THE UNIVERSITY OF HULL

" Studies on the Mitochondrial DNA of
Tetrahymena."

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Summary.

The mitochondrial DNA from the ciliate protozoan Tetrahymena furgasoni str. W/ATCC. was isolated and mapped using six restriction enzymes. The linear molecule was seen to be approximately 35 Md. in size, slightly larger than the mtDNA seen in other Tetrahymena strains.

The T. furgasoni mtDNA molecule also showed a heterogeneity in the length of its terminal regions, a characteristic of Tetrahymena mtDNA.

The position of the mitochondrial rRNA genes were mapped on the molecule by hybridization studies using isolated mitochondrial rRNA's. The T.furgasoni mtDNA molecule possesses two large rRNA genes, one at each end of the molecule in sub-terminal locations, and a single small rRNA gene. A third, incomplete large rRNA gene was also found. The presence of this extra, incomplete rRNA gene may indicate why T.furgasoni mtDNA is slightly larger than the mtDNA from other Tetrahymena strains.

The terminal Hind III restriction fragment from the mtDNA of T.pyriformis was cloned using the vector pJB 8. Three copies of the fragment were cloned. These three recombinant molecules were different in size, a difference which was associated with the size of the original terminus of the mtDNA molecule. DNA sequencing studies showed the difference in length to be associated with a variation in the number of copies of a 31 bp repeat sequence present at the original terminus of the mtDNA molecule.

The significance of this mtDNA terminal structure is discussed with respect to the completion of replication of the linear mtDNA molecule, and to the generation of the terminal length heterogeneity of the linear mtDNA molecule. The structural characteristics of the Tetrahymena mtDNA terminus are compared with the structures seen at the termini of other linear genetic elements from a variety of sources.

INTRODUCTION

Mitochondrial Biogenesis: General Introduction

In 1949, an unusual type of non-mendelian inheritance was reported in yeast. The appearance of a small-colony mutant phenotype appeared to be maternally inherited. The suggestion was made that a cytoplasmically inherited genetic element or particle must be present (Ephrussi et al., 1949) to account for this characteristic.

These "petite" mutants were found to lack a functional respiratory chain. The localisation of cytochrome oxidase activity to the mitochondria labelled these organelles as a possible site for this cytoplasmically inherited element or its expression (Slonimski and Ephrussi, 1949).

In 1958, studies on cell protein synthesis indicated that the mitochondria possess their own distinct protein-synthesizing machinery producing a limited number of proteins (M'clean et al., 1958). In order to investigate this phenomenon, work on elucidating the properties of the translation system and identifying the particular products in question was initiated. This led to the discovery that mitochondria possess their own ribosomes which are distinct from those of the cytoplasm in size, structure and antibiotic sensitivity and upon which specific mitochondrial proteins are translated.

It has been shown that mitochondria contain their own double-stranded DNA molecules (Nass et al., 1963). Studies on the mitochondrial DNA (mtDNA) of the yeast petite (respiratory deficient) mutants showed them to have an altered buoyant density when compared with the wild-type mtDNA (Mounolou et al., 1966). Further work on these petite mutants has now identified

the mitochondrially expressed functions affected by the petite mutations as being encoded by the mtDNA molecule itself. These mitochondrial genes are transcribed and translated on the mitochondria's own translational apparatus to produce products vital to mitochondrial function.

Size and Structure of mtDNA molecules

Mitochondrial DNA has been seen in every eukaryote investigated to date. The size and structure of the mtDNA appears to vary considerably across the eukaryotic kingdom (for a review see Wallace, 1982).

Animal mtDNA

The mtDNAs of animal cells show little variation in size and structure. All are approximately 5 μm contour-length circles. Many different phyla have been examined, spanning the animal kingdom. The only notable variation seen to date appears between species of *Drosophila*. The mtDNA of Drosophila appears to vary between 5.0 μm circles seen in, for example, *Drosophila erectus*, and 6.2 μm circles seen in *Drosophila melanogaster*. Intermediate sizes are seen in other Drosophila species (Fauron & Wolstenholme, 1976). This diversity is accounted for by a variation in the size of an A + T rich region about the replicative origin in Drosophila mtDNAs (Fauron *et al*., 1980).

Protist mtDNA - Unicellular Eukaryotes

The size and nature of protist mtDNAs tend to vary widely, as may be expected with such an arbitrary taxonomic classification. The algae *Chlamydomonas reinhardtii* possesses the smallest mtDNA molecules with circles of 10 Md (Mega-daltons) molecular weight. A yeast *Brettenomyces custerii* possesses the largest

mtDNA molecules with circles of 70 Md (Clark-Walker et al., 1981). Closed circular mtDNAs are common, ranging in size between these two extremes according to the species. In many cases covalently closed circular mtDNAs have proved to be difficult to isolate intact. Linear mtDNAs have been found in the ciliate protozoans Tetrahymena (Flavell & Follet, 1970) and Paramecium (Goddard & Cummings, 1975) and possibly in one yeast Hansenula mrakii (Wesolowski & Fukuhara, 1981).

In the Trypanosomatidae, a single giant mitochondria is seen. The mtDNA found in this organelle is an extensive network of large and small circles (maxi and mini-circles) linked together to form the complex "kinetoblast" structure. The maxicircles have been shown to be similar in composition to the mtDNAs characterised in other organisms. The function of the mini-circles remains unknown (Englund et al., 1982).

Plant mtDNA

Many plants possess large mtDNA molecules both linear and circular in structure, ranging in size from about 70 thousand base pairs (Kbps) to hundreds of Kbps in length with numerous molecular sizes present in a single species (Dale, 1981; Levings & Pring, 1979; Ward et al., 1981). The proportion of circular molecules recovered does appear to vary with the method of preparation.

Restriction digest studies on these heterogeneous populations show numerous fragments present in greater-than equimolar proportions, indicating possible repetitive elements present in these large molecules (Ward et al., 1981).

Plant mtDNAs appear to be both complex and "fluid". Two cultured cell lines drawn from the same strain can produce

identical restriction digest products, yet will appear as two distinct populations differing in the size of the whole mtDNA molecules. The smaller mtDNAs show homology to the larger molecules present in these heterogeneous populations.

Two suggestions have been made to explain these observations:

(i) The plant mtDNA molecules originated from a single sized population which has amplified, duplicated and re-arranged into a complex heterogeneous population of molecules;

(ii) an original heterogeneous population of molecules has exchanged and recombined to a large degree so that all members of the population now show considerable homology.

Studies on the translation products of the plant mitochondrial system indicate that a larger number of polypeptides may be produced compared with the number produced in animal or fungal systems. This may mean that these large plant mtDNA molecules may in part be comprised of genes not seen in other systems (Leaver et al., 1982).

Mitochondrial DNA Function

Genes coded for by the mtDNA

The use of antibiotics and inhibitors specific for points in the respiratory chain, and for mitochondrial protein synthesis allowed the identification of the components of the yeast mitochondrial system affected by the petite mutations. Supported by biochemical studies this enabled the construction of a genetic map for the mitochondrially encoded characteristics. An important part of this work was the production of genetic crosses between petite mutants. This combination of the

yeast's unique ability to survive without mitochondrial respiratory function and the existence of mitochondrial genetics made such studies possible.

The development of sophisticated tools available to the molecular biologist has permitted the construction of physical maps of these different petite mutant mtDNA molecules, finally resulting in the primary structure of the genetically identified loci on the mtDNA molecule. Recombinant DNA clones of these characterised genes have been used as probes to map the loci of these genes on other mtDNA molecules from other sources. Complete nucleotide sequences are now available for human (Anderson et al., 1981) murine (Clayton et al., 1982) and bovine (Anderson et al., 1982) mtDNA molecules. Large stretches of the mtDNA molecule from yeast and Drosophila have also been sequenced.

A comparison of the location of the various genes coded for by the Human and Yeast mtDNA molecules is shown in Fig. 1.

Both molecules have been found to encode the following genes:

the large and small mitochondrial ribosomal RNAs (rRNAs);

the mitochondrial transfer-RNAs (tRNAs);

ATPase subunit No. 6;

Cytochrome oxidase subunits I, II and III; and

Apocytochrome 'b'.

The following two small genes, which are not seen in animal mtDNAs, are seen in fungal mtDNAs:

the Var I gene, coding for a ribosome-associated protein;

the ATPase subunit No. 9 gene (DCCD binding subunit).

Fig 1.

Diagrammatic representations of the mtDNA molecules from yeast and man are shown, to indicate their differences in organisation of the same mitochondrial genes. The broken lines on the yeast mtDNA molecule indicate large tracts of the molecule containing sequences whose function is as yet unknown.

Clear gaps between shaded regions of the yeast genes indicate intron regions.

Fig 1.

Mitochondrial DNA molecules from man and yeast, showing the mitochondrial genes and their organisation.

denotes a tRNA gene.

Sequence analysis has shown tracts of homologous sequence in mammalian and yeast mtDNA molecules not accounted for above. These code for as yet unidentified proteins which are translated in the mitochondria. They are referred to as "unidentified reading frames", and are numbered 1 to 6 (Anderson *et al*., 1982; Clayton *et al*., 1982).

Mitochondrial Genome Autonomy

It has been shown that no integrated copy of the mitochondrial DNA molecule is present in the nuclear genome (Tabak *et al*., 1972; Flavell & Trampé, 1973). However, a gene showing homology to the ATPase subunit 9 gene has been found in the nuclear genome of *Neurospora crassa* (Agsteribbe *et al*., 1982*a*). The nuclear gene has been shown to be expressed. The mitochondrial gene is transcribed, but not translated (Agsteribbe *et al*., 1982*b*).

Fragments of the Var I gene, the mtDNA replicative origin and a piece of the apocytochrome *b* gene have been found to have homologous sequences in the nuclear genome of yeast, present as a tight cluster of "apparently 'sewn-together" fragments (Farrelly & Butow, 1983). Other studies on *N. crassa* (Lambowitz *et al*., 1983) and sea urchin (Britten *et al*., 1983) suggest that short sequences of nuclear DNA homologous to sequences in the mtDNA molecule are perhaps not uncommon. The small or fragmented nature of these segments of DNA may indicate the inclusion into the nuclear genome of pieces of mtDNA resulting from mitochondrial damage.

Gene organisation on the mtDNA molecule

In *Saccharomyces cerevisiae* large portions of the mtDNA molecule have been shown not to be involved in coding for the

structural regions of the genes mentioned. Much of this extra DNA forms introns (intervening sequences) in the yeast mitochondrial genes (see Fig. 1) (Arnberg et al., 1980). The smaller Aspergillus nidulans mtDNA molecule also possesses introns in its mitochondrial genes, but there are fewer introns present than in yeast mitochondrial genes and they are shorter in length than the corresponding introns in the yeast gene (Kochel et al., 1982; Brown et al., 1982).

The ordering of the named mitochondrial genes is different in A. nidulans, S. cerevisiae and N. crassa mtDNAs. The ordering of the mitochondrial genes in various mammalian mtDNAs are identical. As Fig. 1 indicates, the ordering of fungal and mammalian mitochondrial genes differ. Regions of Drosophila mtDNA so far characterised show a different gene order to the mammalian mtDNAs (de Bruijn, 1983).

Animal and fungal mtDNAs differ in other respects also. Mammalian mtDNAs show no introns in the mitochondrial genes. Fungal mtDNAs show extra sequence elements present between the mitochondrial genes, the function of these extra elements is unknown (Bernardi, 1980, 1982). Drosophila mtDNA possesses very few extra bases between the mitochondrial genes (de Bruijn, 1983). Mammalian mtDNAs show no extra bases between the mitochondrial genes, being very efficient in the usage of space on the mtDNA molecule (Anderson et al., 1982; Clayton et al., 1982).

The essential difference between the mtDNA molecules of the organisms studied in depth appears to be a difference in the organisation of the same mitochondrially encoded functions.

Mitochondrial Gene Expression

Transcription. Mitochondrial RNA polymerase is encoded in the nuclear genome, synthesized in the cytosol and imported into the mitochondria. In mammalian systems the mtDNA molecule is transcribed as a single, genome length transcript. Both strands are transcribed (Murphy et al., 1975). The tRNA genes appear to act as spacers in the transcript and may serve to function as cleavage sites during processing of the transcript (Attardi et al., 1980). Stable transcripts are produced from most of the H-strand (precursor) transcript. Large and small rRNAs and tRNAs are produced directly by cleaving the large precursor transcript. Base modifications and additions are made post-transcriptionally. mRNA transcripts are polyadenylated (Ojala et al., 1980). The L-strand transcript is largely degraded and apart from tRNAs only one stable mRNA transcript is seen. This is transcript No. 18 (mitochondrial transcripts are numbered in order of decreasing size). This small, polyadenylated transcript is present in considerable quantities and its function is unknown (Amalric, 1978).

In yeast, at least five possible promoter sites have been identified on the mtDNA molecule (Levens et al., 1980). Stable transcripts for particular genes have been identified. Large precursor mRNAs are also present, with an accompanying series of smaller mRNAs conforming to the stages of intron removal and mRNA processing (Schweyen et al., 1982).

Translation. Mitochondrially encoded genes are translated on the mitochondria's own translation system. Mitochondrial ribosomes contain mitochondrially transcribed rRNAs. All the ribosomal proteins are synthesized in the cytosol and imported into the mitochondria, except the Var I protein.

The other protein components and translation factors required are also imported (Barath & Küntzel, 1972; Küntzel, 1967). Mitochondrial ribosomes are smaller than their cytoplasmic counterparts with smaller rRNAs and fewer protein subunits. Animal and protist mitochondrial ribosomes lack a 5 s rRNA. Mitochondrial ribosomes show a sensitivity to chloramphenicol, an insensitivity to cycloheximide and generally show more relationship to prokaryote ribosomes than to eukaryote ribosomes.

Twenty-two tRNAs are coded for in the mammalian mtDNA molecule (Anderson et al., 1982; Clayton et al., 1982) and twenty-four tRNAs are coded for in the yeast mtDNA molecule (Bonitz et al., 1980).

Sequence analysis of these mtDNA molecules has shown that the genetic code found in the mtDNA systems investigated to date varies from that of the "universal" genetic code.

Mitochondrial Genetic codes

The wobble hypothesis (Crick, 1966) requires a minimum of thirty-two tRNAs to read the necessary number of codons. Mitochondrial systems appear to achieve a reduction in the number of required tRNAs (Bonitz et al., 1980; Barrel et al., 1980) by the presence of an unmodified uridine base in the (5') wobble position in some of the tRNA anticodons (Roe et al., 1982). Any tRNAs possessing a modified uridine in the anticodon wobble position will read only two possible bases in the (3') codon position. Any tRNAs possessing an unmodified uridine in the anticodon (5') wobble position will read all four bases in the (3') codon position. In bovine tRNAs a uridine present

in the wobble position is always unmodified, but modified bases immediately adjacent (5') to it appear to induce the same effects upon the wobble position base.

Differences within the mitochondrial code are also seen in different species. Yeast possesses two arginine codons, (tRNAs) mammalian systems possess only one. Drosophila appear to possess their own mitochondrial codon, AGA, for serine. This functions as an arginine codon in the universal code and in fungal mitochondrial systems. AGA codes a stop codon in mammalian mitochondria, etc.

Whether this simplification of the genetic code indicates that the mitochondrial system is in "genetic" isolation, or that selective pressure is encouraging the reduction in size of the mtDNA molecule is not known.

DNA Replication: General introduction

It has been shown that DNA polymerases are incapable of *de novo* synthesis of DNA. These enzymes require a primer upon which to extend the new polymerising DNA strand.

After strand separation of the parent DNA molecule a short RNA-containing strand is produced by an RNA polymerase or "primase". DNA synthesis is initiated on this primer. As the growing DNA strand nears the primer ahead of it the DNA polymerases' 5'-3' exonuclease activity removes this RNA primer. The discontinuous DNA strands are then ligated together to form a new daughter strand (Hirose *et al.*, 1972; Anderson *et al.*, 1977; Tseng & Goulian, 1977).

Mitochondrial DNA Replication

In mammalian mtDNA, a characteristic triple-stranded region is seen at the replicative origin.

The two strands of the mtDNA duplex are separated over a short distance in this region, and a new third strand pairs with the L-strand of the original duplex. The original H-strand in this region is not base-paired, resulting in the characteristic "D-loop" structure (Clayton, 1982). The D-loop structure acts as the replication origin with synthesis of the new DNA strand being initiated on the short extra (E-strand) third strand of the structure. Replication of the H-strand continues in a 'continuous" manner until an origin of replication for the L-strand is reached. Both strands now replicate as continuously polymerising strands until completion.

The precise nature of the priming event remains unknown, but since most non-replicating mammalian mtDNA molecules contain covalently incorporated ribonucleotides it is reasonable to assume that priming involves RNA synthesis. Recent evidence has shown the existence of ribonucleotides in the E-strand of the D-loop structure (Clayton, 1982).

In Drosophila mtDNA, no D-loop structures are seen, the origin of replication is flanked by A + T rich regions which vary in length between Drosophila species. The exact mechanism of the priming event is unknown.

Replication in yeast mtDNA is even less understood. Three replication origins are thought to exist in this large molecule (Blanc & Dujon, 1982).

DNA Replication in Linear Genetic Elements

As described, most mtDNAs occur as circular molecules. In the replication of a circular DNA molecule it is possible for all primers to be removed. However, a linear replicating

molecule would require additional events for the completion of its replication cycle (Watson, 1972).

The ciliate protozoans Tetrahymena and Paramecium have both been shown to possess linear mtDNA molecules. As the replication fork approaches the end of the molecule, that strand growing 5′-3′ continuously can be completed without further priming. The complementary strand which must be growing discontinuously must inevitably require some further priming event at its 5′ "outside" end. If this priming event involves an RNA strand, its removal would leave an uncompleted, single-stranded DNA region at the terminus of the molecule.

A naturally-occurring linear genetic element may also require other properties not applicable to circular molecules. Unlike the ends of DNA molecules created by chromosome damage or restriction digest, the ends of linear genetic elements, e.g. chromosome ends, are non-recombinogenic (McClintock, 1942; Orr-Weaver et al., 1981). This stable or inertial state implies that a terminal structure is present which is distinct from the termini created by DNA breakage.

Viral Linear DNA Replication

Viral linear DNAs have been extensively characterised. They show three types of structure present at the termini of the DNA molecules which overcome the defined lack of a terminal DNA primer.

(i) Provision of a priming deoxynucleotide at the terminus attached to a terminally-associated protein. As seen in the adenovirus, or in bacillus 'phage ø 29 (Rekosh et al., 1977; Forte & Fangman, 1979).

(ii) Presence of direct repeat sequences at the termini of the DNA molecule allowing circularisation of the molecule, as

seen in the Herpes simplex virus (Mocarski & Roizman, 1982).

(iii) Cross-linking of the strands at the ends of the molecule to create a hairpin terminal structure, as in the Vaccinia virus (Baroudy et al., 1982).

Linear Eukaryotic Genetic Elements

A structural model for a eukaryotic chromosome end (telomere) has been proposed which suggests that terminal interstrand cross-links exist. During replication, the strand replicating in a 5′-3′ continuous manner would continue to replicate around the end of the hairpin, effectively priming the other strand at the terminus.

Breaks then occur on one strand allowing the two newly replicated molecules to separate. The single-stranded regions so produced at the termini then fold back upon themselves to form the new terminal hairpin structure (Cavalier-Smith, 1974; Bateman, 1975).

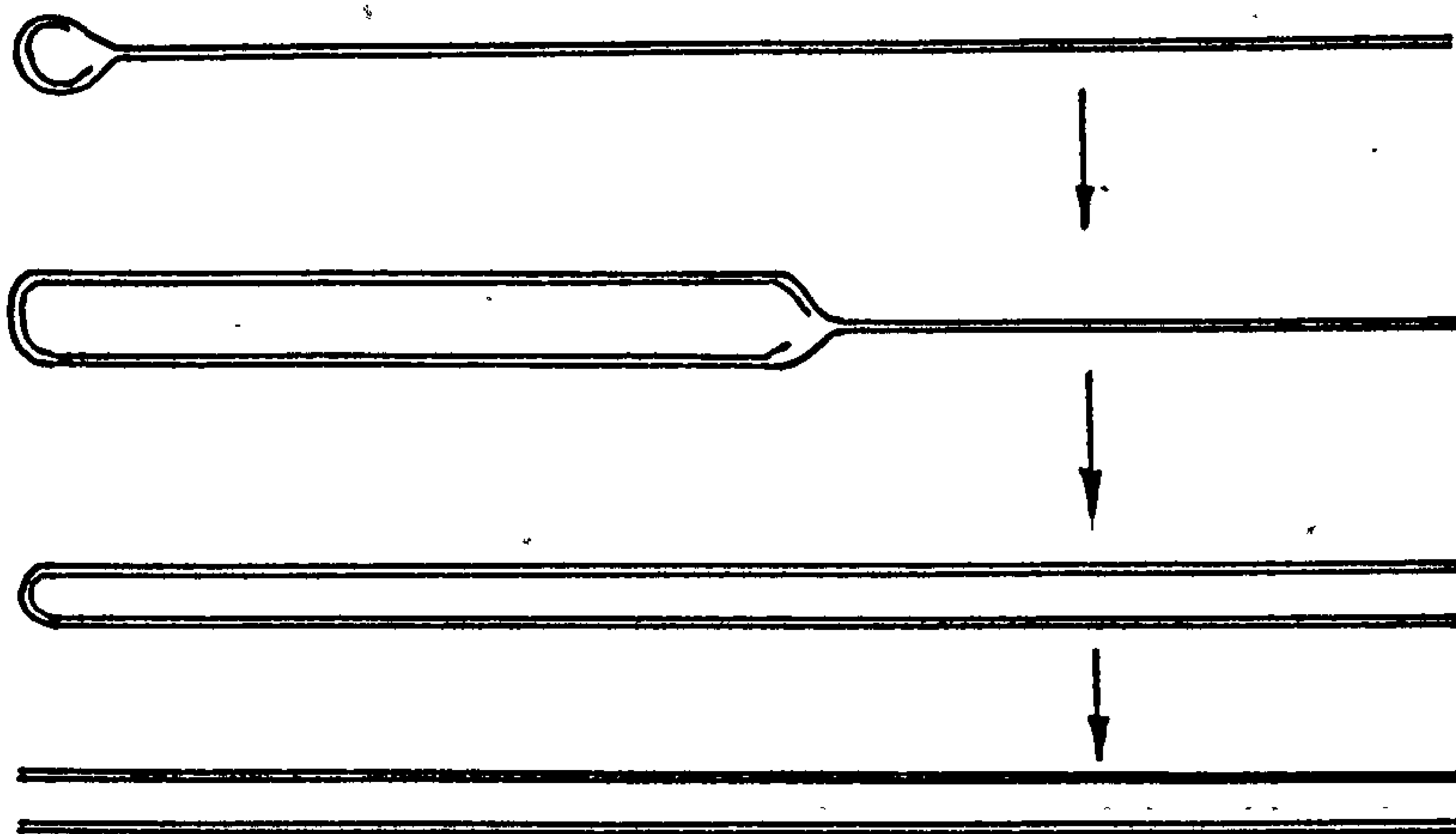
Such hairpin structures could label the ends of these molecules as being non-recombinogenic.

The structure of the termini of a number of eukaryotic linear genetic elements have been seen to bear a resemblance to this model (see Discussion).

Replication of Linear mtDNAs

Paramecium mtDNA

Paramecium mtDNA has been shown to be a linear duplex DNA molecule (Goddard & Cummings, 1975). Electron microscopic studies showed the replication of the molecule to proceed from one end via "lariat" structure replicative intermediates. This implies that a cross-link is present at the end of the molecule concerned with replication initiation.



Partial denaturation and sequence analysis studies have shown an A + T rich region at the origin of replication. The strand-strand cross-link removes the described problem of replication completion at this end of the molecule (Cummings & Pritchard, 1982).

Tetrahymena mtDNA

The mtDNA of Tetrahymena has been shown to be a linear duplex DNA molecule of ~30 x 10^6 daltons (Flavell & Follet, 1970; Suyama & Miura, 1968). Numerous attempts have been made to isolate circular molecules without success (Borst et al., 1974; Borst & Upholt, 1974; Jones, unpublished). Studies on the restriction endonuclease digest products of these molecules have shown the linear molecules to be non-circularly permuted (Goldbach et al., 1977), and are not the result of random breakage of a circular molecule. Electron microscopic studies have shown the replication origin to be near the middle of the molecule and that replication proceeds bi-directionally to the ends of the molecule (Borst et al., 1974; Borst & Upholt, 1974).

with the replication origin fixed in the middle of the molecule, and with no replicative intermediates longer than genome-length molecules observed, a mechanism for the completion of the replication cycle distinct from that seen for paramecium mtDNA must be present.

Studies on the hybridization of mitochondrial rRNAs to the mtDNA molecule using electron microscopic and isotopic labelling methods have shown the molecule to possess two copies of the large rRNA (21s rRNA) gene, one at each end of the molecule (Goldbach et al., 1978a,b). A single copy of the small rRNA (14s rRNA) gene is located 6-7 Md away from the nearest 21s rRNA gene. The location of these genes appears to be well conserved between various strains of Tetrahymena (Goldbach et al., 1978a; Norton, Holt & Jones, unpublished).

Electron microscopic studies have shown the mtDNA molecules to possess a large degree of length heterogeneity. This corresponds to the presence of two DNA fragments in the restriction digest pattern of the molecule which show a heterogeneity in length. These fragments have been "mapped" to the termini of the molecule (Goldbach et al., 1977).

A mechanism for the completion of the termini of the molecule during replication was proposed by Goldbach et al. (1979). It was suggested that short direct sequence repeats existed at the termini of the molecule along with single-stranded breaks or nicks. This mechanism also accounted for the observed length heterogeneity of the terminal restriction fragments (see Discussion).

The evidence presented for this mechanism was of structures observed in partial denaturation studies analysed by electron microscopy. The mechanism awaits further verification.

Introduction to Studies on Tetrahymena furgasoni strain W/ATCC mtDNA

When the mtDNA from *Tetrahymena furgasoni* str. W/ATCC was digested with Eco RI restriction endonuclease, the digest products produced were identical to those obtained by Goldbach *et al.* (1977) for *Tetrahymena pyriformis* str. GL/B mtDNA. This strain GL/B had shown an *extreme* heterogeneity in length of the terminal restriction fragments, and appeared to possess three copies of the large rRNA gene. Both these characteristics labelled this strain as one of interest.

It was proposed to construct a restriction map of strain W/ATCC mtDNA and to map the locations of the rRNA genes on the molecule. It was also decided to attempt to construct a library of cloned restriction fragments of this strain, as a start to further studies on the regions of the molecule not involved in coding for the rRNA genes.

Introduction to Studies on Tetrahymena pyriformis str. T mtDNA

The mtDNA molecule from this strain has been mapped and characterised (Norton, Holt & Jones, unpublished). The length heterogeneity of the terminal restriction fragments is neither extreme nor unusually short in this strain compared to other strains, giving the appearance of being an "average case".

It was proposed to attempt the further characterisation of the termini of the mtDNA molecule from this strain. Many of the models proposed for a mechanism of terminal replication completion in this and other systems suggest the existence of repeated sequence elements at the termini of these linear

molecules. In the case of Tetrahymena mtDNA the model also suggests that a variation in the number of such repeat sequence elements present at the termini is responsible for the observed length heterogeneity associated with the terminal restriction fragments.

To find evidence as to whether the termini of T mtDNA molecules possess such repeat elements it was proposed to attempt to clone the terminal restriction fragments of the molecule in a suitable molecular cloning vehicle. Once achieved, it was proposed to use this source of material to determine the nucleotide sequence of the terminal regions of the molecule using recently developed rapid DNA sequencing techniques.

Materials and Buffers.

Recombinant DNA Experiments

Safety and containment

After consultation with the Genetic Manipulation Advisory Group (G.M.A.G.) (Medical Research Council, Park Crescent, London) the proposed work was categorised as "requiring only good microbiological practice". This is defined in the document "Guidelines for Microbiological Safety", issued by the Consultative Committee for Microbiology.

The advised guidelines for work in this category were followed in all the recombinant DNA and microbiological experiments described in this project.

List of Abbreviations

The abbreviations used in the text are those recommended in the Biochemical Journal "Instructions to Authors". A summary of the abbreviations used which are not included in this publication is given below.

Amp^r	Ampicillin resistance
bp	base pair
DTT	Dithiothreitol
EDTA	Diaminoethanetetracetic acid, disodium salt
H-strand	Heavy (more dense) strand
Kbp	Kilo (10^3) base pair
L-strand	Light (less dense) strand
Md	Mega (10^6) daltons
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
p (prefix)	plasmid
poly C	Polycytidylic acid
poly G	Polyguanydilic acid
Rf	Replicative-form
rRNA	Ribosomal RNA
SDS	Sodium dodecyl-sulphate
SSC	Standard saline citrate
TEMED	N,N,N′,N′, tetramethylethylene diamine
Tet^r	Tetracycline resistance
TCA	Trichloroacetic acid
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol.

Tetrahymena Strains used in this Study

Tetrahymena furgasoni strain W/ATCC, was obtained from the American type culture collection* (often referred to in the text as simply "W/ATCC").

Tetrahymena pyriformis strain T, was obtained from the Culture Collection of Algae and Protozoa, Cambridge† (often referred to in the text as simply str. T).

* Present address: Rockville, Maryland, U.S.A.

† Present Address: 36, Storey's Way, Cambridge, England.

Interconversion of units used in mapping DNA molecules.

1 Md. = 1.5 Kbp.

Bacterial Strains and Cloning Vectors used in this Study

E. coli strains	Description of genotype	Reference
ED 8767	met hsd_k $S^-R^+M^+$, Sup E, Sup F, rec 56	Murray *et al.*, (1977)
JM 103	Δlac pro, thi, StrA, Sup E, endA, sbc B15, hsd R4, F′tra D36, pro AB lac I^q ZΔ M15	Messing *et al.*, (1981)

Plasmid cloning vectors	Reference
pAT 153	Twigg & Sherrat (1980)
pJB 8	Ish-Horowitz & Burke (1981)
pJC 79	Hohn & Collins (1980)
pUC 9	Messing & Vieira (1982)

'Phage cloning vectors (sequencing vectors)	Reference
M13 mp 8	Messing & Vieira (1982)
M13 mp 9	Messing & Vieira (1982)

Commonly used Media and Buffers

Growth Media

Tetrahymena Growth Medium

1% (w/v) Proteose/peptone (Difco)

0.25% (w/v) Yeast extract (Difco)

20 ml/litre 50X Salts solution

0.5 ml/litre Ferric citrate solution (0.67 mg/ml)

50X Salts Solution

50 g KH_2PO_4

50 g Na_2HPO_4

100 g NaCl

2.5 g $MgCl_2.6H_2O$

water to 1 litre

L-Broth (Bacterial growth medium)

1.0% (w/v) Bacto Tryptone

0.5% (w/v) Bacto Yeast Extract

1.0% (w/v) NaCl

pH to 7.5

L-agar

1.5% (w/v) Lab-'M' agar added to L-broth

L-Broth/L-agar plus antibiotics

Antibiotics were dissolved in water and passed through a pre-sterilized membrane filter. Antibiotics were added to L-broth immediately before use. Antibiotics were added to L-agar at 55°C, immediately before casting and pouring plates or stabs.

Antibiotic	Concentration used
Ampicillin	50 μg/ml
Tetracycline	20 μg/ml
Chloramphenicol	150 μg/ml

Minimal medium agar plus thiamine

10.5 g	K_2HPO_4
4.5 g	KH_2PO_4
1.0 g	$(NH_4)_2SO_4$
0.5 g	Na_3citrate.$2H_2O$
0.2 g	$MgSO_4.7H_2O$*
2 g	Glucose*
10 mg	Thiamine HCl (Vitamin B1)*
15 g	Bacto agar
	water to 1 litre

* These components were added separately, after filter sterilization, at 55°C immediately before casting and pouring plates or stabs.

Electrophoresis Buffers

Agarose/Agar gel Electrophoresis Buffer. 6x Stock solution

Tris Base	116.28 g
NaOAc (anhydrous)	39.36 g
Na_2EDTA	17.88 g
Glacial Acetic Acid	40 ml
water to 6 litres	

Ethidium bromide (10 mg/ml) added to ·5 μg/ml before use.

Sequencing gel electrophoresis buffer. 10x Stock solution

Tris Base	108 g
Boric acid	55 g
Na_2EDTA	9.3 g
water to 1 litre	

General purpose restriction endonuclease buffer, HX.

10x Stock solution.

NaCl	1.0 M
Tris/HCl, pH 7.5 at 20°C	0.5 M
$MgCl_2$	100 mM

Restriction endonuclease Hind III Buffer 10x Stock solution.

NaCl	0.5 M
Tris/HCl, pH 7.5 at 20°C	100 mM
$MgCl_2$	100 mM

Restriction endonuclease Sau 3A buffer 10x Stock solution.

NaCl	0.5 M
Tris/HCl, pH 7.5 at 20°C	60 mM
$MgCl_2$	60 mM

Large Fragment E. coli DNA Polymerase I buffer

10x Stock solution.

Tris/HCl, pH 7.5 at 20°C	70 mM
$MgCl_2$	70 mM
NaCl	200 mM
DTT	10 mM

Ligation Buffer 2x Stock solution

Tris/HCl, pH 7.5 at 20°C	130 mM
$MgCl_2$	13 mM

diluted immediately before use to include:

DTT	10 mM	(final concentration)
ATP	1 mM	(final concentration)
BSA	100 µg/ml	(final concentration)

Other Commonly used buffers

Standard saline citrate (SSC) 10x Stock solution

1.5 M NaCl

0.15 M Na_3 Citrate

Nucleic acid suspension buffer (10T, 0.5E)

10 mM Tris/HCl, pH 7.5, at 20°C

0.5 mM Na_2EDTA, pH 7.7

All other specific buffers and solutions are described in the relevant section of the Methods and Techniques, along with the other relevant experimental details for the procedure in question.

METHODS AND TECHNIQUES

Growth and maintenance of Micro-organisms

Tetrahymena strains

Maintenance. Stock cultures of Tetrahymena were kept in 10 ml screw-cap tubes containing 5 ml of 1% Difco Proteose/ Peptone/0.1% Difco Yeast Extract (w/v). These were sub-cultured every three to four weeks and incubated at room temperature (15-20°C).

Growth for experimental purposes. Starter cultures were grown in 250 ml conical flasks containing 100 ml of Tetrahymena growth medium. These were incubated at 28°C with shaking.

Batch cultures for preparative purposes were grown in 2-litre conical flasks containing 800 ml of Tetrahymena growth medium. These were incubated at 28°C with shaking.

Double-distilled de-ionized water was used throughout. Flasks were plugged with cotton wool.

Bacterial Strains

Maintenance. Bacterial strains were maintained on nutrient agar stabs for storage, and on nutrient agar plates when in general use.

E. coli strain JM 103 was maintained on minimal medium plus thiamine agar. E. coli strain ED 8767 was maintained on L-agar. If the ED 8767 culture was carrying plasmids, all antibiotics to which the plasmid endowed resistance were included in the media. Plates were grown overnight and stored at 37°C until required.

Growth of Bacterial strains. For experimental purposes all bacterial cultures were grown in L-broth. If the ED 8767 cultures carried plasmids, then the appropriate antibiotics were added to the medium.

Isolation of Mitochondrial Nucleic Acids from Tetrahymena

Isolation of mitochondria. All operations were carried out at 0-4°C. All centrifugations were done in an MSE "Mistral 6L" centrifuge fitted with a 6 x 380 ml rotor.

Cultures of Tetrahymena were grown as described until they reached late exponential phase, about 5 x 10^5 cells/ml, and the flasks cooled in iced water. Cells were harvested by centrifugation at 2,500 rpm for 10 min. The cell pellet was resuspended in 1/10th the original volume of extraction buffer (0.35 M mannitol, 50 mM Tris/HCl, pH 7.5 at 20°C, 3 mM EDTA) and re-centrifuged. The washed cell pellet was weighed and resuspended in extraction buffer at 20 ml per gram of cells. (A typical cell yield for cells in late exponential phase was 3-4 g/litre of culture.)

The cell suspension was passed through a "bel cream maker". The homogenate was centrifuged at 5,500 rpm for 15 min and the supernatant removed. The pellet was gently creamed into suspension using a "cold finger" (narrow test tube filled with crushed ice) and finally resuspended in a 40x volume of extraction buffer (40 ml per gram of cells). This suspension was centrifuged for 10 min at 1,800 rpm and the supernatant decanted off. The supernatant was re-centrifuged and decanted, and the mitochondria pelleted from the second supernatant by centrifugation at 5,500 rpm for 15 min. (Flavell & Follet, 1970.)

The resulting crude mitochondrial pellet was resuspended in extraction medium, to 1 ml per gram of original cell yield, and 1 M $MgCl_2$ added to 10 mM. Deoxyribonuclease I (1 mg/ml) (Sigma DN-100, Bovine Pancreatic Deoxyribonuclease I) was added

to 5.0 μg/ml and the mixture stood on ice for 20 min. Sufficient 200 mM EDTA was added to chelate the Mg^{2+} ions, followed by 50 ml of extraction buffer. The mitochondria were once again pelleted by centrifugation at 5,500 rpm for 15 min. (For preparations of mitochondrial RNAs the DNAase step was omitted.)

Extraction of mitochondrial nucleic acids

All operations were carried out at 0°-4°C. All centrifugations were done in an MSE 4 x 50 ml rotor.

mtDNA preparations. The DNAase treated mitochondrial pellet was resuspended in 0.1 x SSC in a volume of 1 ml per gram of original cell yield. SDS solution (10% w/v) was added to a final concentration of 1% SDS, quickly followed by a volume of phenol solution equal in volume to that of the lysate. The lysate was phenol extracted with gentle occasional mixing for 15 min. The phases were separated by centrifugation at 4,000 rpm for 10 min. The aqueous phase was chloroform extracted for 5 min and the phases separated by centrifugation at 4,000 rpm for 3 min. Mitochondrial nucleic acids were recovered from the aqueous phase by ethanol precipitation.

mtRNA preparations. The mitochondrial pellet was resuspended in 0.1 x SSC solution in a volume of 1 ml per gram of original cell yield. Di-ethyl pyrocarbonate (10 μl) (Sigma) was added per ml of suspension, followed by 1 ml of a sterile suspension of 10% w/v "Macaloid" (National Lead Co., Texas, USA). The mitochondria were lysed by the addition of SDS solution (10% w/v) to 1% w/v final concentration and phenol and chloroform extracted as for mtDNA preparations. Mitochondrial nucleic acids were recovered from the final aqueous phase by ethanol precipitation.

Purification of mitochondrial nucleic acids

Ethanol precipitates of mitochondrial nucleic acids were collected by centrifugation at 12,000 rpm for 15 min in a Sorval SS-34 rotor. The ethanol was removed and the pellet dried under vacuum. 1 ml of 10T 0.5E buffer was added and the precipitate resuspended on ice for 1 h. For mtDNA preparations 20 μl of 1 mg/ml RNAase (Bovine Pancreatic Ribonuclease "A" (Sigma)) was added.

Mitochondrial nucleic acids were finally purified on sucrose gradients prepared as described on p49.

mtDNA gradients were run for 17 h at 24,000 rpm to isolate Tetrahymena mtDNA sedimenting at ~ 34S.
mtRNA gradients were run for 17 h at 29,000 rpm to isolate Tetrahymena mt rRNAs sedimenting at 21S and 14S.

Isolation of Bacterial plasmid DNAs

"Mini-plasmid" preparations

All centrifugations done in "mini-plasmid" preparations were done using an Eppendorf 'microfuge'.

Single colonies were picked from agar plates with a sterile cocktail stick and inoculated into 5 ml of L-broth, plus appropriate antibiotics, contained in 20 ml Universal bottles. These were incubated at 37°C overnight with shaking.

Method 1. 2 x 1.5 ml volumes of overnight culture were harvested in a 1.5 ml Eppendorf vial (microfuge x 40 s) and the pellet resuspended in 200 μl of sucrose solution (25% sucrose, 50 mM Tris/HCl, pH 8.0, at 20°C). 40 μl of lysis solution (3 ml of 10% TX-100, 75 ml of 250 mM EDTA, pH 8.0, 15 ml of

1 M Tris/HCl, pH 8.0 at 20°C, 7 ml of H_2O) were added and mixed in. The lysate was stood on ice for 15 min. Chromosomal DNA and cell debris were collected by centrifugation (microfuge) for 12 min. The pellet was carefully removed and the supernatant extracted with 300 µl of chloroform. The aqueous phase was separated by centrifugation for 1 min, and 40 µl aliquots from each sample compared for plasmid content by electrophoresis. Those preparations containing recombinants of interest were phenol extracted, chloroform extracted and ethanol precipitated as described.

Method 2. (Ish-Horowitz & Burke, 1981). 1 ml of overnight culture was harvested by centrifugation (microfuge x 40 s). The supernatant was removed and the pellet gently resuspended in 100 µl of glucose solution (50 mM glucose, 25 mM Tris/HCl, pH 8.0 at 20°C, 10 mM EDTA). The suspension was incubated at 20-25°C for 5 min. 200 µl of 0.2 M NaOH, 1% SDS was added and mixed in. Lysate was stood on ice for 5 min. The precipitated, denatured chromosomal DNA, proteins and SDS were pelleted by centrifugation for 4 min. The supernatant was collected and 0.6 volume of propan-2-ol mixed with it. After 2 min at room temperature the precipitated plasmid DNA was collected by centrifugation for 1 min. The pellet was washed with 70% EtOH and dried under vacuum. Plasmid DNA pellets were resuspended in 100 µl of 10T 0.5E buffer. 5 µl aliquots from each sample were compared for plasmid content by gel electrophoresis.

Amplified preparations of plasmid DNAs

This method was used for large-scale plasmid DNA preparations, such as the preparation of cloning vectors.

Plasmid-bearing cells were streaked out on L-agar plates, plus appropriate antibiotics, and incubated overnight at 37°C. Single colonies were inoculated into 1 litre flasks containing 150 ml of L-Broth plus appropriate antibiotics. Flasks were incubated at 37°C with shaking until the A_{650} of the culture reached 1.0. Chloramphenicol was added to a concentration of 150 μg/ml and the culture shaken at 37°C overnight. Cells were harvested by centrifugation (5,000 g for 10 min) and washed in 10 mM NaCl. Plasmid DNA was then prepared using either of the methods described. A 40x scale-up of the quantities described was used.

<u>Preparation of plasmid DNAs in pools of 5 or 10</u>

This technique was used to screen large numbers of bacterial colonies for the presence of large recombinant plasmid molecules.

Colonies were picked from agar plates and inoculated into 1 ml of L-broth, plus appropriate antibiotics, contained in 0.5 oz. sample vials. These were arranged in grid-marked trays in groups of fifty. The tops were sealed with adhesive film and shaken overnight at 37°C. Five or ten individual 1 ml cultures were combined, mixed and 1 ml of each "pool" used for a mini-plasmid DNA preparation as described.

After comparative electrophoresis, any pool(s) containing plasmids of interest were further investigated by mini-plasmid preparation on individual colonies.

<u>Purification of plasmid DNAs</u>

5 μl of 1 mg/ml RNAase 'A' solution (boiled for 10 min) was added to resuspended "mini-plasmid" DNA preparations and incubated at room temperature for 45 min. This was then phenol extracted, chloroform extracted and ethanol precipitated as

described. (see p48 - 49.)

Plasmid DNA was recovered by 'microfuging' for 5 min. The ethanol was removed and the plasmid DNA pellet dried under vacuum. The pellet was resuspended in 100 µl of 10T 0.5E buffer. These samples were found to be sufficiently clean for restriction digests to confirm the size of the insert DNAs in recombinants. The volume of RNAase solution added was scaled up for large plasmid preparations, as was the extraction steps. Large scale plasmid DNA preparations were purified finally on CsCl and/or sucrose gradients.

Isolation of M13 'phage RF (Replicative form) DNA

This method was used on the small scale to prepare samples of double stranded RF DNA for restriction digest analysis to confirm the origins of the insert DNA in M13 recombinants. This method was used on the large scale to prepare samples of double stranded RF DNA for use as M13 'phage cloning vectors.

Suspected recombinant plaques, or stock 'phage suspensions, were transferred to L-broth. A loopful of overnight JM 103 culture was added and the culture incubated for 6-8 h at 37°C with shaking. Preparations for investigating recombinant plaques were inoculated into 5 ml of L-broth in a 20 ml universal bottle. Preparations for M13 'phage cloning vectors were inoculated into 150 ml of L-broth in a 1 litre flask. The bacterial cells were harvested by centrifugation and washed twice in 10 mM NaCl. The resulting pellet was used to prepare and purify the RF (plasmid form) DNA using the plasmid DNA preparations described.

Horizontal gel electrophoresis

Agar or agarose gels were cast on glass plates after the method of Schaffner. (Molecular cloning, a Laboratory Manual. CSH. 1982. T.Maniatis, E.F.Fritsch, J.Sambrook, eds.)

Mini-gels. These were cast on 50 mm x 75 mm microscope slides. Loading slots were formed with a plasticard former near one end of the plate. Each measured 0.8 mm x 5 mm and held a volume of approximately 15 µl. Five slots were formed per gel. These gels were used for rapid estimations and were run in a small gel electrophoresis tank to minimise the running time.

Large-gels (analytical or 'test' gels). These were cast on 20 x 25 cm glass plates. Loading slots (12) were formed with a plastic former near one end of the plate. An extra row of slots was formed half-way down the gel if large sample numbers were required. Each slot measured 1 cm x 1.5 mm and held approximately 70 µl.

Preparation and casting of agar/agarose gels

Agar or agarose was dissolved in water by boiling for 5 min. 6x Electrophoresis buffer was added to a 1x final concentration and the mixture cooled to 55°C before casting.

Glass plates were placed in suitable moulding trays and levelled. Slot formers were placed in position and the gel mixture poured on. When the gel had set (about 10-15 min) the slot formers were removed, the gel plus glass plate excised as one from the mould and stored in 1x electrophoresis buffer until required.

Loading and running conditions

Electrophoresis tanks were levelled before use. Gels were placed in the tank and sufficient 1x buffer added to cover the gel.

Samples were mixed with ¼ volume of 30% sucrose, 100 mM EDTA,

0.05% Bromophenol Blue and gently pipetted into the loading slots. Gels were run at 50 V (dc) until the blue marker had migrated sufficiently. After completing the run, the gel was slid off the glass plate and on to a stainless steel sheet. The nucleic acids were visualized using u.v. light.

Gel photography

To record results, gels were photographed on Kodak plus-x-pan professional film, using u.v. illumination and an orange filter (Wratten No. 16). Negatives were developed to high contrast and prints made in the normal manner.

Types of Agar/Agarose used

Concentration and type		Use
1%	Lab 'M' agar	Mini-gels, large and analytical gels.
1.25%	Lab 'M' agar	Separation of small restriction fragments on large gels.
2%	Lab 'M' agar	Separating and estimating RNAs.
0.8%	Agarose (Sigma type II low EEO)	Recovery of restriction fragments.

Estimation of the size of Restriction Fragments

Eco RI and Hind III digests of λDNA were run alongside restriction digests to provide a calibration of each gel. The mobility of the λDNA fragments were measured on photographic prints of the gel and plotted against the log of the molecular weight for the λDNA fragments. Values for the λDNA fragments were those of Schroeder et al. (1981). The mobilities of digest fragments of unknown size were measured from the gel photograph and their molecular weight estimated from the λDNA calibration curve.

Techniques for the recovery of DNA from Agarose gels

DNA recovery using Hydroxyapatite columns. Hayashi et al. (1976).

The DNA fragments of interest were visualised using a long-wavelength u.v. light and excised with a clean blade. The gel slices were frozen in a glass vial. Upon thawing, solid potassium iodide was added and the mixture incubated at room temperature with occasional gentle mixing. Further additions of potassium iodide were made until no gel fragments remained un-disaggregated. A 0.5 ml hydroxyapatite column was prepared using Biogel HTP (Bio-Rad). (This was fined twice and suspended in 50 mM sodium phosphate, pH 6.8 (50 mM Pi) before packing.) Columns were packed in 10 ml disposable pipette tips, plugged with a small amount of glass wool and 40 mesh glass beads. Disaggregated gel samples were loaded on to the column, and washed with:

(i) 1 column volume of saturated KI solution

(ii) 3 column volumes of 50 mM Pi solution.

The complete removal of all iodide was confirmed by a starch/ peroxide spot test on the column washings.

DNA was eluted from the column with 1 ml of 400 mM Pi solution. Samples were dialysed for 18 h against 0.5 mM Tris/ HCl, pH 7.5 at 20°C, 2.5 μM EDTA with three changes of 4 litre volumes of buffer in the 18 h period. Samples were lyophilysed overnight and taken up in 50 μl of H_2O.

DNA recovery by electroelution. McDonnell et al. (1977).

The DNA fragments of interest were visualised and excised as before. Gel slices were loaded into dialysis bags half-filled with electrophoresis buffer, and the ends of the bags closed with clips. The dialysis bags were placed in an

electrophoresis tank, parallel to the electrodes and the DNA eluted by electrophoresis at 50V for 2-3 h. The gel slices were removed from the dialysis bags and rinsed in 0.5 ml of electrophoresis buffer. This rinse buffer and the buffer from the dialysis bag in question were combined.

The dialysis bag was washed out using phenol solution. This phenol wash was then used to phenol extract the combined electrophoresis and rinse buffers in question. The aqueous phase was chloroform extracted and the DNA precipitated using ethanol as described.

DNA recovery using the Freeze/Squeeze Technique

This method is a modification of the method of Thuring et al. (1975). The DNA fragments of interest were visualised and excised as before. Gel slices were placed in 1.5 ml Eppendorf vials. These were frozen and thawed twice by immersion in liquid nitrogen and thawing at room temperature. The vials were centrifuged in an Eppendorf 'microfuge, for 5 min and the supernatant removed by pipette. This was phenol extracted, chloroform extracted and ethanol precipitated as described.

Transfer of DNA from gels to nitrocellulose filters by the "Southern Blot". Southern (1975)

The gel strip containing the DNA fragments of interest was soaked in 0.5 M NaOH, 1.5 M NaCl for 15 min. The gel strip was drained and then soaked in 0.5 M Tris/HCl, pH 7.5 at 20°C, 3 M NaCl for 15 min. The gel strip was drained and placed on a sheet of Whatman 3 MM filter paper pre-soaked in 20x SSC solution. The 3 MM filter paper was supported on a glass plate, with the edges of the 3 mm dipping into a reservoir of

10 ml of 20 x SSC solution. All regions of the 3 MM not in contact with the gel were masked off with cling-film. A strip of nitrocellulose filter (Millipore) was pre-soaked in 2 x SSC solution, drained, and placed on top of the gel slice. Ten sheets of Whatman 3 MM paper were placed on top of the nitrocellulose strip and weighted down with two similarly sized pieces of 4 mm glass plate. The assembly was left undisturbed overnight.

The completed transfer was carefully dismantled and the nitrocellulose filter strip washed gently in 2 x SSC solution and dried under vacuum. The filter was finally baked at 80°C for 2 h under vacuum.

General purification Techniques for Nucleic Acids

Solvent extractions

Phenol solution. This was prepared as follows. AR grade phenol was purified by distillation. Melted phenol was neutralised with 1 M Tris base and cooled to 0-4°C. The phenol was then water saturated, frozen and stored at -20°C until required.

Chloroform solution. The chloroform solution used in solvent extractions was 24:1, Chloroform:Iso-amyl-alcohol (v/v). Large-scale extractions. These were done in glass tubes closed with ground glass stoppers. After extraction the phases were separated by centrifugation in 50 ml "glass" centrifuge tubes in a swing-out rotor.(4 X 50 ml. rotor, MSE 6L centrifuge.)
Small-scale extractions. These were used repeatedly in the step-wise processes involved in the molecular cloning protocols. Small-scale extractions were done in Eppendorf vials. Aqueous and organic phases were mixed by gently "vortexing". The phases

were separated by centrifugation in a 'microfuge for 3 min.

Alcohol Precipitations

Nucleic acids were precipitated from solutions by the addition of 0.1 volumes of 1 M NaOAc, pH 6.8 and two volumes of EtOH. If the initial DNA concentration of the solution was less than 50 μg/ml the precipitation was stored at $-20^{o}C$ overnight. If the initial DNA concentration of the solution was greater than 50 μg/ml the precipitation was cooled to $-20^{o}C$ for at least 15 min before collection. Nucleic acid precipitations were collected by centrifugation and dried under vacuum before resuspension to a concentration of 50-150 μg/ml.

To remove small amounts of contaminating proteins, nucleic acids were precipitated by the addition of 7.5 M NH_4OAc solution to 2.5 M and 2 volumes of propan-2-ol. Under these conditions most proteins remain in solution.

Sucrose gradients

Sucrose gradients were assembled in 25 ml MSE polypropylene centrifuge tubes, and were run in an MSE 3 x 25 ml swing-out rotor (MSE 65 Ultracentrifuge). Gradients were 17.5 ml in volume and varied between 15% w/v and 25% w/v sucrose concentration between the top and the bottom of the gradient. (These gradients were an exponential approximation of an isokinetic gradient.) The gradients were made up in a buffer of 100 mM NaCl, 10 mM Tris/HCl, pH 7.5 at $20^{o}C$, 1 mM EDTA.

Nucleic acid samples were layered on to the gradients in a 2 ml volume. Gradients were run at the speed required by the sedimentation coefficients of the individual samples. Upon completion of the centrifuge run the gradient centrifuge tubes

were pierced at the base and the gradients collected in 1 ml samples whilst monitoring A_{254} continually. Those fractions of interest were either pelleted by centrifugation at ~300,000 g overnight (MSE 3 x 6.5 ml rotor) or precipitated with ethanol.

CsCl gradients

A stock solution of 65% CsCl in water was prepared. 8.292 g of stock CsCl solution (ρ = 1.9052, measured by refractive index) was placed in a 10 ml MSE polycarbonate centrifuge tube. The DNA sample, totalling 1.953 g was added followed by 90 µl of 1 M Tris/HCl, pH 8.0 at 20°C and 20 µl of 250 mM EDTA. 110 µl of 10 mg/ml ethidium bromide were added and the tube contents mixed. (The tube and its contents were protected from light after the addition of the Ethidium bromide.) The tube was loaded into a 10 x 10 ml angle rotor and centrifuged at 54 Krpm for 5 h (MSE 65 Ultracentrifuge) at 20°C. The centrifuge speed was then reduced to 42 Krpm and the sample centrifuged for at least 20 h.

Upon completion of the centrifuge run the centrifuge tube was supported in a clamp and the DNA visualised using a long wavelength u.v. light. The band(s) of interest were removed using a syringe.

Recovered samples were extracted three times with an equal volume of iso-amyl-alcohol. CsCl was removed by dialysis against three changes of 4 litre volumes of H_2O. Nucleic acids were finally collected by ethanol precipitation.

General experimental techniques involving enzymes.

DNA digests with restriction endonucleases

Digests were done at 37°C for 1 h. The DNA concentration was usually of the order of 50 µg/ml. A general purpose buffer

was used with most enzymes, referred to as HX buffer. This was added to digest mixtures as a 10x stock solution to a final concentration of 1x. BSA (5 mg/ml) was added to a final concentration of 100 µg/ml. DTT (100 mM) was added to a final concentration of 5 mM. For use with some commercial enzymes, namely Sau 3A and Hind III, specific buffers were made up according to the suppliers' recommendations as 10X stock solutions (see Buffers and Media).

Non-commercial enzymes were used for mapping studies. These were prepared in the laboratory by R. W. Holt following the schemes of Pirrotta & Bickle (1980).

Digestion of plasmid and M13 'phage cloning vectors

Restriction enzyme digests were done under the conditions described above. Commercially obtained enzyme preparations were used throughout.

Wherever possible the completeness of digestion was checked by electrophoresis of 1 µg samples of the digest.

Completed digests were phenol-extracted, chloroform-extracted and ethanol-precipitated before use.

Double-enzyme digests of plasmid and M13 'phage cloning vectors

The DNA sample was divided into two. Single enzyme digests were done using the two different enzymes. Limit digestion was confirmed by electrophoresis of 1 µg samples. The single enzyme digestion products were then digested with the same amount of the second enzyme which had been shown to completely digest an equal quantity of the ccc DNA.

In this way the completeness of digestion with each enzyme was confirmed. Completed digests were phenol-extracted, chloroform-extracted and ethanol precipitated before use.

Alkaline Phosphatase Treatment

Vectors which had been digested with a single restriction endonuclease were treated with alkaline phosphatase to reduce the likelihood of the vector ligating to itself in the ligation reaction.

Digests were done at 55°C for 15 min. 1 M Tris/HCl, pH 9.0 at 20°C was added to 100 mM before digestion. Bovine intestinal Type VII alkaline phosphatase (Sigma) was added at 0.2 units/μg DNA.

The treated DNA was phenol extracted four times to remove the phosphatase activity. The final aqueous phase was chloroform extracted and the DNA ethanol precipitated.

Homopolymer tailing with terminal transferase

The addition of poly dG or poly dC tails to linear DNA molecules was made by reaction with terminal deoxynucleotide transferase under the conditions specified by the supplier (BRL). 500 mM Potassium cacodylate pH 7.2, 10 mM $CoCl_2$, 1 mM DTT, 1 mM [^{3}H]dNTP. The DNA concentration was of the order of 50 μg/ml. Eight units of terminal deoxynucleotide transferase were added per p mol of termini present. The reaction was incubated at 37°C and the extent of polymerisation of homopolymer tails monitored by estimating the incorporation of isotopically labelled nucleotides into acid precipitable material.

Addition of poly dG Tails (Vector molecules)

The dNTP used was deoxy[8-^{3}H]guanosine-5′-triphosphate (Amersham International). This was diluted to a specific activity (s.a.) of 2 Ci/mmol before use.

Addition of poly dC-tails (Insert molecules)

The dNTP used was deoxy[5-^{3}H]cytidine-5′-triphosphate (Amersham International). This was diluted to a s a of 2 Ci/mmol before use. Small aliquots were taken from the reaction mixture at timed intervals and the incorporation of label into acid precipitable material was estimated.

Approximately 20 residues were added, per end, to both vector and inset DNAs. Vector molecules were always Pst I cut cloning vectors. The addition of dG residues effectively re-builds the restriction site. Insert and vector DNAs were annealed using the method of Clark & Carbon (1975).

Treatment with S_1 nuclease

Potentially single-stranded regions of nucleic acids were degraded with S_1 nuclease, a single stranded specific nuclease. Digestion was for 30 min at 37°C. The DNA concentration was of the order of 50 μg/ml. 0.01 Units of nuclease S_1 (Sigma) were added, per μg of DNA. Reactions were done in a buffer of 30 mM NaOAc pH 4.5, 250 mM NaCl, 1 mM $ZnSO_4$. Digested samples were phenol-extracted, chloroform-extracted and ethanol precipitated before further use.

Treatment with Bal 31 exonuclease

The enzyme Bal 31 exonuclease was used to progressively degrade linear DNAs. This exonuclease degrades both strands of a duplex and has many applications in DNA mapping and sequencing studies. Digestions were done under the conditions recommended by the supplier (BRL). The DNA concentration was of the order of 50-100 μg/ml, the actual concentration used was dependent upon the size of the linear molecule in question. Digests were done in the following recommended buffer:

12 mM $CaCl_2$, 12 mM $MgCl_2$, 600 mM NaCl, 20 mM Tris/HCl, pH 8.1, at 20°C, 1 mM EDTA. 0.25 Units of Bal 31 exonuclease were added per p mol of ends present. Samples were removed at timed intervals and pipetted directly into 0.25 volume of 100 mM EDTA. Samples were compared for their degree of degradation by comparative electrophoresis. Suitable samples were phenol-extracted, chloroform-extracted and ethanol precipitated before further use.

DNA "filling", with Large Fragment E. coli DNA Polymerase I (Klenow fragment)

This method was used to fill-in the cohesive ends created by digestion with restriction endonuclease Bam H1. The method was also used to prepare strain T mtDNA for cloning studies. The protocol is that described in the "BRL protocol for M13 mp7 cloning and sequencing" (1981).

The DNA concentration was 100 µg/ml in the reaction. A 10x buffer concentrate was added to 1x buffer final concentration (see Table of buffers and media). 0.2 Units of Large Fragment E. coli DNA Polymerase I (BRL) were added per µg of DNA, and the mixture incubated at room temperature for 5 min. All four dNTPs were added to 125 µM each, and the mixture incubated at room temperature for 30 min. 200 mM EDTA solution was added to 20 mM. The reaction was phenol-extracted, chloroform-extracted and the DNA ethanol precipitated before further use.

"Nick translation" of DNA, using E. coli Polymerase I

This technique, used to incorporate high levels of labelled nucleotides into DNA samples was a modification of the method of Rigby et al. (1977). The reaction was done under the following conditions:

DNA concentration of 10-20 μg/ml, 50 mM Tris/HCl, pH 7.8 at 20°C, 2.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 25 μg/ml BSA, 5 μM dCTP, dGTP and dTTP. [α-^{32}P].dATP, s/a ~ 1 Ci/μmol was added at 1 μCi per μg of DNA present. 5 μl Of a non-commercial preparation of E. coli Polymerase I, prepared according to Jovin et al., (1969) was added and the reaction incubated at 14°C for 2 h. The reaction was stopped by the addition of 2 volumes of 5 mM EDTA. This mixture was phenol-extracted, chloroform-extracted and the DNA ethanol precipitated before further use.

Ligation reactions

In all cases vector and insert DNAs were ethanol precipitated together and resuspended in the ligation mixture. Ligation buffer was made up as a 2x stock solution (see buffers and media).

Cohesive-end Ligations. When the vector and insert DNAs possessed cohesive termini, e.g. restriction fragments such as Eco RI Fragments, 0.05 units of ligase (Weiss units) were added per p mol of termini present. Ligations were incubated at 12°C for at least 6 h.

2-Step Ligations. When one end of the vector and insert molecules possessed a cohesive termini, and the other end was blunt (flush). Blunt or flush ends were present at the site of restriction enzyme cuts using Hae III or Sma I. The "filled" or degraded ends of DNA fragments were also assumed to be largely of this form.

Vector and insert DNAs were ligated together under the conditions described for cohesive termini. The ligation mixture was then diluted five-fold with ligase buffer and the ligase concentration increased 100-fold. This mixture was incubated at 22°C overnight.

Estimation of isotope incorporation into acid precipitable material

The sample volume was pipetted into a glass tube with ~2 µg of calf-thymus DNA. The nucleic acids were precipitated by the addition of 1 ml of ice-cold 10% TCA, 20 mM sodium pyrophosphate and stood on ice for five minutes. The precipitate was collected by filtering the mixture through a glass-fibre filter (Whatman GFC) using a water pump to provide suction. The precipitate was washed with 10 ml of the TCA/PPi solution, and finally with 2 ml of ice-cold ethanol. Filters were transferred to scintillation vials and dried under vacuum. For samples containing [^{32}P]labelled nucleic acids, 10 ml of water was added to the vials and the activity estimated by counting in a liquid scintillation counter (Kerenkov counting, ^{3}H channel). For samples containing [^{3}H]- or [^{35}S]-labelled nucleic acids, 10 ml of toluene/PPO scintillation cocktail (diphenyloxazole, 5 g/l in toluene) was added to the vial and the activity estimated by counting in the relevant channel of a liquid scintillation counter.

The estimated amount of material present in the precipitation was used to calculate specific activities.

Isotopic labelling of RNA with T4 polynucleotide kinase

Goldbach et al. (1978b)

Purified, undegraded RNA (5 µg) was ethanol precipitated in a 1.5 ml Eppendorf vial. Following collection and drying the precipitate was resuspended in 40 µl of 50 mM Na_2CO_3 and incubated for 80 min at 50°C. 20 µl of (100 mM HCl, 150 mM Tris/HCl, pH 7.5, at 20°C) were added, followed by

1 μl of 1 M $MgCl_2$ and 1 μl of 100 mM DTT. Between 0.2 and 0.5 mCi of adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP) s/a ~0.5 Ci/mmol, in EtOH) were dried under vacuum. ([γ-^{32}P]ATP was prepared by the method of Maxam & Gilbert (1980).) The degraded RNA solution was added to the labelled ATP and the nucleotide triphosphate gently resuspended. 10 Units of T4 polynucleotide kinase (BRL) were added and the reaction incubated at 37°C for 1 h.

A column of Sephadex G-25 was packed in a 1.5 ml disposable pipette tip and equilibrated with 6 x SSC solution. The column was spun to dryness in a bench centrifuge. The reaction was pipetted on to the top of the column and the column re-centrifuged. The elutant was collected in an Eppendorf vial. The incorporation of label into the RNA was estimated by measuring the incorporation into acid precipitable material.

This method was also used in attempting to incorporate [^{35}S]-labelled orthophosphate into RNA. Adenosine 5′-([γ-^{35}S]Thio)-triphosphate ([γ-^{35}S]ATP.γS) was used (Amersham International).

The rate of incorporation of this isotope into acid precipitable material is very slow compared to [^{32}P]-labelled moieties. In the interests of economy its use was discontinued.

Incubation procedure for the Hybridization of isotope-labelled RNA to DNA on nitrocellulose filters

The nitrocellulose filter carrying the DNA fragments was placed in a glass tube fitted with a loosely-fitting glass rod, and closed at one end. The remaining free volume was ~2.5 ml. Approximately 2-4 μg of [^{32}P]-labelled RNA was mixed with 2 ml of 6 x SSC, 0.05% SDS and syringed into the glass tube. The glass tube assembly (the hybridization chamber) was placed in

a 100 ml measuring cylinder, partially filled with the SSC/SDS solution. The cylinder was closed with a stopper and incubated at 65°C overnight.

The filter was removed from the hybridization chamber by withdrawing the glass rod and gently flushing out the glass tube with SSC/SDS solution at 45°C. The filter was gently washed in 6 x SSC at room temperature for five minutes, followed by two fifteen minute washes in 2 x SSC solution. The excess buffer was blotted off and the filter dried under vacuum. The dried filter was sealed in a polythene bag and autoradiographed by contact with "Kodak X-Omat" X-ray film. A speed increase of 4-5 fold was achieved using an intensifying screen and flash-pre-sensitised film if required (Dupont Cronex screen, film pre-exposed by flash to an optical density of 0.1 .)

<u>Preparation of competent cells</u> Morrison (1979)

A single colony was picked from a streaked-out agar plate and grown in L-broth overnight at 37°C. 150 ml of pre-warmed L-broth were inoculated to A_{650} of ~0.05 with the overnight culture and incubated at 37°C with shaking until the A_{650} reached 0.5. The culture was cooled to 4°C and the cells harvested by centrifugation at 5,000 g for 6 min. The cell pellet was washed in 100 mM $MgCl_2$ and re-centrifuged. This washed cell pellet was resuspended in 40 ml of cold 100 mM $CaCl_2$ and stood on ice for 20 min. The calcium treated cells were pelleted by centrifugation and gently resuspended in 8 ml of cold $CaCl_2$/glycerol solution (43 ml of 100 mM $CaCl_2$ + 7 ml glycerol).

Aliquots of 0.5 ml were dispensed into pre-cooled polypropylene-screw-top ampoules and stored in liquid nitrogen until required.

Transformation of competent cells

Ampoules containing frozen competent cells were thawed in ice-water. The DNA sample, containing $\leqslant$ 1 μg of DNA was pipetted into a pre-cooled glass tube. 200 μl of thawed competent cell suspension was added. The two components were gently mixed and stood on ice for 20 min. This transforming mixture was heat-shocked by immersing the glass tube in a 42°C water bath for 2-3 min. The transformation mixture was immediately added to 5 ml of pre-warmed L-broth.

Plating of transformants bearing plasmid vectors

The transformed culture was incubated at 37°C with shaking for 90 min. 100 μl quantities of the culture were either plated directly from the 5 ml of L-broth or concentrated by pelleting and resuspension in a small volume. Transformants were plated on L-agar + antibiotic plates using a glass spreader to distribute the cells evenly on the plate. The plates were incubated overnight at 37°C. Colonies were picked off these transformation plates using a sterile cocktail stick and inoculated on to L-agar + antibiotic plates upon which 100 numbered spots were visible. These plates were incubated overnight at 37°C. In this way stock plates carrying 100 transformant colonies were used to catalogue and store potential recombinants. Plates were sealed with parafilm and stored at 4°C until required.

Antibiotic sensitivity screening of transformants

Colonies were picked from the 'stock' plate and inoculated on to an L-agar plate containing the screening antibiotic. This screening plate had a grid of numbered spots visible on it in the same manner as the stock plate. Colonies were inoculated

on to the same numbered spot on the screening plate as the stock plate. Screening plates were incubated at 37°C overnight. Colonies failing to grow on the screening plate were further investigated using the same numbered colony on the stock plate.

Plating M13 'phage transformed cells

Competent JM 103 cells were transformed with the DNA sample in question and 100 μl quantities from the 5 ml of L-broth culture were plated immediately.

100μl quantities of the culture were added to 5 ml of molten 0.6% L-agar at 45°C. The agar also contained:

(a) 0.6 mg of IPTG (isopropyl-β-D thiogalactopyranoside), (Sigma) added immediately before use as a 12 mg/ml solution in water.

(b) 1.0 mg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), added immediately before use as a 20 mg/ml solution in dimethyl formamide (Sigma).

(c) Five drops of an overnight culture of E. coli JM 103 in L-broth.

The agar mixture was briefly vortexed and evenly spread on top of L-agar plates. Once the agar had set, plates were incubated overnight at 37°C.

Rapid method for the comparison of M13 'phage recombinants

Clear plaques, indicating potential recombinants were picked from the transformation plate with a sterile cocktail stick and inoculated into 5 ml of L-broth. These were incubated at 37°C for 6 h with shaking.

1 ml of each culture was 'microfuged for 1 min (Eppendorf 'microfuge). 40 μl of each supernatant was removed and mixed with 10 μl of 30% sucrose/1% SDS. These samples were loaded

directly on to a 1% agar gel and electrophoresed for three hours. A blue plaque, indicating a complete vector M13 'phage DNA molecule devoid of any insert, was treated in the same way to act as a control. Useful recombinants were noted as those samples which showed sufficient increase in the size of the s/s DNA compared to the vector s/s DNA.

Preparation of single-stranded DNA from M13 'phage, for use as sequencing templates

This method follows the recommended protocol in the "BRL M13 mp 7 cloning and sequencing protocol (1981)".

Plaques were picked from soft agar/transformation plates and inoculated into 5 ml of L-broth. These were incubated for 6 h at 37°C with shaking. 1 ml of each culture was 'microfuged for 10 min. The supernatants were decanted off and 200 µl of 20% polyethylene glycol, 2.5 M NaCl added. The supernatant/PEG mixes were stood at room temperature for 15 min. Precipitated 'phage were collected by 'microfuging for 5 min. The supernatants were completely removed and the pellets resuspended in 100 µl of TES buffer (20 mM Tris/HCl, pH 7.5,@20°C, 10 mM NaCl, 0.1 mM EDTA). These 'phage suspensions were phenol-extracted, chloroform-extracted and the s/s DNA ethanol precipitated. Following collection and drying the precipitated s/s DNAs were resuspended in 50 µl of TES buffer.

Sub-cloning of insert DNAs from isolated recombinants

The DNA fragments of interest were cut from the parent clone with the relevant restriction enzymes and recovered from agarose gels by the squeeze/freeze technique. The new vector was prepared in the normal manner (see Page 51).

Vector and insert DNAs were then ligated together as described in the Methods for Ligation Reactions, at a DNA concentration of 30 μg/ml.

DNA Sequencing Methods

These methods are based upon the "dideoxy-chain termination reaction" (Sanger et al., 1977) as applied to the M13 'phage cloning system (Messing et al., 1981). The recently constructed M13 strains mp 8 and mp 9 were used in this study (Messing & Vieira, 1982).

The M13 Cloning System. The s/s DNA 'phage M13 contains a multiple restriction site within a β-galactosidase gene. When inserts are ligated into these sites the β-galactosidase gene is split. If a β-galactoside compound is incorporated into the growth medium, it can be used as an indication of the presence or absence of an intact β-galactosidase gene.

Intact vector (No insert) ⟶ β-galactosidase produced ⟶ cleaves "indolyl β-galactoside to produce blue coloured plaques.

Recombinant clone (insert) ⟶ No β-galactosidase produced ⟶ no cleavage of β-galactoside ⟶ colourless plaques.

The M13 Sequencing System. A synthetic primer strand specific for a sequence adjacent to the multiple restriction site in the M13 s/s DNA is annealed to it. In the presence of the necessary nucleotide triphosphates, the enzyme Large Fragment E. coli polymerase I (Klenow Fragment), hereafter referred to as "Klenow polymerase", polymerises a new strand. Primed by the synthetic primer and reading the template DNA through the multiple restriction site, the newly synthesizing DNA makes a complementary copy of the insert DNA sequence. The presence of di-deoxy-nucleotide triphosphates in the reaction mixture causes base-specific termination of the DNA strand.

Sequencing using [^{32}P]-labelled dATP

This method is based on the "New England Biolab protocol #409 (1981)".

Annealing of template and primer DNAs. 5 µl of s/s M13 'phage DNA (~100 µg/ml) were mixed with 3 µl of H_2O, 1 µl of 10x Biolab sequencing reaction buffer (0.5 M NaCl, 0.1 M Tris/HCl, pH 7.5 at 20°C, 0.1 M $MgCl_2$, 0.01 M DTT) and 1 µl of New England Biolab synthetic primer DNA (see Fig. 2).

The tube was sealed and placed in a boiling tube which was sitting in a boiling water bath. After 3 min the boiling tube was removed from the water bath and allowed to cool at toom temperature. In this way the two DNAs were gently allowed to anneal.

Addition of labelled dATP. 30 p moles of deoxyadenosine 5'-[α-^{32}P]triphosphate, s/a 500-1000 Ci/mmol (Amersham International) were dried under vacuum. The annealed template/primer DNA mixture was added to it and the labelled dATP gently resuspended. 1.5 µl of Klenow polymerase (New England Biolab) (4 u/µl) was diluted to 6 µl with enzyme diluent (1.0 M potassium phosphate pH 6.5, 1 mM DTT, 50% glycerol). 2 µl of this dilution was added to the template/primer/labelled dATP mixture.

Sequencing reactions

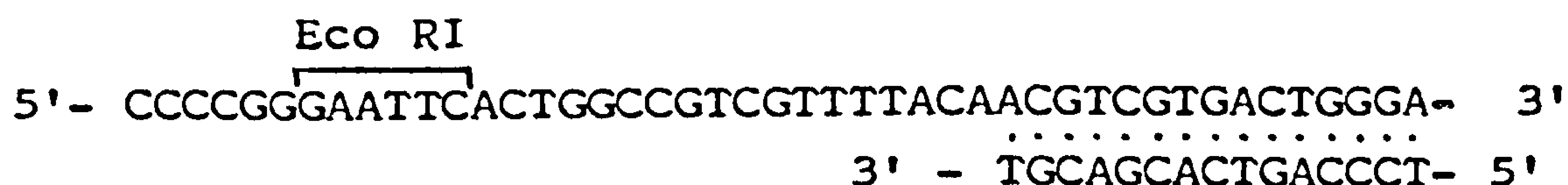
Four 1 ml Eppendorf vials were labelled A, C, G and T respectively. 3 µl of the relevant nucleotide mixture was added to each vial (see Table 1). 2 µl of the prepared reaction mixture (template/primer/labelled dATP/enzyme) was added to each vial, mixed and incubated at 24°C for 15 min. 8 µl of a chase solution (0.25 mM uniform dNTPs) was added to the remainder

Fig 2.

Structure and binding sites of synthetic DNA primers on the plus strand of M13 'phage sequencing vectors.

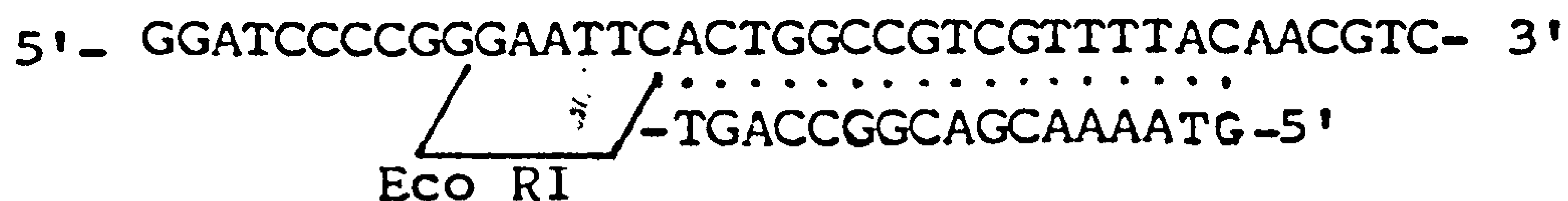
New England Synthetic Pentadecamer.

Shown attached to M13 mp9 plus strand.



Amersham Synthetic Heptadecamer.

Shown attached to M13 mp9 plus strand.



The two primers were found to be interchangeable between the two protocols for sequencing reactions. The New England primer was always used to check the structure of the cloning site to confirm the nature of the M13 recombinant in question.

of the Klenow polymerase dilution and 1.5 µl of this mixture added to the four vials A, C, G and T. These were incubated at 24°C for a further 15 min.

10 µl of stop solution (0.1% Bromophenol blue, 0.1% Xylene cyanol FF, 10 mM EDTA, in formamide) was added to each vial. The vials were placed in a boiling water bath for 3 min. 2-3 µl samples were loaded immediately on to a sequencing gel for separation.

Sequencing using [^{35}S]-labelled dATP (Biggin et al., 1983)

This method is based on the "Amersham International" Protocol (1982) (for N4502). The use of radioisotopes of sulphur possesses certain advantages over phosphorous isotopes:

(a) A longer half-life for the isotope;

(b) The lower energy β-particles produce a sharper, more easily read autoradiogram.

Annealing of template and primer DNAs

5 µl of s/s M13 'phage DNA (~100 µg/ml) were mixed with 2.5 µl of H_2O, 1 µl of 'Amersham International synthetic primer DNA' (see Fig. 2) and 1.5 µl of 10x Amersham sequencing reaction buffer (100 mM Tris/HCl, pH 8.5, at 20°C, 100 mM $MgCl_2$). The tube was sealed and incubated at 55°-60°C for 2 h.

Addition of labelled dATP. 2 µl of a solution of deoxadenosine 5'-([α-^{35}S]Thio)-triphosphate (s/a 650 Ci/mmol) containing ~15 µCi was added to the template/primer mixture. (The labelled deoxynucleotide was supplied in 20 mM DTT by Amersham International) 1 µl (1 unit) of Klenow polymerase was also added (Amersham International).

Sequencing reactions. Four 1 ml Eppendorf vials were labelled A, C, G and T, respectively. 2 µl of the relevant nucleotide

mixture were pipetted in to each vial (see Table 1). 2.5 μl of the annealed template/primer/dATP/enzyme mixture were added to each vial and reactions incubated at 30°C for 20 min. 2 μl of a chase solution (0.5 m$\underline{M}$ uniform dNTPs) was added to each vial and incubated for 15 min at 30°C. 4 μl of stop solution were added to each vial. The vials were placed in a boiling water bath for three minutes. 2 μl volumes were loaded immediately on to a sequencing gel for separation.

Table 1

Table of Nucleotide Mixtures Used in Sequencing Reactions

Sequencing using [α-^{32}P]dATP

	"A" mixture	"C" mixture	"G" mixture	"T" mixture
ddNTP	60 μM ddATP	150 μM ddCTP	150 μM ddGTP	150 μM ddTTP
dATP	-	-	-	-
dCTP	40 μM	4 μM	40 μM	40 μM
dGTP	40 μM	40 μM	4 μM	40 μM
dTTP	40 μM	40 μM	40 μM	4 μM

Sequencing using [α-^{35}S]dATP.αS

	"A" mixture	"C" mixture	"G" mixture	"T" mixture
ddNTP	50 μM ddATP	50 μM ddCTP	150 μM GTP	250 μM TTP
dATP	-	-	-	-
dCTP	62.5 μM	4 μM	83 μM	83 μM
dGTP	62.5 μM	83 μM	4 μM	83 μM
dTTP	62.5 μM	83 μM	83 μM	4 μM

Dideoxynucleotide triphosphate concentrations were occasionally increased to improve the intensity of the shorter terminating chains, by increases of 2x to 3x of the above figures.

Polyacrylamide sequencing Gel Electrophoresis

Preparation of glass plates. Identical glass plates measuring 40 cm x 20 cm x 4 mm were used to support the gel.

"Siliconised" plate. The plate was washed and rinsed in distilled water then finally in ethanol and dried. The "inside" surface was sprayed with silicone mould release aerosol and excess reagent removed with a tissue. The plate was gently polished with a tissue soaked in methanol and finally wiped dry.

"Clean" plate. The plate was washed and rinsed, finally in ethanol, and dried. The "inside" surface was wiped with a tissue soaked in chloroform and allowed to dry.

The plates were then sandwiched together. They were separated along their "long" edges with 1 cm wide strips of 0.3 mm plasticard. The plates were sealed along three sides with adhesive tape.

Acrylamides' Stock solution. A stock solution of 38% acrylamide, 2% N′,N-methylene-bis-acrylamide was deionized by stirring with MB1 resin for 20 min (10 g/l of resin to solution). This suspension was filtered and the acrylamides' solution stored at 4°C until required.

Preparation of polyacrylamide gels

A gel mixture was made up as follows:

6% Acrylamides (from 40% acrylamides' stock solution)

8 M Urea

0.6 mg/ml ammonium persulphate

1x Tris/Borate sequencing electrophoresis buffer (from 10x stock) (see Media and Buffers).

The mixture was de-gassed under vacuum and cooled to 13°C. TEMED (N,N,N′,N′-tetramethylethylene diamine) was added at

1 μl per ml of gel mixture and quickly mixed in.

This mixture was pipetted into the prepared glass-plate "sandwich" and a channel former of 0.3 mm plasticard inserted in the top of the gel assembly. Bulldog clips were attached to the sides of the "sandwich" and the poured gel supported at an angle of 20° to the horizontal for at least 1 h, with the siliconized plate uppermost.

The channel former was removed and the loading slots rinsed with 1x Tris/borate electrophoresis buffer. The tape closing the base of the gel "sandwich" was cut away and the assembly mounted in a vertical gel electrophoresis apparatus. Both top and bottom buffer reservoirs were filled with 1x Tris/borate electrophoresis buffer. Loading slots were cleared of accumulating urea immediately before loading, by flushing with buffer.

Samples were loaded using 5 μl graduated capillary pipettes, drawn down on one end in a hot flame. The gel was electrophoresed for ~ 1 h at 1.5 kV. Sequential runs of the same samples were done on the same gel to extend the range of readable sequences. Upon dissembly of the apparatus the "siliconised" plate was removed, leaving the gel adhered to the "clean" plate.

Gels containing sequencing reactions done using [α-^{32}P]dATP were covered with cling-film and autoradiographed by contact with "Fujifilm X-ray film" for 24 h.

Gels containing sequencing reactions done using [α-^{35}S]-dATP αS were fixed, dried and autoradiographed as described below.

<u>Fixing and drying of polyacrylamide gels for Autoradiography</u>

The gel, still attached to the clean plate, was immersed in a tray of 10% acetic acid, 10% methanol and fixed for 15 min with occasional gently agitation. After removal from the tray,

all excess fixing solution was drained away by gradually tilting the plate to 45° to the horizontal.

A sheet of Whatman No. 1 paper was cut to the same size as the gel and placed in contact with the gel. Good adhesion was encouraged by pressing with a roller. The paper plus gel was gently peeled away from the glass plate. The exposed gel surface was covered with cling-film and the transfer placed cling-film-side-down on a pre-heated photographic print drier. When the assembly was dry, the cling-film was peeled away and the dried gel autoradiographed by direct contact with "Fujifilm" X-ray film.

RESULTS

Fig 3.

The diagram shows growth curves for *T.furgasoni* str.W/ATCC. Initial inocula were made to a cell density of above 1 X 10^4 cells/ml, into flasks of growth medium used for standard batch growth. (see p 37.)

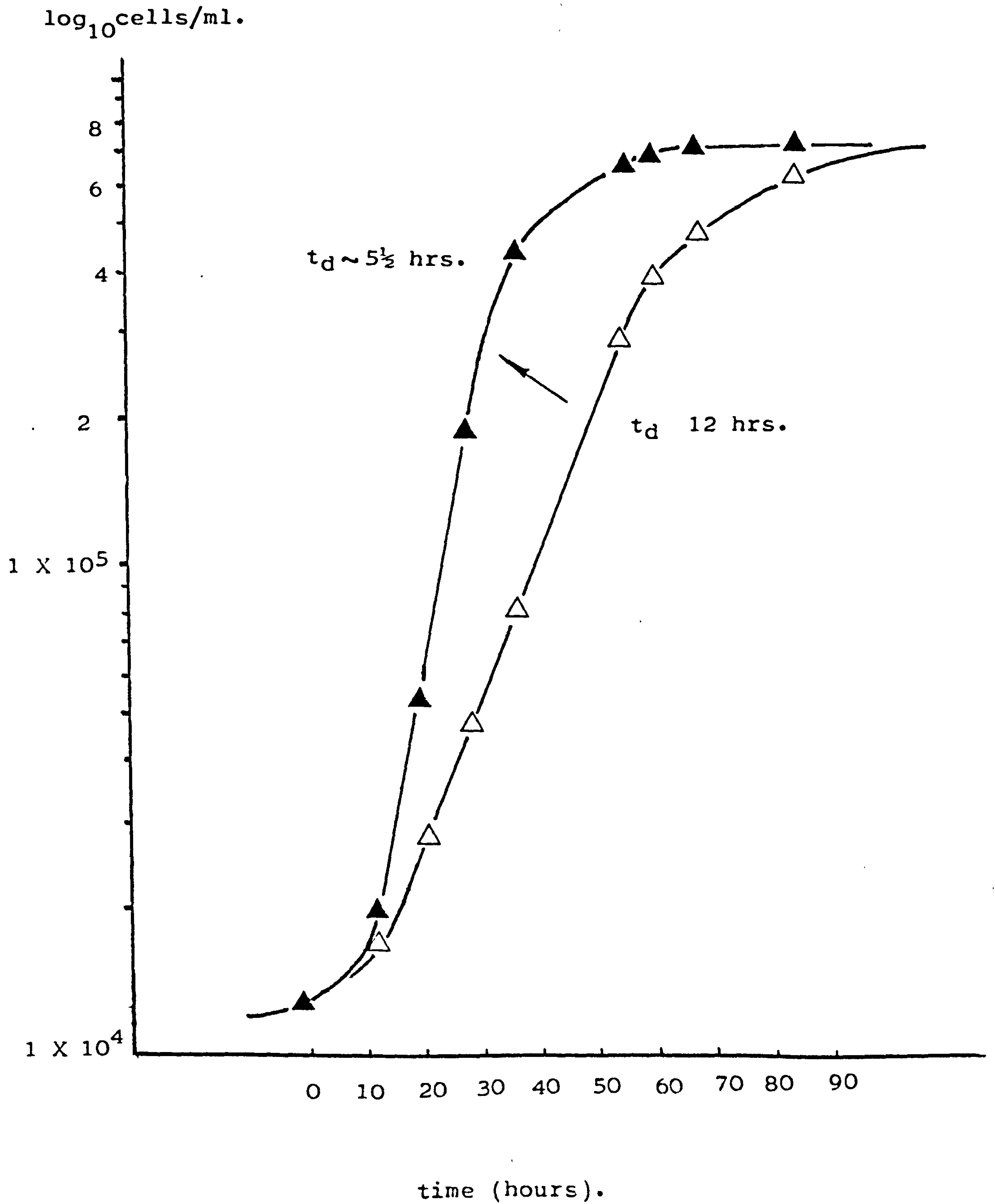
Cell densities were measured by counting samples of the culture in a suitable cell counting chamber at regular intervals.

The curve plotted through the open points (△) represents the growth curve of *T.furgasoni* soon after sub-culturing of the stock culture began.

The curve plotted through the filled points (▲) represents the growth curve of *T.furgasoni* after 3 months regular sub-culturing. the shift in doubling time from 12 hrs to 5½ hrs was noted.

Fig 3.

Growth curves for *Tetrahymena furgasoni* str. W/ATCC.



Studies of T. furgasoni str. W/ATCC mtDNA

Samples of T. furgasoni str. W/ATCC mtDNA were prepared from exponentially growing cells.

This strain showed an increase in growth rate over a 3-month period, during which time a starter culture was sub-cultured frequently. Growth curves for W/ATCC are shown in Fig. 3. It was concluded that a faster growing population was being selected for under these conditions. This phenomenon has been observed previously in other Tetrahymena strains when exponentially growing cells were sub-cultured regularly (I. G. Jones, personal communication). When the faster growing cell line of str. W/ATCC was returned to stock culture it maintained its increased growth rate, compared with the original stock culture, for two years.

Purification of W/ATCC mtDNA. W/ATCC mt DNA was purified on sucrose gradients and found to sediment at 34 s. This sedimentation coefficient is similar to that observed for the mtDNA of other Tetrahymena strains (Flavell & Follet, 1970) (Goldbach et al., 1977) (Holt & Jones, unpublished).

A typical sucrose gradient trace of a W/ATCC mtDNA gradient is shown in Fig. 4.

Studies with restriction endonucleases on W/ATCC mtDNA

Samples of W/ATCC mtDNA prepared from both fast and slow growing cell lines were digested with Eco RI restriction endonuclease.

The two digests were analysed by gel electrophoresis on a horizontal agar gel and photographed as described. No difference in the appearance of the two digests was apparent.

Samples of W/ATCC mtDNA were subsequently prepared from the faster growing cell line. Samples of W/ATCC were digested

Fig 4.

The diagram shows the absorbance profile, measured at 254 nm, of sucrose gradients of mtDNA and mtRNA preparations.

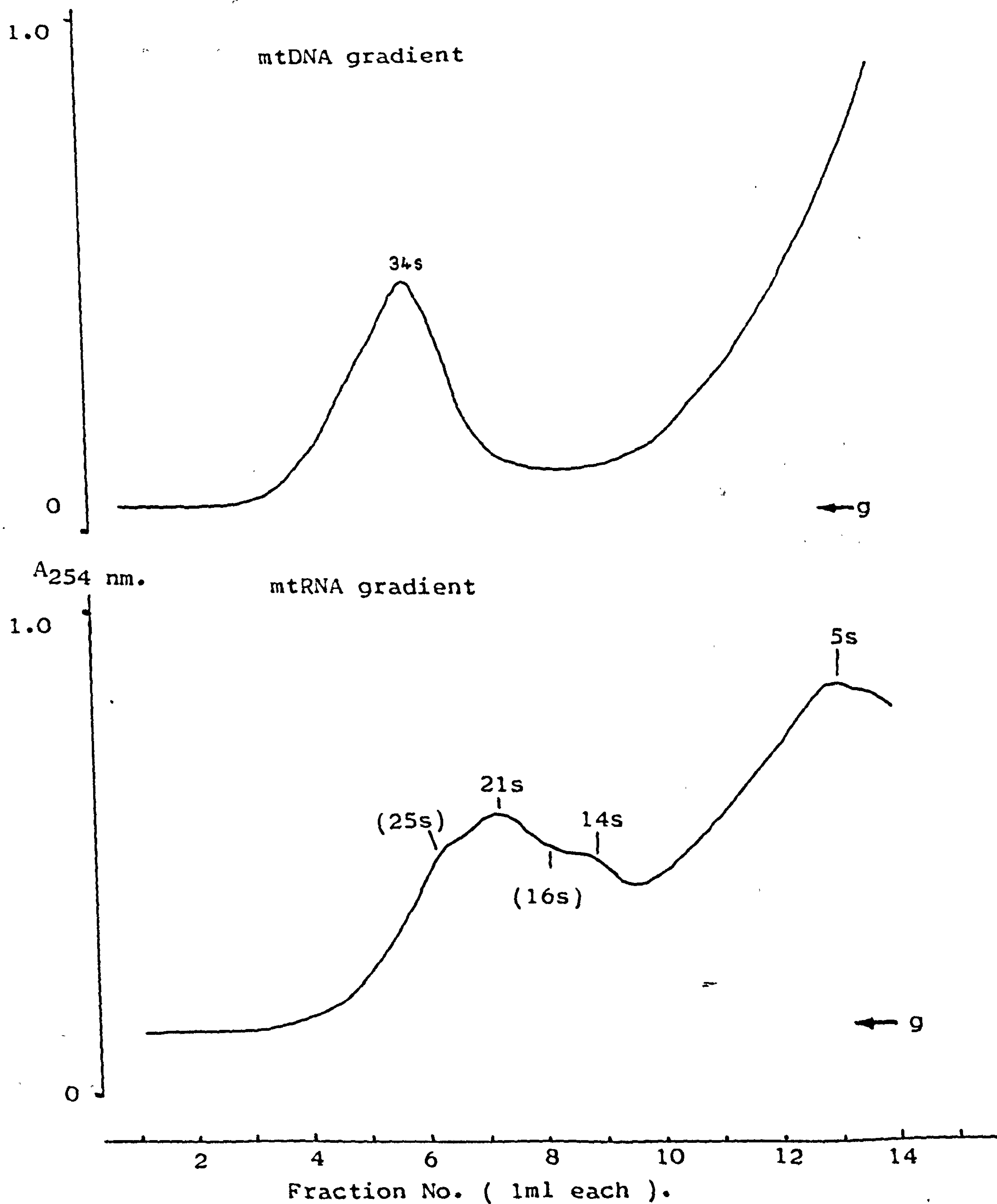
The mtDNA peak centred at 34s represents approx. 30µg of mtDNA.

The trace of the mtRNA peaks, recorded using a less-sensitive flow cell, represents approx. 150 µg of RNA.

By knowing the duration and speed of the centrifuge run, and the characteristics of the gradients, the position of the individual molecular species on the gradients could be predicted. The validity of these predictions were confirmed by gel electrophoresis of aliquots of the appropriate gradient fractions , prior to collection of the molecular species in question by precipitation or pelleting.

Fig 4.

Sucrose gradient fractionations of mitochondrial nucleic acid from T. furgasoni str. W/ATCC.



with all the restriction endonucleases available in the laboratory at the time.

The standard restriction endonuclease nomenclature used is that found in the reviews by Smith & Nathans (1975) and Roberts (1979).

The W/ATCC mtDNA molecule was found to be cut with a number of enzymes. Details are given in Table 2.

Greater numbers of sites were found for those enzymes specific for hexonucleotide recognition sites containing 4 A-T pairs. Lesser numbers of sites were found for those enzymes specific for G-C-rich sites. This result implies that the W/ATCC mtDNA possessed a high A + T base composition. The number of sites seen for these enzymes reflects similar results seen in the restriction digest studies on other Tetrahymena strains (Holt & Jones, unpublished) (Norton, 1980). The base composition of these other Tetrahymena strains have been determined at ~26% G + C (Goldbach et al., 1977) (Holt & Jones unpublished).

System of nomenclature used to describe restriction endonuclease digest products of W/ATCC mtDNA

The following system of nomenclature has been adopted to describe the products obtained upon digestion of W/ATCC mtDNA with the restriction endonucleases used to map the molecule.

(a) The complete collection of restriction fragments present in a restriction digest, be it a single or multiple enzyme digest, are referred to as the "pattern" for that digest (with reference to the characteristic appearance or "pattern" of the restriction fragments after gel electrophoresis).

Table 2

Restriction enzyme sites on the W/ATCC mtDNA molecule

The molecule was found to be cut by the following enzymes:

Enzyme	Recognition Site	No. of sites in the W/ATCC mtDNA molecule
Eco RI	G AATTC	7
Bcl I	T GATCA	11
Xba I	T CTAGA	10
Hind III	A AGCTT	11
Hae III	GG CC	4
Pst I	CTGCA G	4
Bgl II	A GATCT	13
Alu I	AG CT	Many
Taq I	T CGA	Many

No sites were found for the following enzymes:

Sal I	G TCGAC
Kpn I	GGTAC C
Xho I	C TCGAG
Bam Hl	G GATCC

(b) Any restriction fragment which is apparently discrete in size will be referred to as a "discrete" fragment.

(c) Any restriction fragment which is heterogeneous in size will be assumed to be a terminal fragment and will be referred to as a "terminal fragment".

(d) Each fragment is assigned a letter code denoting the enzyme used to produce that fragment:

Enzyme	Letter Code
Hae III	Ha
Hind III	H
Xba I	X
Bcl I	B
Eco RI	R
Pst I	P

(e) Each "discrete" fragment produced from a single enzyme digest is assigned a number indicating its position in the digest pattern in order of decreasing size, e.g. P2 will be the second-largest Pst I fragment produced.

(f) Any fragment which appears to be present in the digest pattern at greater than equimolar amounts compared to the other fragments is assigned the suffix "D" to denote a double intensity band on the gel, or "T" to denote a triple intensity band on the gel.

(g) A new fragment produced in a double-enzyme digest, which is not seen in either of the single enzyme digest patterns, is assigned the letter codes of both the enzymes in question. This will indicate that the fragment possessed different restriction sites at the ends of the fragment. Such

fragments are also assigned a number corresponding to their estimated molecular weight expressed in Megadaltons, e.g. RH 1.8 would be a fragment of 1.8 Md produced in an Eco RI/Hind III double digest, with this fragment not being present in either the Eco RI or Hind III single digest patterns.

(h) Terminal restriction fragments are not included in the numbering system for the discrete fragments since their extreme heterogeneity of length may confuse their position in the pattern. These fragments are assigned the extra letter code "F" and a number 1 or 2 in order of decreasing size, e.g. BF 2 is the smaller of the terminal restriction fragments produced in a Bcl I digest.

In the derivation of the restriction map a convention will be adopted of "left" and "right", to describe the orientation of the molecule (relative to the reader as the diagram appears on the page). These conventions are purely arbitrary and are merely designed to simplify this discussion.

Derivation of the Restriction Site Cleavage Map for W/ATCC mtDNA

A physical map of the sites for the enzymes Eco RI, Bcl I, Xba I, Hind III, Hae III and Pst I was constructed. The sizes of the restriction fragments produced by digestion of W/ATCC mtDNA are shown in Fig. 5.

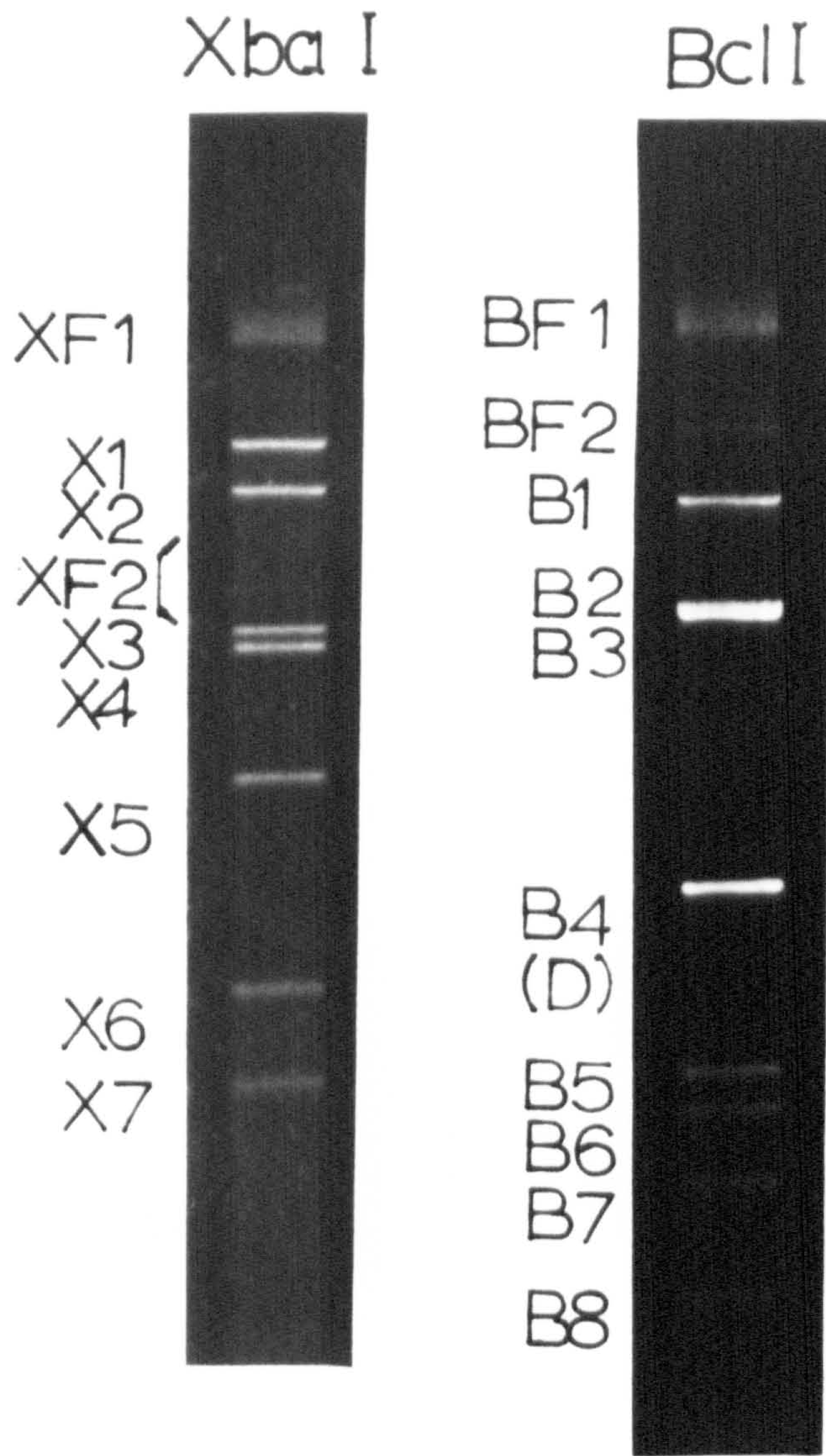
A particular feature of note seen in these restriction digest patterns was the extreme degree of length heterogeneity in certain fragments, e.g. in Xba I and Bcl I digests, see Plate 1. The apparent non-existence of certain terminal restriction fragments will be discussed later e.g. in the Hind III digest.

Plate 1.

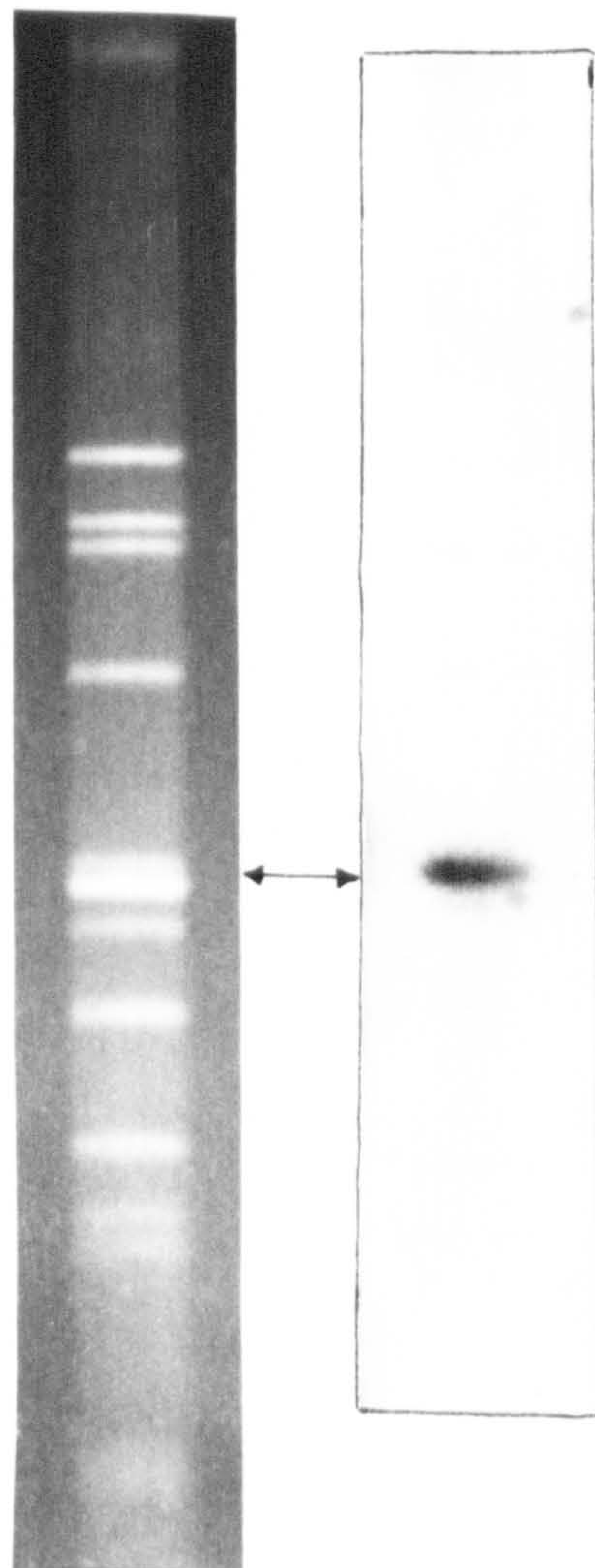
The gel electrophoresis patterns for Xba I and Bcl I single enzyme restriction digests are shown. Of particular note are the smeared or "fuzzy" restriction fragments XF1 & 2, and BF1 & 2. These fragments display a large heterogeneity in length.

The gel electrophoresis pattern produced by an Xba I/Bcl I double restriction digest on W/ATCC mtDNA is shown alongside the accompanying Southern blot which has been hybridized with labelled 14s rRNA. The significance of the hybridization of the 14s rRNA to fragment XB 1.15 is discussed on p 106.

Plate 1.



Digests of w/atcc mtDNA with Xba I and Bcl I.



Double digest of w/atcc mtDNA with Xba I and Bcl I. The autoradiogram shows the hybridization of labelled 14s rRNA.

XB 1·15

Fig 5.

The size of the restriction fragments produced by digestion of W/ATCC mtDNA with the named restriction endonucleases are shown on this diagram. The approximate sum of the size of these fragments is shown a the bottom of each column. The actual appearance of the Eco R1, Pst I, and Hae III digest patterns are shown below. (The photographs of the Xba I, Bcl I, and Hind III patterns are shown on plates 1 & 2.)

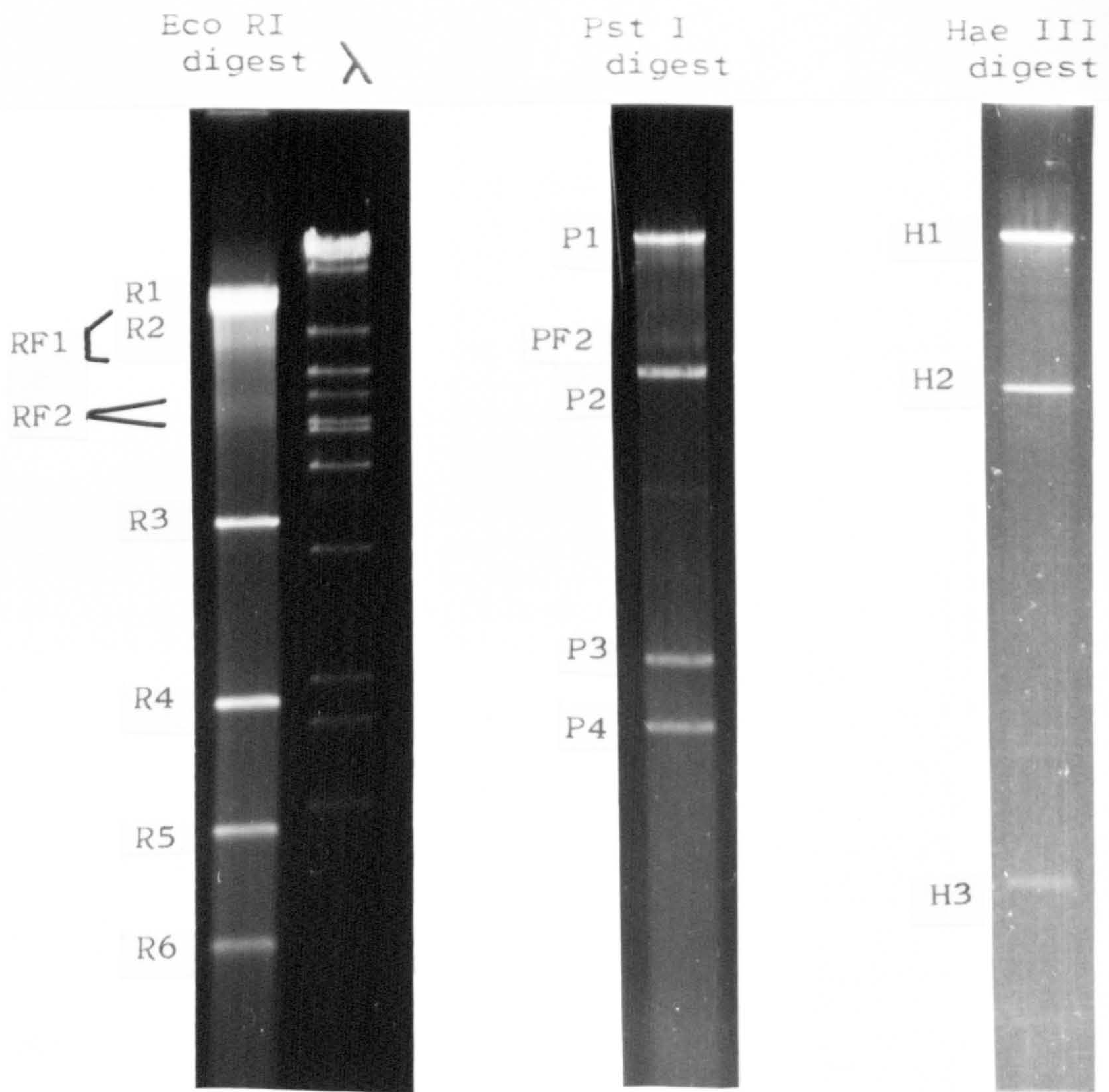
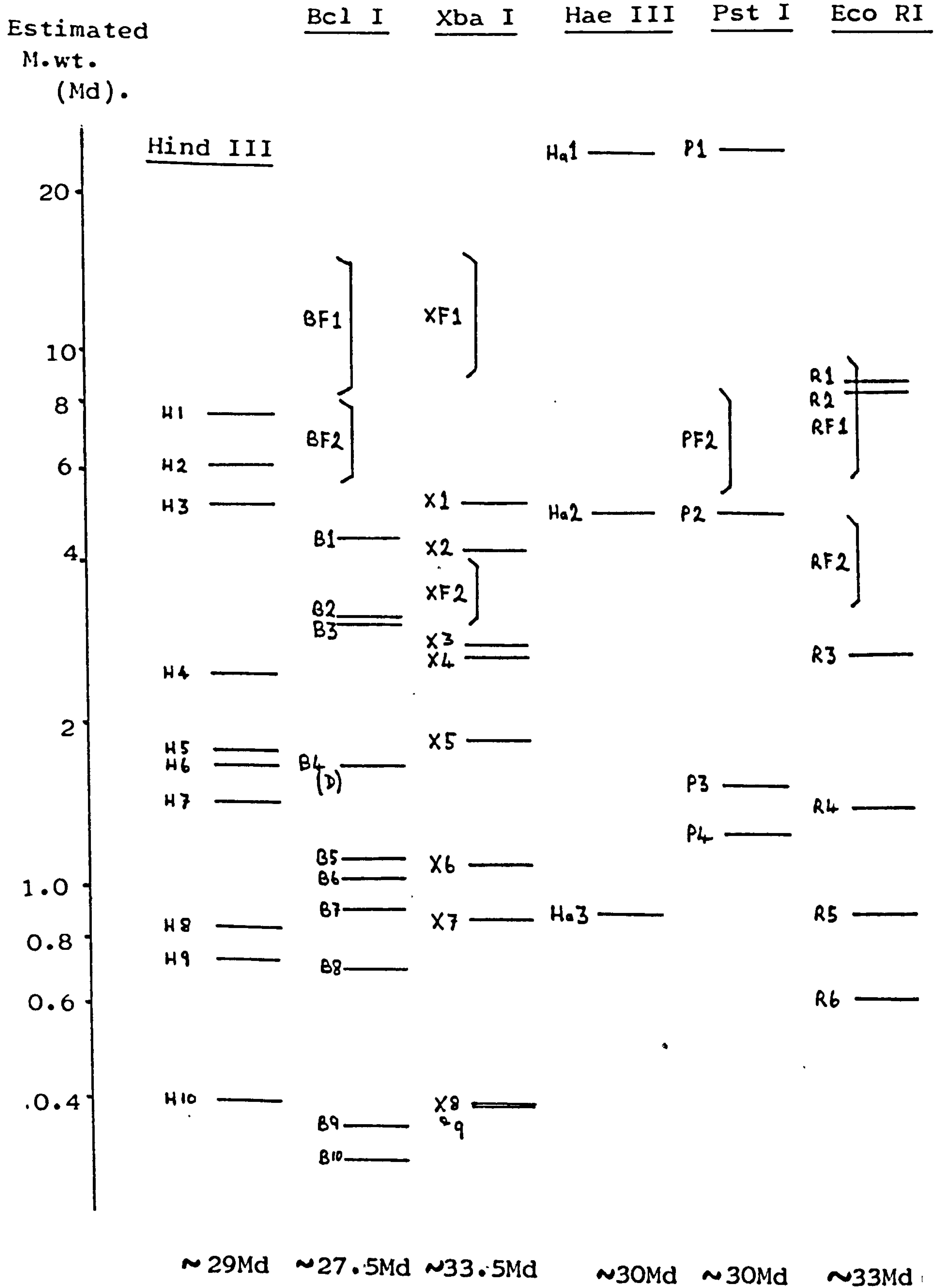


Fig 5. Single restriction enzyme digests.

Restriction digest fragment patterns produced upon digestion of T. furgasoni str. W/ATCC mtDNA with the named restriction enzymes.



Experience with other Tetrahymena strains has shown that they also possess restriction fragments showing a heterogeneity in length. These heterogeneous-length fragments were subsequently mapped to the termini of the molecule.

This assumption that these fragments, showing length heterogeneity, originated from the termini of the molecule was used in the derivation of the restriction site map. The validity of this assumption will be discussed later.

The enzyme Hae III produces the least number of fragments of all the enzymes used. To initiate the mapping of restriction sites, double digests were done using Hae III in conjunction with the other five enzymes named.

Double digests with Hae III. The double digest products produced upon digestion of W/ATCC mtDNA with the five named enzymes in conjunction with Hae III are shown in Fig. 6. All digests were repeated with increased amounts of enzymes to ensure 'limit' digestion.

In all the double digests, the fragments Ha1 and Ha 2 were lost. New fragments were found in the double digest patterns to account for the lost fragments.

Ha2 fragment (5.0 Md)

Enzyme in double digest	New fragments
Eco RI	RHa 2.4, RHa 2.6
Hind III	HHa 4.5, HHa 0.5
Xba I	XHa 3.2, XHa 1.8
Bcl I	BHa 4.1, BHa 0.9
Pst I	PHa 4.2, PHa 0.8

In the Pst I/Hae III double digest the new fragments PHa 4.2 and PHa 0.8 account for the loss of a fragment of 5.0 Md. A single fragment of 5.0 Md is also retained in the

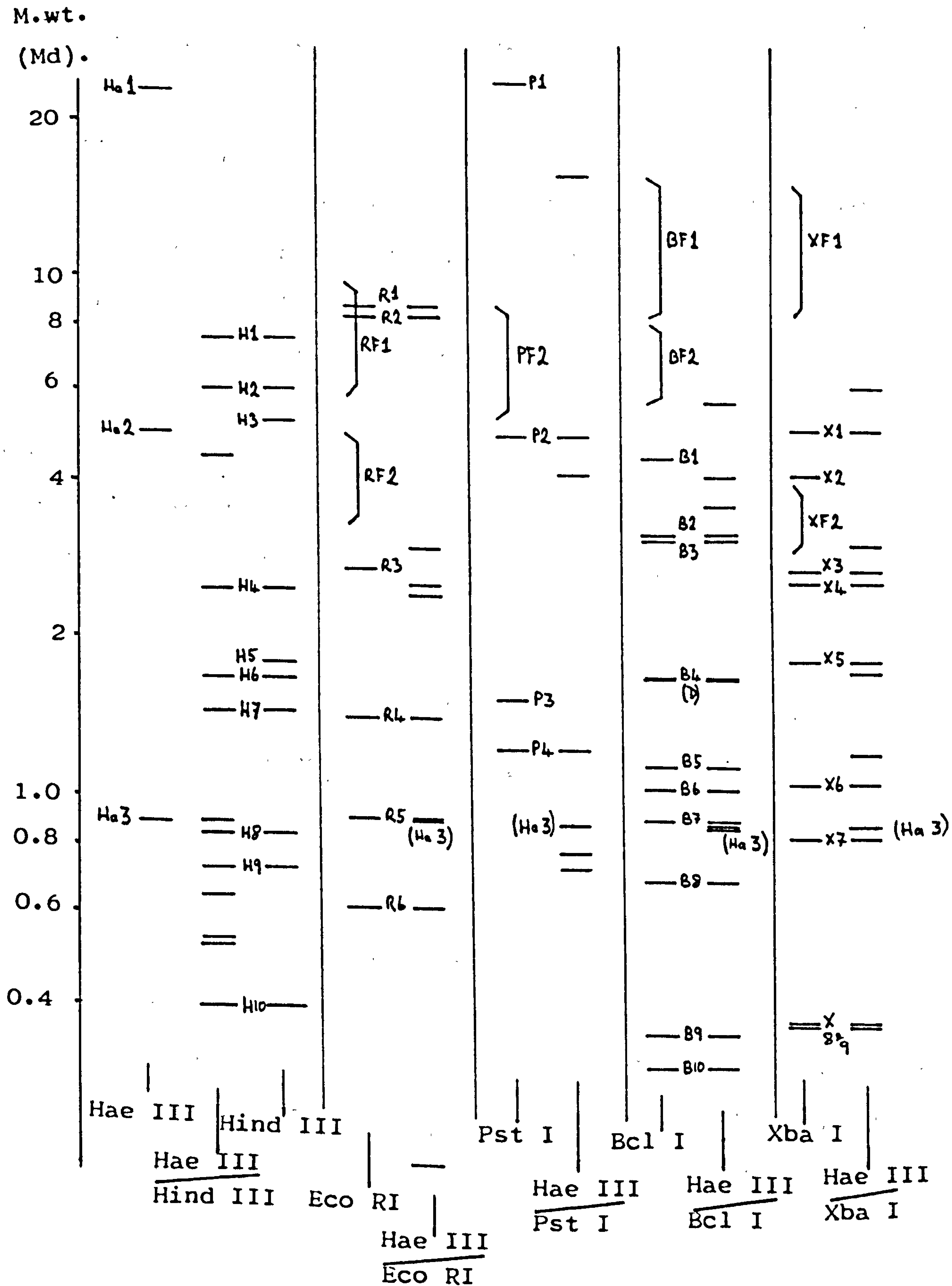
Fig 6.

The diagram shows the size of the double digest products produced by digestion of W/ATCC mtDNA with the named restriction enzymes in conjunction with Hae III. In each case a single restriction digest pattern for the enzyme in question is shown for comparison.

Fig 6

Hae III double digests

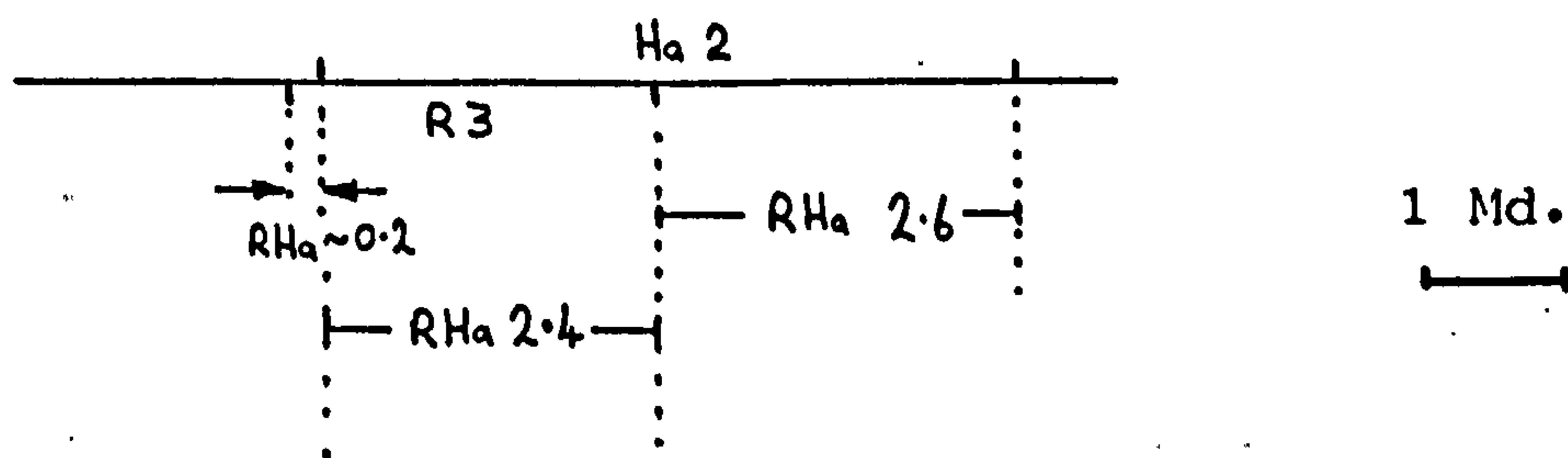
Restriction digest fragment patterns produced upon double digestion of T. furgasoni str. W/ATCC mtDNA with the named restriction enzymes in conjunction with Hae III.



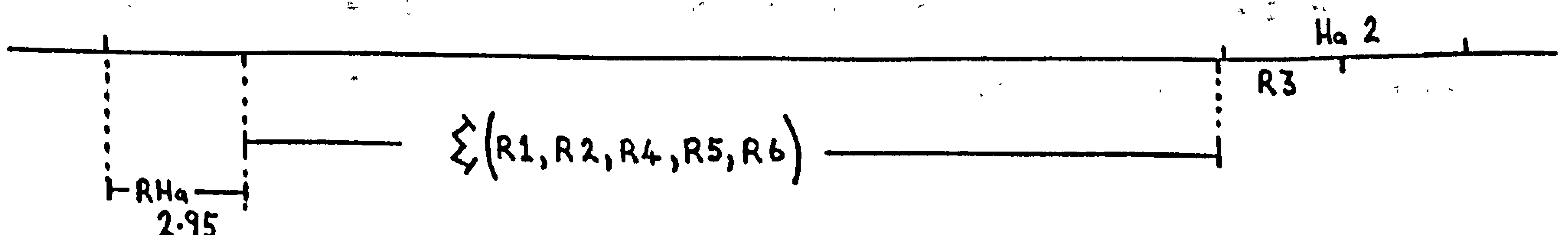
double digest and could be either P2 or Ha2, (see Fig. 6). To resolve this ambiguity, fragments Ha2 and P2 were recovered from single enzyme digest patterns on agarose gels and treated with the alternate enzyme (Ha2 was digested with Pst I, P2 was digested with Hae III). Ha2 digested to give new fragments PHa 4.2 and PHa 0.8, no digestion of P2 with Hae III was observed.

It has now been established that all five enzymes possess sites within the Ha2 fragment. The relative location of these sites will now be determined.

In the Eco RI/Hae III double digest pattern, the fragment R3 is lost (see Fig. 6). This means that the R3 fragment possesses a Hae III site. The lost R3 fragment could be accounted for by the new fragments RHa 2.4 and RHa ~0.2 in the Eco RI/Hae III double digest pattern.



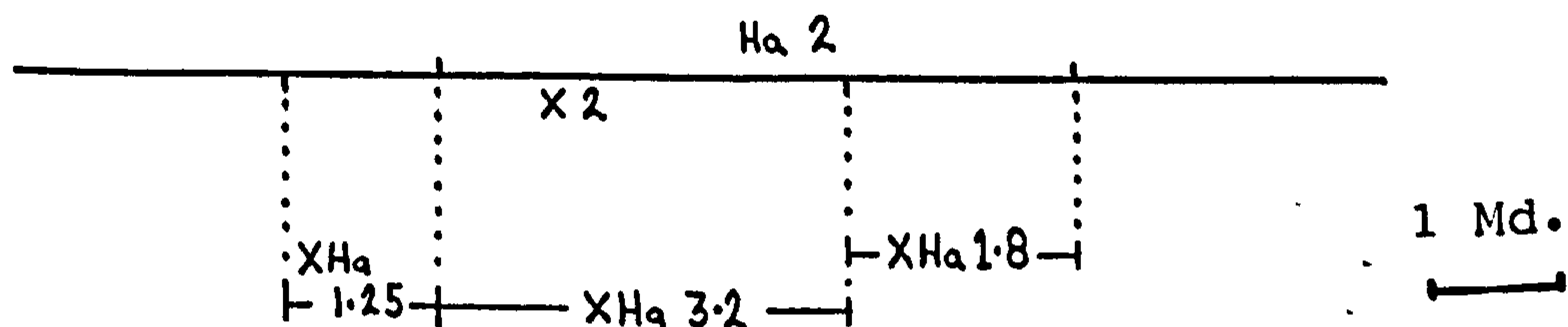
The lost Ha 1 fragment could be accounted for if all the remaining Eco RI discrete fragments 'mapped' within Ha 1. The difference between the extreme "left" Eco RI site and the Hae III site marking the boundary of the Ha 1 fragment, would account for the remaining unaccounted for new fragment RHa 2.95.



The origins of the fragments RHa 2.95 and RHa 2.6 have been accounted for from the Hae III map, but not from the Eco RI map. The only fragments from the Eco RI pattern not yet considered are the terminal fragments RF1 and RF 2. Since RHa 2.95 and RHa 2.6 are seen to map on either side of the region containing R1 to R6 inclusive (sum of the discrete fragments), it is reasonable to assume that the new fragments RHa 2.95 and RHa 2.6 originate from RF1 and RF2 in the EcoRI pattern, since by definition, the terminal restriction fragments will map on either side of the collected discrete restriction fragments.

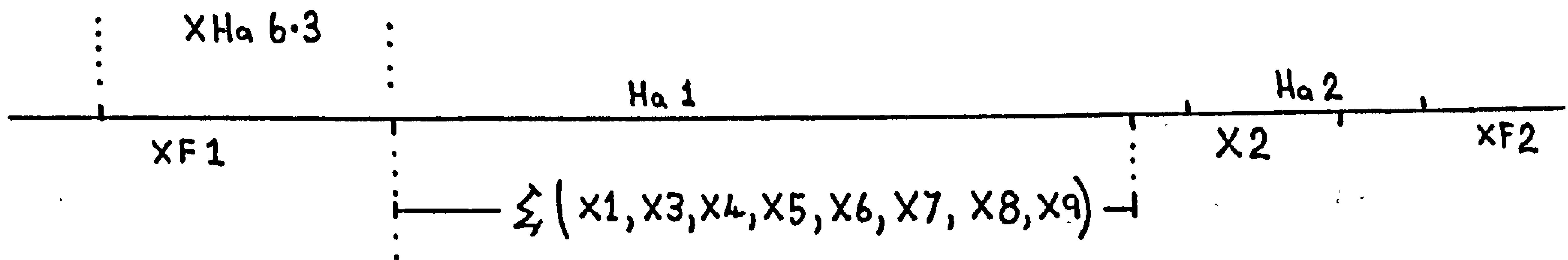
This process of mapping a fragment, which is lost in an Hae III double digest, over the identified Hae III sites will now be applied to the other Hae III double digest products.

Double digests with Xba I and Hae III. The discrete Xba I fragment lost in the Xba I/Hae III double digest is X2 (see Fig. 6). The new fragments accounting for its loss are XHa 3.2 and XHa 1.25. The lost Ha 2 fragment can be accounted for by the new fragments XHa 3.2 and XHa 1.8. This data can be mapped as follows:



The remaining Xba I/Hae III double digest product not accounted for is the new fragment XHa 6.3. This is produced by the extreme left-hand site of the Xba I map cutting well within the Ha 1 fragment. (Ha 1 is the only discrete fragment

in the Hae III pattern sufficient in size to produce XHa 6.3.)

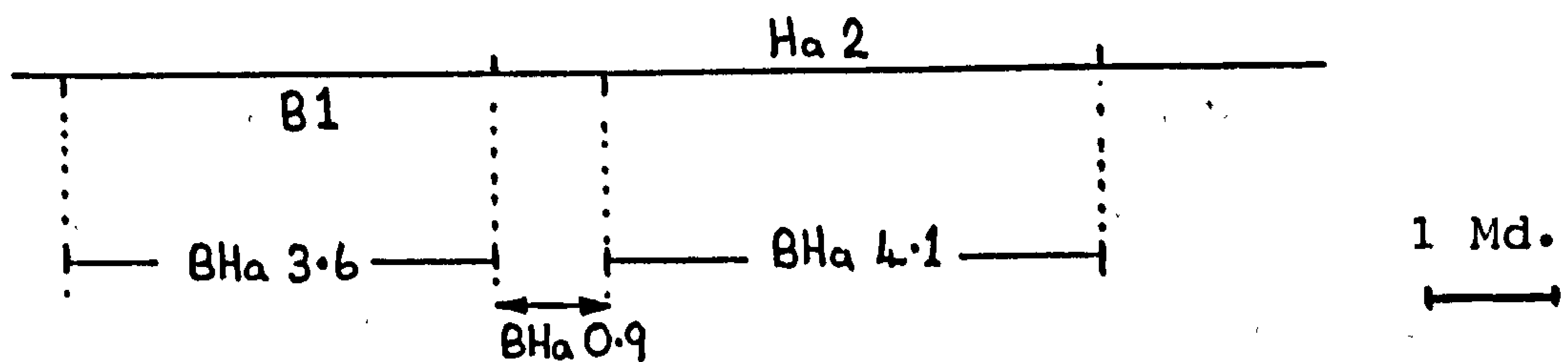


Since the fragments XHa 6.3 and XHa 1.8 map outside the sum of the discrete XbaI fragments, they must originate from the terminal fragments, XF1 and XF2. As in the Eco RI double digests the terminal fragments from the Xba I pattern are lost in the Xba I/Hae III double digest.

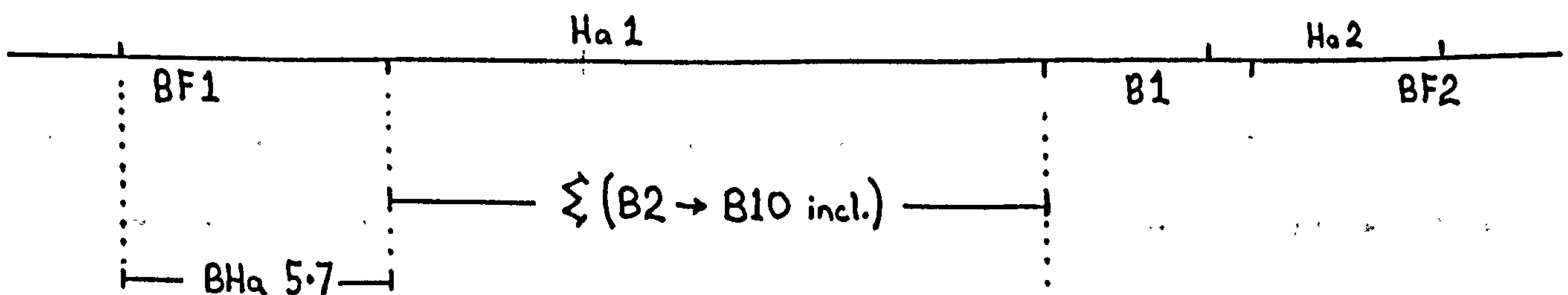
Double digests with Bcl I and Hae III. The discrete fragment lost from the Bcl I single digest pattern is B1, when the enzyme Hae III is included in the digest.

B1 is accounted for by BHa 3.6 and BHa 0.9

Ha 2 is accounted for by BHa 0.9 and BHa 4.1 (see Fig. 6).



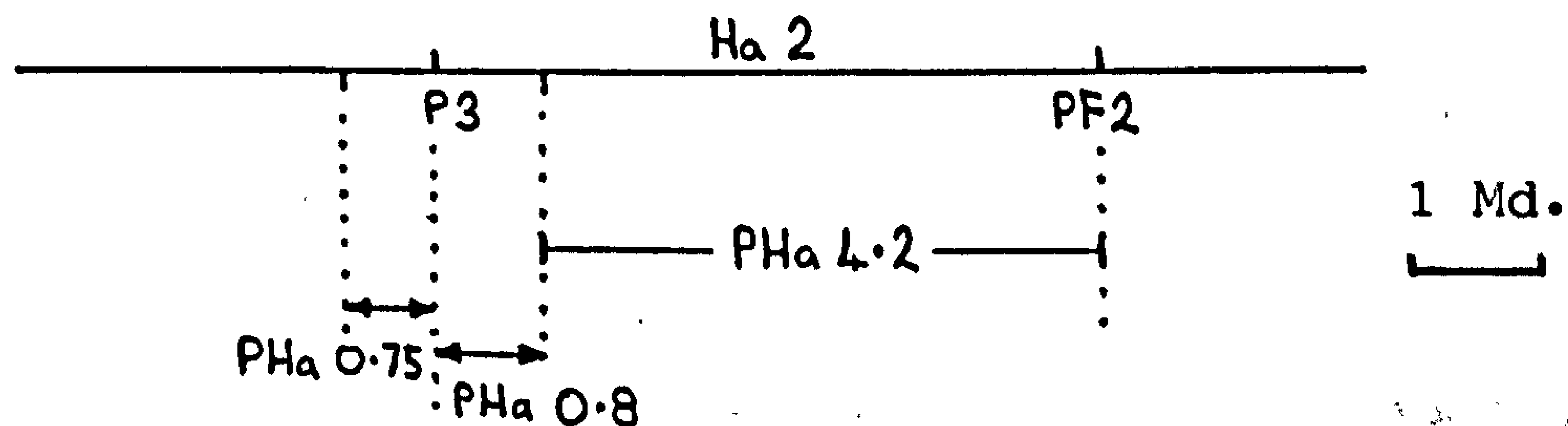
The remaining new fragment BHa 5.7 is produced by the extreme left-hand site of the Bcl I map cutting well within the Ha 1 fragment.



The new fragments BHa 5.7 and BHa 4.1 originate from the terminal fragments BF1 and BF2 in the Bcl I single digest pattern.

Double digests with Pst I and Hae III. The large restriction fragment P1, appears at first to be a large discrete fragment. The mapping of the Pst I sites on the gradually expanding restriction map centres around a key assumption, that is that the large Pst I fragment P1 is actually a very large terminal fragment.

The fragments lost from the Pst I single digest pattern when Hae III is included, are P1, PF"2" and P3. The fragments P3 and Ha 2 can both be accounted for by new fragments:

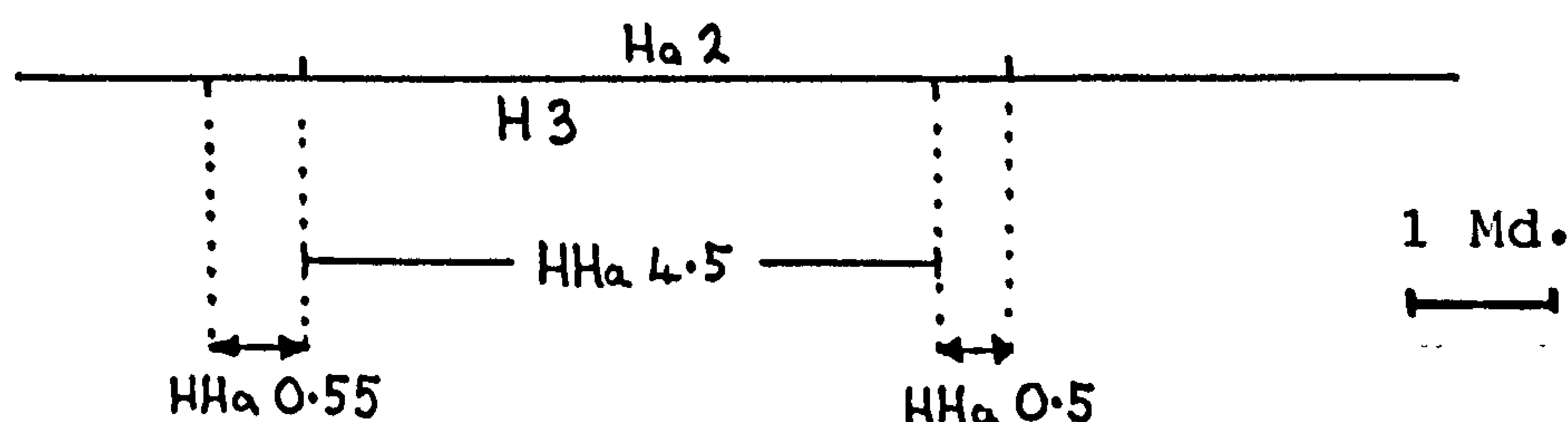


As in the previous three cases, the remaining single digest Pst I fragment can all map to the "left" of this region shown above. The remaining new fragment PHa ~ 16.0 would occur from the extreme left Pst I site (which compared to the other Eco RI, Xba I and Bcl I maps isn't very far left at all) mapping well within the Ha 1 fragment, in fact ~16 Md away from the site bordering the 'left'-hand end of the Ha 1 fragment.

This assumption that the P1 fragment is in fact "terminal" is not as far-fetched as it first appears. The fragment is very large in size. The length heterogeneity of the terminal fragments from other digest patterns (Eco RI, etc.) show length

heterogeneities of 3-4 Md. The "percentage" heterogeneity of a very large terminal fragment would therefore be very much smaller than that for the more accurately sized terminal fragments from other digest patterns. The very nature of the reduced mobility of a large restriction fragment would result in any length heterogeneity being difficult to detect (see Fig. 7).

Double digests with Hind III and Hae III. Two discrete Hind III single digest fragments are lost in the Hind III/Hae III double digest, H3 and H5. H3 and Ha 2 can be accounted for from some of the double digest new fragments (see Fig. 6):



The sum of the discrete fragments present in the Hind III single digest pattern amounts to a greater amount of material than in the previous discrete maps produced. The sum of the discrete Hind III fragments, minus H3, could map to the left of H3 in the above diagram and reach far enough along the molecule to reach the Hae III site at the left-hand end of the Ha 1 fragment. This means that there are two possible locations in which the H5 fragment could map:

(a) to the right of H3, (see the last diagram); or

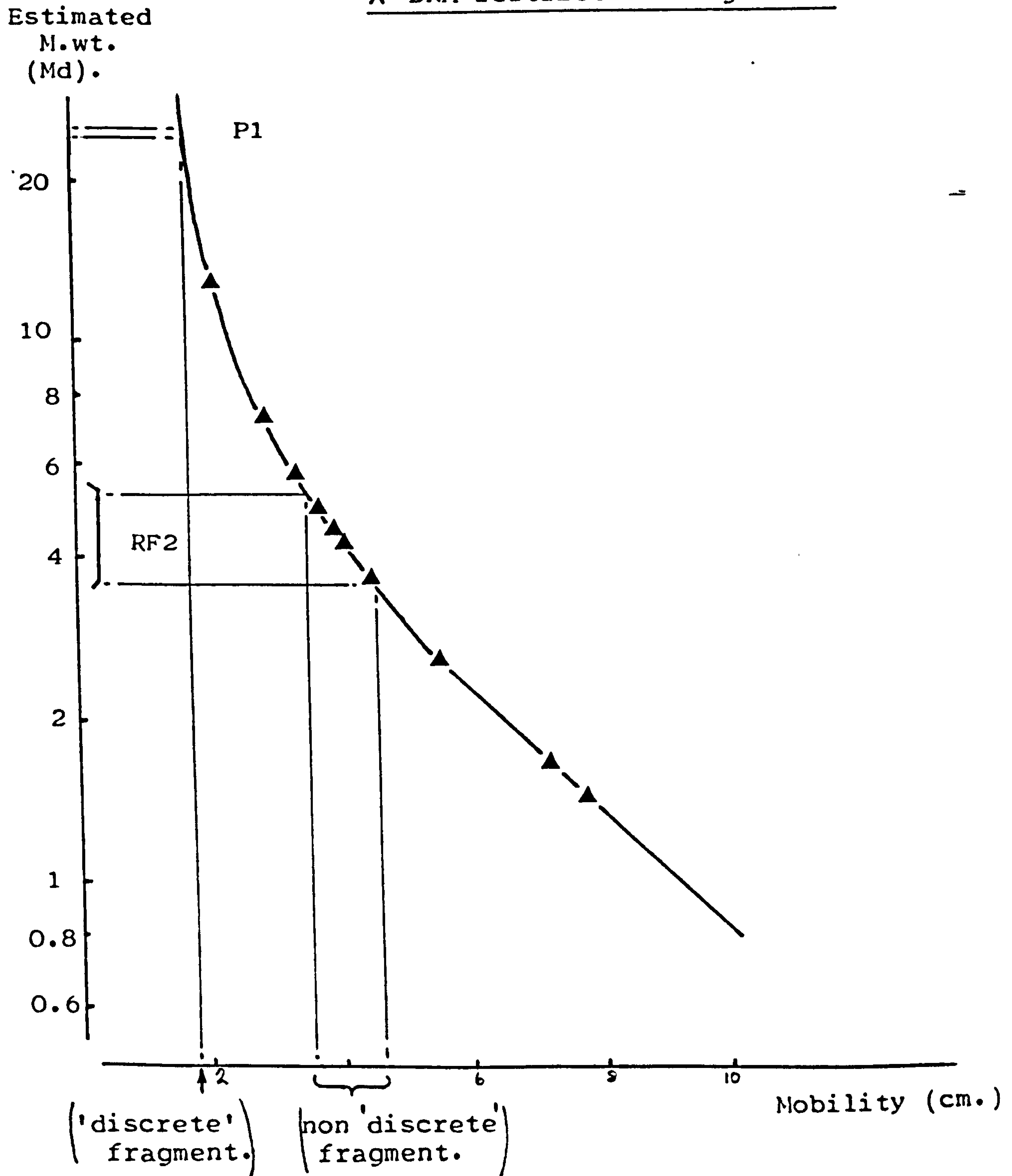
(b) at the other end of the molecule at the end of the Ha 1 fragment.

Fig 7.

This diagram illustrates the supposed difference in appearance of the restriction fragments P1 and RF2. Both fragments are plotted with a length heterogeneity of 2 Md, to show the difference in appearance that such a heterogeneity would produce. (The photographs of these digest patterns are shown opposite p80.). The lambda calibration curve on the diagram is typical, the points being plots of the lambda restriction fragment values. (Schroeder & Blattner, 1981).

Fig 7.

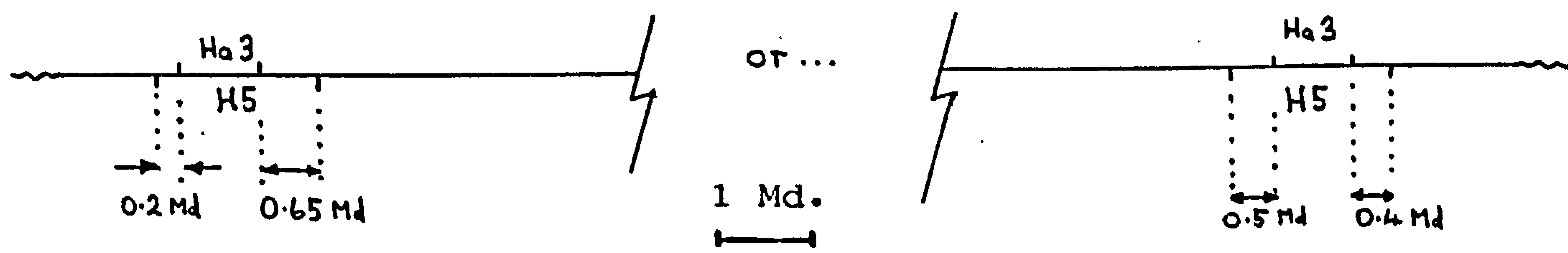
Gel electrophoresis calibration curve of λ DNA restriction fragments.



The heterogeneity in length of fragments P1 and RF2 is shown, as being 2 Md.

This spreads the smaller RF2 fragment over a considerable area of the digest pattern resulting in its 'fuzzy' or non discrete appearance. The same length heterogeneity of 2 Md does not spread the very large P1 fragment, giving it the appearance of a fragment which is discrete in size.

The discrete fragment Ha 3 has not yet been mapped. It must map outside the mapped fragments Ha 1 and Ha 2.



The only unaccounted for double digest products (new fragments) in the Hind III/Hae III double digest pattern are small. Only by including Ha 3 in the mapping of H5 can this region of the map be resolved. If Ha 3 did not map within H5, large double digest products of Mol. wt. ~1.3 Md would be produced. These are not seen.

The version of the map given as the 'left-hand possibility' for the mapping of H5 and Ha 3 is the permutation which agrees with the size of the double digest products seen in the double digest pattern (see Fig. 6).

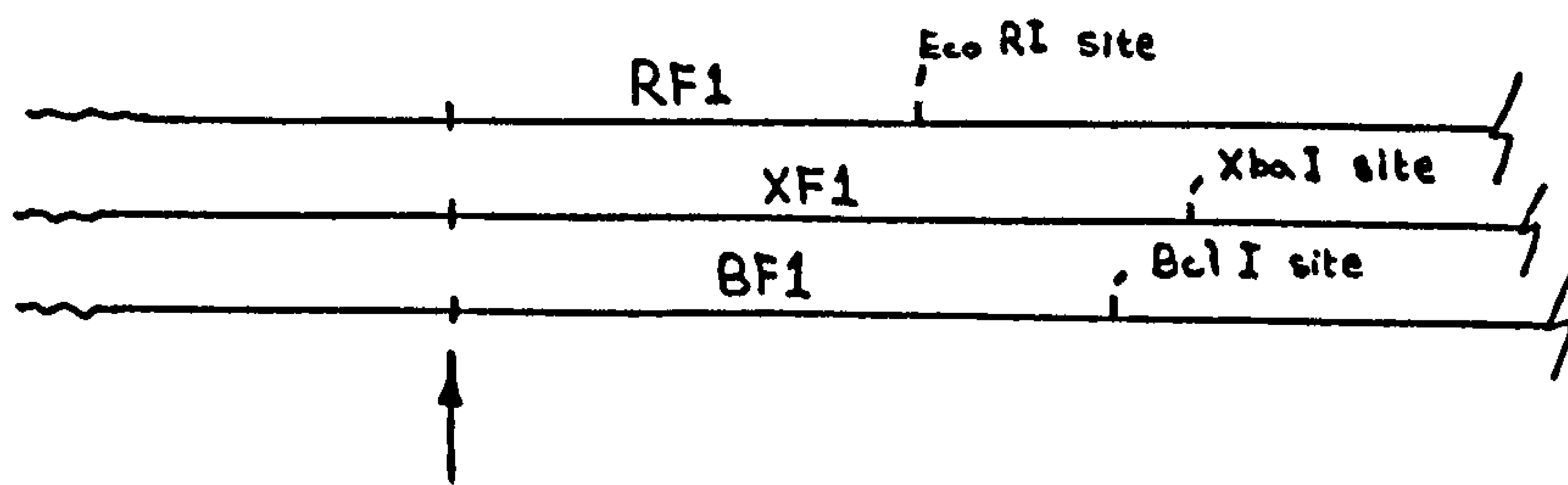
Mapping the terminal regions of the molecule

It was noted that the fragments showing length heterogeneity, and assumed to be the terminal restriction fragments, showed a distinct minimum size detectable on gel electrophoresis. This characteristic was particularly noticeable in fragments BF 1 and XF 1 (see Plate 1).

In following this lead, as a possible line of investigation to indicate properties of the termini, the minimum size of all the detectable terminal restriction fragments were noted on the map, along with the estimated maximum size of the fragments.

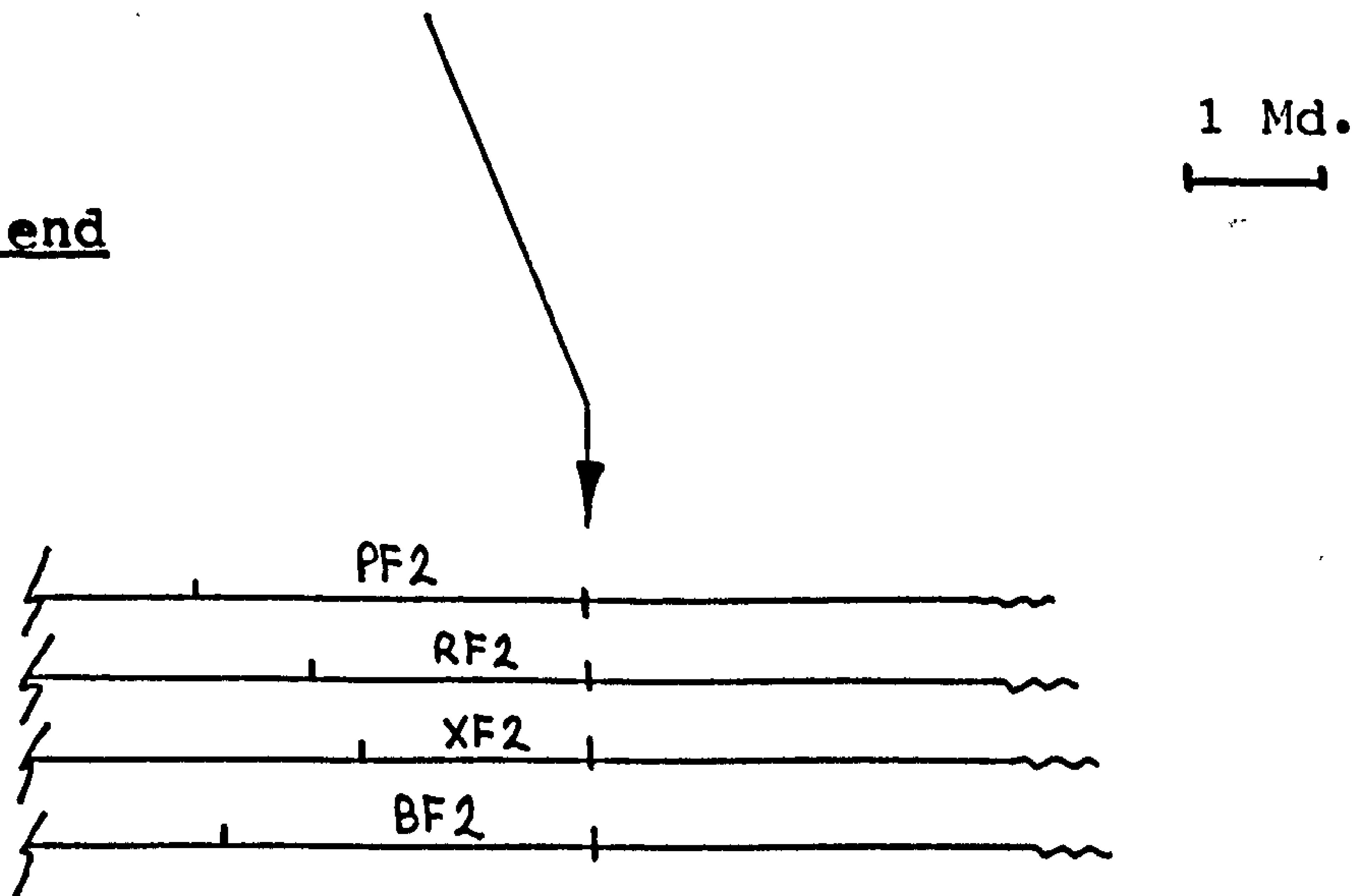
The maximum size, as expected, gave the variable limits of the molecule and varied in position depending upon the relative merits of the gel in question. Wherever they were detectable the estimated "minimum" size of the terminal fragments, which for XF 1 and Bcl 1 were always sharply defined, never altered.

<u>Left-hand end</u>



loci of minimum
length of terminal
restriction fragments.

<u>Right-hand end</u>



The mapping of all these minimum sizes on the same loci implies that there is a minimum size beyond which the molecule is not reduced. This implies that there is a distinct point beyond which the terminal length heterogeneity becomes apparent.

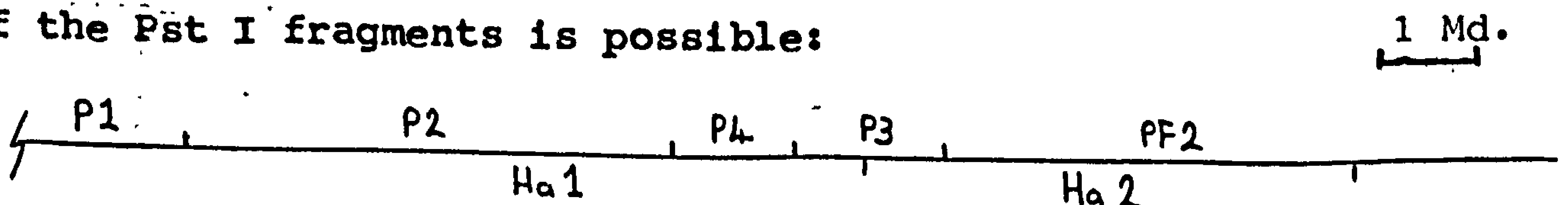
The terminal restriction fragments for the Hind III and Hae III single digest patterns have not been identified. These fragments were not detectable.

Predicted characteristics of the Hae III and Hind III terminal restriction fragments.

The position of the mapped discrete fragments from the Hind III and Hae III digest patterns suggests that the terminal restriction fragments from these digest patterns would be small in size compared to those seen in the digest patterns of, for example, Eco RI and Bcl I. In these other restriction patterns it was noted that as the size of the terminal restriction fragment is reduced, then the greater is the effect of the length heterogeneity in spreading the fragment over a larger area of the gel. This is particularly noticeable in comparing BF 2 and XF 2 (see Fig. 5 and Plate 1). It is therefore assumed that the small size of the Hae III and Hind III terminal restriction fragments has resulted in their being spread over such a large expanse of the gel pattern as to render then undiscernable from the background.

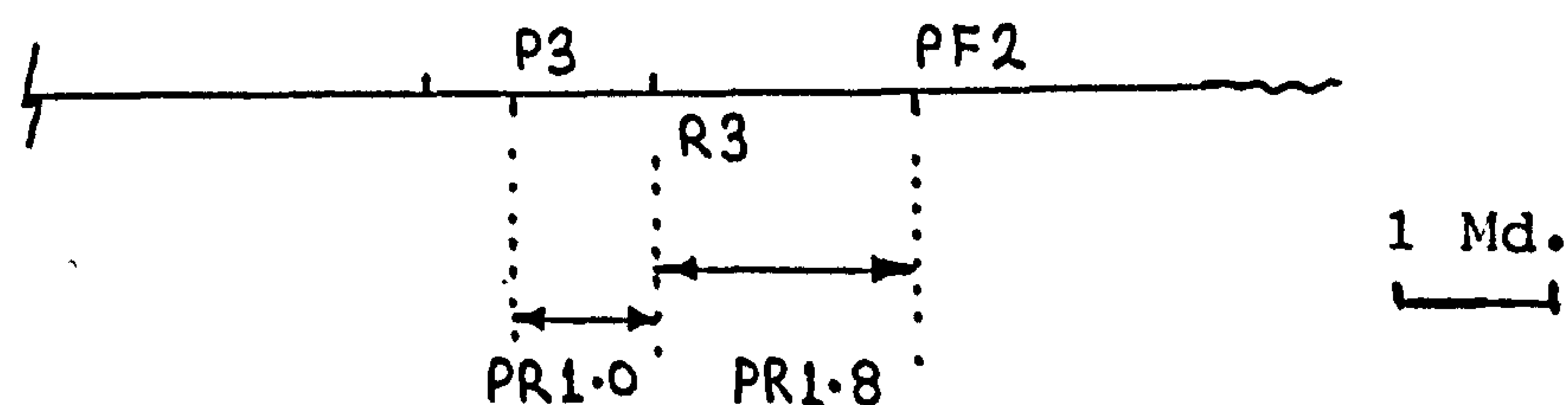
Now that the ends of the molecule have been defined, the rest of the restriction sites can be filled in between the established limits.

Completion of the Pst I map. In a few Pst I single digests it was noted that a partial digest product of mol. wt. 2.8 Md was present. This size would correspond to the partial digest product of P3 + P4. To allow P2 to be retained in a Hae III/Pst I double digest, only one possible permutation of the Pst I fragments is possible:

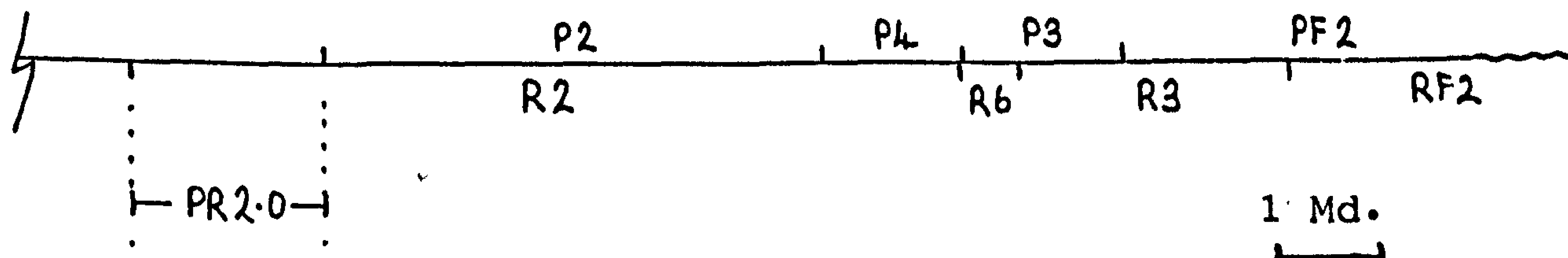


The completed maps for the Hae III and Pst I sites will now serve as a framework on which to build the remainder of the maps for the other restriction enzymes.

Double digests with Eco RI and Pst I. In an Eco RI/Pst I double digest the discrete fragments R2 and R3 are lost from the single digest Eco RI pattern (see Fig. 8). R3 has already been located by Hae III/Eco RI double digest information. By comparing the mapped R3 fragment with the completed Hae III and Pst I maps, the new fragments PR 1.0 and PR 1.8 are accounted for:



Since R2 is lost in the Eco RI/Pst I double digest, it must map over the remaining Pst I sites. Since the only unaccounted for new fragment is PR 2.0, there is only one permissible mapping of this region:



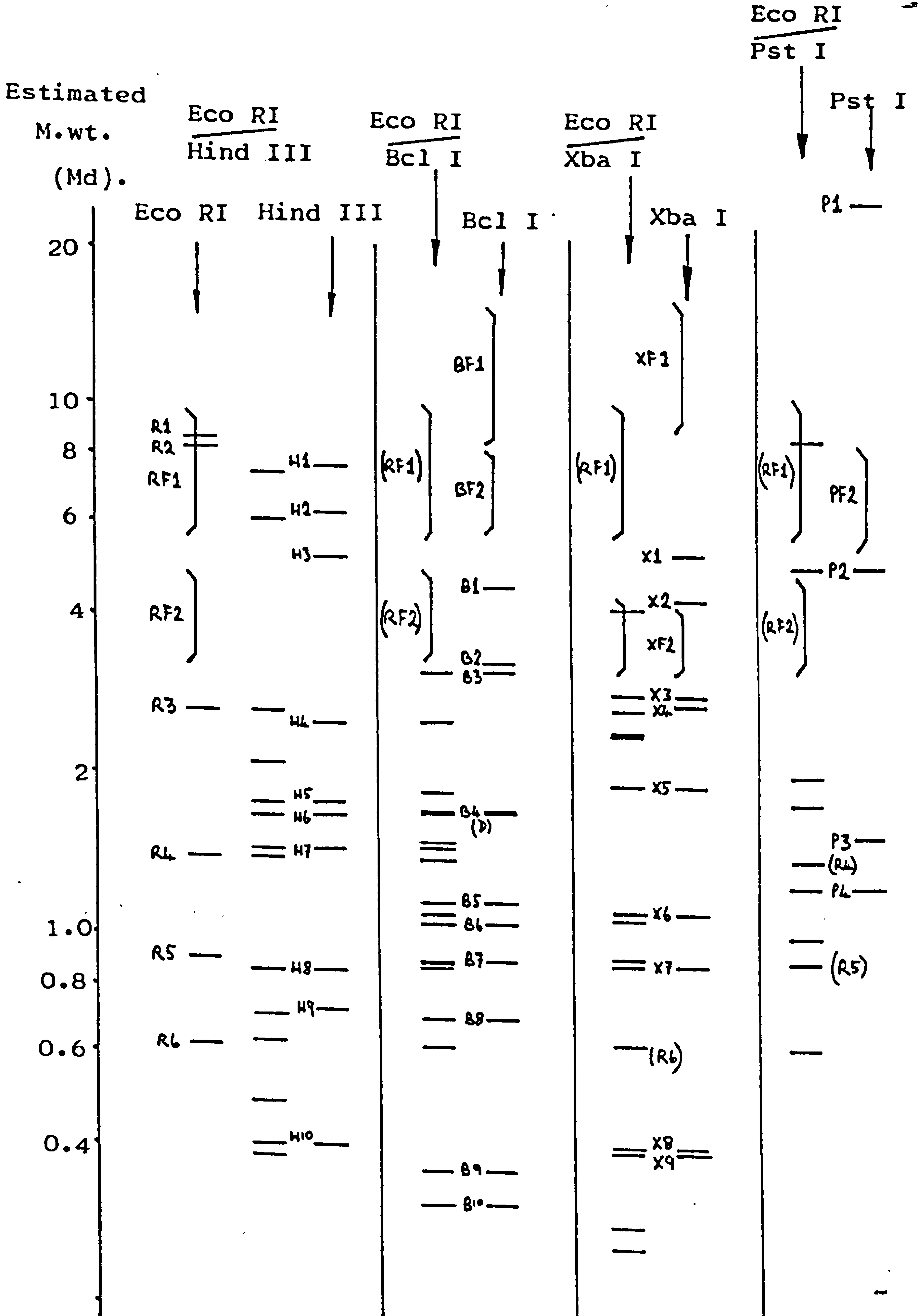
A "spacer" of ~0.6 Md is required to position the R2 fragment over the Pst I sites, in order to produce PR 2.0. This size of 0.6 Md is conveniently the size of R6, suggesting that it maps in this position.

Fig 8.

The diagram shows the size of the double digest products produced by digestion of W/ATCC mtDNA with the named restriction enzymes in conjunction with Eco RI. In each case a single restriction digest pattern is shown for comparison.

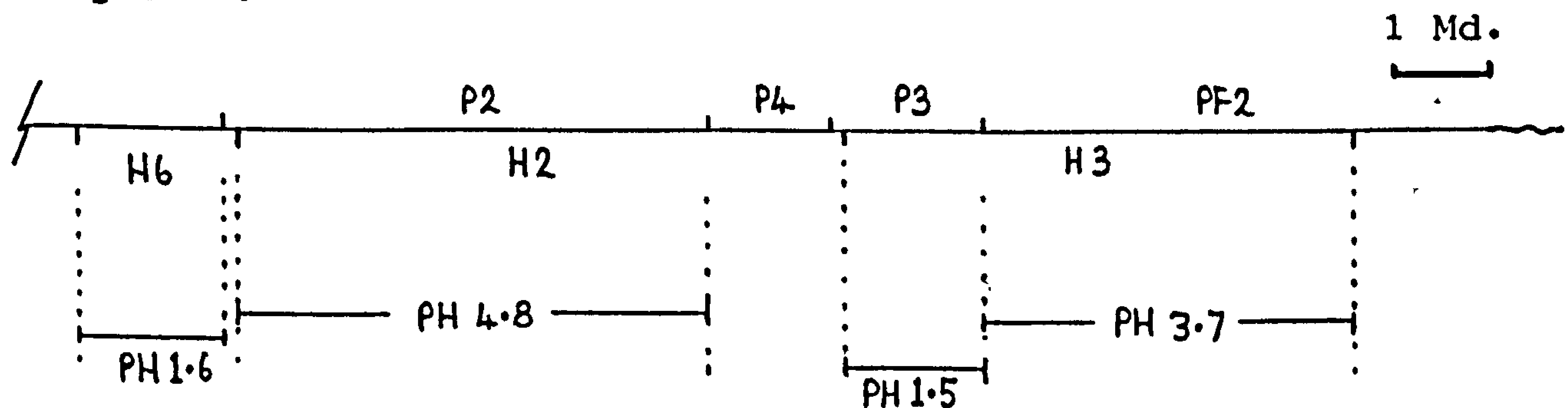
Fig 8. **Eco RI double digests.**

Restriction digest fragment patterns produced upon double digestion of *T. furgasoni* str. W/ATCC mtDNA with the named restriction enzymes in conjunction with Eco RI.



Double digests with Hind III and Pst I. In the Hind III/ Pst I double digest the fragments H2, H3 and H6 are lost from the single digest pattern. H3 has already been mapped from Hae III/Hind III double digest information.

This location of H3 accounts for the new fragments PH 1.5 and PH 3.7 in the Pst I/Hind III double digest. Only one permutation of the remaining H2 and H6 fragments in this region is permissible, considering the size of the remaining new fragments:



In a few single Hind III digests, a partial digest product of mol. wt. ~7.9 Md was seen. The presence of this partial digest product was concurrent with an observed reduction in the intensity of fragments H6 and H2 compared to their surrounding fragments in the digest pattern. This indicates that H2 and H6 map alongside each other. This evidence supports the suggestion made in the Pst I/Hind III digest information that H2 and H6 map adjacent to one another.

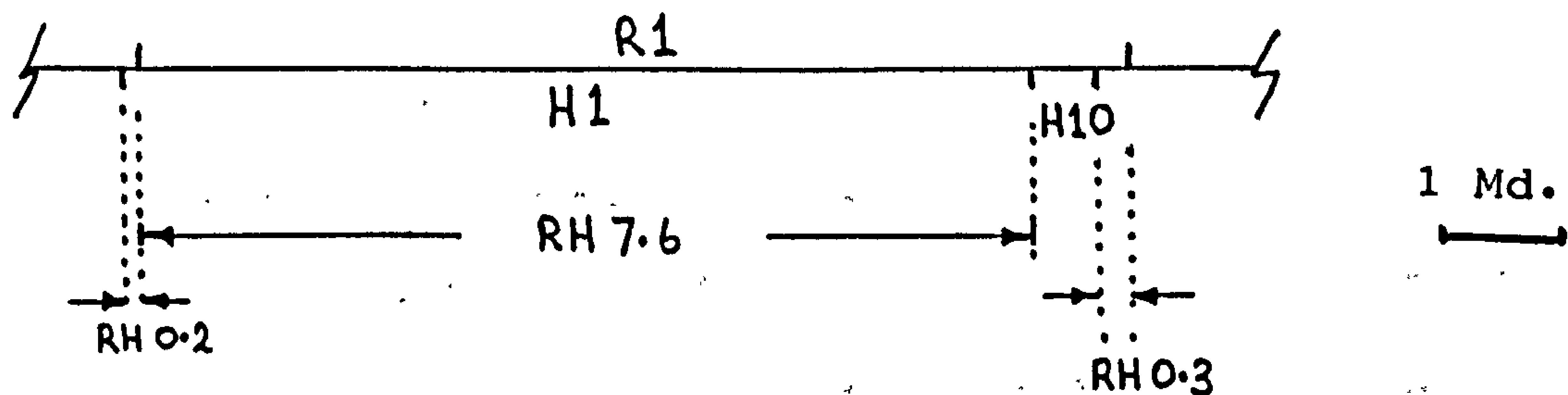
Double digests with Hind III and Eco RI. In the Eco RI/ Hind III double digest a new fragment RH 7.6 was produced (see Fig. 8). The only fragments sufficient in size to produce such a new fragment are R1 and H1 (since R2 and H2 are accounted for and their relative positions are known from the Pst I double digest information).

When R1 was recovered from agarose gels containing single digests, and redigested with Hind III the following fragments resulted:

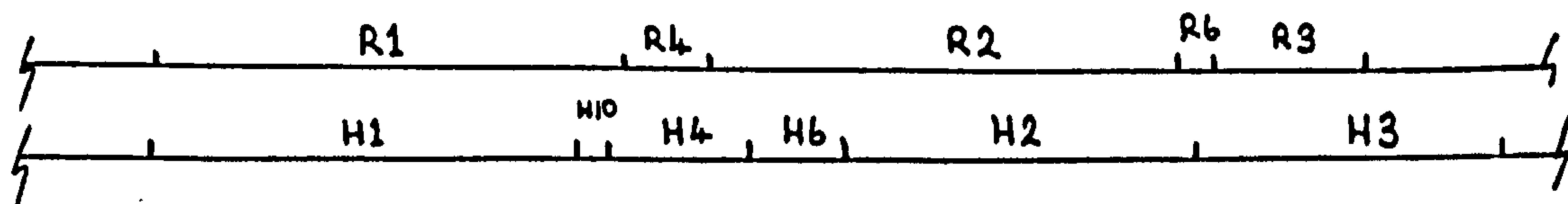
RH 7.6, RH 0.3, H10.

When H1 was recovered from agarose gels containing single digests, and redigested with Eco RI, the fragments RH 7.6 and RH 0.2 resulted.

This data can be mapped as follows:



At this point, attempts were made to connect the two mapped regions of the discrete restriction sites, the R1/H1 region and the R2/H2/Ha 2 region. It was noted that if the as yet unmapped fragments R4 and H4 were placed between the R1/H1 and R2/H2 regions, then these two regions fit together.

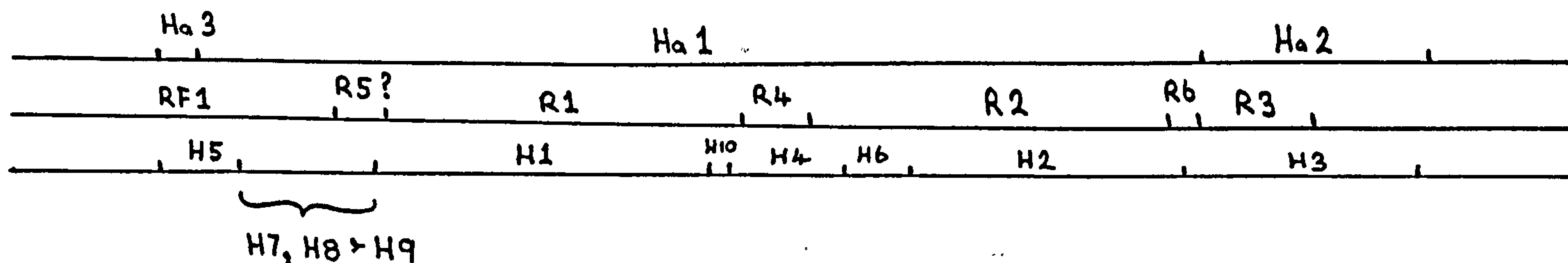


This evidence is supported by studies done whilst mapping the restriction sites in the Xba I map. To avoid confusion this map will not be presented here, but will fall into place later.

<u>Completion of the map for Eco RI and Hind III sites</u>

The sites mapped so far, with their accompanying fragments

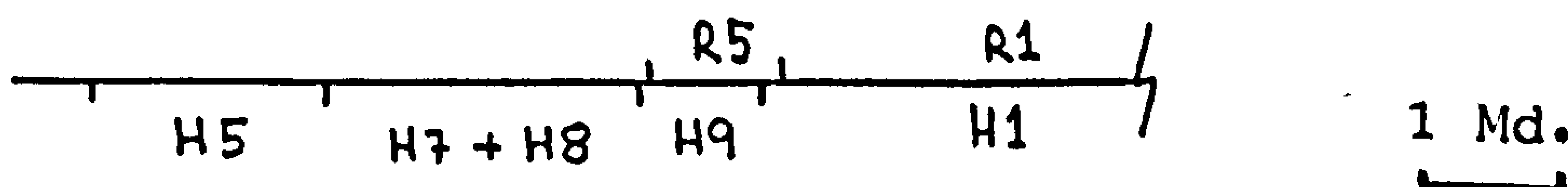
from single digest patterns, are shown below:



The difference between the most extreme left-hand site in the Eco RI map (mapped by Hae III digests) and the R1 fragment conforms to a mapping distance of ~0.92 Md. This is the estimated size of R5.

The difference between the mapped Hind III fragments H5 and H1 conforms to a mapping distance of ~3.2 Md. This is the estimated size of the remaining, unmapped Hind III fragments H7, H8 and H9.

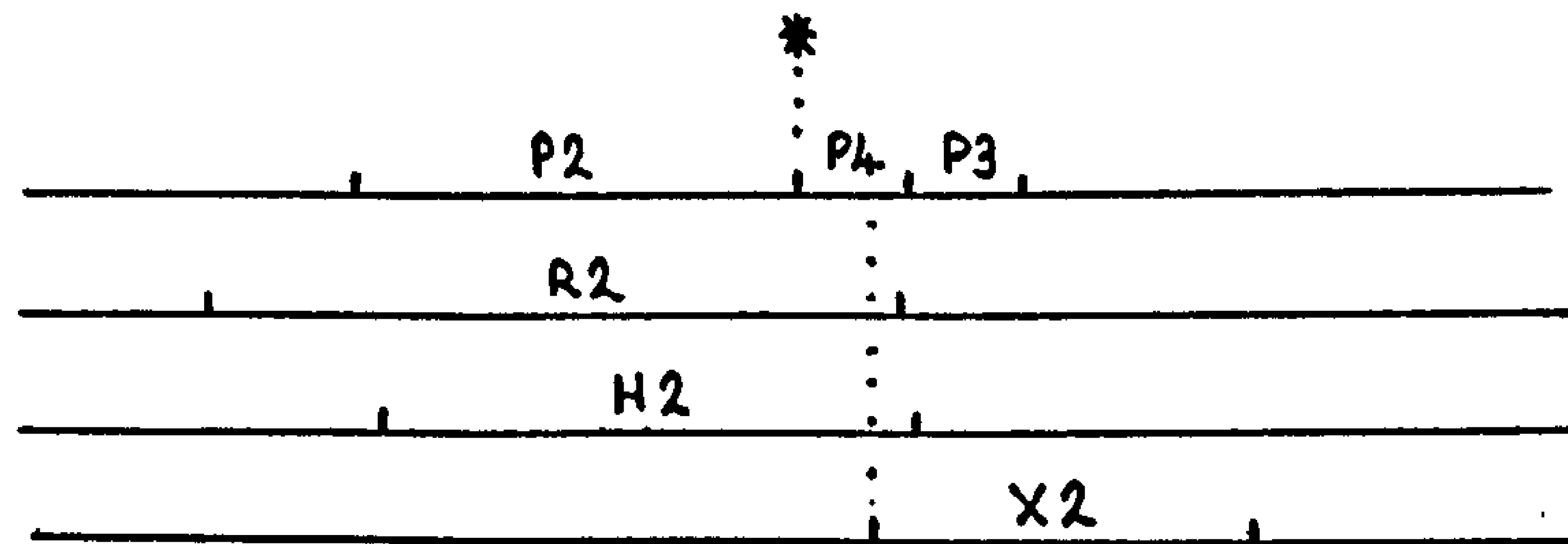
Since both H9 and R5 are lost in an Eco RI/Hind III double digest (see Fig. 8), these fragments can only map in one possible orientation:



<u>Further mapping of the Xba I sites</u>. In an Xba I/Pst I double digest, fragments X2, X4 and X6 are lost. X2 has already been mapped using Hae III double digest information and is seen to occupy the two right-hand most Pst I sites. The remaining Xba I fragments, X4 and X6, must each map over one of the remaining two Pst I sites.

Consider the Pst I site marked by an asterisk in the following diagram. The Xba I fragment which occupies this

site will lie within H2, since both X4 and X6 are considerably smaller than H2.



In an XbaI/Hind III double digest, X4 is lost, whilst X6 is retained (see Fig. 9). It is therefore concluded that X6 maps over the marked Pst I site.

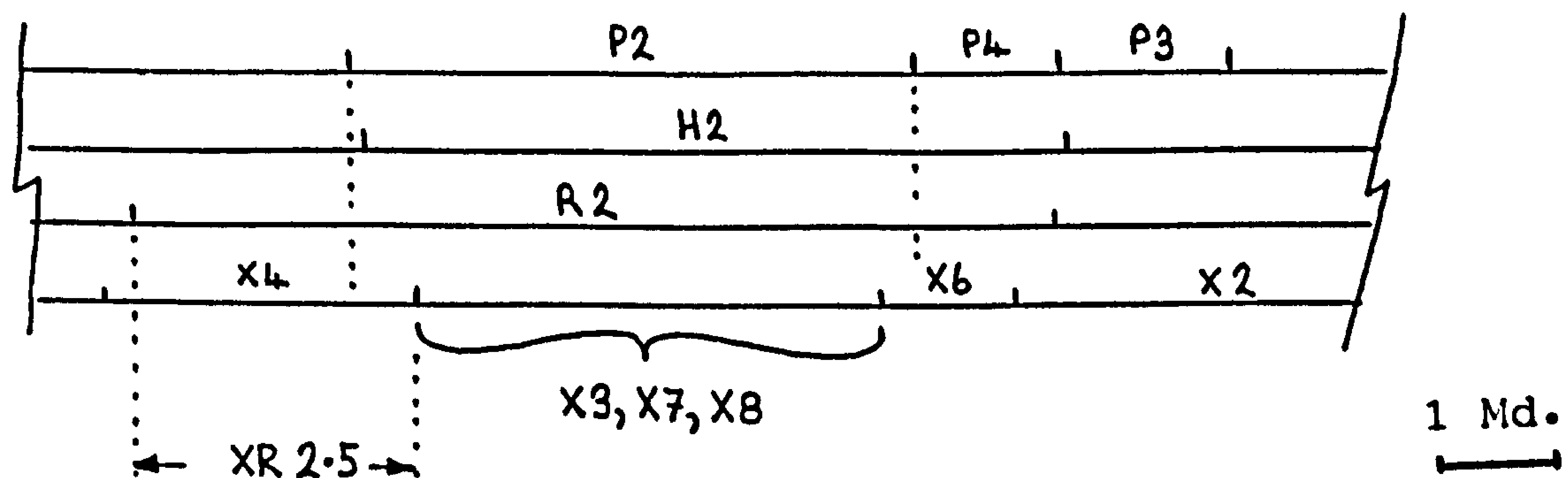
When fragment R2 was recovered and digested with Xba I, the fragments produced were:

XR 2.5, XR 0.45, X3, X6, X7, X8.

When fragment H2 was recovered and digested with Xba I, the fragments produced were:

XH 0.4, XH 0.3, X3, X6, X7, X8.

Considering the condition that X4 must lie over the remaining Pst I site, this data can be mapped as follows:

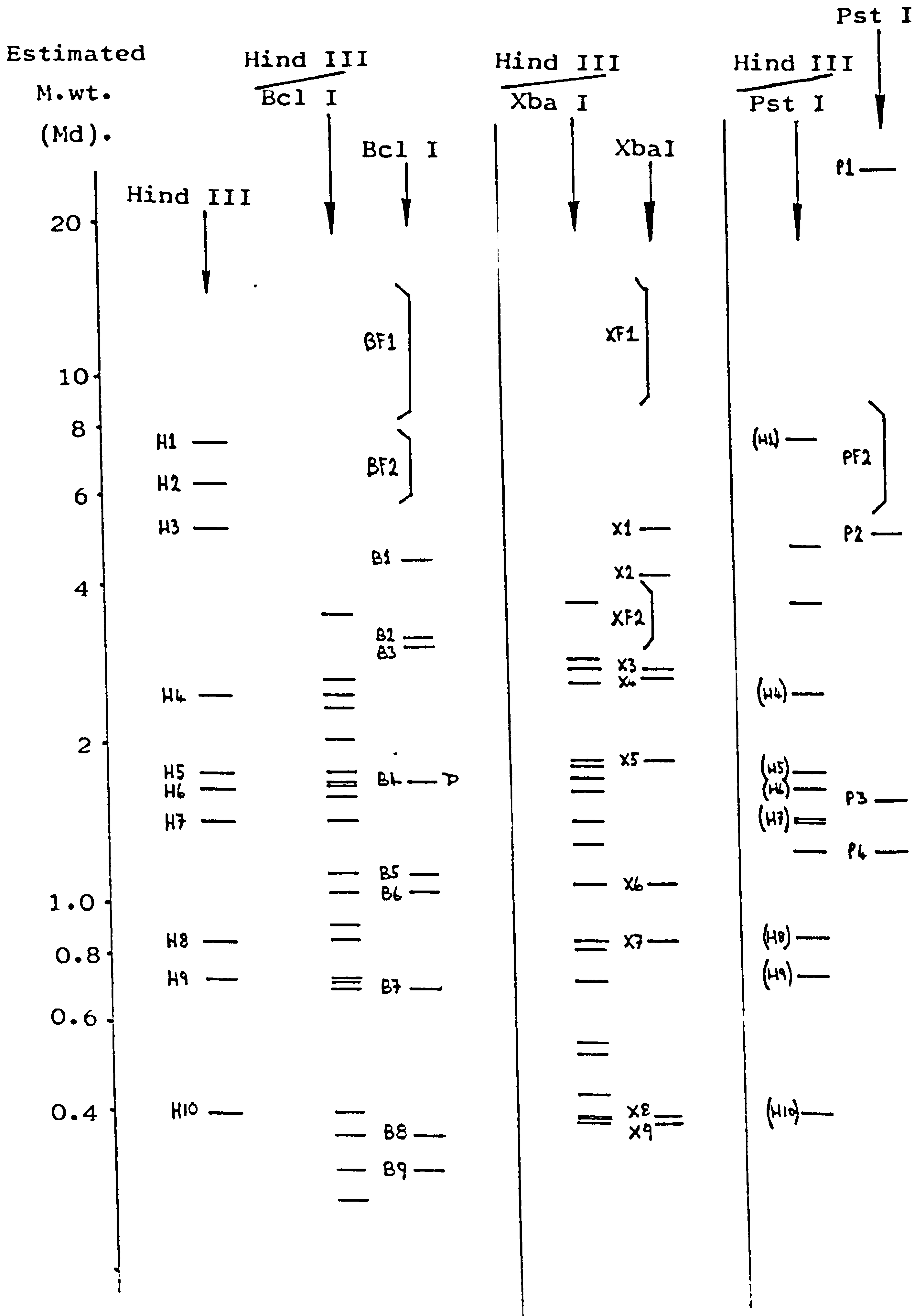


When fragment X1 was recovered and digested with Eco RI, the fragments XR 4.1 and XR 1.1 were produced. The only Eco RI fragment of sufficient size to produce XR 4.1 is R1,

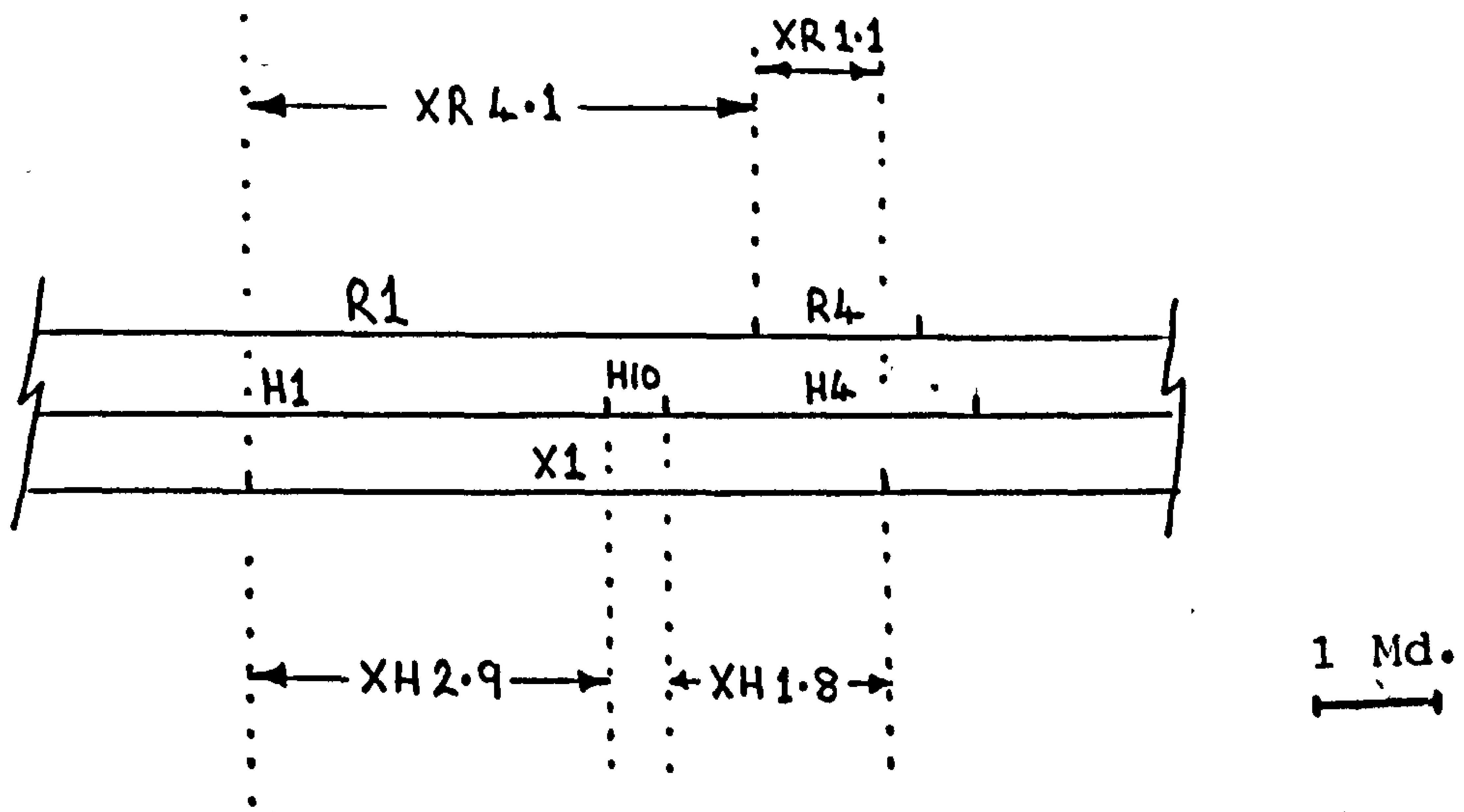
Fig 9.

The diagram shows the size of the double digest products produced by restriction digests of W/ATCC mtDNA with the named restriction enzymes in conjunction with Hind III. In each case a single restriction digest pattern for the enzyme in question is shown for comparison.

Fig 9. Restriction digest fragment patterns produced upon double digestion of *T. furgasoni* str. W/ATCC mt DNA with the named enzymes in conjunction with Hind III.



since R2 has been accounted for above. The only remaining Eco RI fragment of sufficient size to produce XR 1.1 is R4. This implied that R4 must map adjacent to R1, and supports the suggestions made in joining the "partial" maps of the R2/H2 and R1/H1 regions. In the same way, when X1 was recovered and digested with Hind III the fragments XH 2.9, XH 1.8 and H10 were produced. Since H2 and H3 are already accounted for, XH 2.9 must originate from H1. Since H4 is the only non-accounted for fragment in this region, XH 1.8 must originate from H4.



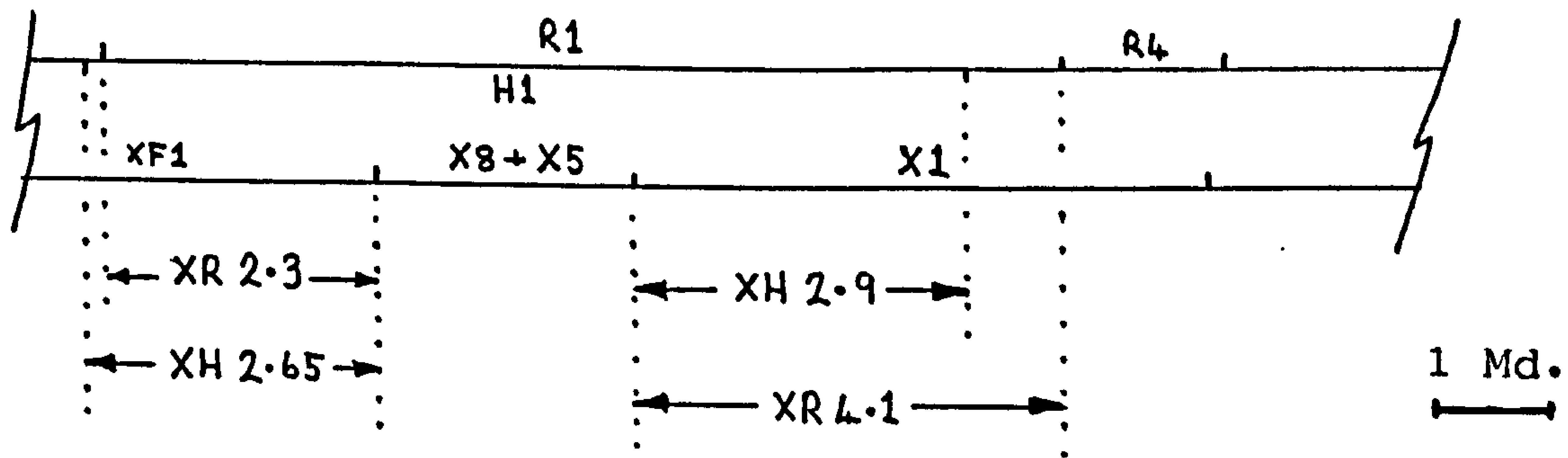
When fragment R1 was recovered and digested with Xba I, the following fragments resulted:

XR 2.3, XR 4.1, X9, X5.

When fragment H1 was recovered and digested with Xba I, the following fragments resulted:

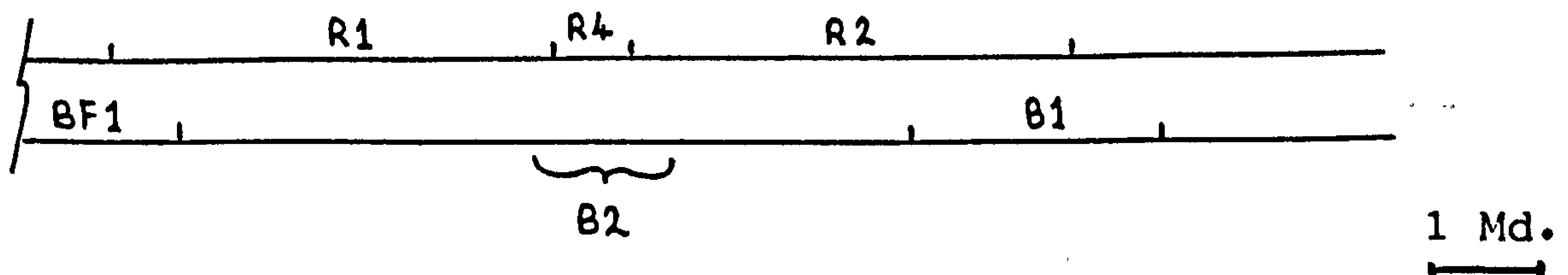
XH 2.65, XH 2.9, X9, X5.

If the digest products of the recovered X1 fragment, digested with Eco RI and Hind III, are taken into account, the data can be mapped as overleaf:



The most left-hand Xba I site coincides with the site placed by Hae III double digest information as the extreme left-hand site in the Xba I map. XR 2.3 and XH 2.65 therefore originate from the XF 1 fragment in the Xba I pattern.

Further mapping of Bcl I sites. The position of B1, and the extreme left-hand Bcl I site have been identified by Hae III double digest information.



From the diagram above it can be seen that the remainder of the Bcl sites are contained within fragments R1, R2 and R4. With the exception of B1, the only Bcl I fragment lost in the Eco RI/Bcl I double digest is B2 (see Fig. 8). B2 must therefore map over the R4 region. Since R4 is retained in the Eco RI/Bcl I double digest, R4 must map within B2.

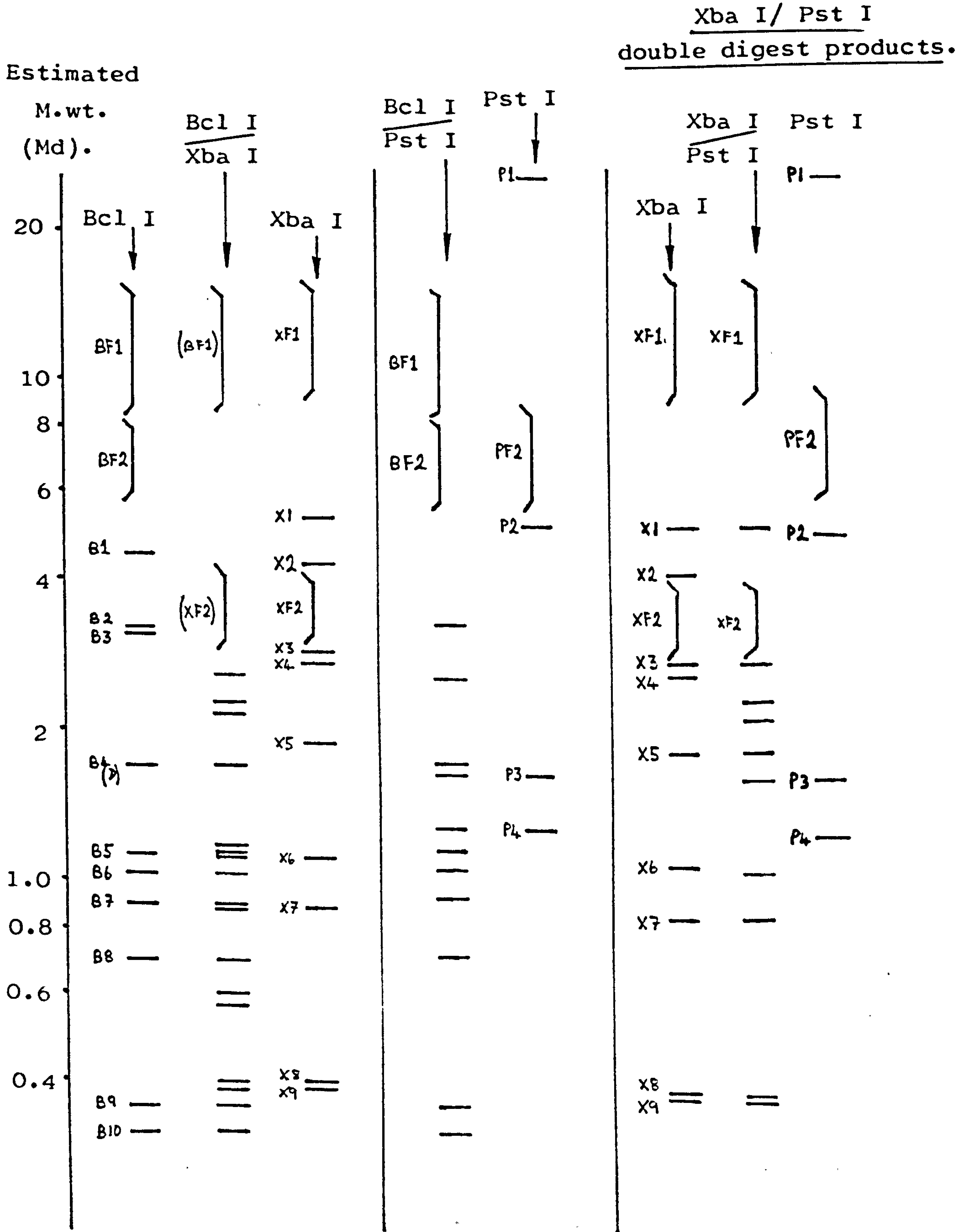
In a Pst I/Bcl I double digest the only Bcl I fragments lost were B1 and B3 (see Fig. 10). The already mapped B1 maps over three of the Pst I sites. If the condition is that B3 be retained in an Eco RI/Bcl I double digest, only one location for B3 is possible.

Fig 10.

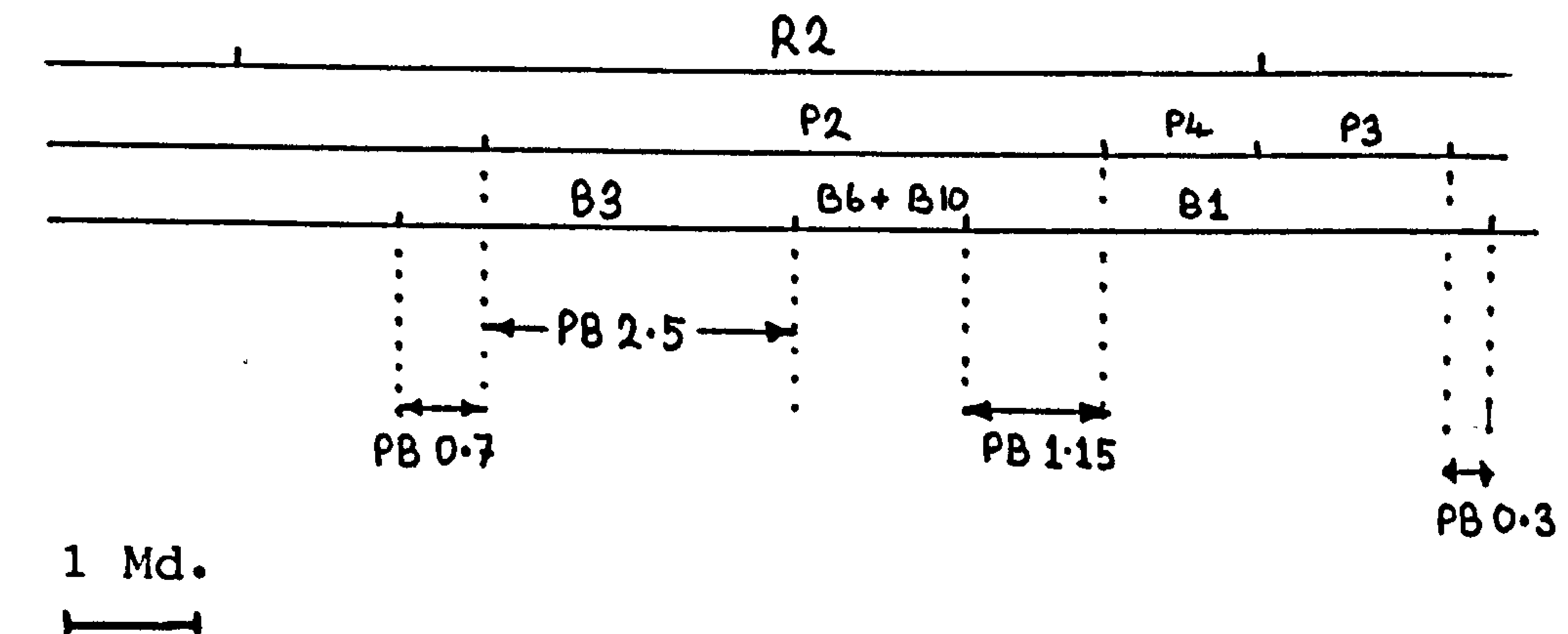
The diagram shows the size of the double digest products produced by digestion of W/ATCC mtDNA with the named restriction enzymes in conjunction with Bcl I. The Xba I/Pst I double digest pattern is also shown. In each case a single restriction digest pattern is shown for comparison.

Fig 10. Bcl I double digests.

Restriction digest fragment patterns produced upon double digestion of T. furgasoni str. W/ATCC mtDNA with the named enzymes in conjunction with Bcl I.



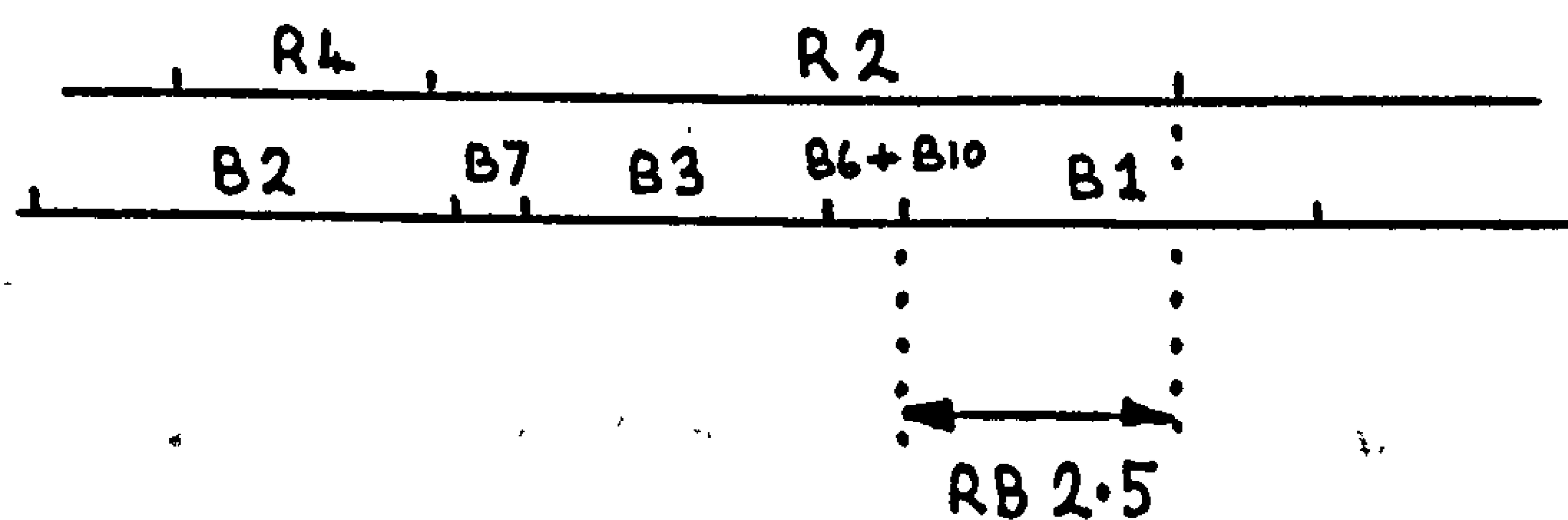
Only by using B6 as a "spacer", can the observed double digest (new) fragments be accounted for:



When fragment R2 was recovered and redigested with Bcl I, the following fragments were produced:

B3, RB 2.5, B6, B7, RB ~0.2, B10.

By adding this data to the diagram above, the fragments B6, B7 and B2 are mapped; since R4 is adjacent to R2, and B2 maps over R4. The small fragment B10 could map anywhere in this region, its small size would not alter any of these positive mapping positions.

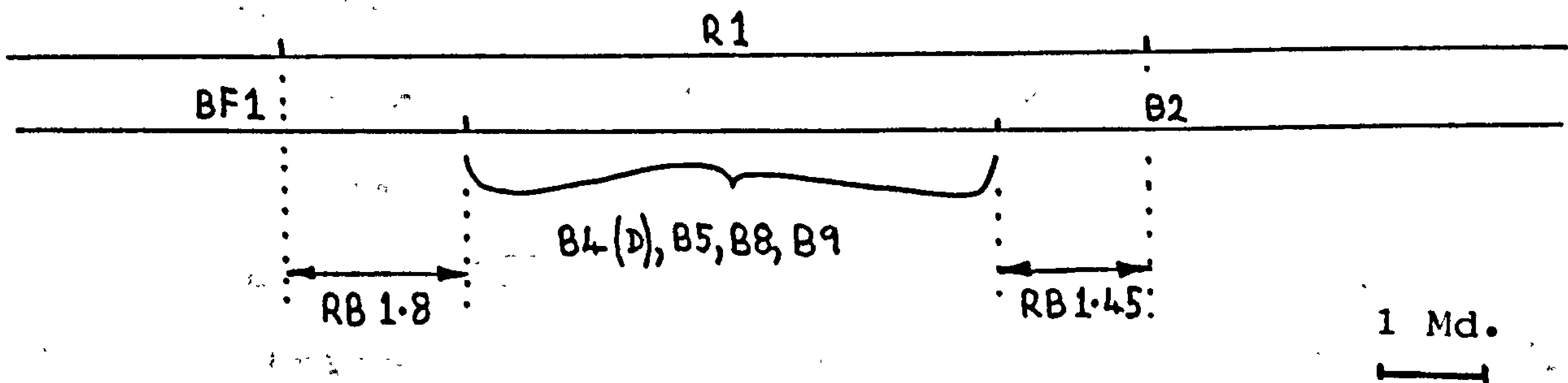


When fragment R1 was recovered and digested with Bcl I, the following fragments were produced:

RB 1.8, B4(D), RB 1.45, B5, B8, B9.

The extreme left-hand site in the Bcl I map has been defined by Hae III double digest information, thus giving the

origins of RB 1.8. The now mapped B2 fragment gives the origins of RB 1.45.

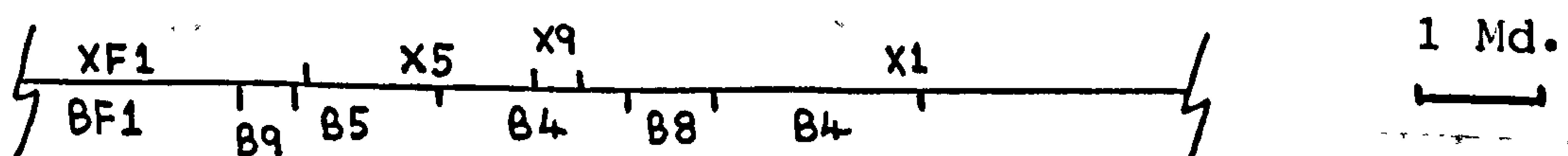


Completion of the Bcl I and Xba I maps

From the digest information obtained by digestion of the recovered R1 fragment, the as yet unmapped fragments X5, X9; B9, B5, B4(D), and B8 all lie within a defined region.

This region is bordered on the left by the extreme left-hand restriction sites for the Xba I and Bcl I maps, defined by Hae III double digests and exhaustively qualified in redigest studies with Hind III and Eco RI recovered fragments. The defined region is bordered on the right by the fragments X1 and B2, both of which have been qualified by recovered fragment digestion.

Only a limited number of permutations can possibly fit in this region. Only one of these permutations, shown below, agrees with the double digest restriction pattern of a Bcl I/Xba I double digest, (see Fig. 10).



This map agrees with considerable constraints imposed by the observed results.

(a) Fragments B9, X9, B8 and one B4 are retained in the Xba I/ Bcl I double digest.

(b) The resulting new fragments agree with the double digest products for the Xba I/Bcl I double digest.

(c) When fragment X5 was recovered and digested with Bcl I, Fragments XB 1.1 and XB 0.7 were produced.

(d) When fragment B5 was recovered and digested with Xba I, fragment XB 1.1 was produced.

This completes the mapping of the sites of the six named restriction endonucleases on the W/ATCC mtDNA molecule.

A complete map of the sites discussed is shown in Figure 11.

Preparation and Isotopic Labelling of Mitochondrial rRNAs from W/ATCC

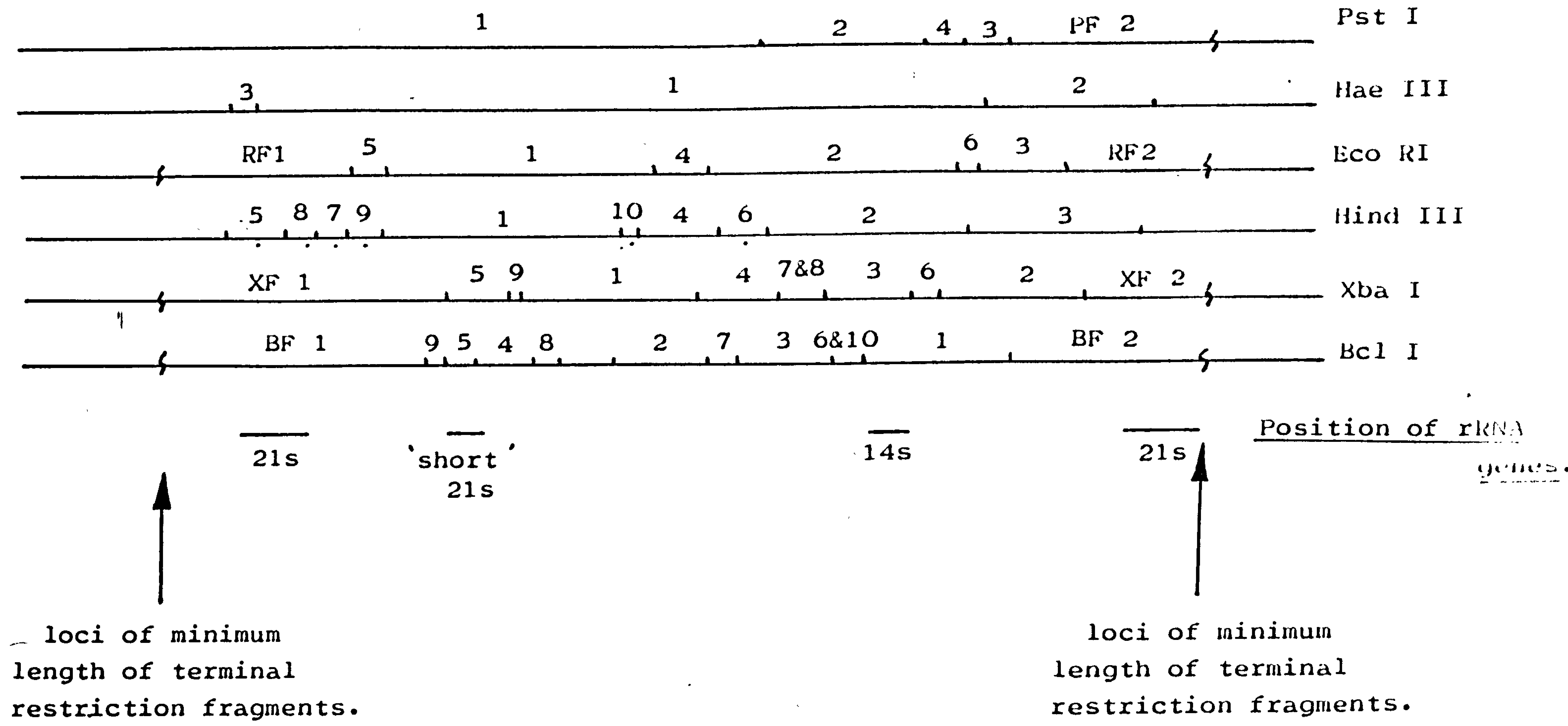
Mitochondrial RNAs were prepared from exponentially growing cells and purified on sucrose gradients. A typical sucrose gradient trace for a W/ATCC rRNA preparation is shown in Fig. 4. Small amounts of contaminating RNA species carried over from the cytoplasm were always present. The final purified 21 s rRNA contained small amounts of cytoplasmic 25 s (large subunit) rRNA, and the 14 s rRNA contained small amounts of 16 s (small subunit) rRNA, plus any degraded 21 s and 25 s rRNAs. (Faint bands conforming to the hybridization of contaminating large rRNAs were seen when the 14 s rRNA gene was being mapped. These contaminant activities were removed by a preincubation of the digest filter with unlabelled 21 s rRNA.)

The isolated 21 s and 14 s rRNAs were partially degraded and labelled as described using the method of Goldbach *et al.*, (1978).

Fig 11.

The diagram shows the completed restriction map for W/ATCC mtDNA. The estimated position of the rRNA genes are shown.

Fig 11. Restriction map of *T. furgasoni* str. W/ATCC mtDNA.



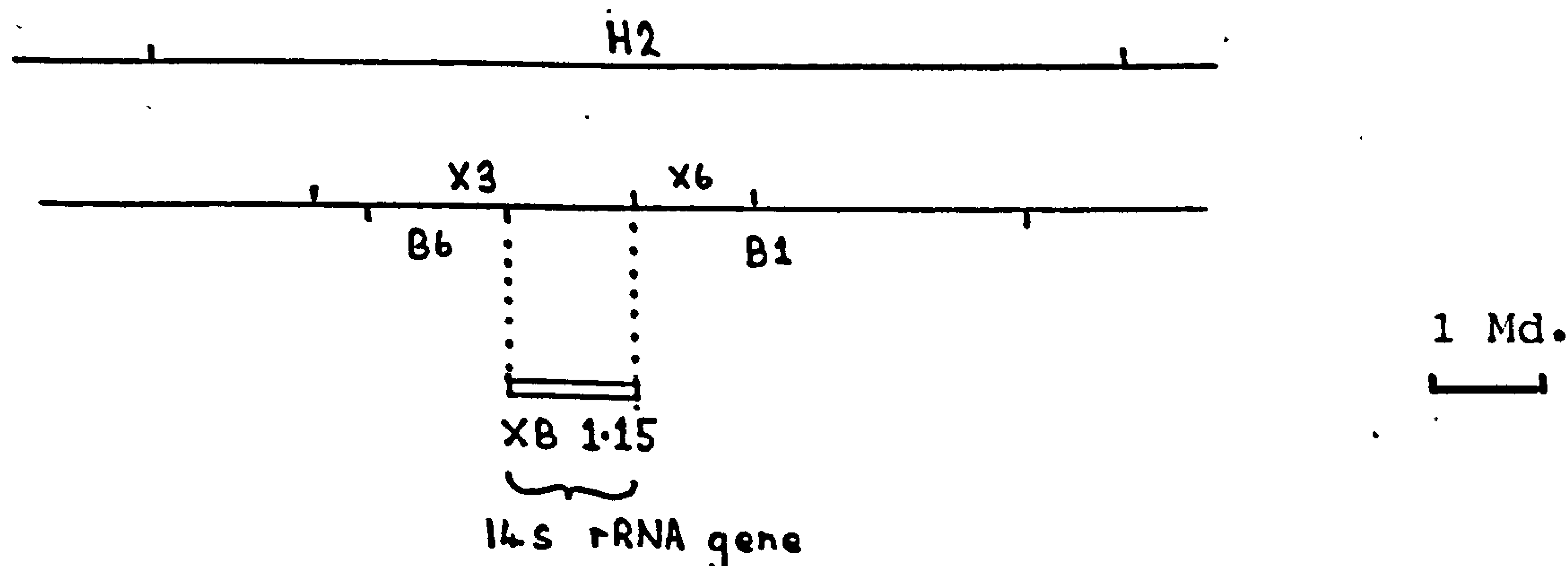
A specific activity of 0.2–0.5 µCi/µg was usually achieved. For experiments requiring higher specific activities, such as hybridization to the very widely distributed terminal fragments, the RNA was degraded to smaller size by a 2 h alkaline incubation.

Labelled rRNAs were incubated with str. W mtDNA restriction digest products immobilized on nitrocellulose filters (see protocol for incubation procedure, p. 57).

RNA Hybridization Studies with W/ATCC mtDNA

14 s rRNA. 14 s rRNA was seen to hybridize to fragment H2 on a Hind III digest pattern (data not shown). In an effort to narrow the region of hybridization of the 14 s rRNA, the labelled 14 s rRNA probe was incubated with a filter carrying the Xba I/Bcl I double digest pattern. As can be seen from the map of these digests, this particular double digest breaks the H2 region into as many fragments as possible using the mapped restriction sites.

Only the fragment XB 1.15 showed a hybridization signal. This fragment is produced by the overlap of fragments X3 and B1 (see Plate 1).

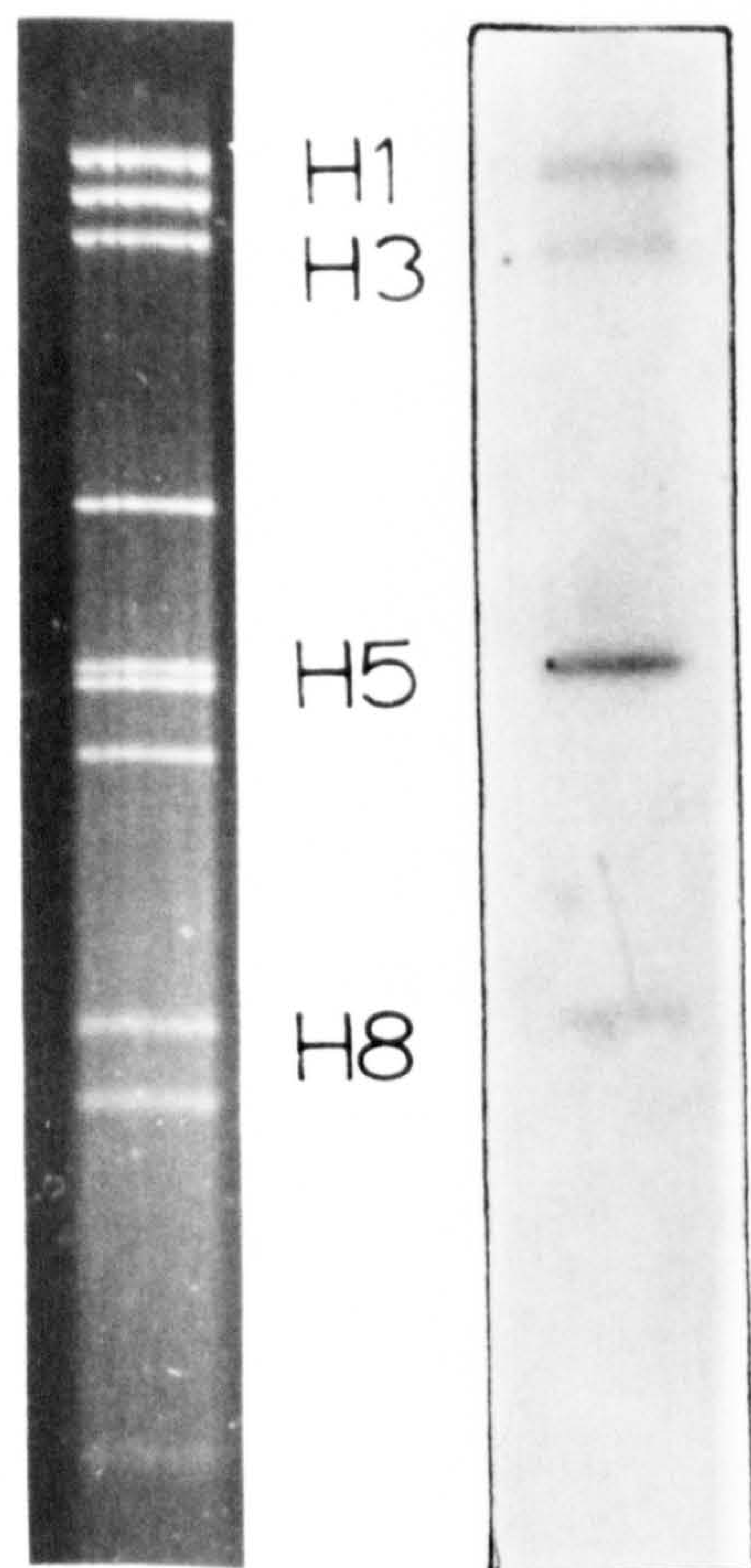


21 s rRNA. 21 s rRNA was seen to hybridize to fragments H1, H3, H5 and H8 from a Hind III digest pattern (see Plate 2).

Plate 2.

The gel electrophoresis pattern produced by a Hind III restriction digest on W/ATCC mtDNA is shown alongside the accompanying Southern blot which has been hybridized with labelled 21s rRNA. The significance of the hybridization of 21s rRNA to fragments H1, H3, H5 and H8 is discussed on p 107.

Plate 2.



Hybridization of labelled 21s rRNA to a Hind III digest of w/atcc mtDNA.

As can be seen from the restriction map of this digest pattern, the H3 hybridization signal indicates that a 21 s rRNA gene is present at the right-hand end of the molecule. The region of hybridization contained within the H1 fragment is separated from the H5 and H8 hybridizing region by the non-hybridizing fragments H7 and H9. This suggests that two separate regions of DNA sequence complementary to 21 s rRNA are present at the left-hand end of the molecule. This suggestion is supported by the data obtained upon incubation of 21 s rRNA with an Eco RI digest pattern filter. Hybridization was seen to the RF2 terminal fragment, confirming the hybridization region seen in H3. At the left-hand end of the molecule hybridization was seen to fragment R1 and fragment RF1, but not to fragment R5 which separates the two fragments.

When a Bcl I digest pattern was tested both the terminal fragments gave a hybridization signal. Fragment B5 also showed a hybridizing region with 21 s rRNA, the fragment B9 did not. (see Plate 3).

Fine Structure of 21 s rRNA Hybridizing regions

(a) Right-hand end

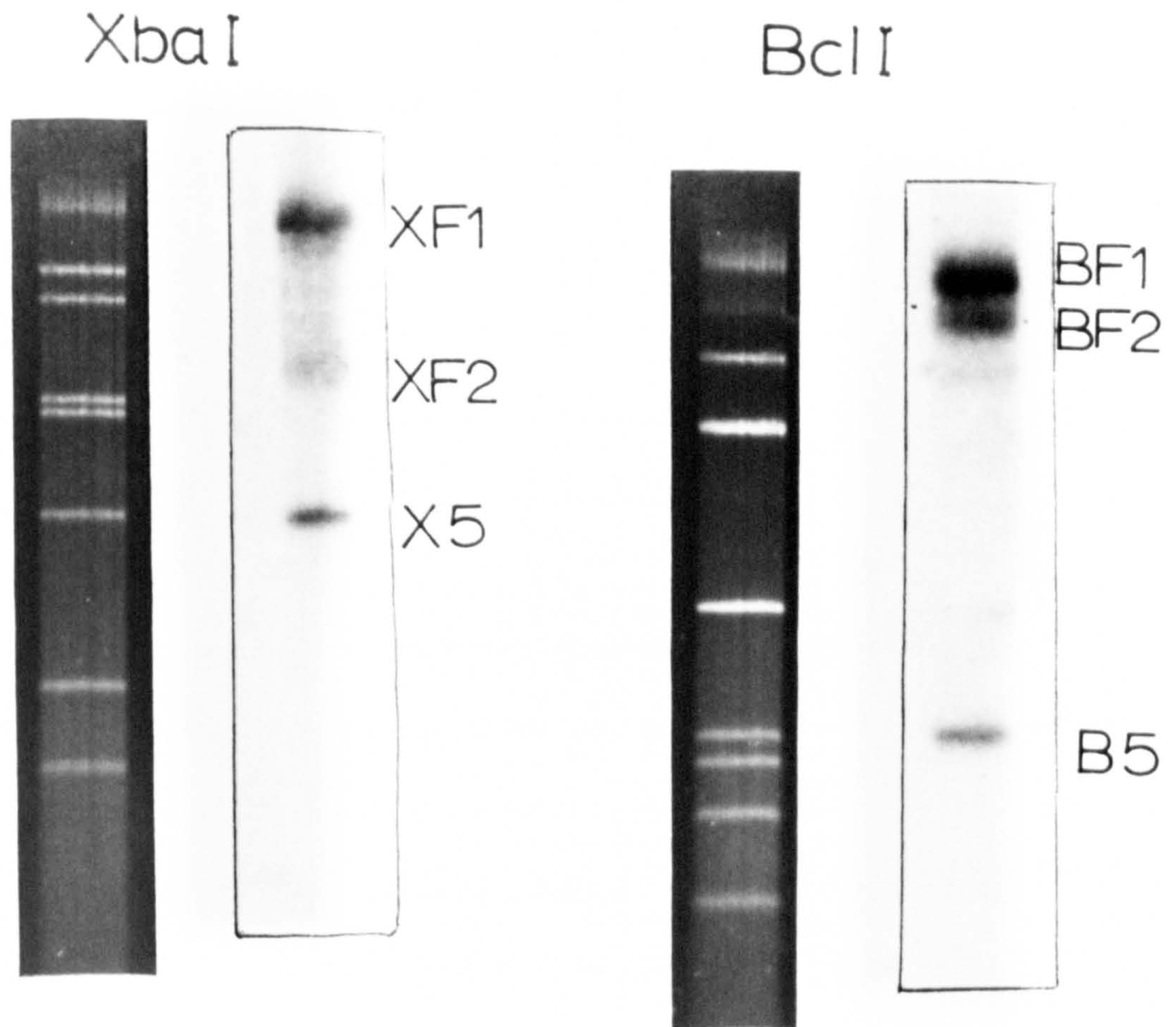
As described, no hybridization is seen with fragment X2. Therefore the 21 s rRNA gene must map to the right of X2. When an Xba I/Hind III double digest pattern was tested, hybridization occurred with the fragment XH 1.3, which originates from the overlap of fragments H3 and XF 2 (see Plate 4).

No further definition of the limits of the 21 s rRNA gene at this location can be made since the Hae III digest terminal fragments are, as already described, undetectable.

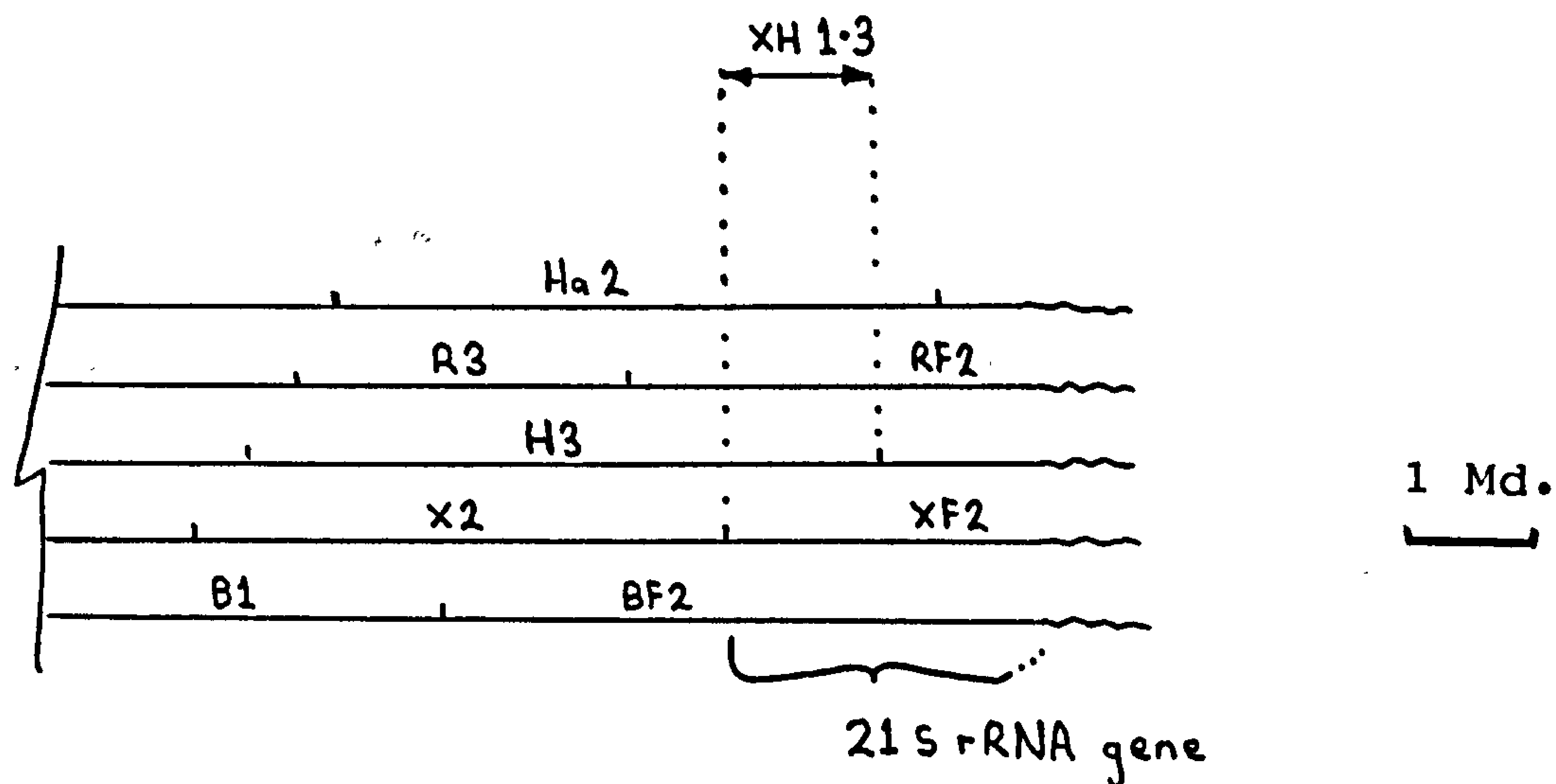
Plate 3.

The gel electrophoresis patterns produced by Xba I and Bcl I restriction digests of W/ATCC mtDNA are shown alongside the accompanying Southern blots, which have been hybridized with labelled 21s rRNA. The significance of the hybridization of the 21s rRNA to the indicated restriction fragments is discussed on page 107.

Plate 3.



Hybridization of labelled 21s rRNA to XbaI and BclI digests of w/atcc mtDNA.



(b) Left-hand end.

The hybridization of 21 s rRNA with fragments H5 and H8 shows the presence of a 21 s rRNA gene. As already described the Hind III digest pattern terminal fragments are not detectable, the outer limits of the gene cannot therefore be defined.

The 21 s rRNA hybridizing region contained within H1 and R1 was further defined by testing the hybridization of labelled 21 s rRNA with an Xba I/Bcl I double digest pattern filter. Only fragment XB 1.1 showed a hybridization signal (see Plate 4). This fragment has been confirmed as originating from the overlap of X5 and B5 (see final mapping of the Xba I and Bcl I restriction maps, p. 104). The non-hybridization of fragments XB 0.7 to the right of XB 1.1, and of fragment B9 to the left of XB 1.1 suggests that the 21 s rRNA gene in this region is contained in a fragment of $\leqslant$ 1.0 Md.

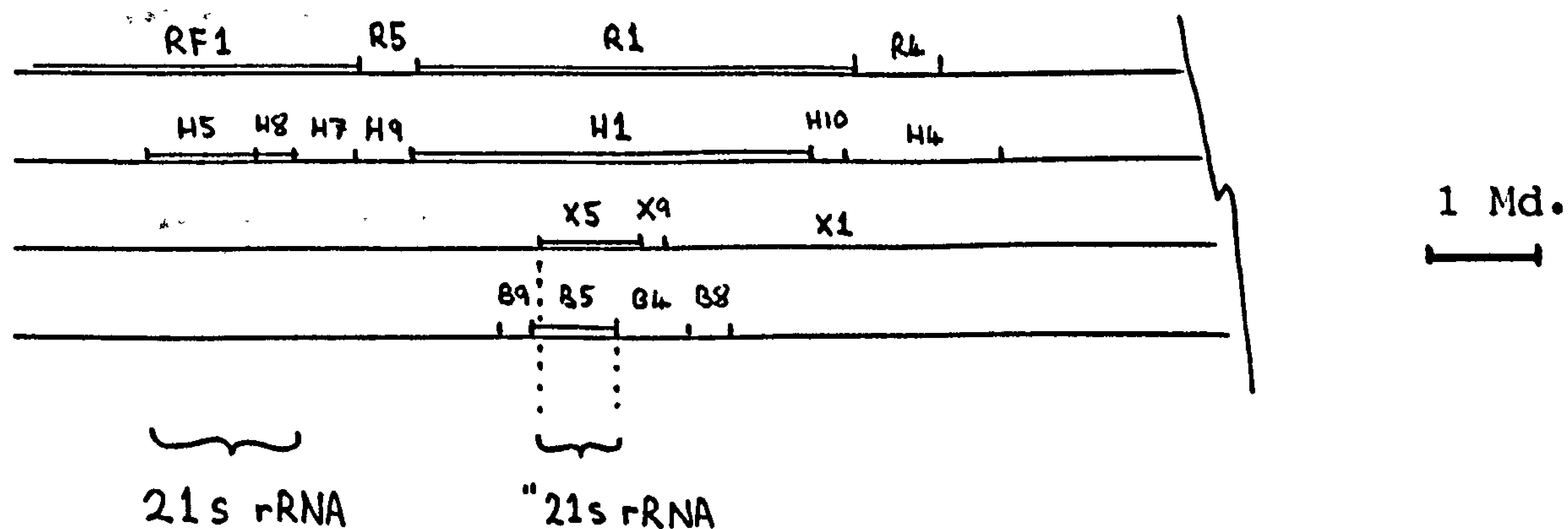
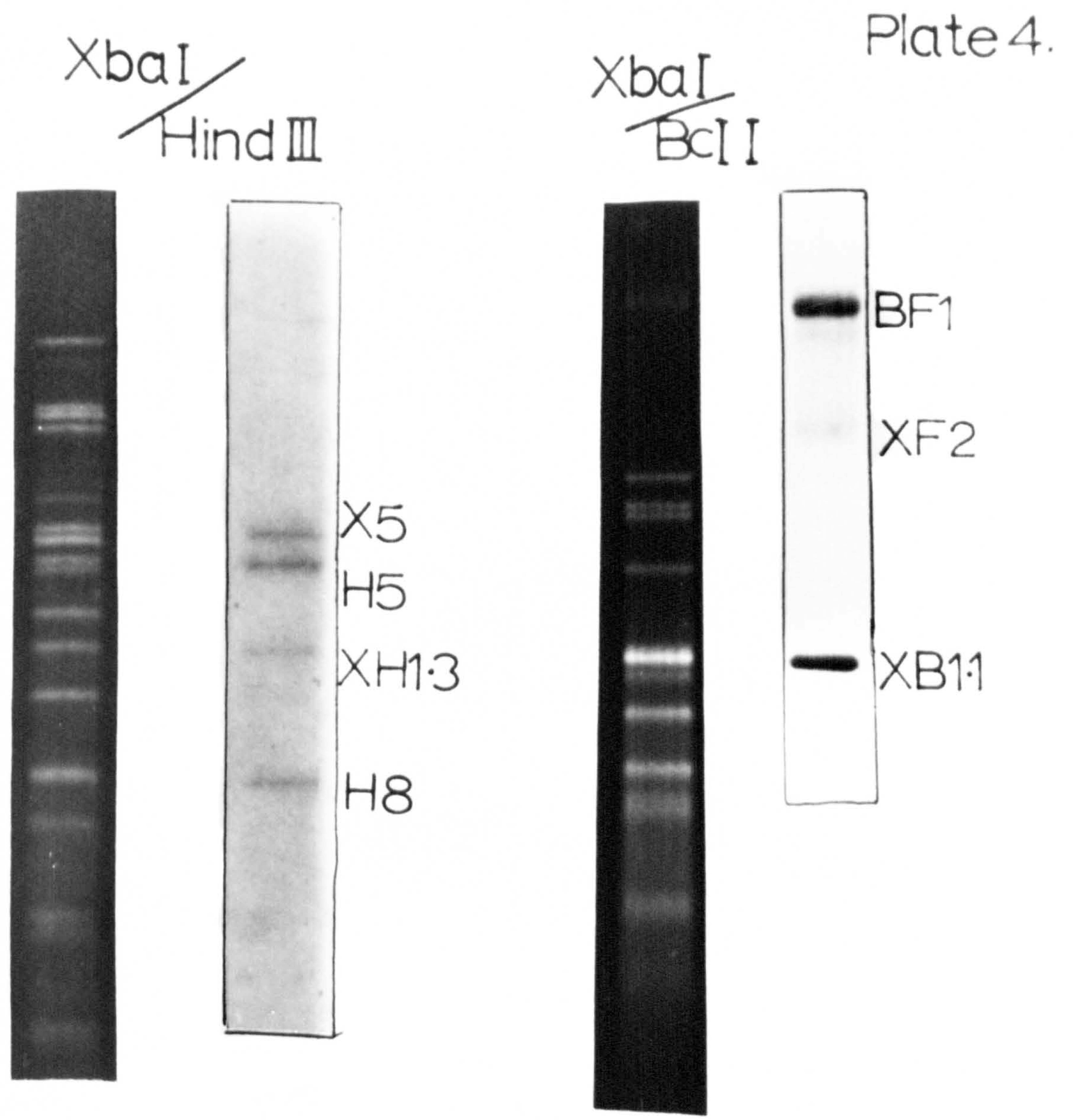


Plate 4.

The gel electrophoresis patterns for double restriction digests of W/ATCC mtDNA digested with Xba I/Hind III and Xba I/Bcl I are shown alongside the accompanying Southern blots hybridized with labelled 21s rRNA. The significance of the indicated hybridizing restriction fragments is discussed on p 107 - 108.



Hybridization of labelled 21s rRNA to XbaI HindIII and XbaI BclI double digests of w/atcc mtDNA.

Estimates on the size of the 21 s rRNA from Tetrahymena mitochondria indicate a molecular weight of about 0.91 Md (Sherer, 1973), Schutgens et al., (1973). The corresponding gene (ds DNA) will therefore be of the order of ~1.82 Md.

The small size of the middle 21 s rRNA hybridizing region indicates the presence of either a region of high sequence homology with 21 s rRNA, or the presence of an incomplete 21 s (large subunit) rRNA gene.

Sucrose gradient fractionation of a mtDNA sample

Analysis by restriction digest

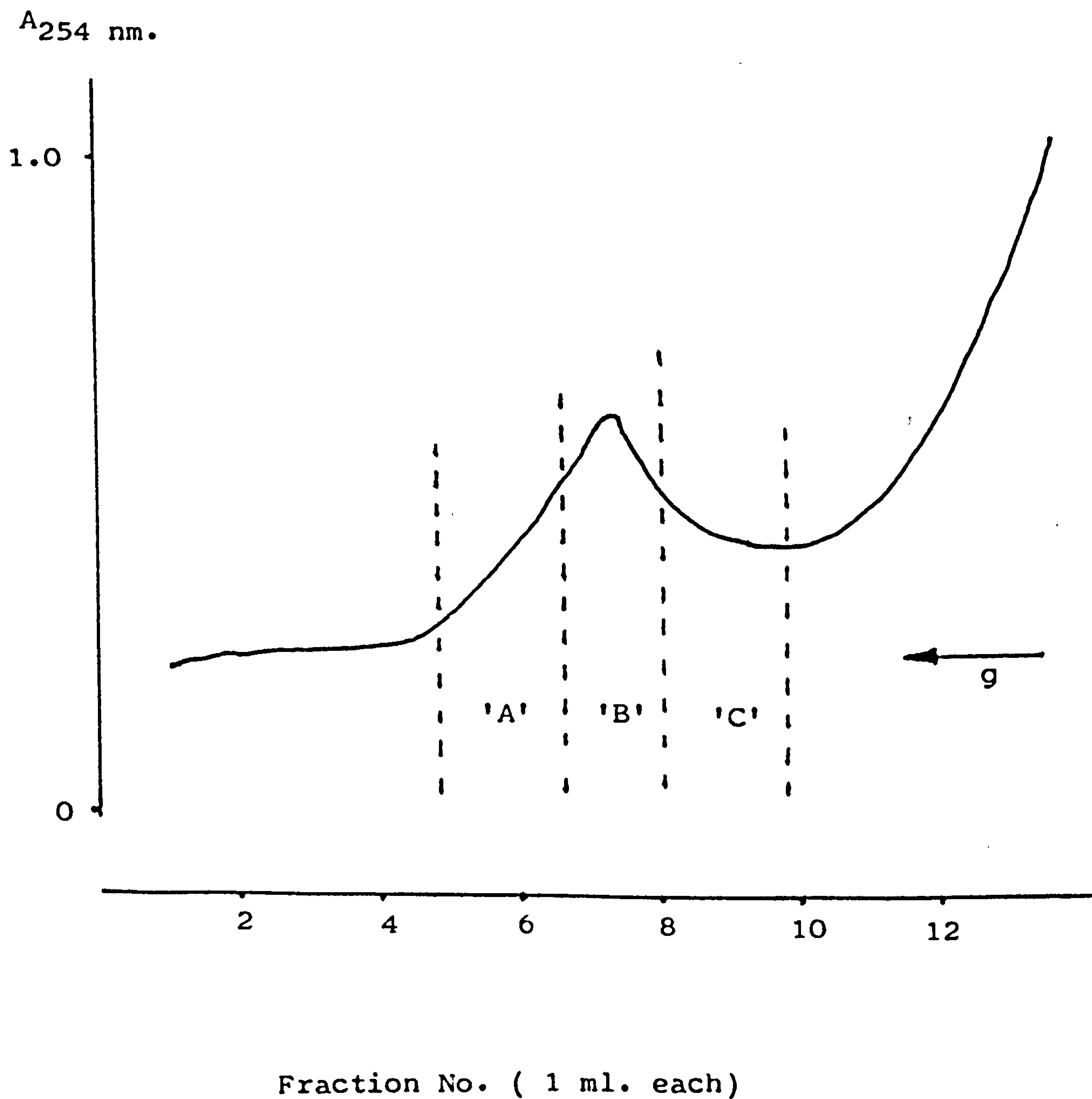
A sample of W/ATCC mtDNA was run on a sucrose gradient. The peak corresponding to the mtDNA was collected in three fractions as shown in Fig. 12. Samples of the three fractions were digested with Eco RI and analysed by gel electrophoresis as described.

No apparent difference was seen between the digest products of the three fractions.

Fig 12.

Fractionation of str. W/ATCC mtDNA on a sucrose gradient.

to investigate the possibility that the 'spread' of the mtDNA on sucrose gradients may be due to a differing sedimentation coefficient of molecules of differing length.



Fraction No. (1 ml. each)

Molecular cloning Experiments with W/ATCC mtDNA

It was seen from the restriction map of W/ATCC mtDNA, that of the enzymes used the Hind III sites cover the largest portion of the molecule. To facilitate the production of material in quantity for further studies on the W/ATCC mtDNA molecule it was decided to attempt to clone the discrete fragments produced by Hind III digestion of the mtDNA, in a suitable molecular cloning vehicle.

Cloning Rationale

The cloning vectors available in the laboratory at the time were the bacterial plasmid cloning vectors pBR 325, pBR 322 and pAT 153, all related derivatives of the Col El replicon. The Hind III site in these vectors is located in the tetracycline resistance gene of the plasmid. It was proposed to attempt to clone the Hind III fragments from W/ATCC mtDNA into this Hind III site. Any host cells transformed to ampicillin resistance would indicate the presence of the plasmid. Any host cells which also bear tetracycline resistance can be ignored, as any insert in the tetracycline resistance gene should destroy its tetracycline resistance-conferring properties. Host cells which are ampicillin resistant and tetracycline sensitive are therefore likely to contain inserts in the vector molecule at the Hind III site.

Throughout the experiments described, approximately 20% of the tetracycline sensitive cells showed no inserts in the Hind III site of the vector molecules. This was seen as some effect of the cutting and re-ligating of the Hind III site on the tetracycline resistance gene's activity, or

perhaps the presence of very small, undetectable Hind III fragments.

Of the three plasmid vectors available at the time, pAT 153 was chosen. This plasmid was produced by the removal from pBR 322 of two Hae II fragments which contain sequences concerned with the control of plasmid copy number. With this control relaxed, the pAT 153 plasmid possesses a higher copy number compared with the pBR 322 plasmid, resulting in an increased yield of material in both the preparation of vector and the preparation of subsequent recombinant plasmids (Twigg & Sherrat, 1980) (see Fig. 13).

Cloning of the Hind III Fragments from W/ATCC mtDNA

Procedure

Preparation of the vector. A Hind III cut, phosphatased vector was prepared from purified pAT 153 using the methods and techniques described for plasmid vector digestions (see p. 51).

The vector was tested for its ability to re-ligate. Uncut pAT 153 gave a transformation efficiency of ~15,000 colonies/μg (control) with competent ED 8767 cells.
Hind III cut pAT 153, re-ligated at 10 μg/ml DNA concentration, under the conditions described for cohesive-end ligation; gave a transformation efficiency of ~8,000 colonies/μg with the same batch of competent ED 8767 cells as used above.
Hind III cut, phosphatased pAT 153, re-ligated at 10 μg/ml as above gave a transformation efficiency of ~400 colonies/μg with the same batch of ED 8767 competent cells.

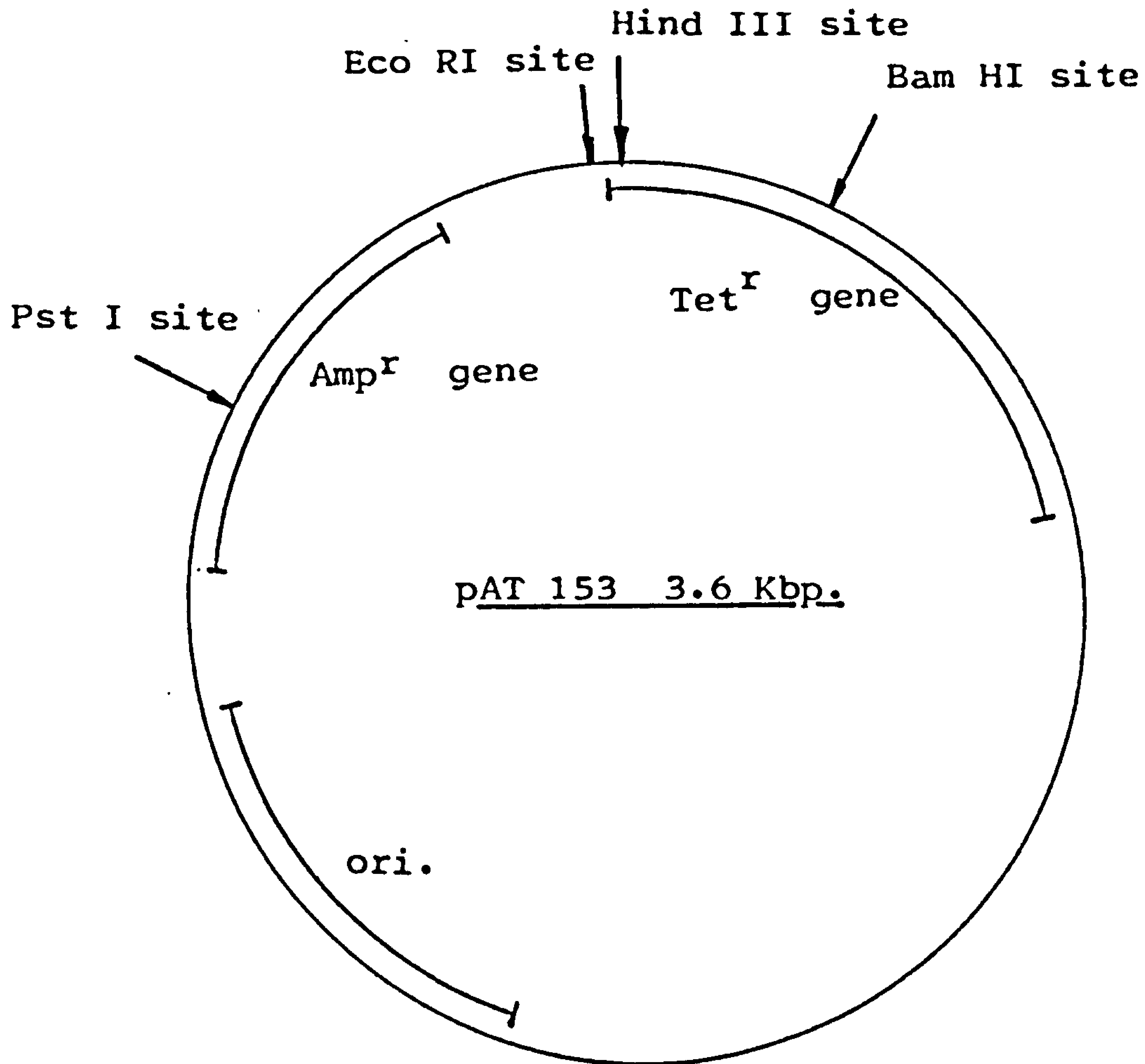
Preparation of insert DNA. Purified W/ATCC mtDNA was digested with Hind III and the enzyme inactivated by heating

Fig 13.

The diagram shows the structure of the plasmid vector pAT 153. The useful characteristics of the vector, relevant genes coding antibiotic resistance and unique restriction sites, are shown.

Fig 13. Structure of the cloning vector pAT 153 .

(Twigg and Sherrat , 1980)



The Hind III site lies within the tetracycline- resistance gene. Any insertion into this site will destroy the tetracycline - resistance conferring properties of the gene. By screening transformed E. coli for ampicillin resistance and tetracycline sensitivity, the presence of a plasmid bearing an inserted Hind III fragment can be detected.

the digest to 70°C for 5 min.

Ligation. Vector and insert DNAs were ligated together at a 1:1 molar ratio of vector:insert fragments, and at a total DNA concentration of ~100 μg/ml. The ligation conditions were those described for cohesive-end ligations. The ligation mixture was transformed into competent ED 8767 cells and plated on to L-agar plus ampicillin plates. Ampicillin resistant cells (Transformants) were screened for tetracycline resistance using the replica-plating technique described. Tetracycline sensitive colonies were grown and used to prepare samples of plasmid DNA using the "mini-plasmid preparation" methods described (see p. 40). Recombinant plasmids were digested with Hind III to determine the size and origins of the inserts.

Results. Just over 50% of the transformants showed tetracycline-resistance. Approximately 20% of the tetracycline sensitive colonies showed no insert present.

Single insert recombinants were found which contained the W/ATCC Hind III fragments H5, H6, H7, H8, H9 and H10. Fragment 5 seemed to be the major ligation and transformation product. Recombinants containing the single fragment H5 occurred more frequently than all the other single fragment recombinants combined. Only one recombinant of H6 was isolated, from a population of 151 confirmed recombinants.

"Double" recombinants were also found which had ligated more than one insert fragment into the Hind III site of the vector.

Double recombinants isolated were:

H7 + H9

H5 + H10

H7 + H8

H5 + H9

H8 + H5

Not all these double recombinants can be explained as partial digestion products of the W/ATCC molecule ligated into the vector. The H5 + H10 and H5 + H9 "pairs" are not adjacent to one another on the W/ATCC mtDNA restriction map. Therefore they must have ligated together in the ligation mixture.

With the exception of the low efficiency of H6 insertion and transformation, these results are in keeping with the predicted ligation products under the conditions used, as defined by Dugaiczyk et al., (1975).

Efficient cloning of the H6 fragment

The experiment to clone the Hind III restriction fragments from W/ATCC mtDNA was repeated. The protocol was as described above except that the Hind III digest of W/ATCC mtDNA was not heat-treated, but phenol-extracted, chloroform extracted and the DNA precipitated in ethanol as described in the protocol for small-scale nucleic acid purification (see p. 48).

The results from this experiment were similar to those described for the initial cloning experiment except in that the number of recombinants containing the H6 fragment was similar to the number of recombinants containing the H5 fragment. These two recombinants were present in far greater quantities than the smaller Hind III fragment recombinants containing H7 to H10, as before. Such a result is expected

since H5 and H6 are similar in size. It is concluded that H6 may be in some way heat labile compared with fragments H5, and H7 to H10, and that this susceptibility to heat reduces the number of H6 fragments participating and competing in the ligation reaction if the W/ATCC mtDNA Hind III digest is heated.

Attempts to clone the large Hind III fragments from W/ATCC mtDNA

The experiment was repeated as before, except in that the Hind III digest was not heat-treated but solvent extracted and ethanol precipitated as described above. The vector and insert DNAs were ligated under the same conditions as before, excepting that the DNA concentration used was 10 μg/ml.

Tetracycline-sensitive colonies were grown up, and collected in 'pools' of five for the preparation of plasmid DNA samples as described in the protocol for 'preparation of plasmid DNA in pools of 5 or 10'. In this way large numbers of tetracycline-sensitive colonies were screened for the presence of large recombinants, using the H5 + H8 double recombinant as a marker (the largest recombinant isolated at the time).

No recombinants larger than H5 + H8 were isolated from over 3,000 tetracycline-sensitive colonies screened.

The Hind III fragments H1, H2, H3, H4 and H5 were recovered from agarose gels and used in individual ligations. The conditions of low DNA concentration (10 μg/ml), and equimolar ratio of vector:insert were maintained, using the conditions described for cohesive-end ligations. All other protocols were as described for the previous cloning experiment to isolate large recombinants containing H1 to H4 fragments.

Numerous recombinants containing H5 were isolated, though at a much reduced efficiency when considering the amount of isolated, recovered fragment which was used. No recombinants containing H1 to H4 were seen.

All three methods of DNA recovery described in the Methods and Techniques Section were attempted. The continued poor efficiency of isolation of H5 containing recombinants (control) indicated that the methodology was partially responsible for the inability to isolate the large Hind III fragments.

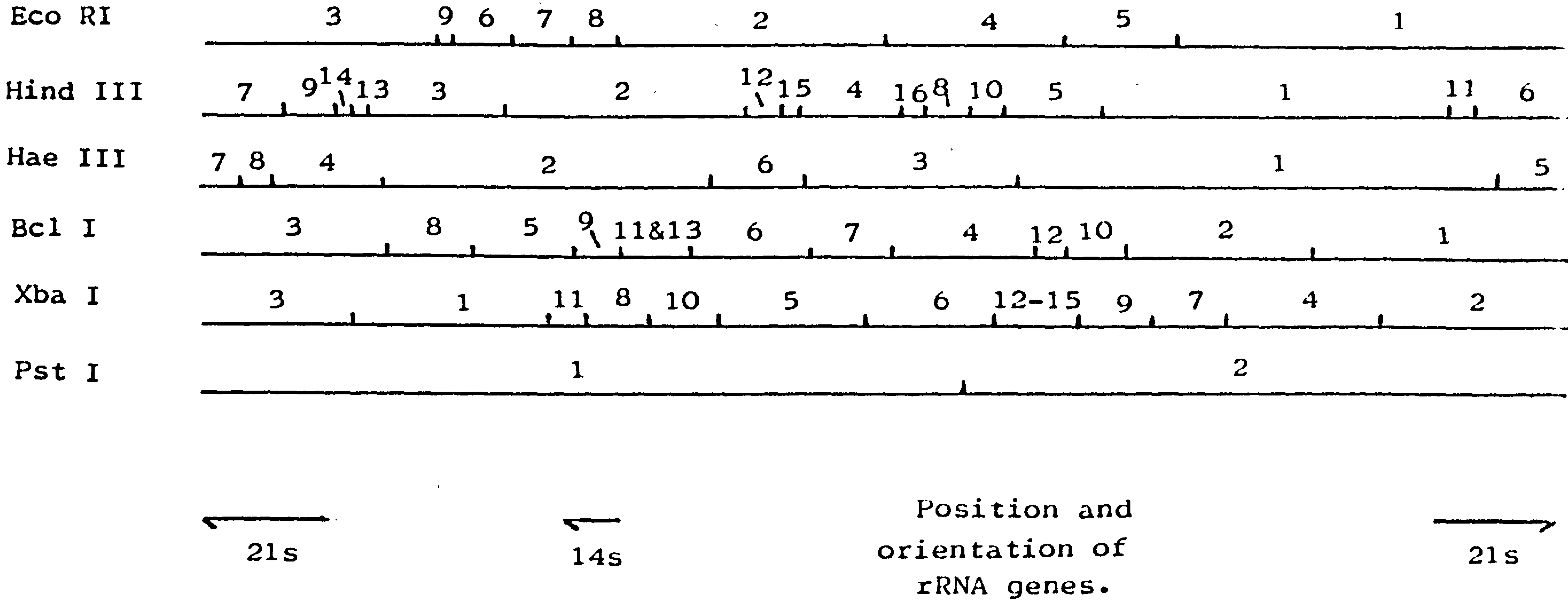
Fig 14.

The diagram shows the restriction map of *T.pyriformis* str.T., as deduced by Norton (1980), and Maycock, Holt and Jones.(unpubl.) The indicated sizes of the terminal restriction fragments are the size of these fragments measured to the middle of the "fuzzy" bands present on the gel photographs.(A typical restriction digest of str.T. mtDNA with Eco RI is shown overleaf.)

The position of the RNA genes shown on the diagram were mapped by Norton (1980). The orientation of these genes ,shown by the direction of the arrows, are those deduced by Goldbach *et al.*, (1978b). (see p 120).

Fig 14. Restriction site map for *T. pyriformis* str. T. mtDNA.

(Norton , 1980.)
(Holt & Jones, Unpubl.)



Position and orientation of rRNA genes.

Studies on Tetrahymena pyriformis str. T mtDNA

The mtDNA molecule from *T. pyriformis str. T,* has been mapped with several restriction endonucleases. The location of the rRNA genes have also been established (Norton, 1980; Holt & Jones, unpublished).

A summary of this information is shown in Fig. 14. This restriction map has been found to be identical with that obtained by Goldbach *et al.*, (1978*b*) for *T. pyriformis str. ST*. The polarity of the rRNA genes shown in Fig. 14 are those deduced by Goldbach *et al.*, (1978*b*) for strain ST.

The cell-line of *T. pyriformis str. T* used in these studies was a clonal population raised from one isolated cell by N. A. Maycock in 1980.

Samples of str. T mtDNA were prepared and purified as described in the protocols for preparation and purification of Tetrahymena nucleic acids (see p. 38). The mtDNA was found to sediment at approximately 34 s on sucrose gradients. The digest pattern obtained after digestion of str. T mtDNA with Eco RI appeared identical to that obtained by Norton in 1980.

Str. T mtDNA was used in attempts to further define the nature of the mtDNA terminus, working from the already established restriction map of the molecule.

Experiments on whole mt DNA

Treatment with Bal 31 exonuclease

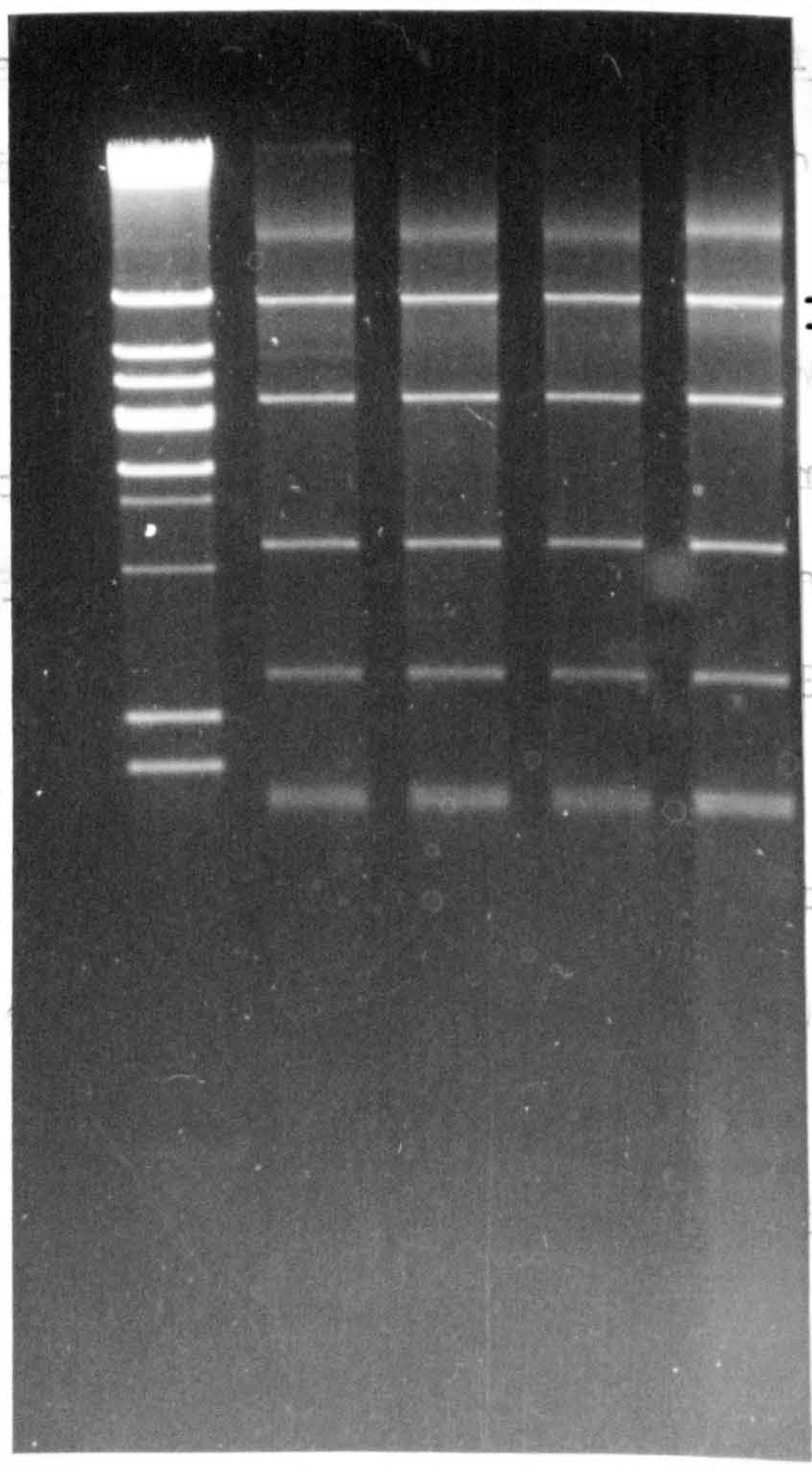
Str. T mt DNA (8 μg) was digested with Bal 31 exonuclease (BRL). Samples containing 2 μg of str. T mtDNA were taken at 10 min intervals and pipetted directly into 200 μl of 10 m*M* EDTA. Samples were phenol-extracted, chloroform extracted and ethanol precipitated as described in the protocols for nucleic acid

The photograph shows TmtDNA samples digested to varying degrees with Bal 31 exonuclease, then digested with Eco RI. (see p120.) The progressive degradation of the R1 and R3 terminal restriction fragments can be seen.

Bal 31 digested TmtDNA.

5' 10' 15' Control (no Bal 31 digestion.)

λ



purification. Following collection and drying, samples were resuspended and digested with Eco RI. The resulting digest patterns were compared.

The R1 and R3 fragments conforming to the terminal fragments from the mtDNA molecule were seen to be reducing in size and intensity as the digestion proceeded.

This result indicates that an open-ended structure of some kind is present at or near the termini of the molecule.

Treatment with S_1 nuclease

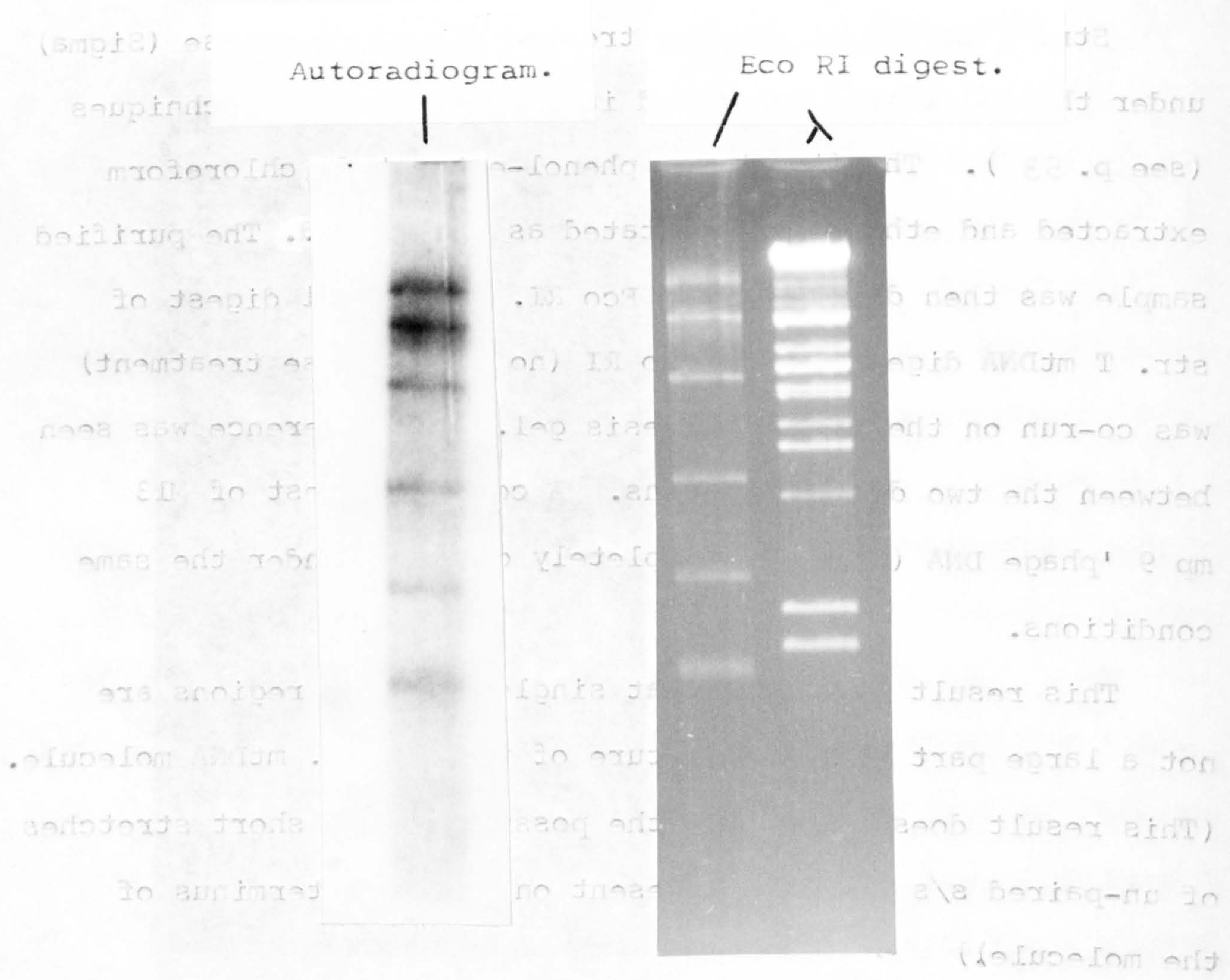
Str. T mt DNA (5 μg) was treated with S_1 nuclease (Sigma) under the conditions described in the Methods and Techniques (see p. 53). The digest was phenol-extracted, chloroform extracted and ethanol precipitated as described. The purified sample was then digested with Eco RI. A control digest of str. T mtDNA digested with Eco RI (no S_1 nuclease treatment) was co-run on the electrophoresis gel. No difference was seen between the two digest patterns. A control digest of M13 mp 9 'phage DNA (s/s) was completely degraded under the same conditions.

This result indicates that single-stranded regions are not a large part of the structure of the str. T. mtDNA molecule. (This result does not remove the possibility of short stretches of un-paired s/s DNA being present on the very terminus of the molecule.)

Nick translation of str. T mtDNA

Str. T. mtDNA (3 μg) was nick-translated using [α-^{32}P]-dATP as described in the Methods and Techniques (see p. 54). The labelled DNA was digested with Eco RI and the resulting

The gel electrophoresis pattern shows an Eco RI digest of TmtDNA labelled by nick translation. the accompanying photograph shows the same digest pattern transferred to nitrocellulose and autoradiographed. (see p121 - 122).



digest pattern transferred from the electrophoresis gel to a nitrocellulose filter using the Southern technique (see p. 47). This filter was then autoradiographed as described in the Technique for hybridization filters (see p. 58). The autoradiogram showed a pattern identical to that of the Eco RI restriction digest pattern.

It was concluded that since no effort was made to "nick" the DNA before or during the reaction, the nick-translation system was largely taking advantage of nicks already present in the mtDNA molecule.

This result suggests that the terminal regions of the molecule are not excessively nicked or gapped compared with the rest of the molecule.

Cloning studies on the Str. T mtDNA Terminal Restriction fragments

Cloning rationale

The restriction site map for Str. T mtDNA is shown in Fig. 14. It can be seen that the terminal restriction fragments produced by the enzymes Bcl I, Xba I, and Eco RI are all large. Since difficulties had been encountered in attempting to clone any large fragment from Tetrahymena mtDNA, it was decided not to attempt to clone these terminal fragments.

Both Hae III and Hind III produce terminal restriction fragments of a size similar to already cloned Tetrahymena mtDNA restriction fragments. Hae III enzyme produces a blunt (flush) end on cutting a DNA molecule (",-GG↓CC-," being the recognition site). Hind III enzyme produces a cohesive end on cutting a DNA molecule, ("-A↓AGCTT-", being the recognition site).

It was decided to attempt to clone the Hind III terminal restriction fragments since the use of a cohesive terminus would afford an easy method for ligating the "inside" end of a terminal restriction fragment, and would give an indication of the polarity of the cloned terminal restriction fragment.

Choice of a Cloning Vector

The structure of the termini of the mt DNA molecule is unknown. In attempting to clone these terminal restriction fragments, assumptions had to be made as to the types of structure that may have been present.

It was assumed that it would be possible to resolve the termini of the molecule to a blunt or flush-ended structure, which would be available for blunt-end ligation into a suitable cloning vector. This cloning vector would therefore require a blunt (flush) cutting site, and a Hind III site. The vector available at the time which would allow the production of these cloning sites in a useful manner was p JB 8 (see Fig. 15).

Removal of Cloned inserts for Further Study. Any recombinants resulting from the successful ligation of insert and vector DNAs could be characterised and the inserts removed in the following manner:

Cut with Hind III	-	site of the original "inside" end of the terminal restriction fragment.
Cut with Eco RI	-	immediately adjacent to the filled Bam H1 site, and therefore only a few based away from the blunt-end cloning site.

PAGE

NUMBERING

AS ORIGINAL

Fig 15.

The diagram at the top of the page shows the steps used in the original construction of vector pJB8.

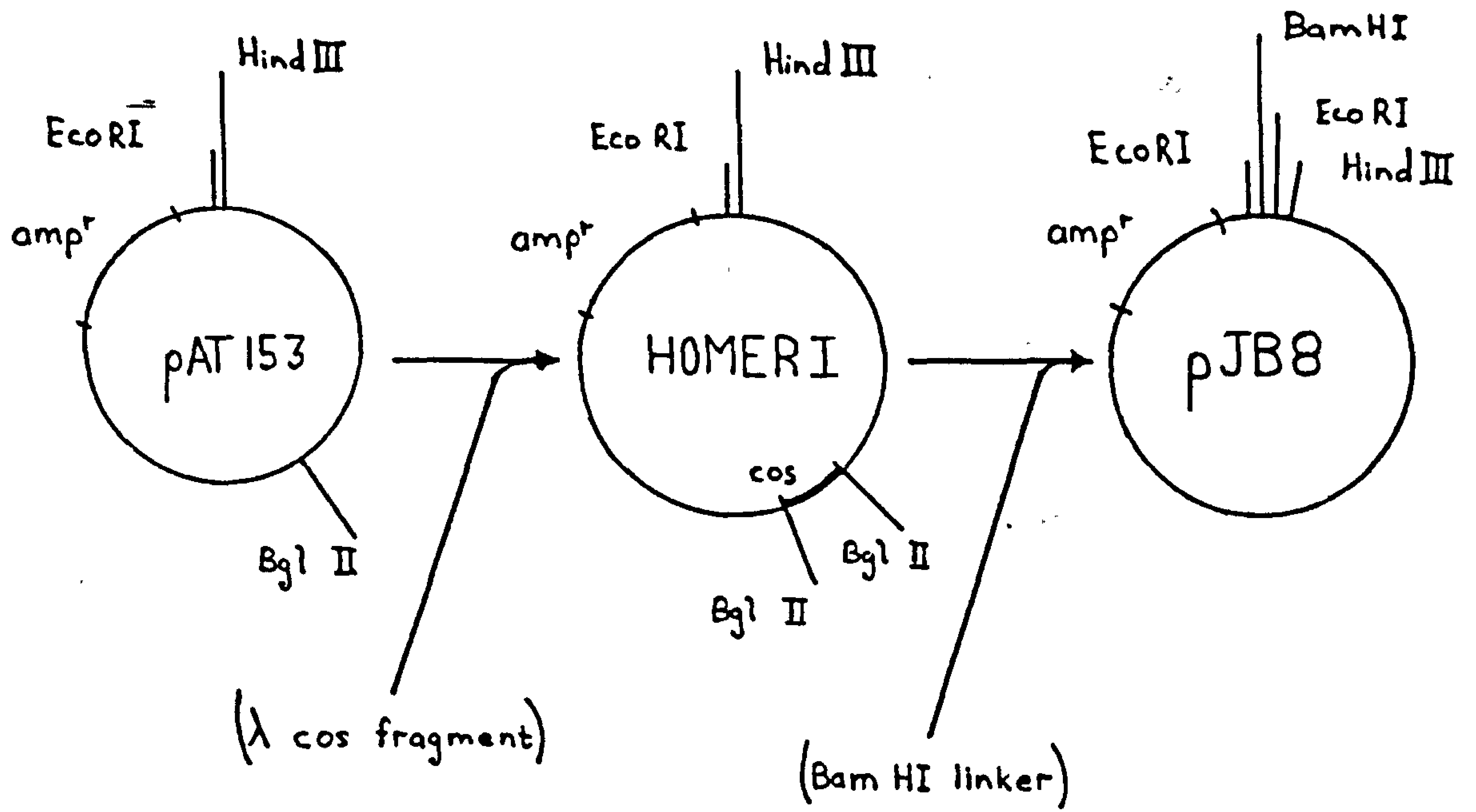
(Ish-Horowitz & Burke, 1981)

The diagram at the bottom of the page shows the step-wise reactions required to create a blunt end/Hind III ended cloning vector using pJB8. The relevant protocols are described in the methods and techniques. (p50 - 55).

1). Cut with Bam HI.

2). Fill the Bam HI cohesive ends with Klenow polymerase. (Large fragment E.coli DNA polymerase I.)

3). Cut with Hind III.



Eco RI
Eco RI
Hind III
Bam HI
Eco RI
Eco RI
Hind III
1).
Eco RI
Eco RI
Hind III
2).
Eco RI
3).
Hind III
cohesive end.

Procedure

Preparation of the vector. The blunt end/Hind III pJB 8 vector was prepared following the scheme described in Fig. 15. The relevant protocols are those described in the Methods and Techniques section.

Preparation of the Insert DNA. Purified str. T. mt DNA was "filled" using the Klenow polymerase (large fragment *E. coli* DNA Polymerase I), as described on page 54 . The DNA was then digested with Hind III, phenol extracted, chloroform extracted and ethanol precipitated.

Ligation of Vector and Insert DNAs

Vector and insert DNAs were ligated together at a total (initial) DNA concentration of 100 μg/ml under the conditions described for 2-step ligations. A 1:1 ratio of vector:insert was used, where the measurable parameter was the number of Hind III cohesive termini present. (The number of vector Hind III termini present ≡ number of insert Hind III termini present.) Samples of the ligation mixture were used to transform competent ED 8767 cells. Transformants were plated on L-agar plus ampicillin (pJB 8 possesses an ampicillin resistance gene derived from its pAT 153 parent vector, see Figs. 13 and 15). Samples of plasmid DNA were prepared from transformants. Recombinants were tested for their adherence to certain characteristics which would indicate whether they were genuine recombinants containing the terminal Hind III restriction fragments from T mtDNA (see below).

Experimental control for the confirmation of the structure of the vector

Unlike the cleavage of a circular DNA molecule with a restriction enzyme, the filling-in reaction described to turn a

Bam H1 cohesive terminus into a blunt-end affords no simple control to confirm the completion of the reaction. The following test was done to indicate the nature of the termini produced by this treatment.

Bam H1 cut vector was re-ligated under cohesive-end conditions at a DNA concentration of 50 μg/ml. When transformed into competent ED 8767 cells this gave a transformation efficiency of ~4,000 colonies/μg. When a sample of this Bam H1 cut vector was "filled", then treated as before, the transformation efficiency dropped to ~150 colonies/μg. When a sample of the Bam H1 cut, "filled" vector was ligated under blunt-end conditions at 50 μg/ml DNA concentration, it gave a transformation efficiency of ~1,800 colonies/μg. (Blunt-end ligation conditions are those defined as the additional steps required in the 2-step ligation, without the dilution step.)

These results were taken as an indication that blunt-ended molecules had been formed due to the action of the "filling" system on the Bam H1 cohesive termini.

<u>Characteristics required of recombinant plasmids to be identified as clones of the $^{str.T.}$ Hind III terminal restriction fragments</u>

The following criteria were used to screen for the required recombinants:

(a) The presence of intact restriction sites: The presence of an Eco RI site and a Hind III site which, when cut, produce a vector-sized fragment and an insert. This criterion was the first in the screening and removed the ligation products involving damaged vector molecules.

(b) The size of the insert: After being cut from the vector with the two enzymes mentioned above, the insert must fall within the size limits defined by the str. T mtDNA Hind III terminal fragments H6 and H7.

(c) The presence of a 21 s rRNA hybridizing region in the insert. As defined by the map shown in Fig. 14, a copy of the H6 or H7 fragment will bear a large part of the 21 s rRNA gene from that end of the molecule. To test recombinants for adherence to this parameter, Hind III/Eco RI double digests of suspected clones were transferred to nitrocellulose filters and incubated with isotopically labelled str. T mitochondrial 21 s rRNA. (This was prepared, purified and labelled in the same manner as W/ATCC 21 s rRNA.)

Results

A number of recombinants fitted the first screening parameter, possibly due to the presence of broken or damaged str. T Hind III fragments being ligated to the vector. A quantity of these first "screened" recombinants also fitted the second parameter. Most of the recombinants which passed the first test but failed to adhere to the second parameter's condition were those showing very small inserts. Only two recombinants were isolated from these experiments which fitted all three parameters. These two recombinants will hereafter be referred to as No. 76 clone and No. 67 clone.

Attempts to isolate a Hind III terminal restriction fragment clone from a specific termini

Whole, purified str. T mtDNA was "filled" using the Klenow polymerase as described on Page 54. The DNA was

digested with Eco RI and the terminal fragments R1 and R3 recovered from agarose gels using the electroelution method described. The recovered fragments were digested with Hind III and ligated into the Hind III, blunt-end pJB 8 vector as before, with the necessary adjustments made to the amount of vector present to ensure a molar ratio of vector-Hind III termini:insert-Hind III termini, of 1:1.

One recombinant was isolated from these experiments which satisfied the criteria defined for identifying clones of the terminal restriction fragments. This recombinant was isolated from a ligation involving the recovered R3 fragment, and therefore purports to be a clone of the H7 fragment (see Fig. 14). This clone will hereafter be referred to as No. 2 clone.

Further characterisation of the terminal restriction fragment clones Nos. 2, 67 and 76

Samples of the three p JB 8 terminal restriction fragment clones were digested with the enzymes Hind III and Sau 3A (recognition site: ↓-GATC-). A control digest of pJB 8 cut with the two enzymes was co-run on the same electrophoresis gel.

All three digests produced a fragment of 0.7 Md, and a fragment whose size indicated the clone of origin. (See diagram overleaf).

The digest products were transferred to nitrocellulose filters by the Southern Blot technique and incubated with isotopically labelled str. T. mitochondrial 21 s rRNA. Strong hybridization was seen with the 0.7 Md Sau 3A/Hind III fragment. Weak hybridization was seen with the terminal Sau 3A fragment, characteristic in size for the clone in question. (see Plate 5).

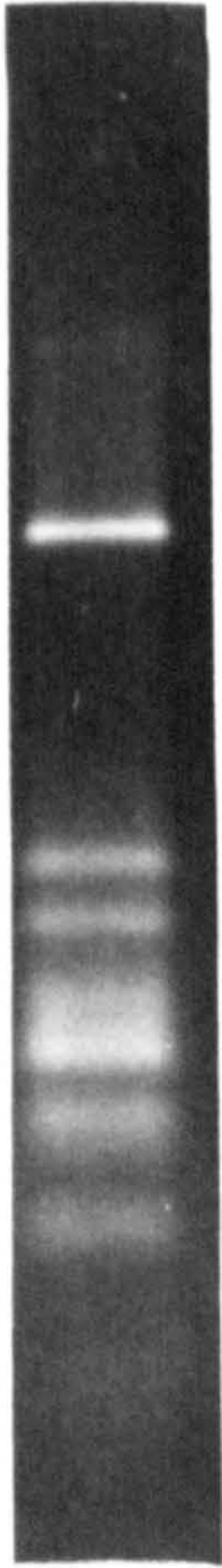
Plate 5.

The restriction digest patterns obtained from the digestion of pJB8 recombinant clones No.2 and No.67 with Sau 3A and Hind III are shown. The accompanying control digest of the vector alone is also shown. This indicates that, in each case, the insert DNA has been cut into two pieces. (see diagram on p 130).

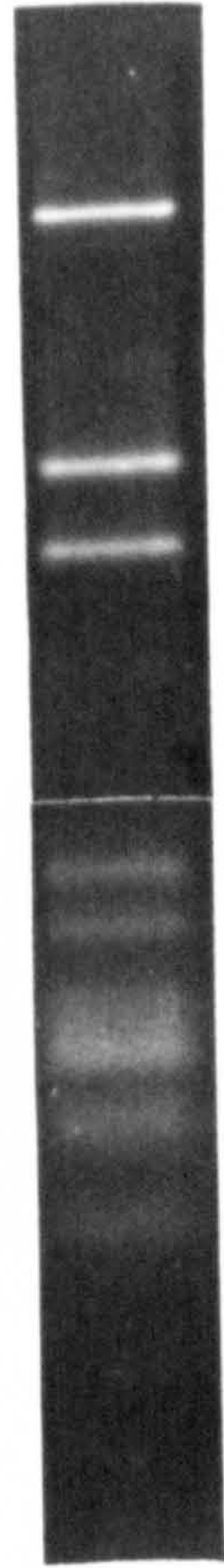
The hybridization of labelled 21s rRNA to the Southern blots of these digest patterns are also shown.

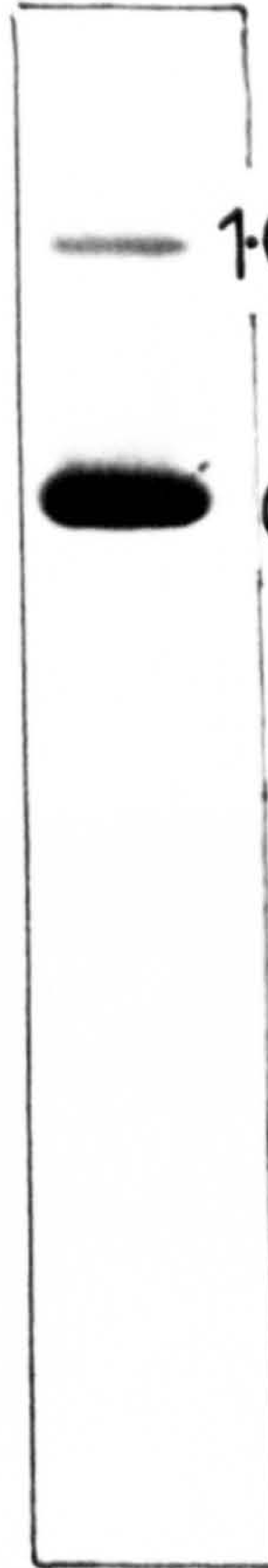
Plate 5.

pJB8 (control)



No.2

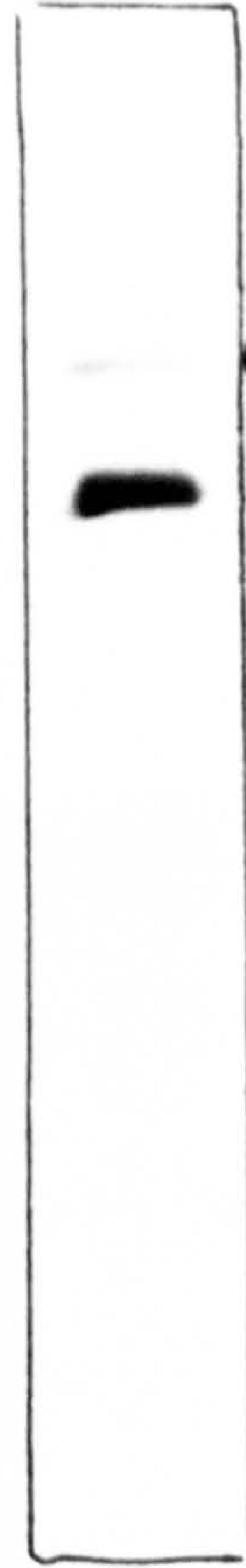




1·05Md

0·7Md

No.67



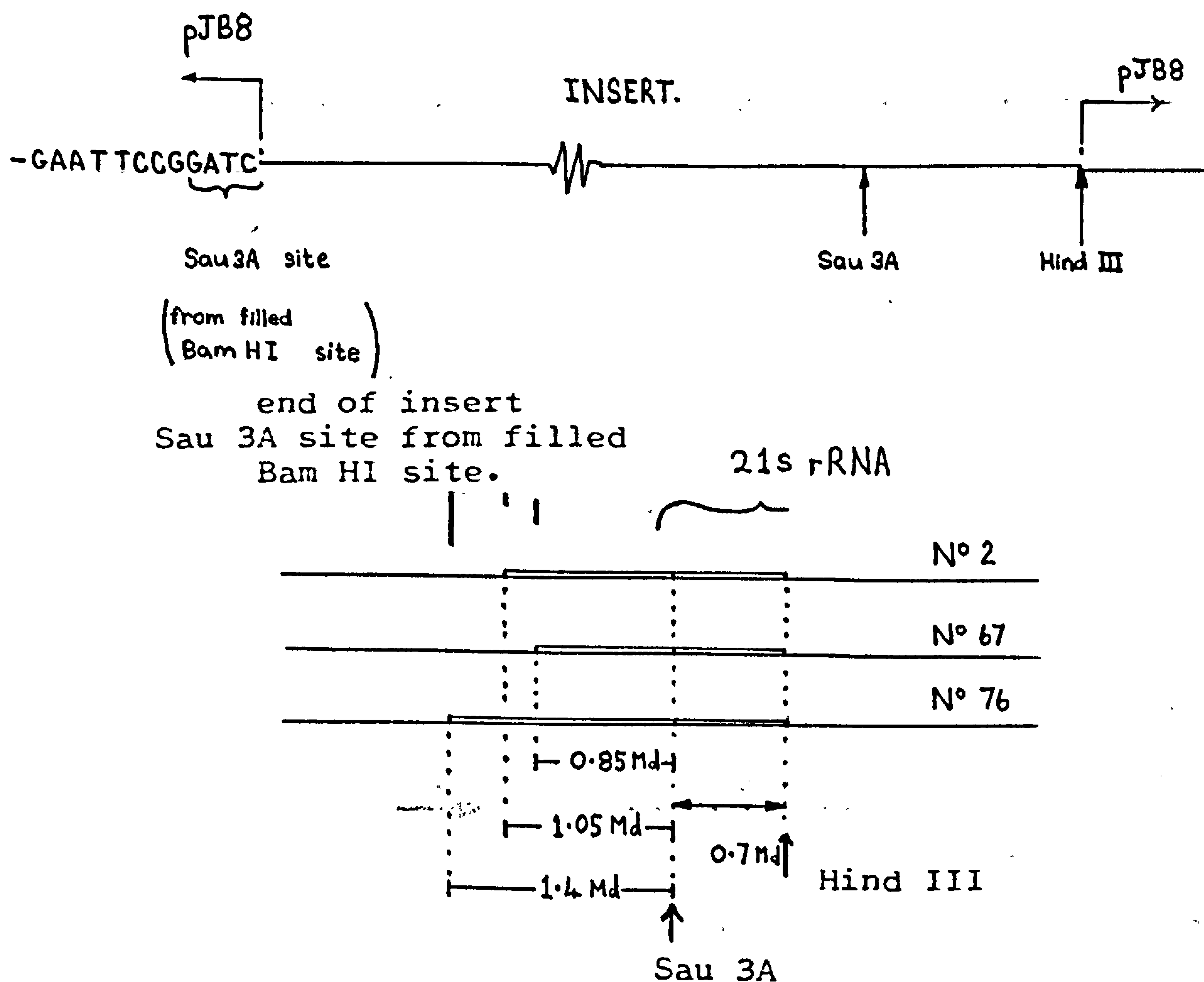
0·85Md

0·7Md

Hybridization of labelled 21s rRNA to Sau 3A Hind III double digests of clones No.2 and No.67 (pJB8 clones of the H7 terminal restriction fragment from T mtDNA).

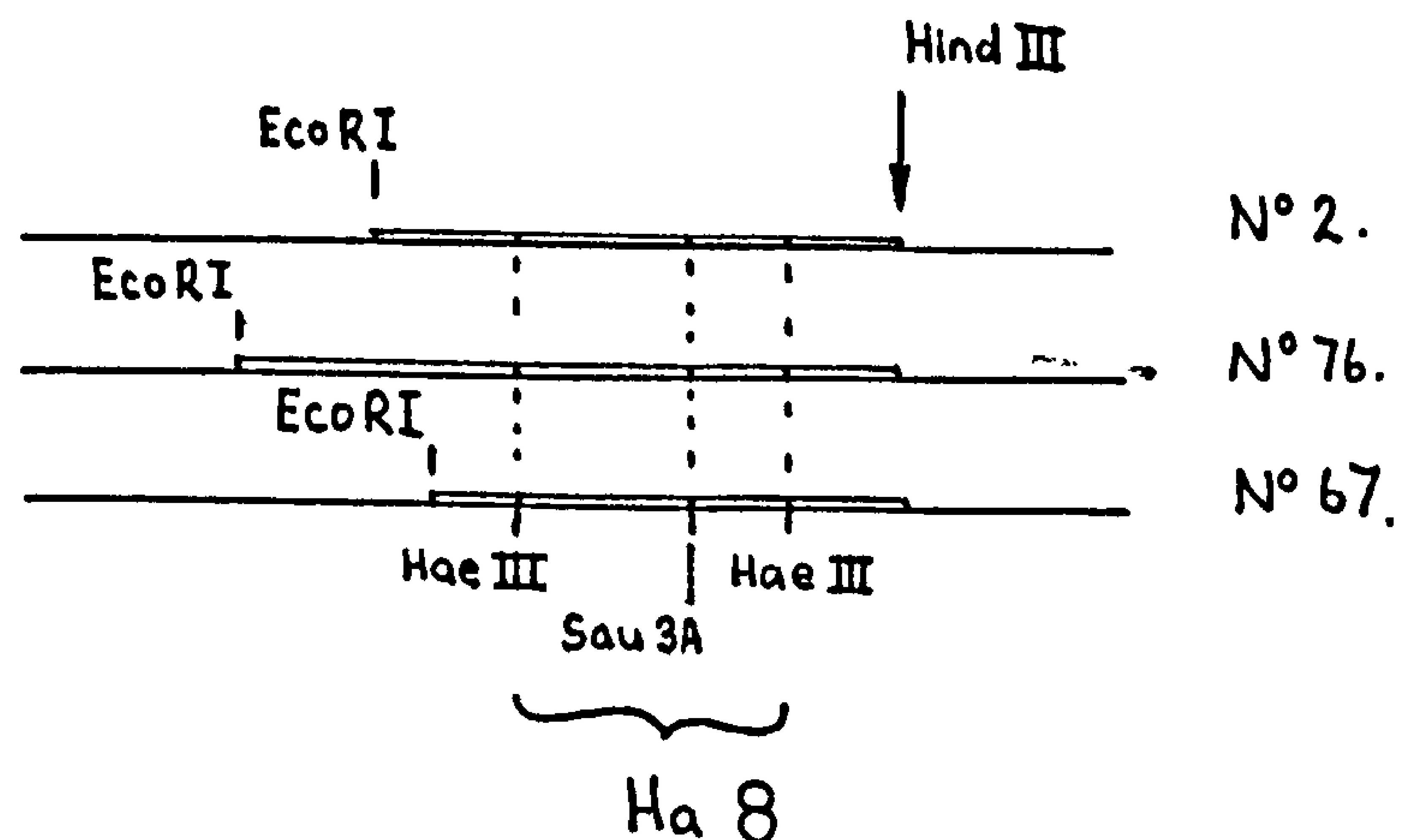
The diagram (right) shows the mapping of the Hind III and Sau 3A sites on the three terminal restriction fragment clones. Sau 3A will cut at the filled Bam HI site at the junction of the vector and insert DNA's. The insert DNA will therefore be excised from the clone by a Sau 3A/ Hind III double digest, with any additional cuts within the insert DNA being made by the Sau 3A .

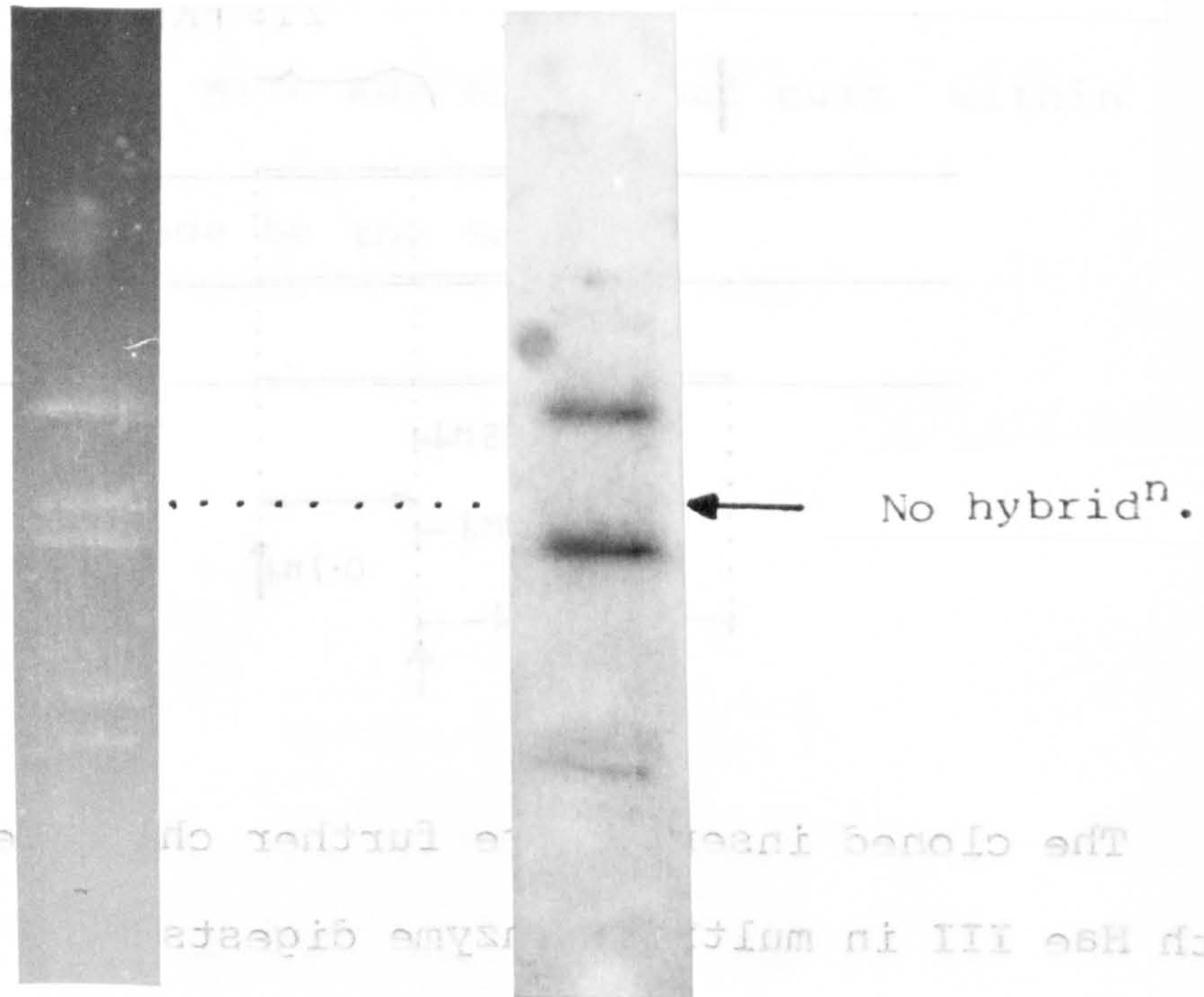
The diagram (right) shows the mapping of the Hae III sites on the insert DNA's of the three terminal restriction fragment clones.



The cloned inserts were further characterised by digestion with Hae III in multiple enzyme digests with the two enzymes Hind III and Sau 3A.

The three clones gave similar digest patterns which only differed in the size of one fragment which was characteristic of the clone in question.





No hybridization is seen to the fragment containing

the origional terminus of the molecule. (see arrow.)

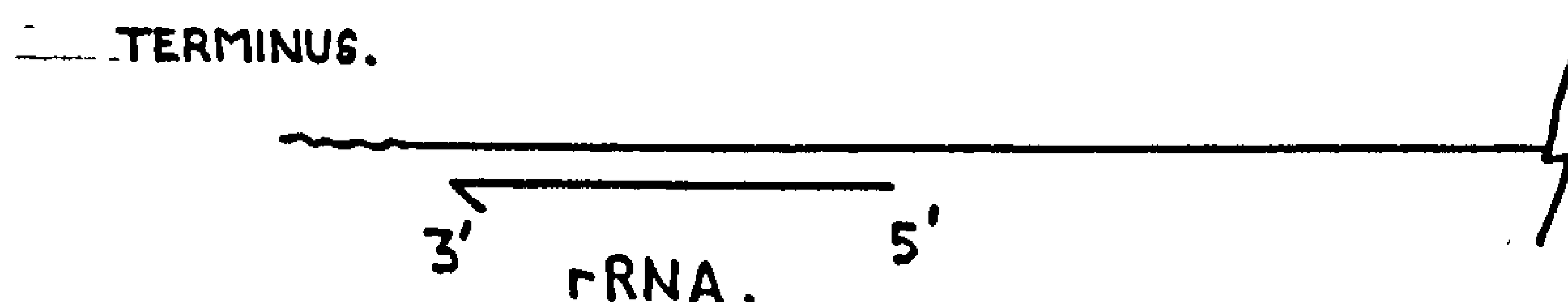
The Hae III fragment produced in these digests conforms to the size and position of the Ha 8 discrete restriction fragment from the Hae III map of str. T mtDNA (see Fig. 14). The identical nature of the non-terminal regions of these three clones indicates that all three are copies of the H7 terminal restriction fragment from str. T mtDNA. Any heterogeneity in length of members of the population of H7 fragments appears to be confined to the very terminal region itself.

Limits of the 21 s rRNA gene in the cloned H7 fragment

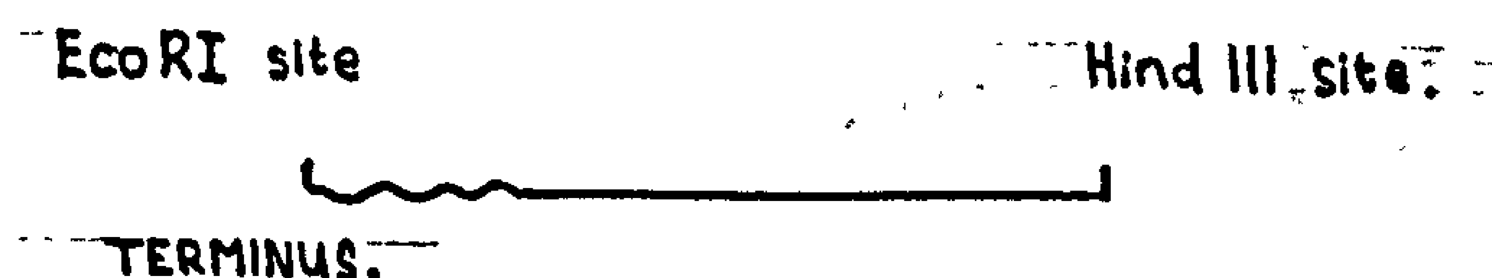
The Sau 3A/Hind III/Hae III digest pattern produced upon digestion of clone No. 2 was transferred to a nitrocellulose filter and incubated with isotopically labelled str. T mitochondrial 21 s rRNA. Hybridization was seen with the three "internal" fragments but not with the fragment conforming to the original terminus of the molecule (see opposite.)

Confirmation of the polarity of the 21s rRNA gene (from str. T mtDNA) using M13 'phage cloning vectors

The polarity of the 21s rRNA gene mapping at the "R3" end of the str. T mtDNA molecule is given by Goldbach et al. (1978b) as being:



Clone No. 76 is a copy of the H7 terminal restriction fragment from str. T mtDNA which contains a portion of this gene (see Fig. 14). This recombinant insert has the orientation:



Samples of No. 76 clone were digested with Eco RI and Hind III and the insert DNA fragment recovered from agarose gels using the freeze/squeeze technique. The insert DNA was then sub-cloned into Eco RI/Hind III cut vectors of M13 strains mp 8 and mp 9. RF DNA preparations were done on recombinant plaque cultures as described on Page 43. The step involving the washing of the cell pellet was omitted. The resulting RF DNA samples were contaminated with the s/s 'phage DNA from that strain (mp 8 or mp 9). These contaminated samples were used for Eco RI/Hind III digests to confirm the origins of the insert DNA. The gel channels containing the RF DNA digest plus s/s DNAs were excised and the DNAs transferred to

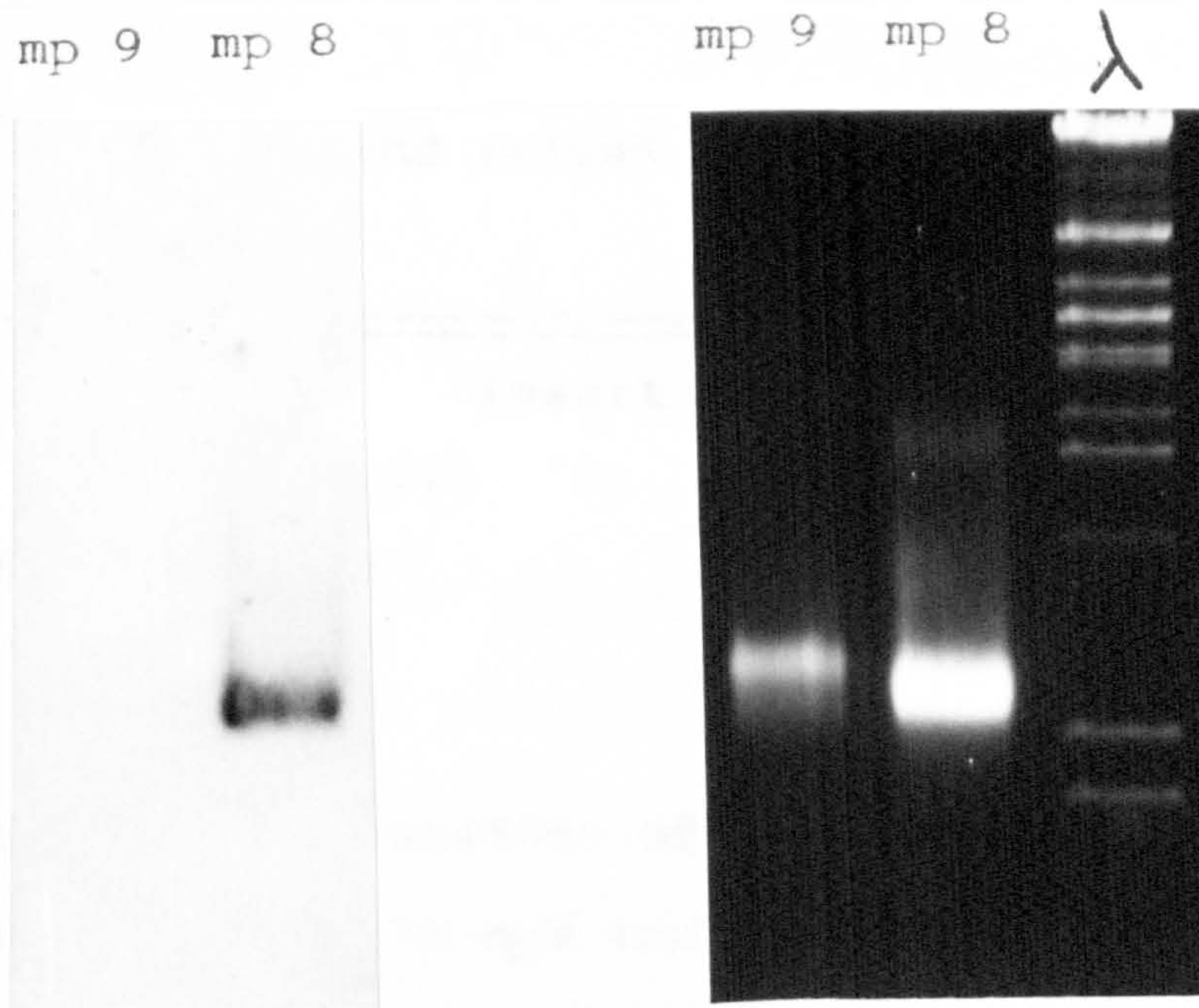
nitrocellulose filters using the Southern Blot technique as described on page 47. Filters were incubated with isotope-labelled str. T 21s rRNA as described on page 57. (This 21s rRNA was prepared and purified as described on page 56.)

Both the mp 8 and mp 9 clones showed a hybridization of the 21 s rRNA to the insert DNA fragment from the RF DNA (double stranded).

The 21s rRNA also hybridized to the contaminating s/s DNA from the mp 8 clone.

The 21 s rRNA did not hybridize to the contaminating s/s DNA from the mp 9 clone.

This result confirms the polarity of the 21s rRNA gene as that proposed by Goldbach <u>et al</u>. (1978<u>b</u>), see Fig. 16.

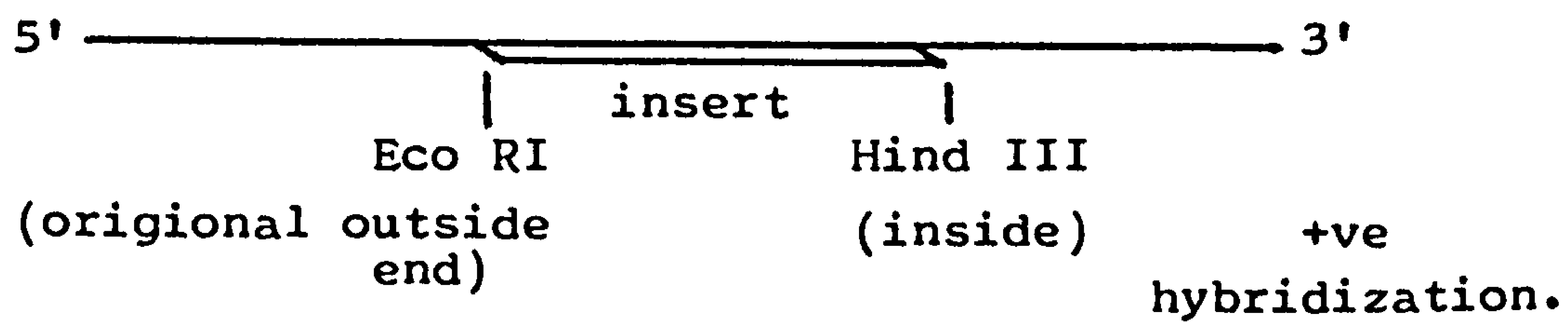


The photograph shows the s/s DNA from M 13 clones of No. 76 insert in mp 8 and mp 9. The accompanying autoradiogram shows the hybridization of labelled 21s rRNA to the s/s DNA from the mp 8 clone only.

Fig 16. <u>Confirmation of the orientation of the 21s rRNA genes from T. pyriformis str. T.</u>

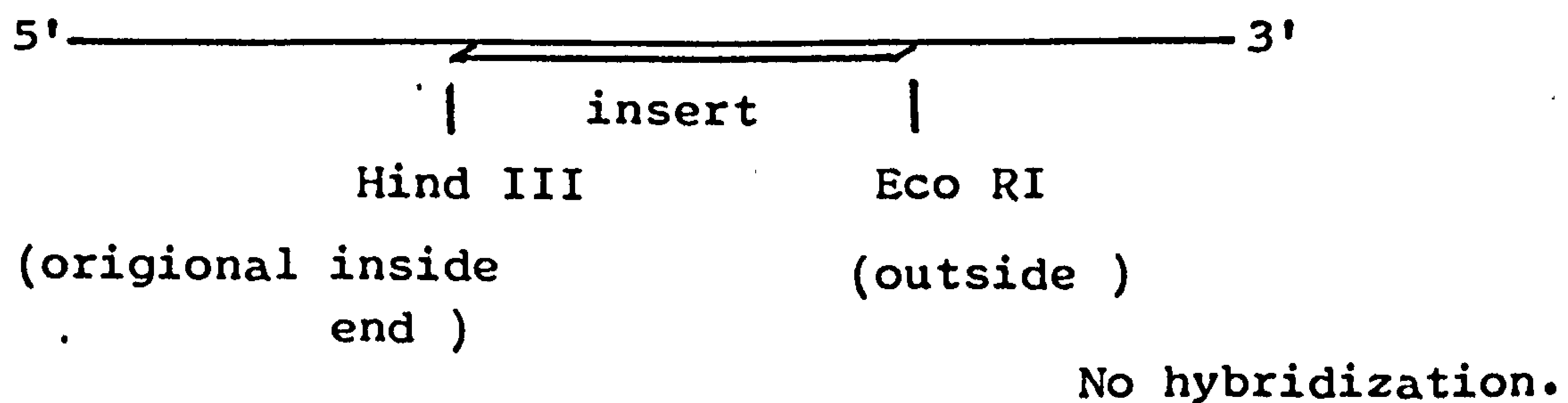
M13 mp8 clone of the Eco RI / Hind III fragment.

(plus strand (viral strand.)).



M13 mp9 clone of the Eco RI / Hind III fragment.

(plus strand (viral strand)).



The opposite orientation of the multiple cloning sites in M13 strains mp8 and mp9 results in the two complementary strands of the insert DNA being present in the s/s viral DNA's. The labelled 21s rRNA hybridizes to its complementary strand.

Sub-cloning of the H7 terminal clones Nos. 2, 67 and 76, from pJB8 vector into the M13 'phage sequencing vector

Samples of all three pJB8 clones were digested with Eco RI and Hind III and the insert DNAs recovered from agarose gels using the freeze/squeeze technique. The recovered inserts were then sub-cloned into Eco RI/Hind III digested RF DNA vectors of M13 mp 9 RF DNA as described on page 61 .

Variation in size of the M13 sub-clone recombinants

Only recombinants whose inserts' origins were confirmed by restriction digests on the clones' RF DNA were used to prepare s/s DNAs for use as sequencing templates.

In the sub-cloning of the insert DNAs from No. 2 and No. 76 clone, recombinants were isolated which contained inserts smaller than those recovered from the parent pJB8 clones. In each case the Eco RI and Hind III sites were intact, allowing the removal of the reduced-size insert. This intact nature of these restriction sites suggests that simple degradation of the recovered insert DNAs will not account for this observed reduction in size. (This behaviour of a reduction in insert size is apparently common in M13 clones where the insert DNA is large (>1 Kbp).) The size of the reduced-size inserts were all different from one another. No two reduced-length inserts were found to be identical after overnight gel electrophoresis. The frequency of this insert reduction event was quite high. In No. 76 sub-clones, approximately 30% of the M13 recombinants showed a reduction in length of the insert DNA.

In No. 2 sub-clones, approximately 10% of the M13 recombinants showed a reduction in the length of the insert DNA.

No reduced-length inserts were seen in the sub-cloned M13 recombinants from No. 67 clone.

<u>The nature of the M13 recombinants showing reduced-size inserts</u>.

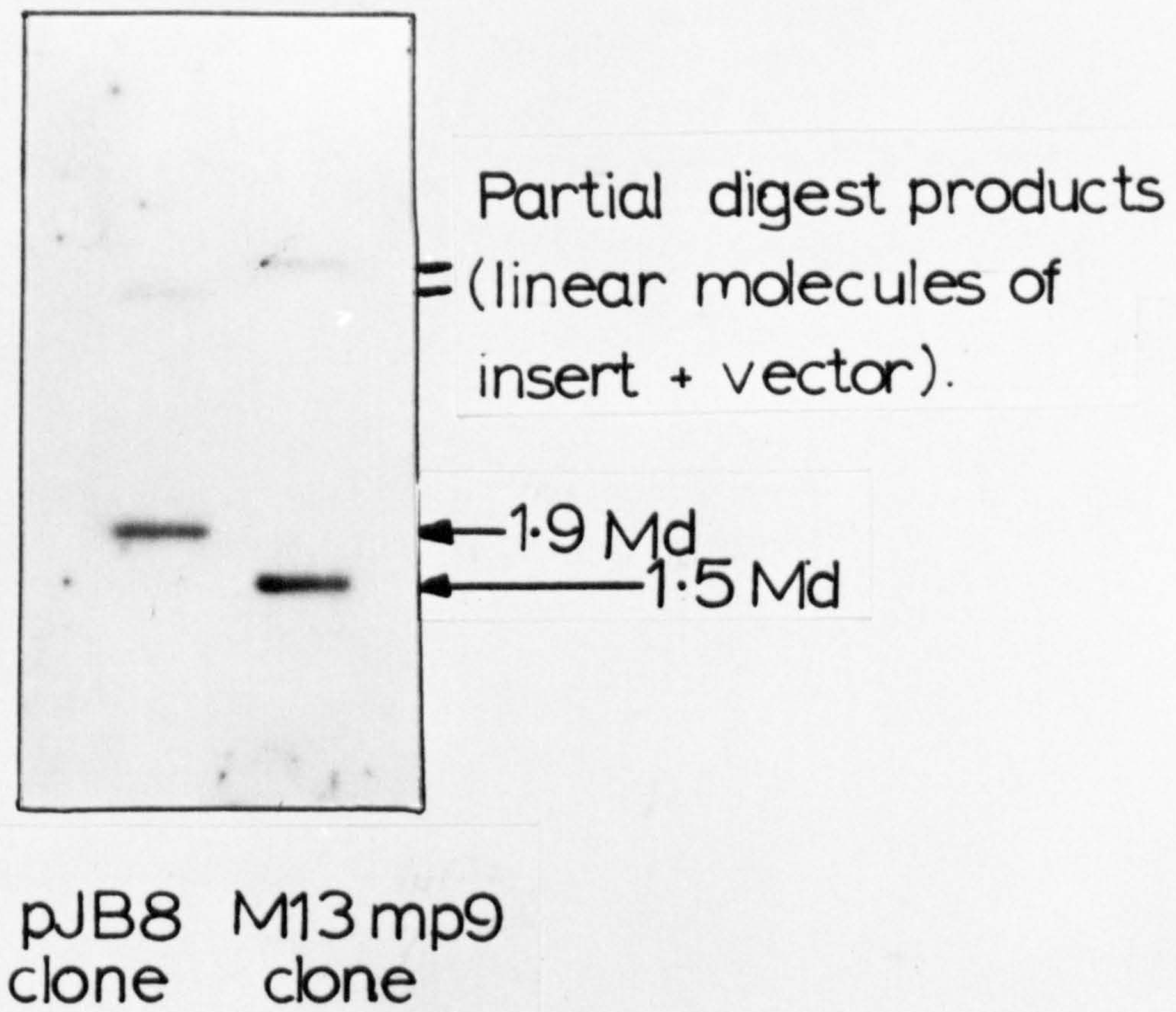
An RF DNA sample prepared from an M13 mp 9 recombinant of the No. 2 clone insert, which possessed a reduced-length insert, was digested with Eco RI and Hind III. A sample of the parent clone (No. 2, in pJB8) was also digested.

The two sets of digest products were separated by gel electrophoresis and transferred to a nitrocellulose filter using the Southern Blot technique. The filter was then incubated with isotope-labelled str. T 21s rRNA as described on page 57 . Both the full sized and reduced size inserts gave a strong hybridization signal with the labelled 21s rRNA (see Plate 6).

These results suggest that an internal deletion occurred in the insert DNAs when sub-cloned into the M13 'phage vector. The loss of 0.4 Md (~0.6 Kbp) does not appear to reduce the intensity of the hybridization signal for the insert DNA, implying that the 21 s rRNA gene region of the insert fragment is probably not involved.

The variable magnitude of the deletion event may suggest that large numbers of sequences capable of participating in some form of recombination event are present in the insert fragments. No conclusion can be drawn from the variation in the frequency of this size reduction event, with the variation in size of the terminal clone in question since, as stated, smaller M13 recombinants are not found to be as susceptible as larger recombinants.

Plate 6.



The autoradiogram above shows the hybridization of labelled 21s rRNA to i). aHind III/Eco RI digest of the original pJB8 clone of No.2 clone.

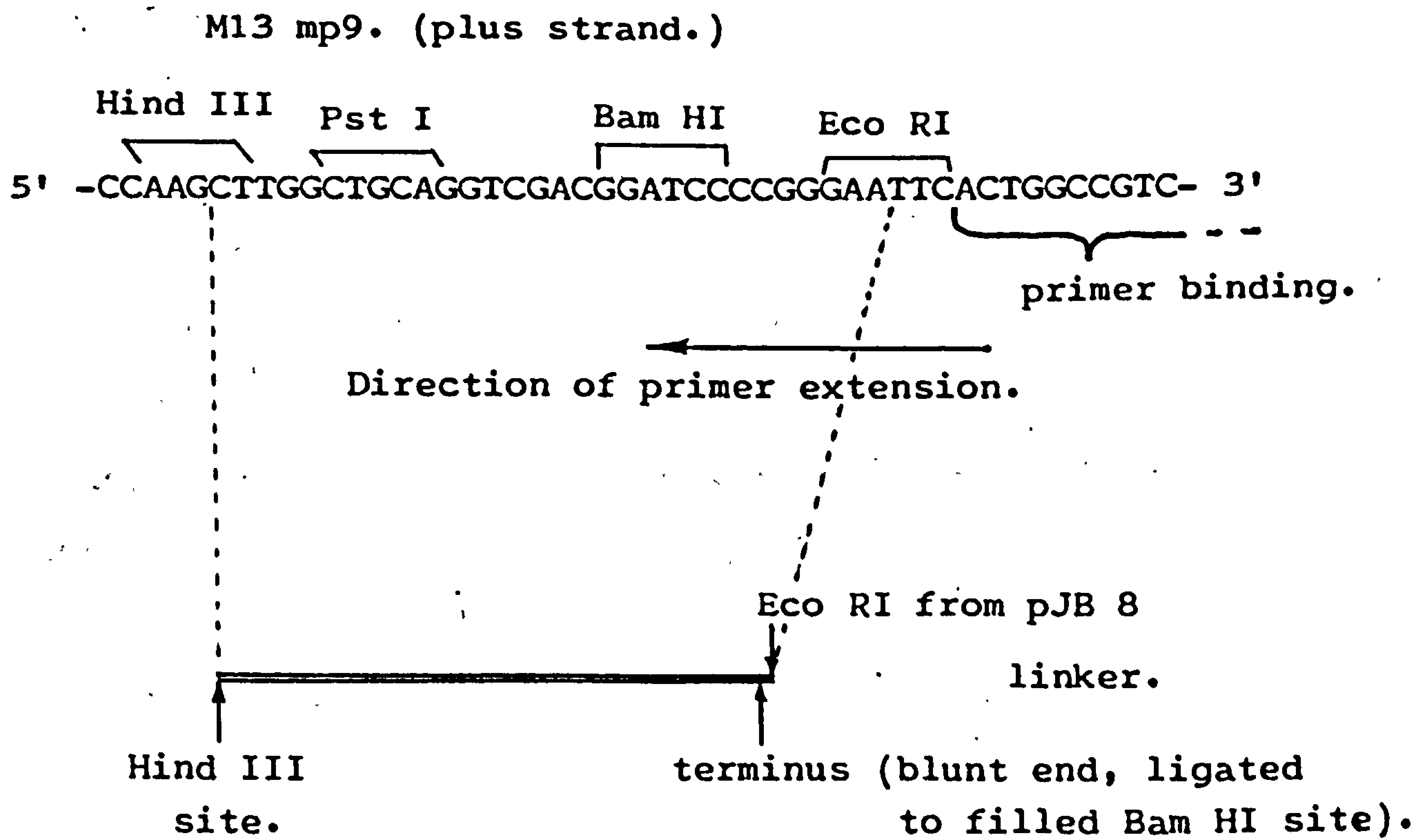
ii). a Hind III/Eco RI digest of a reduced - size No.2 clone, found when the insert was sub-cloned into M 13 mp9.

The insert has been reduced in size by 0.4 Md whilst retaining the 21s rRNA hybridizing region.

Fig 17.

The diagram shows the multi-restriction cloning site region of vector M 13 mp9, indicating the rationale for choosing this vector to provide sequence information about the "origional terminus" of the cloned terminal restriction fragments for str.T. mtDNA.

Fig 17. Multi' cloning site in M13 mp9.



The primer extension sequencing reaction will read from the Eco RI site towards the Hind III site in the vector M13 mp9. If the terminal restriction fragment clone is ligated into mp9 using these sites, then the sequencing reaction will read through the Eco RI and filled Bam HI sites and into the origional terminus of the mtDNA fragment.

Sequencing studies on the H7 terminal clone

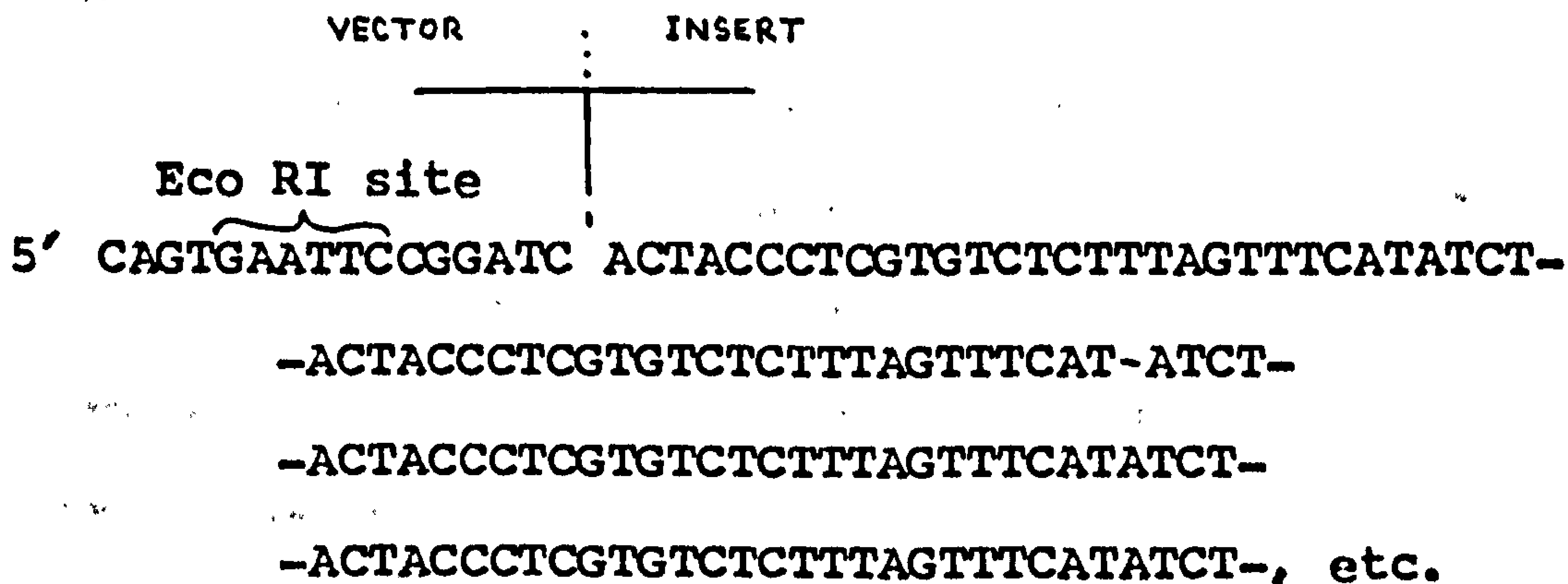
The s/s DNAs from M13 mp 9 recombinants containing the sub-cloned inserts from pJB8 clones No. 2, No. 67 and No. 76 were used as templates to sequence the insert DNA regions conforming to the original terminus of the str. T mtDNA molecule (see Fig. 17).

Sequencing reactions of the dideoxy- chain termination type were done using the s/s DNAs as templates, and [α-^{32}P]dATP as described in the sequencing protocol (see page 64). The sequence of the regions in question were obtained from the autoradiograms (see Sanger et al., 1977).

Results

All three of the terminal fragment H7 clones possessed the same sequence at their original "outside" end. The sequence seen did not vary by as much as a single base between the three clones.

The sequence is as follows:



The sequence is not readable with certainty beyond this point. The pattern created by the repeating 31 base sequence is visible as a distinctive repeating pattern stretching to the limits of the gel, or the sequence reaction products. In the case of the No. 67 clone sequencing autoradiogram, the repeating unit appears to end after 10-11 repeats (see Plate 7). This would conform to ~330 bases from the outside end of No. 67 insert, reading "inwards". This point is concurrent with the position of the first Hae III site in the insert fragment (see Page 130).

The 31 base sequence contains no palindrome sequences.

The 31 base sequence contains no useful restriction sites.

All three sequences, obtained from the three clones in question, start at the same point in the 31 base sequence repeat. This point is immediately after the vector's "filled Bam Hl site", and conforms to the sequence present at the original terminus of the mtDNA molecule. The significance of this observation will be discussed later.

<u>Extent of the cloned H7 fragment covered by the sequence repeats</u>

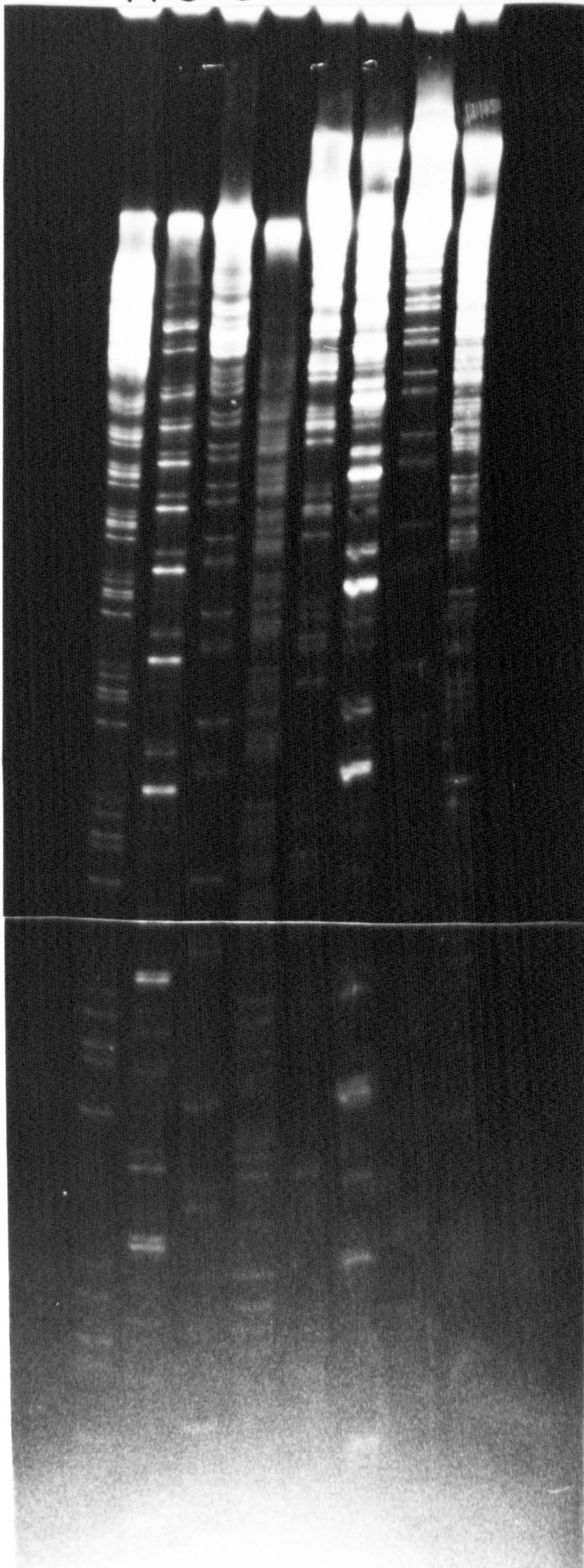
From the sequence-repeat pattern visible on the sequence autoradiograms from clones No. 76 and No. 2, a large portion of the H7 restriction fragment can be accounted for as being composed of a repeating 31 base sequence. Each of these clones showed the repeating pattern continuing for approximately 15 to 16 copies of the repeat. Further copies <u>may</u> be present, but their presence is not definite as the autoradiograms are very faint beyond this point.

Plate 7.

The photograph shows an autoradiogram taken from the sequencing studies on No.67 clone, (terminal Hind III restriction fragment clone.) using the vector M 13 mp 9.

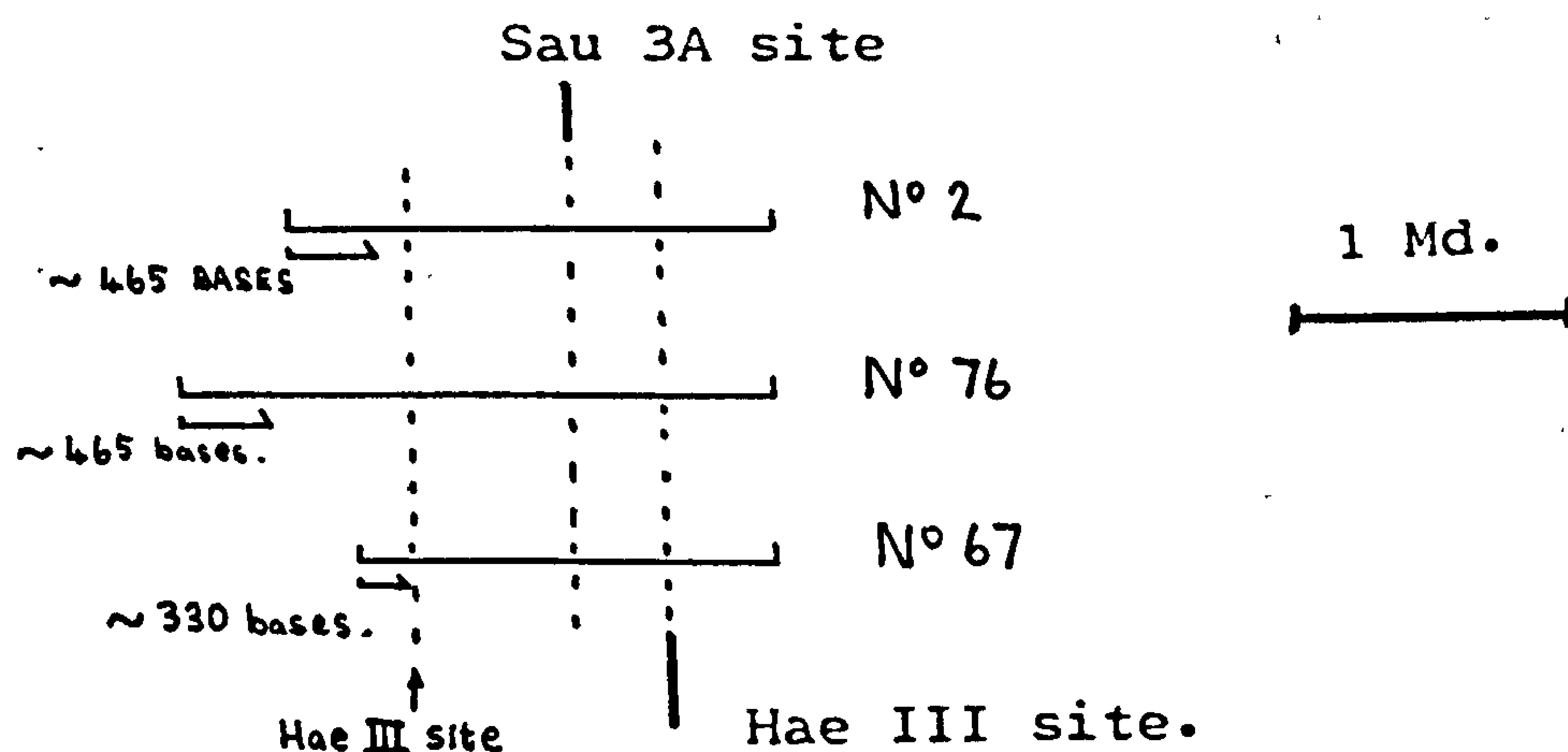
The repeating unit of the 31 bp sequence is clearly visible as a repeating pattern continuing for approximately 10 repeats.

Plate 7



Fifteen repeats of a 31 base sequence = 465 bases.

This amount of the H7 fragment possessing a sequence repeat is shown in the following diagram for both No. 2 and No. 76 clones. The portion of the No. 67 clone possessing 10-11 repeats of the sequence is also shown.



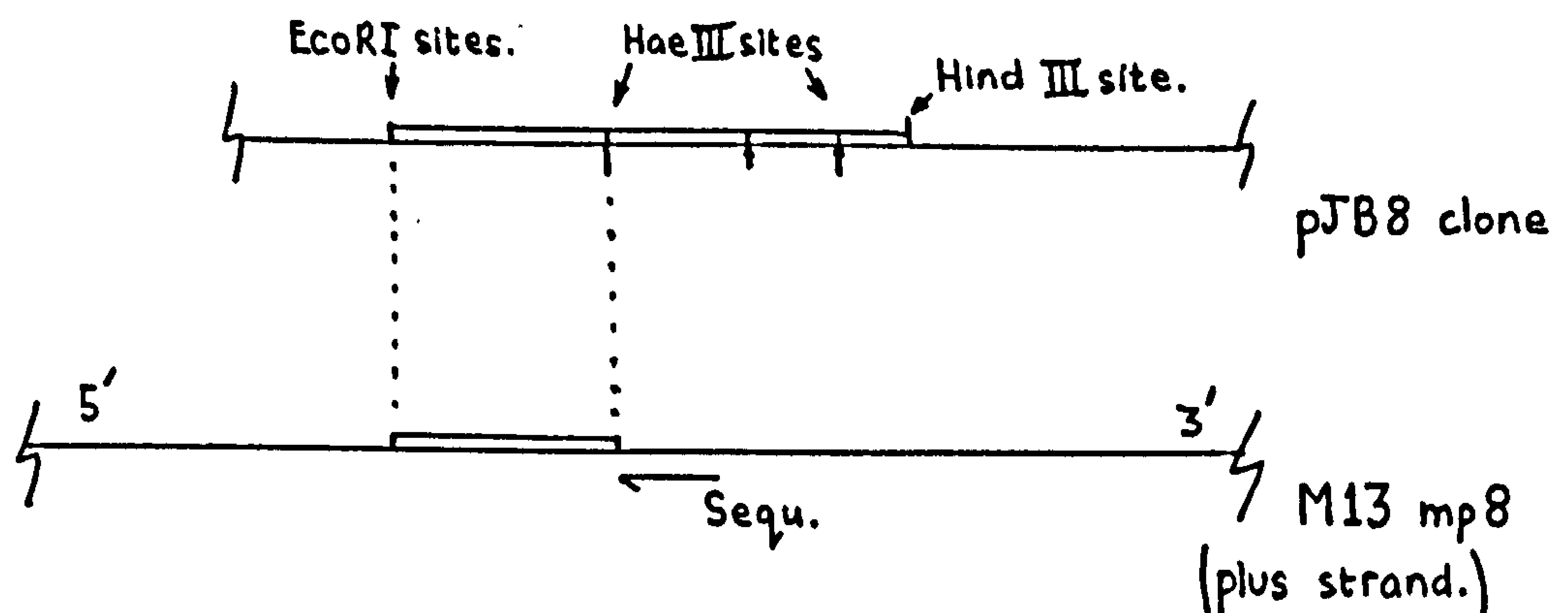
This indicates that apart from a short un-investigated region of about 150 bases, the H7 fragment appears to be composed of 31 base sequence repeats at its "terminal" (outside) end (relative to the original mtDNA molecule). The absolute conserved nature of the repeat sequence cannot be assured without further sequencing studies, but no great deviation from the quoted sequence is suspected. In the No. 76 autoradiogram, no obvious change in the repeating pattern is apparent beyond the 15th repeat. The sequence repeats are therefore envisaged as stretching from the terminus of the mtDNA molecule to a region at or about the first Hae III site (for the H7 terminus).

<u>The nature of the repeated sequence/regular sequence junction</u>

The Eco RI/Hae III fragment from the No. 76 clone insert was excised from the parent clone by digestion with Eco RI and

Hae III, and recovered from agarose gels. The recovered fragment was sub-cloned into an M13 mp 8 RF DNA vector (digested with Eco RI and Sma I) as described in the protocol for sub-cloning (see Page 61) using a two-step ligation.

In this orientation, the primer extension of the sequencing reaction will read through the Hae III site and into the insert, reading sequence from the Hae III site towards the Eco RI site. This orientation is opposite to that used in the sequencing of the terminal regions of the three terminal H7 clones, and will therefore be reading on the opposite (complementary) strand.



Single stranded DNA was prepared from suitable recombinant plaques and used as template DNA to sequence the Hae III end of the insert using the dideoxy-chain termination reaction with [α-^{35}S] dATP.αs as described in the sequencing protocol (see page 66).

The sequence from the Hae III end of the Eco RI/Hae III fragment in mp 8 was obtained from the sequence gel autoradiogram and was as given overleaf.

```
5'-GGCCAGTCGGAAGCTTGGCTGCAGGTCGA-
      vector  :      insert
  -CGGATCCCC | CCTTATTATTAATATTATTAATAAC-
  -ACATTATTACAAATGAAACTAAAGAGACACGAGGGTAGTAGATA-
                  -TGAAACTAAAGAGACACGAGGGTAGTAGATA-
                  -TGAAACTAAAGAGACACGAGGGTAGTAGATA-
                  -TGAAACTAAAGAGACACGAGGGTAGTAGATA-
                  -TGAAACTAAA,   etc.
```

The sequence is not readable with certainty beyond this point. A repeating unit of 31 bases is present. This repeat forms a recurring pattern in the upper reaches of the sequencing gel which is seen stretching to the limits of the autoradiogram (see Plate 8).

The repeated 31 base sequence is entirely complementary to that sequence obtained when sequencing from the opposite

Plate 8.

The photograph shows an autoradiogram taken from the sequencing studies on the Eco RI / Hae III fragment from No.76 clone, (terminal Hind III restriction fragment clone.) using the vector M 13 mp 8.

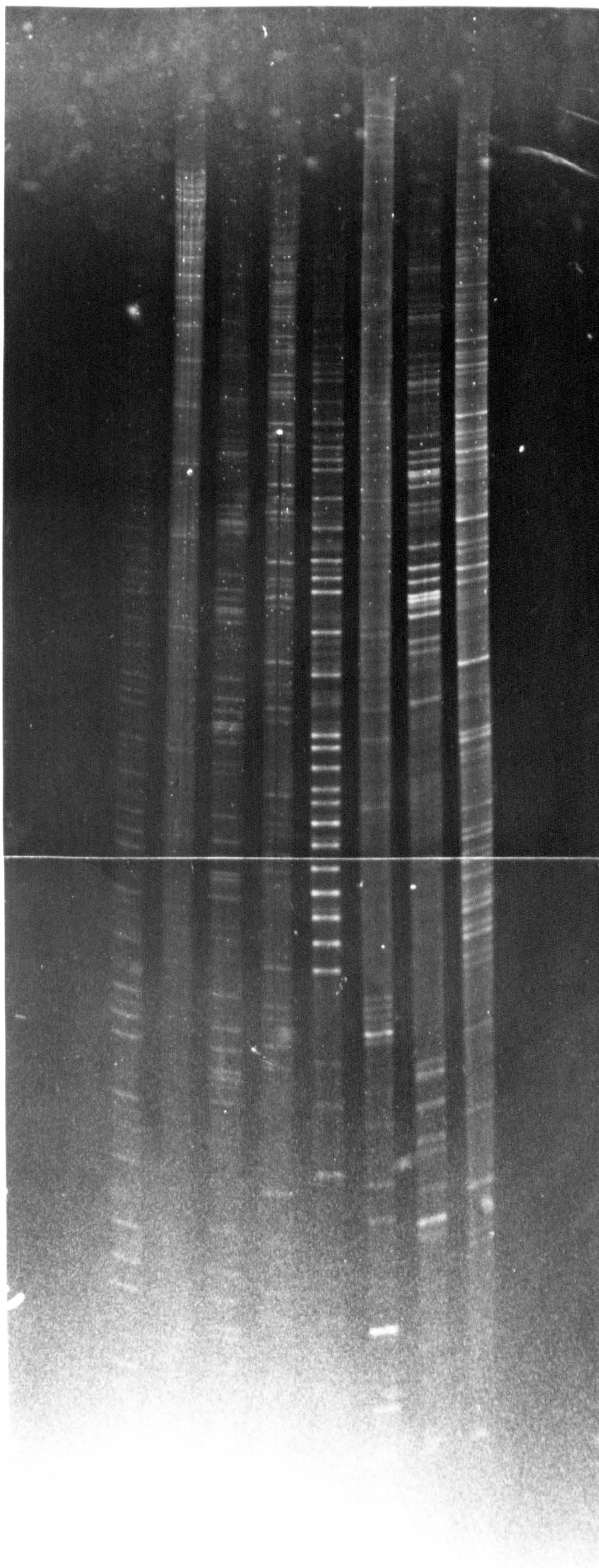
The following features are noteworthy...

i). The 31 bp repeating unit seen as a repeating pattern in the upper reaches of the sequencing gel.

ii). The stretch of A-T repeats following the Hae III site.

iii).The run of six consecutive 'C' residues in the 'C' reaction channel, confirming this clone as being the blunt-end ligationproduct of a Sma I-cut mp 8 vector ligated to a Hae III fragment.

Plate 8



end of the Eco RI/Hae III fragment. (Sequencing on the Eco RI/Hind III insert was on the opposite strand.)

```
         Eco RI end                                    Hind III end
mp 9 5′ ——ACTACCCTCGTGTCTCTTTAGTTTCATATCT————————————3′
mp 8 3′ ——TGATGGGAGCACAGAGAAATCAAAGTATAGA————————————5′
         Eco RI end                                    Hae III end
```

This data confirms the earlier statement as to the point of commencement of the sequence repeats being close to the Hae III site.

Since no copy of the Hae III recognition site is present in the repeated sequence, the Hae III site is considered to represent part of the discrete, non-terminal regions of the mtDNA molecule. The sequence repeats will now be regarded as that part of the molecule being "terminal" in character.

It is felt that no worthwhile statement can be made as to the possible functions of the A-T region lying between the sequence repeats and the Hae III site. (The base composition of the *Tetrahymena* mtDNA molecule is 75% (A + T).) For such a short stretch of DNA, any discussion would not be meaningful.

Cloning experiments with Bal 31 exonuclease

It was decided that the region of the cloned H7 terminal fragment lying between the "outermost" (relative to the whole mtDNA molecule) Hae III site and the Sau 3A site was of interest. This region contains the end of the 21s rRNA gene (see mapping end hybridization of 21s rRNA in the characterisation of the three terminal clones, page 130). This end of the 21s rRNA gene is not thought to be adjacent to the Hae III site. It was proposed to attempt to sequence this region of the clone

(a) To investigate the nature of the sequence between the end of the 21s rRNA gene and the outermost Hae III site.

(b) To screen this sequence for copies of the repeat sequence which is present at the original terminus of the cloned H7 fragment.

Cloning rationale. It was proposed to use the enzyme Bal 31 exonuclease to produce a set of sub-clones which varied in length, with their degraded "ends" mapping over the region of interest in the clone. By sequencing from these "ends" inwards into the clone a sequence of 2-300 bases would be obtained from each sub-clone. In this way the region would be sequenced using the sub-clones.

Procedure

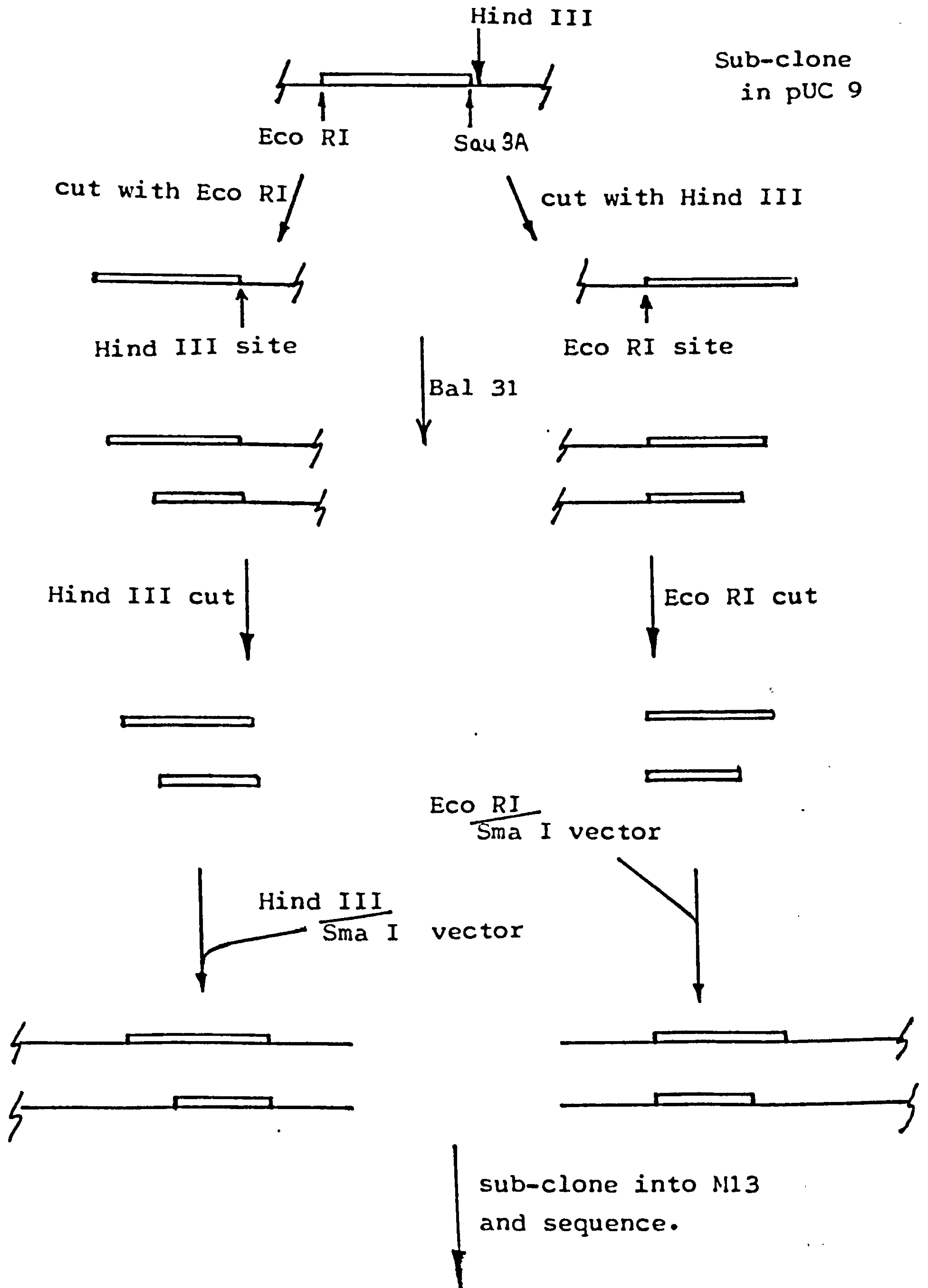
Sub-cloning the region of interest

The Eco RI/Sau 3A fragment of interest was excised from the parent clone by sequential digestion with Sau 3A and Eco RI (the enzymes require different buffers, see page 34) and recovered from agarose gels. This recovered fragment was sub-cloned into a Bam H1/Eco RI cut vector of "pUC 9" using the methods described in the sub-cloning protocol (see Page 61) with a cohesive-end ligation.

Fig 18.

The figure opposite shows a flow diagram for the Bal 31 sub-cloning experiments. The protocols for the various restriction enzyme and Bal 31 digestions, and the sub-cloning of DNA fragments are detailed under those relevant headings in the methods and techniques section. This experiment is discussed on p 146.

Fig 18. Scheme for producing a series of sub-clones of a cloned insert using Bal 31 exonuclease.



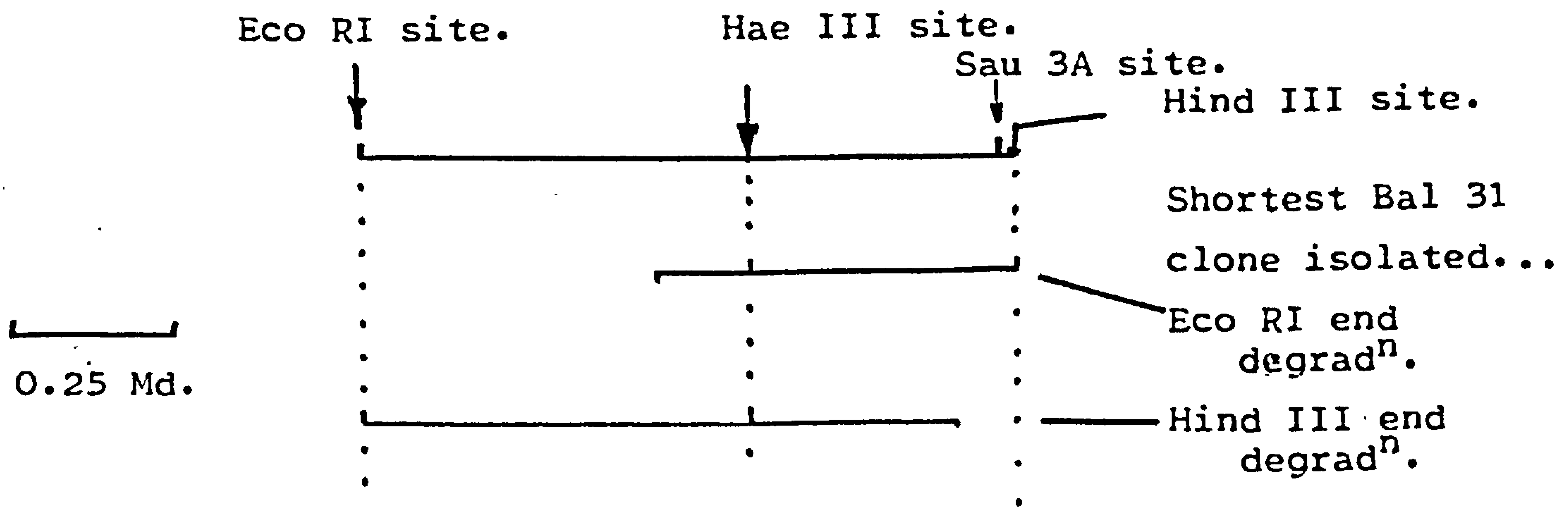
The size of the recombinant inserts were found by digesting recombinant plasmid DNAs with Eco RI and Hind III.

To obtain "Bal 31" clones whose degraded ends varied in position over the region of interest, starting at the other end of the Sau 3A/sub-cloned fragment, the role of the two enzymes Eco RI and Hind III was reversed. (If successful, this second set of clones could be used to confirm some of the sequence determinations from the first set of Bal 31 clones by reading the complementary strand.)

This cloning rationale is shown diagramatically in Fig. 18.

Results

Although the Bal 31 digestion conditions and ligation conditions were considered identical for the two sets of "Bal 31" cloning experiments, a difference in the size range of the sub-clones isolated was seen.



The sub-clones obtained after degrading the molecule from the Hind III site (Sau 3A end) were not usefully degraded to a sufficient degree.

The sub-clones obtained after degrading the molecule from the Eco RI site were much more extreme in their size range,

but did not reach the area of interest.

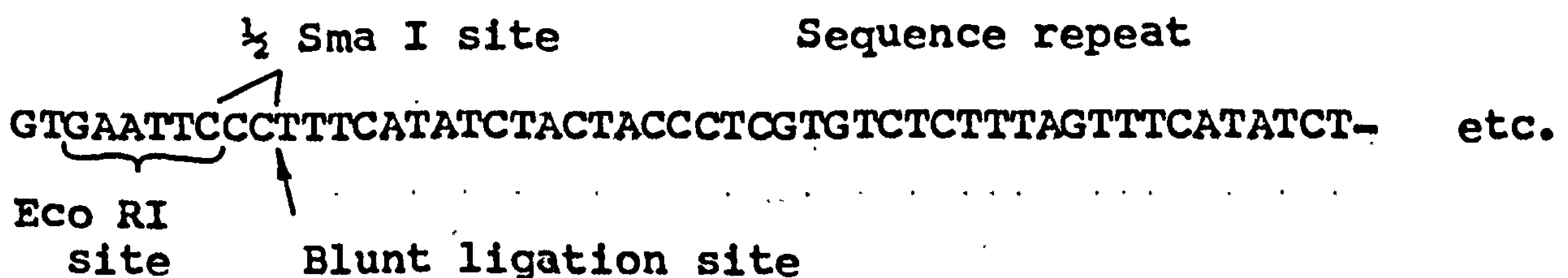
Varying the conditions of the Bal 31 treatment gave no improvement in the range of clones obtained from either experiment.

The shortest clone obtained from degrading the Eco RI end of the insert was seen to be a similar size (over this region) to the clone No. 67 insert.

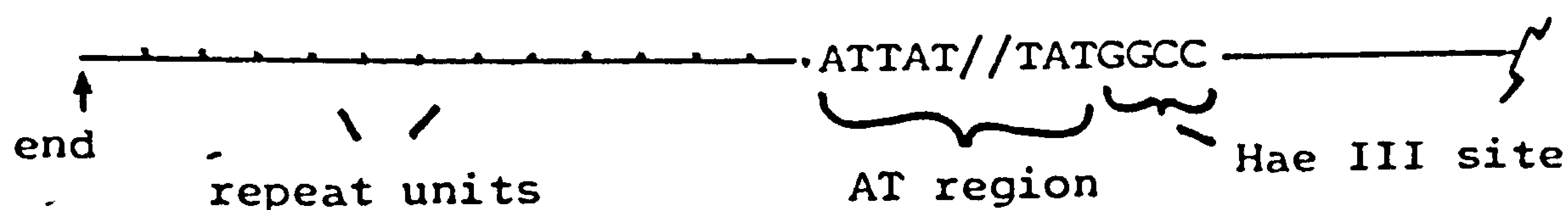
This Bal 31 clone was sub-cloned into M13 mp 9 using the Eco RI and Hind III sites. Vector preparation is given on Page 51, the sub-cloning protocol is given on Page 61.

Single stranded 'phage DNA was prepared from identified recombinant M13 phage and used as the template DNA to obtain sequence data from the inserts' Eco RI end (adjacent to the degraded end). The dideoxy-chain termination reactions used [α-^{35}S].dATP.αS as described in the sequencing protocol on Page

A 31-base sequence repeat identical to that seen in the sequencing of the original terminal regions of the cloned H7 fragment was seen in this Bal 31 clone. The blunt-end ligation point in the degraded molecule was at the 2nd 'T' triplet in the sequence:



This evidence supports the suggestion made earlier that the terminal sequence repeats stretch from the A-T region near the outermost Hae III site to the terminus of the molecule.

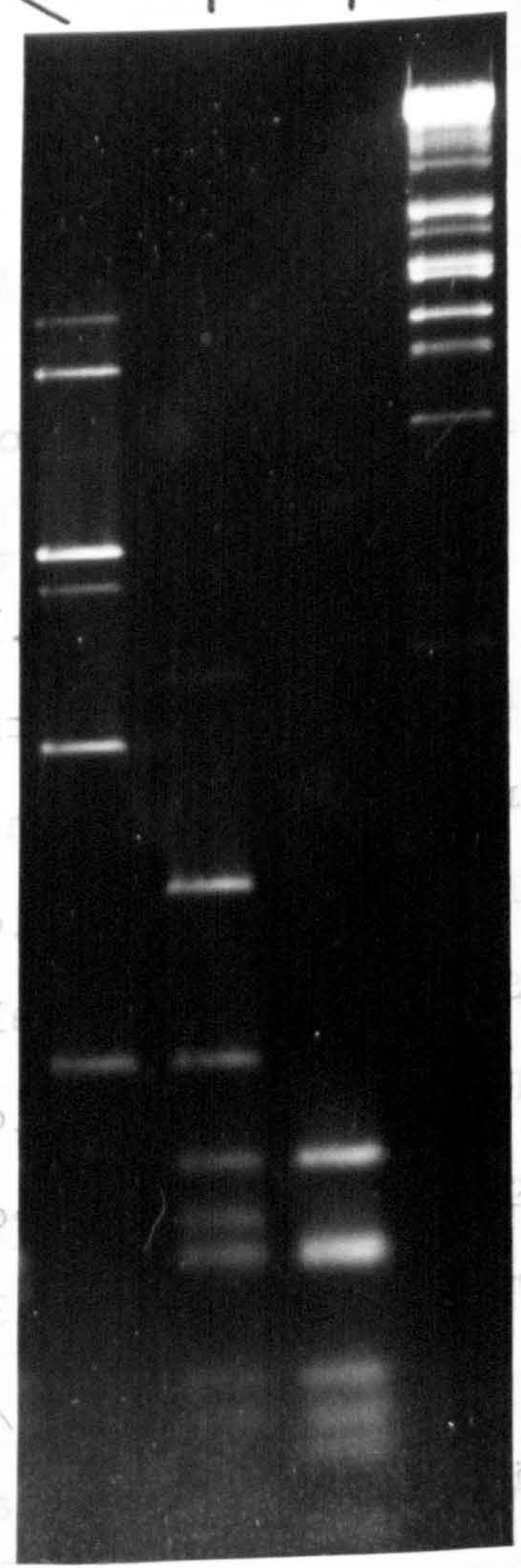


Further attempts to isolate specific terminal restriction fragment clones using S_1 nuclease

Purified str. T mtDNA was treated with S_1 nuclease (see Page 53). This treatment has been used by other workers to resolve the terminal structures which may be present on linear DNA molecules, prior to cloning. (Emery & Weiner, 1981). The S_1 treated material was digested with Eco RI and the R1 and R3 terminal restriction fragments were recovered from agarose gels using the electro-elution technique (see Page 46). The recovered R1 and R3 fragments were Hind III digested.

The blunt end/Hind III vector used was prepared by digesting pUC 9 with Sma I and Hind III. Vector and insert DNAs were ligated together at a 1:1 molar ratio of number of T mtDNA fragments present:Number of vector molecules present, at 30 μg/ml total DNA concentration under the conditions described for two-step ligations.

Ligation mixtures were used to transform competent ED 8767 cells, which were plated on L-agar plus ampicillin as described on Page 59. Recombinants were screened for their adherence to



The photograph shows an Eco RI/Hind III digest pattern of the recombinant clone isolated after cloning using the S1 nuclease treatment. (see p 152). The second photograph shows the same clone digested with Eco RI/Hind III/HaeIII.

the description of a terminal restriction fragment clone, as given in the section describing the cloning of the H7 fragment (see Page 127).

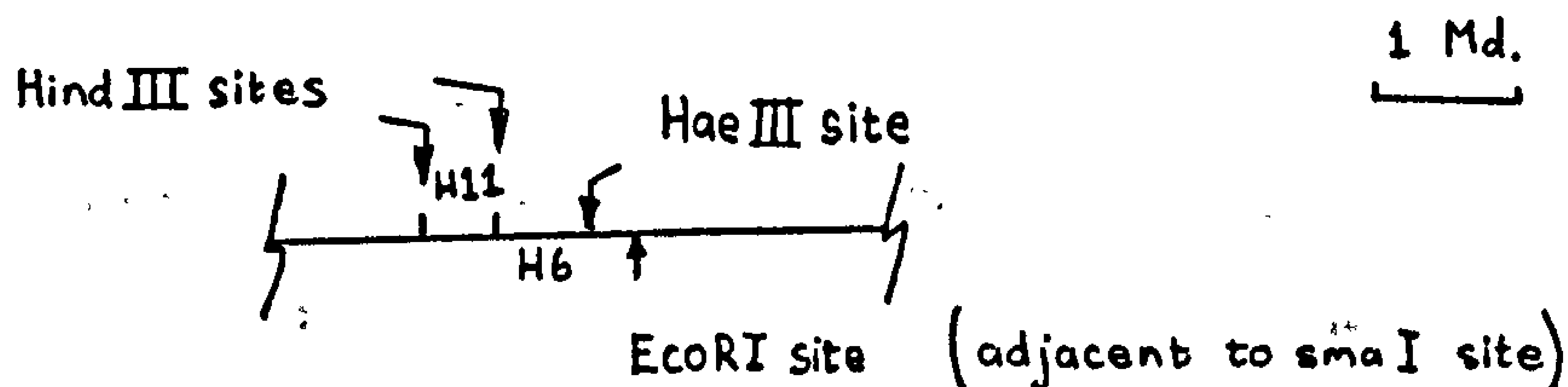
Two recombinants were isolated from these experiments which showed characteristics consistent with the stated parameters. Both these recombinants originated from the recovered R1 fragment experiment.

Further characterisation of the "S_1" recombinant clones

Upon digestion of the recombinant plasmid DNAs with Eco RI and Hind III one recombinant showed itself to possess two inserts. Single enzyme digests with the two enzymes mentioned showed that one of the fragments was the H 11 fragment from str. T mtDNA. (see opposite.)

It is supposed that the insert cloned into the vector was a partial digest product from the Hind III digest of the str. T mtDNA, and was composed of H11 and the terminal fragment H6 still attached (uncut).

Multiple digests of this clone with Eco RI, Hind III and Hae III showed a Hae III site to be present in the large insert purporting to be H6.

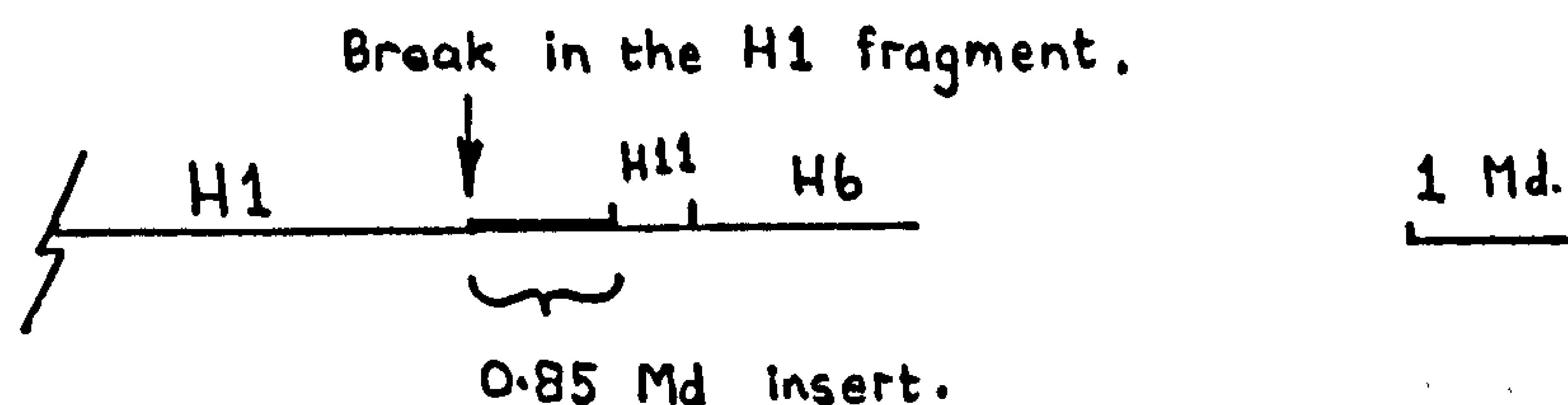


This mapping of the insert DNA agrees with the digest products predicted by the map of Norton (1980) for str. T mtDNA. The Eco RI/Hind III/Hae III digest products were transferred to a nitrocellulose filter using the Southern Blot technique and incubated with isotope-labelled str. T 21s rRNA as described

on Page 57 .

The H11 fragment and the Hind III/Hae III fragments both gave a hybridization signal with the 21 s rRNA. The small "original terminus" fragment did not. The size of this cloned copy of the H6 fragment implied that the clone was not large enough to contain the regions of the molecule suspected to display the length heterogeneity (from the map by Norton, 1980).

The second S_1 clone isolated showed no digestion of the insert DNA with Hae III. This result disqualifies it from being a H6 recombinant. The presence of the intact Hind III site and a 21s rRNA hybridizing region, as required by the selection parameters, suggests its origin is as follows:



The broken end of a damaged piece of the right-hand end of the H1 fragment could have been ligated into the Blunt end/Hind III vector.

Sequence studies on the Eco RI/Hae III fragment of the H6 terminal fragment clone

The H6 terminal clone was digested with Eco RI and Hae III and the small Eco RI/Hae III fragment from the insert DNA recovered from agarose gels using the freeze-squeeze technique. The recovered fragment was sub-cloned into an Eco RI/Sma I cut vector of M13 mp9 RF DNA as described in the sub-cloning protocol, using a two-step ligation.

Single-stranded 'phage DNA was prepared from identified recombinants and used as the template DNA to obtain sequence

from the insert DNA, using the dideoxy-chain termination reaction with [α-^{35}S].dATP.αS as described in the sequencing protocol on Page 66.

Nature of the sequence from the original terminus of the H6 terminal restriction fragment clone

No repeating unit of any form was seen in the sequence of the original terminus (Eco RI end of the Eco RI/Hae III fragment) of the H6 clone. No copy of the 31 base repeating sequence seen at the other terminus of the molecule (H7 clone) was present in the first 400 bases.

The mapping of this clone indicates that no portion of the 21s rRNA gene is contained in this region of the clone.

No A-T region was seen in the sequence.

These results emphasize the dissimilarity of the two sub-terminal regions of the str. T mtDNA molecule seen in the different mapping of the Hae III and Hind III sites in the map of Norton (1980).

As time was pressing, and since this region of the molecule contained nothing of obvious interest to this project, no further attempts were made to clarify or confirm the sequence.

Other rationales used in attempting to clone the terminal restriction fragments of str. T mtDNA

Direct cloning. Attempts were made to clone the str. T mtDNA Hind III terminal restriction fragments using a blunt end (Bam H1 cut, filled)/Hind III cut vector of pJB8, without "filling" the target str. T mtDNA prior to cutting with Hind III.

These experiments were run concurrently with experiments in which the target DNA was filled and from which useful recombinants were isolated. In all respects except the filling reaction, the two concurrent experiments were identical. No recombinants containing the desired fragments were seen.

Homopolymer tailing. Whole str. T mtDNA was "filled", using the Klenow polymerase, then "C-tailed", using the terminal deoxynucleotide transferase reaction as described in the enzyme protocol on Page 53. Approximately 20 "C" residues were attached, per end to the mtDNA molecule. This "C-tailed" mtDNA molecule was then digested with Hind III.

The vector molecule was plasmid pUC9, digested with Pst I, and "G-tailed", using the terminal deoxynucleotide transferase reaction as described in the enzyme protocol on Page 52. Approximately 20 "G" residues were attached, per end to the linearised plasmid vector. This "G-tailed" vector was then digested with Hind III.

Vector and target DNAs were annealed together following the scheme of Clarke & Carbon (1975). This annealed mixture was then ligated at a total DNA concentration of ~10 μg/ml under the conditions described for cohesive termini. No recombinants were isolated which adhered to the stated

parameters describing a terminal Hind III restriction fragment (see Page 127).

Attempts to clone the entire mtDNA molecule.

See "Experiments with cosmid vector systems".

Experiments with cosmid vector systems

In vitro packaging extracts for packaging cosmid vector ligation products into infective lambda particles were prepared from the Barbara Hohn *E. coli* strains BHB 2688 and BHB 2690 using a modification of the procedure of Sternberg *et al.* (1977) (modified by V. Pirrotta). A "freeze-thaw lysate" was prepared using strain BHB 2688, and a sonicated extract was prepared from strain BHB 2690. The DNA in question was packaged using these extracts in a putrescine/spermidine buffer. Packaged DNAs were then infected into *E. coli* str. ED 8767 following the procedure of B. Hohn (1979).

(a) To attempt to clone a large portion of the T mtDNA molecule

Str. T mtDNA was partially-digested with Hind III and ligated with Hind III cut cosmid vector pJC 79 (Hohn & Collins, 1980) at high DNA concentrations under the conditions defined for cohesive termini. The ligation mixture was packaged and infected into ED 8767 cells. These were plated on L-agar plus ampicillin as described on Page 59 . (pJC 79 possesses the ampicillin resistance gene, the tetracycline resistance gene and the Col El derived replication origin from pBR 322, see Hohn & Collins, 1980.) A few apparent recombinants were isolated, all large plasmids. When digested with Hind III, all the plasmid DNAs showed only vector DNA present. It was concluded that either the ligation conditions favoured the

ligating together of vector molecules, or that any recombinants formed with large pieces of the mtDNA molecule were non-permissible in the E. coli ED 8767 host or the in vitro packaging system.

(b) To attempt to clone the entire str. T mtDNA molecule

Whole str. T mtDNA was "filled" using the Klenow polymerase and "C"-tailed as described on Page 53, using terminal deoxynucleotide transferase. Approximately 20 "C" residues were attached per end, pJC 79 was digested with Pst I and "G"-tailed with approximately 20 "G" residues per end. The two DNAs were annealed together following the scheme of Clarke & Carbon (1975), and packaged into lambda particles. These were used to infect ED 8767 cells which were plated as described in the above experiment (see Experiment (a) with Cosmids). No transformants were seen.

In both the experiments described using cosmid vectors, control packaging reactions with lambda DNA gave efficiencies of packaging of $\sim 3 \times 10^7$ plaques per µg of DNA.

D I S C U S S I O N

Studies on Tetrahymena furgasoni str. W/ATCC mtDNA.

General discussion

Restriction site mapping of the str. W/ATCC mtDNA molecule

Many methods have been described for determining a restriction site map for a DNA molecule (see Smith & Nathans, 1975; and Methods in Enzymology, Vol. 65, 1980). Many of these methods use techniques which would be either impractical or economically very costly when applied to the analysis of a DNA molecule the size of Tetrahymena mtDNA. For these reasons the techniques used in the laboratory at the time for mapping these Tetrahymena mtDNA molecules were those of "multi-enzyme digests" and "partial digest product analysis" (see Danna, 1980). Both these techniques rely upon the estimation of the size of the restriction fragments in question.

Estimation of the size of restriction fragments. These were estimated by comparing the electrophoretic mobility of the fragment(s) in question with that of λDNA restriction fragments. The standard values for the size of the λ fragments were those of Schroeder et al. (1981). These are a set of optimised figures which place all the plots on the curve when plotting mobility vs. log molecular weight. (Slight deviations from the curve do occur if non-optimised figures are used, due to local base-composition variation in the λDNA fragments.) It has been reported that A+T rich DNA fragments have lower electrophoretic mobility than G+C rich fragments (Sanders et al. 1975). In this respect the quoted estimations for the size of W/ATCC restriction fragments (~25% G+C) may be a little high, due to the comparison with λDNA fragments, which are about 50% G+C.

Size of restriction fragments used when mapping the mtDNA molecule

To allow for localised base composition variation the estimated size of the restriction fragments were "adjusted" ±~5% when mapping the mtDNA molecule.

Ordering of the restriction fragments from Tetrahymena furgasoni str. W/ATCC mtDNA. The inherent slight imprecision in the estimation of fragment size, and the large number of fragments produced by the enzymes mentioned weighed against the use of partial digest studies. The large number of small fragments which mapped close together, e.g. from Bcl I and Hind III digests, and the number of large fragments of similar size, e.g. Eco RI and Hind III digests, produced considerable ambiguities.

Double enzyme digest information was used to establish basic relationships between restriction sites. The regions between these "primary mapping points", were defined by recovering fragments and digesting with other enzymes. The large degree of recovery and redigestion has therefore confirmed much of the map implying that it is internally consistent with only the absolute size of the restriction fragments in question.

Mapping of the terminal restriction fragments. The restriction fragments which displayed a heterogeneity in length were assumed to originate from the termini of the molecule. (The molecule was assumed to be linear in structure.) In previous studies which mapped the mtDNA molecules from other Tetrahymena strains these fragments showing length heterogeneity were proven to originate from the ends of the molecule.

The Tetrahymena furgasoni mtDNA molecule was assumed to be a linear molecule on the following grounds:

Restriction enzyme digest products indicate a molecule of 32 Md plus is present. (see Fig.5, p80). The mtDNA sedimented at 34s on sucrose gradients, as seen in other Tetrahymena strains. A molecule of 32 Md plus, sedimenting at 34s would conform to a model of a linear molecule.

It was therefore assumed that the str. W/ATCC mtDNA molecule complied with the model for the structure of the *Tetrahymena* mtDNA molecule seen in other strains, and that the restriction fragments displaying a heterogeneity in length originated from the termini of the molecule.

Mapping the rRNA genes on the Tetrahymena furgasoni str. W/ATCC mtDNA molecule

The position of the 14s rRNA gene on the map of W/ATCC mtDNA closely resembles that of other mapped strains of Tetrahymena, in being approximately 6-7 Md distant from the nearest 21s rRNA gene.

The position of the 21s rRNA genes on the map of W/ATCC mtDNA show a diversity from the position of the 21s rRNA genes mapped on the mtDNA of other strains (Norton, 1980; Holt & Jones, umpublished results; Goldbach *et al*., 1978*b*).

The distance between the outermost 21s rRNA genes is about 27-28 Md. (~41.5 Kbp). This is greater than the separation observed in the mtDNA of other Tetrahymena strains.

Strain	Separation of 21s rRNA genes	Reference
T. pyriformis str. T	~24.5 Md (~37 Kbp)	(Norton, 1980)
T. pyriformis str. A	~25 Md (~37.8 Kbp)	(Holt & Jones, unpublished)
T. pyriformis str. E	~24.5 Md (~37 Kbp)	(Maycock, Holt & Jones, unpublished)

This increased separation of the 21s rRNA genes is concurrent with the increased size of W/ATCC mtDNA compared with these strains.

The small size of the internal 21s rRNA region suggests that it may be an incomplete 21s rRNA gene.

A possible explanation of these notable features of W/ATCC mtDNA could be that the ancestral W/ATCC mtDNA molecule was similar in size and structure to that of other Tetrahymena strains. At some point a stretch of DNA containing a fragment of the 21s rRNA gene, or some similar sequence, became inserted into the molecule resulting in the increased length of the molecule and the increased separation of the external 21s rRNA genes. Alternatively, the internal 21s rRNA region may have successfully been deleted in other Tetrahymena strains, a process not yet completed in W/ATCC mtDNA.

Comparison of the mtDNAs from *T. furgasoni* str. W/ATCC and *T. pyriformis* str. GL/B

The restriction pattern of the Eco RI digest of str. W/ATCC mtDNA appears to be the same as that obtained by Goldbach *et al.* (1977) for the Eco RI digest of *T. pyriformis* str. GL/B mtDNA. Both strains also appear to possess three 21s rRNA genes. The mtDNA of *T. pyriformis* str. GL/B was mapped by Goldbach *et al.*, using electron microscopic techniques.

Differences noted between the two strains. The map of str. W/ATCC mtDNA places the twin 21s rRNA region remote from the 14s rRNA gene, and shows one of the genes to be possibly incomplete.

The model of the str. GL/B mtDNA molecule proposed by Goldbach *et al.*, places the twin 21s rRNA gene region adjacent to the 14s rRNA gene and shows the twin 21s rRNA genes to be of the same size.

The size of the mtDNA molecule in str. GL/B was estimated at 40-50 Md and sedimented at 36s (Goldbach *et al.*, 1977).

No molecules greater than 40 Md in size are estimated from the data on str. W/ATCC mtDNA, though the actual limits of the molecule are not definable. Str. W/ATCC mtDNA sedimented at 34s.

Without further data on both strains it is not possible to make any meaningful statement as to their relationship or similarities other than to say that they appear to be different.

Problems associated with the stock cultures of Tetrahymena available from the Culture Collections

At this point it is worthwhile to define the problems involved in attempting to compare the maps and information deduced for Tetrahymena mtDNA molecules, with the results of other groups.

Goldbach et al. (1977) characterised the mtDNAs of seven strains of Tetrahymena using Eco RI restriction digests and electron microscopy.

The Tetrahymena strains obtained by our laboratory show very little resemblance to those strains of the same name characterised by Goldbach et al., e.g. Str. 'GL' (our laboratory) resembles a str. 'A' from the same culture collection. As described, our str. W/ATCC gives a similar Eco RI digest pattern to the GL/B investigated by Goldbach, yet appears to map differently, etc.

This apparent lack of consistency between culture stocks has been observed by other workers looking at iso-enzyme distribution characteristics in Tetrahymena (Borden et al., 1973).

Either Tetrahymena is an extremely changeable and "plastic" organism, or the stock-keeping practices of the Culture Collections are rather inconsistent.

Cloning of the Hind III restriction fragments from str. W/ATCC mtDNA

The difficulties experienced in attempting to clone large restriction fragments from Tetrahymena mt DNA, were also experienced by other members of the laboratory. By contrast, large restriction fragments have been successfully cloned

from the mtDNA of the protozoan <u>Acanthamoeba</u> (Holt & Jones, personal communication) and from DNAs from other sources.

Although the ligation conditions were adjusted to those favouring the incorporation of large restriction fragments, these considerations were obviously not sufficient. However, other factors may be influencing the ligation reaction:

(a) The high A+T base composition of Tetrahymena mtDNA may affect the ligation due to partial melting of stretches of the DNA fragments in the ligation mixture.

(b) The mtDNA from <u>Tetrahymena</u> appears to possess numerous nicks or breaks along its length (Goldbach <u>et al</u>., 1979), and see nick-translation experiment. These may result in very few whole, undamaged molecules being present in the ligation mixture. (The larger the fragment, the greater the likelihood of it being damaged.)

(c) Insertion of the un-cloned mtDNA fragments, with whatever qualities they possess, into the tetracycline resistance gene of pAT 153 may result in a non-permissible gene fusion product, if read through from the tetracycline resistance gene promoter is possible. Alternatively it is possible that the un-cloned fragments may contain sequences which are themselves non-permissible in the <u>E. coli</u> host. This has been suggested to explain certain negative results in cloning problems experienced by other groups using <u>E. coli</u> hosts e.g. (Van de ploeg <u>et al</u>., 1982).

Studies on the termini of the Tetrahymena pyriformis mtDNA molecule

General Discussion

Difficulties involved in cloning terminal restriction fragments.

Examples are quoted in this discussion which describe the termini of linear genetic elements from a number of sources. Most of these studies have been done on material recovered directly from the organism, without the need to revert to cloning procedures. In these cases the quantity and quality of the material isolated was adequate due to the amplified nature of the linear genetic elements in question.

In the studies on the Physarum and Dictyostelium rDNAs, cloning of the termini was attempted. The material used for these cloning studies was also suitable for sequence studies on the naturally occurring 'nicks' in the termini, implying that the DNA was available in good condition and in quantity. Considering this availability and high quality of the material the number of useful recombinants recovered was low:

Dictyostelium rDNA terminal clones: 15 clones isolated.
Physarum rDNA terminal clones: 7 clones isolated.

Cloning of non-terminal restriction fragments would have given a greatly increased number of recombinants. It is therefore felt that the numerous step-wise processes used in cloning the termini of linear DNA molecules are responsible for the low number of recombinants isolated.

The difficulty involved in processing and cloning sufficient amounts of Tetrahymena mtDNA terminal restriction fragments is thought to be an extreme example of these problems. The suspected fragmentary nature of the Tetrahymena mtDNA, and

the difficulty in amassing it in quantity are thought to have contributed to the low number of recombinants isolated.

Discussion on the identity of the cloned terminal restriction fragments

The combination of the size of the cloned fragments, their content of Hae III sites and the presence and location of a 21s rRNA hybridizing region identify them as being identical to the said regions at the termini of the mtDNA molecule. No part of the vector molecule conforms to these parameters. Both vector and insert DNAs were purified before use, with no other DNAs present in the ligation mixture.

Considering these factors the identity of the terminal clones appears to be validated to a high degree.

<u>Validity of the identity of the sub-cloned M13 clone inserts</u>

The origin of the M13 recombinant inserts, sub-cloned for use in sequence determinations, were all confirmed by re-digestion of the RF DNAs of the clones. Only full sized insert recombinants were used for sequencing studies.

The presence of recombinant sub-clones reduced in insert size raises the possibility that "internal" recombinations may have removed parts of the sequence in the sequenced sub-clones. Due to the resolving power of the gel electrophoresis system used, only very small deletions could have gone unnoticed (<100 bp).

There are three possible types of insert present, according to this argument, in the clones which were used for sequence determination:

(a) The inserts sequenced were not altered by recombination in the M13 vector.

(b) The inserts sequenced had been shortened by a small amount (<100 bp) in the M13 vector, where the lost fragment was of the same structure as the repeating sequence elements.

(c) The inserts sequenced had been shortened by a small amount (<100 bp) in the M13 vector, where the lost fragment was not of the same structure as the repeating elements.

If a copy of the repeated sequence had been lost, it would be of little consequence. The suspected mode of DNA replication does not rely upon the absolute number of repeats present, but upon there being sufficient copies present to

ensure a successful base-pairing (Goldbach _et al._, 1979).

If some other small structural element had been lost, the presence of such a short stretch of dissimilar sequence amongst such a large quantity of repeating sequences would not invalidate the evidence for the proposed replication completion mechanism.

Cloning of the H6 terminal restriction fragment

The only isolated recombinant thought to contain the H6 terminal restriction fragment from str. T mtDNA was thought to be too short to contain the terminal regions of the molecule which exhibit the heterogeneity in length. Sequencing studies on this clone support this view.

Since this recombinant was isolated from an experiment involving the use of an S_1 nuclease, it cannot be ignored that this treatment may have degraded the molecule in a manner which was not detectable upon gel electrophoresis of the treated mtDNA.

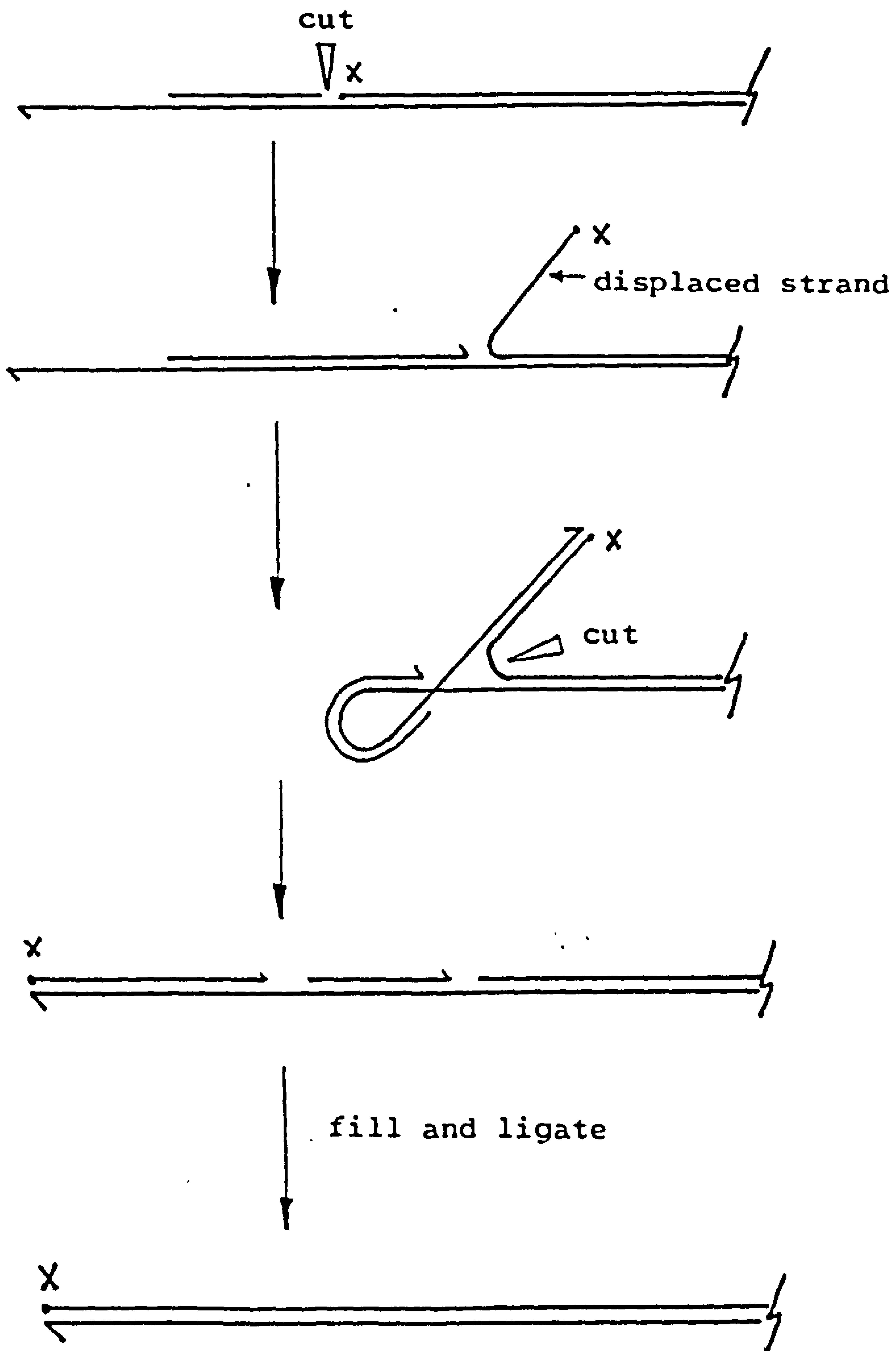
It is felt that further attempts to clone the terminal regions of this and other _Tetrahymena_ mtDNA molecules should avoid the use of degradative processes in resolving the termini of the molecule, and should favour the "filling" and repair type reactions to ensure the conservation of the terminal structure.

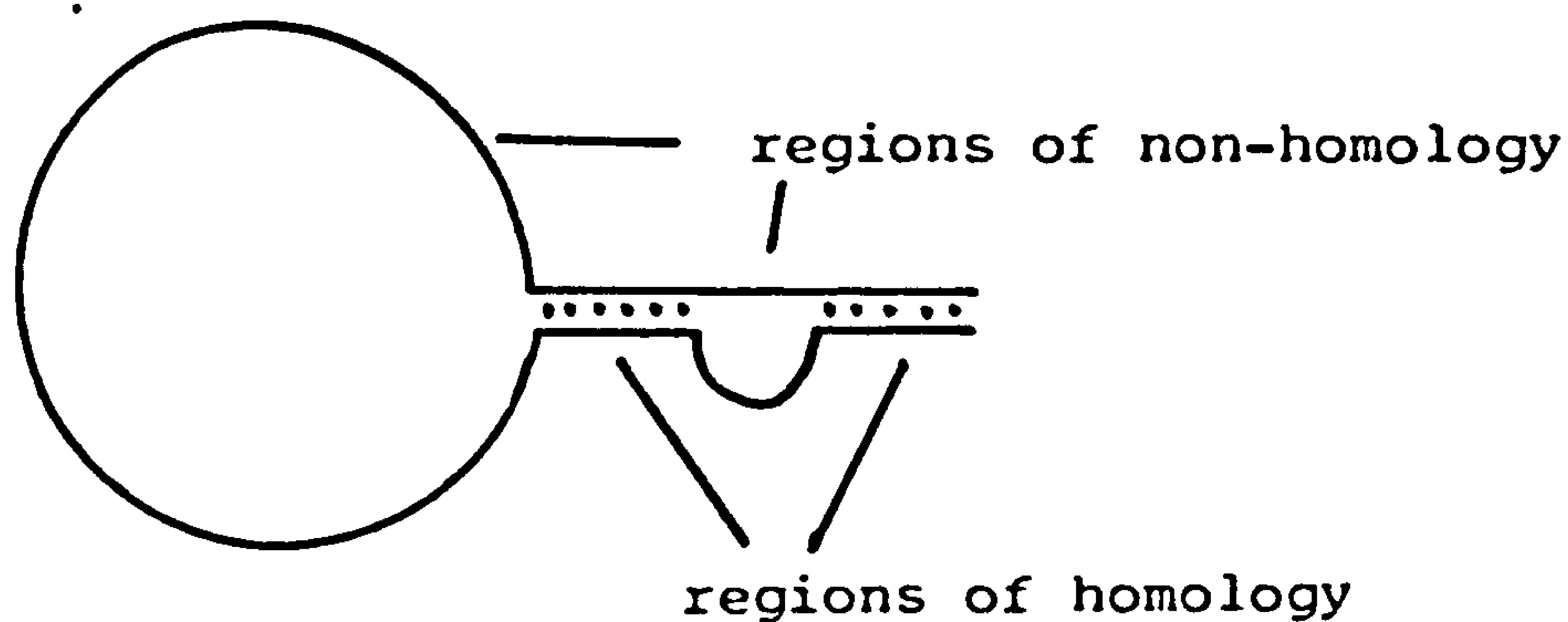
Dissimilarity of the terminal regions of the str. T mtDNA molecule

The dissimilarity of the Hae III sites in the terminal restriction fragments H6 and H7 emphasizes a dissimilarity between the ends of the str. T mtDNA first suspected from

Fig 19. <u>A possible mechanism for replication completion in Tetrahymena mtDNA.</u> (Goldbach *et al.*, (1979))

The mechanism relies upon the assumption that numerous tandem repeat units exist in the terminal regions of the molecule. It can be seen from this diagram that the point 'X' at which the 'pairing' strand is nicked and displaced forms the new terminus of the molecule.


incomplete molecule
cut
x
x
displaced strand
x
cut
x
fill and ligate
X
completed terminus.



When Tetrahymena mtDNA molecules were denatured and allowed to renature under certain conditions, "pan - handle" structures formed due to the existence of inverted repeat structures at the termini of the molecule. (Goldbach *et al*., 1977.) These "pan-handles were imperfect with a region of non-homology lying between two regions of homology.

This may indicate a region of dissimilarity between the ends of the molecule, lying between two duplicated regions at the ends of the molecule.

electron micrographs of renatured mtDNA molecules (Goldbach *et al*., 1977). The dissimilarity appears to be located between two regions of high homology located *near* the end and *at* the end of the molecule.

The size and position of the sub-terminal region of homology conforms to being the 21s rRNA genes. The homology of the terminal regions of the renatured mtDNA molecules indicates that similar structures are probably present at each end of the molecule.

This point obviously requires confirmation.

Comparison of the structure of the mtDNA terminus with the requirements of a proposed mechanism for replication completion of the mtDNA molecule

A mechanism for the completion of the replication cycle for *T. pyriformis* mtDNA was proposed by Goldbach *et al*., (1979). This mechanism relied upon the postulated existence of short tandem repeats at the termini of the molecule. The suggested mechanism is shown diagramatically in Figs. 19 and 20.

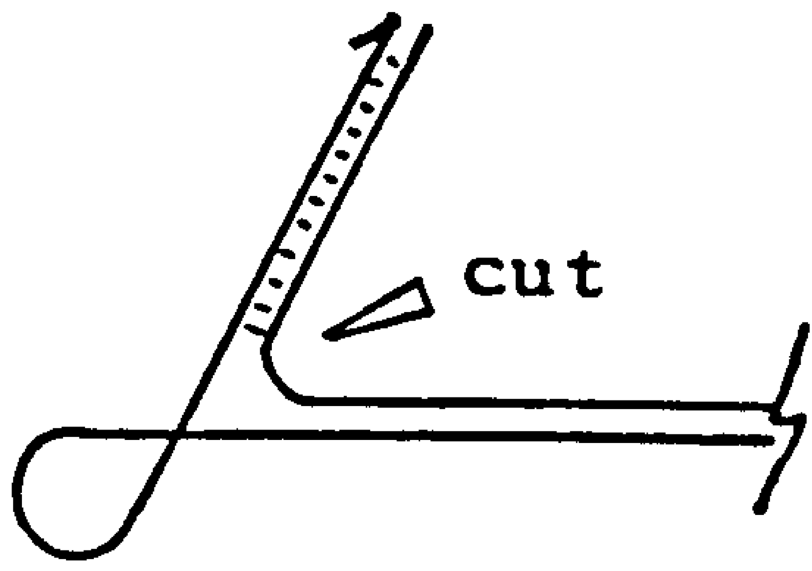
A repeating unit of 31 bases has been seen in the cloned restriction fragments from the termini of the mtDNA molecule. Their existence in tandem array supports the proposed model for the replication completion of the mtDNA molecule. The ease of digestion of the termini of the str. T mtDNA molecule with Bal 31 exonuclease suggests that the ends of the molecule are "open ended", lacking terminal crosslinks or hairpin structures. This model of the terminus of the mtDNA molecule is in keeping with the model proposed by Goldbach *et al*. (1979).

Generation of terminal length heterogeneity. The Goldbach mechanism (1979) went on to propose that the observed heterogeneity

Fig 20.

A possible mechanism for the generation of length heterogeneity in the terminal regions of the Tetrahymena mtDNA molecule. (Goldbach et al .,(1979)).

The replication completion mechanism relies upon the existence of tandemly repeated sequence elements being present in the terminal regions of the molecule. It seems reasonable to suppose that the numerous repeat sequence elements could align in a number of different combinations, producing a continually varying population of terminal lengths.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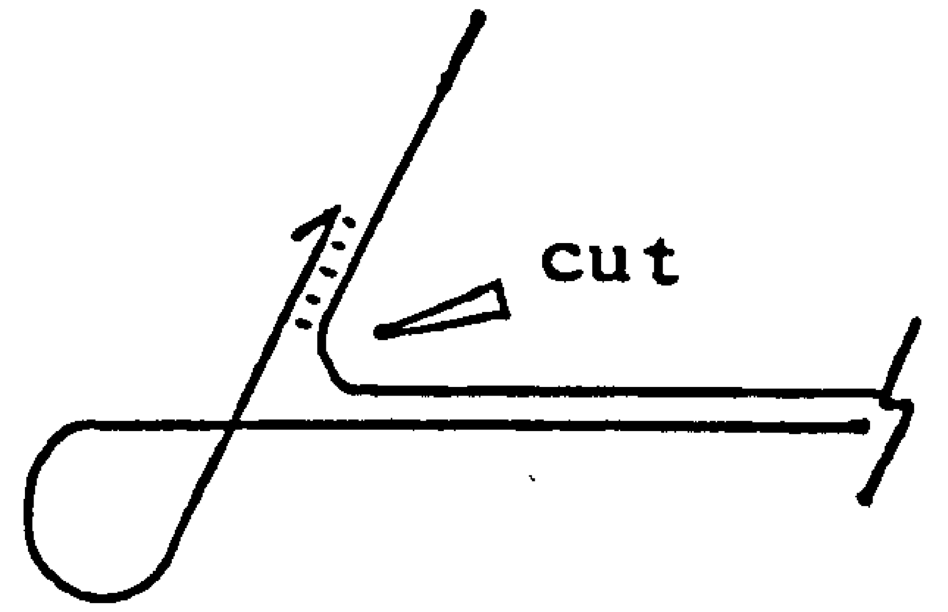
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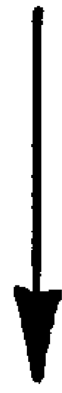
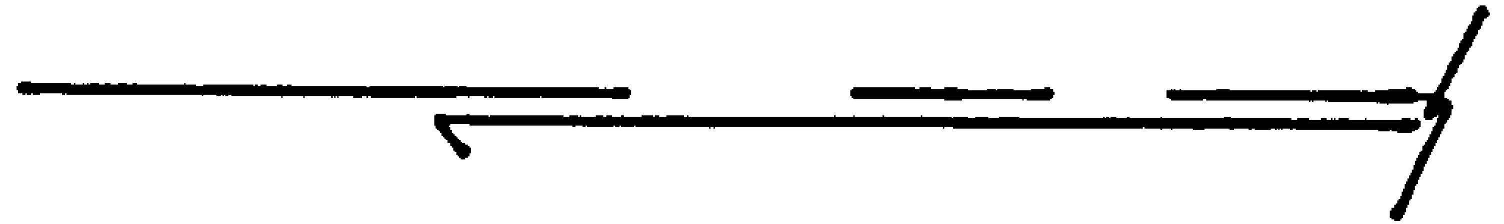
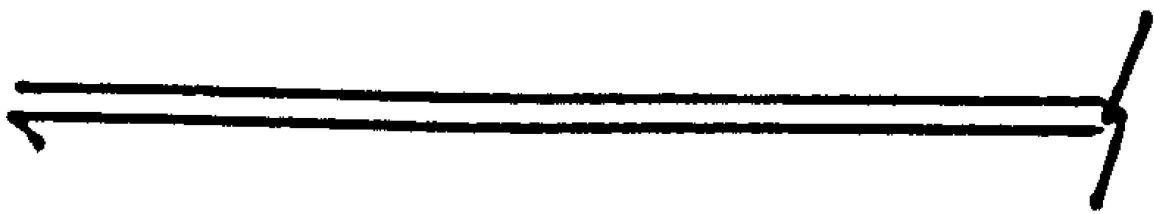
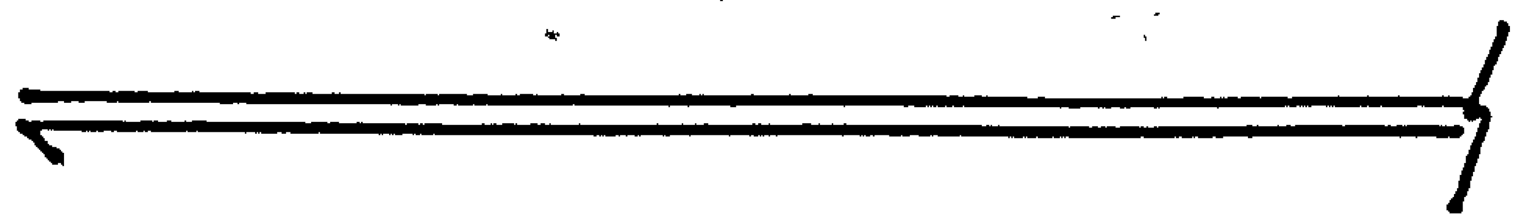








in length of the terminal restriction fragments from Tetrahymena mtDNA could be due to a variation in the number of copies of the tandemly repeated structure present on the termini of the molecule. The three terminal clones isolated were identified as copies of the same terminal restriction fragment. Restriction digests and rRNA hybridization studies revealed the difference in size to be associated with a difference in the size of the original terminal regions of the fragment. In the two clones compared, their difference is accounted for by a difference in the number of copies of the 31 base repeat present on the original terminal end of the restriction fragment.

Nature of the sequence at the "original terminus" of the H7 terminal restriction fragments

In all three clones isolated, which conformed to copies of the H7 fragment from str. T mtDNA, the repeat sequence began at the same point in the repeated sequence. If these three clones are a representative sample of the H7 terminal restriction fragment molecules, then the actual terminus of the mtDNA molecule must be clearly defined.

The only question which casts doubt on this idea is whether the three clones are a representative sample of the population of terminal restriction fragment molecules.

Has the cloning procedure been selective?

The Goldbach model for replication completion suggested that three possible types of structure could result from a completed cycle of the mechanism:

(i) A flush-ended end to the molecule;

(ii) A 3′ extended end to the molecule;

(iii) A 5′ extended end to the molecule.

Using the described 'filling' reaction to prepare the target str. T mtDNA for cloning, only the flush-ended and 5′ extended structures would be cloned. In this way, if all three structures were present, only two of them would be cloned. The Goldbach mechanism for mtDNA replication completion does suggest that its purpose is to effectively turn 3′ extended molecules into 5′ extended molecules (which can then be completed by "continuous" DNA synthesis in a 5′→3′ manner), so the non-cloneability of the 3′ extended molecules may not be a problem, as they would be effectively "intermediates" in the replication completion cycle and not a final product.

Discussion of the Goldbach replication mechanism, and the nature of the terminus of the molecule

Figure 19 shows the Goldbach model for replication completion of the *Tetrahymena* mtDNA molecule. It can be seen from this diagram that the actual 5′ strand terminus of the molecule is specified by the cut 'X' which initiates the production of the displaced strand.

The identical nature of the point of commencement of the terminus of the cloned mtDNA termini, within the repeat sequence element (all three terminal clones possess the same base at their original terminal end) implies that this 'X' cut occurs at a sequence specific point in the repeated sequence. This may serve to constantly regenerate the terminus of the molecule.

It is concluded that the sequencing data obtained from the three terminal clones of the H7 fragment from str. T mtDNA, supports the mechanism proposed by Goldbach *et al*. (1979) for the replication completion of the *Tetrahymena* mtDNA

molecule, and further suggests that the single strand breaks seen in the proposed mechanism may be produced in a sequence specific manner.

Comparison of the structure of the Tetrahymena mtDNA terminus with the termini of other linear genetic elements

Few linear genetic elements have been characterised sufficiently to show the exact nature of their replication mechanisms. The best characterised are the viral linear DNA systems which show a variety of methods for replication completion (see Introduction and p.185 of this Discussion).

The structure of the termini of a number of eukaryotic linear DNA elements have been partially characterised. The following discussion will briefly describe these molecules and compare their structural characteristics with those seen in the *Tetrahymena* mtDNA terminus.

Notable features of the Tetrahymena mtDNA terminus

The terminus of the *Tetrahymena* mtDNA molecule possesses a number of copies of a 31 base repeating sequence arranged in tandem repeating units. The number of copies of these repeats varies from molecule to molecule giving rise to a heterogeneity in length of the molecules terminal regions. The actual terminus is thought to be 'open' in structure, lacking terminal cross-links. The actual terminus of the molecule may be specified by an event intrinsic to the replication mechanism involving a strand break at a sequence specific point.

Notable features of the Paramecium mtDNA terminus

The replication of *Paramecium* mtDNA has been characterised (see Introduction). The existence of a lariot-loop replication intermediate indicates that a terminal cross-link is present at that particular end of the molecule, which resolves the problem of replication completion. (No information is

available as to the replication completion at the other end of the *Paramecium* mtDNA molecule.)

Ciliate nuclear systems

Brief introduction

Ciliate protozoa such as *Tetrahymena* possess an unusual mode of gene expression and organisation of their "genetic material". Following the mating event (conjugation) the germinal nucleus replicates to produce a second nucleus. The germinal nucleus now remains effectively inactive. The new nucleus undergoes processing involving chromosome breakage, gene amplification and degradation of certain stretches of DNA to convert the required genetic information into numerous linear DNA structures. This processing involves the conversion of ~70% of the unique sequences in *Tetrahymena* into linear elements contained in the now transcriptionally active "macronucleus". (For an account of *Tetrahymena* biology, biochemistry and physiology see Hill, (1972).) The processing of nuclear DNA is seen to an extreme degree in a related ciliate class, the "hypotrichous ciliates". (For an account of ciliate taxonomy see Corliss (1979).) In these ciliates 95% of the unique sequences found in germinal "micronucleus" are destroyed in the processing events producing the macronucleus. The transcriptionally active DNA in these ciliates is now present as very short "gene-sized" segments of DNA.

During the vegetative growth of the ciliates, the linear DNA elements present in the macronucleus replicate autonomously, and therefore require special mechanisms to complete the replication of the termini of these molecules (Cech & Brehn, 1981).

rDNA systems

The genes coding for the ribosomal RNAs (rDNA) are amplified in a number of simple eukaryotic systems. In these simple eukaryotes the ribosomal RNA genes are found on extrachromosomal linear molecules, which are palindromic in structure. The ease of identification (by RNA hybridization), purification (distinct size of the linear molecule) and the increased concentration (amplified) of these stretches of DNA have encouraged their use as an easily acquired model system for looking at the termini of linear genetic elements.

Notable features of the Holotrichous ciliate rDNA termini

The holotrichous ciliates *Tetrahymena* and *Glaucoma* possess tandem repeats of the sequence $-C_4A_2-$ at the termini of their rDNA molecules (Blackburn & Gall, 1978; Katzen *et al.*, 1981). The number of repeats present on a particular molecule may vary between 20 and 70 copies, possibly explaining an observed heterogeneity in the length of the terminal regions of the rDNA molecule. Single stranded breaks or gaps are seen in the terminal regions of the molecule occurring at a specific point in the sequence (-C-A-↓-A-C). These breaks are resistant to ligation. A cross-link exists at the very terminus of the molecule.

The repeating C_4A_2 hexonucleotide is generated from a single macronuclear copy during macronuclear development, being added to the linear elements during processing. The single copy of the hexonucleotide is remote from the rDNA element in the micronuclear gene (King & Yao, 1982).

Stability of the Tetrahymena rDNA terminus in yeast

The terminal restriction fragment from the *Tetrahymena* rDNA has been ligated to a yeast chromosomal DNA digest and

introduced into yeast cells. Stable, linear plasmids were seen, comprising the Tetrahymena rDNA terminus and a yeast telomere (chromosome end) (Szostak & Blackburn, 1982). The survival and stability of the linear plasmid indicates that the essential characteristics for chromosome-end replication were present.

A clonal population showed that the rDNA/yeast "plasmid" generated a population of molecules displaying a heterogeneity in size of the linear "plasmids", due to a variation in the number of copies of the $-C_4A_2-$ repeat present on any one molecule. Single stranded interruptions were found at the same frequency as those seen in the Tetrahymena rDNA termini.

When a "synthetic" hairpin (terminal cross-link) molecule was introduced into yeast cells, it did not survive.

These results suggest a strong conservation of certain features of a "chromosome-end" between Tetrahymena and yeast.

Notable features of the cellular slime mould "Dictyostelium" rDNA termini

The termini of the rDNA molecule possesses a sub-terminal 29 bp repeat sequences, of 2½ copies present in a tandem duplication. (No variation in copy number.) A terminal repeat of 29 bp is also present, of the form $(C_nT)_m$, where n varies to give an irregular -C-T- sequence with a repeating unit of 29 bp. The number of copies of the repeat may vary from molecule to molecule. (This point is uncertain since the enzyme S1 nuclease was used to resolve the termini of the molecule for cloning and may have degraded the structure.) A terminal crosslink is also suspected (Emery & Weiner, 1981).

Notable features of the acellular slime mould "Physarum"

rDNA termini

The rDNA molecule possesses between six and ten copies of a 140 bp inverted repeat sequence. These inverted repeats could form hairpin structures which are observed on electron micrographs as hairpin structures present at the termini of extrachromosomal DNA molecules. Single strand gaps are seen specifically at the sequence -CCCTA-, which is seen in most of the potentially hairpin-forming sequences. Completion of replication is thought to occur by recombination between hairpin structures of adjacent hairpins or rDNA molecules (Bergold *et al*., 1983).

Comparison with the Tetrahymena mtDNA terminus

The structure of the termini of the linear DNA molecules described vary considerably but appear to possess certain common basic features.

The presence of repeated sequence elements. Repeated copies of a sequence are seen in all the quoted examples. If a base-pairing event is required in the completion of replication of the molecules' end, as the Cavalier-Smith, Bateman & Goldbach models all suggest, the presence of more than one copy of the pairing sequence will increase the likelihood of a successful base-pairing event. In the case of the rDNA termini from *Physarum*, the presence of a number of the terminal hairpin structures will increase the likelihood of a successful recombination.

Variation in the number of copies of the repeated sequence element present. This property is seen in all the examples quoted except *Paramecium* mtDNA. The variation in number of

copies of a sequence repeat may reflect two possible properties of terminal replication completion mechanisms:

(i) The necessitous generation of extra terminal sequences to augment loss due to damage of the end of the molecule.

(ii) The generation of a heterogeneosu population of size of the terminal regions due to an inherent feature of the replication completion mechanisms.

The presence of numerous copies of an identical repeat could result in numerous possible arrangements of a base-paired replication structure, as seen in the mechanisms quoted. In this way the mechanisms may be described as perhaps being "slack". The variation in the precise binding of one copy of the sequence with its complementary copy would also explain the apparent total conservation of these repeat sequences, without a single base change in any repeat. The only exception to this appears to be in the hairpin structures of the Physarum rDNA, though, as already described, these molecules complete their replication cycle by recombination events, not specific base-pairing events of different strands of the DNA molecules.

The regeneration of a variable copy-number, for the repeat sequence elements in the Tetrahymena rDNA terminus cloned in yeast, implies that such a "slack" mechanism is present, and that the production of a variable population is inherent in these molecules' mode of replication.

Presence of terminal crosslinks. The presence of terminal crosslinks or hairpins on many of these molecules agrees with the Bateman model for the structure of a chromosome end.

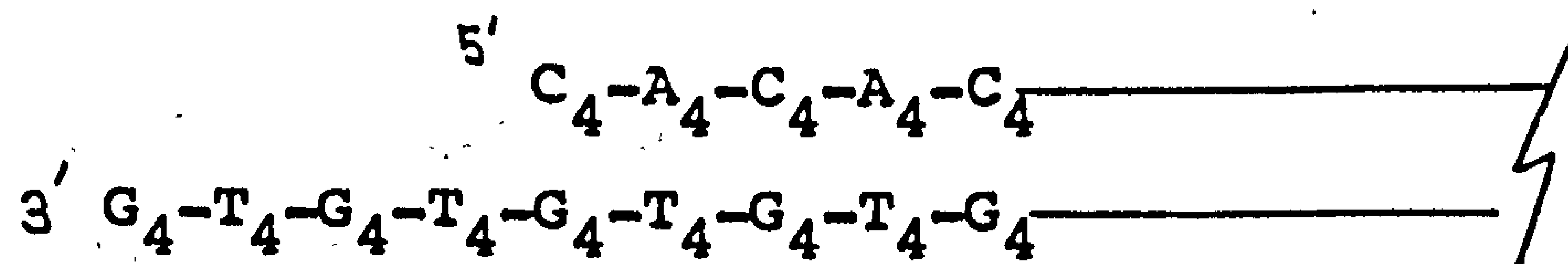
This model does suggest that the hairpin structure not only serves to complete replication of the molecule, but also to label the ends as being non-recombinogenic.

The *Tetrahymena* mtDNA molecule is not thought to contain a terminal crosslink. An alternative mechanism for the replication completion of this molecule has been proposed (Goldbach *et al.*, 1979) for which we now have some further evidence.

As to the recombinogenic labelling role of a hairpin structure, no information is available as to the occurrence or otherwise of such events in ciliate mitochondria. No meaningful statement can therefore be made. However, the *Tetrahymena* mtDNA molecule is not the only linear DNA structure which does not possess a terminal crosslink.

Notable features of the Hypotrichous ciliate rDNA

The termini of the rDNAs of four hypotrichous ciliates have been characterised. All four show a $-C_4A_2-$ repeating unit, with a 3′ stranded extension. No variation in the number of copies of the repeat are seen (Klobutcher *et al.*, 1981)



These terminal repeats have been shown to be added to the linear DNAs during macronucleus development, from a single copy of the $-C_4A_4-$ sequence found in the micronucleus (Boswell *et al.*, 1982). The small size of the macronuclear linear genetic elements in hypotrichs has permitted the characterisation of molecules other than the rDNA. These studies indicate that the described terminal features are present on most, if not all, of the macronuclear linear genetic elements in hypotrichous ciliates.

These hypotrichous ciliate linear DNA molecules therefore lack a terminal crosslink, and a lack of variation in the number of repeated sequence elements present. The lack of a terminal cross-link disqualifies the system from the Bateman model for replication completion.

The open-ended, 3′ extended nature of the termini could suggest a mechanism of replication similar to that proposed for Tetrahymena mtDNA. However, no copy of the $-C_4A_4-$ repeat sequence is seen in the micronuclear copy of the genes comprising the linear "gene-sized elements" (Boswell et al., 1982). A different mechanism for replication completion must therefore be active in these ciliates. That mechanism may not be simply deducible from a purely structural analysis of the terminus itself. Other possible factors may be important, such as in the mechanism proposed for the adenovirus DNA replication completion (see Introduction). Such a protein primer would not be detected by the DNA structure analysis studies done to date (Rekosh et al., 1977).

Essential features of the replication mechanism of the Herpes simplex virus

Although the Herpes simplex virus is not strictly a eukaryotic linear genetic element, it does replicate in the eukaryotic cell with certain notable features of interest to this discussion.

The viral molecule (DNA) replicates by a rolling circle mechanism. Genome-length molecules are cleaved off by a sequence specific endonuclease, producing a staggered break

in the polymer. The staggered break results in a cohesive terminus. These cohesive termini enable the viral DNA molecule to circularise prior to its own replication. The staggered breaks occur at a sequence specific point in a large tandemly repeated sequence. The number of copies of the sequence repeat appear to vary from molecule to molecule (Mocarski & Roizman, 1982).

General Conclusion

The structure of the terminus of the mtDNA from *Tetrahymena* has been partially characterised. The terminal structure shows strong resemblances to the termini of other linear genetic elements which have been characterised from a number of sources.

It is felt that this work may prove useful in furthering the understanding of the structure of "chromosome ends" in general and also in defining the essential requirements for the replication of these structures.

Proposals for further work on the mtDNA of Tetrahymena

These proposals can be usefully divided into two headings:

(a) Investigations into the structure of the termini of the Tetrahymena mtDNA molecules

(i) The H6 terminal restriction fragment from str. T mtDNA requires cloning in its entirety to confirm the existence of sequence repeats at both ends of the molecule.

(ii) Further clones of the H7 terminal restriction fragment could be isolated in order to confirm the nature of the actual terminus of the molecule as specified by a sequence specific cut.

(iii) The structure of the termini of other Tetrahymena mtDNA molecules could be investigated with an aim to comparing the structures of the termini.

(iv) It might prove interesting to construct a hybrid linear molecule, as in the yeast telomere/Tetrahymena rDNA terminus, to see whether the mtDNA terminus is compatible with nuclear linear replication completion systems.

(b) Investigations into the structure of the mtDNA of Tetrahymena, as an organelle genome

If the recombinant clones of the yeast mt genes used to map other mtDNAs could be obtained, the nature of the, as yet, uncharacterised mtDNA molecule could be deduced. Apart from the obvious usefulness in comparing the gene content and organisation of the Tetrahymena mt genome with other species, this work might shed some light upon the evolution of the Tetrahymena mtDNA from strain to strain, and possibly implicate the origins of the mtDNA molecule which shows only a passing resemblance to animal and fungal mtDNAs.

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