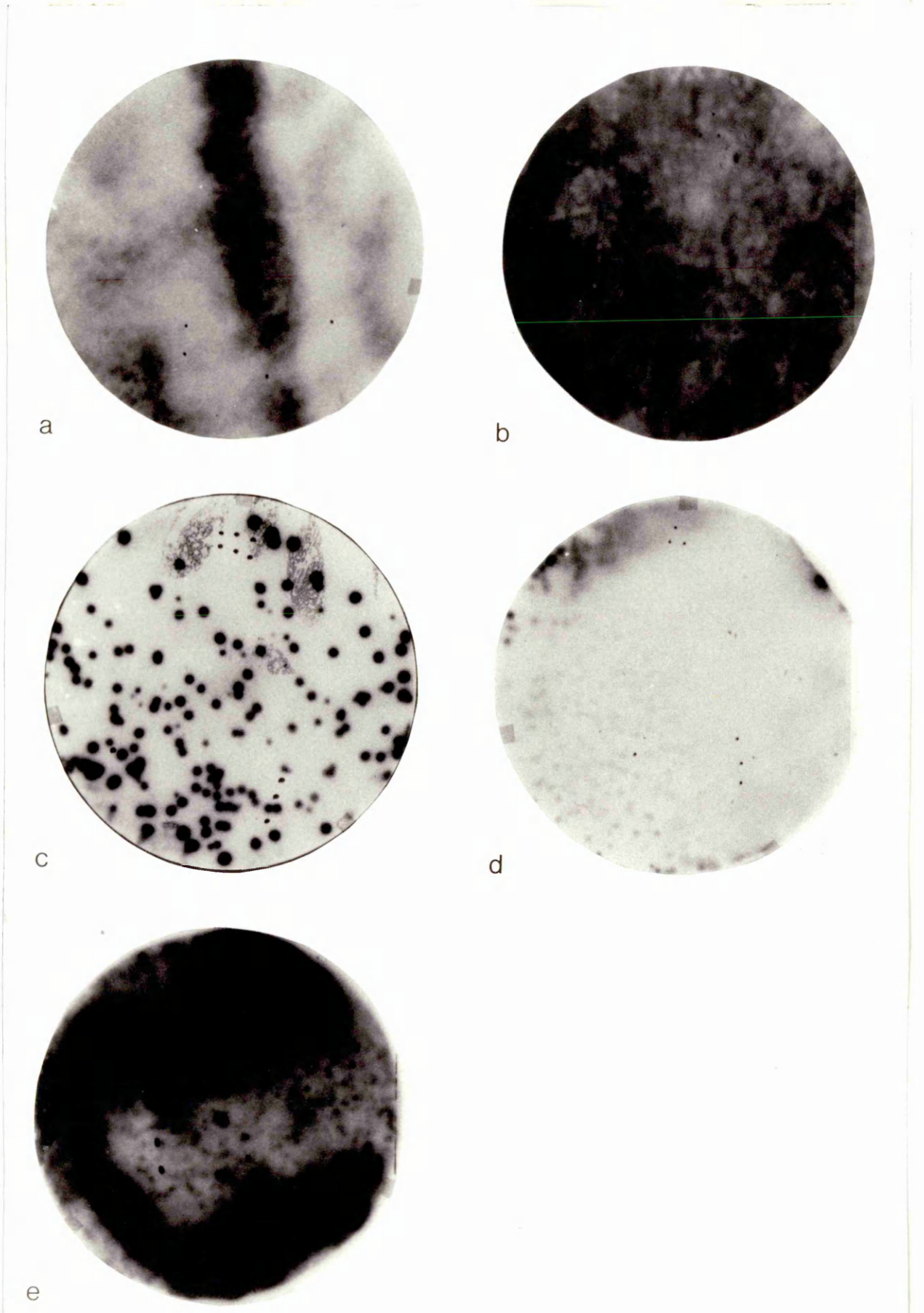
b. Plaque filter replicas of recombinant phage from the EMBL3 human lymphocyte DNA library were prepared, hybridised and subjected to autoradiography as described above. One such autoradiograph is shown.

c. A plaque filter replica of λmA36 phage was prepared according to Section 2.8 using a filter sterilised by γ-irradiation. Hybridisation was performed at 65°C using the nick-translated cDNA insert of p749 (Shani et. al., 1981) as probe. After 18 hours, the filter was washed and subjected to autoradiography as described above. The resultant autoradiograph is shown.

d. A plaque filter replica of λmA36 phage was prepared according to Section 2.8 using a filter sterilised by autoclaving. The filter was hybridised as described in c. above. After 18 hours, the filter was washed and subjected to autoradiography as described above. The resultant autoradiograph is shown.

e. A plaque filter replica of λmA36 phage was prepared according to Section 2.8. Hybridisation was performed at 65°C using nick-translated whole plasmid p749 as probe. After 18 hours, the filter was washed and subjected to autoradiography as described in c. above. The resultant autoradiograph is shown.

Figure 3.1



much faster on agar than those filters sterilised by γ-irradiation. This suggested possible alterations to the filter membrane occurred during autoclaving, which subsequently resulted in reduced efficiency of transfer of DNA. The high background was considered possibly to be due to binding of the pBR322 vector of pHS2 to sequences in the transferred bacterial lawn DNA.

Having resolved these technical problems a trial screening was carried out on the EMBL3 human genomic DNA library using the p749 cDNA insert as a probe. This was done to determine whether the library was satisfactory, using a probe already shown to be capable of selecting recombinants from another library, before proceeding to screen with pHS2. Actin-like clones had previously been picked out of a mouse lambda library with a frequency of about 1 in 10,000 (D.P. Leader, personal communication). Two controls were included in this (and subsequent) screening experiments. The first was a plaque-filter replica of λmA36 phage hybridised to nick-translated p749 cDNA insert. This was to check that the hybridisation conditions were correct each time (Figure 3.2.a). The second was a plaque-filter replica of EMBL3 vector phage hybridised to the probe being used for screening (i.e. p749 cDNA insert in this case). This was to check that any hybridisation was not to the EMBL3 vector DNA (Figure 3.2.b).

100,000 plaques from the EMBL3 library were screened according to Section 2.8 using nick-translated p749 cDNA insert as probe. Thirteen putative positive actin clones were observed, four of which are presented in Figure 3.2.c. To determine whether they were actual positives, the plaques were picked, replated and screened again (Section 2.8.6). An example of how one of the positives rescreened is depicted in Figure 3.2.d. All thirteen clones gave positive

Isolation of putative actin clones using p749 cDNA insert as probe.

Plaque-filter replicas of recombinant phage were prepared as described in Section 2.8 and hybridised to p749 cDNA insert. After 18 hours hybridisation at 65°C, the filters were washed and subjected to autoradiography at -70°C for 18 hours (Sections 2.8.3, 2.8.4 and 2.8.5).

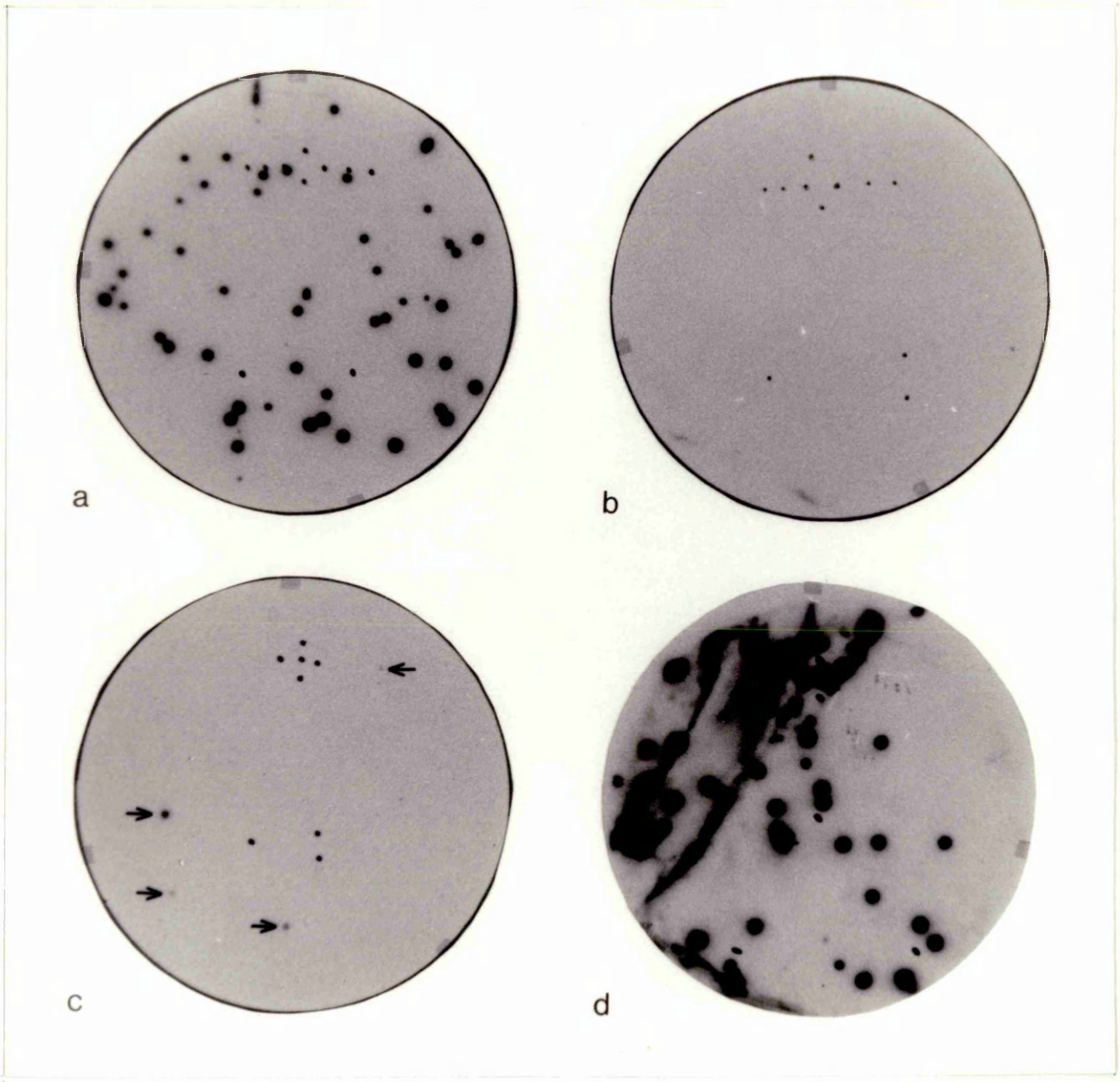
a. A plaque-filter replica of λmA36 phage hybridised to p749 cDNA insert.

b. A plaque-filter replica of EMBL3 vector phage hybridised to p749 cDNA insert.

c. A total of 100,000 recombinant phage from the EMBL3 human lymphocyte DNA library were screened with p749 cDNA insert. Four out of the thirteen putative positives isolated are presented. They are indicated by arrows.

d. Phage from each putative positive was replated and rescreened with p749 cDNA insert. An autoradiograph showing the result of rescreening one positive plaque is shown.

Figure 3.2



signals on rescreening and this indicated a frequency of approximately 1 in 8,000 positive actin clones isolated, quite similar to that obtained previously screening the mouse library.

3.1.2 Isolation of clones corresponding to probe pHS2

Having demonstrated the ability of probe p749 to select positive clones from the human genomic library, screening was resumed using pHS2 in order to try to isolate human heat shock genomic clones.

400,000 plaques from the EMBL3 human genomic library were screened using nick-translated pHS2 cDNA insert (approximately 350 base pairs in size). A total of thirty-five putative positives were observed. Figure 3.3 shows the six of these which had the strongest hybridisation signals. Upon rescreening five of these again gave positive signals (Figure 3.4). The frequency of positive plaques obtained on this basis was therefore 1 in 80,000. The positives were then plaque-purified as described in Section 2.8.6. Figure 3.5 shows one cycle of plaque purification for one of the positive clones isolated. The positive clones were given trivial names initially, but were ultimately named λHr02, λHr03, λHr04, λHr05 and λHr06 respectively for reasons that are explained later. These designations are used throughout for consistency.

3.2 Physical mapping of genomic clones

Phage DNA was prepared from each positive clone (Section 2.9) and used in subsequent clone characterisation experiments.

3.2.1 Determination of the size of the human DNA inserts

In order to calculate the size of the DNA insert within each clone, it was necessary to digest with a restriction enzyme that would cut out the insert leaving the phage arms intact. Sal I which is present in the polylinker (see Figures 2.1 and 2.2) was used for this purpose. Bam HI, used to prepare the lambda arms, cannot be used as these latter were ligated to DNA that had been cleaved with Sau 3AI.

Figure 3.3

Isolation of clones using pHS2 cDNA insert as probe

Plaque-filter replicas of recombinant phage from the EMBL3 human lymphocyte DNA library were prepared and hybridised to nick-translated pHS2 cDNA insert (Section 2.8). After hybridisation for 18 hours at 65°C, the filters were washed at 65°C and subjected to autoradiography at -70°C for 18 hours (Sections 2.8.3, 2.8.4 and 2.8.5).

A total of 400,000 plaques were screened and 35 putative positives isolated. Six of these which had the strongest hybridisation signals are presented (a-f). They are indicated by arrows. Two control filters were included : (g) a plaque-filter replica of λmA36 hybridised to nick-translated p749 cDNA insert and (h) a plaque-filter replica of EMBL3 vector phage hybridised to pHS2 cDNA insert.

Figure 3.3

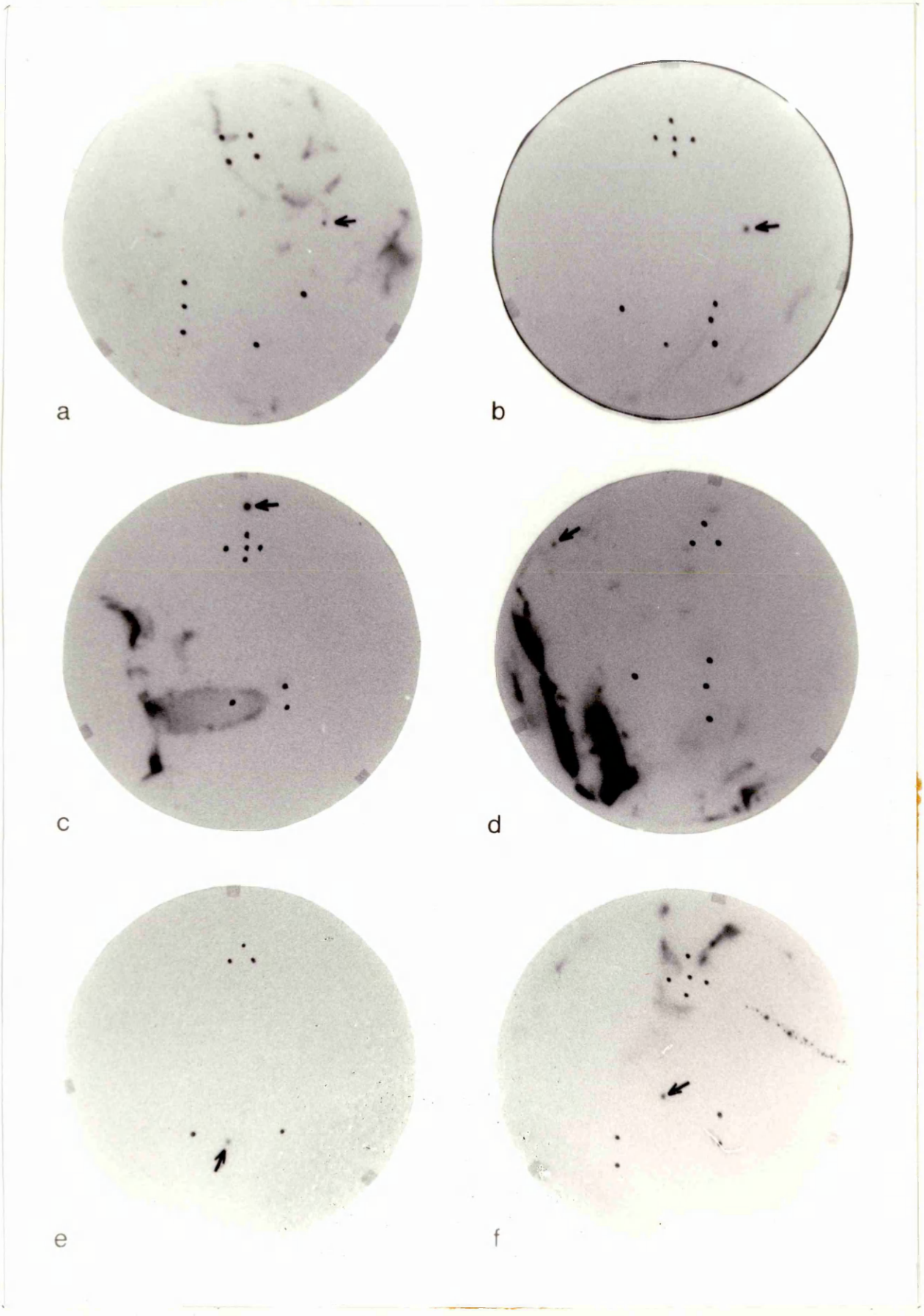


Figure 3.3

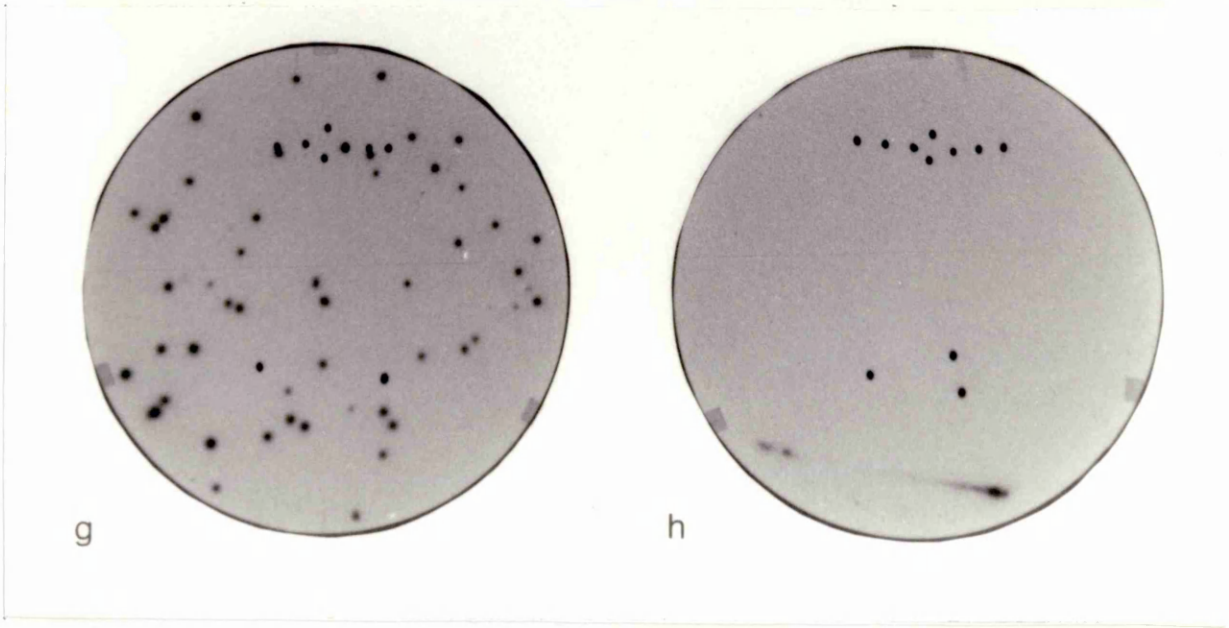


Figure 3.4

Rescreening of 'positive' phage selected with clone pHS2

The 'positive' phage detected were picked, replated and rescreened according to Section 2.8.6, again using pHS2 cDNA insert as probe. The autoradiographs obtained upon rescreening are presented. Autoradiographs a-f correspond to clones on autoradiographs a-f of Figure 3.3, respectively.

Figure 3.4

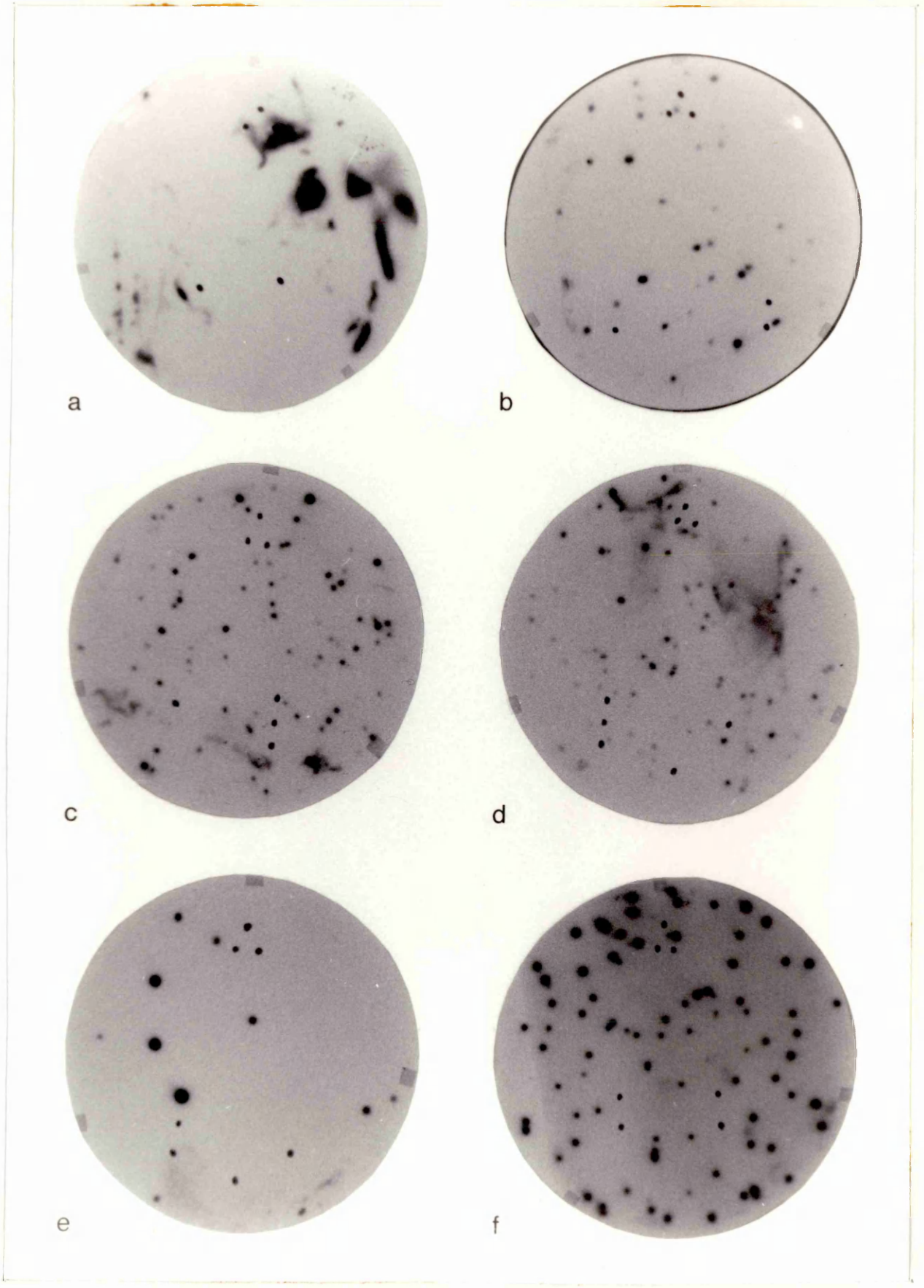
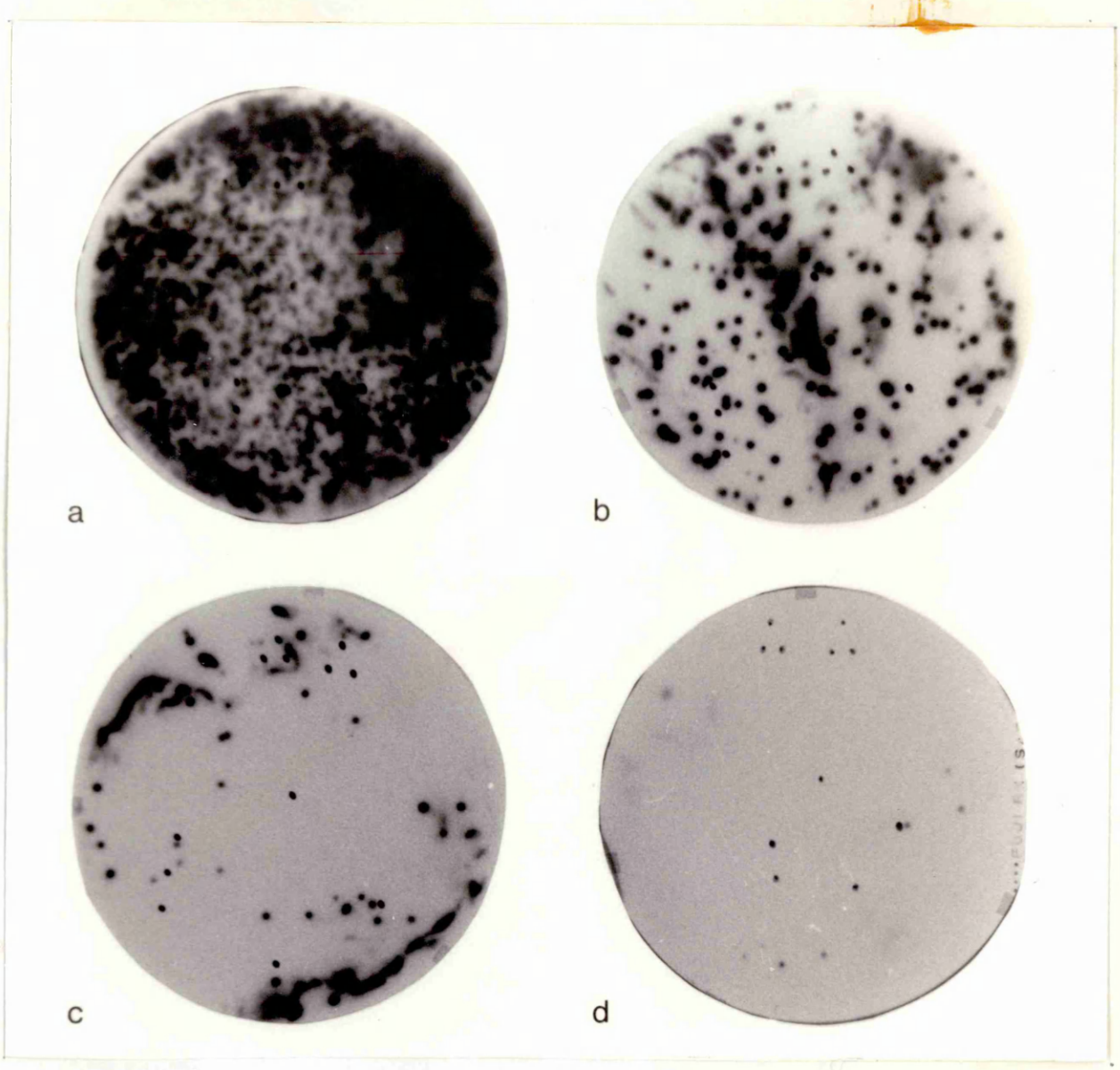


Figure 3.5

Plaque purification of clone λHr02

Plaque purification of each positive clone was carried out according to Section 2.8.6. An example of the final plaque purification cycle of one of the clones, λHr02, is presented. Ten-fold serial dilutions were plated : a. 10^{-1}, b. 10^{-2}, c. 10^{-3} and d. 10^{-4}. All the plaques on each plate gave a positive signal. A well-separated plaque was chosen from the plate corresponding to autoradiograph d.

Figure 3.5



Using the distance of migration, the size of fragments generated from Sal I digests of the genomic clones (Figure 3.6) were determined from a semi-logarithmic plot of known DNA sizes markers (a Hind III digest of bacteriophage lambda in this case: Section 2.12). Table 3.1 shows the size of fragments produced. The sizes of the insert in clones λHr02, λHr03, λHr04, λHr05 and λHr06 were 11 kb, 15.5 kb, 13.5 kb, 18 kb and 19 kb respectively. Clone λHr04 contained one Sal I site which divided the insert into 2 fragments of 7 kb and 6.5 kb in size. The two fragments, 9 kb and 20 kb, seen in all the digests corresponded to the right and left phage arms of EMBL3 (Figure 2.1). In addition a fragment of greater than 23 kb was also observed. This was considered to be due to the left and right phage arms annealing at their cohesive ends.

3.2.2 Selection of clones for further study

To determine which of these clones, λHr02, λHr03, λHr04, λHr05 and λHr06 should be studied further, a selection was made according to their degree of homology with probe pHS2. The rationale of this was that those clones with the highest degree of homology stood a greater chance of containing the gene encoding this (rather than some other related sequence which might not be functional). In order to determine the relative extents of homology between pHS2 and each of the isolated clones the following experiment was performed.

The gel depicted in Figure 3.6 was Southern blotted and hybridised to nick-translated pHS2 cDNA insert. A series of washes was then carried out on the hybridised filter, increasing the temperature of the wash each time. Figure 3.7 shows the results of this experiment. At 42°C, hybridisation of pHS2 to DNA from all the genomic clones was observed, fragments hybridising corresponding to the human DNA inserts within each clone (in λHr04 this hybridisation was confined to the 7 kb Sal I fragment of the insert). The

Figure 3.6

Determination of insert size for clones λHr02, λHr03, λHr04, λHr05 and λHr06

Sal I digests of clones λHr02 (lane 2), λHr03 (lane 3), λHr04 (lane 4), λHr05 (lane 5) and λHr06 (lane 6) were prepared (Section 2.10.1) and subjected to electrophoresis on a 0.7% (w/v) agarose gel (Loening phosphate buffer : Sections 2.5.2.a and 2.11). A Hind III digest of bacteriophage lambda (lanes 1 and 8) and pHS2 cDNA insert (lane 7) were included as markers. A photograph of the stained gel is presented with the size of the marker fragments indicated at the side.

The size of fragments generated by Sal I are presented in Table 3.1. Those >23 kb represent undigested DNA.

Figure 3.6

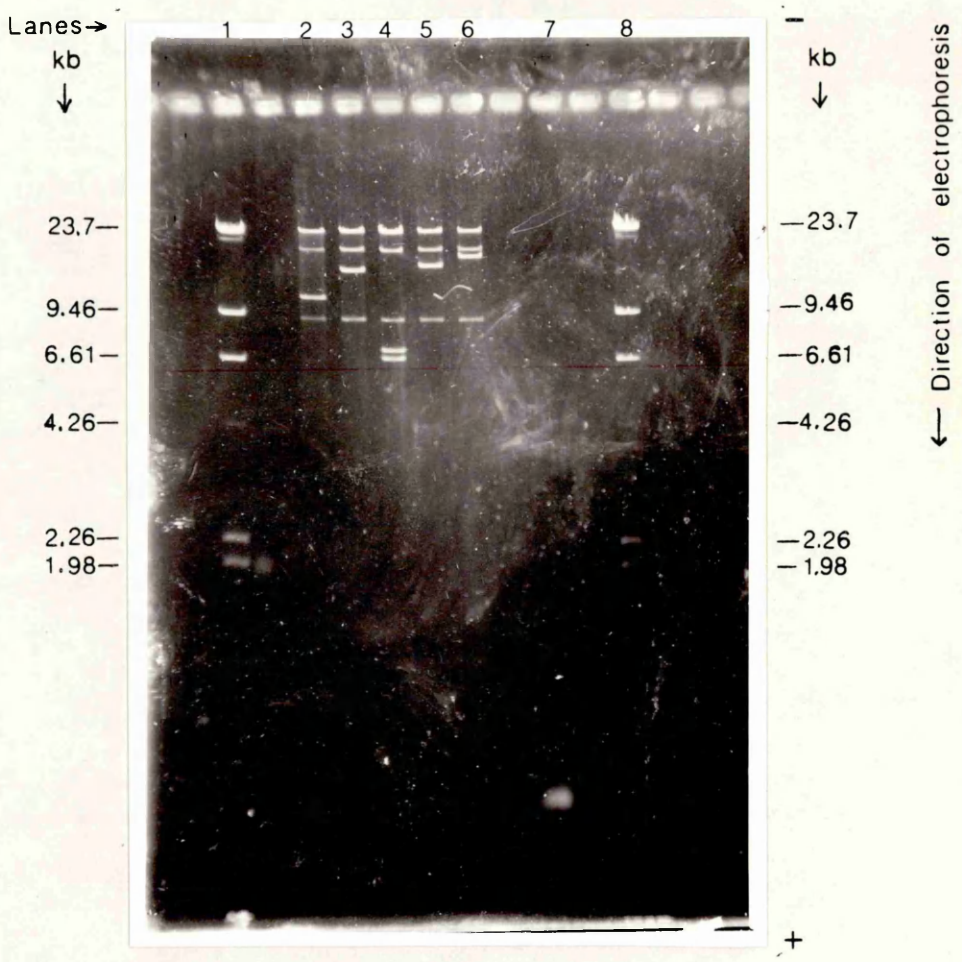


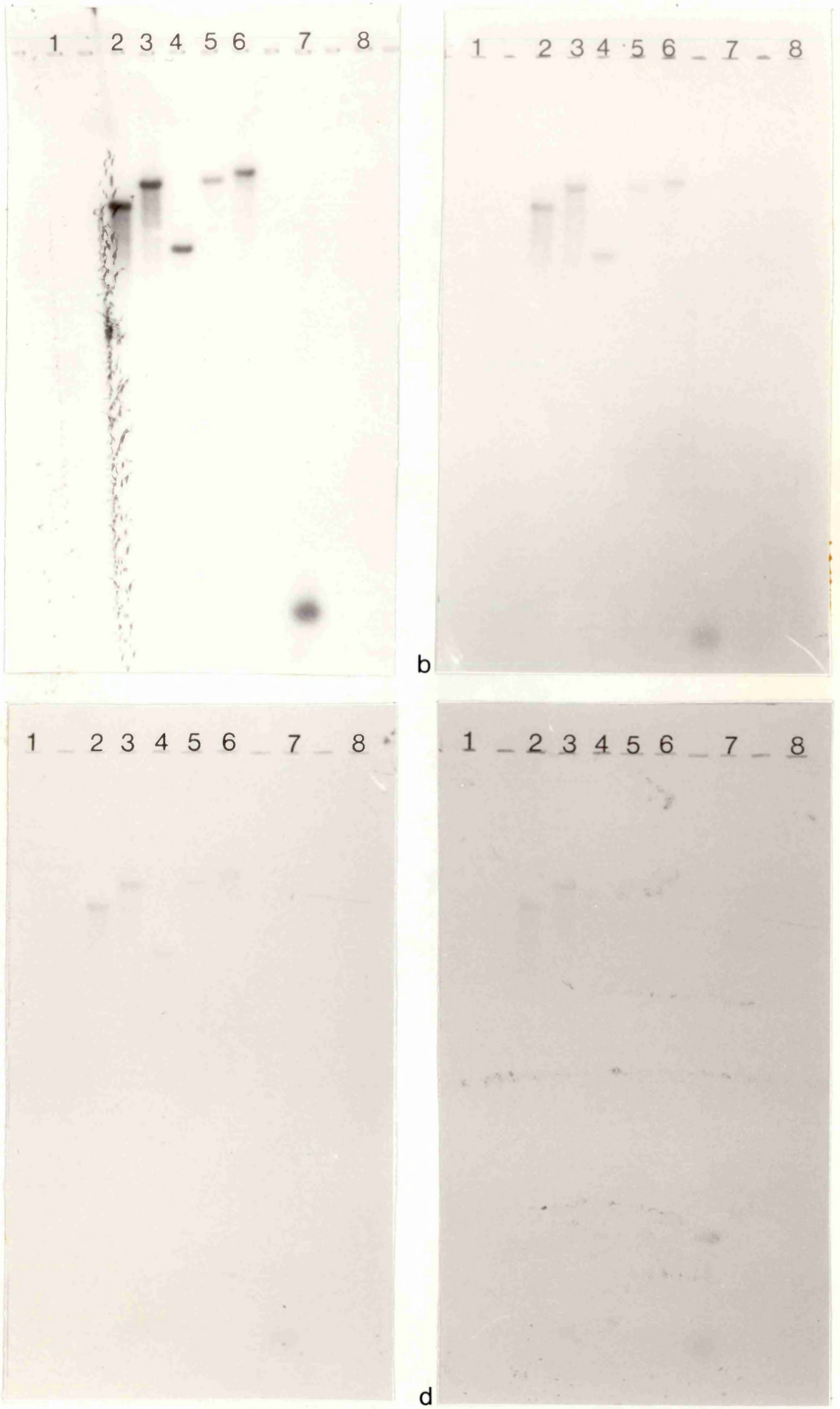
Table 3.1

λHrO2	λHrO3	λHrO4	λHrO5	λHrO6
>23 kb	>23 kb	>23 kb	>23 kb	>23 kb
20 kb	20 kb	20 kb	20 kb	20 kb
11 kb	15.5 kb	9 kb	18 kb	19 kb
9 kb	9 kb	7 kb	9 kb	9 kb
		6.5 kb		

Determination of relative extents of homology between pHS2 and isolated genomic clones

The gel is that depicted in Figure 3.6 : Hind III digest of bacteriophage lamba (lanes 1 and 8), Sal I digests of λHr02 (lane 2), λHr03 (lane 3), λHr04 (lane 4), λHr05 (lane 5), λHr06 (lane 6) and pHS2 cDNA insert (lane 7). This was Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m)(Sections 2.14, 2.15 and 2.17.2.b). After 18 hours hybridisation at 42°C the filter was washed (Section 2.17.2.b) and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiograph is shown in a. The filter was then rewashed at increasing temperatures. Autoradiographs b, c and d correspond to 50°C, 55°C and 62°C respectively. Hybridisation of pHS2 cDNA insert to clones λHr02 (lane 2), λHr03 (lane 3), λHr05 (lane 5) and λHr06 (lane 6) was still clearly observed at 67°C and 72°C, however the bands were too faint to be photographed.

Figure 3.7



hybrids between the pHS2 insert and the DNA from the genomic clones remained stable up to 55°C. Between 55°C and 62°C the hybrid formed between pHS2 and λHr04 was destroyed. This indicated that λHr04 has a lower homology to pHS2 than the other clones. As this clone was isolated at 65°C this result may appear contradictory, however a higher stringency of wash had been used in this case (0.1X SSC compared to 0.2X SSC in plaque isolation). The hybrids with the remaining clones were stable up to 72°C, this being the highest temperature of wash studied.

Clones λHr02, λHr03, λHr05 and λHr06 were therefore chosen for further study as they exhibited greatest homology to pHS2.

3.2.3 Restriction endonuclease mapping of genomic clones

Restriction endonuclease maps were then constructed for each of the selected clones λHr02, λHr03, λHr05 and λHr06 using several different mapping techniques. These techniques included single and double digestion linked to hybridisation, and partial digestion mapping.

3.2.3.a Single digestion mapping

To determine the number of restriction sites within λHr02, λHr03, λHr05 and λHr06 for each enzyme, and the size of fragments generated, a series of single digests were carried out as an initial step in mapping.

Single digests of each clone were performed as described in Section 2.10.1 using the following restriction enzymes with six base-pair recognition sites: Bam HI, Bgl II, Cla I, Eco RI, Hind III, Kpn I, Pst I, Sal I, Sma I, Sph I, Sst I and Xba I. The digests for different enzymes with a single clone were subjected to electrophoresis on 0.7% (w/v) agarose gels. The gels obtained are illustrated in Figures 3.8, 3.9, 3.10 and 3.11. The size of the fragments produced in each digest were determined as described in

Figure 3.8

Single restriction digestion analysis of clone λHr02

Aliquots of λHr02 DNA (1 μg) were cleaved with the following restriction enzymes : Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14) and Sph I (lane 15). (Section 2.10.1). The resulting digests were subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11) in parallel with a Hind III digest (lanes 1 and 12) and a Hind III/ Eco RI digest (lanes 11 and 16) of bacteriophage lambda. Photographs of the stained gels are shown. The size of the fragments produced in each digest are presented in Table 3.2.

Figure 3.8

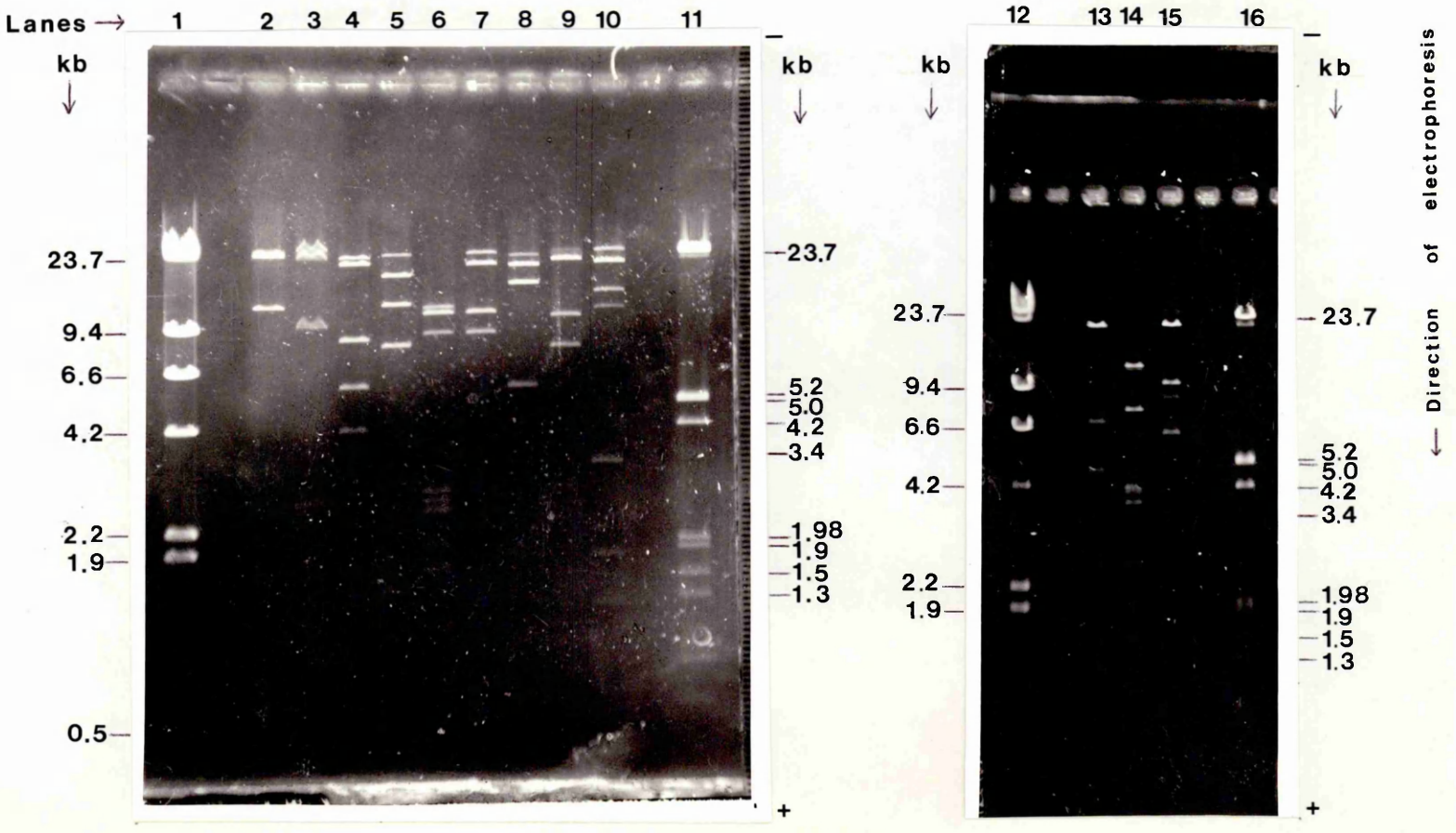

Lanes →
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
−
+
Direction of electrophoresis

Figure 3.9

Single restriction digestion analysis of clone λHr03

Aliquots of λHr03 DNA (1 μg) were cleaved with the following restriction enzymes : Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14) and Sph I (lane 15). (Section 2.10.1). The resulting digests were subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11) in parallel with a Hind III digest (lanes 1 and 12) and a Hind III/ Eco RI digest (lanes 11 and 16) of bacteriophage lambda. Photographs of the stained gels are shown. The size of the fragments produced in each digest are presented in Table 3.3.

Figure 3.9

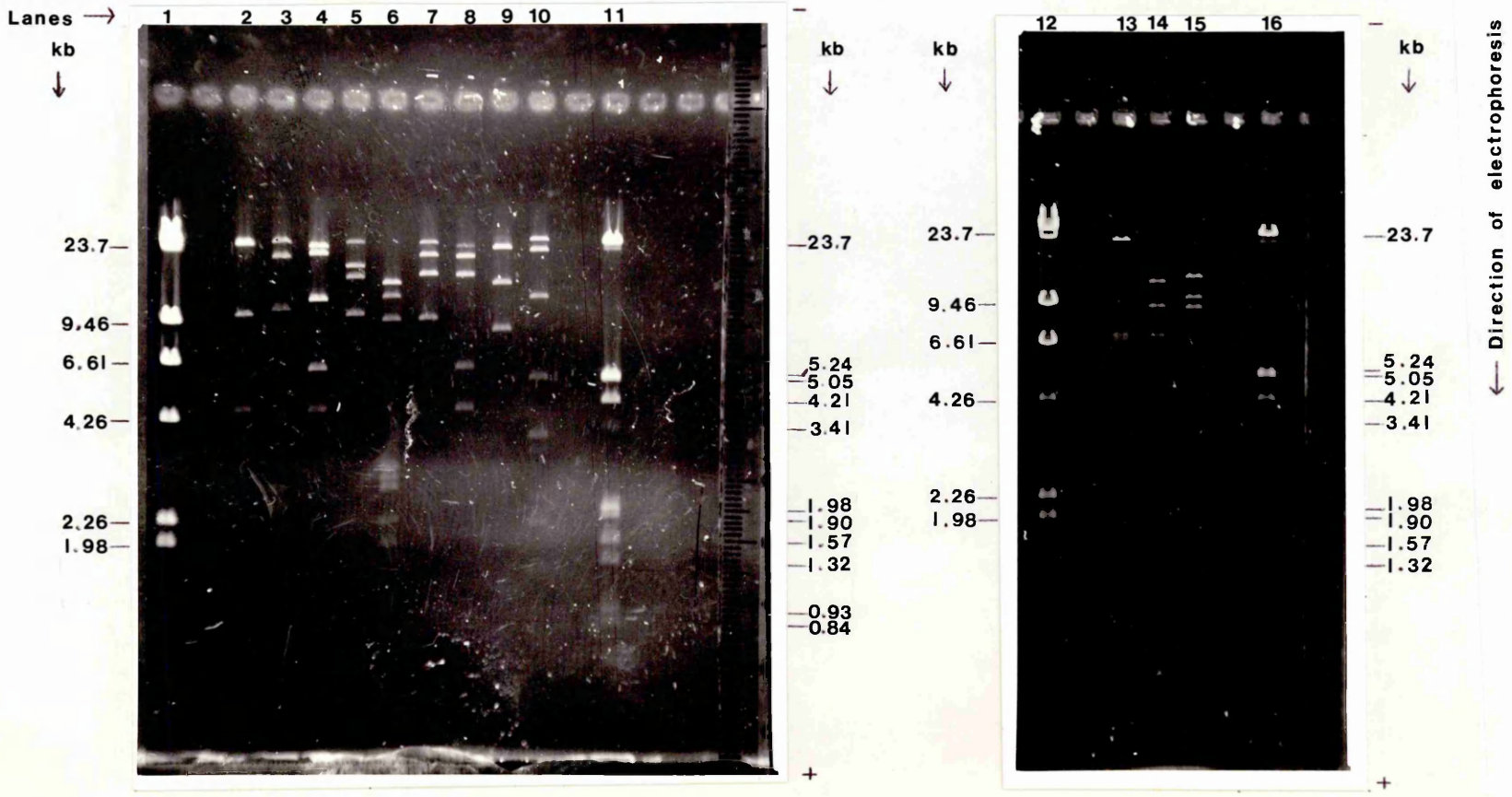

Lanes →
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16
kb
23.7
9.46
6.61
4.26
2.26
1.98
5.24
5.05
4.21
3.41
1.57
1.32
0.93
0.84
−
+
Direction of electrophoresis

Figure 3.10

Single restriction digestion analysis of clone λHr05

Aliquots of λHr05 DNA (1 μg) were cleaved with the following restriction enzymes : Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14) and Sph I (lane 15). (Section 2.10.1). The resulting digests were subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11) in parallel with a Hind III digest (lanes 1 and 12) and a Hind III/ Eco RI digest (lanes 11 and 16) of bacteriophage lambda. Photographs of the stained gels are shown. The size of the fragments produced in each digest are presented in Table 3.4.

Figure 3.10

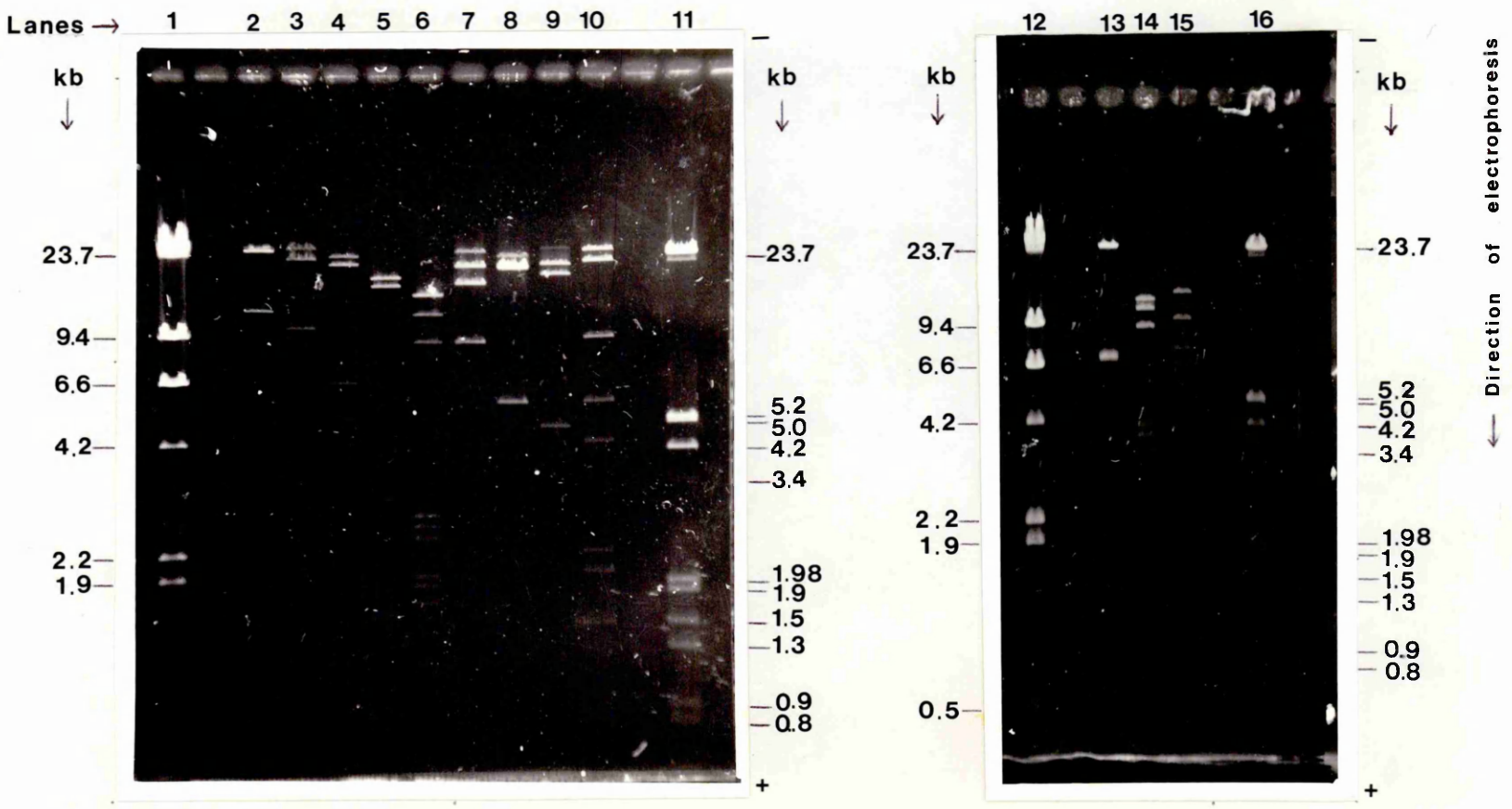

Lanes →
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
5.2
5.0
3.4
1.98
1.5
1.3
0.9
0.8
−
+
Direction of electrophoresis

Figure 3.11

Single restriction digestion analysis of clone λHr06

Aliquots of λHr06 DNA (1 μg) were cleaved with the following restriction enzymes : Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14) and Sph I (lane 15). (Section 2.10.1). The resulting digests were subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11) in parallel with a Hind III digest (lanes 1 and 12) and a Hind III/ Eco RI digest (lanes 11 and 16) of bacteriophage lambda. Photographs of the stained gels are shown. The size of the fragments produced in each digest are presented in Table 3.5.

Figure 3.11

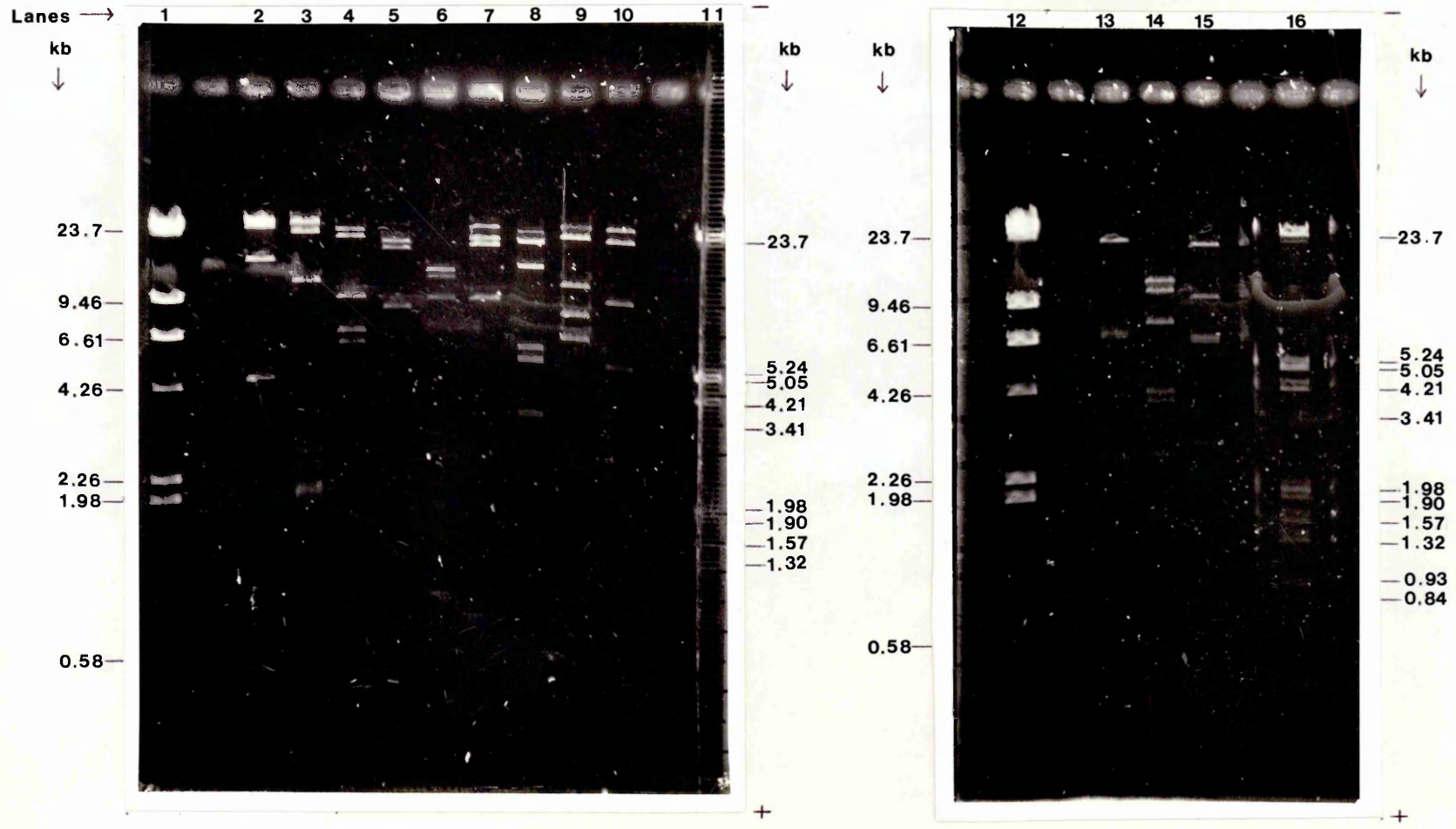

Lanes
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16
kb
23.7
9.46
6.61
4.26
2.26
1.98
0.58
5.24
5.05
4.21
3.41
1.90
1.57
1.32
0.93
0.84
Direction of electrophoresis

Table 3.2

Size of fragments generated during single digestion of λHr02

The size of the fragments produced in each restriction digest (shown in Figure 3.8) are presented.

Table 3.2

Restriction enzyme	Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I
size of fragments in kb	>23	>23	12	>23 p	23 p	23 p
	11	7.4	7.7	23	19	16.6
		4.9	6.2	9.4	8.3	11
		2.3	4.0	2.7	6	8.3
		1.2	3.9	2.4	4.2	1.5
			3.5	1.9		1.1
			2.3			
			2.0			
			1.6			
			1.4			
Total length of clone	>34	>38.7	44.6	>37.5	37.5	38.5
Estimated no. of sites	1	4	9	4	3	4
Restriction enzyme	Pst I	Sal I	Sma I	Sph I	Sst I	Xba I
size of fragments in kb	11	>23 p	>23 p	>23	23	>23 p
	10	20	20	9.4	10	22 p
	9	10.5	15	8.4	8.3	18
	3.1	8.9	6.0	6.4		11
	2.8			1.9		3.7
	2.7					1.9
	2.1					1.4
	1.95					
	1.2					
Total length of clone	43.6	38.6	41	49.1	41.3	36
Estimated no. of sites	8	2	2	4	2	4

p : partials omitted when calculating clone length

Table 3.3

Size of fragments generated during single digestion of λHr03

The size of the fragments produced in each restriction digest (shown in Figure 3.9) are presented.

Table 3.3

Restriction enzyme	Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I
size of fragments in kb	>23	>23	11	>23 p	24 p	>23 p
	9.8	7	8.4	20	22	18
	4.7	6.8	8.8	10.5	12	16
		2.5	6.2	4.5	6.2	10
		1.4	4.0	2.8	4.6	1.5
			3.9	2.5	1.05	1.1
			2.5	2.1		
			1.5			
Total length of clone	>37.5	>40.7	44.3	42.4	45.8	46.6
Estimated no of sites	2	4	7	5	4	4
Restriction enzyme	Pst I	Sal I	Sma I	Sph I	Sst I	Xba I
size of fragments in kb	14	>23 p	>23 p	17	>23	>23 p
	12	21	21	9.4	14	23
	9.2	16	16	8.4	9	12
	3.2	9.6	6.8	6.6		5.15
	3.0		5.0	5.0		3.0
	2.8					2.3
	2.3					
	1.8					
Total length of clone	48.5	46.6	43.8	46.4	46	47.4
Estimated no. of sites	7	2	3	4	2	4

p : partials omitted when calculating clone length

Table 3.4

Size of fragments generated during single digestion of λHr05

The size of the fragments produced in each digest (shown in Figure 3.10) are presented.

Table 3.4

Restriction enzyme	Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I
size of fragments in kb	>23	>23	13	23	23 p	17
	11	7	11	15	20	15
	3.3	1.2	9.4	9.6	6.8	4
		1.0	6.6	2.7	5.3	1.6
			4.3	2.3	4.5	1.5
			4.1		3.6	
			2.6		1.65	
			2.1		1.4	
Total length of clone	>37.3	>32.2	53.1	29.6	43.2	39.1
Estimated no. of sites	2	3	7	3	6	4
Restriction enzyme	Pst I	Sal I	Sma I	Sph I	Sst I	Xba I
size of fragments in kb	25.5 p	>24 p	23	14	20	>23 p
	11	19.5	19	10	18	20
	9	17	6	8.2	5	9
	2.9	8.8		7.4		5.9
	2.7			4.8		4.7
	2.5			6.4		2.3
	2.0			2.2		2.0
	1.8					1.4
	1.65					
Total length of clone	33.5	38.8	48	55	42	45.3
Estimated no. of sites	7	2	2	6	2	7

p : partials omitted when calculating clone length

Table 3.5

Size of fragments generated during single digestion of λHr06

The size of the fragments produced in each restriction digest (shown in Figure 3.11) are presented.

Table 3.5

Restriction enzyme	Bam II	Bgl II	Cla I	Eco RI	Hind III	Kpn.I
size of fragments in kb	24	25	16	>23 p	>23 p	26
	17	6.8	12.5	25	25	22
	4.7	3.2	8	14	11.5	10
		2.6	4.7	2.9	7.4	1.7
		1.3	4.2	2.5	6.4	1.2
			2.8	2.0	4.8	
			2.2	1.15		
			1.7	0.9		
Total length of clone	45.7	38.9	52.1	48.4	55.1	60.9
Estimated no. of sites	2	4	7	6	4	4
Restriction enzyme	Pst I	Sal I	Sma I	Sph I	Sst I	Xba I
Size of fragments in kb	17.5	>23 p	>23 p	23	>23 p	>23 p
	15.5	25	26	12	25	25
	11.5	22	17.5	6.8	14	10
	3.2	11.5	6.2	3.1	9.2	5.2
	2.9		5.8	2.3	6.8	3.7
	2.7		3.8			3.3
	2.4					2.8
	1.8					1.7
	1.5					1.2
	1.3					
Total length of clone	60.3	58	59.3	47.2	55	52.9
Estimated no. of sites	9	2	4	4	3	7

p : partials omitted when calculating clone length

Section 2.12 and are presented in Tables 3.2, 3.3, 3.4 and 3.5. The overall length of each clone, including the phage arms, was determined using the results of the Sal I digests. Ignoring the upper Sal I fragments (which are a result of left and right phage arms annealing at their cohesive ends) the sum of the sizes of the remaining 3 fragments in each Sal I digest of clones λHr02, λHr03, λHr05 and λHr06 gave overall lengths of 40 kb, 44.5 kb, 47 kb and 48 kb respectively. The sum of the sizes of fragments produced by the digestions with each of the other restriction endonucleases were then determined, and compared to the reference size determined from the Sal I fragments. Some of the values were larger than expected indicating the presence of partial digestion products. Because of this partial digestion in some cases and the possibility that some small fragments were overlooked because they were not resolved from one another, only an approximate estimate of the number of restriction enzyme sites present in each clone could be obtained by this method. Nevertheless the results were of value in determining a preliminary map of each clone and in locating the region of pHS2 hybridisation (see below).

3.2.3.b Location of the region of pHS2 hybridisation

In order to define the region of pHS2 hybridisation within each clone more accurately, the gels depicted in Figures 3.8, 3.9, 3.10 and 3.11 were Southern blotted and hybridised to nick-translated pHS2 cDNA insert. Figures 3.12, 3.13, 3.14 and 3.15 show the autoradiographs obtained and the size of fragments which gave a positive hybridisation are presented in Table 3.6.

The size of hybridised fragments in λHr02, λHr03 and λHr06 show some striking similarities with one another, whereas clone λHr05 appears to be quite different. For clones λHr02, λHr03 and

Figure 3.12

Location of the region of pHS2 hybridisation in λHr02

The gels are those depicted in Figure 3.8 : Hind III digest of bacteriophage lambda (lanes 1 and 12), λHr02 digested with Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14), Sph I (lane 15) and a Hind III/Eco RI digest of bacteriophage lambda (lanes 11 and 16). These were Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m. per filter)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filters were washed (Section 2.17.2.a) and subjected to auto-radiography for 18 hours at -70°C. The resultant autoradiographs are presented. Table 3.6 indicates the size of fragments which hybridised to pHS2 cDNA insert.

Figure 3.12

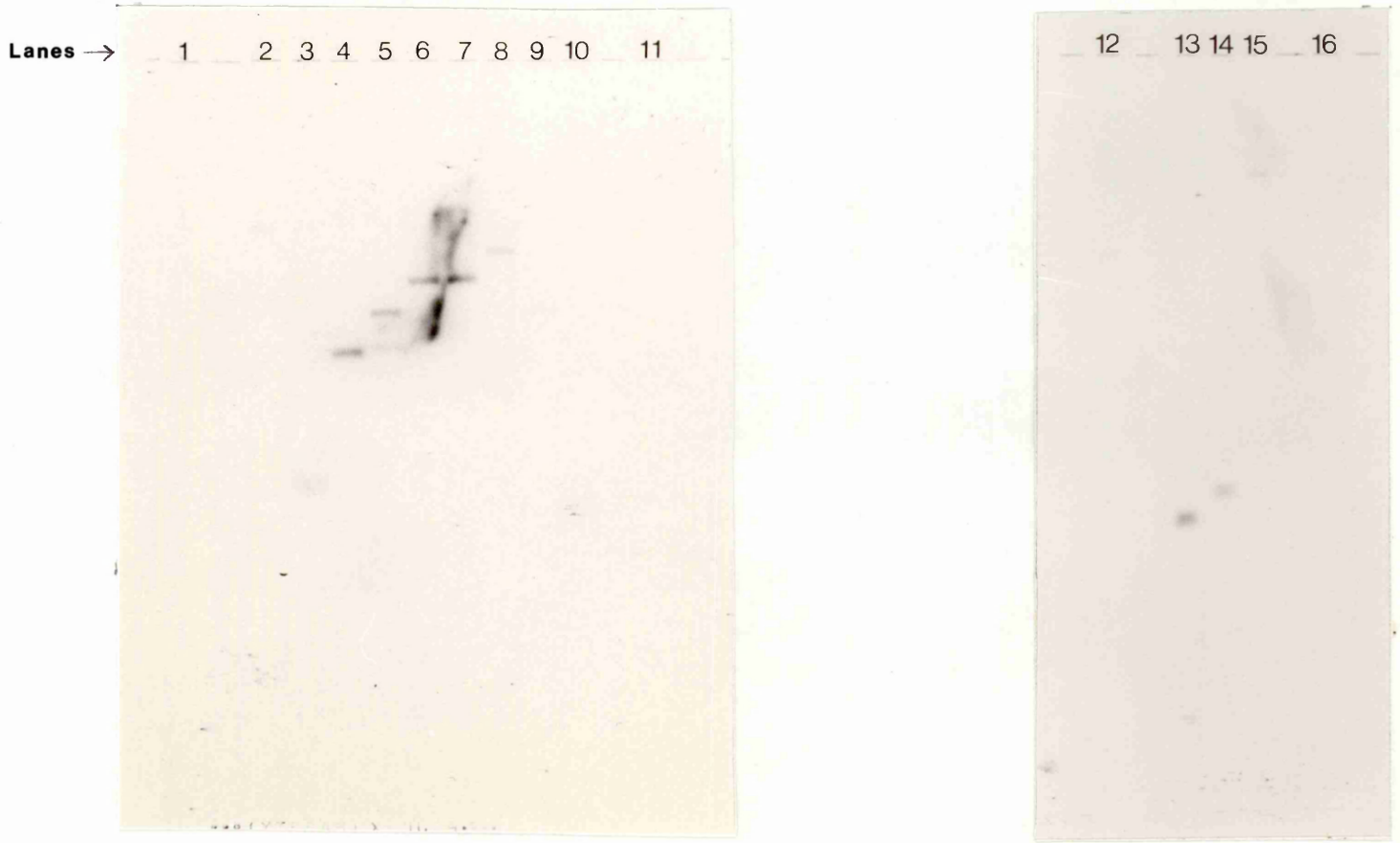
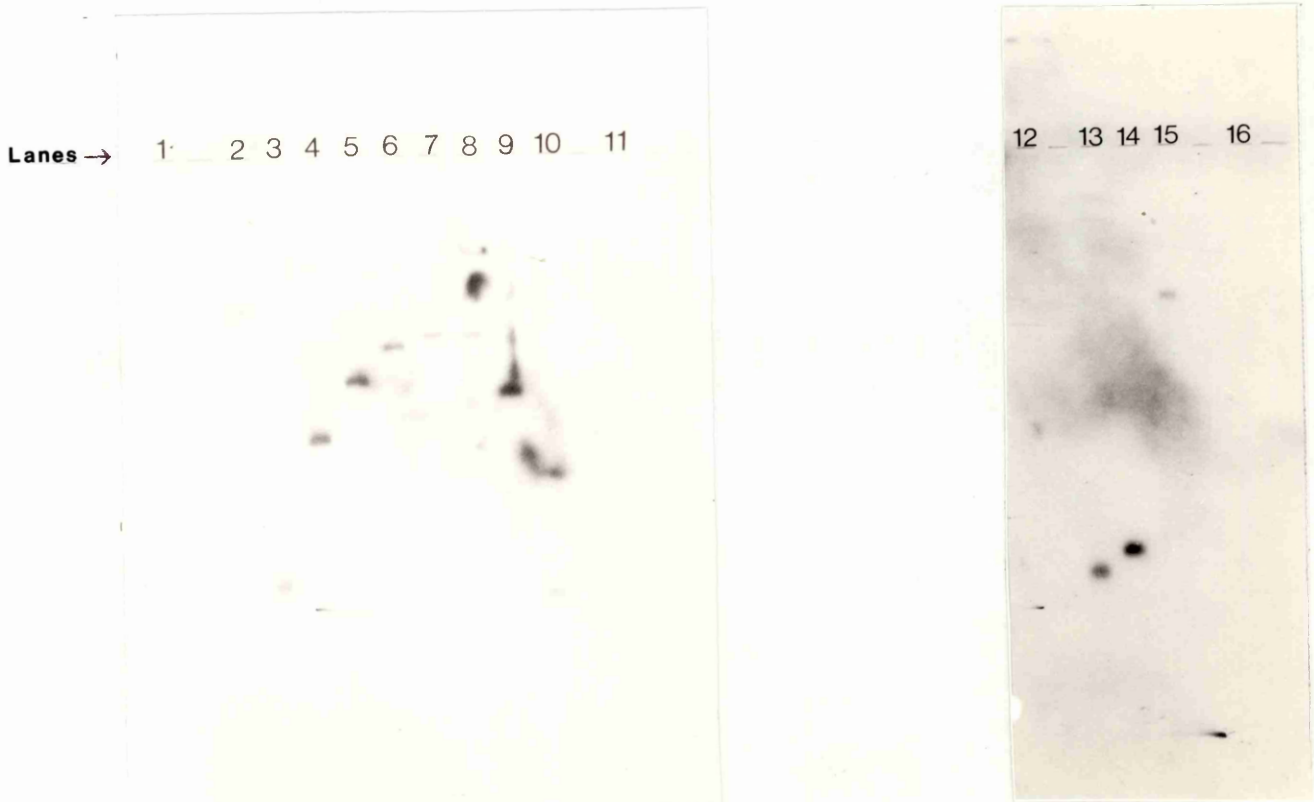


Figure 3.13

Location of the region of pHS2 hybridisation in λHr03

The gels are those depicted in Figure 3.9 : Hind III digest of bacteriophage lambda (lanes 1 and 12), λHr03 digested with Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14), Sph I (lane 15) and a Hind III/Eco RI digest of bacteriophage lambda (lanes 11 and 16). These were Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m. per filter)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filters were washed (Section 2.17.2.a) and subjected to auto-radiography for 18 hours at −70°C. The resultant autoradiographs are presented. Table 3.6 indicates the size of fragments which hybridised to pHS2 cDNA insert.

Figure 3.13



Location of the region of pHS2 hybridisation in λHr05

The gels are those depicted in figure 3.10 : Hind III digest of bacteriophage lambda (lanes 1 and 12), λHr05 digested with Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 4), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14), Sph I (lane 15) and a Hind III/Eco RI digest of bacteriophage lambda (lanes 11 and 16). These were Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m. per filter)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filters were washed (Section 2.17.2.a) and subjected to auto-radiography for 18 hours at -70°C. The resultant autoradiographs are presented. Table 3.6 indicates the size of fragments which hybridised to pHS2 cDNA inert.

Figure 3.14

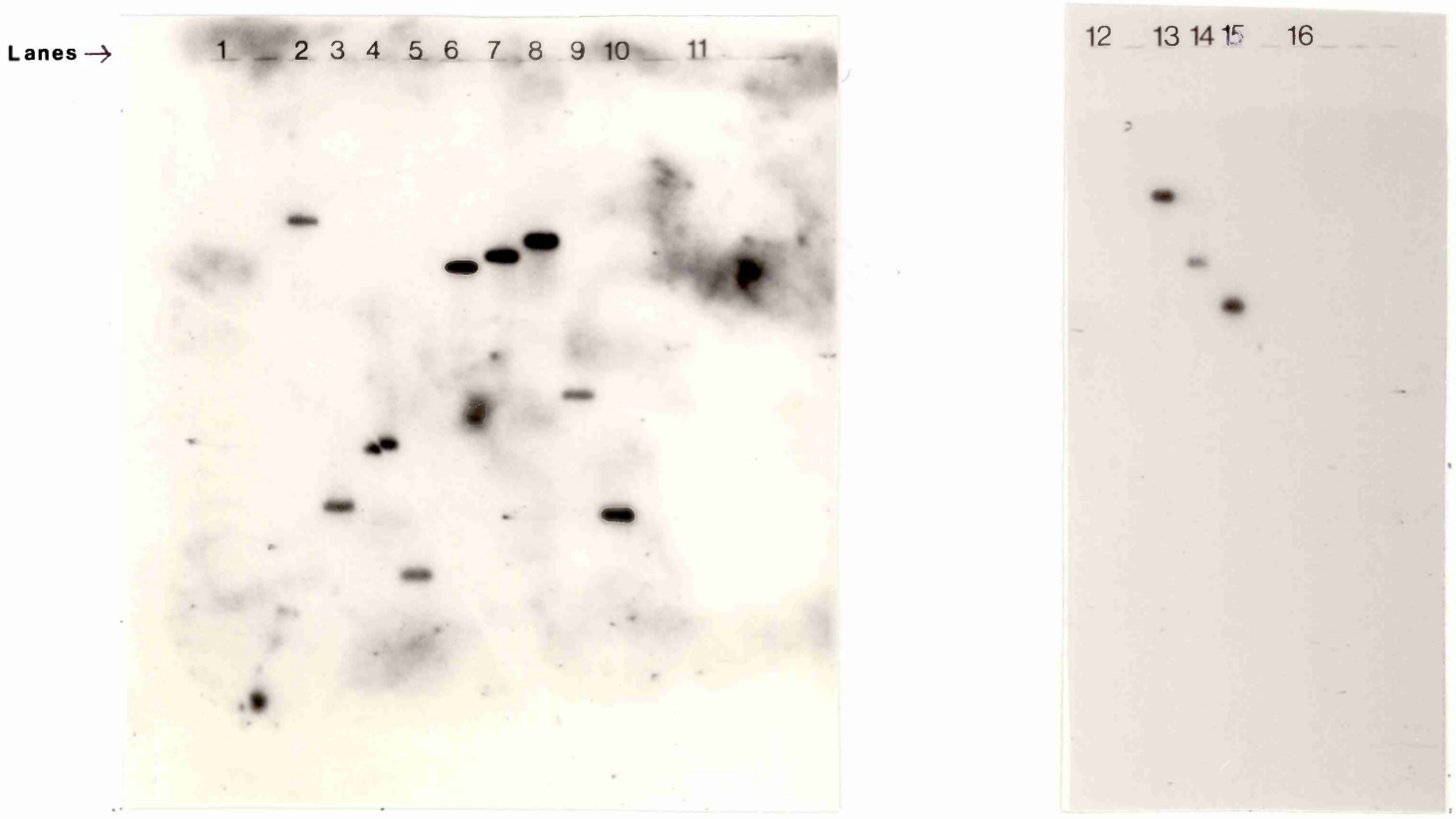


Figure 3.15

Location of the region of pHS2 hybridisation in λHr06

The gels are those depicted in Figure 3.11 : Hind III digest of bacteriophage lambda (lanes 1 and 12), λHr06 digested with Bam HI (lane 2), Eco RI (lane 3), Hind III (lane 5), Kpn I (lane 5), Pst I (lane 6), Sal I (lane 7), Sma I (lane 8), Sst I (lane 9), Xba I (lane 10), Bgl II (lane 13), Cla I (lane 14), Sph I (lane 15) and a Hind III/ Eco RI digest of bacteriophage lambda (lanes 11 and 16). These were Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m. per filter)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filters were washed (Section 2.17.2.a) and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiographs are presented. Table 3.6 indicates the size of fragments which hybridised to pHS2 cDNA insert.

Figure 3.15

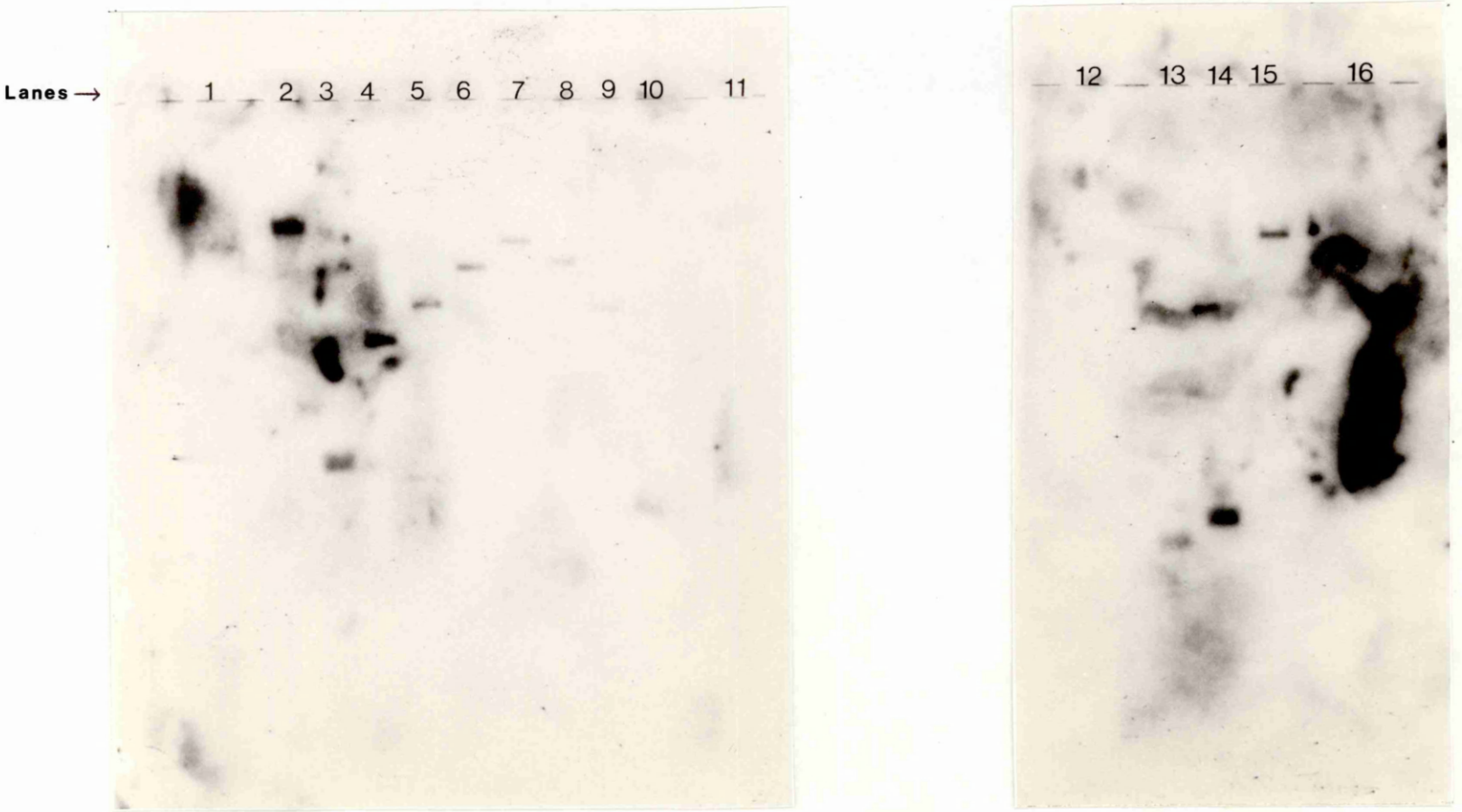


Table 3.6

Size of the single digestion fragments which hybridised to pHS2

The size of the single digestion fragments, from clones λHr02, λHr03, λHr05 and λHr06, which hybridised to the pHS2 cDNA insert are presented.

Table 3.6

Enzyme	λHr02 (kb)	λHr03 (kb)	λHr05 (kb)	λHr06 (kb)
Bgl II	1.2	1.4	>23	1.3
Bam HI	>23	>23	23	>23
Cla I	1.4	1.5	13	1.7
Eco RI	2.4	2.5	2.3	2.5
Hind III	6	6.2	3.6	6.4
Kpn I	8.3	10	1.6	10
Pst I	10.5	14	14	17.5
Sal I	10.5	16	17	22
Sma I	15	16	19	17.5
Sst I	8.3	9	18	9.2
Xba I	1.9	2.3	2.3	1.7

λHr06 similarities were seen between the Bgl II fragments (1.2 kb, 1.4 kb, 1.3 kb), Cla I fragments (1.4 kb, 1.5 kb, 1.7 kb), Hind III fragments (6 kb, 6.2 kb, 6.4 kb) and Sst I fragments (8.3 kb, 9 kb, 9.2 kb) with the possibility that these fragments were, in fact, identical. For λHr05, the size of these fragments was noticeably different: Bgl II fragment(>23 kb), Cla I fragment(13 kb), Hind III fragment(3.8 kb)and Sst I fragment (17 kb). Eco RI produces similar fragments in all four clones whereas the remaining digests show differences between them.

To establish whether these similar fragments (for clones λHr02, λHr03 and λHr06) were in fact identical in size, the single digests were re-examined, subjecting the digests for a particular enzyme with the different genomic DNA to electrophoresis on the same gel in parallel. This is outlined in more detail in Section 3.2.3.d.

3.2.3.c Partial digestion mapping

To establish the order of the restriction sites with respect to each other, another mapping technique which involved the use of partial digests was employed. The method of Rackwitz et. al., (1984) was chosen in preference to the more commonly used method of Smith and Birnstiel, (1976). There were two reasons for this. Firstly, it removed the requirement of having to find unique sites for cleavage after end-labelling (which can often be more difficult for long DNA molecules like phage DNA), and secondly, it removed the background problems of labelling reactions (e.g. labelling nicks or gaps). The disadvantage of all partial mapping procedures is that the resolution of the map decreases with distance from the labelled site (i.e. between the middle and end of the DNA). Partial mapping should therefore be carried out from both ends of the molecule,

however, this could not be performed in this study as only one of the synthetic oligodeoxynucleotides, which are used in the method of Rackwitz et. al., (1984), was available.

Samples of partial-digested DNA from clones λHr03, λHr03, λHr05 and λHr06 (prepared using the restriction enzymes Bam HI, Bgl II, Cla I, Eco RI, Hind III, Kpn I, Sal I, Sma I, Sph I, Sst I and Xba I) were hybridised to labelled ON-R (Section 2.13) and subjected to electrophoresis on 0.5% (w/v) agarose gels. The autoradiographs obtained from these gels (when dried) are shown in Figures 3.16, 3.17, 3.18 and 3.19.

A ladder of fragments was observed for each digest, with non-hybridised ON-R and unincorporated $\gamma[^{32}$ P]ATP seen at the base of each autoradiograph. Restriction sites were noted as they were encountered, reading from the bottom of the autoradiographs upwards (i.e. from the labelled terminus) and the size of each fragment generated, calculated as previously described in section 2.12. Tables 3.7, 3.8, 3.9 and 3.10 list the fragments observed. To determine the distance between two restriction sites the size of the smaller partial fragment was subtracted from the size of the larger. Certain fragments, on the autoradiographs, were seen to have a lesser or greater intensity than others, which suggested that not all restriction sites were equivalent substrates. A region of DNA bounded by two Sst I sites in clones λHr03, λHr03 and λHr06 (see Tables 3.7, 3.8 and 3.10) possessed a similar pattern of restriction sites, which gave further support to the possibility of these clones containing some identical fragments. However λHr05 was still seen to be different as it did not reveal this ordering of sites.

3.2.3.d Additional single and double digestion analysis of the genomic clones

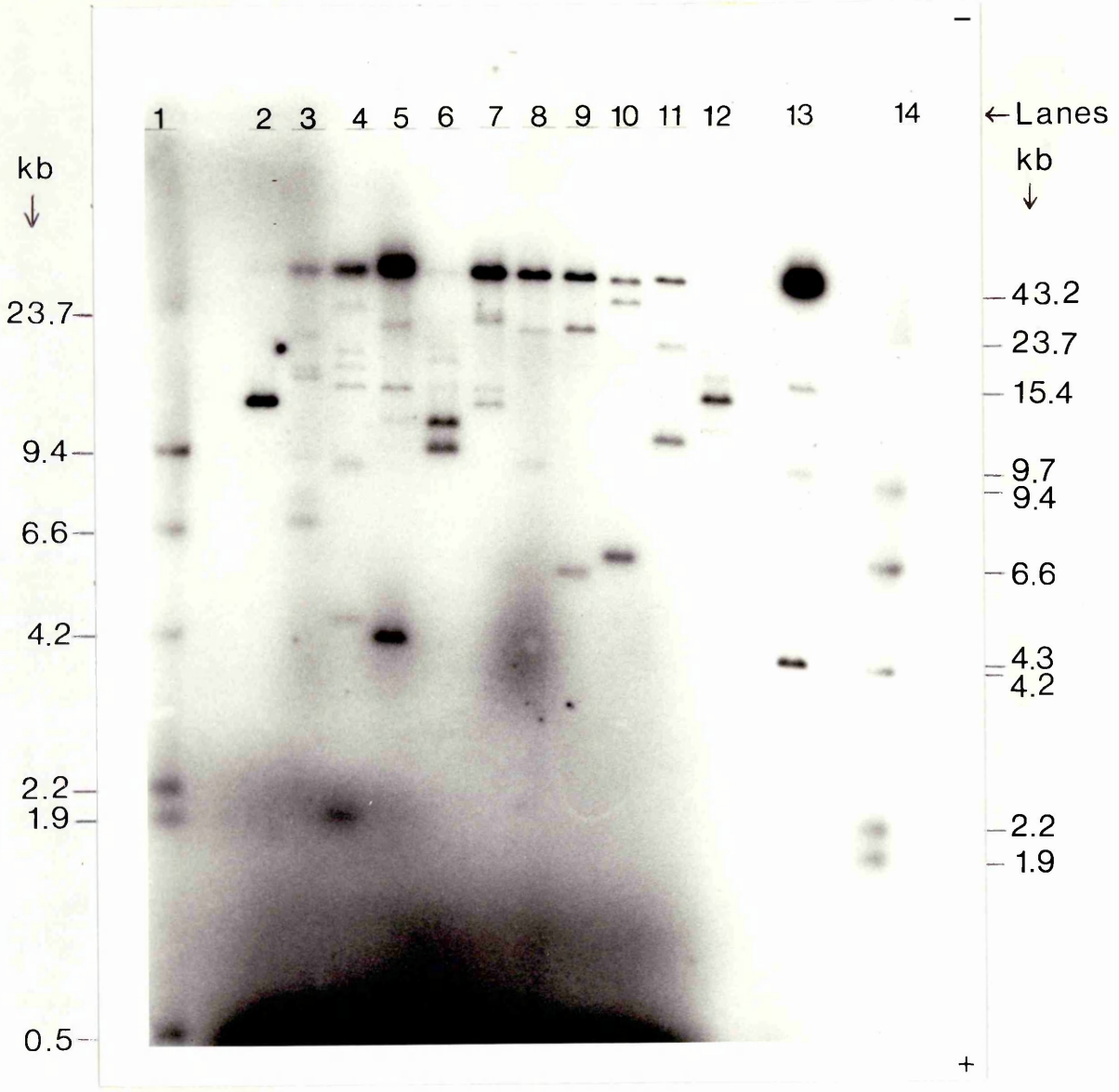
As a result of the evidence from partial digestion and single

Figure 3.10

Partial restriction digestion analysis of clone λHr02

Aliquots of λHr02 DNA (0.75μg) which had been partially digested with the following restriction enzymes : Bam HI (lane 2), Bgl II (lane 3), Cla I (lane 4), Hind III (lane 5), Eco RI (lane 6), Kpn I (lane 7), Sal I (lane 8), Sma I (lane 9), Sph I (lane 10), Sst I (lane 11) and Xba I (lane 12), were hybridised to ^{32}P-labelled ON-R (200,000 Cherenkov c.p.m.) and subjected to electrophoresis, in parallel with a ^{32}P-labelled Hind III digest of bacteriophage lambda (lanes 1 and 14) and a Hind III digest of EMBL3 DNA hybridised to ^{32}P-labelled ON-R (lane 13), on a 0.5% (w/v) agarose gel (Section 2.13). The gel was then dried under vacuum and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiograph is presented and the size of fragments observed, listed in Table 3.7.

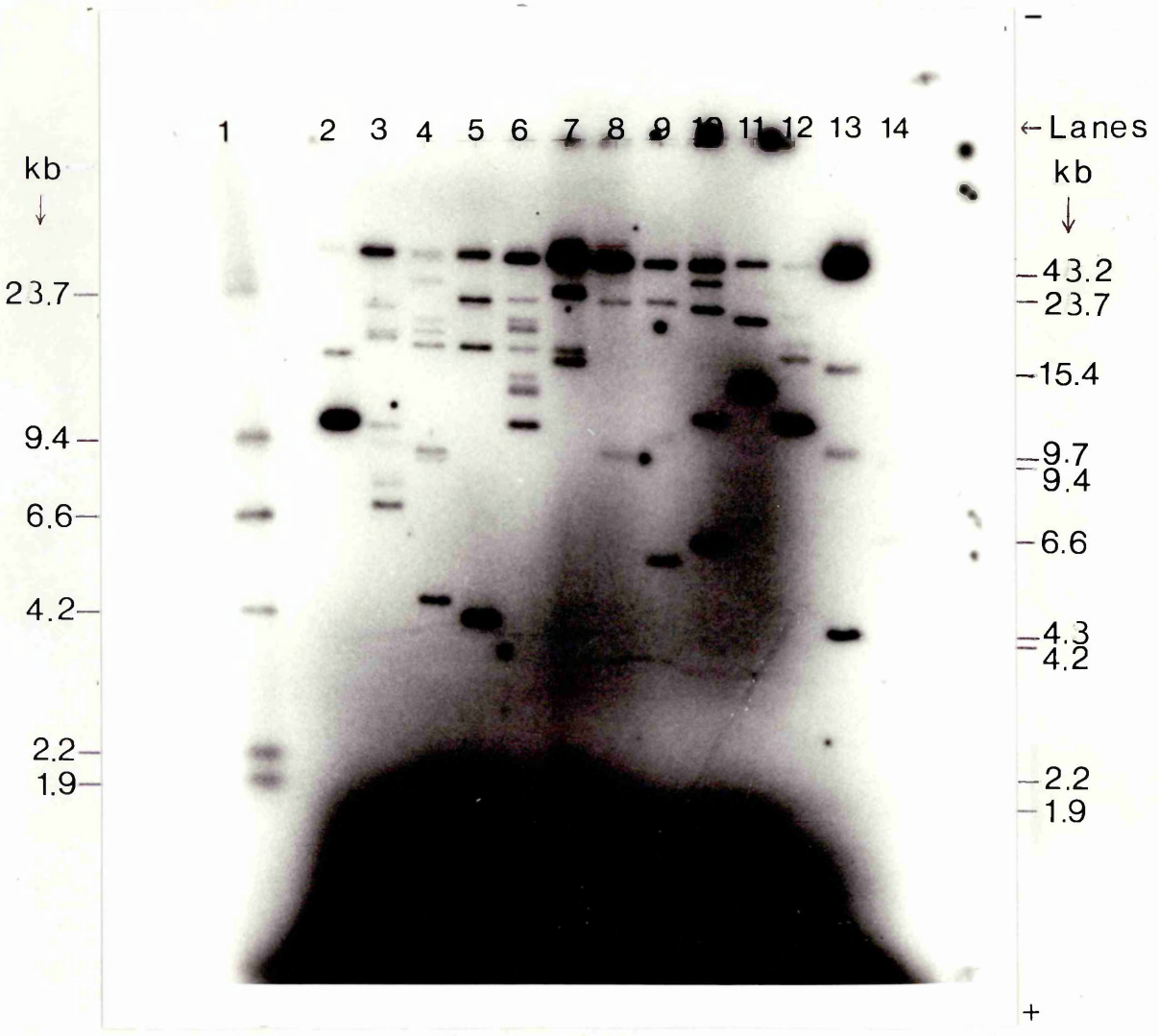
Figure 3.16


1 2 3 4 5 6 7 8 9 10 11 12 13 14
←Lanes
kb
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
43.2
23.7
15.4
9.7
9.4
6.6
4.3
4.2
2.2
1.9
−
+

Partial restriction digestion analysis of clones λHr03

Aliquots of λHr03 DNA (0.75μg) which had been partially digested with the following restriction enzymes : Bam HI (lane 2), Bgl II (lane 3), Cla I (lane 4), Hind III (lane 5), Eco RI (lane 6), Kpn I (lane 7, Sal I (lane 8), Sma I (lane 9), Sph I (lane 10), Sst I (lane 11) and Xba I (lane 12), were hybridised to ^{32}P-labelled ON-R (200,000 Cherenkov c.p.m.) and subjected to electrophoresis, in parallel with a ^{32}P-labelled Hind III digest of bacteriophage lambda (lanes 1 and 14) and a Hind III digest of EMBL3 DNA hybridised to ^{32}P-labelled ON-R (lane 13), on a 0.5% (w/v) agarose gel (Section 2.13). The gel was then dried under vacuum and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiograph is presented and the size of fragments observed, listed in Table 3.8.

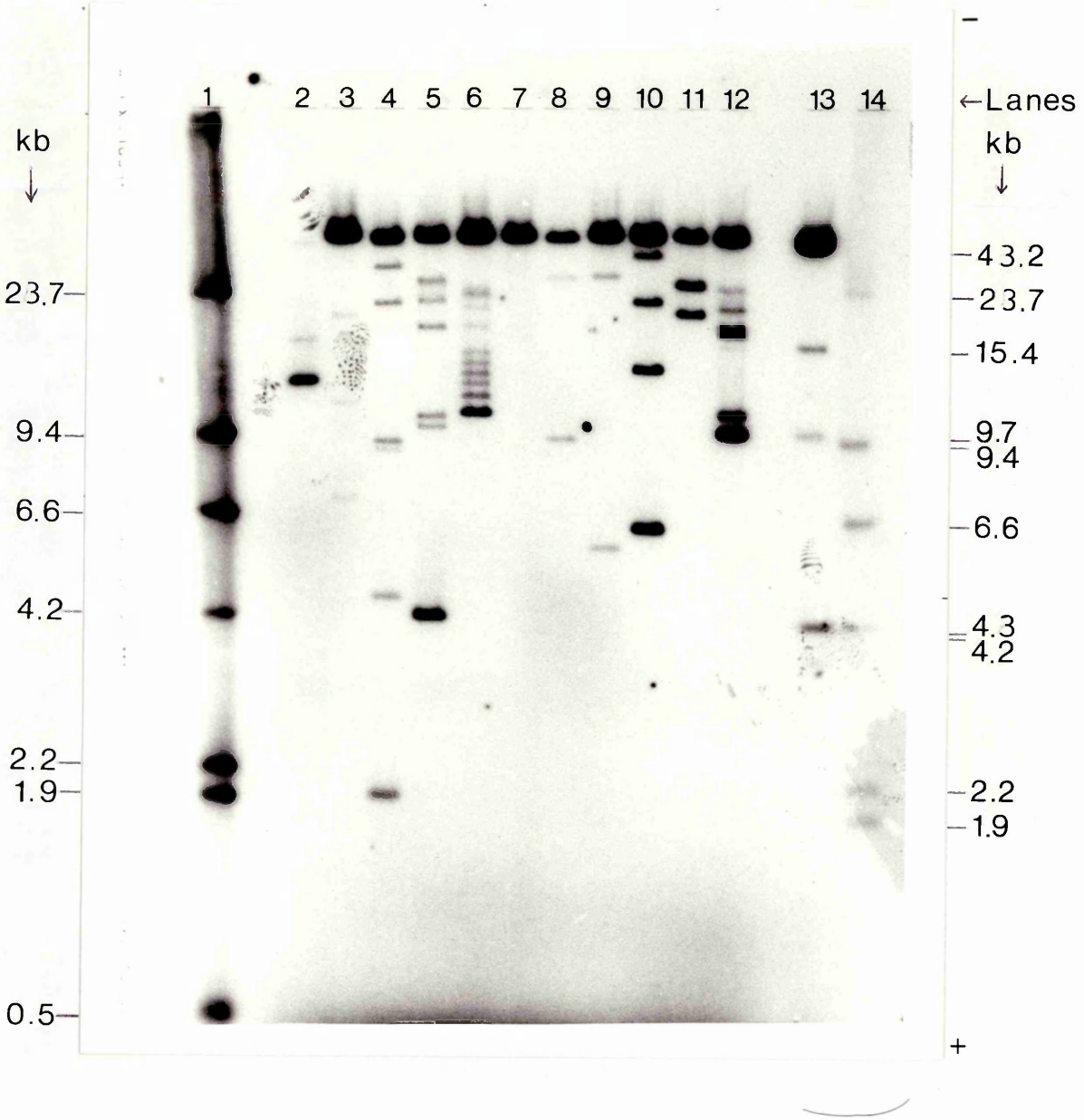
Figure 3.17


1 2 3 4 5 6 7 8 9 11 12 13 14
← Lanes
kb
23.7
9.4
6.6
4.2
2.2
1.9
kb
43.2
23.7
15.4
9.7
9.4
6.6
4.3
4.2
2.2
1.9
−
+

Partial restriction digestion analysis of clone λHr05

Aliquots of λHr05 DNA (0.75μg) which had been partially digested with the following restriction enzymes : Bam HI (lane 2), Bgl II (lane 3), Cla I (lane 4), Hind III (lane 5), Eco RI (lane 6), Kpn I (lane 7), Sal I (lane 8), Sma I (lane 9), Sph I (lane 10), Sst I (lane 11) and Xba I (lane 12), were hybridised to ^{32}P-labelled ON-R (200,000 Cherenkov c.p.m.) and subjected to electrophoresis, in parallel with a ^{32}P-labelled Hind III digest of bacteriophage lambda (lanes 1 and 14) and a Hind III digest of EMBL 3 DNA hybridised to ^{32}P-labelled ON-R (lane 13), on a 0.5% (w/v) agarose gel (Section 2.13). The gel was then dried under vacuum and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiograph is presented and the size of fragments observed, listed in Table 3.9.

Figure 3.18


1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
−
←Lanes
kb
43.2
23.7
15.4
9.7
9.4
6.6
4.3
4.2
2.2
1.9
+

Partial restriction digestion analysis of clone λHr06

Aliquots of λHr06 DNA (0.75μg) which had been partially digested with the following restriction enzymes : Bam HI (lane 2), Bgl II (lane 3), Cla I (lane 4), Hind III (lane 5), Eco RI (lane 6), Kpn I (lane 7), Sal I (lane 8), Sma I (lane 9), Sph I (lane 10), Sst I (lane 11) and Xba I (lane 12), were hybridised to ^{32}P-labelled ON-R (200,000 Cherenkov c.p.m.) and subjected to electrophoresis, in parallel with a ^{32}P-labelled Hind III digest of bacteriophage lambda (lanes 1 and 13) and a Hind III digest of EMBL3 DNA hybridised to ^{32}P-labelled ON-R, (lane 14), on a 0.5% (w/v) agarose gel (Section 2.13). The gel was then dried under vacuum and subjected to autoradiography for 18 hours at -70°C. The resultant autoradiograph is presented and the size of fragments observed, listed in Table 3.10.

Figure 3.19

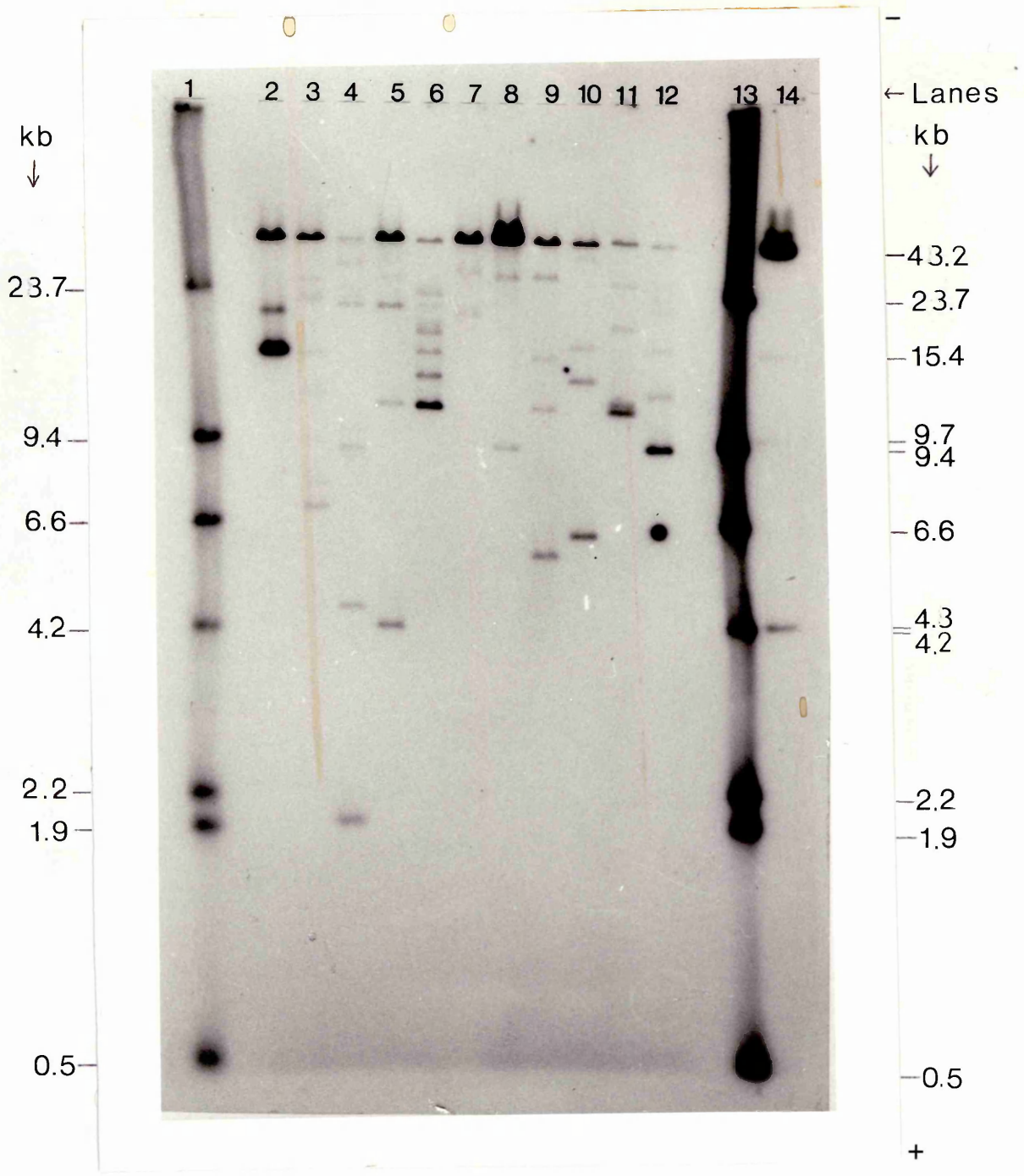

1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
−
←Lanes
kb
43.2
23.7
15.4
9.7
9.4
6.6
4.3
4.2
2.2
1.9
0.5
+

Table 3.7

Partial restriction digestion products of λHr02 which hybridised to ON-R

The size of the partial digestion products of λHr02 which hybridised to oligodeoxynucleotide ON-R (shown in Figure 3.16) are presented.

Table 3.7

Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I	Sal I	Sma I	Sph I	Sst I	Xba I
38	38	38	38	38	38	38	38	38	38	38
								37		
		34								
					33					
					30					
				29						
							28			
						26				
	25									25
									24	
			23							23
		21.5								
			19							
	18	18								
										17.5
	15.5									
		15	15	15	15					
										14.5
					14					
13.5										
			12.5							
										12
									11.5	
			11							
	10.5	10.5				10.5				
	9.8									
	9.0									
								7.2		
							6.8			
		5.4								
				5						
		2.3								

Table 3.8

Partial restriction digestion products of λHr03 which hybridised to ON-R

The size of the partial digestion products of λHr03 which hybridised to oligodeoxynucleotide ON-R (shown in Figure 3.17) are presented.

Table 3.8

Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I	Sal I	Sma I	Sph I	Sst I	Xba I
48	48	48	48	48	48	48	48	48	48	48
								37		
		33								
					32					
					30					
							28			
						27.5		27.5		
				26.5						
			26							26
				25						
	24									
									22	
		21	21							
			20							
	19.5	19.5								
										18.25
	18									
		17.5	17.5	17.5	17.5					
										17
					16.5					
16										
			14.5							
									14	
			13.5							
								12		
11.5			11.5				11.5			11.5
	11									
	10.5									
		10				10				
		9.6								
	9									
	8.6									
								6.8		
	6.6									
							6.2			
		4.8								
				4.6						
		2								

Table 3.9

Partial restriction digestion products of λHr05 which hybridised to ON-R

The size of the partial digestion products of λHr05 which hybridised to oligodeoxynucleotide ON-R (shown in Figure 3.18) are presented.

Table 3.9

Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I	Sal I	Sma I	Sph I	Sst I	Xba I
38	38	38	38	38	38	38	38	38	38	38
								37		
		33.5								
					32					
							30			
						29				
									28	
			27	27						
			26							
					25					25
				24						
								22.5		
		22	22		22					22
					20.5				20.5	
	20									
			19		19					19
				18.5						
18										18
					17					17
			16							
			14.5							
			14					14		
13.5										
			13							
			12.5							
	12									
			11.5	11.5						11.5
	11									
	10.5			10.5						10.5
		10				10				
		10.6								
	8.9									
	8.4									
	7.8									
								6.6		
							6			
		4.9								
				4.4						
		1.9								

Table 3.10

Partial restriction digestion products of λHr06 which hybridised to ON-R

The size of the partial digestion products of λHr06 which hybridised to oligodeoxynucleotide ON-R (shown in Figure 3.19) are presented.

Table 3.10

Bam HI	Bgl II	Cla I	Eco RI	Hind III	Kpn I	Sal I	Sma I	Sph I	Sst I	Xba I
58	58	58	58	58	58	58	58	58	58	58
								42		
		38								
					33					
				30.5		30.5	30.5			
	30									30
									29	
			28							
		27								27
			25							
	23	23								
										22.5
	22									
		21.5	21.5	21.5	21.5					21.5
					21					
20.5										
			19							
									18.5	
			18							
								17.5		17.5
16			16							
	15.5									
							15			
			13.75							
								13.5		
	12.5									12.5
				12					12	
							11.75			
									11.5	
			10.75							
		10.5				10.5				
	10									
		9.9								
		9.4								
	8.6									
	8.2									
	7.3									
								6.4		
							5.9			
		4.6								
				4.2						
		2								

digest/Southern blot analysis, a re-examination of the single digests was performed to establish whether clones λHr02, λHr03 and λHr06 did contain identical fragments. Complete digests were subjected to electrophoresis on 0.7% (w/v) agarose gels, this time grouping the digests for a particular enzyme in parallel (Figure 3.20). Additional digests of λHr05 (Figure 3.21) and double digests of λHr02, λHr03 and λHr06 were also carried out to aid mapping the distance between the sites more accurately.

Excluding the fragments produced by the phage arms (Figure 2.1), it was seen that clones λHr02, λHr03 and λHr06 do possess identical fragments e.g. the 8.3 kb Sst I fragment, 6.5 kb Hind III fragment, 1.3 kb Bgl II fragment and 1.7 kb Cla I fragment. These results strongly indicate that these clones have a large region in common with identical restriction sites for the enzymes used. It was concluded that these were most probably overlapping clones derived from the same region of genomic DNA, while recognising that the formal possibility remained of the clones being derived from different copies of a repeated region. λHr05 does not possess fragments identical to those described for λHr02, λHr03 and λHr06 and appears to have a different restriction map.

Pooling the data from single, double and partial digestion mapping the following restriction maps were constructed (Figure 3.22). The region of pHS2 hybridisation is indicated on each map.

3.2.4 Comparison of genomic organisation of sequences homologous to pHS2 with restriction maps of the genomic clones

The genomic organisation of sequences homologous to the cDNA insert of pHS2 was examined by Southern blot/hybridisation analysis of Bam HI, Eco RI, Hind III and Sst I digests of HeLa DNA using the cDNA insert of pHS2 as probe. The resulting autoradiograph (Figure 3.23) indicated that the sequence homologous to pHS2 was located on

Figure 3.20

Additional single and double restriction digests of the genomic clones

Additional single and double digests (some of which are shown) of the genomic clones were subjected to electrophoresis on a 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Section 2.5.2.a and 2.11).

a. Hind III digest of bacteriophage lambda (lane 1), Bgl II digests of λHr02, λHr03, λHr05 and λHr06 (lanes 2, 3, 4 and 5 respectively), Cla I digests of λHr02, λHr03, λHr05 and λHr06 (lanes 6, 7, 8 and 9) Hind III digests of λHr02, λHr03, λHr05 and λHr06 (lanes 10, 11, 12 and 13) and a Hind III/Eco RI digest of bacteriophage lambda (lane 14).

b. Kpn I digests of λHr02, λHr03, λHr05 and λHr06 (lanes 2, 3, 4 and 5), Sst I digest of λHr02, λHr03, λHr05 and λHr06 (lanes 6, 7, 8 and 9), a Hind III/Eco RI digest of bacteriophage lambda (lane 10) and a Hind III digest of bacteriophage lambda (lane 1).

Figure 3.20

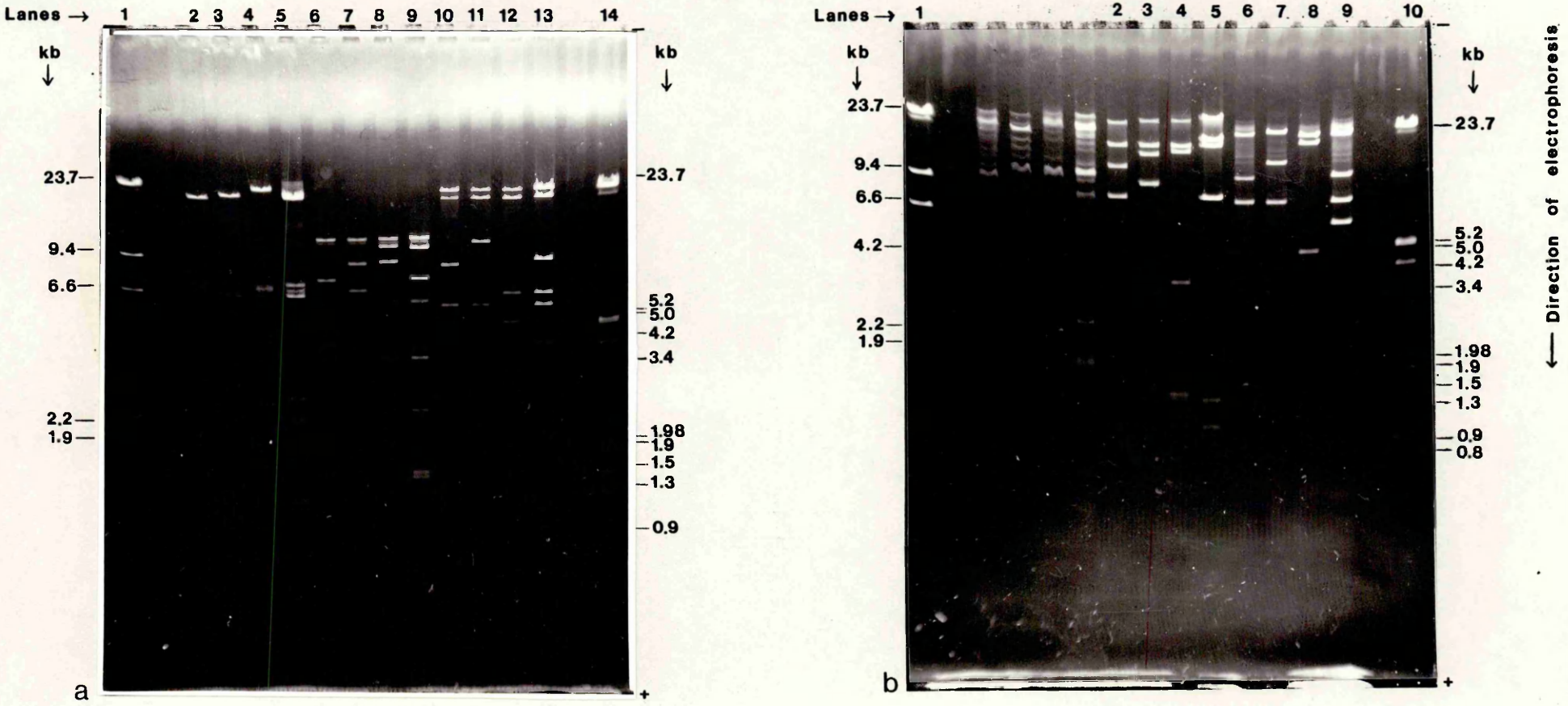

Lanes → 1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.7
9.4
6.6
2.2
1.9
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
0.9
a
Lanes → 1 2 3 4 5 6 7 8 9 10
kb
23.7
9.4
6.6
4.2
2.2
1.9
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
0.9
0.8
b
Direction of electrophoresis

Figure 3.21

Additional restriction digests of λHr05

Aliquots of λHr05 DNA (1μg) were cleaved with the following restriction enzymes : Bgl II (lane 2), Bgl II/Sph I (lane 3), Cla I (lane 4), Cla I/Sst I (lane 5), Hind III (lane 6), Hind III/Bam HI (lane 7), Sst I/Sph I (lane 8) and Sph I (lane 9), and subjected to electrophoresis, in parallel with a Hind III digest (lane 1) and Hind III/Eco RI digest (lane 10) of bacteriophage lambda, on a 0.7% (w/v) agarose gel (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The stained photograph is shown. The size of the fragments generated were calculated and used to map λHr05 more accurately.

Figure 3.21

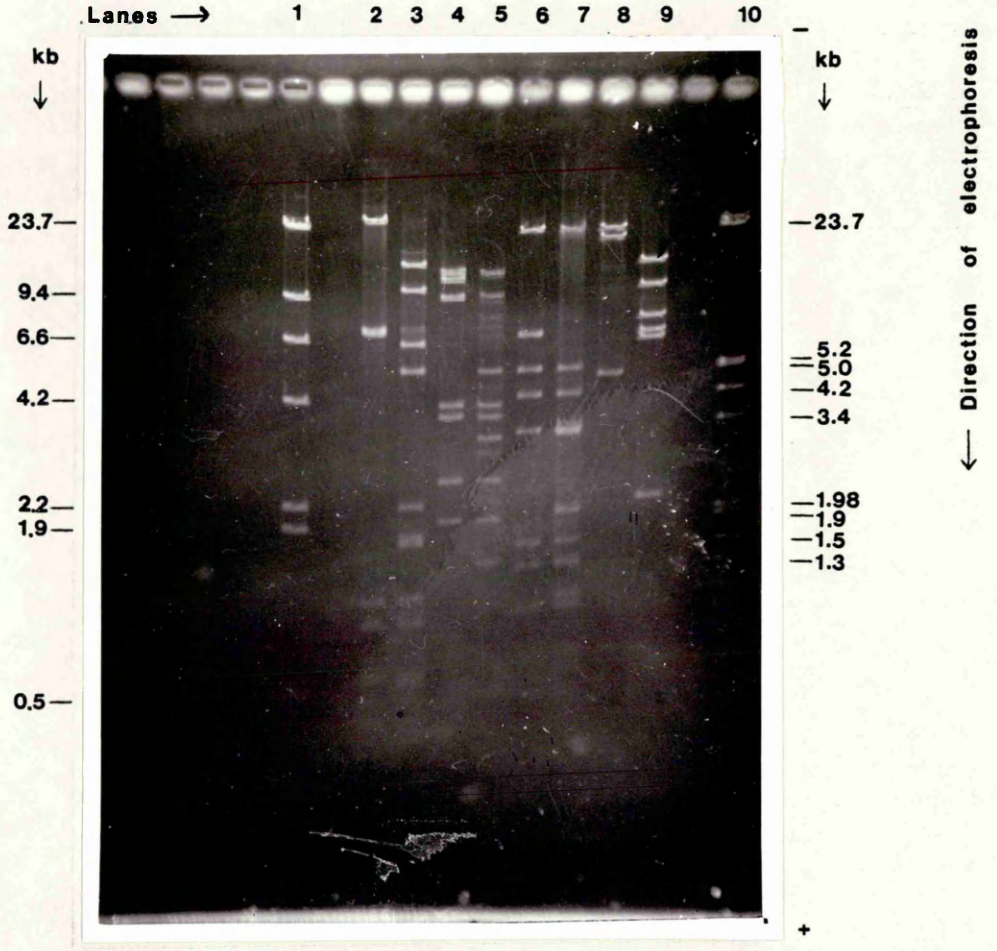

Lanes ⟶
1
2
3
4
5
6
7
8
9
10
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
kb
23.7
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
Direction of electrophoresis

Figure 3.22

Restriction endonuclease maps of λHr02, λHr03, λHr05 and λHr06 insert DNA

Final restriction endonuclease maps of the genomic clones λHr02, λHr03, λHr05 and λHr06 constructed from single, double and partial digestion data (see Section 3.2.3 for details).

Key :

B : Bam HI

C : Cla I

G : Bgl II

H : Hind III

K : Kpn I

Sp : Sph I

Ss : Sst I

Scale : 1 cm = 1 kb

pHS2 region of hybridisation : □

Figure 3.22

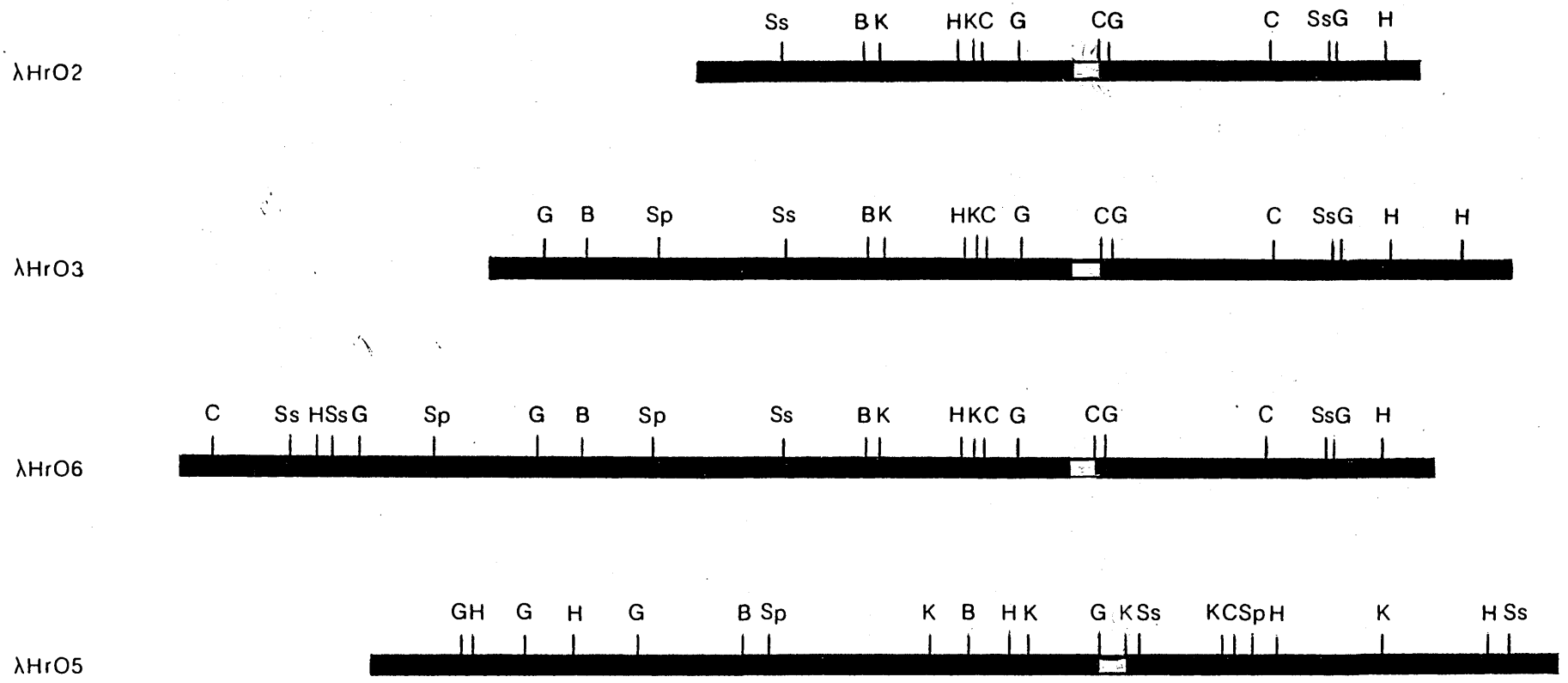
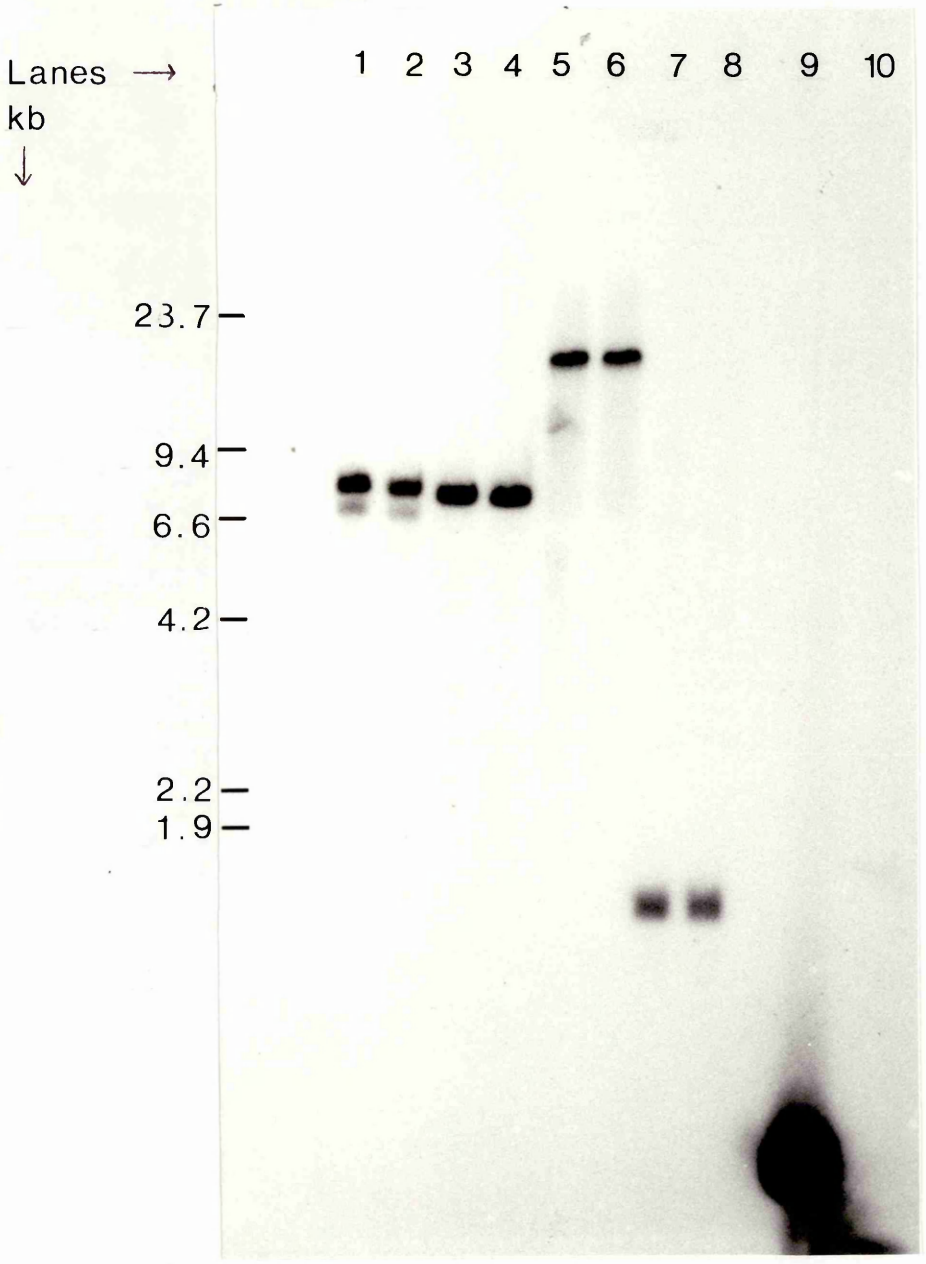

λHrO2
Ss
B K
HKC
G
CG
C
SsG
H
λHrO3
G
B
Sp
Ss
BK
HKC
G
CG
C
SsG
H
H
λHrO6
C
Ss
HSs
G
Sp
G
B
Sp
Ss
BK
HKC
G
CG
C
SsG
H
λHrO5
GH
G
H
G
B
Sp
K
B
HK
G
K
Ss
KCSp
H
K
H
Ss

Figure 3.23

Southern blot analysis of HeLa genomic DNA probed with pHS2 cDNA insert

Aliquots of HeLa DNA (20μg)(Section 2.25) were cleaved with the following restriction enzymes : Bam HI (lanes 1 and 2), Eco RI (lanes 3 and 4), Hind III (lanes 5 and 6) and Sst I (lanes 7 and 8)(Section 2.10.1). These digests were subjected to electrophoresis on a 0.5% (w/v) agarose gel (TEA electrophoresis buffer : Sections 2.5.4.a and 2.11) in parallel with a Hind III digest of bacteriophage lambda (lane 10) and pHS2 cDNA insert (lane 9). The gel was then Southern blotted (Section 2.17.1) and the filter hybridised to nick-translated pHS2 cDNA insert (10^7 Cherenkov c.p.m.)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filter was washed (Section 2.17.2.a) and subjected to autoradiography at -70°C for 18 hours. The resultant autoradiograph is shown.

Figure 3.23



7.8 kb and 7 kb Bam HI fragments (although the former may be a partial digest of the latter), a 7.3 kb Eco RI fragment, a 17 kb Hind III fragment and a 1.4 kb Sst I fragment, which were quite different to the values obtained for clones λHr02, λHr03, λHr05 and λHr06 (see Table 3.6). The high intensity and low background of the hybridised fragments in the genomic DNA also suggested that the region of DNA containing the pHS2 sequence might be present in multiple copies, again contrasting with the number of genomic clones isolated.

These results were also very different from those obtained by Southern blot analysis of HeLa DNA using, as probe, Drosophila heat shock clone 56H8 (Moran et. al., 1979), which contains a single copy of a Drosophila hsp 70 gene. Hybridisation with 56H8 indicated that heat shock sequences were present on 7.3 kb, 5.6 kb and 3.6 kb Bam HI fragments; 4.5 kb, 3.6 kb and 3.0 kb Eco RI fragments; 6.2 kb, 3.9 kb, 2.9 kb and 2.1 kb Hind III fragments and a 1.1 kb Pst I fragment (work done in collaboration with A. Paton. Figure 3.24) and the intensity of hybridisation indicated that they were present in fewer copies than the fragments hybridising to clone pHS2 (Figure 3.23).

It was therefore decided to determine the nucleotide sequence of the insert in pHS2.

3.3 Determination of nucleotide sequence of cDNA clone pHS2.

3.3.1 Subcloning strategy for pHS2

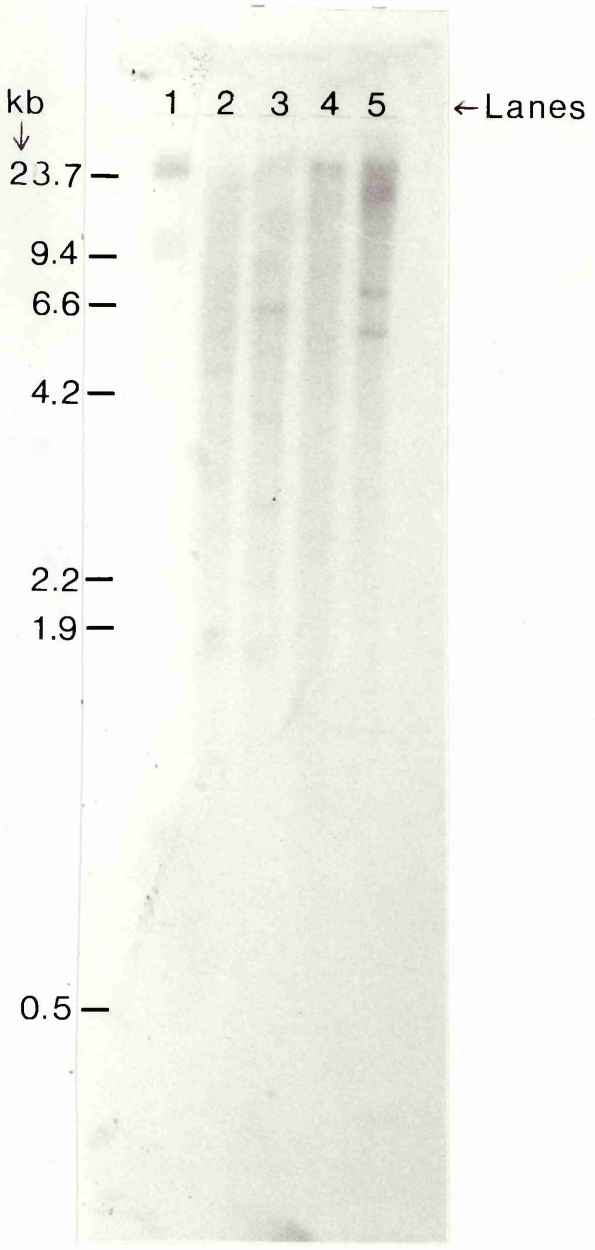
The cDNA insert was removed from pHS2 by restriction digestion with Pst I followed by electrophoresis and electroelution (Sections 2.10, 2.11 and 2.14). It was transferred into the Pst I site of vector pUC18 to facilitate sequencing by the method of Maxam and Gilbert, (1980). This DNA was transformed into JM109 cells and positive clones selected on amp./X-gal./IPTG/ampicillin plates. DNA from each subclone was subjected to electrophoresis on a 1% (w/v) agarose

Figure 3.24

Southern blot analysis of HeLa genomic DNA probed with *Drosophila* probe 56H8

Aliquots of HeLa DNA (20μg)(Section 2.25) were cleaved with the following restriction enzymes: *Eco* RI (lane 2), *Hind* III (lane 3), *Pst* I (lane 4) and *Bam* HI (lane 5)(Section 2.10.1). These digests were subjected to electrophoresis on a 0.5% (w/v) agarose gel (TEA electrophoresis buffer : Sections 2.5.4.a and 2.11) in parallel with a *Hind* III digest of bacteriophage lambda (lane 1). The gel was then Southern blotted (Section 2.17.1) and the filter hybridised to nick-translated 56H8 cDNA insert (10^7 Cherenkov c.p.m.)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation at 65°C for 18 hours the filter was washed (Section 2.17.2.a) and subjected to auto-radiography at -70°C for 18 hours. The resultant autoradiograph is shown.

Figure 3.24



gel, transferred to nitrocellulose and subclones selected by hybridising the filter to nick-translated pHS2 cDNA insert. The subclone isolated, pA12 (Figure 3.25), was subjected to restriction digestion with Pst I to check that the insert size corresponded to that in pHS2 (Figure 3.25).

3.3.2 Sequence determination

A large scale preparation of this subcloned DNA was performed and the sequence of the insert determined by the chemical method of Maxam and Gilbert, (1980). The strategy used to obtain the sequence is shown in Figure 3.26 and the resulting sequence presented in Figure 3.27. A computer search using the program WORDSEARCH was done using initial sequence data to find out if pHS2 was homologous to any known sequence in the nucleotide sequence databank, Genbank, and the result is shown in Figure 3.28. Clone pHS2 showed only five mismatches from the 324 nucleotide segment of rat 28S rRNA extending over nucleotide 3355-3678. After careful examination of the sequencing gels at the points of mismatch and redetermination, the final sequence (which is the one actually presented in Figure 3.27) had only one mismatch at position 245. In contrast, direct comparison with Drosophila (and later human) hsp 70 genes revealed no significant homology.

It was thus clear that pHS2 contained a cDNA copy of part of human 28S rDNA (see Discussion) and this implied that the genomic clones contained rDNA sequences. However three factors argued strongly that the genomic clones did not correspond to the human tandem rDNA repeat. These are considered in more detail in the Discussion but in brief were: 1. different restriction maps from human rDNA repeat, 2. different restriction fragments hybridising to pHS2 than the reported fragments for genomic DNA, and 3. the frequency of occurrence in the genomic library inconsistent with expected number

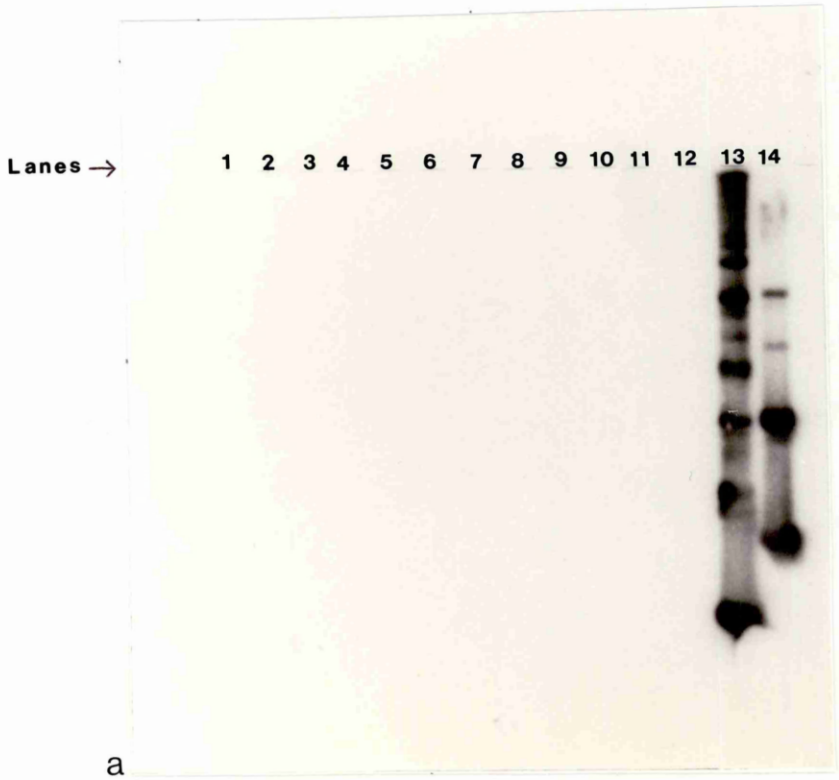
Figure 3.25

Analysis of pUC18 subclones containing pHS2 cDNA insert

a. pHS2 DNA digested with Pst I was subcloned into the Pst I site of pUC18. Subcloned DNA was transformed into JM109 cells and positive subclones selected on Amp./X-gal./IPTG plates (Section 2.4.7). Plasmid DNA preparation (50 mls) was carried out on each positive colony (Section 2.21.1). Aliquots of each positive subclone DNA (1μg) were subjected to electrophoresis (lanes 2-13 are subclones 1-12 respectively), in parallel with a Hind III digest of bacteriophage lambda (lane 1), and pHS2 DNA (lane 14), on a 1% (w/v) agarose gel (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The gel was Southern blotted (Section 2.17.1) and the filter hybridised to pHS2 cDNA insert (10^6 Cherenkov c.p.m.) (Sections 2.14, 2.15 and 2.17.2.a). After 18 hours hybridisation at 65°C, the filter was washed (Section 2.17.2.a) and subjected to autoradiography at -70°C for 18 hours. The resultant autoradiograph is shown.

b. The positive subclone isolated, pA12 DNA, was digested with Pst I and the insert isolated (Section 2.15). The insert (lane 1) was subjected to electrophoresis in parallel with a Hae III digest of pAT153 DNA (lane 2) on a 4% (w/v) polyacrylamide gel. The stained gel is shown.

Figure 3.25



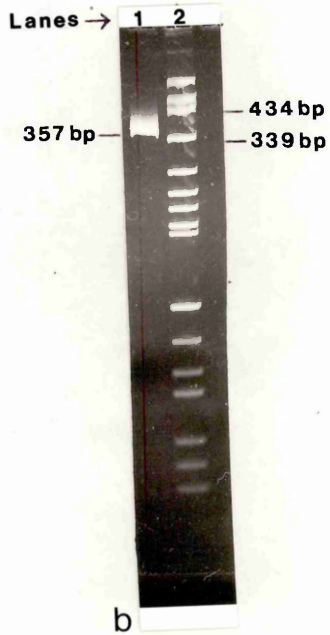


Figure 3.26

Restriction map of pHS2 cDNA insert and sequencing strategy

The precise positions of the restriction sites shown were determined by sequencing. Arrows show the direction of sequence determination. Arrows above the dotted line indicate the upper DNA strand and those below the lower. The length of each arrow corresponds to the number of nucleotides sequenced.

Figure 3.26

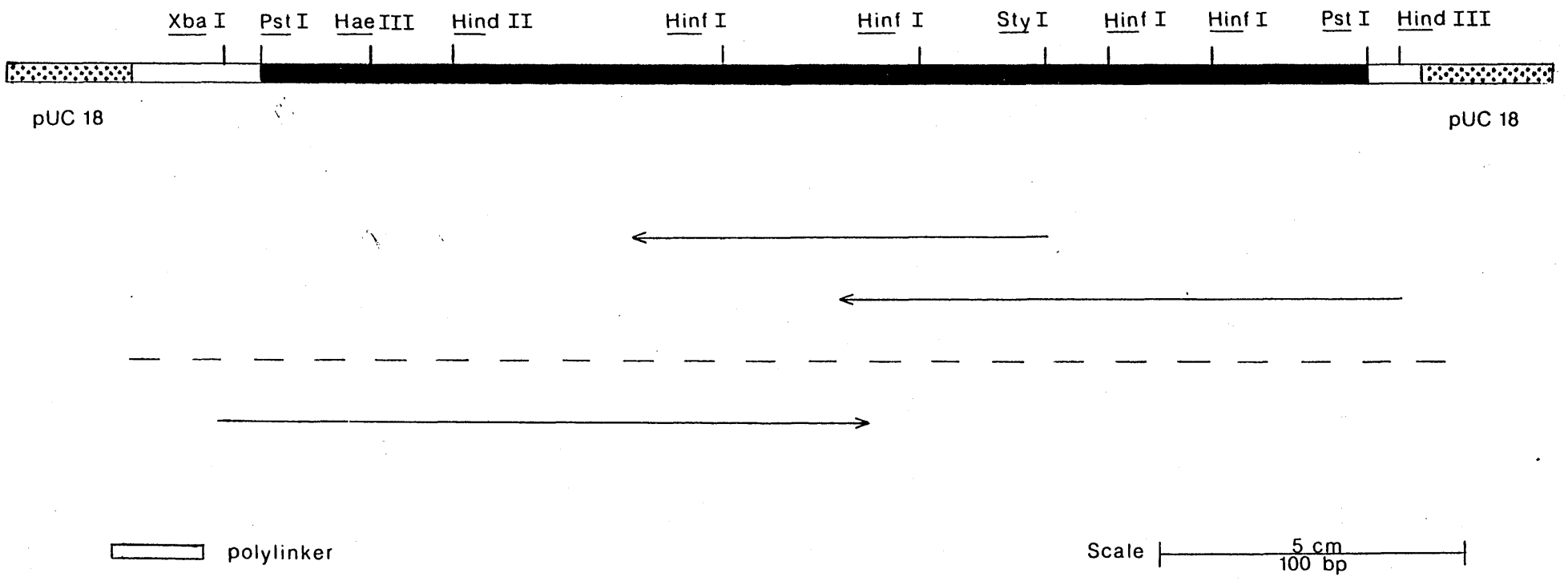
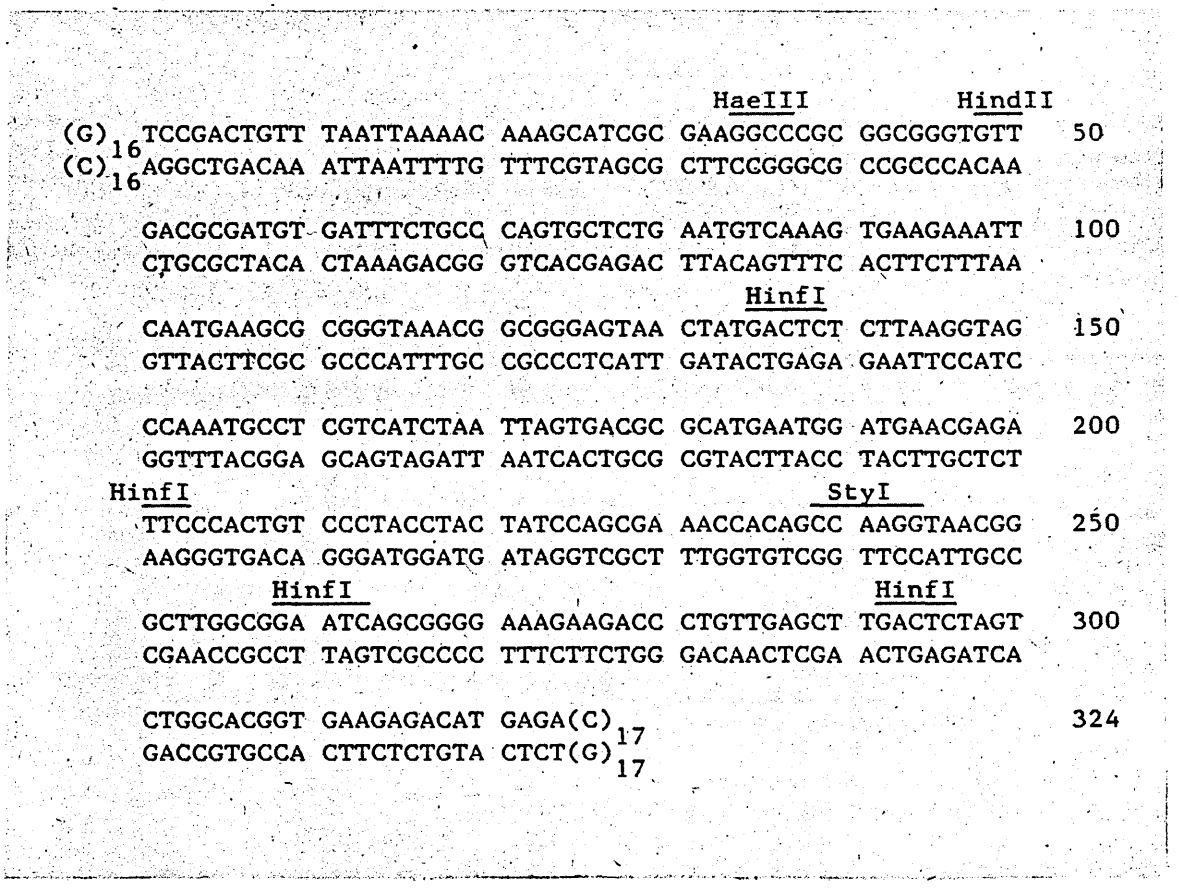

Xba I
Pst I
Hae III
Hind II
Hinf I
Hinf I
Sty I
Hinf I
Hinf I
Pst I
Hind III
pUC 18
pUC 18
polylinker
Scale
5 cm
100 bp

Figure 3.27

Nucleotide sequence of pHS2 cDNA insert

The nucleotide sequence of the cDNA insert of pHS2 (Cato et. al., 1981) was determined by the method of Maxam and Gilbert, (1980) (Section 2.22). The final DNA sequence is presented.

Figure 3.27

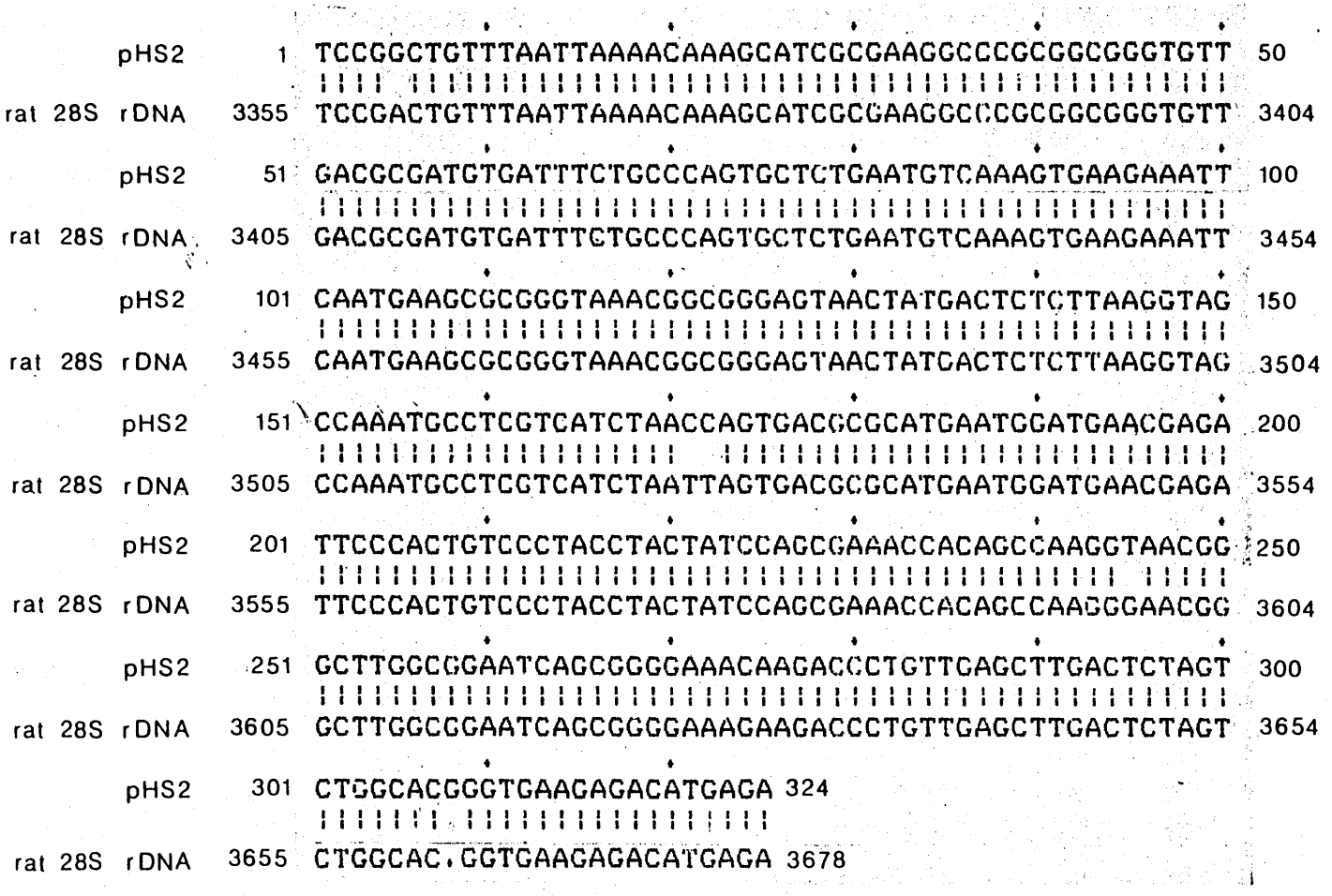

HaeIII
HindII
(G)16 TCCGACTGTT TAATTAAAAC AAAGCATCGC GAAGGCCCGC GGCGGGTGTT 50
(C)16 AGGCTGACAA ATTAATTTTG TTTCGTAGCG CTTCCGGGCG CCGCCCACAA
GACGCGATGT GATTTCTGCC CAGTGCTCTG AATGTCAAAG TGAAGAAATT 100
CTGCGCTACA CTAAAGACGG GTCACGAGAC TTACAGTTTC ACTTCTTTAA
HinfI
CAATGAAGCG CGGGTAAACG GCGGGAGTAA CTATGACTCT CTTAAGGTAG 150
GTTACTTCGC GCCCATTTGC CGCCCTCATT GATACTGAGA GAATTCCATC
CCAAATGCCT CGTCATCTAA TTAGTGACGC GCATGAATGG ATGAACGAGA 200
GGTTTACGGA GCAGTAGATT AATCACTGCG CGTACTTACC TACTTGCTCT
HinfI
StyI
TTCCCACTGT CCCTACCTAC TATCCAGCGA AACCACAGCC AAGGTAACGG 250
AAGGGTGACA GGGATGGATG ATAGGTCGCT TTGGTGTCGG TTCCATTGCC
HinfI
HinfI
GCTTGGCGGA ATCAGCGGGG AAAGAAGACC CTGTTGAGCT TGACTCTAGT 300
CGAACCGCCT TAGTCGCCCC TTTCTTCTGG GACAACTCGA ACTGAGATCA
CTGGCACGGT GAAGAGACAT GAGA(C)17 324
GACCGTGCCA CTTCTCTGTA CTCT(G)17

Result of WORDSEARCH of pHS2 cDNA insert against sequences in Genbank

The nucleotide sequence of the cDNA insert of pHS2 (initial data) was subjected to a WORDSEARCH against sequences in the Genbank database. The best match found is shown. It is homologous to a portion of rat 28S rDNA (sequence and numbering from Chan et. al., 1983). Vertical lines indicate identity.

The sequence shown here was subsequently revised in the light of additional data the final corrected sequence is shown in Fig. 3.27.

Figure 3.28


pHS2 1 TCCGGCTGTTTAATTAAAACAAAGCATCGCGAAGGCCCGCGGCGGGTGTT 50
rat 28S rDNA 3355 TCCGACTGTTTAATTAAAACAAAGCATCGCGAAGGCCCGCGGCGGGTGTT 3404
pHS2 51 GACGCGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAAGTGAAGAAATT 100
rat 28S rDNA 3405 GACGCGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAAGTGAAGAAATT 3454
pHS2 101 CAATGAAGCGCGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAG 150
rat 28S rDNA 3455 CAATGAAGCGCGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAG 3504
pHS2 151 CCAAATGCCTCGTCATCTAACCAGTGACGCGCATGAATGGATGAACGAGA 200
rat 28S rDNA 3505 CCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATGAACGAGA 3554
pHS2 201 TTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGTAACGG 250
rat 28S rDNA 3555 TTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGGAACGG 3604
pHS2 251 GCTTGGCGGAATCAGCGGGGAAACAAGACCCTGTTGAGCTTGACTCTAGT 300
rat 28S rDNA 3605 GCTTGGCGGAATCAGCGGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGT 3654
pHS2 301 CTGGCACGGGTGAAGAGACATGAGA 324
rat 28S rDNA 3655 CTGGCAC.GGTGAAGAGACATGAGA 3678

of rDNA repeats. As a result of this it was felt to be of some considerable interest to continue the analysis of these genomic clones and the remaining part of the Results section of this thesis describes this analysis.

3.4 Further analysis of the genomic clones

3.4.1 Selection of subclones

As the genomic clones λHr02, λHr03, λHr05 and λHr06 did not correspond to the rDNA repeat (see 3.3.2 and Discussion) an investigation of the region hybridising to pHS2 and determination of the extent of 28S rDNA sequence within these clones was performed. Subclones were constructed to aid this study.

Using the restriction maps (Figure 3.22) suitable enzymes were chosen which produced small fragments containing the pHS2 region of hybridisation. Bgl II and Cla I digests of λHr02 DNA and a Kpn I digest of λHr05 were subcloned into the vector pUC18, transformed into JM109 cells and positive subclones selected on amp./X-gal./IPTG plates. DNA from each subclone was subjected to electrophoresis on 1% (w/v) agarose gels, Southern blotted and hybridised to the cDNA insert of pHS2 to select positive subclones. The subclones isolated (Figure 3.29) were mapped by restriction digestion analysis to ensure the insert sizes were correct (Figures 3.30,3.31 and 3.32). Of the three subclones selected, pHr02B (a Bgl II subclone of λHr02), pHr02C (a Cla I subclone of λHr02) and pHr05K (a Kpn I subclone of λHr05), only one showed the correct insert size: pHr02C (1.7 kb). Subclones pHr02B and pHr05K contained inserts of 4.3 kb and 8 kb which were larger than the expected 1.3 kb and 1.5 kb fragments. As pHr02C also contained the expected restriction sites it was thus decided to use this subclone in initial nucleotide sequencing studies.

To determine which area of subclone pHr02C to investigate first,

digest of bacteriophage lambda in lanes 7-9 respectively. The positive subclone pHr05K is in lane 5.

Figure 3.29

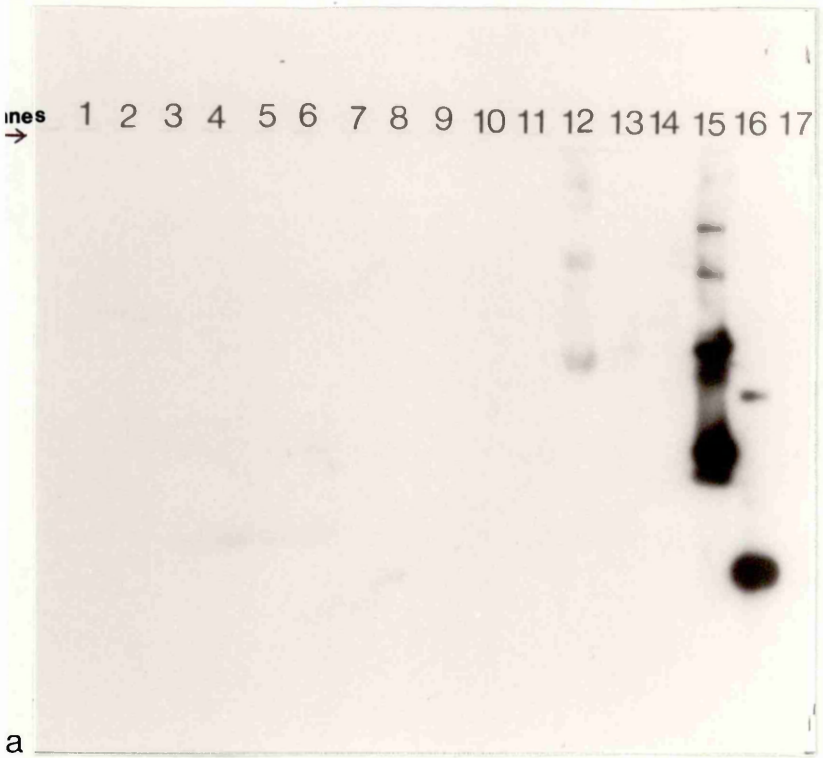
Southern blot analysis of pUC18 subclones containing DNA from genomic clones λHr02 and λHr05 using pHS2 cDNA insert as probe

a. λHr02 DNA digested with Bgl II was subcloned into the Bam HI site of pUC18. Subcloned DNA was transformed into JM109 cells and positive subclones selected (Section 2.19). Plasmid DNA preparation (50 mls) was carried out on each positive colony (Section 2.21.1). Aliquots of each positive subclone DNA (1μg) were subjected to electrophoresis (lanes 2-14 are subclones 1-13 respectively), in parallel with a Hind III digest of bacteriophage lambda (lane 1 & 17), pHS2 DNA (lane 15) and Bgl II digested λHr02 (lane 16), on a 1% (w/v) agarose gel (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The gel was Southern blotted (Section 2.17.1) and the filter hybridised to pHS2 cDNA insert (10^6 Cherenkov c.p.m.)(Sections 2.14, 2.15 and 2.17.2.a). After 18 hours hybridisation at 65°C, the filter was washed (Section 2.17.2.a) and subjected to autoradiography for 18 hours at -70°C. The positive subclone pHr02B is in lane 12.

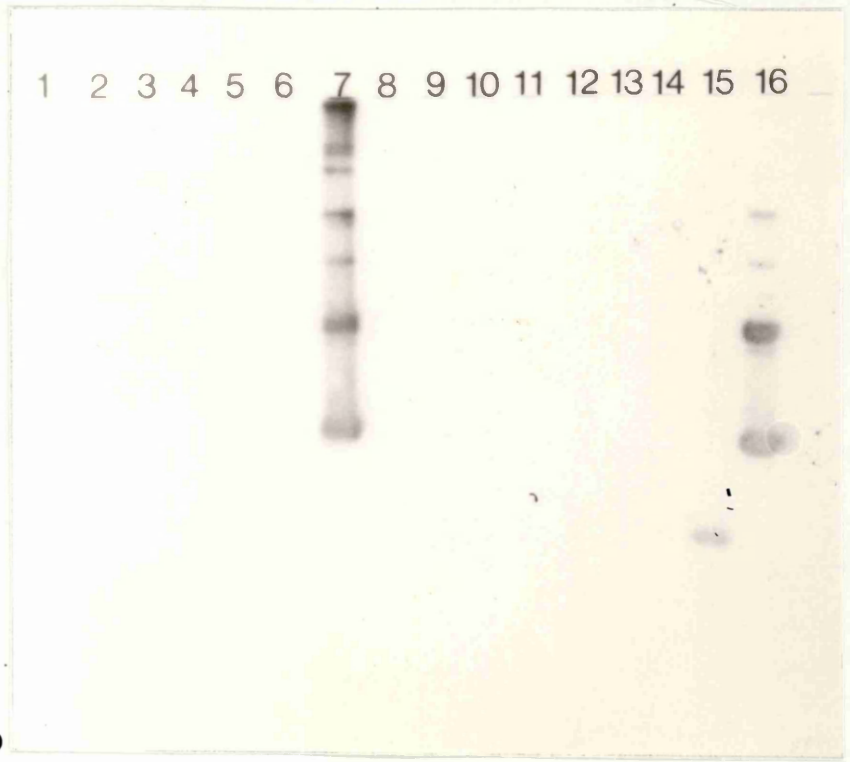
b. λHr02 DNA digested with Cla I was subcloned into the Acc I site of pUC18. Subcloned DNA was transformed and positive colonies selected for further study as described in a. Lanes 2-14 on the autoradiograph correspond to subclones 1-13. Marker DNAs include Hind III digest of bacteriophage lambda (lane 1) λHr02 DNA digested with Cla I (lane 15) and pHS2 DNA (lane 16). The positive subclone pHr02C is in lane 7.

c. λHr05 DNA digested with Kpn I was subcloned into the Kpn I site of pUC18. Subcloned DNA was transformed and positive colonies selected for further study as described in a. Lanes 1-6 on the autoradiograph correspond to subclones 1-6. Marker DNAs include λHr05 digested with Kpn I, pHS2 DNA and a Hind III

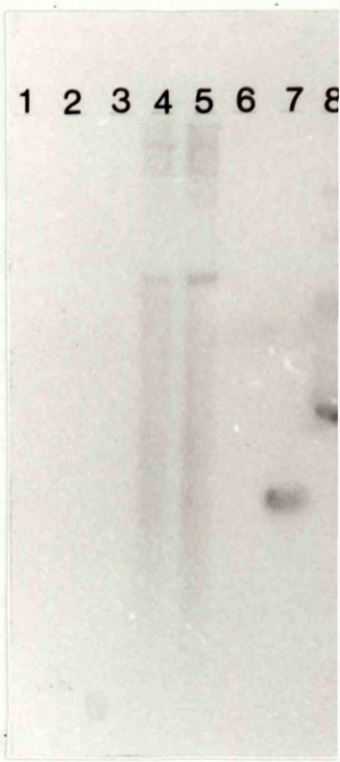
Figure 3.29



a



b



c

Figure 3.30

Single and double restriction digestion analysis of pHr02B

Aliquots of pHr02B DNA (1μg) were cleaved with the following restriction enzymes : Sty I (lane 4), Sty I/Eco RI (lane 5), Eco RI (lane 6), Sty I/Asp 718 (lane 7), Asp 718 (lane 8), Sty I/Sst I (lane 9), Sst I (lane 10), Asp 718/Sph I (lane 11), Sph I (lane 12), Asp 718/Hind III (lane 13), Hind III (lane 14), Xba I (lane 19), Xba I/Sst I (lane 20), Sst I (lane 21), Hind III (lane 22), Hind III/ (lane 22), Hind III/Sst I (lane 23), Eco RI/Hind III (lane 24), Eco RI (lane 25), Pst I (lane 26), Sst I/Pst I (lane 27), Asp 718/ Pst I (lane 28) and Asp 718 (lane 29)(Sections 2.10.1 and 2.10.2). The digests were subjected to electrophoresis, in parallel with a Hind III digest (lane 1) and a Hind III/Eco RI digest (lanes 16 and 31) of bacteriophage lambda, undigested pUC18 DNA (lane 3 and 18), undigested pHr02B (lanes 15 and 30) and Bam HI digested PUC18 DNA (lanes 2 and 17), on 1% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The stained gels are shown. The restriction map of the inserted DNA derived from the analysis of the above digests is also shown.

Key :

E : Eco RI

H : Hind III

K : Kpn I

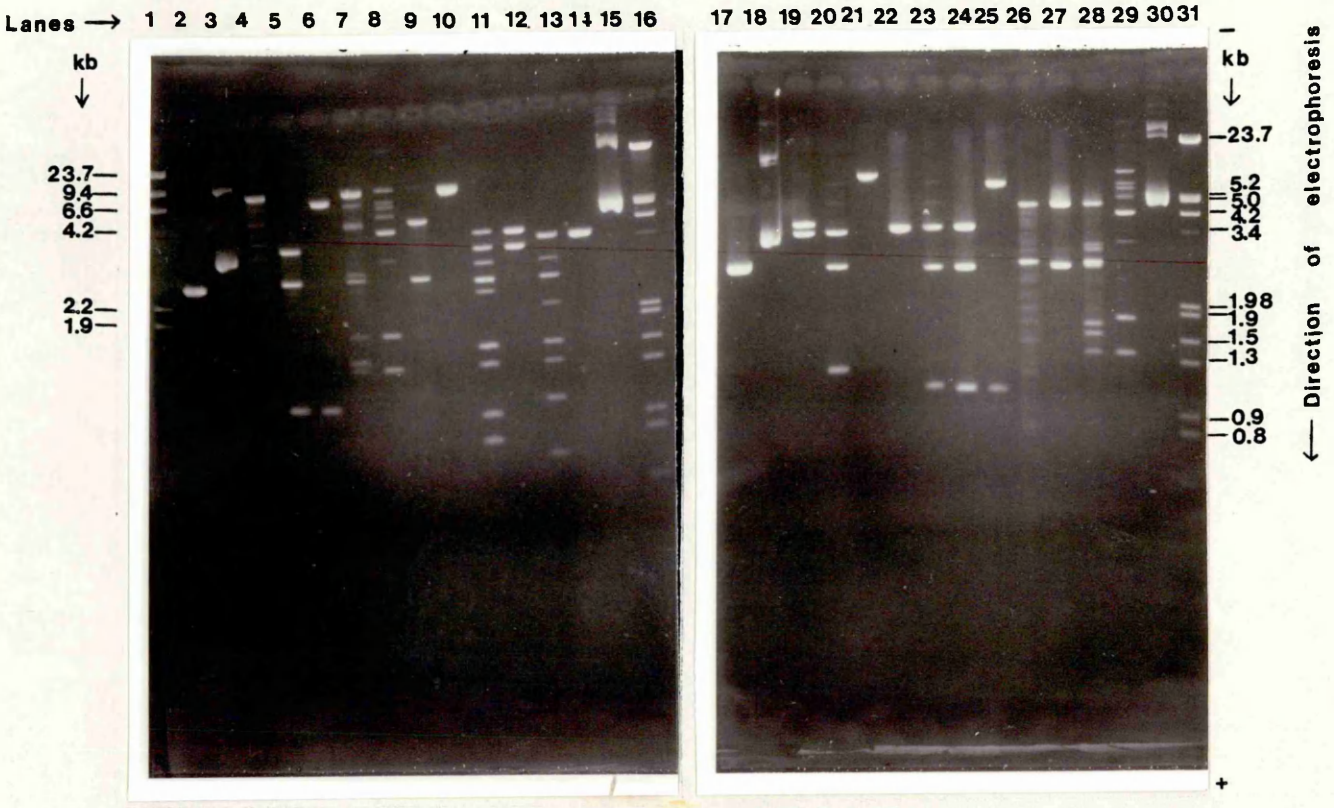
Sp : Sph I

St : Sty I

X : Xba I

Scale : 1 cm = 0.5 kb

Figure 3.30


Lanes → 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
kb
23.7
9.4
6.6
4.2
2.2
1.9
kb
23.7
5.2
5.0
4.2
3.4
1.98
1.9
1.5
1.3
0.9
0.8
Direction of electrophoresis
−
+

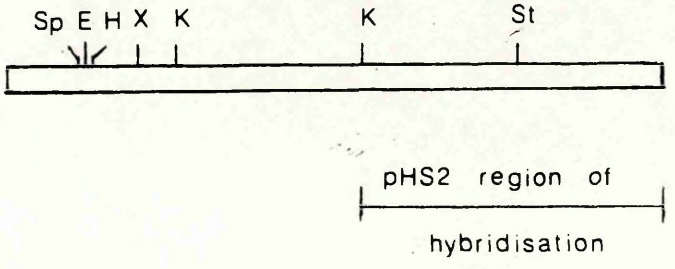

Sp E H X K
K
St
pHS2 region of
hybridisation

Figure 3.31

Single and double restriction digestion analysis of pHr02C

Aliquots of pHr02C DNA (1µg) were cleaved with the following restriction enzymes : Eco RI/Hind III (lane 3), Eco RI (lane 4), Xba I/Eco RI (lane 5), Xba I (lane 6), Xba I/Hind III (lane 7), Hind III (lane 8), Hind III/Bam HI (lane 9), Bam HI (lane 10), Sty I/Xba I (lane 11), Sty I (lane 12), Pst I (lane 13), Bgl II (lane 14), Asp 718 (lane 15) and Sst I (lane 16), (Sections 2.10.1 and 2.10.2). The digests were subjected to electrophoresis, in parallel with a Hind III digest of bacteriophage lambda (lane 1), Cla I digested pUC18 DNA (lane 2) and undigested pHr02C DNA (lane 17), on a 1% (w/v) agarose gel (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The stained gel is shown. The restriction map of the inserted DNA derived from the analysis of the above digests is also shown.

Key :

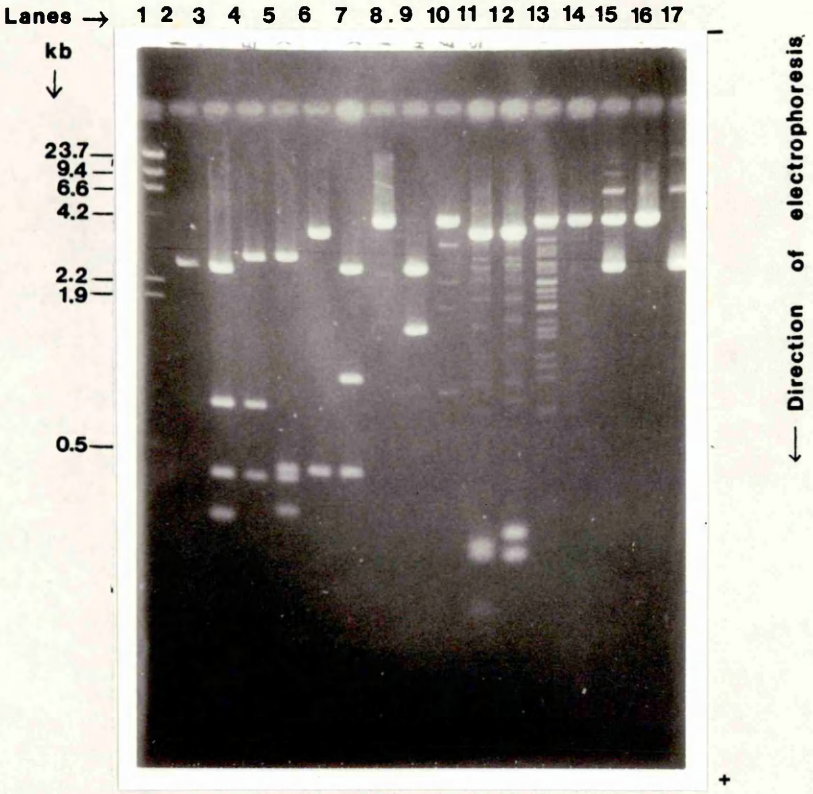
E : Eco RI

St : Sty I

X : Xba I

Scale : 2.5 cm = 0.5 kb

Figure 3.31


Lanes → 1 2 3 4 5 6 7 8.9 10 11 12 13 14 15 16 17
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
−
+
← Direction of electrophoresis

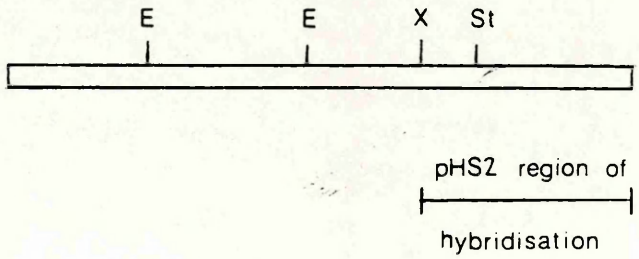

E
E
X
St
pHS2 region of
hybridisation

Figure 3.32

Single and double restriction digestion analysis of pHr05K

Aliquots of pHr05K DNA (1µg) were cleaved with the following restriction enzymes : Hind III (lane 4), Hind III/Eco RI (lane 5), Eco RI (lane 6), Eco RI/Xba I (lane 7), Xba I (lane 8), Bam HI (lane 9), Bam HI/Eco RI (lane 10), Sma I (lane 11), Cla I (lane 12), Bgl II (lane 13), Sst I (lane 14), Sph I (lane 15) and Pst I (lane 16)(Section 2.10.1 and 2.10.2). The digests were subjected to electrophoresis, in parallel with a Hind III digest of bacteriophage lambda (lane 1), undigested pHr05K DNA (lane 17), undigested pUC18 DNA (lane 3) and Kpn I digested pUC18 DNA (lane 2), on a 1% (w/v) agarose gel (Loening phosphate electrophoresis buffer : Section 2.5.2.a and 2.11). The stained gel is shown. The restriction map of the inserted DNA derived from the analysis of the above digests is also shown.

Key :

E : Eco RI

H : Hind III

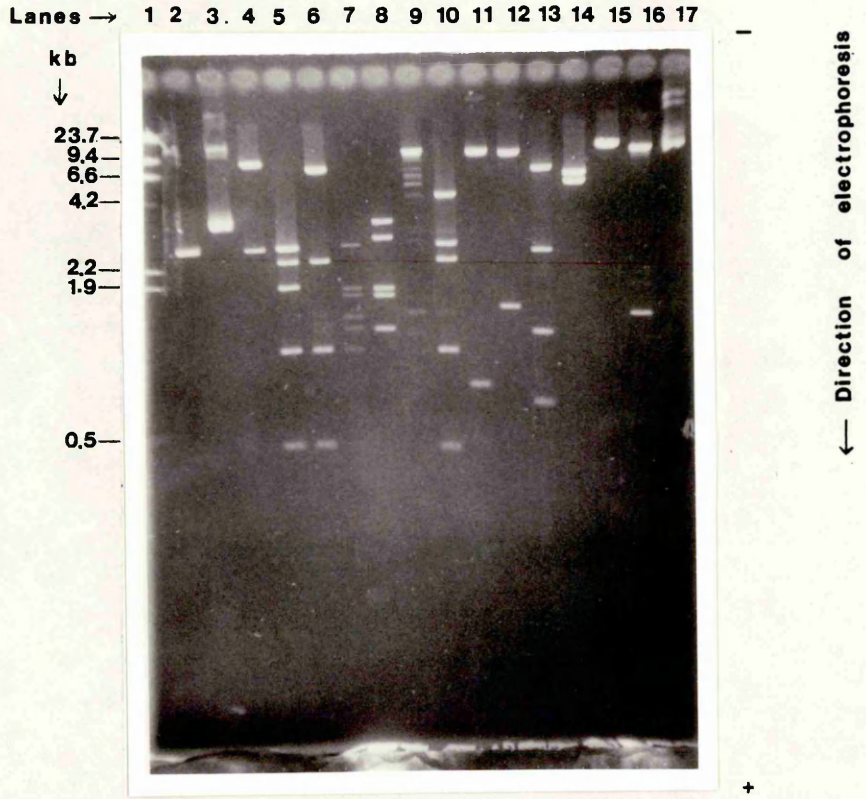
P : Pst I

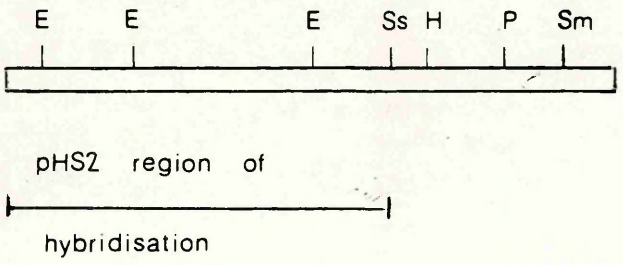
Sm : Sma I

Ss : Sst I

Scale : 1 cm = 1 kb

Figure 3.32


Lanes → 1 2 3. 4 5 6 7 8 9 10 11 12 13 14 15 16 17
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
−
+
Direction of electrophoresis


E
E
E
Ss
H
P
Sm
pHS2 region of
hybridisation

part of the gel depicted in Figure 3.31 was Southern blotted and hybridised to the pHS2 cDNA insert. The resultant autoradiograph (Figure 3.33) indicated that the pHS2 region of hybridisation was located within a 0.5 kb Xba I fragment (Figure 3.31). Therefore sequencing was initiated in and around this area.

3.4.2 Nucleotide sequence of pHr02C

Sequence data were obtained for a 0.85 kb Eco RI fragment of pHr02C (Figure 3.34) using the chemical cleavage method of Maxam and Gilbert, (1980)(Section 2.22). This fragment includes the 0.5 Xba I fragment which had previously been shown to contain the pHS2 region of hybridisation (Figure 3.33). Restriction enzymes which produced 5' overhangs were chosen for initial digestion and end-labelling of the DNA and both Klenow and polynucleotide kinase methods of end-labelling were used to obtain sequence from both strands of the DNA fragment.

Three fragments were initially isolated from pHr02C: a 0.33 kb Eco RI/Xba I fragment, a 0.24 kb Xba I/Sty I fragment and a 0.85 kb Bam HI/Eco RI fragment (Figure 3.34), and sequencing was carried out in the following directions: Eco RI → Xba I, Xba I → Eco RI, Xba I → Sty I, Sty I → Xba I and Bam HI → Eco RI (the first enzyme stated in each case being the site which was end-labelled). The resulting sequence data revealed some less common but useful restriction sites for further sequencing. The nucleotide sequence from the Eco RI/Xba I and Bam HI/Eco RI fragments also helped position two Sty I sites (Sty I sites 1 and 3 in Figure 3.34) which had been difficult to place during restriction mapping. Using this information the following fragments were generated: a 0.55 kb Nco I/Eco RI fragment, a 0.35 kb Dra II/Xba I fragment, a 0.17 kb Dra II/Xba I fragment and a 0.15 kb Sty I/Hinf I fragment. These fragments were then sequenced in the following directions: Nco I → Eco RI, Dra II → Xba I(0.35 kb),

Figure 3.33

Southern blot analysis of subclone pHr02C

The left hand portion of the gel in Figure 3.31 is presented : Hind III digest of bacteriophage lambda (lane 1), Cla I digested pUC18 DNA (lane 2), Eco RI/Hind III (lane 3), Eco RI (lane 4), Xba I/Eco RI (lane 5), Xba I (lane 6) and Xba I/Hind III (lane 7). This was Southern blotted (Section 2.17.1) and hybridised to nick-translated pHS2 cDNA insert (10^6 Cherenkov c.p.m.)(Sections 2.14, 2.15 and 2.17.2.a). After hybridisation for 18 hours at 65°C, the filter was washed (Section 2.17.2.a) and subjected to autoradiography at -70°C for 18 hours. The resulting autoradiograph is presented.

Figure 3.33

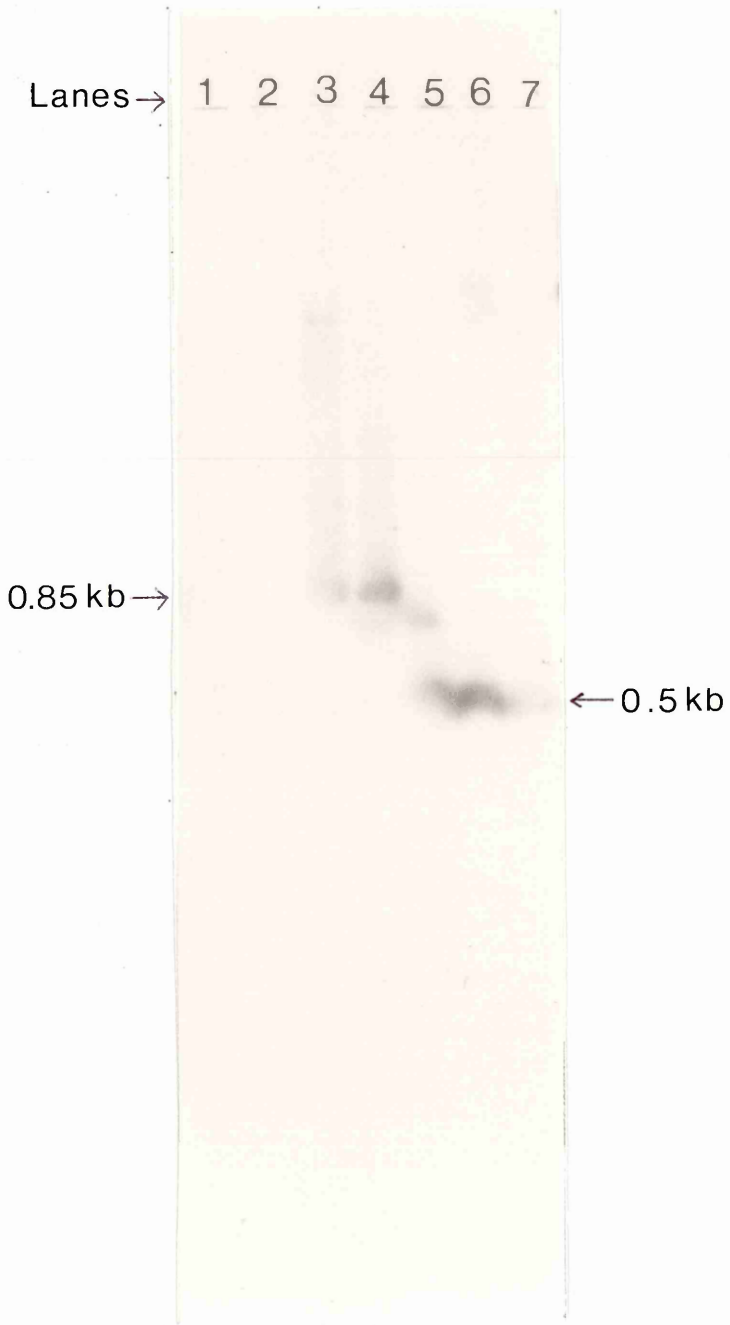
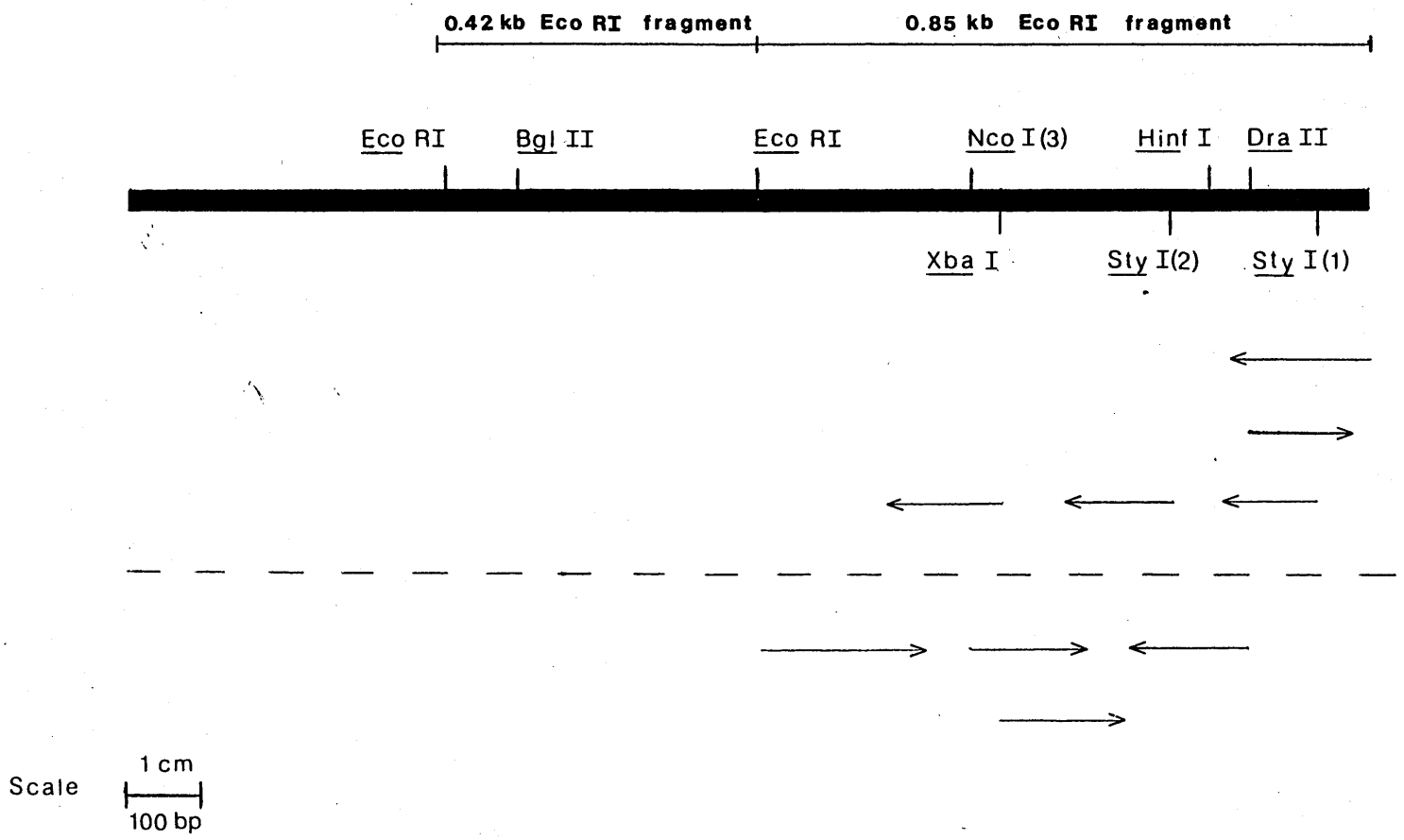


Figure 3.34

Restriction map of pHr02C insert DNA and sequencing strategy

The exact positions of the restriction sites shown were determined by restriction mapping and DNA sequencing. The arrows below the restriction map indicate the direction of sequence determination. Arrows above the dotted line indicate the upper DNA strand and those below the lower DNA strand. The length of the arrows correspond to the number of nucleotides sequenced.

Figure 3.34


0.42 kb Eco RI fragment
0.85 kb Eco RI fragment
Eco RI
Bgl II
Eco RI
Nco I(3)
Hinf I
Dra II
Xba I
Sty I(2)
Sty I(1)
Scale
1 cm
100 bp

Dra II → Xba I (0.17 kb) and Sty I → Hinf I. The nucleotide sequence from these fragments bridged the remaining gaps. A summary of the sequencing strategy and the extent to which the nucleotide sequence of each fragment was determined is depicted in Figure 3.34. All parts of the 0.85 kb Eco RI fragment of pHr02C were sequenced at least twice and 70% of it on both strands. All autoradiographs for sequencing gels were read on three separate occasions to eliminate reading errors. Discrepancies between separate readings were resolved by going back to the autoradiograph in question. Occasionally a region of a sequence was difficult to read but a film on the other strand led to the unambiguous resolution of the sequence. The nucleotide sequence for this region is presented in Figure 3.35.

3.4.3 Extent of 28S ribosomal DNA homology

As is described in more detail in the Discussion (see Figure 4.5), homology of the pHr02C subclone with human 28S rDNA was confined to a 451 base pair region of the 823 base pair sequence determined. It was thus of interest to determine whether any further homology with 28S rDNA existed upstream or downstream of this site in the remaining unsequenced portion of pHr02C,or in the neighbouring regions present in the genomic clone λHr02 from which pHr02C was derived (and in the other assumed overlapping clones λHr03 and λHr06, and for λHr05). This was done by Southern transfer/hybridisation experiments of the subclone and genomic clones using, as probes, fragments of the human 28S rDNA clone, pA4 (Erickson et. al., 1981) obtained from Professor B.E.H. Maden. The restriction map of this clone is presented in Figure 3.36 with the fragments, which were isolated and used as probes, shown below the map: a 1.43 kb Bam HI fragment and a 4.3 kb Eco RI/Bam HI fragment (these fragments lie outwith the area recognised by pHS2).

To answer the question of whether any homology existed between

Figure 3.35

Nucleotide sequence of pHr02C

The nucleotide sequence of the 0.85 kb Eco RI fragment shown in Figure 3.34 is presented.

The actual regions sequenced are indicated in blue.

Figure 3.35

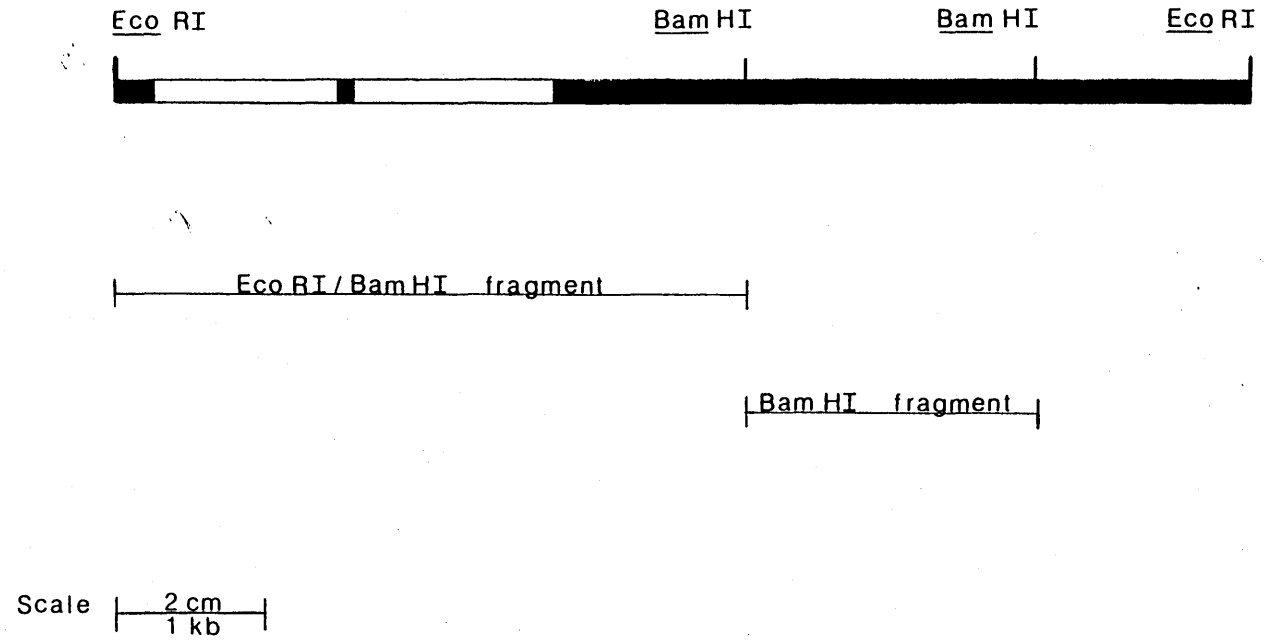
```
1   CCGTAACGGA GGATGTTTTT CAGAATGTGG TTGGGATTGA TGGATGGGAG GTAGAACGAG TTCGAGTTGA AAAGGTTTTG TGTGCCATGT GAAAAGGTTA   100
    GGCATTGCCT CCTACAAAAA GTCTTACACC AACCCTAACT ACCTACCCTC CATCTTGCTC AAGCTCAACT TTTCCAAAAC ACACGGTACA CTTTTCCAAT

101 GCATCTATTA CGTAGACGAG AGAAATTCAT TGGAAATTTG AGAAGGAGAT TGAGCATAAT GAAACTTGTT TTGGAAAAAT ATGTTGTTAT TAATGTGGAG   200
    CGTAGATAAT GCATCTGCTC TCTTTAAGTA ACCTTTAAAC TCTTCCTCTA ACTCGTATTA CTTTGAACAA AACCTTTTTA TACAACAATA ATTACACCTC

201 GTGGGCAAGA ATGAGAATAA TCAGTAGCAA TGAGGTGTCA ATAATTTGAT ACTGTCTACA TGGAAGACGG CGACCAGAGC CATGGAAGTC AGAATGAAAA   300
    CACCCGTTCT TACTCTTATT AGTCATCGTT ACTCCACAGT TATTAAACTA TGACAGATGT ACCTTCTGCC GCTGGTCTCG GTACCTTCAG TCTTACTTTT

301 ATGATAAATG TGAAAACATT CTAGAGAAGA AATGAATACG CGAAGGCCCG TGGTGGGTGA TGACATGATG TGATTTCTGC CCAGTGCTCT GAATGTCAAA   400
    TACTATTTAC ACTTTTGTAA GATCTCTTCT TTACTTATGC GCTTCCGGGC ACCACCCACT ACTGTACTAC ACTAAAGACG GGTCACGAGA CTTACAGTTT

401 GTGAAGAAAT TCAATGAAGG ACGGGTAAAC GGCGGGAGTA ACTATGACTC TCTTAAGGTA GCCAAATGCA TAGTCATCTA ATTAGTGACG TTCATGAATG   500
    CACTTCTTTA AGTTACTTCC TGCCCATTTG CCGCCCTCAT TGATACTGAG AGAATTCCAT CGGTTTACGT ATCAGTAGAT TAATCACTGC AAGTACTTAC

501 GATGAACGAG ATTCCCACTG TCCCTACCTA CTATCCAGCG AAACCACAGC CAAGGTAACG GGCTTGGTGG AATCCGCGGG GAAAGAAGAC CCTGTTGAGC   600
    CTACTTGCTC TAAGGGTGAC AGGGATGGAT GATAGGTCGC TTTGGTGTCG GTTCCATTGC CCGAACCACC TTAGGCGCCC CTTTCTTCTG GGACAACTCG

601 TTGACTCTAG TCTGGCACGG TGAAGAGCCA TGAGAAGTGT AGAATAAGTG GGAGGCCCCT GGGCCCCCCT GCCCAGCAAG GGGACAGAGT GGGGCAAGGC   700
    AACTGAGATC AGACCGTGCC ACTTCTCGGT ACTCTTCACA TCTTATTCAC CCTCCGGGGA CCCGGGGGGA CGGGTCGTTC CCCTGTCTCA CCCCGTTCCG

701 CAGAGGTGAA ATACCACTAC TCTGATTGTT TATTCACTGA CCCGTGAGGT GCCCCAAGGG GCTCTTGCTT CTGGCGCCGA GTGCCCGGCC ACATGCACAT   800
    GTCTCCACTT TATGGTGATG AGACTAACAA ATAAGTGACT GGGCACTCCA CGGGGTTCCC CGAGAACGAA GACCGCGGCT CACGGGCCGG TGTACGTGTA

801 GCCAAATTGT AAAGACCATC GAT  823
    CGGTTTAACA TTTCTGGTAG CTA
```

Figure 3.36

Restriction map of human ribosomal DNA clone, pA4

The restriction map of the insert DNA in human ribosomal DNA clone pA4 (Erickson et. al., 1981) is presented. The Bam HI and Bam HI/Eco RI fragments used as probes are indicated below the map.

Figure 3.36


Eco RI
Bam HI
Bam HI
Eco RI
Eco RI / Bam HI fragment
Bam HI fragment
Scale
2 cm
1 kb

28S rDNA and the 0.42 kb Eco RI fragment to the left of the sequenced region (Figure 3.34), Southern blots were prepared of pA4, λHr06 and HeLa DNA digested with Eco RI and probed with ^{32}P-labelled pHS2 cDNA insert and the 0.42 kb Eco RI fragment of pHr02C.

As can be seen from the autoradiograph (Figure 3.37), the Eco RI fragment of pHr02C recognised the identical fragment in λHr06 but did not hybridise to anything in pA4 or HeLa DNA. In contrast, the pHS2 cDNA insert hybridised to the 7.3 kb Eco RI fragment of the rDNA repeat in HeLa DNA and pA4 and a 2.0 kb fragment in λHr06.

These results, together with additional sequence data from the Hind III site in pHr02C (not shown), suggested that no other sequences corresponding to 28S rDNA were present in pHr02C.

In order to determine whether any homology existed outwith the region of pHS2 hybridisation in the genomic clones, Bgl II and Cla I digests of λHr02, λHr03 and λHr06 DNA and Bgl II and Asp 718 (isochizomer of Kpn I) digests of λHr05 DNA were prepared and used in an additional Southern blot/hybridisation experiment. These digests fragmented the genomic clones into sufficiently small regions to be able to identify which area, if any, contained homology with 28S rDNA.

As had been observed before, pHS2 hybridised to a 1.3 kb Bgl II and 1.7 kb Cla I fragment in λHr02, λHr03 and λHr06, and a 1.5 kb Asp 718 and >23 kb Bgl II fragment in λHr05 (Figure 3.38). The rDNA probes from pA4 do not however recognise any sequences in the genomic clones suggesting that only an isolated fragment of 28S rDNA is present in λHr02, λHr03, λHr05 and λHr06.

3.4.4 Nature of the sequences surrounding 28S rDNA fragment

In order to further investigate the nature of the sequences surrounding the 28S rDNA fragment, an additional Southern blot of the genomic clones was prepared and probed with nick-translated

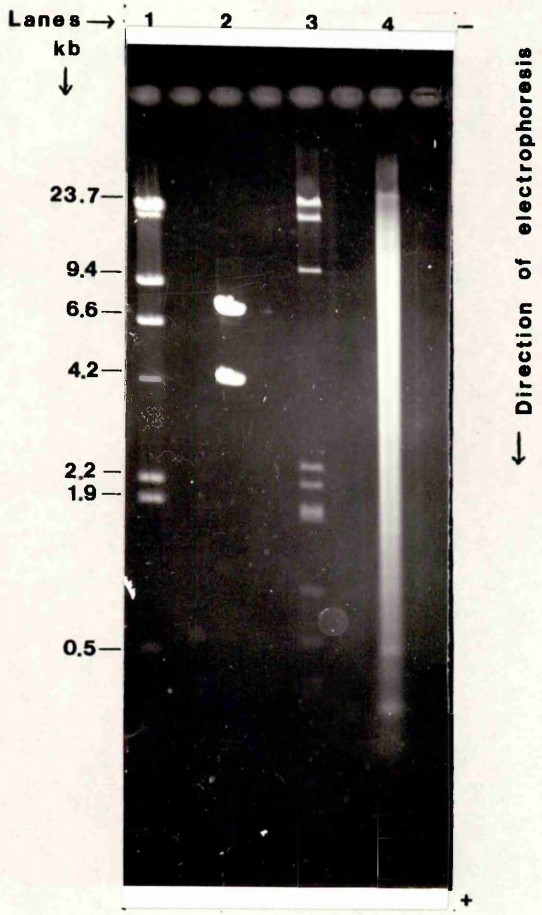
Figure 3.37

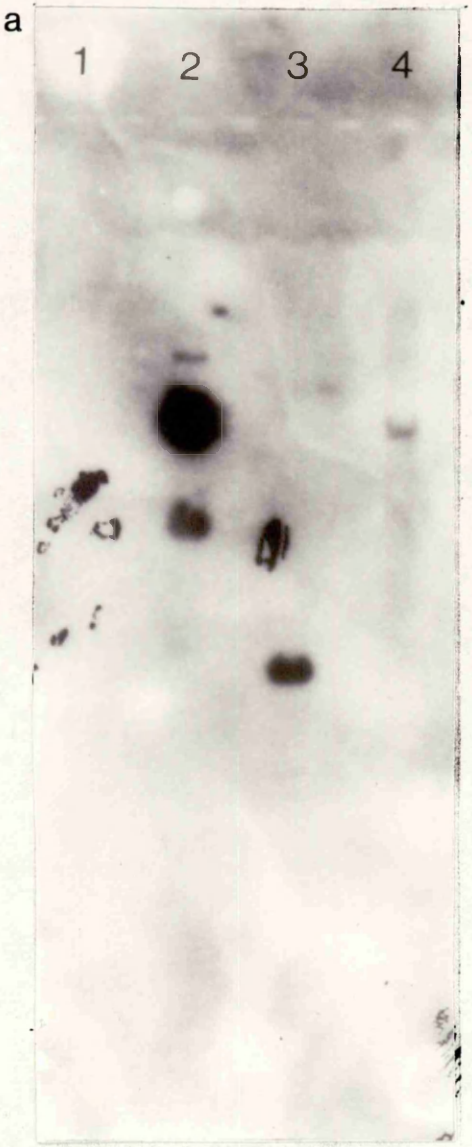
Analysis of relatedness to 28S rDNA of sequences flanking the orphon rDNA

The Eco RI fragment of Figure 3.34, flanking the orphon rDNA was nick-translated and hybridised to rDNA in order to determine possible further rDNA sequence in λHrO2. A comparison was made with pHS2 which hybridises to 28S rDNA sequence.

The following single digests were prepared : Hind III digest of bacteriophage lambda (lane 1), Eco RI digest of pA4 (lane 2), Eco RI digest of λHrO6 (lane 3) and Eco RI digest of HeLa DNA (lane 4)(Section 2.10.1) and subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). The gels were Southern blotted (Section 2.17.2.a) and hybridised to the following nick-translated probes (Sections 2.14, 2.15 and 2.17.2.a) : a. pHS2 cDNA insert and b. 0.42 kb Eco RI fragment of pHrO2C. After hybridisation for 18 hours at 65°C the filters were washed (Section 2.17.2.a) and subjected to autoradiography at -70°C for 18 hours. The autoradiographs and one of the photographs obtained are shown.

Figure 3.37


Lanes →
1
2
3
4
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
−
+
← Direction of electrophoresis


a
1
2
3
4

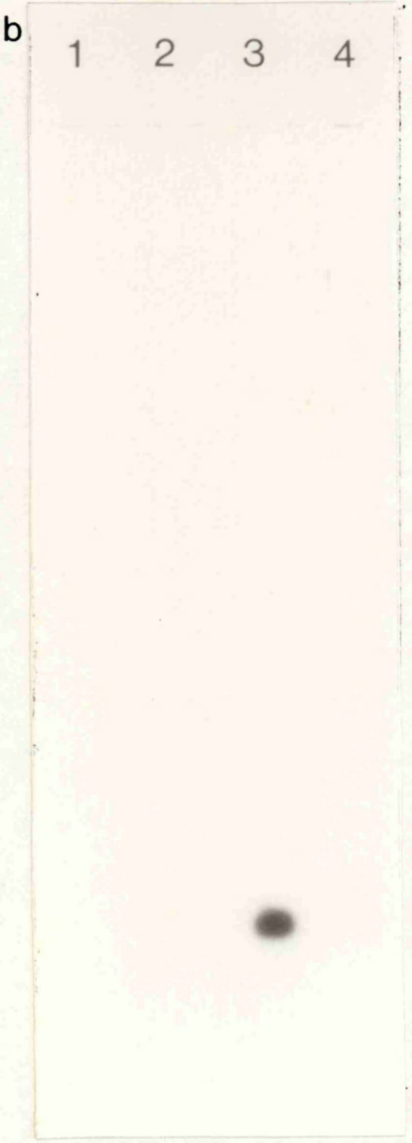
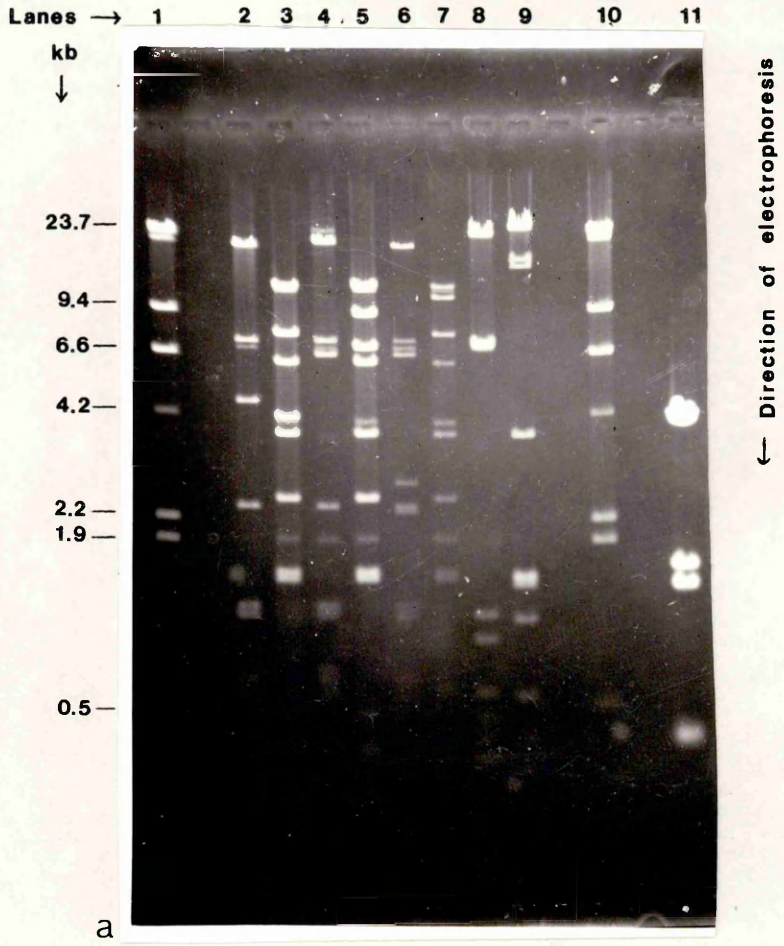

b
1
2
3
4

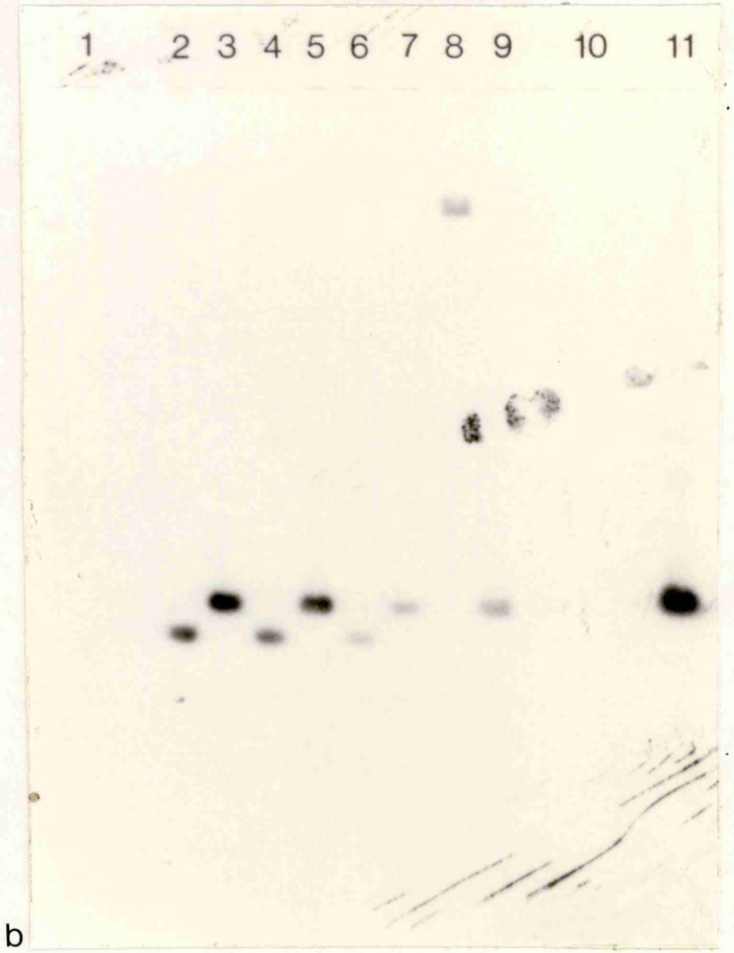
Figure 3.38

Southern analysis of genomic clones with 28S rDNA probes

The following single digests were prepared : Hind III digest of bacteriophage lambda (lane 1), Bgl II digest of λHr02 DNA (lane 2), Cla I digest of λHr02 DNA (lane 3), Bgl II digest of λHr03 DNA (lane 4), Cla I digest of λHr03 DNA (lane 5), Bgl II digest of λHr06 DNA (lane 6), Cla I digest of λHr06 DNA (lane 7), Bgl II digest of λHr05 DNA (lane 8), Asp 718 digest of λHr05 DNA (lane 9), Hind III digest of bacteriophage lambda (lane 10) and a Bam HI/Eco RI digest of pA4 (lane 11). They were subjected to electrophoresis on 0.7% (w/v) agarose gels (Loening phosphate electrophoresis buffer : Sections 2.5.2.a and 2.11). A photograph of one of the stained gels is shown in a. The gels were Southern blotted (Section 2.17.2.a) and hybridised to the following nick-translated probes (Sections 2.14, 2.15 and 2.17.2.a) : b. pHS2 cDNA insert, c. Bam HI fragment of pA4 and d. Bam HI/Eco RI fragment of pA4. After hyridisation for 18 hours at 65°C the filters were washed (Section 2.17.2.a) and subjected to autoradiography at -70°C (18 hours). The autoradiographs are presented.

Figure 3.38


Lanes → 1 2 3 4 5 6 7 8 9 10 11
kb
23.7
9.4
6.6
4.2
2.2
1.9
0.5
← Direction of electrophoresis
a


1 2 3 4 5 6 7 8 9 10 11
b

c

d

HeLa DNA. The rationale of this experiment was that any fragments of the genomic clones which contained highly-repetitive sequences would hybridise with greater intensity and thus be distinguisable from the rest.

From the autoradiograph (Figure 3.39) it can be seen that there are sequences upstream and downstream of the fragment homologous to 28S rDNA which are highly-repetitive. The fragments involved in hybridisation are shown in Figure 3.40. The extent and precise location of these sequences cannot be determined without further experiments.

Figure 3.39

Southern analysis of sequences surrounding the 28S rDNA fragment

A gel similar to that shown in Figure 3.38 was prepared : Hind III digest of bacteriophage lambda (lane 1), λHr02 digested with Bgl II (lane 2), Cla I (lane 3), λHr03 digested with Bgl II (lane 4), Cla I (lane 5), λHr06 digested with Bgl II (lane 6), Cla I (lane 7), λHr05 digested with Bgl II (lane 8), Asp 718 (lane 9), Hind III digest of bacteriophage lambda (lane 10) and a Bam HI/Eco RI digest of pA4 (lane 11). This was Southern blotted (Section 2.17.1) and the filter hybridised to nick-translated genomic HeLa DNA (10^6 Cherenkov c.p.m.)(Sections 2.15 and 2.17.2.a). After hybridisation for 18 hours at 65°C the filter was washed (Section 2.17.2.a) and subjected to autoradiography for 48 hours at -70°C. The autoradiograph is presented.

Figure 3.39

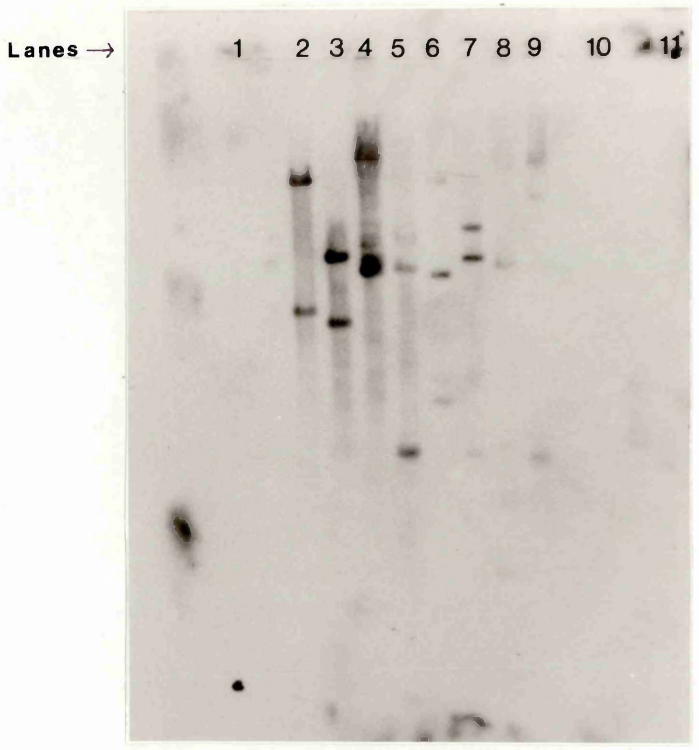


Figure 3.40

Areas containing repetitive sequences in genomic clones λHr02, λHr03, λHr05 and λHr06

Restriction maps of the genomic clones studied are presented. Indicated below each map are the fragments which hybridised to nick-translated HeLa DNA (results from Figure 3.39). Shaded regions represent the genomic clone DNA inserts. Non-shaded regions represent the EMBL3 phage arms.

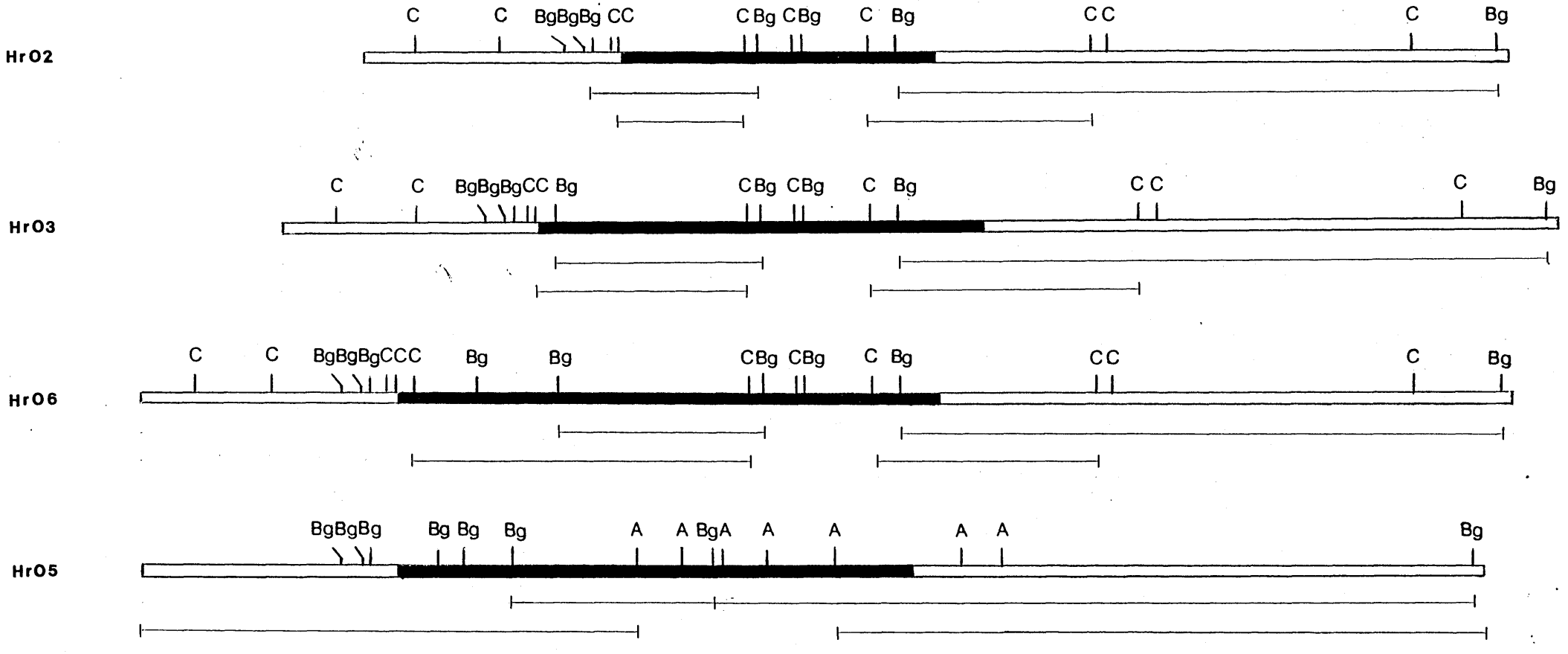
Key :

A : Asp 718

Bg : Bgl II

C : Cla I

Figure 3.40


HrO2
C C BgBgBg CC CBg CBg C Bg CC C Bg
HrO3
C C BgBgBgCC Bg CBg CBg C Bg CC C Bg
HrO6
C C BgBgBgCCC Bg Bg CBg CBg C Bg CC C Bg
HrO5
BgBgBg Bg Bg Bg A A BgA A A A A Bg

CHAPTER FOUR

DISCUSSION

4. DISCUSSION

4.1 Nature and origin of clone pHS2

Contrary to the conclusion drawn by Cato et. al., (1981), it has been clearly established from this work that their clone, pHS2, does not contain a cDNA sequence corresponding to a heat-shock mRNA. This fact is based on several pieces of evidence, the most conclusive being the nucleotide sequence of the pHS2 cDNA insert. An initial computer comparison of this nucleotide sequence with sequences in the Genbank database revealed homology with a 324 base-pair segment of 28S rDNA of rat (Chan et. al., 1983) corresponding to nucleotides 3355-3678 (Figure 3.28), which upon subsequent comparison with the final sequence of pHS2 (Figure 3.27) showed only one mis-match. This was at position 245 where a T replaced the G seen at position 3599 of the 28S rDNA sequence of rat. As emerged later, pHS2 also shows 323 out of 324 base-pair homology with a segment of human 28S rDNA sequence (Gonzalez et. al., 1985) extending from nucleotide 3600-3923 (Figure 4.1). The one mis-match observed is at position 3844 of the human 28S rDNA sequence, where a G again replaces the T seen at position 245 of the cDNA sequence of pHS2. This point of difference observed in the cDNA sequence of pHS2 could be a consequence of any one of three possible factors: a nucleic acid sequencing error, polymorphism, or reverse transcriptase artefact. The first possibility can be discounted as this region has been sequenced three times and on each occasion shows a clear T at this position (Figure 4.2). G has been observed at position 3844 in two other human rDNA clones (B.E.H. Maden, personal communication) and also in both rat and human sequences referred to above. There is no evidence therefore to support the idea of polymorphism at this position in members of the human rDNA repeat, although this possibility cannot be excluded.

Figure 4.1

A comparison of pHS2 and human 28S rDNA nucleotide sequences

The nucleotide sequence of the cDNA insert of pHS2 is presented in comparison with a portion of human 28S rDNA (sequence and numbering from Gonzalez et. al., 1985). One point of mis-match is observed at position 245 of the cDNA insert sequence of pHS2.

Figure 4.1

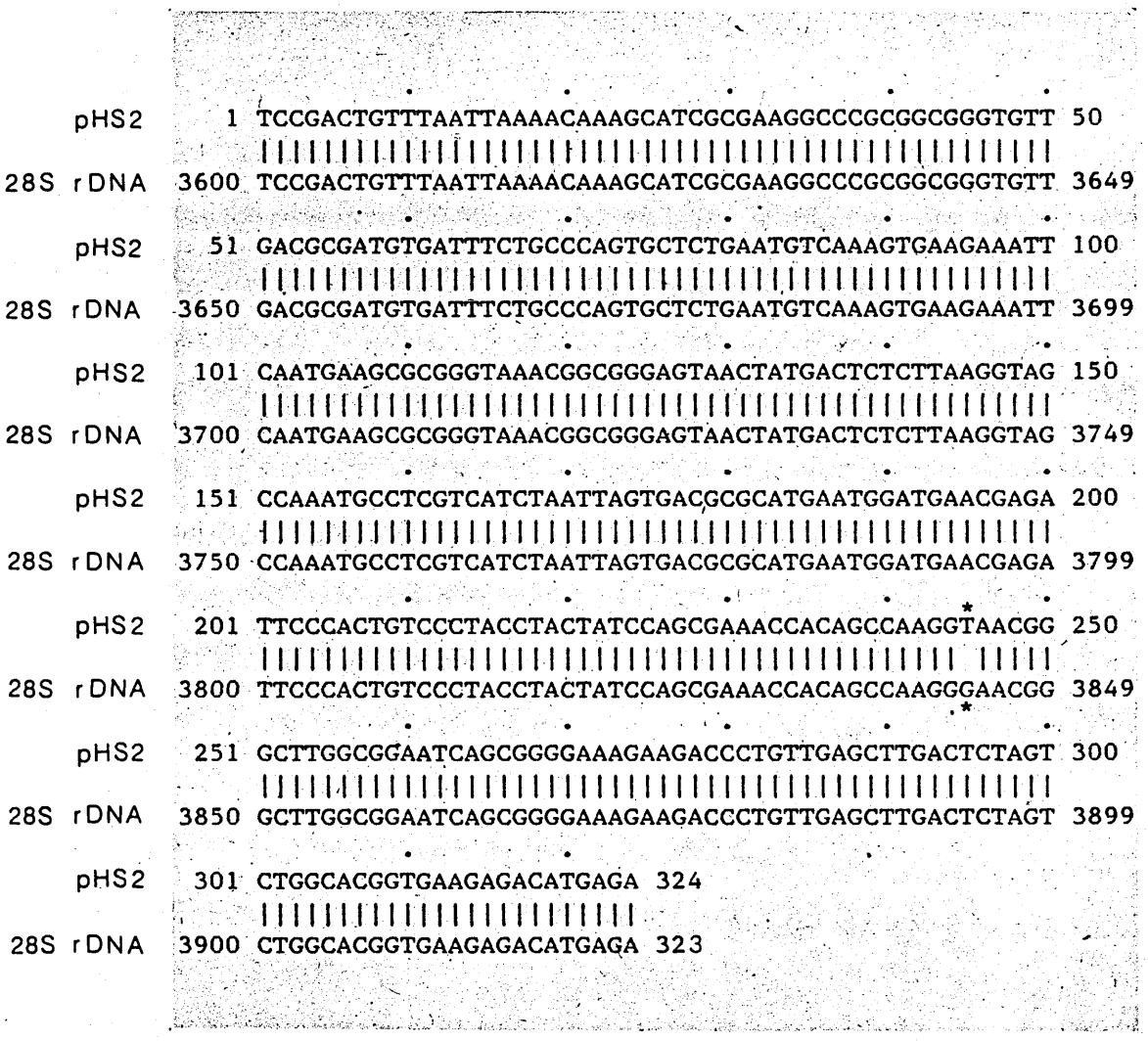

pHS2 1 TCCGACTGTTTAATTAAAACAAAGCATCGCGAAGGCCCGCGGCGGGTGTT 50
28S rDNA 3600 TCCGACTGTTTAATTAAAACAAAGCATCGCGAAGGCCCGCGGCGGGTGTT 3649
pHS2 51 GACGCGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAAGTGAAGAAATT 100
28S rDNA 3650 GACGCGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAAGTGAAGAAATT 3699
pHS2 101 CAATGAAGCGCGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAG 150
28S rDNA 3700 CAATGAAGCGCGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAG 3749
pHS2 151 CCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATGAACGAGA 200
28S rDNA 3750 CCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATGAACGAGA 3799
pHS2 201 TTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGTAACGG 250
28S rDNA 3800 TTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGGAACGG 3849
pHS2 251 GCTTGGCGGAATCAGCGGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGT 300
28S rDNA 3850 GCTTGGCGGAATCAGCGGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGT 3899
pHS2 301 CTGGCACGGTGAAGAGACATGAGA 324
28S rDNA 3900 CTGGCACGGTGAAGAGACATGAGA 323

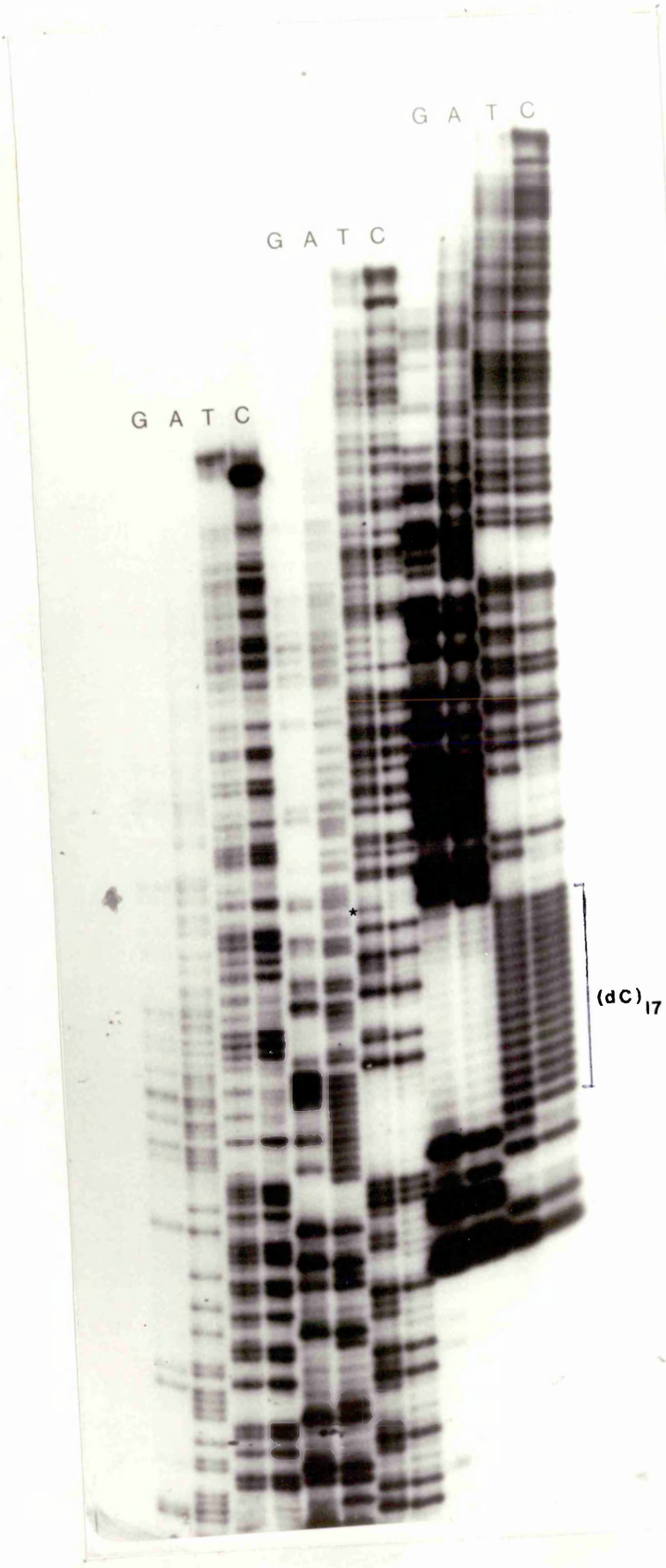
Figure 4.2

Region of mis-match between pHS2 and human 28S rDNA nucleotide sequences and evidence of poly dC tails in the cDNA insert sequence of pHS2

The autoradiograph showing the single point of difference (T rather than G) of the pHS2 cDNA insert from the corresponding region of human 28S rDNA sequence (Gonzalez et. al., 1985) is presented. This mis-match is marked by an asterix at its left-hand side.

One of the poly dC tails found on either side of the pHS2 cDNA insert is also indicated.

Figure 4.2



Reverse transcriptase, however, has been reported to have an error rate of 10^{-3} *in vitro* (Gopinathan *et. al.*, 1979). It thus seems more likely that the difference in sequence was caused by this enzyme during the construction of the cDNA clone. Indeed other workers (Fields and Winter, 1981; Minty *et. al.*, 1982) have reported similar cDNA artifacts assumed to be caused by reverse transcriptase.

Although this cDNA sequence clearly corresponds to 28S rDNA, could it just be fortuitous that this is the case, pHS2 actually representing part of the hsp 70 gene? A comparison of both strands of this cDNA sequence and the nucleotide sequence of the *Drosophila* hsp 70 gene (Ingolia *et. al.*, 1980) and later the human hsp 70 gene (Hunt and Morimoto, 1985) suggested that pHS2 does not possess significant homology to the hsp 70 gene. Southern blot analysis of HeLa DNA with *Drosophila* hsp 70 probe 56H8 (Figure 3.24) and pHS2 (Figure 3.23) also confirms this, as these probes did not hybridise to common fragments in the human genome.

It is evident that the sequence of pHS2 presented here is not compatible with it being part of the hsp 70 gene. It is necessary therefore to ask whether the insert sequenced was in fact pHS2. It is well known how easily cross contamination of plasmid DNA can occur, and at the time this work was performed, work on *Xenopus* rDNA was going on in a common laboratory (Furlong and Maden, 1983). A contamination from this source can definitely be ruled out, however, as pHS2 is clearly a cDNA clone with dG and dC tails evident at either side of the inserted sequence (Figure 4.2). Maden and co-workers, on the other hand, were only working with subclones of genomic rDNA. It is true that there are differences between the restriction map of pHS2 presented here and that published by Cato *et. al.*, (1981): the *Ava* II site of the latter is missing from Figure 3.26, the *Hae* III

site is at the opposite side from that shown in Figure 3.26, and there are additional Hinf I sites in Figure 3.26, although the Hind II site and one of the Hinf I sites are consistent. It seems most likely, however, that the discrepancies arise from difficulties in mapping these abundant sites in pBR322 clones. Moreover the results of Northern blot analysis of pHS2 are compatible with those published by Cato et. al., (1982).

The question thus arises of how could a portion of 28S rDNA have been cloned into pBR322 in the first place? It seems clear that pHS2 is a cDNA copy of a fragment of 28S rRNA. Thus the mRNA used in the construction of the cDNA library must have been contaminated with rRNA, fragments of which had the secondary structure potential to prime reverse transcription. Possible sites for reverse transcription in 28S rRNA around the area recognised by pHS2 are shown in Figure 4.3. In each case double-stranded regions caused by palindromic sequences could act as primers for reverse transcriptase. Indeed other cDNA clones derived from rRNA have been reported. Jain et. al., (1985) isolated such clones while screening a cDNA library for ferritin sequences. Upon sequencing, these clones were also found to correspond to the 3' end of 28S rRNA (although the precise region was not reported) and were presumed to have arisen from self-primed rRNA fragments.

Small fragments of rRNA may have been generated either in vivo, as a result of the heat shock, or in vitro during isolation of poly $(A)^+$ RNA. In yeast there is evidence that a ribonuclease that is associated with ribosomes increases in activity during heat shock (Schultz-Harder, 1983). If such a ribonuclease became active in HeLa cells, small fragments of rRNA could have been generated and subsequently carried through during preparation of the mRNA. There are, however, no reports of such a ribonuclease activity induced by heat

Figure 4.4

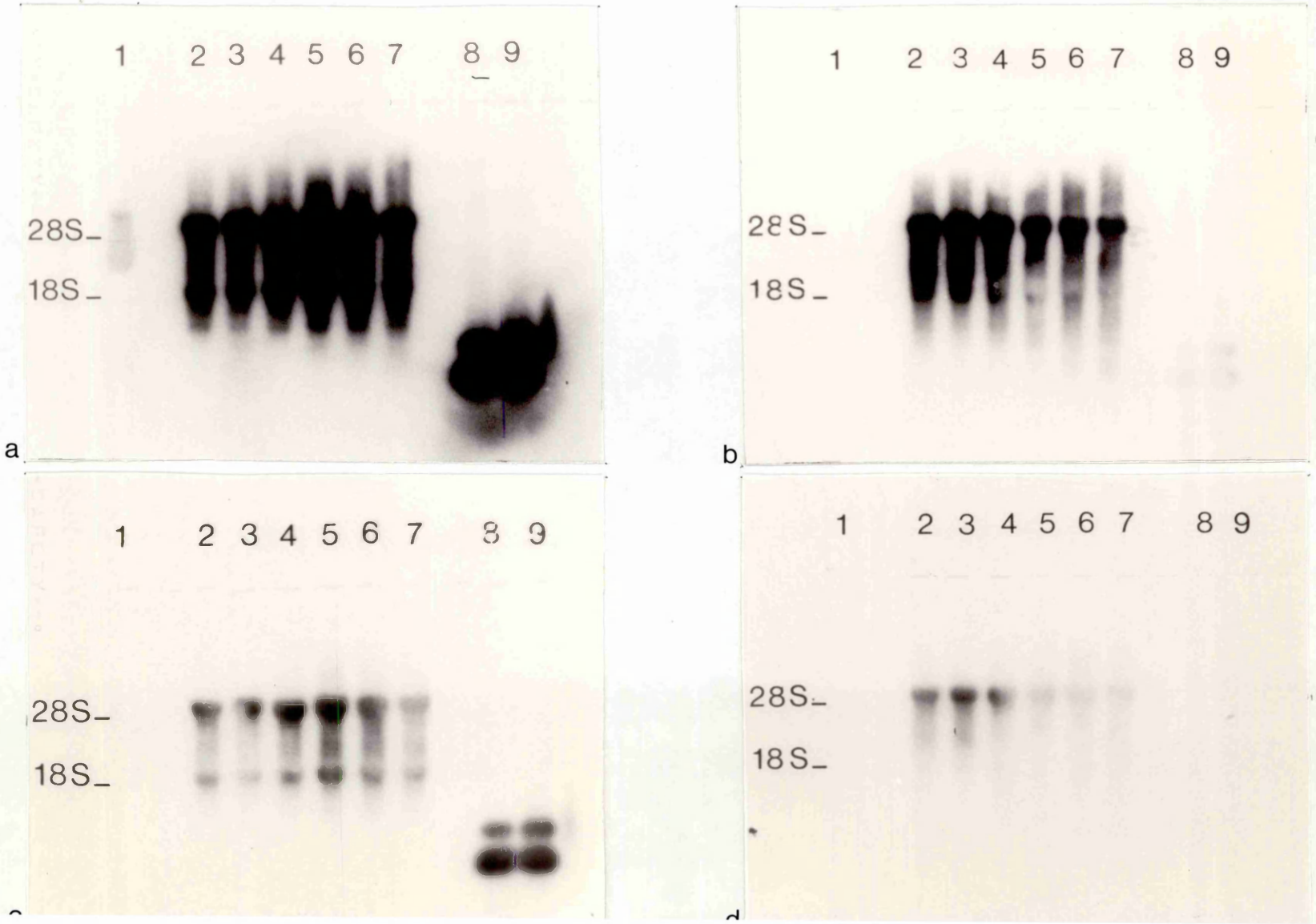
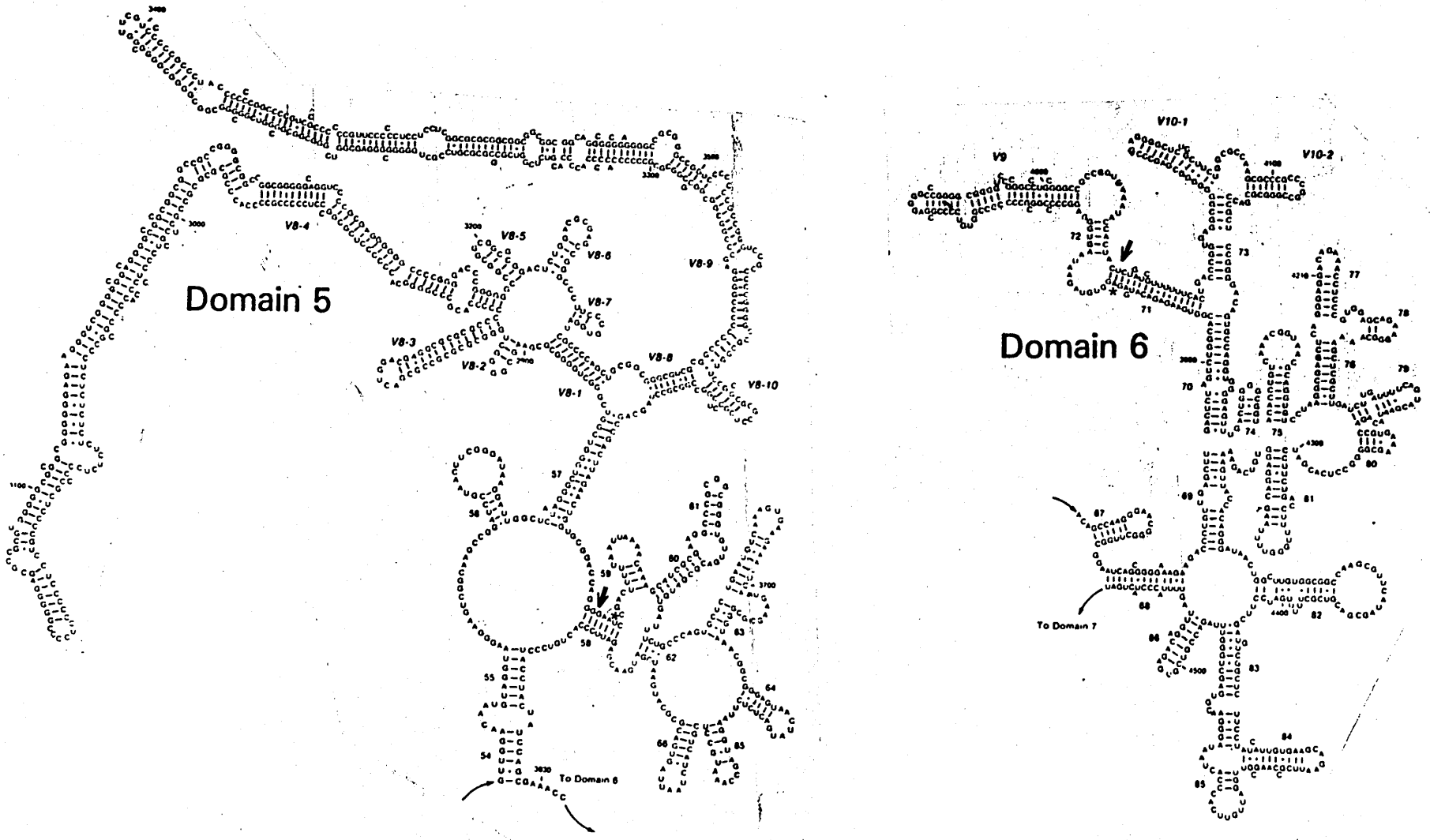

1 2 3 4 5 6 7 8 9
28S
18S
a
1 2 3 4 5 6 7 8 9
28S
18S
b
1 2 3 4 5 6 7 8 9
28S
18S
1 2 3 4 5 6 7 8 9
28S
18S
d

Figure 4.3

Potential regions for priming reverse transcription in 28S rDNA

Two of the seven domains from the predicted secondary structure of human 28S rDNA are presented (obtained from R.D. Schmickel : personal communication). They contain the region recognised by pHS2 cDNA insert (from * to *). Potential regions for priming reverse transcription are indicated by bold arrows on the diagram.

Figure 4.3


Domain 5
V8-1
V8-2
V8-3
V8-4
V8-5
V8-6
V8-7
V8-8
V8-9
V8-10
To Domain 6
Domain 6
V9
V10-1
V10-2
To Domain 7

shock in HeLa cells. An experiment was therefore performed using human genomic rDNA clone pA4 (Erickson et. al., 1981) and pHS2 to probe a Northern blot of total cytoplasmic HeLa RNA following heat shock to see if degraded fragments of rRNA would be detected. However the results gave no indication of degradation of rRNA during heat shock (Figure 4.4). It therefore seems more likely that the contaminating rRNA may have become fragmented in the course of isolation of the poly(A)$^+$ RNA or during the construction of the cDNA library. Although one would not expect rRNA to bind to oligo(dT) cellulose because it lacks a poly A tail or A-rich regions, it is well established that it is extremely difficult to get 100% pure poly(A)$^+$ RNA using such a column (Aviv and Leder, 1972).

As pHS2 is a cDNA clone derived from 28S rRNA, Cato et. al., (1981 and 1982) must clearly have misinterpreted the original results which led them to conclude that it was a heat-shock cDNA clone. Their original Northern blot analysis of cytoplasmic and nuclear RNA indicated that pHS2 corresponded to RNA of 7.3 kb in size with a precursor of 15.3 kb. They accounted for the large size of the cytoplasmic RNA by proposing that a rather long 3'- or 5'- untranslated region was present. As it is now known that human hsp 70 mRNA is 2.6 kb in size (Wu et. al., 1985), it seems that the 6.3 kb and 15.3 kb RNA species seen by Cato et. al., (1982) are most probably 28S rRNA and a precursor of 28S rRNA. Upon re-examination of the published Northern blot it is evident that the 6.3 kb RNA species which hybridised to pHS2 has a mobility similar to 28S rRNA. A curious feature of one of the Northern blots presented in this work (Figure 4.4.c) is that pHS2 also hybridises slightly to 18S rRNA, and indeed Cato et. al., (1982) also observed some hybridisation in the 18S rRNA region. It is possible that the cDNA insert of pHS2 may also possess some homology to 18S rRNA in the manner shown for 16S

Figure 4.4

Northern blot analysis with pHS2 and pA4 of cytoplasmic HeLa RNA obtained after heat shock

Total unfractionated cytoplasmic RNA was isolated from HeLa cells (Section 2.25) after the following treatments : none : 37°C throughout (lane 2), 10 minutes at 45°C followed by recovery at 37°C for 0 hours (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6) or 4 hours (lane 7). RNA (2μg) from each time point was denatured and subject to electrophoresis on 1% (w/v) agarose/2.2M formaldehyde gels (Section 2.18) and then transferred to nitrocellulose paper (Section 2.18). The filters were then hybridised to nick-translated 1. pHS2 cDNA insert and 2. Bam HI pA4 fragment (10^6 Cherenkov c.p.m.). After 18 hours hybridisation at 42°C the filters were washed at 65°C and then subjected to autoradiography (18 hours). The resultant autoradiographs are presented (a and b which correspond to probes 1. and 2. above).

The positions of migration of 18S and 28S are indicated at the side of the autoradiographs. Size markers pUC18 (lane 1) and pHS2 cDNA insert (lane 8 and 9) were also included.

Autoradiographs c and d show filters a and b respectively, rewashed at a higher temperature : 70°C (to remove some of the background hybridisation).

and 23S rRNA (Tapprich and Hill, 1986). In this latter case 16S rRNA contains a 9 base-pair region which is involved in the association of 30S and 50S subunits, presumably by binding to a similar region in 23S rRNA. A more trivial explanation of the results of Figure 4.4.c is that the GC tails on either side of the cDNA insert may be binding to GC-rich regions in 18S rRNA. This latter explanation would be consistent with the lack of hybridisation of human ribosomal probe pA4 to the 18S rRNA (Figure 4.4.d).

The other evidence which led Cato et. al., (1982) to suggest that pHS2 contained a cDNA insert corresponding to part of heat-shock mRNA came from hybrid-released translation experiments. These experiments indicated that pHS2 selected RNA which could be subsequently translated to produce a protein of 70,000 molecular weight. It was concluded that this protein was hsp 70 by superimposing the fluorogram of the two-dimensional gel of the polypeptide synthesised in vitro by the eluted RNA, over a fluorogram of the two-dimensional gel analysis of the products of translation of poly(A)$^+$ RNA from heat-shocked cells. It must be stated that the ^{35}S-labelled protein on the former fluorogram is very faint in the published figure. However it is possible that the mRNA corresponding to hsp 70 may contain small regions of sequence complementary to 28S rDNA. As a result, heat shock mRNA might hybridise to pHS2 and, upon elution, be translated to produce the authentic heat shock protein. Although a synthetic oligonucleotide corresponding to 21 bases of the leader sequence of the hsp 70 gene of maize (Rochester et. al., 1986) has been shown by Southern blot analysis to hybridise slightly to 18S rRNA, no homology between pHS2 and published human heat shock sequences (Hunt and Morimoto, 1985; Voellmy et. al., 1985) has yet been found.

4.2 Nature of genomic clones isolated with pHS2

It was apparent that the human genomic clones isolated from the lambda library using pHS2 as a probe must contain sequences complementary to 28S rDNA. However analysis of these genomic clones revealed properties which were not characteristic of the human 28S rDNA repeat observed previously in studies of genomic rDNA (Arnheim and Southern, 1977; Hollar et. al., 1977 ; Tanhauser et. al., 1981).

Nucleotide sequence analysis of part of subclone pHr02C showed that the rDNA detected in genomic clone λHr02, and possibly λHr03 and λHr06 (all thought to be overlapping clones with respect to their restriction maps), by hybridisation to probe pHS2, consisted of a 451 base pair fragment with 451 out of 478 base pair homology to a region extending from nucelotides 3627-4105 in human 28S rDNA (Gonzalez et. al., 1985)(Figure 4.5). Figure 4.5 indicates the positions of the base changes and deletions in this sequence and some of these points are shown in more detail in Figure 4.6.

It was, however, evident before undertaking the sequencing of the genomic clone λHr02 that this rDNA fragment was not a member of the human rDNA repeat. Southern blotting studies showed that whereas pHS2 hybridised to the expected Eco RI and Sst I fragments of the rDNA repeat in HeLa DNA (Figure 3.23), smaller Eco RI and larger Sst I fragments were seen in the genomic clones (Table 3.6). As has been observed by other workers (Schmickel et. al., 1980; Erickson et. al., 1981), the length of the hybridised Bam HI fragment (Figure 3.23) appears to vary. While the presence of a variable region (Wellauer and Dawid, 1979; Erickson et. al., 1981) in the non-transcribed spacer downstream of the 28S rDNA gene accounts for this variation in length (Figure 4.7), it seems likely that the 7.8 kb fragment observed in Figure 3.23 is a partial digestion product. If these two Bam HI fragments seen in Figure 3.23 represented discrete regions due to

Figure 4.5

Comparison of pHr02C (H28S-01) with human 28S rDNA.

The nucleotide sequence of the 0.85 kb Eco RI fragment of subclone pHr02C (insert DNA from genomic clone λHr02) containing orphon rDNA (H28S-01) is presented. Comparison is made with a portion of human 28S rDNA (sequence and numbering from Gonzalez et. al., 1985).

The region extending from 798-823 of the H28S-01 sequence of λHr02 is also homologous to a portion of the Hind III sequence of Manuelidis, (1981) extending from nucleotide 146-170.

Figure 4.5

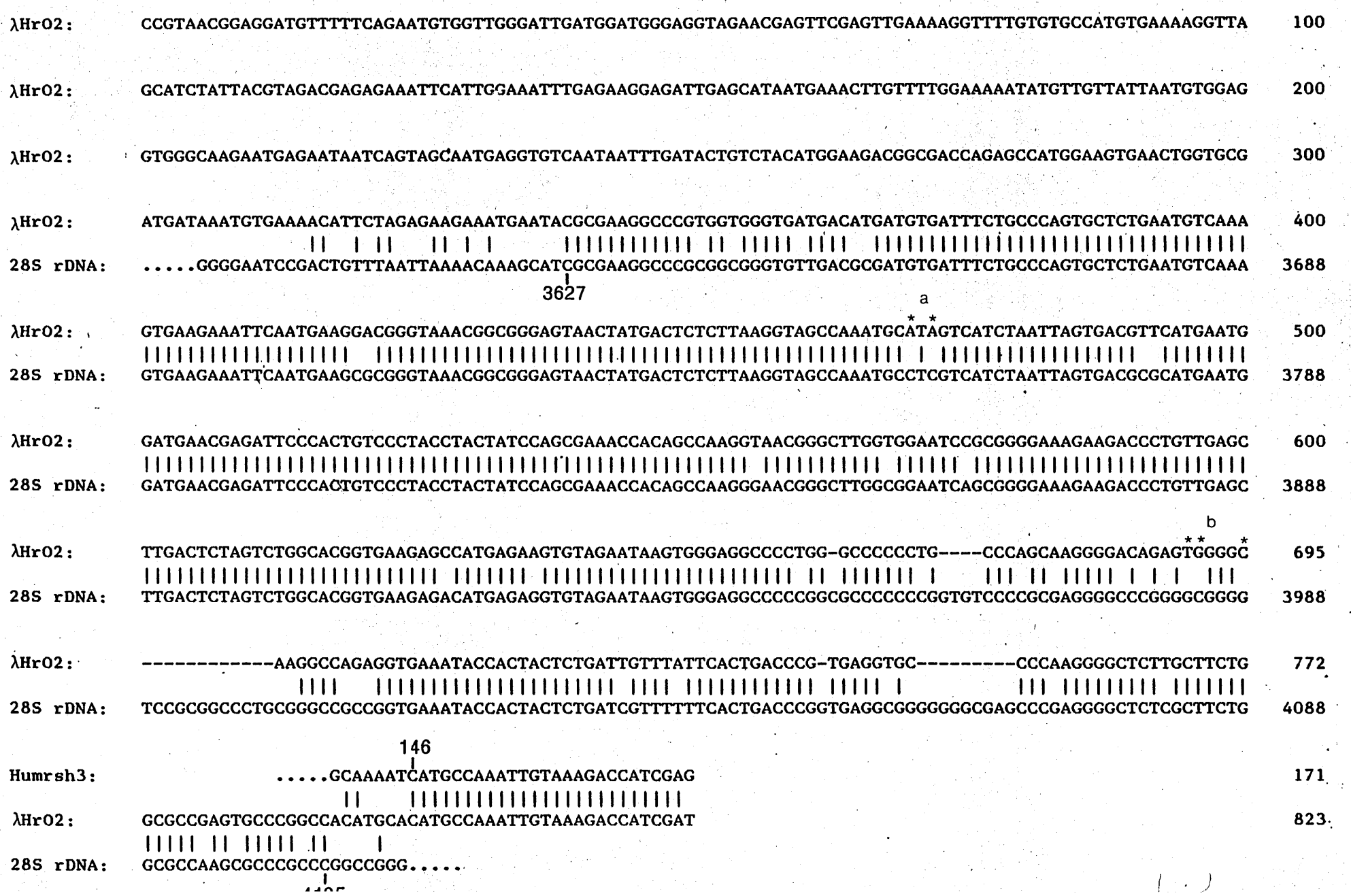

λHr02: CCGTAACGGAGGATGTTTTTCAGAATGTGGTTGGGATTGATGGATGGGAGGTAGAACGAGTTCGAGTTGAAAAGGTTTTGTGTGCCATGTGAAAAGGTTA 100
λHr02: GCATCTATTACGTAGACGAGAGAAATTCATTGGAAATTTGAGAAGGAGATTGAGCATAATGAAACTTGTTTTGGAAAAATATGTTGTTATTAATGTGGAG 200
λHr02: GTGGGCAAGAATGAGAATAATCAGTAGCAATGAGGTGTCAATAATTTGATACTGTCTACATGGAAGACGGCGACCAGAGCCATGGAAGTGAACTGGTGCG 300
λHr02: ATGATAAATGTGAAAACATTCTAGAGAAGAAATGAATACGCGAAGGCCCGTGGTGGGTGATGACATGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAA 400
28S rDNA: .....GGGGAATCCGACTGTTTAATTAAAACAAAGCATCGCGAAGGCCCGCGGCGGGTGTTGACGCGATGTGATTTCTGCCCAGTGCTCTGAATGTCAAA 3688
3627
a
λHr02: GTGAAGAAATTCAATGAAGGACGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAGCCAAATGCATAGTCATCTAATTAGTGACGTTCATGAATG 500
28S rDNA: GTGAAGAAATTCAATGAAGCGCGGGTAAACGGCGGGAGTAACTATGACTCTCTTAAGGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATG 3788
λHr02: GATGAACGAGATTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGTAACGGGCTTGGTGGAATCCGCGGGGAAAGAAGACCCTGTTGAGC 600
28S rDNA: GATGAACGAGATTCCCACTGTCCCTACCTACTATCCAGCGAAACCACAGCCAAGGGAACGGGCTTGGCGGAATCAGCGGGGAAAGAAGACCCTGTTGAGC 3888
b
λHr02: TTGACTCTAGTCTGGCACGGTGAAGAGCCATGAGAAGTGTAGAATAAGTGGGAGGCCCCTGG-GCCCCCCTG----CCCAGCAAGGGGACAGAGTGGGGC 695
28S rDNA: TTGACTCTAGTCTGGCACGGTGAAGAGACATGAGAGGTGTAGAATAAGTGGGAGGCCCCCGGCGCCCCCCCGGTGTCCCCGCGAGGGGCCCGGGGCGGGG 3988
λHr02: ----------AAGGCCAGAGGTGAAATACCACTACTCTGATTGTTTATTCACTGACCCG-TGAGGTGC---------CCCAAGGGGCTCTTGCTTCTG 772
28S rDNA: TCCGCGGCCCTGCGGGCCGCCGGTGAAATACCACTACTCTGATCGTTTTTTCACTGACCCGGTGAGGCGGGGGGGCGAGCCCGAGGGGCTCTCGCTTCTG 4088
146
Humrsh3: .....GCAAAATCATGCCAAATTGTAAAGACCATCGAG 171
λHr02: GCGCCGAGTGCCCGGCCACATGCACATGCCAAATTGTAAAGACCATCGAT 823
28S rDNA: GCGCCAAGCGCCCGCCCGGCCGGG.....

Illustration of some of the points of mutation in the nucleotide sequence of pHr02C

Autoradiographs illustrating some of the points of mutation in the nucleotide sequence of pHr02C (H28S-01) relative to the human 28S rDNA sequence (Gonzalez et. al., 1985) are presented. Autoradiograph a corresponds to region a on Figure 4.5. Autoradiograph b corresponds to region b on Figure 4.5.

Figure 4.6

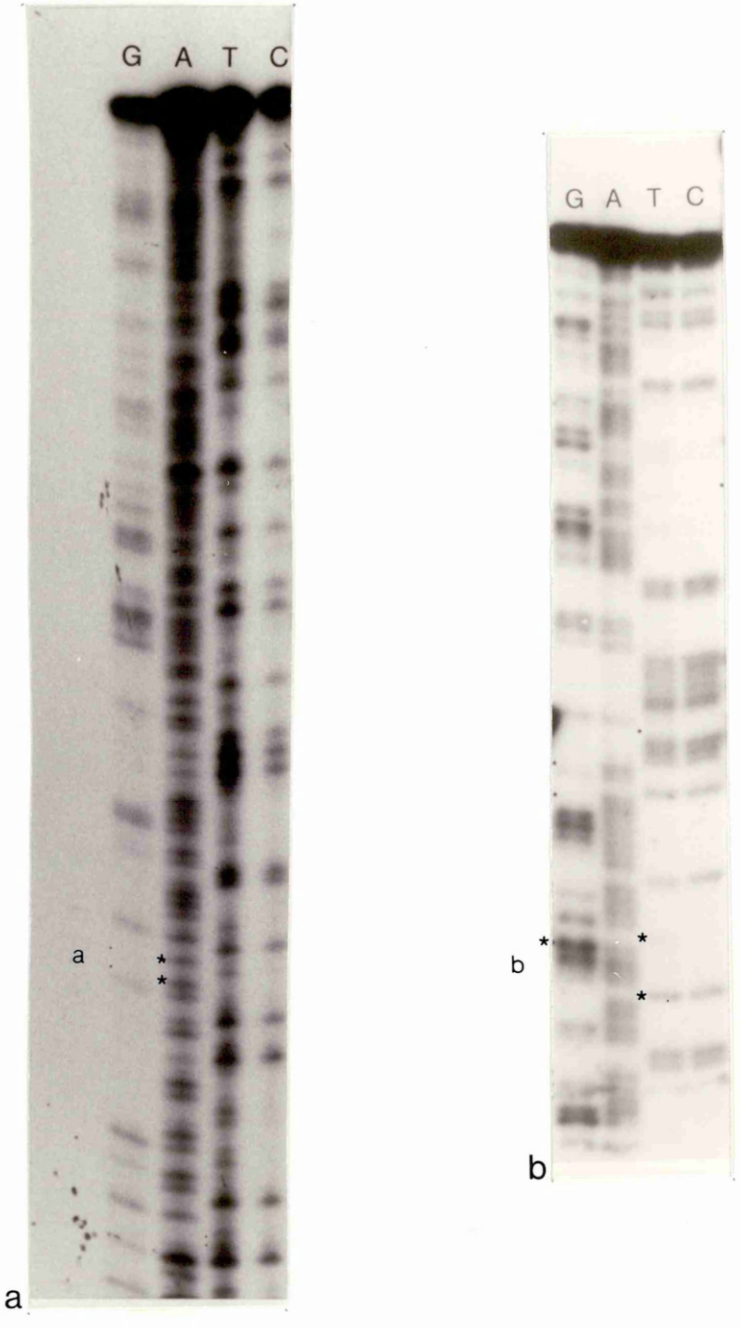
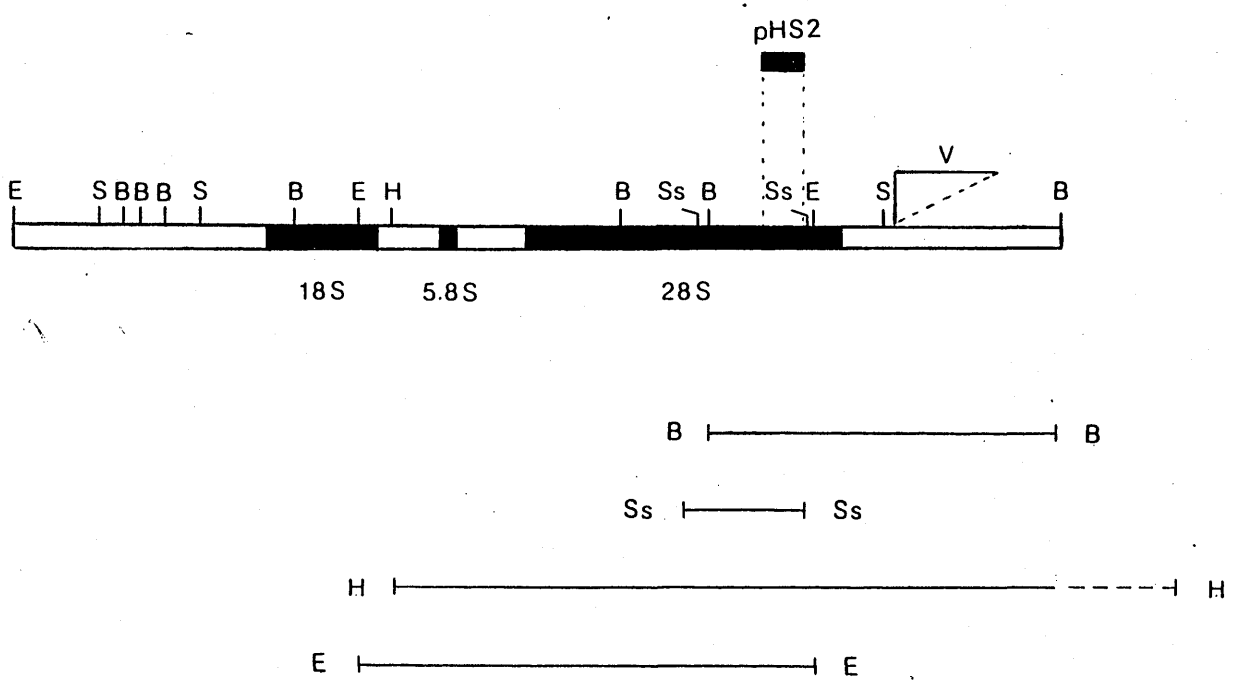


Figure 4.7

Restriction map of the human rDNA repeat

The restriction map presented is taken from Erickson et. al., (1981). The region recognised by pHS2 is indicated by the shaded area (above the map). Shown below the map are the regions of the rDNA repeat which hybridised to pHS2 during Southern blot/ hybridisation analysis of HeLa DNA using pHS2 cDNA insert as probe (see also Figure 3.23).

Figure 4.7



B : Bam HI
E : Eco RI
H : Hind III
S : Sal I
Ss : Sst I
V : Variable region

polymorphism, then two fragments should have also been observed in the Hind III digestion, as the Hind III fragment which hybridised also encompasses this non-transcribed spacer region (Figure 4.7). Clearly this was not the case. However, even taking into consideration the presence of this variable region, the size of the Bam HI and Hind III fragments observed in both HeLa DNA (Figure 3.23) and the genomic clones (Figure 3.22) are still too different to represent the same region.

Wilson et. al., (1979) have shown that the 7.3 kb Eco RI fragment (which contains the region recognised by pHS2: Figure 4.7) of the rDNA repeat appears to remain constant in size between different tissues. Thus the difference in size of Eco RI fragments between the genomic clones described here and the rDNA repeat does not seem to be a tissue-specific feature which might explain a difference between clones derived from lymphocyte DNA and total genomic HeLa DNA.

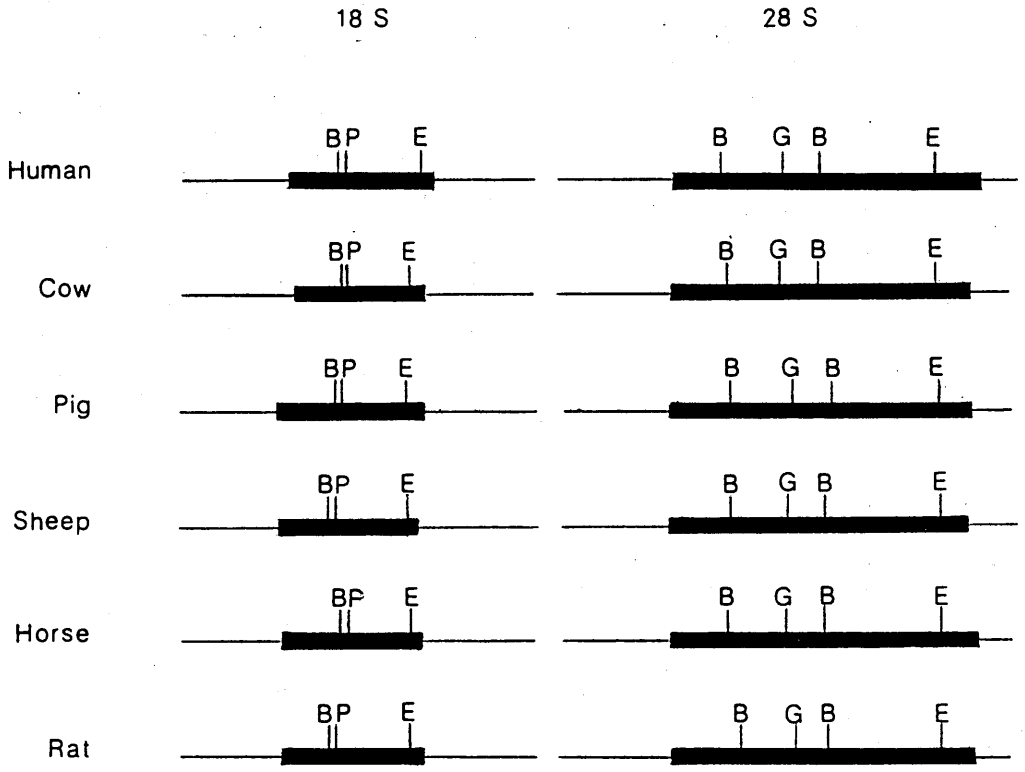
In higher vertebrates, a conservation of certain restriction sites has been observed within 28S rDNA (Tanhauser et. al., 1986). These sites include an Eco RI site present at the 3' end and a Bgl II site positioned in the middle of these 28S rDNA genes (Figure 4.8). In addition, one of two Bam HI sites is also conserved, being located approximately 850 base pairs 5' to the Bgl II site, while the other is positioned between the Bgl II and Eco RI sites. Comparison of these conserved restriction sites within 28S rDNA revealed no obvious similarities with the restriction endonuclease maps of genomic clones λHr02, λHr03 and λHr06 (designation described below) in the area immediately surrounding the region of hybridisation of pHS2. This was consistent with a comparison of the restriction maps of the genomic clones with a more detailed restriction

Figure 4.8

Conserved sites within vertebrate 28S rRNA genes

This figure is taken from Tanhauser et. al., (1986) and shows conserved restriction sites within 28S rRNA genes of vertebrates.

Figure 4.8



Key

B : Bam HI

E : Eco RI

G : Bgl II

P : Pst I

Scale : 1 kb
1.3 cm

map predicted from the nucleotide sequence of human 28S rDNA (Gonzalez et. al., 1985) which also revealed no similarity (Figure 4.9).

How then does one account for the fact that no members of the human rDNA repeat were isolated during the original screening of the lambda library? It is clear that a higher proportion of rDNA clones, containing members of the tandem rDNA repeat, should have been isolated as it has been estimated by hybridisation methods that there are 100-450 copies of ribosomal genes per diploid genome (Schmickel, 1973; Gaubatz and Culter, 1975; Young et. al., 1976). It is possible that the mode of construction of the EMBL3 library (Frischauf et. al., 1983) has selected against this tandem rDNA repeat. The number, grouping or endonuclease sensitivity of Sau 3AI sites within the tandem rDNA repeat may have resulted in Sau 3AI fragments too small or too large to be cloned into the EMBL3 vector.

This rDNA pseudogene fragment in λHr02 does not appear to be immediately flanked by other rDNA sequence (see below). The question arises, however, could this pseudogene be associated with the tandem repeat, as for 5S rRNA (Jacq et. al., 1977), or is it a dispersed copy, an 'orphon.' The name 'orphon' was coined to describe dispersed members of both protein-coding and non protein-coding tandemly-repeated multigene families (Childs et. al., 1981). Evidence which confirms it to be an orphon is as follows. A comparison of the 3' and 5' flanking sequences with sequences in the Genbank database has shown that this rDNA pseudogene is clearly bound by non-ribosomal DNA. Although this rDNA fragment is very near to the 3' end of the insert in subclone pHr02C, it has been established that the 3' flanking sequence exhibits homology to a region extending from 146-

Figure 4.9

A comparison of the restriction map of 28S rDNA with those of λHr02, λHr03, λHr05 and λHr06.

The restriction map of 28S rDNA was predicted from the nucleotide sequence of Gonzalez et. al., (1985). A comparison of this with the restriction map of λHr05 and composite map of λHr02, λHr03 and λHr06 is shown. Dotted areas indicate the region of homology with pHS2.

Key

B : Bam HI

C : Cla I

G : Bgl II

H : Hind III

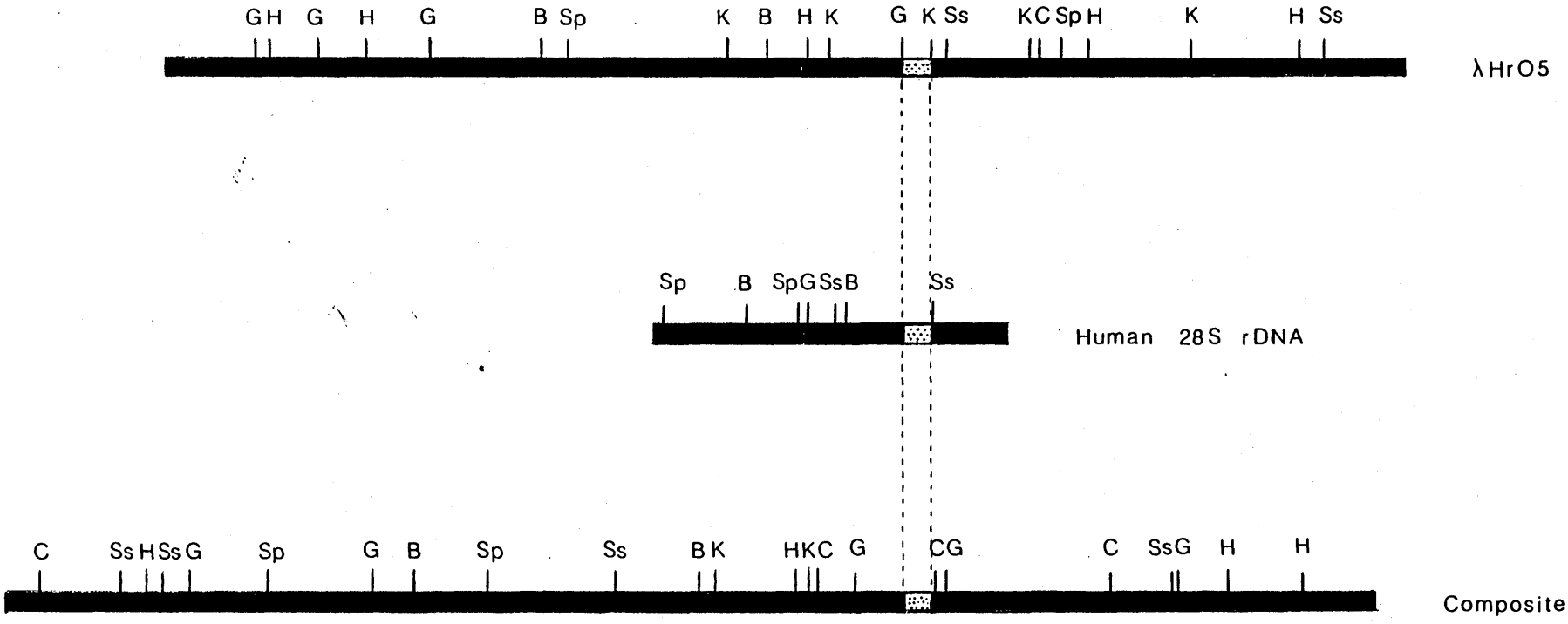
K : Kpn I

Sp : Sph I

Ss : Sst I

Scale 1cm = 1kb

Figure 4.9


G H G H G B Sp K B H K G K Ss K C Sp H K H Ss
λHrO5
Sp B Sp G Ss B Ss
Human 28S rDNA
C Ss H Ss G Sp G B Sp Ss B K H K C G C G C Ss G H H
Composite map
λHrO2, λHrO3 ar
λHrO6

170 of the 1.9 kb Hind III repeat described by Manuelidis,(1982) (Figure 4.5). The 1.9 kb Hind III sequence is repeated approximately 3500 times in the human genome and is now recognised to be part of the 6-7 kb long interspersed repeat L1Hs (LINE) family (Singer and Skowronski, 1985). Analysis of the 5' flanking region of the orphon does not reveal homology to any known sequence, however. Further comparison of the restriction map of the genomic clone λHr02, and also those of λHr03 and λHr06, upstream and downstream of the area recognised by pHS2, with the map predicted from the 28S rDNA sequence of Gonzalez et. al., (1985) also shows no similarities. This was consistent with Southern blot evidence. Restriction digests of the lambda clones probed with labelled fragments derived from the genomic rDNA clone pA4 (Erickson et. al., 1981) also show no homology (Figure 3.38). The probes used were a Bam HI fragment encompassing nucleotides 1405-2839 of 28S rDNA, 5' to the region corresponding to the pseudogene; and an Eco RI/Bam HI fragment which contains the 3' end of the 18S rRNA gene, the transcribed spacer and the 28S rRNA gene up to nucleotide 1405 (Figure 3.36). Neither of these probes hybridised to the genomic clones, even though they were of high enough specific activity to detect the rDNA repeat in an Eco RI digest of total HeLa cell DNA (not shown). Although it is possible that areas of the rDNA not probed for are present in the genomic clones, it is clear that this pseudogene is not in the immediate proximity of any of the tandem human rDNA repeats.

As this rDNA pseudogene in λHr02 satisfied the criteria for being an orphon, it was designated H28S-01 (human (H) 28S rDNA (28S) orphon 1 (01)). The genomic clones were designated λHr02-6 (bacteriophage lambda clones (λ) of human genomic DNA (H) containing a ribosomal orphon (r0). The numbers were arbitrary and the sub-clones, e.g. pHr02C (plasmid clones (p) containing, e.g. a Cla I

fragment (C) of genomic clone λHr02 (Hr02)).

The clone λHr05 also appears to contain a dispersed pseudogene as, although it has not been subjected to nucleotide sequence analysis, it was found that it too did not hybridise to either of the two probes from pA4 mentioned above (Figure 3.38). The restriction map is dissimilar to that of the other lambda clones studied in this work but neither does it bear any resemblance to that of human 28S rDNA (Gonzalez et. al., 1985)(Figure 4.9).

While λHr04 has not been subjected to restriction mapping, nucleotide sequence analysis, nor been probed with fragments from pA4, it is possible that it too contains a dispersed rDNA sequence. However the fact that it possesses even less homology to pHS2 than the other genomic clones, as seen from Southern blot studies (Figure 3.7), sugests that it is an even more diverged rDNA pseudogene.

Although no further rDNA sequence appears to be present in the genomic clones described here, there do appear to be regions of highly repeated DNA present in the vicinity of the pseudogenes contained within these clones. Whether these represent other regions of the LINE family has yet to be established, although an initial comparison of restriction maps suggests this may not be the case (Figure 4.10).

Possible ways by which H28S-01 (and the orphons within the other genomic clones) may have been generated are as follows. Childs et. al., (1981) have suggested that their histone H3 orphon may have arisen through a DNA-mediated mechanism. Tandem repeat multigene families are known to undergo frequent unequal crossing-over, which serves to homogenise these genes (Fedoroff, 1979; Kedes, 1979; Petes, 1980; Szostak and Wu, 1980). Childs et. al., (1981) proposed that as a consequence of a possible misalignment of the

Figure 4.10

Comparison of the restriction map of L1 element, L1Hs, with the composite map of λHr02, λHr03 and λHr06.

The restriction map of L1Hs is taken from Singer and Skowronski, (1985). A comparison of this with the composite map of λHr02, λHr03 and λHr06 is shown. Region of homology is bounded with vertical dotted lines.

Key

B : Bam HI

C : Cla I

E : Eco RI

G : Bgl II

H : Hind III

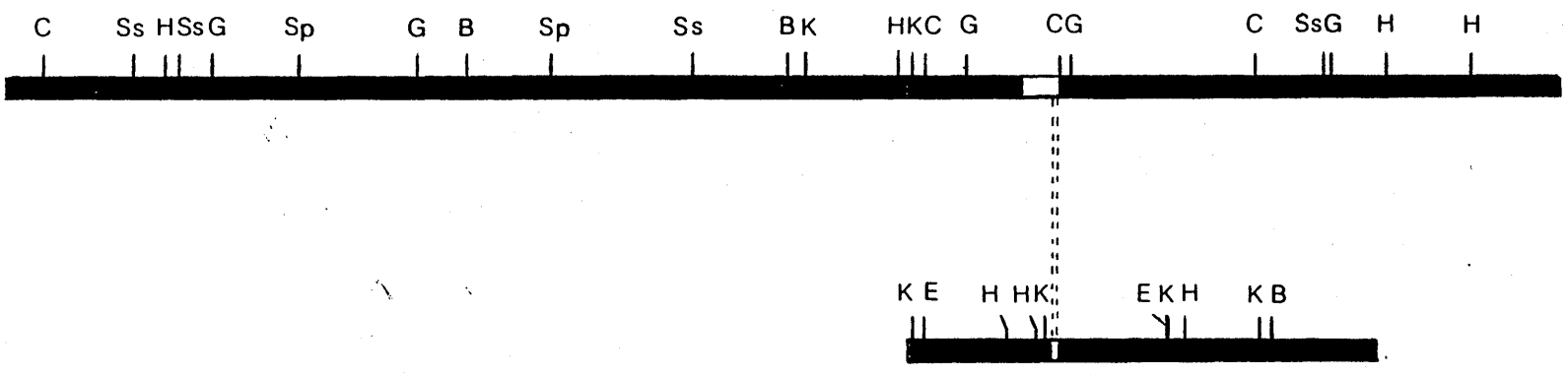
K : Kpn I

Sp : Sph I

Ss : Sst I

Scale : 1cm = 1kb

Figure 4.10



Composite map

λHrO2 λHrO3

λHrO6

Composite map

primate L1 ele

histone tandem repeats during recombination, looped-out segments might be formed which might then be integrated non-specifically into the genome, as has been seen when exogenous DNA fragments are appplied to mammalian cells (Wigler et. al., 1977; Maitland and McDougall, 1977). It is possible that this may be the case for H28S-01 as it is from an area within 28S rDNA.

A different mechanism, suggested for the generation of certain other orphons, involves reverse transcription of mRNA. Both a Drosophila histone pseudogene (Maxson et. al., 1983) and an early histone H2B orphon from sea urchin Strongylocentrotus purpuratus (Liebermann et. al., 1983) appear to have been generated by this mechanism. Both have a poly(A)tract at their 3' ends (even though histone mRNAs are not normally polyadenylated) and the S. purpuratus orphon is flanked by six-nucleotide direct repeats which indicate that it integrated at a staggered chromosomal break. An alternative possibility for the origin of H28S-01 is that it has arisen through such an RNA mediated mechanism, although in this case it may have been derived through reverse transcription of an rRNA fragment, which was subsequently integrated into the genome. The fact that pHS2 is a cDNA copy of 28S rRNA strongly argues that fragments of rRNA have the secondary structure potential to prime reverse transcription in vitro, and there is no reason to doubt that under suitable conditions they could do likewise if present in vivo. It is known that certain reversible stress conditions prevent conversion of nucleolar 45S ribosomal precursor RNA to mature cytoplasmic RNA (Warcoquier and Scherrer, 1969) and under such conditions rRNA fragments of the size of orphon 28S rDNA might occur as intermediates in the degradation of 45S pre-RNA. By reverse transcription such fragments could give rise to orphons if inserted into the germ

line. While Northern blot analysis (Figure 4.4) suggests that no fragments of the size of H28S-01 appear to be generated during heat shock, they may be formed during normal degradation processes of rRNA molecules.

Both the possible mechanisms described above involve non-specific integration into DNA. The target for this in the present case may have been part of the human L1Hs (LINE) family (Hind III repeat) which flanks one side of H28S-01. However as there are no L1Hs sequences immediately flanking the other side, and no direct repeats (which are characteristic of transposition) it cannot be certain that this was the case here. H28S-01 has clearly been subjected to a number of mutational events since its generation and it is possible that as a consequence of such events flanking direct repeats it may have originally possessed have been lost..

Do pseudogenes such as H28S-01 have a function? Some workers have suggested that by shuffling preformed sequences about the genome (exons have been particularly discussed in this respect) new functions can be acquired more quickly by the organism. Many such events may be unproductive however, and give rise to functionless sequences that accumulate mutations which lead to their eventual disappearance from the genome. Pseudogenes such as H28S-01 would seem more likely to belong to this latter category.

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