

PROTEINS SYNTHESISED BY CLONED DNA
ISOLATED FROM THE GENETICALLY
'SILENT' ter C REGION OF E. COLI K12.

by

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ABSTRACT.

A region of the E.coli K-12 chromosome has been cloned by the method of chromosome "walking". In total about 38kb of DNA, or almost 1% of the genome, has been isolated upon recombinant plasmid molecules.

The cloned DNA spans the 31 min region of the E.coli genetic map, in which only one genetic marker, trg, has been previously identified. This section of the chromosome lies within a much larger region encompassing the DNA replication terminus and contains very few mapped genes.

The proteins synthesised by recombinant plasmids were investigated using two semi in vitro methods of protein synthesis. DNA isolated from this genetically "silent" region showed that the region was not gene sparse and carries active genes which code for several polypeptides of a large range of molecular weights, accounting for approximately 50% of the coding capacity of the region.

A physical map of the cloned region is presented; and polypeptides synthesised by the region are roughly located upon this map.

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INTRODUCTION.

CHAPTER 1.

1.1 THE E.COLI CHROMOSOME: ITS GENE POPULATION AND THEIR DISTRIBUTION.

The chromosome of E.coli K12 is a circular DNA molecule which, according to the latest edition of the genetic linkage map (Bachmann, 1983) is approximately 4,600 kilobases in length. On this map the location of 1100 genes has been determined. Although the total number of genes encoded by the E.coli chromosome is not known, estimates of the genetic capacity of E.coli have been obtained from the size of its genomic DNA, protein content, RNA composition and consideration of gene distribution.

By taking the average molecular weight of an E.coli protein to be about 40,000 daltons (O'Farrell, 1975) and assuming an average amino acid molecular weight of 110 daltons, it can be calculated that a DNA molecule the size of the E.coli chromosome could code for over 4,000 individual proteins. The protein content of whole cell homogenates of E.coli has been separated by high resolution two dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975). Over 1100 polypeptides were resolved by this technique (O'Farrell, 1975; O'Farrell et al, 1977) and possibly a total of 1500 individual proteins are produced by E.coli cells grown under various conditions such as, different media and temperatures (Bloch et al, 1980; Neidhardt et al, 1983).

Other studies involving growth of E.coli under anaerobic conditions did not result in an increase of the number of polypeptides which are resolved by the O'Farrell gel system, but did show an alteration in the relative amounts of some gene products, a few of which were

identified biochemically (Smith & Neidhardt, 1983). Pulse labelling of size fractionated asynchronous or synchronously growing cells, using radioactive methionine, indicated that E.coli lacked any detectable periodically synthesised proteins (Lutkenhaus et al, 1979). At least 750 proteins, which were resolved in O'Farrell two dimensional gels, are synthesised continuously throughout the cell cycle. Although, these workers do point out that due to technical limitations of the O'Farrell system, periodically synthesised proteins may have escaped detection.

Thermal renaturation studies have been used to investigate the amount of genetic information carried by the E.coli chromosome and transcribed into messenger RNA (mRNA) (Hahn et al, 1977). These workers calculated that the E.coli genome contains sufficient information to code for about 2,300 non-identical mRNA species and essentially all of the equivalent of one strand of the DNA is transcribed. The mRNA was characterised into three abundance classes. High abundance mRNA consisted of 240 species which correlated well with the number of the most abundant class of protein resolved by O'Farrell two dimensional gels. This class contains proteins that are found at 10% to 0.05% of total labelled protein. Hahn et al (1977), equate an intermediate class of mRNA of around 1300 species, with about 900 protein species present at 0.01% abundance or less, in two dimensional gels. A rare abundance class of around 700 mRNA species, if translated, would constitute much less than $1 \times 10^{-5}\%$ of the total protein and would not be resolved by the O'Farrell system. Fully repressed genes which occasionally escape repression would be in the latter abundance class (Hahn et al, 1977).

Hahn et al, (1977) accounting for the discrepancy between the mRNA and protein abundance of the intermediate class, suggested that a number of basic proteins may be missing, also, the average size of 1700 nucleotides for mRNA is possibly under-estimated or some other RNA species is present in E.coli. Concerning the rare abundance class, the presence of mRNA due to errors of transcription cannot be excluded (Lutkenhaus et al, 1979). All these factors serve to reduce the total of 2300 genes estimated by Hahn et al (1977). However, if the average molecular weight of an E.coli protein is around 40,000 daltons this would require a mRNA molecule of about 1200 nucleotides, resulting in an increase in the total number of genes to over 3000. This number is reduced by about 1000 if the rare abundance class of mRNA is ignored.

Failure to observe many more proteins produced by E.coli is unlikely to be due to the resolving power of the gels perse, as they could theoretically resolve 7000 proteins (O'Farrell). Furthermore, the probability of two individual proteins migrating to exactly the same position on an O'Farrell type gel and not be resolved, is very low, especially if double isotopic labelling is used, then this chance event is almost negligible (McKonkey, 1979).

In order to correlate the mapped genetic loci of E.coli with the proteins that are observed in two dimensional gels, a collaborative effort by many workers has set out to biochemically characterise as many as possible of the proteins resolved by the O'Farrell system and to assign each protein to a genetic locus (Bloch et al 1980; Phillips et al, 1980; Neidhardt et al, 1983). The most recent

publication presented the characterisation of 164 individual proteins as part of a gene-protein index of E.coli (Neidhardt et al, 1983). The gene protein index also contains a rough mapping of the structural genes of 800 proteins. These were expressed by plasmids carrying segments of the E.coli chromosome and located to a region by either complementation of a mutant gene, or, production of a known gene product. It is hoped that the entire chromosome will be mapped out in this way, while awaiting biochemical and genetical characterisation of each individual protein.

Phillips et al (1980) listed 136 individual proteins identified from O'Farrell two dimensional gels, and characterised these proteins according to their physiological function. This type of analysis was performed to indicate the number and nature of potentially identifiable proteins. In a similar approach but using broader based classifications, genetic loci were taken at random from three editions of the genetic linkage map of E.coli K12 (Bachmann et al, 1976; Bachmann & Low, 1980; Bachmann, 1983) and placed into the four following categories; Intermediate Metabolism, this includes genes involved in biosynthesis of amino acids, nucleotides, sugars and fatty acids, also, genes encoding amphibolic, anapleurotic and energy metabolism enzymes; Macromolecules and Proteins, genes involved in synthesis, processing, repair and modification of nucleic acids and proteins; Cell Envelope, in addition to membrane proteins and cell wall components, included also are cell division, transport, chemotaxis and motility genes; Unassigned, genes which could not readily be placed in any of the other categories. Table 1.1 summarises the results of this analysis.

TABLE 1.1.

YEAR OF EDITION.	INTERMEDIARY METABOLISM.		MACROMOLECULAR SYNTHESIS.		CELL ENVELOPE.		MISCELLANEOUS.	
	NO	% TOTAL	NO.	% TOTAL	NO.	% TOTAL	NO.	% TOTAL
1976.	315	51	157	25.5	109	17.7	35	6
1980	397	44.7	257	28.9	132	16.7	87	9.8
1983	445	44.5	271	27.1	190	19.0	93	9.3

Comparison of the genetic composition of three successive editions of the E.coli genetic map. The number of genes in each of the four categories (described in the text), and this number expressed as a percentage of the total number of genes surveyed is shown.

By far, the Intermediate Metabolism category contains the largest proportion of the known genes, just over half the total of the gene population of the 1976 edition of the linkage map and 45% in both the 1980 and 1983 editions. Genes which fell into the Macromolecules and Proteins category, increased from 25.5% to 29% of the total genes and then dropped to 27%, over the three successive editions of the genetic map. The initial increase in this case was due to the identification of a large number of genes coding for ribosomal proteins which made a significant contribution to the total number of genetic loci. However, from the 1983 edition of the map, only 14 new loci were added to the Macromolecules and Proteins category, out of 110 of the newly discovered genes.

On the basis of this analysis, it would appear as though most of the loci, which code for the types of genes that defined the Macromolecules and Proteins category, have been discovered. The same numerical argument applies to the Unassigned category, based on the finding that only 6 new genes from the 1983 map were placed in this class. Also, as information is gained in the individual genes of the Unassigned category, it may be expected that most of these genes will be absorbed into one of the other categories.

It is only the Cell Envelope category that has shown an increase in the share of the gene population of the 1983 map and is at its highest proportion, at 19%, over the three maps. From Table 1.1, it can be calculated that 42 out of 110 new genes from the 1983 map were placed into this category, compared with 39 out of 273 new genes on the 1980 map. It may be worthwhile to break down the Cell Envelope category into subclasses to determine if any particular type of genes has made a significant contribution to this increase.

What is likely to be the nature of potentially identifiable genes, based on the above analysis? Around 45% of the new genes may be expected to fall into the Intermediary Metabolism category. Very few will be placed into either the Macromolecules and Proteins or Unassigned categories. Cell Envelope category genes will continue to increase their share of the total gene population. The next edition of the genetic linkage map is awaited to confirm, or otherwise, these predicted trends in newly discovered genes.

Not only will it be interesting to discover how many and what types of genes are yet to be identified, but also, whether they are located within, or close to, regions on the chromosome that already possess genes, or if they will be found to lie in the genetically 'silent' regions (Bachmann et al, 1976).

The mapped genetic loci of E.coli K12 are not distributed randomly along the chromosome. A striking result is obtained when the distribution of genes upon the genetic map is expressed as a plot of the number of genes per unit map length (Bachmann et al, 1976). This reveals major peaks of high gene density which rise and fall sharply, indicating that these crowded regions are interspaced by relatively silent regions possessing few, if any, of the known loci.

Alternating regions of high and low gene density were found to be statistically significant for 606 genes (Bachmann et al, 1976).

From map positions of over 300 new loci added to a more recent linkage map (Bachmann & Low, 1980) it is noteworthy that the majority of the genes entered the major peaks in a plot of gene frequency (Newman, PhD. Thesis).

Alignment of the known homologous genes mapped upon the chromosome of E.coli K12 and Salmonella typhimurium results in about 86% of their genetic maps lying in register (Riley & Anilionis, 1978). This high degree of similarity between the closely related E.coli K12 and Salmonella typhimurium chromosomes, allowed Riley & Anilionis, (1978) to conduct a comparison of nonhomologous regions, from which they speculate upon several individual cases of gene duplication, deletion, insertion and transposition.

One interesting and relevant observation by these workers, is that nearly all of the inhomologies, whether present upon the E.coli K12 or the Salmonella typhimurium chromosomes, correlate remarkably well with genetically silent regions of the respective genetic maps. Furthermore, the largest silent region of each chromosome spans the DNA replication terminus, and an approximately 10 minute section of the genetic map from this region can only be aligned when it is completely inverted in one of the chromosomes (Riley & Anilionis, 1978).

In E.coli K12 Bachmann et al, (1976), pointed out that many of the genes for macromolecular synthesis are clustered according to their function and located in regions of high gene density. For instance, many of the genes responsible for ribosomal RNA, transfer RNA, elongation and termination factors, ribosomal proteins, RNA and DNA polymerases, cell division and envelope synthesis, are found in major gene frequency peaks at 72, 84, 89 and 2 mins. (Bachmann et al, 1976).

The phenomenon of alternating gene dense and silent regions has also been observed in Bacillus subtilis (Henner & Hock, 1980)

a bacterium genetically unrelated to the Enterobacteriaceae.

Although the number of loci that have been mapped for Bacillus subtilis is not as many as for E.coli K12, it appears that essential genes are also located in gene dense clusters upon the Bacillus subtilis chromosome.

Two specific features were found in good accord regarding the layout of the E.coli K12 versus the Bacillus subtilis genomes (Henner and Hock, 1980). Firstly, clusters of ribosomal protein and ribosomal RNA genes are located near the origin of replication in both species. Second, the largest silent region of Bacillus subtilis also resides close to the DNA replication terminus. Henner & Hock, (1980) suggest that the presence of genetically silent replication termini in both species, increases the likelihood that these regions play a role in chromosome organisation.

In E.coli K12 a gene density plot shows elements of symmetry centred close to the replication origin. An increased gene dosage effect, due to genes lying close to the origin being replicated before those further away, may have a bearing on this arrangement and certain gene duplications around the genome indicate that the chromosome may be divided into two functionally and topologically distinct half-genomes (Bachmann et al, 1976). Zipkas & Riley, 1975, and later evidence provided by Riley et al, (1978), noted either a 90° or 180° periodicity of pairs of gene loci, particularly those associated with glucose catabolism for which four symmetrically placed groups were found. This led these workers to speculate that two successive doublings of a primordial chromosome occurred during evolution of the E.coli K12 chromosome.

However, other groups of biochemically related genes and gene duplications were not found to be spacially related (Zipkas & Riley, 1975; Riley et al, 1978). In particular, many exact or near-exact gene duplications bear no relation to each others position in the chromosome (Charlier et al, 1979; Bachmann & Low, 1980; De Martelaere et al, 1981). In contrast the layout of known loci of Bacillus subtilis suggest that this genome does not have an axis of symmetry (Henner & Hock, 1980) . Also the major clusters possessing essential genes are distributed more evenly throughout the chromosome compared to E.coli K12.

Because of gene clustering, Bachmann et al (1976) suggest that there may be a topological relationship between genetically dense regions versus silent regions and the folded structure of the condensed nucleoid. It is imagined that gene dense regions, being physiologically more active, would be preferentially exposed to the cells transcriptional and translational machinery, whereas, the flanking silent regions would be buried in the interior of the nucleoid . There is no direct evidence to support this model, but, in the next section, we see that the E.coli chromosome probably does possess a degree of physical organisation.

1.2 PHYSICAL STRUCTURE OF THE CHROMOSOME.

In the previous section we saw that the multifarious gene population of the bacterial genome is not dispersed randomly along the DNA molecule. This led to suggestions that a relationship exists between the location of a gene upon the chromosome and the overall topology of the genome.

Although there is no direct evidence indicating that physically distinct regions of the chromosome are organised within the nucleoid, either to

allow preferential expression or to bring functionally related genes into proximity, the DNA is condensed into a uniformly sized structure (Worcel & Burgi, 1972).

The theme of an organised, or structured, genome is carried further in this section. It is easily imagined that the folded chromosome within the cell is arranged in an organised fashion. The DNA of one complete E.coli K12 chromosome is a circular molecule with a contour length of more than 1000m (Cairns, 1963). Within the bacterial cell the DNA is packaged into a condensed structure about 0.5 μ m in diameter and has been shown to have this form by fluorescence spectroscopy (Hecht et al, 1975) scanning electron microscopy and autoradiography (Woldringh & Nanninga, 1976). Somehow DNA replication and subsequent segregation of daughter chromosomes occurs efficiently and faithfully to ensure proper inheritance of the genetic material.

By gently lysing cells Worcel & Burgi (1972) obtained intact nucleoids and showed that uniformly shaped folded chromosomes could be isolated using sucrose density gradients. An effect on the sedimentation rate of isolated nucleoids was observed upon treatment with various reagents; ethidium bromide, this interchelates between base pairs of the DNA helix and progressively reverses negative supercoiling with increasing ethidium concentration; DNase, which attacks the DNA molecule itself; RNase, an enzyme specific for polyribonucleotides, another major nucleic acid of the cell and intimately associated with DNA. All these treatments resulted in an unfolding, to varying degrees, of the isolated nucleoids and due to increased drag through sucrose solution, lowered their sedimentation rates. This led Worcel & Burgi to suggest

that folded chromosomes were composed of an RNA core surrounded by DNA arranged into many independently negatively supercoiled domains. Supporting evidence for this model, with the inclusion of stabilising proteins, was found by other workers (Stonington & Pettijohn, 1971; Pettijohn & Hecht, 1973; Drlica & Worcel, 1975; Hecht et al, 1977).

Association of RNA with the nucleoid undoubtedly stabilises extracted folded chromosomes (Pettijohn & Hecht, 1973; Stonington & Pettijohn, 1971). Most, if not all, the DNA bound RNA molecules are nascent mRNA or tRNA species (Hecht & Pettijohn, 1976) and no enrichment for a unique class of chromosome-stabilising RNA was observed amongst the pool of heterologous RNA molecules extracted from isolated nucleoids.

Recently an investigation into the domain hypothesis of chromosome structure, produced evidence for the existence in vivo of independently supercoiled regions of DNA (Sinden & Pettijohn, 1981). They demonstrated the stable maintenance within cells of partially relaxed chromosomes, which is consistent with a domain model. A partially relaxed nucleoid consists of a mixture of unconstrained and supercoiled domains. Rifampicin treatment did not produce any difference in torsional tension of the nucleoid compared with untreated cells, thus ruling out dependence of nucleoid structure in vivo for nascent RNA molecules (Sinden & Pettijohn, 1981).

Whilst the nature of isolated nucleoids was being expounded upon, other workers, inspired by the "Worcel-Pettijohn" condensed chromosomes sought after histone-like proteins which could interact with DNA in an analogous manner as eukaryotic histones.

The chromatid (Kornberg, 1980) of higher organisms has been extensively studied and found to be organised by the binding of histones (Kornberg, 1977) into a chain of nucleosome beads connected by linker DNA (Kornberg & Klug, 1981). Nucleosomes of higher cell nuclei, as visualised by the electron microscope, appear as a beaded fibre 120Å in diameter and were originally imagined to be a repeating chain of 200 to 250bp bundles joined by short thin bridges (Oudet et al, 1975; Griffith, 1975; Kornberg, 1974). Griffith, 1976, extended this work to E.coli K-12 folded chromosomes and reported structures similar in size and appearance to condensed eukaryotic DNA. However, since a search to detect significant amounts of E.coli histone protein failed (Raaf & Bonner, 1968), and that one histone-like protein from E.coli K12 (Rouvier-Yaniv & Gros, 1975) was present in insufficient amounts to fully complex the E.coli K12 chromosome, Griffith, 1976, suggested the structural organisation of E.coli DNA may be due to a complex of Mg^{2+} , polyamines and perhaps a very heterogenous class of weakly bound proteins.

Currently there are at least three or four histone-like DNA binding proteins that have been identified in extracts of E.coli K12. These proteins generally resemble histones in their amino acid composition. They are abundant in the cells, heat stable (Geider & Hoffmann, 1981) and show strong evolutionary conservation of structure. This has been shown for one of the histone-like proteins, HU (Haselkorn & Rouviere-Yaniv, 1976) which immunologically cross-reacted with a similar sized DNA binding protein from a blue-green bacterium, cyanobacter.

HU protein, renamed DNA binding protein II (Geider & Hoffmann, 1981) is the best studied of the E.coli histone-like proteins and has been

isolated by other workers; NS protein has been shown to be HU protein from amino acid sequence data (Mende et al, 1978; Laine et al, 1980). The protein consists of two 90 amino acid long polypeptides that differ in 30% of amino acid residues. These two components HU and H_B (also called NS1 and NS2) exist as a 1:1 dimer in solution (Rouviere-Yaniv & Kjeldgaard, 1979). Also a 9kD protein, HLP II, was shown by Lathe (1980) to be identical to HU protein as well as the histone-like protein BH2, of Varshavsky et al. (1977).

Protein II is associated with the nucleoid in E.coli (Rouviere-Yaniv, 1977) and binds to the viral DNA of SV40, condensing it twofold and forming nucleosome-like structures showing characteristic binding in the electron microscope (Rouviere-Yaniv, 1977).

Another histone-like protein of E.coli, H₁ protein (Hubscher et al, 1980) behaves as a dimer of 28kD which can bind 75 residues of single or double stranded DNA. H₁ protein resembles histone H2A in amino acid composition and is neutralised by histone H2A antibodies.

A genetic locus for one histone-like protein, HLP I, has been located to 4 mins on the E.coli K-12 chromosome (Lathe et al, 1980). HLP I is the product of the fir A gene which codes for a 17kD protein (Lathe et al, 1980) this interacts with rifampicin resistant RNA polymerase molecules and suppresses partially or completely antibiotic resistance phenotype, Rif^r. Lathe et al, (1980) determined that another 17kD histone-like protein BH2 (Varshavsky et al. 1977) was not identical to HLP I.

Collectively all of the E.coli K-12 histone-like proteins could complex about half of the genome (Kornberg, 1982). However, even though so far the E.coli K12 histone-like protein is only found in half the abundance relative to DNA, as that of eukaryotic histones, this may be sufficient to stabilise a nucleosome structure (Kornberg, 1980).

A picture may be now unravelling of the E.coli K12 folded chromosome existing as a DNA molecule organised into several domains of supercoiling, Each domain possibly stabilised by proteins which have the ability to bind to and reduce the contour length of the DNA molecule forming a compact structure, in a fashion analogous to eukaryotic histones (Kornberg, 1980). Eukaryotic DNA associated with histones and contained in a chromatid is highly condensed. Transcriptionally active regions of the chromosome differ from the bulk chromatin in that they are altered in a highly specific manner. DNase I and blot hybridisation have shown the regions of active transcription to contain exposed sites that are preferentially cut. If a parallel is ~~drawn~~ between actively transcribed eukaryotic DNA and the gene dense, more active, regions of the E.coli genome, this adds weight to the Bachmann et al (1976) hypothesis of the relationship between genetically dense and silent regions. That is, the gene dense regions are placed at the surface the nucleoid freely available to cells transcriptional and translational machinery, whereas 'silent' regions are buried within the nucleoid, having a structural role in maintaining the organisation of the folded chromosome.

1:3 NUCLEOID-MEMBRANE ASSOCIATION.

In addition to the approach of molecular dissection of folded chromosomes described in the previous section, another way that workers have looked at nucleoids is to see if a relationship can be found between its overall structure and the general physiology of the cell. Since the absolute structure of folded chromosomes is unknown, one has to explore any interactions of the nucleoid with other cellular components in order to explain the apparent highly organised process of cell growth and division. Could gene dense and silent regions be parts of the genome which are more, or less, likely to be specifically involved with other cellular components?

As early as 1963 Jacob et al, as part of their replicon model, proposed specific DNA membrane attachments to ensure proper strand segregation of replicated chromosomes into their respective daughter-cells.

Various approaches have been tried to study the nature of interactions between chromosomal DNA and membrane proteins, as well as to dissect isolated folded chromosome - envelope complexes into individual components.

Stonnington & Pettijohn, 1971, found that folded chromosomes attached to cell envelope fragments could be isolated from gently lysed cells. Later radioactively pulse labelled DNA was found associated with the cell envelope isolated as part of folded chromosomes (Fuchs & Hanawalt, 1970; Parker & Glaser, 1974). Both the latter groups discussed membrane attachment of replication forks as being a likely explanation. But

Drlica et al, (1978) found pulse labelled DNA was not selectively bound to the cell envelope and criticised the methods employed by earlier workers as they produced fragmented DNA which could be free to associate non-specifically with the cell membrane.

Also Gómez-Eichelmann & Bastarrachea (1975) traced labelled DNA to both cytoplasmic and outer membranes, but reported that the finding was non-specific and may have occurred during the membrane isolation procedure.

Other workers, also, have attempted to purify and study E.coli DNA-envelope complexes (Worcel & Burgi, 1974; Heidrich & Olsen, 1975; Portalier & Worcel, 1975).

Doworsky & Schaechter (1973) studied the effect of ripampcin on the amount of DNA bound by membrane complexes, and in a further study by this group, Abe et al, (1977) digested DNA-membrane complexes with single strand specific endonucleases. Both treatments resulted in a significant reduction of the amount of DNA found associated with the cell envelope. These workers calculated that the chromosome possesses about twenty sites that are in some way different from the rest of the DNA molecule in their sensitivity to ripampcin or single-strand endonucleases. It is not known if these sites, determined by the two different methods, are the same, or whether the sites have fixed positions or not, on the chromosome. Drlica et al, (1978) concluded from their study of membrane associated DNA, that the folded chromosome is not a static structure and with the exception of the origin region, and possibly the replication terminus, they favour a strong but dynamic interaction of the folded chromosome with the cell membrane.

One of the main difficulties in studying nucleoid-membrane association is the relevance of isolated membrane complexes to the in vivo state of their components. Artifacts created during purification steps have complicated the interpretation of results. For instance, basic counterions in the isolation media, employed to extract nucleoids, can influence the results obtained (Craine & Rupert, 1978). High salt (Pettijohn et al, 1973) or low salt plus spermidine (Kornberg, 1974) produce different amounts of bound protein (Portalier & Worcel, 1976). Craine and Rupert, (1978), suggest that when lysozyme is used, a narrow range of ionic strength must also be used, to avoid lysozyme-induced non-specific binding of membrane proteins to DNA. Artifactual enrichment of DNA associated proteins may occur under certain physical conditions and could also be caused by the lysis procedure, during which preferential removal of susceptible proteins may happen (Meyer et al, 1976).

Association of the cell envelope with several parts of the chromosome thus remains an open question. However, one region of the E.coli genome, which carries the DNA replication origin, has been found tightly bound to DNA membrane complexes. A weight of evidence clearly indicates that the cell envelope is closely associated with the replication origin (Fielding & Fox, 1970; Parker & Glaser, 1977; Abe et al, 1977; Craine & Rupert, 1978; Nicolaidis & Holland, 1978; Wolf-Watz & Masters, 1979).

In Bacillus subtilis, DNA membrane complexes, enriched for DNA sequences from the replication origin and terminus region, have been highly purified (Sueoka & Quinn, 1968; O'Sullivan & Sueoka, 1972; Yamaguchi & Yoshikawa, 1975, 1977; Sueoka & Hammers, 1977; Winston & Sueoka, 1980). A high degree of purity has been obtained for the

E.coli origin membrane complex (Abe et al, 1977) and this carries, almost exclusively, a 26 megadalton section of the chromosome which spans the replication origin (Yamaki et al, 1980).

Wolf-Watz & Masters (1979) supplied evidence for specific binding of DNA to the outer membrane, and showed an enrichment for the replication origin within the membrane associated DNA. Outer membrane attachment of DNA sequences from the origin region was also found by other workers (Craine & Rupert, 1978; Nicolaidis & Holland, 1978; Nagai et al, 1980).

Wolf-Watz & Norquist (1979) studying purified outer membrane proteins, found one particular protein of 31K molecular weight, which seemed to be involved in specific binding of DNA to the outer membrane. In a further study, the 31K outer membrane protein was found at increased levels in strains lysogenic for lambda as phages, and harbouring plasmids carrying origin region DNA (Wolf-Watz & Masters, 1979). It was suggested that the 31K protein is synthesised within the origin region, possibly by the het gene, and that this protein mediates attachment of the replication origin to the outer membrane, accounting for an enrichment of origin DNA in membrane complexes.

Yamaki et al (1980) suggested that one or more proteins are involved in binding of origin DNA in membrane complexes, and this binding is highly specific for a 5.9 megadalton EcoRI fragment that carries the replication origin. Later, these workers narrowed down the binding region to a 460 base-pair stretch of DNA carrying the origin and that binding was mediated by two proteins of 55 K and 75K apparent molecular weight (Hendrickson et al, 1982). Wolf-Watz (1984) supported the finding that a 460 base-pair origin region was involved in outer membrane binding

and, in addition, located a second site within the 5.9 megadalton EcoRI fragment which mediates binding of the origin region to the outer membrane.

Jacq et al (1980) reports that a membrane derived DNA binding protein, B', with an apparent molecular weight of 60K, recognises two sites in or close to ori C. This protein binds to single strands of opposite polarity at the two sites. They suggest that protein B' is a good candidate to promote separation of daughter chromosomes by attachment of one of the DNA strands to a newly synthesised B' protein which is separated by membrane growth from the 'old' B' protein carrying the other daughter DNA strand. A role in initiation of bidirectional replication via binding of single stranded DNA is also suggested. It may be worth noting here that the dna G gene product, DNA primase, has a molecular weight also of 60K (Koningsberg & Godson, 1983) and is an enzyme which transcribes template DNA to produce RNA primer during DNA replication (Zechel et al, 1975).

There is little doubt from the literature cited that the replication origin of E.coli is closely associated with the outer membrane. Furthermore, it is only the origin region, and no other part of the chromosome, which strongly and specifically interacts with components of the cell envelope to form stable complexes.

The replication origin was shown by gene density plots (Bachmann et al, 1976) to lie in a gene dense region. The suggestion by these workers that gene dense regions may be located at the surface of the condensed nucleoid, would be particularly relevant to part of the chromosome which binds to the outer membrane of the cell. Especially

if, in accordance with the Jacob et al (1963) model, the origin-outer membrane binding played a role in segregating replicated chromosomes into daughter cells, as suggested by Wolf-Watz & Masters (1979), Wolf & Watz (1984) and Jacq et al (1980).

In other words, in order for the origin region to bind to the outer membrane and perform a partitioning function (Wolf-Watz, 1984), it would seem unlikely that it will be buried inside a condensed nucleoid. Particularly since Craine & Rupert (1978) using initiation mutants to synchronise DNA replication, have shown that origin binding to membrane complexes persists from initiation to termination of chromosome replication.

The regions in the chromosome where initiation and termination of DNA replication occur, are two chromosome landmarks which differ very much in their genetic layout, they are discussed in the next section.

1.4 INITIATION AND TERMINATION OF DNA

REPLICATION IS LOCATED IN TWO DISTINCT REGIONS

OF THE E.COLI CHROMOSOME.

Replication of the E.coli chromosome is initiated at the genetic locus ori C (Hiraga, 1976) and proceeds bidirectionally from this single origin (Masters & Broda, 1971; Bird et al 1972; Prescott & Kuempel 1972; Rodrigues et al 1973; Hohlfield & Vielmetter, 1973; Jonasson 1973). A map position of 83.5 min on the genetic map between the unc and asaA genes was determined for ori C (Von Meyenburg et al, 1977; Yasuda & Hirota 1977; Fayet & Louarn, 1978; Marsh, 1978).

The location of ori C in the chromosome places it in one of the most gene dense clusters of the genome (Bachmann et al, 1976). In a region extending 50 kilobases to the left and 10 kilobases to the right of ori C, there are about 30 known genetic loci along with so far about 5 coding regions identified either by DNA sequence analysis or by ability to direct synthesis of polypeptides of unknown function.

Masters (1977) found that genetic markers lying close to ori C were transduced by bacteriophage P1 particularly well. Generally, transduction of markers in gene dense regions proved to be of higher frequency than that of those in gene sparse regions. It was suggested that due to accessibility of these regions they might be favoured in the recombination process.

Accessibility of the origin region with respect to outer membrane binding was discussed in the previous section. It is not known what role, if any, membrane binding plays in initiation of replication.

If the origin region is located on the outside of the nucleoid and because this may make it an exposed piece of DNA, accessible to the cell's transcriptional and translational machinery (see end of section 1.2) it may be for this reason that important genes are clustered around ori C. For instance, ATP synthase is encoded by the unc genes adjacent to ori C.

The positioning of genes around ori C could also be due to a gene dosage effect (Bachmann et al, 1976). As newly initiated replication forks move away from the origin, genes close to ori C will be duplicated before genes lying further round the chromosome. Thus it may be

advantageous for some types of genes to be located close to ori C. It is probably significant, therefore, that most of the ribosomal genes are located in two clusters near to ori C (Bachmann & Low, 1980), a situation which is also found in Bacillus subtilis (Henner & Hock, 1980).

In order to study the origin region more closely, particularly to discover the mechanism and control of initiation of replication, ori C was isolated by molecular cloning.

Fragments of DNA capable of initiating autonomous replication derived from the ori C region of the chromosome have been isolated (Messer et al, 1978; Miki et al 1978; von Meyenburg 1978). Deletion derivatives of the cloned ori C segment located the replication origin to within a 232-245 base pair sequence (Oka et al, 1980). The complete DNA sequence of the minimum replication segment and a portion of the surrounding region is now known (Meijer et al, 1979; Sugimoto et al, 1979).

Alterations introduced into the minimal ori C region by deletion, insertion or substitution, have shown that certain bases are essential and these sequences must be of defined length, as well as being spaced at specific distances apart with non-essential, stopper sequences in between (Hirota et al, 1981).

No coding DNA has been recognised within the ori C sequences, but two back-to-back promoters have been found, it is suggested that these promoters may be sites where RNA polymerase binds as a beginning to initiation of DNA synthesis upon RNA primers (Lothar & Messer, 1981).

However, no symmetrical sequences could be identified which would result in initiation of bidirectional replication from two RNA-DNA junctions to give divergent replication forks (Hirota et al, 1981). This suggests an asymmetrical initiation process.

The biochemical pathway leading to initiation of DNA replication at ori C is currently being dissected, as factors which inhibit or activate the process are characterised (Kornberg, 1982). In vitro replication of ori C plasmids (Fuller et al, 1981; Fuller et al, 1982) has been shown to be dependent on certain replication proteins (Kornberg, 1982). Two of these proteins are coded by initiation genes dna A (Hirota et al, 1970; Carl, 1970; Wechsler & Gross, 1971) and dna C (Wechsler & Gros, 1971; Schubach et al, 1973; Marsh & Worcel, 1977). Both dna A and dna C are conditional lethal mutants, which do not stop DNA replication immediately after a shift to the restrictive temperature, but, after termination of DNA synthesis, no new rounds of replication are initiated.

Two other initiation genes have been identified, dna I (Beyersmann et al, 1974) and dna P (Hirota et al, 1970).

One of these mutants dna I, appears to be indistinguishable from dna A strains and in vitro replication of ori C plasmids, which is inactive when using cell extracts from dna I strains, is suppressed if the dna I strain contains plasmids or bacteriophages carrying the dna A gene. (Kornberg, 1982).

In vitro systems for replicating ori C DNA are apparently free from membrane or particulate elements (Kornberg, 1982), and thus membrane binding of ori C does not seem to be a requirement for initiation of DNA replication in these systems. Kornberg (1982) does not rule out

membrane involvement as an important feature of initiation in vivo.

Two replication forks, initiated at ori C, moving away from each other and travelling around the circular E.coli chromosome, would be expected to collide at a position diametrically opposed to the origin, assuming an equal rate of fork progression in each direction. Termination of replication usually occurs when two replication forks, moving towards each other, arrive at the same time within the terminus region (Kuempel et al, 1973). Early work placed the terminus region close to trp (Masters & Broda, 1971; Bird et al, 1972; Hohlfield & Vielmetter, 1973; Louarn et al 1974).

The DNA replication terminus is now thought to lie in a region extending from trp at 27.5 mins to man at 35.5 mins on the E.coli K12 chromosome. This region contains very few mapped genetic loci and is reputedly the largest genetically 'silent' region upon the genetic linkage map (Bachmann, 1983). That eight minutes does indeed separate these two loci, was confirmed by detailed time of entry studies from bacterial conjugation experiments (Bachmann et al, 1976), but this length was not further confirmed by P1 transduction. A lack of co-transducible markers in this region meant that neighbouring loci were separated by more than the 100kb of DNA capable of being packaged by phage P1.

Initially, the existence of a large genetically 'silent' region which encompassed the replication terminus, was questioned. Workers suggested that chromosome transfer during conjugation may be impeded by the replication terminus and result in an artificially extended map distance (Kuempel et al, 1977; Louarn et al, 1979; Backmann & Low, 1980), thus spreading the spacings between loci. Closing of the gap in co-transduction was finally achieved by isolating strains with transposons inserted in

the terminus region and using these as markers in P1 co-transduction measurements (Bitner & Kuempel, 1981; Fouts & Barbour, 1982). Both these groups of workers concluded that the genetic distance across the terminus, whether determined by co-transduction or conjugal transfer, is quite similar. A conclusion also reached by Bouche (1982) after aligning the genetic map of the terminus region in relation to a 470kb physical map spanning the distance from trp to map.

In order to determine whether the large genetically silent terminus region contains any essential genes and if the replication terminus, ter C (Bachmann & Low, 1980) is itself essential, Henson & Kuempel (1983) and Henson et al (1984) generated within this region, large deletions mediated by the transposon Tn 10 (Kleckner et al, 1979; Ross et al, 1979) using the readily applied method of Bochner et al (1980) for selecting tetracycline sensitive strains which have lost the Tn 10 transposon.

Henson & Kuempel (1983) isolated deletions from a strain carrying Tn 10 inserted at about 32 mins and a second transposon, Tn 9, conferring chloramphenicol resistance, located 0.9 minutes further clockwise on the genetic map (Bitner & Kuempel, 1981). It was

confirmed by DNA hybridisation that tetracycline^S chloramphenicol^S derivatives of this strain were missing the Tn 10 transposon and an amount of the adjacent chromosomal DNA (Henson & Kuempel, 1983). The loss of the chloramphenicol resistance was explained by a deletion extending into the Tn 9 transposon. Deletions of this sort are known to extend to the right hand, ISI, insertion sequence of Tn 9 (Reif & Saidler, 1975) or beyond the end of the transposon (Ross et al, 1979). Henson & Kuempel (1983) did not provide evidence for either of these possibilities, but suggested that probably a minimum of 40kb of terminus

region DNA had been removed between the two transposons.

Other deletions extending from a Tn 10 which had been shown to insertionally inactivate the trg gene (Harayama et al, 1979) (located at 31.4 mins) (Bitner & Kuempel, 1981; Fouts & Barbour, 1982; Bouché et al 1982) through to rac at 29.7 mins (Binding et al, 1981; Bouché et al 1982) were made by Henson et al (1984). These deletions would delete approximately 60kb of DNA.

Neither of the above 40kb or 60kb deletions affected growth of the strains in minimal or rich media, cell morphology appeared normal, nor was there any filamentation or decreased viability. However, one derivative lacking 240kb from rac (29.7 mins) to kim (33.4 mins) did exhibit altered morphology and slow growth (Henson et al, 1984).

It can be concluded from this work that there are no essential genes in a large section of the terminus region and the ter C locus, if it is indeed located in the deleted sequences, is dispensable as a fixed site for termination of chromosome replication.

What evidence is there to indicate that the terminus of DNA replication is a fixed site? By upsetting the normal symmetry of the bidirectional replication mode, workers were able to study the effect of one replication fork reaching the terminus region before the other (Kuempel et al, 1977; Louarn et al, 1977; Kuempel & Duerr, 1978; Kuempel et al, 1978).

Louarn et al, (1977) obtained strains which did not initiate cycles of replication at 42°C because of a temperature sensitive dna A mutation

but could replicate from the replication origin of a plasmid AR132, inserted at different sites on the chromosome. This phenomenon is known as integrative suppression (Lindahl et al, 1971; Nishimura et al, 1971; Beyersmann et al, 1974). Displacements of initiation points up to 26 mins from the normal origin position were found to direct replication bidirectionally, but termination always occurred between att ϕ 80 at 27 mins and att P2H at 43 mins (Louarn, 1977).

Later Louarn et al (1979) studied replication forks which had been initiated at the usual E.coli K-12 origin and demonstrated that rac at about 30 mins was always replicated by clockwise moving replication forks, but another genetic marker, man at 35 mins, was replicated in the opposite direction.

Inhibition of replication forks moving anti-clockwise through the terminus region, was found to be incomplete (Kuempel et al, 1977). A phage P2 derivative lysogenised at 46 mins in a dna A temperature sensitive strain, which did not show integrative suppression, was found nonetheless to direct replication of the chromosome predominantly in a counter-clockwise direction. Retardation of replication forks occurred between aro D at min 37 and rac, but, a much stronger block to replication was detected between rac and trp.

No significant inhibition of clockwise replication forks was observed between trp and rac when replication was initiated from a P2 sig 5 prophage close to gal at 16.5 min (Kuempel & Duerr, 1978). DNA replication from this site was strongly blocked between rac and aro D. In a different approach, replication forks arriving at the terminus region, were found to travel at a uniform rate as indicated by incorporation of a radioactive precursor into the newly synthesised DNA

(Bouche, 1982). Restriction enzyme digestion of the labelled DNA, followed by locating the restriction fragments upon a physical map of the terminus region (Bouche, 1982), enabled Bouche et al, (1979), to describe a region of approximately 100kb where replication forks are slowed down close to ter C, or alternatively, opposing replication forks collide throughout this region. Extrapolation of this data placed ter C at 31.2 ± 0.2 min. (Bouche et al, 1982).

A specific site for termination of DNA replication has been characterised in the conjugative resistance plasmid R6K. This plasmid confers ampicillin and streptomycin resistance to its host cell. It has a molecular weight of 26 megadaltons and replicates in a relaxed mode (Kontomichalov et al, 1970). The replication of R6K is atypical as it is partially bidirectional with a replication terminus so placed that it results in asymmetry of the replicating molecules (Lovett et al, 1975; Crosa et al, 1976).

Replicative forms (plasmids intermediate in their DNA replication) when terminated or inhibited by the R6K terminus, can be seen to be blocked at a specific locus when examined by electron microscopy (Crosa et al, 1976). To determine if a terminus is active and to roughly map the region responsible, molecules are linearised by a restriction endonuclease at a single landmark target. The amount of replicated versus unreplicated DNA of each replicative form is measured to discover whether the population of replicating molecules is randomly scattered, or if one species of particular size predominates due to the inhibitory effect of the terminus upon one replication fork.

Kolter & Helinski (1978) showed that replication of Cole1 type vectors could be inhibited by the R6K terminus. This region was then localised

(Bastia et al 1981a) and cloned into a suitable plasmid vector and further subcloned to narrow down the functional terminus to a 215 base-pair DNA sequence (Bastia et al, 1981a; Germino & Bastia, 1981). Bastia et al (1981a) showed that the R6K terminus inhibits ColE1 type replication in vitro. By demonstrating that inhibition of DNA replication was specific for the terminus DNA sequence in vitro, they eliminated the requirement for binding to a membrane component.

Also no plasmid encoded trans-acting factor is required by the R6K terminus, since it still functions using cell extracts from cells devoid of resident plasmids (Bastia et al, 1981a). The factors which bring about the transient arresting of replication forks at the R6K terminus sequence, are not known. Bastia et al (1981b) searched the 215 base pair terminus sequence for potential secondary DNA structures which could sterically hinder the progression of replication forks, but none was found. Concomitant with the in vitro studies, the terminus sequence does not contain an open reading frame of significant length which could encode a trans-acting function. One of their hypotheses to explain the mechanism of termination, is that the terminus sequence has specific affinity for a protein which binds to the DNA and somehow affects termination. This suggestion has precedence in repressor and DNA binding molecules. If such a mechanism were involved, then the presence of regions similar in function throughout the E.coli K12 terminus may explain what appears to be a long range slowing up effect upon replication forks as they enter into this region. (Kuempel et al, 1977; Bouche et al, 1982).

1.5 CHOICE OF GENETICALLY SILENT REGION TO
STUDY AND METHODS OF INVESTIGATION.

After surveying the E.coli linkage map available at the time (Bachmann et al, 1976) the choice of which genetically silent region to study lay between several regions of low gene density or any of three co-transduction gaps. Developments in recombinant DNA technology suggested that it could be possible to isolate large sections of genomes without any selection for genetic markers.

Since it might, therefore, be possible to isolate DNA from the co-transduction gaps on the E.coli chromosome, one of these was chosen as representative of genetically silent DNA.

My research was directed towards the DNA replication terminus region of E.coli because of three reasons; the genetic map extending from 29 to 35 minutes was the largest genetically silent section of the chromosome (Bachmann et al, 1976); replication forks were known to terminate within this region (Kuempel et al, 1977; Louarn et al, 1979) adding greater importance to this part of the genome; DNA had recently been isolated from within this region (Kaiser & Murray, 1979).

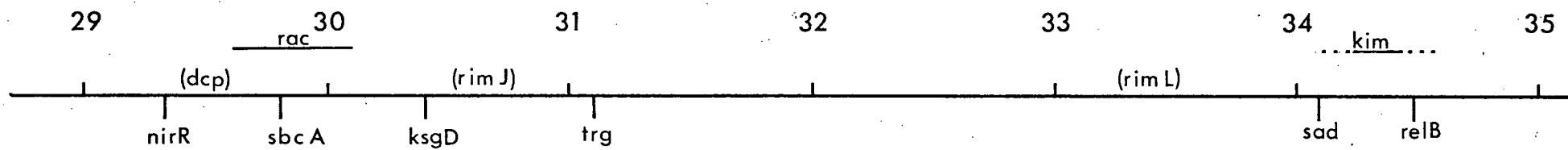
Fig. 1.1 shows the region 29-35 minutes taken from the current genetic linkage map (Bachmann, 1983). Only six loci have been mapped, with three other genes tentatively placed, within this six minute stretch of the map. The types of genes found in this region have varied phenotypes and are well spaced.

The most left hand locus in fig 1.1, nir R (Chippaux et al, 1978) appeared as two closely mapped loci, fnr (Lambden & Guest, 1976)

Fig. 1.1.

Genetic map showing the locations of all known genetic loci in the genetically 'silent' terminus region of E.coli (Bachmann, 1983; Bitner and Kuempel, 1981, Fouts and Barbour, 1982, Bouche et al, 1982).

figure 1-1



and nir A (Newman & Cole, 1978). fnr has been mapped at 29.3 minutes (Bitner & Kuempel, 1982). nir R (fnr, nir A) codes for a gene which is essential for the expression of certain genes involved in anaerobic energy-generating pathways. Mutations at this locus result in deficiencies of nitrate, nitrite and fumarate reductases, hydrogenase and cytochrome C₅₅₂. The pleiotropic product is a positive, regulatory factor which controls the expression of the above anaerobic systems. (Shaw & Guest, 1981; Chippaux et al, 1981). One possibility is that the nir R gene product acts as a sigma subunit directing RNA polymerase towards specific promoters (Shaw & Guest, 1982). These workers identified the gene product as a 31K apparent molecular weight protein.

A gene called dcp has been placed between nir R and rac, Fig 1.1, the parenthesis indicates that the gene has not been precisely mapped (Bachmann, 1983). dcp was mapped to this region by complementation with the episome F'123 (Deutch et al., 1978.) which carries the section of the chromosome extending from trp (27.5 minutes) to rac (Low, 1973). The gene product of dcp is a carboxy-terminal peptidase which may be located in the periplasmic space of the cell envelope and possibly also functions in the cytoplasm.

In Fig 1.1 the position of rac is represented by a line. This is because rac is part of a cryptic prophage capable of undergoing a process similar to zygotic induction when transferred by conjugation to rac minus recipients (Low, 1973; Evans et al, 1979). Approximately 27kb of DNA is precisely excised from the chromosome in rac prophage deleted strains (Kaiser & Murray, 1979).

The locus sbc A is associated with the rac prophage. sbc A mutations suppress rec B rec C double mutants (Lloyd & Barbour, 1974). and were mapped at approximately 29 mins. on the genetic map. In sbc A mutants Kushner et al (1974) have shown that synthesis of exonuclease VIII, the product of the rec E gene, rescues the Ra^+ phenotype of rec B rec C mutants, caused by loss of exonuclease V. The rec E structural gene lies at one end of the rac prophage (Kaiser & Murray, 1979). Expression of rec E may become depressed by sbc A mutations (Kushner et al, 1974). One mutant, sbc A8, is a deletion which extends from within rac through one of its hybrid attachment sites and into the bacterial chromosome (Kaiser & Murray, 1979). It is proposed that rec E may be under control of a rac repressor which is removed by the sbc A8 deletion allowing expression of exonuclease VIII.

Consonant with the existence of a rac repressor Diaz et al. (1979,) have isolated an origin of replication, ori J, which only functions in strains lacking the rac prophage and presumably a rac encoded repressor. Physical mapping studies have located ori J within the rac prophage and adjacent to rec E (Diaz et al, 1979; Kaiser & Murray 1979). A second origin of replication ori X (De Massy et al, 1984) has been located in the E.coli terminus region, by marker frequency analysis using cloned DNA fragments as markers and DNA hybridisation with labelled chromosomal DNA. Replication was not only observed to be initiated from ori C and a plasmid integrated close to the terminus region, but also from an unknown replication origin, designated ori X, at 30-32 minutes. ori X was shown to be distinct from the rac prophage origin ori J.

Two insertions of transposon Tn 10 into the rac locus have been mapped at 29.7 min. and 30 min. by co-transduction with fnr (Binding et al. 1981). By correcting for the size of Tn 10, the genetic distances from fnr were reduced, and the insertions are reported to be at 29.4 min. and 29.8 min. Low (1973) estimated from conjugal transfer studies, that rac was located at approximately 30 - 31 mins.

A gene comparing resistance to kasugamycin ksg D at 30.4 minutes has been mapped by P1 transduction and found to lie 0.6 minutes away from sbc A (Fouts and Barbour, 1981). These workers isolated ksg D, during attempts to insert transposon Tn 5 into ksg B which was thought to reside in the terminus region (Sparling et al. 1973) and had been provisionally placed in this region of the genetic linkage map (Backmann et al. 1976).

Fouts and Barker (1981) subsequently mapped ksg B at 36.5 minutes between man and aro D. The nature of the kasugamycin resistance phenotype conferred by ksg D mutants is not known. Curiously all kasugamycin resistant strains that were isolated by Tn 5 mutagenesis, turned out to be ksg D mutants. Fouts and Barbour (1981) point out that ksg D mutants, both closely linked, and unlinked to Tn 5, were obtained. None of the other kasugamycin resistance genes around the chromosomes were found amongst Tn 5 mutants. Chemical mutagenesis by other workers (Okuyama et al. 1974; Sparling et al. 1973) had not revealed ksg D as a class of kasugamycin resistance mutants.

A suggestion is made that Tn 5 and at least one of its IS50 repeat sequences can promote expression of ksg D. A promoter present on Tn 5 close to its ends may express suitably placed genes next to the transposon (Berg et al, 1980). Also, at least one IS50 of Tn 5 has independent transposition activity (Berg et al, 1981). Therefore, linked and unlinked ksg D mutants express a gene for kasugamycin resistance by transcription from a promoter reading out of Tn 5 or IS50, respectively (Fouts & Barbour, 1981).

Moving clockwise from ksg D on the genetic map (Fig. 1.1) the next known gene trg, maps at approximately 31 mins. by Hfr matings (Harayama et al, 1979) and at 31.3 mins. by P1 transduction (Bitner & Kuempel, 1982). trg has been located at 31.4 min upon a physical map of the terminus region (Bouche, 1980) and may be the closest known gene to ter C. It is also the only terminus region marker that cannot be cotransduced clockwise with any chromosomal locus and defines one end of a cotransduction gap at 31.1 mins. (Bitner & Kuempel, 1981; Fouts & Barbour, 1982).

Initially trg strains were isolated as Tn 5 and Tn 10 insertional mutations which prevented the tactic response of cells to ribose and galactose (Harayama et al, 1979). Chemotactic behaviour is mediated through methylation of transducer proteins (Springer et al, 1977) also called methyl-accepting chemotaxis proteins (MCPs). Harayama et al. (1982) have identified trg as the structural gene for MCP III which was either not produced (Hazelbauer et al, 1981) or had an altered electrophoretic mobility (Koiwai et al, 1980) in mutant trg strains. Identification of the trg gene product was demonstrated unequivocally by transposon mutagenesis. It has an apparent molecular

weight of around 60-70K. (Harayama et al, 1982).

A region designated kim, which shares homology with phage lambda DNA has been mapped at 33.2 to 33.6 minutes relative to the genetic map (Bouche, 1982). Homology with the DNA of phage lambda carried by the E.coli K-12 chromosome (Lowie & McCarthy, 1963; Green, 1963) has been observed as specific restriction endonuclease fragments which hybridise to a lambda 32p - labelled DNA probe (Kaiser & Murray, 1979; Anilionis et al, 1980). Restriction fragments from three regions are thought to comprise the majority of lambda homology within the chromosome. Two of these regions are defective prophages, one is located at rac (Kaiser & Murray, 1979) the other, qsr - prophage, is located within the chromosomal region covered by the episome F'152, which carries the 2.5 - 5 minute region of the chromosome (Anilionis et al, 1980).

Distinct from these two regions Kaiser (1980) proposed that a third defective lambdoid prophage may exist in the chromosome of E.coli K-12. An EcoRI (15.2kb) and a Hind III (28kb) fragment showed homology with lambda DNA, but also the 28kb Hind III fragment hybridised to a lambda rev DNA probe. Bacteriophage lambda rev carries the E.coli K-12 exonuclease VIII gene, rec E (Gillen et al, 1977) and some additional rac prophage DNA (Kaiser & Murray, 1979). Bouche et al (1982) showed that the 15.2kb EcoRI fragment showing homology with lambda DNA was contiguous with a 17.2kb EcoRI fragment. Only homology with lambda rev DNA but not lambda DNA was found with the latter fragment (Kaiser, 1980). By comparison with a physical map of the terminus region (Bouche, 1982) a segment of chromosome was identified as having the appropriate restriction sites and designated kim.

Two loci, rim J and rim L have been mapped by conjugal transfer to within the region shown in fig 1.1. Neither rim J nor rim L could be P1 transduced with either man or trp and therefore must lie at least two minutes away from these markers (Cumberlidge & Isono, 1979; Isono & Isono, 1981). Both rim J and rim L mutants result in thermosensitive modification of the ribosomal proteins S5 and L12, respectively. These proteins are normally N-terminal acetylated, but are not at the restrictive temperature in rim J and rim L mutants. rim J, but not rim L is carried by the episome F'123 (Cumberlidge & Isono, 1979; Isono & Isono, 1981). F'123 extends to about 31 minutes on the genetic map (Low, 1973) therefore rim J and rim L are probably two distinct loci and rim L lies between 31 and 34 minutes.

The sad gene codes for NAD-dependent succinate semialdehyde dehydrogenase. This gene was not discovered in E.coli K12, instead it was isolated as an E.coli C mutant which was unable to grow on 4-hydroxyphenylacetate (Skinner & Cooper, 1982). E.coli K12 does not utilise 4-hydroxyphenylacetate as a carbon source. However, the E.coli C sad mutation is complemented by the sad locus of E.coli K12 and this allowed the E.coli K12 gene to be roughly mapped using F-prime episomes, and more precisely by P1 transduction, to 34.1 minutes (Skinner & Cooper, 1982). The function of the E.coli K12 NAD-dependent succinate semialdehyde dehydrogenase is not known. Skinner & Cooper (1982) suggest that its role is to metabolise succinate semialdehyde, which is toxic, and this is related to induced expression of the sad gene by this metabolite.

The gene furthest clockwise in the 29-34 minute region is rel B at 34 minutes (Diderichsen et al, 1977). E.coli mutants that continue

to synthesise ribonucleic acid (RNA) during amino acid starvation, are said to be 'relaxed' (hence rel) and do not exhibit the 'stringent' control of RNA synthesis shown by wild-type strains (Stent & Brenner, 1961). rel B mutants show a delayed relaxed response and have a characteristic delay of about ten minutes, after amino acid starvation, before RNA accumulation continues (Diderichsen et al, 1977). These workers mapped rel B by P1 transduction with man.

None of the genes described in the above survey are essential, nor do they have easily identifiable phenotypes. To study this region of genetically silent DNA, I decided that the techniques of molecular cloning would be particularly applicable. Once having isolated a section of DNA from the E.coli terminus region, this could be put into protein synthesising systems and any proteins programmed by the DNA could be analysed by polyacrylamide gel electrophoresis.

The cloning strategy known as 'chromosome walking' (see Chapter 3) which involves isolating overlapping restriction fragments, was decided upon. In this way, sections of the chromosome can be cloned without the need of any genetic markers in order to select for the ~~piece~~ of DNA to be cloned. Recombinant plasmids carrying DNA fragments isolated from the rac locus (Kaiser & Murray, 1979), were available to use in the first 'step' of the 'chromosome walking' procedure.

Of the DNA-directed protein synthesising systems which were currently available, two methods were chosen to study proteins synthesised by cloned DNA fragments carried by high copy number plasmids. One of these methods involved introducing a plasmid into an E.coli strain which produces chromosome-less minicells. The minicells containing

entrapped plasmid molecules are purified away from the parental cells and any proteins synthesised within the minicells, coded by the plasmids, are labelled with radioactive amino acids (Reeve, 1979). The preparation of minicells is tedious but the purified minicells can be stored frozen, then thawed, and labelled whenever convenient.

The other method developed by Sancar and Rupp (1979) is less time consuming than minicell preparation, but the reported procedure only yields one lot of cells that are labelled, for each plasmid. This method relies on destroying chromosomal DNA in a strain that is defective in repairing UV damage after a short exposure to ultra violet light, these are called 'maxicells'. Plasmid molecules within the host cell are a much smaller target for irradiation and only one plasmid need survive to replicate to the high copy number state. After time is allowed for degradation of the chromosomal DNA, and death to occur of any cell survivors, from addition of d.-cycloserine, proteins synthesised within the maxicells, programmed by the plasmids, are radioactively labelled.

A simpler method developed by Neidhardt et al (1980) through attempting to improve the maxicell method of Sancar & Rupp (1979) involves labelling of plasmid coded proteins after release of the host cells from inhibition of protein synthesis brought about by treatment with chloramphenicol. However, during the labelling period a number of host cell specified proteins are synthesised. Unless a two-dimensional polyacrylamide gel was used to analyse the gene products, the host proteins would probably interfere with the identification of plasmid coded polypeptides. Furthermore, chloramphenicol sensitive cells must be used, therefore excluding the use of chloramphenicol resistant plasmids, unless other

protein synthesis inhibitors were found to work in this system.

In vitro plasmid-directed protein synthesis using cell extracts (Zubay, 1973) was considered to be too difficult to set up, given that simpler in vivo systems were available. A much simpler modified Zubay type system has now been described (Pratt et al, 1981).

Finally, a semi-in vitro system using E.coli cells that have been irradiated with a large dose of ultra-violet light and then infected with the bacteriophage lambda, has proved very successful in characterising the lambda repressor protein (Ptashne, 1967)

Later, this system was extended to identify chromosomal gene products coded by lambda transducing phages (Jaskunas et al, 1975a, Jaskunas et al, 1975b). The use of phage infection of irradiated cells as an alternative protein synthesising system, to those described above, is limited by the size of DNA fragments which can be inserted into lambda DNA and still be packaged into the phage head. Also, phage must be prepared at high titres in order to infect the irradiated cells at a multiplicity of around ten (Ptashne et al, 1967). The minicell and maxicell methods were preferred to the phage infection system, as there is essentially no limit governing the size of DNA fragments that can be cloned in a plasmid vector and very little preparation of plasmid DNA is required to introduce the plasmids into the E.coli strains used in the minicell and maxicell systems.

1.6 OBJECTIVES.

The objectives of the work presented in this thesis are, to isolate DNA from the genetically silent ter C region of the E.coli chromosome using techniques of molecular cloning; to construct a restriction endonuclease map of the cloned DNA to facilitate sub-cloning of the region; to use the cloned DNA to program protein synthesis and to catalogue the number and sizes of polypeptides that may be found to be expressed by this DNA; to locate any gene products, coded by the cloned DNA, on the restriction map and estimate from the protein synthesis results, the amount of DNA involved in coding and how, if any, this is distributed in this section of genetically silent DNA.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 MEDIA.

Luria broth (LB): 10g Difco Bacto Tryptone, 5g Difco Bacto Yeast Extract, 5g NaCl, per litre distilled H₂O adjusted to pH 7.2.

LB Agar: 10g Difco Bacto Tryptone, 5g Difco Bacto Yeast Extract, 10g NaCl, 15g, Difco Agar, per litre distilled H₂O adjusted to pH 7.2.

M9 Salts: 6g Na₂HPO₄, 3g KH₂PO₄, 1g NH₄Cl, 0.5g NaCl per litre.
These salts were made up at 4 x concentration as a stock solution.

M9 Glucose: 100ml M9 Salts (4 x), 8ml 20% Glucose, 0.4ml 1M Mg SO₄, 0.1ml 0.1mg/ml Thiamine, 300ml distilled H₂O.

This was made up using sterile stock solutions of the ingredients.

Casamino Acids: Made up at 25% with distilled H₂O

MacKonkey/Galactose: 40g Difco MacKonkey Agar base, 50ml 20% Galactose, per litre distilled H₂O.

Hershey Salts without sulphate:

NaCl	5.4g
KCl	3.0g
NH ₄ Cl	1.1g
CaCl ₂ ·2H ₂ O	15mg
MgC ₂ ·CH ₂ O	0.2g
FeCl ₃ ·6H ₂ O	0.2mg
KH ₂ PO ₄	87mg
Tris-Base	12.1g

Adjusted to pH7.4 with concentrated HCl and made up to 1 litre.

Sulphate free Hershey Medium: Per 500ml Hershey Salts.

Glucose	20%	10ml
Threonine	2%	2.5ml
Leucine	2%	2.5ml
Proline	2%	5.0ml
Arginine	2%	5.0ml
Thiamine	0.1%	0.1ml

2.2 ANTIBIOTICS:

Stock solutions of antibiotics were diluted in media and used at the following concentrations.

Ampicilin (Ap)	50mg/ml
Chloromphenicol (Cm)	20mg/ml.
Kanamycin (Km)	25mg/ml
Tetracycline (Tc)	15mg/ml.

2.3 CHEMICALS:

Chemicals were purchased from Sigma or BDH. Radiochemicals were purchased from Amersham; -
 Deoxycytidine 5 - Alpha - P(32) Triphosphate (1mCi/ml, 400Ci/mmol,
 L-S (35) - Methionine (800Ci/m mol)

2.4 ENZYMES:

Restriction enzymes, Hind III, EcoRI , Bam H.I, Sal I,
Pst I and Pvu II were purchased from either Boehringer Mannheim or

New England Bio-Labe. T4DNA Ligase and DNA Polymerase I were also obtained from these sources. DNase I and RNase I were bought from Sigma.

2.5 BUFFERS:

The following stock buffers were made in large quantities.

10 x TE: 100mM Tris-Base, 10mM EDTA (Disodium Salt), adjusted to pH8.0 with HCl.

10 x TEA: 0.4M Tris-Base 0.2M Na Acetate, 0.01M EDTA (Disodium Salt), adjusted to pH8.2 with Acetic Acid.

20 x SSC : 3M NaCl, 0.3M Na Citrate (Trisodium Salt).

2.6 BACTERIAL STRAINS.

<u>NAME</u>	<u>GENOTYPE</u>	<u>FEATURES</u>	<u>SOURCE</u>	<u>REFERENCE.</u>
C600	<u>thr leu</u> <u>ton A lac Y</u> <u>sup 2 s</u>	Good transformation	N. Murray	Appleyard (1954).
DS410	<u>min A min B</u>	Minicell producer.	J. Maule	Reeve. (1977)
CSR603	Multiply marked, <u>rec A uvr A</u> <u>phr</u>	Maxicell strain.	B. Bachmann	Sancur & Rupert. (1978).
C600K	C600 <u>gal K</u>	Galactokinase Mutant.	R. Hayward.	KcKinney (1981).
PLK1110	<u>argA plcA</u> <u>trp supE rpsL</u> <u>zde-234::Tn10</u>	Tn10 insertion 34mins.	P. Kuempel	Bitner & Kuempel (1981)
PLK1291	Multiply marked, <u>zdc-235::Tn10</u>	Tn10 insertion 32 mins.	P. Kuempel	Bitner & Kuempel (1981)
TH1013	<u>minA minB rpsL</u> <u>trg-2::Tn10</u>	Tn10 insertion <u>trg</u> gene		S. Harayama.
TH1024	<u>MinA minB rpsL</u> <u>trg-1:: Tn5</u>	Tn5 insertion <u>trg</u> gene		S. Harayama.

2.7 OVERNIGHT CULTURES:

An overnight culture was set up by taking a single colony from an LB agar plate and used to inoculate a quantity of LB. Routinely 5ml of LB in half-ounce bottles were inoculated and shaken vigorously at 37°C for 14-16 hours.

2.8 PREPARATION OF PLASMID DNA.

All plasmids used for this thesis carry the high copy number ColE1 replicon and were prepared by a modified method of Katz et al (1973). A 100ml overnight culture of the strain carrying the plasmid, and grown in LB containing antibiotic, was spun at 10K for 10 mins. at 4°C. The cell pellet was resuspended in 1.5ml of ice-cold 25% Sucrose, 50m M Tris-HCl pH8.0, by vortex mixing and up-and-down pipetting. All subsequent mixing was by gentle swirling on ice. 0.5ml of 250m M Tris-HCl pH8.0 containing Lysozyme at 10mg/ml, was added and left on ice for 5 minutes. 1ml of RNase at 1mg/ml in TE was added (the RNase solution had been heated at 100°C for 5-10 minutes to inactivate any contaminating DNase). Also 0.5ml of 0.5 M EDTA pH8.0 was added, mixed and left on ice for 5 minutes. To lyse the cells, 2.5ml of Triton mix (see below) was added while gently swirling the mixture. Swirling was continued at intervals and left on ice for a total of 10 minutes. After this time most of the cells have lysed and the viscous mixture became clearer. This lysate was centrifuged at 15000 r.p.m. for 30-45 minutes in a 50ml polycarbonate tube, the cleared lysate was removed with a Pasteur pipette into a 10ml measuring cylinder. The cleared lysate was made up to 9ml using a mixture of the lysis components. To the cleared lysate, in a 25ml beaker, 9g of CsCl and

0.9ml of 5mg/1ml ethidium bromide was added and swirled at room temperature until the CsCl had dissolved. The volume of this solution was sufficient to fill a M.S.E. heat-seal tube and after sealing, a CsCl gradient was formed by centrifuging at 38000 r.p.m. for approximately 60 hours in a Ti 50 rotor. At equilibrium the CsCl gradient contains two well separated DNA bands, which were visualised by long wavelength ultra-violet light. The lower, covalently closed circular plasmid band, was removed by firstly puncturing the top of the heat-seal tube and then inserting a G19 hypodermic needle just below the band and taking off about 0.5ml from the centre of the band. Ethidium bromide was removed by extracting four times with an equal volume of H₂O equilibrated iso-amyl alcohol. Dialysis against 2 x 500ml of TE at room temperature for 2 x 15 minutes, was used to remove the CsCl. DNA prepared in this way is of good enough quality for restriction and ligation. Phenol extraction (section 2.11) was used to 'clean up' the DNA, if required. This method routinely yielded about 0.5mg of DNA, for most plasmids. The DNA concentration was most usefully determined in an agarose gel (section 2.16) by comparing the intensity of the ethidium fluorescence of the plasmid DNA (which was linear by restriction) with a standard linear DNA sample.

Triton Lysis Mix.

2 ml 10% Triton X -100

25ml 0.5 M EDTA pH8.0

10ml 1 M Tris-HCl pH8.0

H₂O to 200ml.

2.9 DNA MINI-PREPS.

The rapid preparation of plasmid DNA by the method of Birnboim & Doly (1979) was employed. The following solutions were prepared:

Lysis Solution:	Lysozyme, 2mg/ml
	Tris-HCl pH8.0, 25m M
	EDTA pH8.0, 10m M
	Glucose, 50m M
Alkaline SDS Solution:	NaOH, 0.2 M
	Sodium Dodecyl Sulphate, 1%
High Salt Solution:	Na Acetate pH4.8, 3 M.
Low Salt Solution:	Na Acetate pH6.0, 0.1 M

Approximately 1.5ml of culture was taken from 2ml overnight cultures in LB plus antibiotic, and put into snap-cap tubes (Sarstedt). The cells were pelleted by spinning in a microcentrifuge for 15-20 seconds. Most of the supernatant was poured off, and the rest was carefully removed with an automatic pipette. The cells were resuspended in 0.1ml of lysis solution by vortexing and left on ice for 30 minutes. 0.2ml of alkaline SDS solution was added and gently mixed by inversion and left standing on ice for 5 minutes. The mixture clears and becomes viscous within this time, showing that cell lysis has occurred. Next, 0.15ml of high salt solution was added and gently mixed before leaving on ice for a further 60 mins. A heavy white precipitate formed which was removed by spinning in a microcentrifuge for 5mins and transferring

the clear supernatant to a fresh snap-cap tube. To this was added 1ml of cold ethanol and placed at -20°C for 30 minutes. The precipitated nucleic acids were recovered by pelleting in a microcentrifuge for 2 minutes. A pasteur pipette which had been drawn out over a bunsen flame, was used to remove and discard the supernatant. The pellet was dissolved in 0.1ml of low salt solution and the nucleic acids were precipitated again with 0.2ml cold ethanol at -20°C for 10 minutes. These were recovered as above, and the pellet was dried in a vacuum desiccator before dissolving in 0.05ml of TE. Usually 10-20 μl of this material, whether restricted or not, could easily be detected in an agarose gel.

The above procedure was later modified to reduce the preparation time. Each of the solutions: lysis solution, alkaline SDS solution and high salt solution, were only given 5 minutes after addition, instead of the times given above.

2. 10 PREPARATION OF CHROMOSOMAL DNA.

Bacterial lysates were prepared by the triton lysis method of Katz et al (1973). 250ml of an overnight LB culture was spun at 101000 r.p.m. for 15 minutes at 4°C . The cells were resuspended in 6ml of 25% sucrose, 50m M Tris-HCl, 40m M EDTA. 1ml of 10mg/1ml Lysozyme in the above buffer, and 1ml of 0.5 M E pH8.0, were added, mixed gently by swirling, and left on ice for 5 minutes. 13ml of triton mix (see section 2.8) was added and left on ice for 10 minutes, occasionally swirling until the mixture became very viscous. As much as possible of the mixture was transferred to a siliconised (rinsed with 'repelcote', Hopkin & Williams, and dried) 50ml glass centrifuge tube (Corex). An

equal volume of distilled and equilibrated phenol (see section 2.11) was added. 'Parafilm' was used to close off the end of the tube and the mixture was rocked by hand until an homogenous solution was obtained. This was spun at 5000 r.p.m. for 5 minutes at room temperature, to separate the phases. The lower phenol layer was removed with a pasteur pipette and the phenol extraction was repeated twice more, or until a colourless lysate, free of extraneous matter, was obtained. After the final separation of the phases, the upper aqueous layer was collected with a pasteur pipette, raised slightly above the surface of the solution, once having drawn up a small amount. The solution lying immediately at the interface was left behind, along with any precipitated material. In a chilled sterile beaker, sitting on ice, ethanol at -20°C was gently layered into the lysate and the precipitated chromosomal DNA was spooled onto a glass rod which had been steeped in concentrated phosphoric acid and rinsed well in distilled H_2O . The spooled DNA was dissolved in 5ml of TE with 0.2ml of 10mg/ml RNase, and incubated at 37°C for 3 hours. In a chilled 10ml sterile beaker, the ethanol precipitation step was repeated.

Finally, the chromosomal DNA was dissolved in 1-2ml of TE. The DNA concentration was determined from OD^{260} measurements of known dilutions of the DNA sample. An OD^{260} of one was taken to equal 50mg/ml. DNA free of protein has an OD^{260} to OD^{280} ratio of 0.5.

2.11: PHENOL EXTRACTION OF PLASMID DNA.

An aliquot of phenol, which had been freshly distilled, equilibrated with distilled H_2O or TE, and frozen at -20°C , was thawed in a 37°C water bath or more rapidly at 70°C . An equal volume of phenol was

added to the DNA solution to be extracted. This was mixed by vortexing for about 30 seconds and the phases were separated by spinning for 1-2 minutes in a microcentrifuge. The upper aqueous layer was removed into a fresh snap-cap tube and the extraction was repeated twice more. To remove the phenol the DNA solution was extracted with ether. An equal volume of ether, which had been equilibrated with H_2O , was vortex with the DNA solution. The upper ether phase was removed and discarded. A total of four ether extractions usually resulted in a clear DNA solution. After ether extraction, the DNA was ethanol precipitated (section 2.12).

2.12: ETHANOL PRECIPITATION OF DNA.

Volumes were usually small enough to perform the following operations in snap-cap tubes (Sarstedt). Large volumes were divided into two or more snap cap tubes. The volume of the solution, containing the DNA, was measured using an adjustable automatic pipette. One tenth volume of 3 M Na Acetate pH6 was added and mixed. 2-3 volumes of cold ethanol were added, mixed and placed at either $-20^{\circ}C$ or $-70^{\circ}C$. Final volumes of 0.3ml, or less, were left for 5-15 minutes and full snap-cap tubes were given 30 minutes. DNA was occasionally stored in this precipitated form. The precipitate was collected by spinning in a microcentrifuge for 5 minutes and the supernatant was aspirated with a pasteur pipette which had been drawn out over a bunsen flame. 0.5ml of ethanol at $-20^{\circ}C$ was run down the side of the tube, so as not to disturb the pellet. This was spun for about 20 seconds and the supernatant discarded, as before. The pellet was dried in a vacuum dessicator.

Hind III/Pst I double digests were usually set up in this buffer and having both enzymes present at 2 units.

- iii. A 'universal' reaction buffer occasionally worked with all the enzymes used in this thesis. The following buffer simplified setting up multiple single digests using different enzymes, as well as double digests. Partial digests were set up using scaled up quantities of DNA and reagents, so that aliquots could be removed at each time interval and heat killed as above.

10 x Universal Reaction Buffer: 33ml 1 M Tris-Acetate pH7.9 (Tris Base to pH7.9 with Acetic Acid), 2.145g Mg Acetate, 6.468g K Acetate, made up to 100ml with distilled H₂O then autoclaved. Added Dithiothreitol to 5mM and Bovine Serum Albumin to 1mg/ml before use.

2.14 LIGATION OF DNA RESTRICTION FRAGMENTS.

Ligation of restricted DNA using T4 DNA Ligase (Wilson et al, 1977) was performed in 10-20 μ l volumes for molecular cloning of DNA fragments into vector molecules (the DNA fragments to be cloned were usually added at 2-3 fold excess over the vector DNA) or, 50 μ l volumes were used to obtain a high percentage (90%) of circularised DNA fragments.

In each case about 0.25 μ g of restricted DNA, which had been treated to inactive the enzyme (see section 2.13), was put in a snap-cap tube



(Sarstedt). To this was added one tenth final volume of 10 x T4 ligase cocktail (see below), sterile distilled H₂O if required, and a quantity of T4 DNA ligase (this amount varied with the source of the enzyme, but generally the concentrated stock enzyme was diluted such that 1µl would completely ligate 1µg of Hind III cut phage lambda CI857 DNA in 1 hour).

The reaction was mixed and taken up and down in a microcentrifuge before incubating at 15°C for 1 hour - 2 days, depending on the efficiency of ligation of a particular reaction mix. Ligated molecules were recovered by transformation of a suitably competent E.coli strain (section 2.15). When plasmid DNA was used for ligation, DNA which was uncut, restricted and ligated was transformed as controls for competence of cells, complete restriction and efficiency of ligation, respectively.

10 x T4 DNA Ligase Cocktail:

0.66ml 1 M Tris pH7.2
 0.1ml 0.1 M EDTA
 0.1ml MgCl₂
 0.1ml 1 M Dithiothreitol
 0.01ml 0.1 M ATP
 0.03ml Distilled H₂O

2.15 TRANSFORMATION.

All E.coli strains, which were used for transformation, were made competent for DNA uptake by cold CaCl₂ treatment (Lederberg & Cohen, 1974). Overnight cultures were diluted 1:20 in LB and grown at 37°C.

to an OD⁵⁴⁰ of 0.5-0.6, then chilled in ice for about 15 minutes. After pelleting the cells by centrifugation, they were resuspended in one half original volume of ice cold 50mM CaCl₂, and left on ice for 15 minutes. The cells were pelleted again and resuspended in one twentieth of the starting volume in ice cold 50mM CaCl₂, and left on ice for at least 30 minutes. While maintaining the cells on ice, they were either used for transformation directly, or glycerol was added to 15% and 0.2ml aliquots of competent cells were frozen at -70°C. To transform, DNA (about 0.05-0.1µg) was mixed with 0.2ml of fresh or thawed competent cells on ice and left for 30-40 minutes. The mixture was then heat shocked at 42°C for 2 minutes and 1ml of LB was added. Expression time for antibiotic resistance genes was allowed by incubating the transformed cells at 37°C for 30-60 minutes before 0.1 or 0.2ml were spread onto antibiotic selection plates.

2.16 AGAROSE GEL ELECTROPHORESIS.

The technique essentially that of Sharp et al (1973) was employed. To make a 0.7% agarose gel, 0.14g of agarose (Sigma, type II) was dissolved by boiling in 200ml of TEA electrophoresis buffer (section 2.5). A Pyrex conical or round bottom flask was used so that the gel solution could be cooled to hand-hot by holding under a running cold tap. 16µl of 5mg/ml ethidium bromide was added and mixed well before pouring. The gel apparatus consisted of a perspex base plate (16.5 x 26cm.) with two sides attached and two removable end pieces clamped into position. One or more combs were placed across the base plate supported at about 1mm clear of the base, by bulldog clips. The number of combs and the size of the teeth was chosen according to the number and volume of the samples, also, how far they were to be run. When the gel had set (it had become

uniformly opaque.) TEA was put onto the gel around the teeth and then the comb(s) was carefully removed. The end pieces were removed and either the base plate containing the gel was immersed in an electrophoresis tank with sufficient TEA to cover the gel, or it was supported between two TEA buffer tanks and two paper wicks set up with blotting paper. In the latter case, the cells were filled with TEA to prevent them drying out. Samples were loaded after mixing with one tenth volume of loading buffer; TEA, 50% glycerol, 0.025% Bromophenol Blue marker dye. Samples were run towards the anode, firstly at 400v until the bromophenol blue had entered the gel, after which they were run at a lower constant voltage. Where the gel was not submerged, the cells were then filled with TEA and 'Saran Wrap' or 'Cling Film' was used to cover the gel. Gels run slowly overnight for good resolution of DNA bands, were set at 75 V. Voltages up to 250 V were used for quicker results. Also, gels poured onto much smaller base plates, microscope slides for example, were used for rapid analysis of a small number of samples, and small sample volumes. These 'mini-gels' were set up by resting the teeth of the comb directly on the plate and allowing surface tension to hold the gel until it had set. Running times were reduced to 5-30 minutes. DNA bands were visualised by ultraviolet light using a transilluminator and a record of the gels was kept as a photographic negative (Ilford HP4 film).

2.17 RECOVERY OF DNA FRAGMENTS FROM AGAROSE GELS.

The method of 'freeze-squeeze' (Thuring et al 1975), was used to recover DNA restriction fragments from agarose gels in order to label them with ^{32}p phosphorous by nick translation (section 2.18). DNA bands visualised by ultraviolet light, were cut from agarose-ethidium bromide gels (section 2., 16) taking as little of the surrounding gel as possible.

A small envelope was made by folding up a piece of Parafilm, the gel slice was placed inside this and frozen at -20°C for at least 15 minutes. The frozen gel slice was squeezed between forefinger and thumb, a small amount of liquid was extracted as the gel slice thawed. This liquid was pipetted into a snap-cap tube (Sarstedt) and 20 μl of distilled H_2O was used to wash the thawed gel. The washings and thawed gel were added to the extruded liquid and this was spun in a microcentrifuge for 5 minutes. The supernatant was put into a fresh snap-cap tube and the efficiency of extraction was checked by comparing the fluorescence due to DNA bound ethidium in the supernatant and the pelleted gel. An equal volume of iso-amyl alcohol, equilibrated with H_2O , was vortexed for a few seconds with the supernatant, to extract the ethidium bromide. The DNA was recovered by ethanol precipitation (section 2.12). Usually the DNA pellet was resuspended in 10 μl of TE. 1-5 μl of this DNA was run on an agarose mini-gel (section 2.16) to check for recovery of the fragment, and 2.5-5 μl was used for nick translation.

2.18 NICK TRANSLATION.

Radioactive DNA probes for DNA hybridisation (section 2.20) were made by the nick translation method (Maniatis et al., 1975). 10-20 μl of 32p dCTP in a snap-cap tube (Sarstedt) were dried down in a vacuum dessicator. To this was added approximately 0.25 μg of DNA in a volume which was less than 40 μl . 5 μl of 10 x Hind III reaction buffer (see section 2.13) and 1 μl each of 2mM cold dATP, dGTP and dTTP, was added. This mixture was made up to 47 μl with distilled H_2O , then, 2 μl of 0.1mg/ml DNase in TE, was added, mixed and allowed to stand at room temperature for 2 minutes before adding 1 μl of DNA polymerase I (1u/ml). The nick translation mix was incubated at 15°C for 1 $\frac{1}{2}$ hours.

After incubation was complete, 50 μ l of Orange G (Sigma) marker dye was added to the reaction mix, this was immediately loaded into a sephadex G-50 (fine) column. The sephadex was equilibrated by autoclaving for 10-15 mins in distilled H₂O. Columns were set up either in 10ml pipettes with a siliconised glass wool plug or in siliconised 'Econo-columns' (0.7 x 15cm, Bio-Rad). Nick translated DNA was eluted with distilled H₂O and its passage down the column was followed using a mini-Geiger counter, collecting the radioactive peak as 6 or 7 drop samples in 3 or 4 snap-cap tubes. The unincorporated radioactive nucleotide eluted with the orange G marker dye. An estimate of the efficiency of nick translation was obtained by Cerenkov counting in a Hewlett-Packard scintillation counter. The snap-cap tubes containing the samples from the column were put into scintillation vials and counted at around 60% gain. A total of $5 \times 10^6 - 10^7$ counts per minute was found to be a useful DNA probe. A hole was pierced at the top of the snap cap tubes and the DNA probe was denatured by heating in a boiling water bath for 5 minutes.

2.19 TRANSFER OF DNA FRAGMENTS FROM AGAROSE GELS TO NITROCELLULOSE FILTERS.

The technique known as 'Southern Transfer' or 'Southern Blotting' (Southern, 1975) was used to transfer DNA restriction fragments from agarose gels (section 2.16) to nitrocellulose filters. After a photographic record of the gel was taken, excess agarose was trimmed off at the wells and also the region of the gel to which fragments of 300 base pairs and less, would migrate. DNA fragments smaller than 300 base pairs do not bind well to nitrocellulose. An orientation mark was cut at one corner of the gel and then it was immersed for 40 minutes in

0.5 M NaOH, 1.5 M NaCl to denature the DNA. The gel was neutralised by immersing in 0.5 M Tris-HCl pH7.4, 3 M NaCl for 2 x 20 minutes. Directly from the neutralising solution, the gel was placed on top of three sheets of blotting paper which were supported by a glass plate, and suspended over a reservoir of 20 x SSC (Section 2.5). The blotting paper dipped into the 20 x SSC and acted as a wick. Strips of polythene at least 1cm wide, were placed just under all four edges of the gel, so that they were insulated from the 20 x SSC soaked blotting paper. The gel was checked for entrapped air bubbles, as these may cause a blank spot or sideways transfer of the DNA fragments.

A piece of nitrocellulose filter (Schleickler & Schull), was cut to the size of the gel plus 5mm larger all round. A clean scalpel blade was used and 'Triflex' gloves were worn throughout. The filter was only handled by the edges as the chalk from the gloves affected its wetting properties. After cutting an orientation mark on the piece of filter to correspond to the gel, it was floated on top of 2 x SSC until wetted and then submerged. Some 20 x SSC was placed on top of the gel and the soaked filter was laid carefully onto the surface of the gel, so that it slightly overlapped all edges. Again, the presence of air bubbles was checked. A stack of Watmann 3mm paper, about 2-3cm high, was cut to the dimensions of the filter. One piece of Watmann 3mm was soaked in 20 x SSC and laid on top of the filter, making sure no air bubbles were present, then 5 other pieces of Watmann 3mm were laid directly on top of the first one. A glass plate was balanced on top of the stack of paper. After 5-10 mins, all but the first sheet of paper were removed one by one. These were checked for active and even capillaration of the buffer, paying particular attention to look for any short circuiting around the edges of the gel. The rest of the

stack of Watmann 3mm, and a 5cm stack of blotting paper cut to size, were set up on top of the gel. On top of the stack of paper was put a pile of paper hand towels, cut approximately to size and then a glass plate. 'Saran Wrap' or 'Cling Film' was stretched over the whole apparatus and a 500ml bottle filled with water was placed on top to act as a weight. Usually, the reservoir contained about 2 litres of 20 x SSC, this ensured that sufficient buffer was available to pass through the gel for up to 24 hours. Transfer was normally continued for 16-24 hours. After transfer, the filter was rinsed in 2 x SSC to remove the high salt, blotted, dry and baked in a vacuum oven at 80°C for 1½-2 hours.

2.20 DNA HYBRIDISATION TO NITROCELLULOSE FILTERS.

A hybridisation buffer containing 50% formamide, in order to perform all DNA/DNA hybridisations at 37°C, (Casey & Davidson, 1977) was used. A 32p- labelled DNA probe (Section 2.18) after denaturation, was either placed on ice to cool rapidly or immediately added to hybridisation solution. DNA hybridisation was usually performed using 5ml, 10ml or 20ml volumes. After allowing for the volume of the DNA probe, hybridisation solution contained 50% formamide, 2 x SSC (0.3 M NaCl 0.03 M Na Citrate) and 0.1% SDS (Sodium Dodecyl Sulphate). The complete hybridisation mixture, along with the baked nitrocellulose filters to be probed, was set up in a polythene bag, doubly heat sealed with a bag sealer, then taped into a plastic tray and shaken at 37°C for about 16 hours. If required, the DNA probe after hybridisation, was saved and stored at -20°C to be used once or twice again. The filters were rinsed in 2 x SSC to remove excess radioactivity, and washed in a large volume (250ml) of 2 x SSC, 0.1% SDS for 2 hours at 37°C with

4 changes of the wash. They were then given 2 x 5 minutes in 2 x SSC at 37°C, and sometimes a quick rinse in distilled water to further reduce any background radioactivity. The washed and blotted dry filters were sealed inside a polythene bag and set up for autoradiography. 'Dupont Cronex' x-ray cassettes with fast tungstate screens, which enhance the autoradiographic response, were used. The polythene bag containing the filters was placed in the base of the cassette and a sheet of x-ray film (Kodak X-Omat H or Fuji RX) was pre-exposed with a flash-gun and placed on top of the polythene bag with the exposed surface against the intensification screen. Autoradiograms were left to expose for several hours or up to four or five weeks at -70°C.

2.21 SETTING UP OF LIBRARIES OF HIND III AND ECORI DNA FRAGMENTS.

50 μ l (approximately 5 μ g) of C600 DNA was digested with 2 units Hind III or EcoRI in a final volume of 60 μ l for 6 hours, at 37°C. The restricted DNA was loaded onto an agarose bead column (Bio-Gel A - 1.5m, Bio-Rad) in order to remove many of the small DNA fragments and thus reduce the total number of different fragments available for cloning. The agarose beads which have an exclusion volume of less than 10,000 - 1.5 MD were hydrated in distilled H₂O and equilibrated in 3 M NaCl, 0.3 M Na acetate pH5.0. A 10ml pipette, plugged with siliconised glass wool, was used to set up columns of about 9ml volume. A thin layer of equilibrated Sephadex G-100 (fine) was used to stabilise the top of the column. Blue dextran marker dye (Sigma) which has an average molecular weight of 2 megadaltons, was run down the column to calibrate the time taken (number of drops) for excluded molecules to be eluted. Restricted DNA was eluted with 3 M NaCl, 0.3 M Na acetate pH5.0 and two-drop samples were collected across the

expected elution peak. 20ul of each sample was run on an agarose gel (Section 2.16) to precisely locate the elution peak and check the efficiency of the partitioning of small DNA fragments.

No DNA below about 2 kilobases was detected in the most concentrated DNA samples of the elution peak. These samples were pooled and the DNA was recovered by precipitation with an equal volume of ethanol at -20°C . After the DNA was pelleted by spinning in a microcentrifuge for 5 minutes and dried in a vacuum dessicator, the DNA pellet was resuspended in 5ul of a restriction reaction mix containing 0.5ug of digested pBR325 (Bolivar, 1978) DNA. To this was added 1ul of 10 x ligase cocktail (Section 2.14), 3ul of distilled H_2O and 1ul of T4 DNA ligase. The molecules were ligated for 16 hours at 15°C . Then 0.2ml aliquots of competent C600 cells (Section 2.15) were transformed with 1ul of the ligation mix and transformants were selected in LB antibiotic plates. Transformants were screened for either, chloramphenicol sensitivity to locate EcoRI recombinant plasmids, or tetracycline sensitivity to detected Hind III recombinants. Colonies harbouring putative pBR325 recombinant molecules, were picked using sterile toothpicks and stabbed in ordered arrays into LB antibiotic plates.

2.22 COLONY HYBRIDISATION.

A modified method of the Grunstein & Hogness (1975) colony hybridisation screening procedure was used. Colonies from the library of cloned Hind III or EcoRI DNA fragments (Section 2.21) were stabbed in ordered arrays onto LB plates containing antibiotic. Master and test plates were made in this way, rather than by replica plating, which, due to smudging of colonies, limited the number which could be screened

upon one plate. A piece of nitrocellulose filter (Schleickler and Schull) cut to just fit inside the agar plate was carefully laid onto the surface of the test plates. Both the master and test plates were incubated for 14-16 hours at 37°C. The master plates were then stored at 4°C. An orientation mark was put on the filters and using forceps they were carefully removed from the agar and placed colony side up on a pad of blotting paper, soaked with the following solutions:

0.5 M NaOH for 7 minutes; followed by 1.0 M Tris-HCl pH7.4 for 2 minutes then a second pad soaked with this solution, 2 minutes; finally, 1.5 M NaCl, 0.5 M Tris-HCl pH7.4 for 4 minutes.

The filters were then blotted dry, sandwiched between Watmann 3mm paper and baked under vacuum at 80°C for 1½-2 hours. If the filters were found, after baking, to have stuck to the Watmann paper (due to dried down cell debris), the filter and adhered paper were dropped into a pool of hybridisation solution (Section 2.20) this quickly allowed the two to be separated. Any attempt to prise them apart created holes in the filter. Approximately 2.5ml of hybridisation solution per filter up to a maximum of 20ml, was successfully employed. Conditions for hybridisation are given in section 2.20.

2.23 PREPARATION OF MINICELLS.

Minicells were purified and labelled with ³⁵S-methionine by the method of Reeve (1977). Transformants (see Section 2.15) of the minicell producing strain, DS410, were used to inoculate a 2 litre overnight culture in LB containing antibiotic. The cells were harvested by centrifuging at 10,000r.p.m. for 10 minutes, 4°C, in 500ml buckets, GS3

rotor (Sorvil). 20ml of the supernatant was retained and used to resuspend the cell pellets by vortexing, up and down pipetting then vigorous stirring with a magnetic flea and stirrer for at least 10 minutes. These and all subsequent operations, were performed at 4°C or on ice. All of the resuspended cells were loaded into 4 x 35ml sucrose gradients. These were prepared by freezing 35ml of 20% sucrose, M9 glucose medium in 50ml clear polycarbonate centrifuge tubes and allowing these to thaw overnight at 4°C. The loaded sucrose gradients were spun at 5,000r.p.m. for 20 minutes in a lightweight, aluminium swing out rotor, HES4. Using a 10ml syringe and a G19 hyperdermic needle in which a right angle bend had been made, the majority of the minicell band was removed from the middle of the gradients. The pooled minicells were pelleted by centrifuging at 13,000r.p.m. for 10 minutes, and then resuspended in 5ml of M9 glucose by vortex mixing for at least 1 minute. Banding of the minicells was repeated using firstly two sucrose gradients and then one, with pelleting and resuspension in 2.5ml M9 glucose after the second banding. Minicells from the last gradient were pelleted at 8000r.p.m. and resuspended in 1ml M9 glucose, 15% glycerol. Aliquots of a fixed number of purified minicells were frozen at -70°C in snap-cap tubes (Sarstedt). To calculate the volume required for each aliquot, the OD⁶⁰⁰ of 0.1ml of the minicell preparation diluted with 0.9ml of M9 glucose, was measured, and a volume which would give an OD⁶⁰⁰ of 0.2 in 1ml, was used.

2.24 ³⁵S-METHIONINE LABELLING OF MINICELLS.

An aliquot of purified minicells was allowed to thaw at room temperature. 1ml of M9 glucose medium was added, mixed by vortexing, and the minicells were pelleted by spinning in a microcentrifuge for 2 minutes. The

minicells were resuspended in 0.1ml M9 glucose and incubated at 37°C for 1 hour to allow degradation of parental cell messenger RNA and expression of plasmid encoded genes. 10µl of ³⁵S - methionine was diluted to one fifth volume in 25% Difco Methionine Assay mix and this was added to the minicells. Incubation was continued at 37°C for a further 3 hours. The labelled minicells were pelleted, as before, and washed in 0.5 M Tris-HCl pH6.8, 25µl of minicell loading buffer (0.6g SDS, 1ml 2-Mercaptoethanol, 4ml glycerol, 1.25ml 0.5 M Tris-HCl pH6.8 3.75ml H₂O) was added to the final minicell pellet, this was heated at 100°C in a water bath for 3 minutes, before loading into a gradient SDS polyacrylamide gel (Section 2.26).

2.25 MAXICELLS.

A method for studying plasmid encoded proteins within ultraviolet light irradiated cells, maxicells (Sancar and Rupp, 1979) was employed. An overnight culture in antibiotic, of a transformant (see Section 2.15) of the E. coli strain CSR603, was diluted 1:50 into M9 salts (x 1) containing 0.4% glucose, 1% casamino acids and 0.1mg/ml thiamine. The cells were grown at 37°C to a concentration of 2×10^8 cells/ml, approximately, OD⁵⁴⁰ of 0.2 in this medium. 10ml of the culture was transferred to a sterile glass petri-dish. After pre-warming a germicidal ultraviolet lamp, the petri-dish was placed under this lamp and the lid was taken off for 5 seconds then quickly replaced. With the particular lamp used, the cells will have had an ultraviolet dose of 5 Joules/m². The irradiated cells were transferred to a 100ml conical flask and shaken at 37°C for 1 hour, after which D-cycloserine (Sigma) was added to a final concentration of 0.1mg/ml. Cycloserine solution was made up fresh

at 10mg/ml in 0.1 M phosphate buffer pH8.0, and filter sterilised. Incubation at 37°C was continued for 14-16 hours, then the cells were collected by centrifuging at top speed in bench centrifuge for 10 minutes. The cells were washed in 2 x Hershey salts, then resuspended in 5ml Hershey sulphate free medium and shaken at 37°C for 1 hour. 5-10 μ l of ³⁵S-methionine was put into a snap-cap tube (Sarstedt) and 1ml of the 'maxicells' in sulphate free medium was added, mixed and incubated at 37°C for 1 hour. The labelled maxicells were harvested by spinning in a microcentrifuge and treated in the same way as labelled minicells (see end of Section 2.24).

2.26 GRADIENT SDS POLYACRYLAMIDE GELS.

Solutions:

Stock Acrylamide: 30g acrylamide, 0.8g NN'Methylene bis acrylamide. Made up to 100ml with distilled H₂O, filtered and stored in a fridge.

Upper Tris (x 4): 6.06g Tris-Base, 4.0ml 10% SDS. Dissolved in 80ml distilled H₂O, adjusted to pH6.8 with concentrated HCl and then made up to 100ml.

Lower Tris (x 4): 18.17g Tris-Base, 4.0ml 10% SDS. Dissolved in 80ml distilled H₂O, adjusted to pH8.8 with concentrated HCl and then made up to 100ml.

Electrophoresis Buffer: 3g Tris-Base, 14.4g Glycine, 10ml 10% SDS made up to 1 litre with distilled H₂O.

COMPOSITION OF GRADIENT GEL SOLUTIONS.

Solution	7%	20%
Stock Acrylamide	3.5ml	10ml
Lower Tris	5.3ml	5.3ml
Distilled H ₂ O	6.5ml	
Amps ¹	25 μ l	25 μ l
10% SDS	0.2 ml	0.2ml
Temed ²	5 μ l	3 μ l

STACKING GEL:

Stock Acrylamide	1.0ml
Upper Tris	2.5ml
Distilled H ₂ O	6.5ml
Amps ¹	40 μ l
Temed ²	15 μ l

1. 10% Ammonium Persulphate made up fresh.
2. N,N,N',N' - Tetramethylethylenediamine.

The gel system, essentially that of Laemmli (1970) was set up as follows:

Two glass plates, 25.5 x 16cm, one of which was knotted to fit the upper buffer tank of an electrophoresis apparatus, were thoroughly cleaned and left upright to dry. The plates, separated by two 0.1cm side spacers slightly greased on their outside edge, were clamped firmly together using 'Bulldog' clips. This was supported vertically

with the bottom end of the plates resting in a narrow, shallow trough. 20% acrylamide gel solution was poured into the trough to seal off the end of the plates, this was left to set. All the ingredients, except Temed, for 7% and 20% gel solutions were put into 100ml Buckner flasks and degassed using a water vacuum pump. Temed was then added to the two solutions and the 20% solution was poured into the outlet chamber of a gradient maker. The connection channel was opened and a small amount of solution was allowed to run into the other chamber to check that the connection was clear. This solution was returned to the outlet chamber and the 7% solution was poured into the empty chamber. A magnetic flea, activated by a magnetic stirrer supported under the gradient maker, stirred the solution in the outlet chamber continuously. A clamp on the outlet tube was removed and the connection channel was opened. The gel was poured under gravity with the outlet tube taped at the top, and mid-point, of the plates, with the opening just inserted between the plates. When the separating gel was poured, iso-butanol was carefully pipetted down the inside edges of the plates, to form an overlay on top of the gel. After about 1 hour a layer of buffer had appeared between the gel and iso-butanol, indicating that the gel had set. The iso-butanol and buffer were discarded. The top of the gel was rinsed several times with distilled H₂O and twice with upper Tris (x 1). Stacking gel, also degassed, was pipetted onto the top of the separating gel and a comb was inserted, avoiding entrapping air bubbles. This set in about 15 minutes, and electrophoresis buffer was placed around the teeth before removing the comb. The trough and excess gel were removed from the bottom of the plates, which were then clamped into position on the upper tank of the electrophoresis apparatus. Electrophoresis buffer was poured into the upper and lower tanks. Entrapped air bubbles were freed from the bottom end of the plates.

Samples and molecular weight standards (low molecular weight calibration kit, Pharmacia Fine Chemicals) were loaded into the wells using a 50 μ l Hamilton syringe. The gel was run towards the anode at 150V, on constant voltage, for 16-18 hours. After which, the bromophenol blue dye had reached the bottom of the gel. The plates were removed from the apparatus and prised apart (the gel usually stuck to one of the plates). Either the separating gel was encouraged to peel off into some fixing and staining solution, or, the glass plate carrying gel was immersed in this solution. To fix and stain the gel, about 500ml of the following solutions was used for 30 minutes at 37°C with gentle stirring:

60ml methanol, 240g trichloroacetic acid, 72g 5-sulphosalicylic acid, 2.24g comassie brilliant blue, made up to 2 litres with distilled H₂O. This solution was re-used several times. The gel was then destained with 2 x 500ml of 5% methanol, 7.5% acetic acid at 37°C for about 2 hours, or until the protein bands, and in particular, the molecular weight standards could clearly be detected. The gel was then dried down for 1-1½ hours, onto a sheet of Watmann 3mm paper using a Bio-Rad gel drier and following the manufacturers instructions. By manouvering the gel onto a glass plate, greatly aided its transfer onto Watmann paper. A piece of 'Saran Wrap' or 'Cling Film' was used to cover the gel while drying, and removed before autoradiography. The autoradiograms were set up in a similar manner as described in Section 2.20, except that the pre-flashed side of the x-ray film was laid adjacent to the surface of the gel.

RESULTS.

CHAPTER 3.

3.1 INTRODUCTION.

Molecular cloning of genetically 'silent' regions has the particular disadvantage of precluding direct selection of recombinant DNA molecules by way of a scoreable phenotype. The DNA may nevertheless be cloned if DNA representing homologous sequences within, or adjacent to the region to be cloned, is available for use as a specific hybridisation probe (Grunstein & Hogness, 1975). Maniatis et al (1978) have shown how overlapping DNA sequences can be used in a stepwise manner to clone large sections of eukaryotic genomes. Since a physical method of detecting the desired cloned fragments is used in the above techniques, this approach was chosen to isolate 'silent' region DNA from the E.coli K12 chromosome.

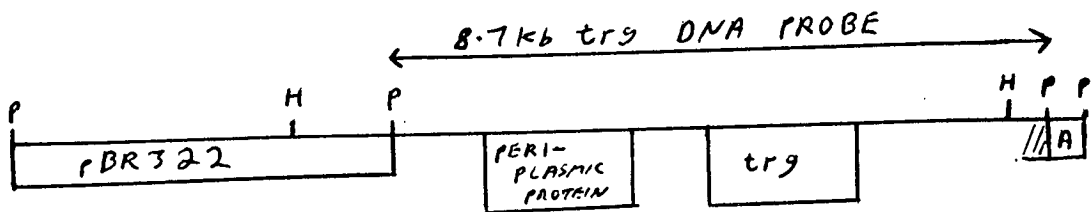
At the beginning of my study into the genetically 'silent' terminus region, DNA associated with the defective lambdoid prophage rac had been isolated in a plasmid cloning vehicle (Kaiser & Murray, 1979) by screening libraries of recombinant plasmids carrying DNA fragments derived from an F-prime factor. My original intention was to use rac DNA, and later, also kim DNA (Kaiser, 1980; Bouche' et al, 1982) as hybridisation probes and as a starting point for 'walking' (Maniatis et al, 1982) along the chromosome towards the potentially interesting ter C locus as rac and kim probably flank the DNA replication terminus (Bouche' et al, 1982). Also, as a source of chromosomal DNA with which to set up libraries of recombinant plasmids I considered using the F-prime factors F'123 and F'506. F'123 carries rac prophage DNA (Low, 1972; Kaiser & Murray, 1979), but does not extend to trg at 31 mins. on the genetic map, whereas F'506 does complement trg mutants (Harayama et al, 1979). In addition, these two F-primes have the same

point of origin to that of Hfr B7 (Low, 1972) and it was hoped that by determining the extent of overlap, if any, of their chromosomal DNA content and by sizing the complete molecules by electron microscopy, information regarding the physical size of the terminus region would have been obtained. But, DNA of these two large plasmids proved to be difficult to isolate and their use as sources of DNA for cloning was abandoned in favour of E.coli chromosomal DNA.

DNA from the rac and kim regions was not, in fact, used to start 'walking' into the ter C region. Instead, a plasmid, p^{TH51}, that had been isolated by complementation of mutants at the trg locus (Harayama et al, 1982) became available and was used as the initial probe. The experiments described below allowed the molecular cloning of chromosomal DNA from the E.coli K12 ter C region. Hybrid, p^{BR325} (Bolivar et al, 1977) plasmids were isolated by screening libraries of C600 colonies containing recombinant vector molecules carrying either Hind III or EcoRI restriction endonuclease fragments of E.coli K12 chromosomal DNA. Hybridisation probes for the colony hybridisation procedure (Grunstein & Hogness, 1975) were made initially from an 8.7 kb Pst I fragment from p^{TH51} known to carry the trg gene (Harayama et al, 1982) then further probes were prepared from the newly obtained ter C region DNA fragments. These fragments were recovered from agarose gels by the 'freeze-squeeze' method (see Materials and Methods).

Restriction endonuclease mapping and DNA/DNA hybridisation techniques were used to physically characterise the cloned ter C region DNA. The restriction map shown in Fig. 3.13 gives the labels assigned to C600 DNA fragments on the chromosome that are described in this text, this was constructed from:

PHYSICAL MAP OF γ TH51



HATCHED REGION CONTAINS A SMALL AMOUNT OF COLEI DNA OF UNKNOWN LENGTH.

IT IS NOW KNOWN THAT ON THE CHROMOSOME THE PST I FRAGMENT LABELLED A, IS ATTACHED TO THE LEFT HAND PST I SITE OF THE trg DNA SHOWN ABOVE. THIS 0.5 KB PST I FRAGMENT IS DESIGNATED H/P-VI IN FIG. 3-7 AND P VI IN FIG. 3-13.

THE DIAGRAM WAS ADAPTED FROM HARAYAMA et al., 1982.

- a) the restriction digest data of three ter C region plasmids, pPM1000, pPM2000 and pPM4000, isolated by chromosome 'walking'; these are described individually in the text.
- b) C600 DNA fragments which hybridised to DNA probes prepared from cloned ter C region DNA.

3.2 HIND III AND EcoRI FRAGMENTS IN C600 DNA WHICH HYBRIDISED WITH THE Pst I trg FRAGMENT.

Fig. 3.1 shows that the 8.7kb PstI fragment of pTH51 hybridised to a 9.6 kb Pst I fragment (PII in Fig. 3.13). This homology is shared between a 23 kb (HI) and a 10.5 kb (HII) Hind III fragment and totally contained within a 20 kb EcoRI fragment (RI). This suggests that there is only one Hind III and one EcoRI cleavage site within the C600 DNA 9.6 kb Pst I fragment (PII) which presumably carries the trg gene. This has been shown for other E.coli K12 strains (Harayama & Hazelbauer 1982; Bouché et al, 1982).

3.3 ISOLATION OF TWO PLASMIDS THAT HYBRIDISED TO A trg DNA PROBE.

Two plasmids sharing homology with the 8.7 kb Pst I trg DNA fragment probe were isolated from a library of pBR325 recombinant molecules carrying C600 DNA Hind III fragments after screening by the colony hybridisation procedure of Grunstein and Hogness (1975) (see Materials and Methods). DNA mini-preps (Materials and Methods), were subjected to agarose gel electrophoresis as shown in Fig. 3.2. One of the

Fig. 3.1.

Restriction fragments of C600 DNA that hybridise to a pTH51³²P-labelled DNA probe. Sizes are given in kb. Tracks; 1. Pst I, 2. EcoRI , 3. Hind III. The Hind III treated DNA is only partially digested and does not show a 10.5kb Hind III fragment that hybridises to a DNA probe of the 8.7kb Pst I fragment of pTH51 (not shown). This autoradiogram was kindly supplied by Ian Oliver (this Lab).

Fig. 3.2.

Agarose gel electrophoresis of Hind III digests of DNA mini-preps prepared from overnight cultures of colonies that showed homology to a DNA probe of the 8.7kb Pst I fragment of pTH51 (tracks 2 and 4,) using the Gr[^]ustein and Hogness colony hybridisation method. Track 1 Hind III digest of lambda CI857 DNA. DNA fragment sizes are in kb. (Tracks 3,5,6 and 7, Hind III digests of mini-prep DNA obtained from colonies lying adjacent to the colonies showing homology to the DNA probe). Experimental procedures are described in the Materials and Methods.

figure 3-1

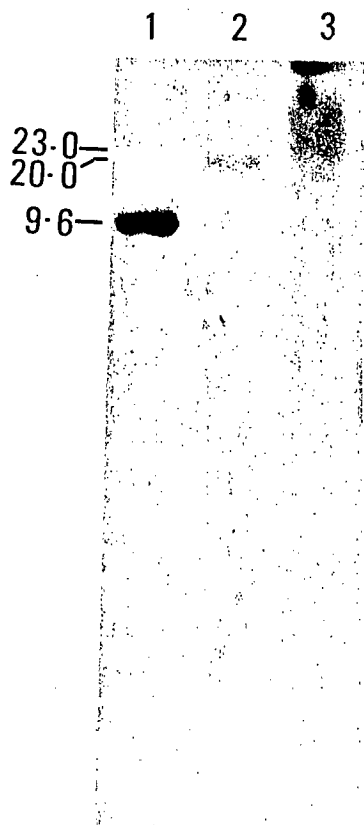
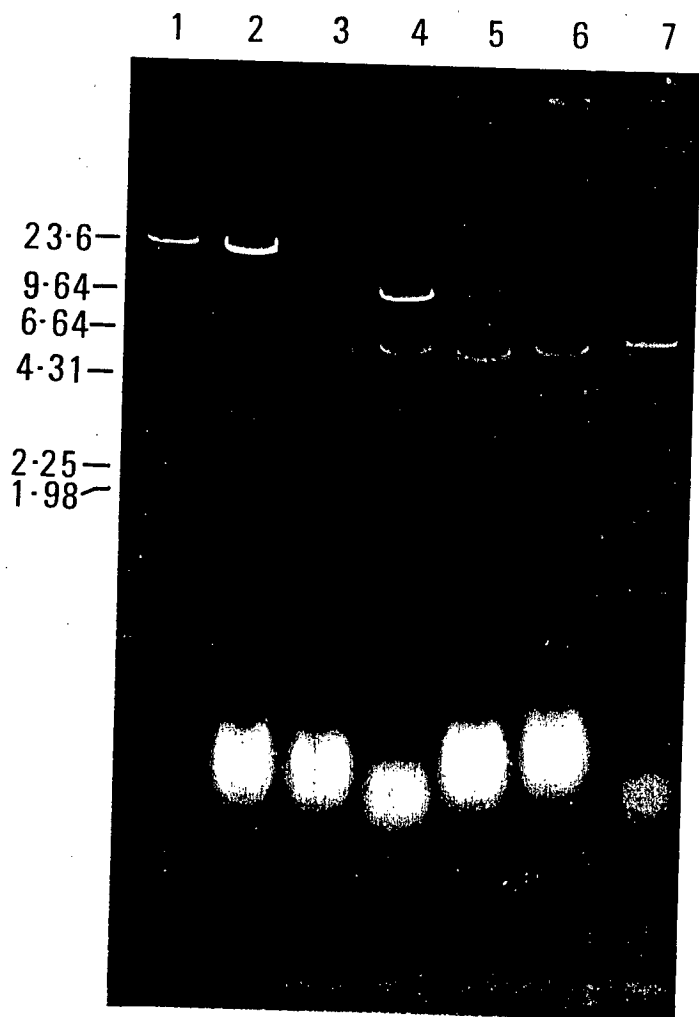


figure 3-2



plasmids contains a Hind III fragment which migrates with a mobility similar to that of the largest Hind III fragment size marker of λ CI857 DNA and is therefore about 23 kb in size; this was designated pPM1000. The other plasmid, pPM2000, has an insert of approximately 11 kb of DNA. Since the sizes of cloned Hind III fragments in pPM1000 and pPM2000 are in accordance with the results obtained previously in Fig. 3.1, they were presumed to be the plasmids desired and investigated further.

3.4 C600 FRAGMENTS WHICH HYBRIDISE WITH pPM1000 & pPM2000 DNA PROBES.

Digests of C600 DNA were tested to find restriction fragments which would hybridise to a pPM1000 or a pPM2000 32 P-labelled DNA probe (Fig. 3.3). As expected, fragments which hybridised to the trg DNA probe (Fig. 3.1) also hybridised to the homologous sequences present in pPM1000 and pPM2000, thus further confirming the origin of the cloned chromosomal DNA.

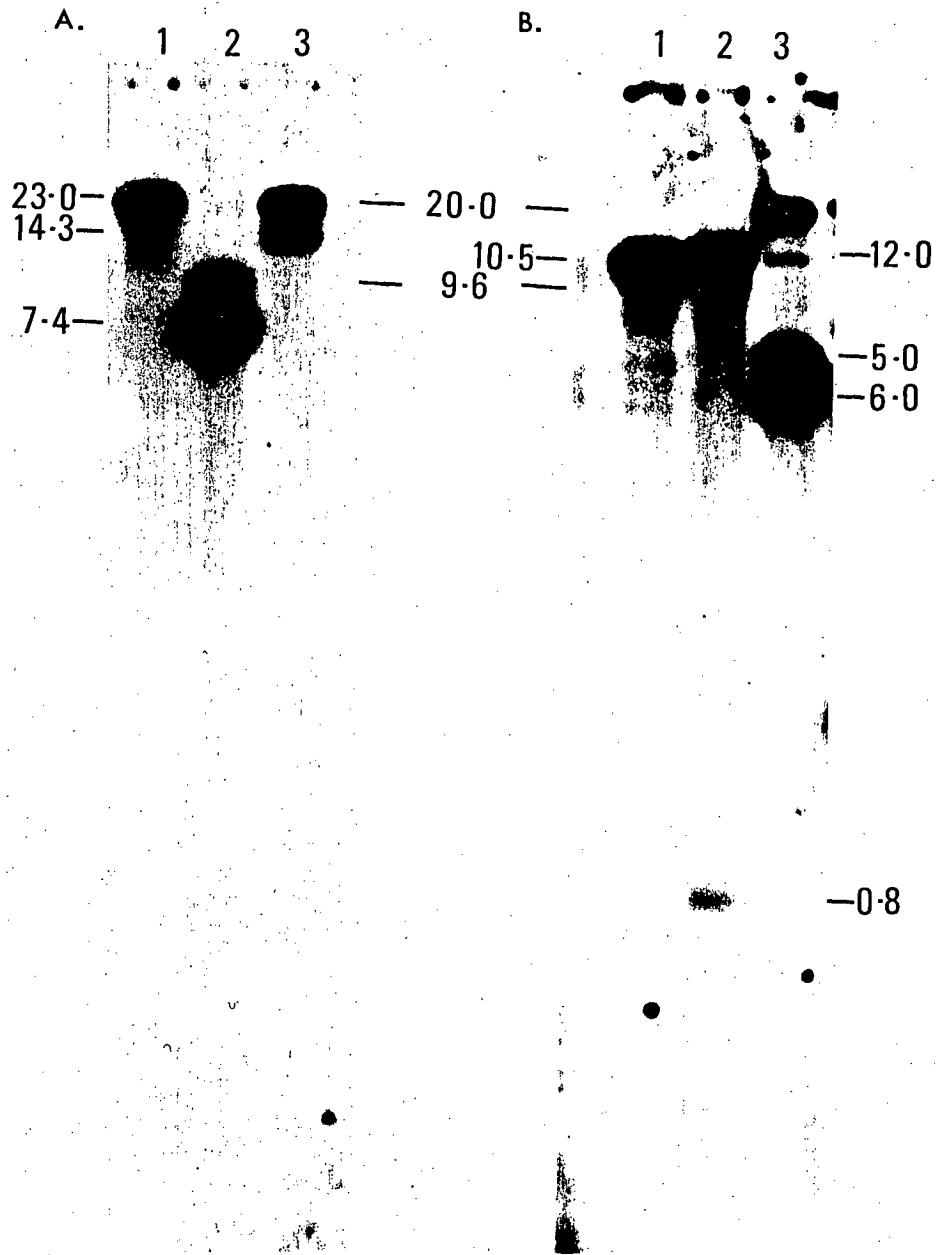
The pPM1000 DNA probe is shown in Fig. 3.3 to hybridise to a 14.3 kb EcoRI fragment (RII) and since all of the 9.6 kb Pst I trg fragment (PII) is contained within a 20 kb EcoRI fragment (RI) then the 14.3 kb (RII) EcoRI fragments must overlap with the end of the 23 kb Hind III fragment (HI) distal to the trg gene. Since no other EcoRI fragment hybridises with the pPM1000 DNA probe, the 20 kb (RI) and 14.3 (RII) EcoRI fragments are likely to be adjacent in the chromosome, Fig. 3.13

Hybridisation of the pPM1000 DNA probe to ^{the} 7.4 kb Pst I fragment appears to be very strong and is probably due to homology with two similarly sized Pst I fragments (PIIIa and PIIIb) on the chromosome. Also, 8.1 kb

Fig. 3.3.

C600 DNA restriction fragments that hybridise to (A). pPM1000 and (B) pPM2000, plasmid DNA probes. Tracks, 1. Hind III, 2. Pst I, 3. EcoRI. Fragment sizes are in kb. See Materials and Methods for experimental procedures.

figure 3-3.



of DNA homology is shared with the Pst I trg fragment (PII) (see following section) and a further 7.4 kb of DNA would be insufficient to cover the 23 kb of DNA carried by pPM1000, whereas an additional 14.8 kb (two adjacent 7.4 kb Pst I fragments) would be just more than enough. In Hind III/Pst I double digests of pPM1000 DNA the presence of a 6.9 kb Hind III/Pst I fragment (Table 3.1) indicates that there is a Pst I fragment in C600 chromosomal DNA which could extend beyond the Hind III site by about 0.5 kb of DNA.

A pPM2000 DNA probe hybridises to three Pst I fragments, 12 kb, 9.6 kb and 0.8 kb (Fig. 3.3). One of these Pst I fragments is the expected trg Pst I fragment (PII) and hybridises more weakly than the 12 kb fragment (PI) due to a smaller overlap of DNA sequence at the right hand end of the 10.5 kb Hind III insert of pPM2000 (see Fig. 3.13). The 0.8 kb Pst I fragment is present in Hind III/Pst I digests of pPM2000 (Table 3.1) and therefore lies internal to the 10.5 kb Hind III fragment and presumably separates the 9.6 kb (PII) and 12 kb (PI) Pst I fragments in the chromosome.

As expected from the reasoning given above with pPM1000, a 20 kb EcoRI fragment (RI) hybridises to the pPM2000 DNA probe. Of the two other EcoRI fragments showing homology (Fig. 3.3), the 5.0 kb fragment (RIV) lies internal to the 10.5 kb Hind III fragment and is present in EcoRI digests of pPM2000 (Table 3.1) leaving the 6.0 kb fragment (RIII) later shown to possess 1.95 kb of homology with the 10.5 kb Hind III fragment (see following sections) and is expected therefore to extend about 4 kb beyond the Hind III site on the chromosome.

3.5 RESTRICTION ENDONUCLEASE MAPS OF pPM1000 AND pPM2000

Detailed physical maps of pPM1000 and pPM2000 were constructed by a combination of restriction mapping and DNA/DNA hybridisation methods. The data obtained is given in Table 3.1 and Fig. 3.4 the complete maps for the enzymes Hind III, EcoRI, Pst I, Sal I, Pvu II and BamHI are shown in Fig. 3.7.

pPM1000.

In Fig. 3.4, an 8.1 kb Hind III/Pst I fragment (H/P-I, Table 3.1, Fig. 3.7) is shown to contain all of the homology with the trg DNA probe in digests of pPM1000 DNA. This defines the extent of the overlap of the 9.6 kb trg Pst I fragment (PII) with the 23 kb Hind III fragment (HI) from the ter C region of the E.coli K12 chromosome (see Fig. 3.13). From the hybridisation of pPM1000 to C600 DNA Pst I digests (described earlier) it can be inferred that the 7.4 kb fragment (H/P-II) of pPM1000 is a Pst I fragment lying internal to the chromosomal DNA insert, whereas the 6.9 kb fragment (H/P-III) has Hind III and Pst I ends. A 0.5 kb Pst I fragment (H/P-VI) which separates the 9.6 kb (PII) and 7.4 kb (PIIIa) Pst I fragments on the chromosome (Fig. 3.13) cannot be seen in Fig. 3.3, but was later subcloned from pPM1000 (see Chapter 4) and its location was mapped by probing the plasmid clone, designated pPM1011, to C600 DNA digests Fig. 3.5.

As expected a 23 kb Hind III fragment (HI) hybridises to the pPM1011 DNA probe. Three Pst I fragments can be seen in Fig. 3.5 to hybridise weakly to this DNA probe. The smallest of these Pst I fragments, 0.5 kb, is the Pst I fragment that has been cloned in pPM1011. The size of the two larger Pst I fragments, 7.9 kb and 10 kb, is consistent

Fig. 3.4.

Digests of pPM1000 and pPM2000 plasmid DNAs and hybridisation with a DNA probe made from an 8.7 Pst I fragment isolated from pTH51. (A). pPM1000. Track 1. Hind III digest of lambda CI857 DNA; tracks 2 and 3, Sal I digest; tracks 4 and 5 Sal I/Pvu II double digest; tracks 6 and 7 Pvu II digest; tracks 8 and 9, Hind III/Pst I double digest. (B). pPM2000. Track 1. EcoRI digest of Lambda CI857 DNA; tracks 2 and 3, Sal I digest; tracks 4 and 5, Sal I/Pvu II double digest; tracks 6 and 7 Pvu II digest; tracks 8 and 9 Hind III/Pst I double digest. (C). Agarose gel of pPM2000 restriction digests: track 1. Hind III digest of lambda CI857 DNA, track 2. EcoRI digest; track 3, Hind III/EcoRI double digest; track 4, Hind III/BamHI double digest; track 5, BamHI digest. Molecular weight standards are given in kb. Digests in (A) and (B) were separated on the same agarose gel and were transferred to a nitrocellulose filter before hybridisation and autoradiography. Experimental procedures are described in the Materials and Methods. The autoradiogram was exposed for about 16 hours.

figure 3-4

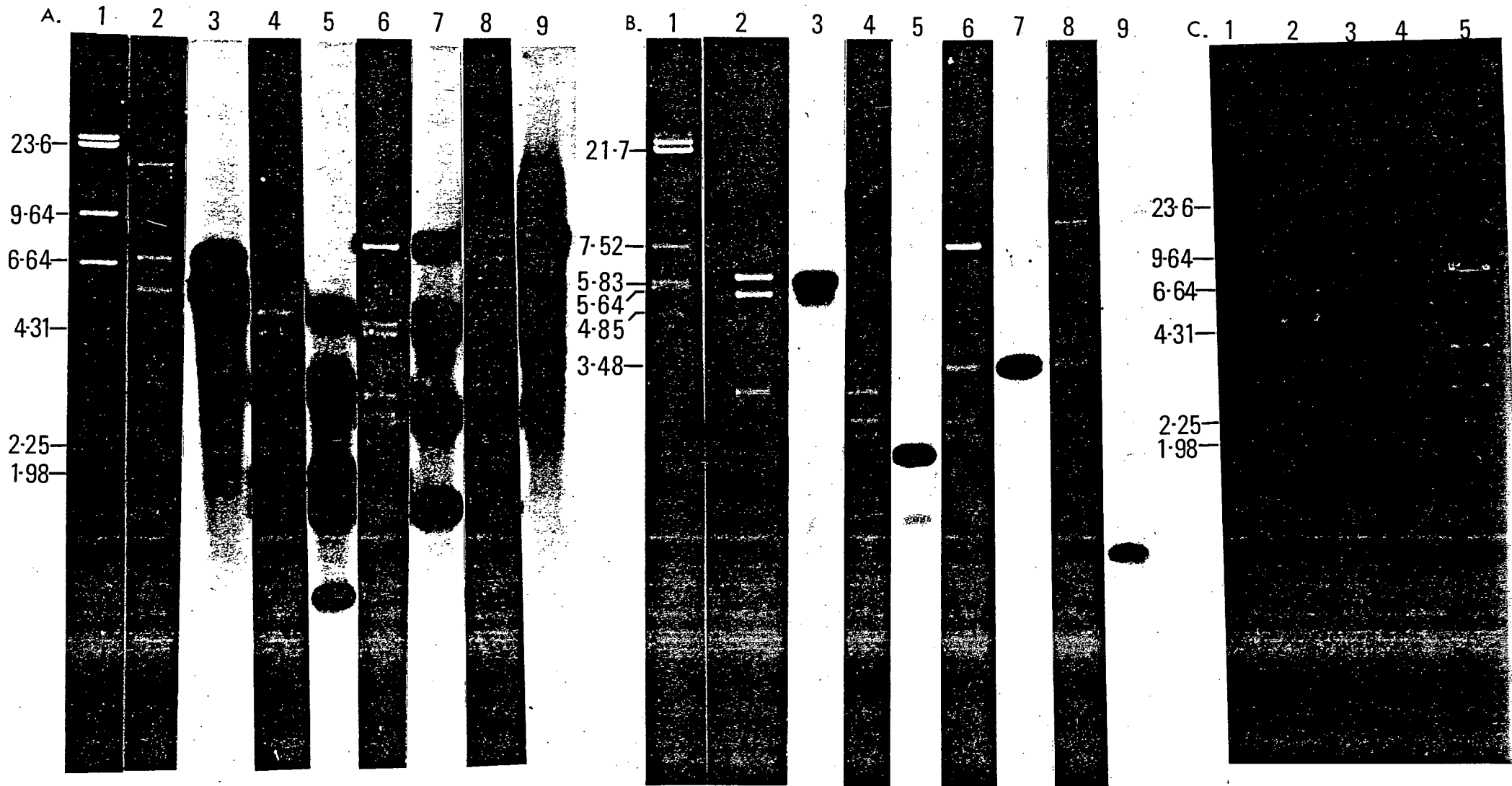


TABLE 3.1.

pPM1000								pPM2000								
H	H/E	E	H/P	Pv	S	Pv/S	B	H	H/E	E	H/P	Pv	S	Pv/S	B	H/B
23.0	16.5	21	8.1	7.55	17.0	4.85	()	10.5	5.0	6.7	8.5	7.5	6.1	3.1	7.8	5.5
6.0	6.5	8.0	7.4	4.55	6.8	4.3	(0.5)	6.0	4.75	5.0	3.55	3.55	5.45	2.6	3.75	3.75
	4.75		6.9	4.3	5.6	3.1			3.55	4.8	2.45	2.6	3.1	2.45	2.85	2.5
	1.25		3.55	3.0		3.0			1.95		1.4	2.45	1.3	2.25	2.3	2.3
			2.45	2.75		2.6d			1.25		0.8	0.7	0.9	1.4d		2.25
			0.5	2.6		1.9								1.3		(0.35)
				1.75d		1.75d								0.9		
				0.6		1.6								0.7		
						1.2								0.5		
						0.6										

Sizes of DNA restriction fragments, given in kb, of the plasmids pPM1000 and pPM2000, obtained from analysis of the agarose gel shown in figure 3.4. (d) doublet of unsolved fragments. Sizes not determined ()

Enzyme abbreviations given in text.

with them resulting from partial digestion of the C600 DNA, giving fragments which carry the 0.5 kb Pst I fragment (PVI) and either, the 7.4 kb (PIIIa) fragment to the right of the (PVI) fragment, or, the 9.6 kb (PII) fragment to the left of the (PVI) fragment, on the chromosome, see Fig. 3.13.

A single Pvu II fragment carries the homology to the 0.5 kb Pst I fragment of pPM1011, Fig. 3.5. This 7.5 kb Pvu II fragment (PvII) is therefore expected to bridge the three Pst I fragments (PII), (PVI) and (PIIIa) on the chromosome as shown in Fig. 3.13.

That a 20 kb EcoRI fragment (RI) shares homology with pPM1011, Fig. 3.5, confirms the overlap between fragment (RI) and the left hand end of fragment (HI) on the chromosome, see Fig. 3.13.

The arrangement of the Sal I sites in Fig. 3.7 allows two (S-II and S-III) out of the three Sal I fragments to hybridise with the 9.6 kb Pst I trg fragment (PII). With one of the Sal I fragments (S-III) see Fig. 3.7) the overlap is greater and explains the stronger hybridisation of the trg DNA probe to the 5.6 kb Sal I fragment, seen in Fig. 3.4. (In one of the two possible orientations of the pBR325 vector molecule, there is barely sufficient DNA for a 5.6 kb fragment to extend from the Sal I site of pBR325 to the chromosomal DNA insert of pPM1000, therefore the opposite orientation is the most likely.)

In order that all of the four Pvu II fragments (Pv-I, II, V and VIIa) which hybridised to the trg DNA probe, can share DNA homology with the 8.1 kb Hind III/Pst I fragment (H/P-I), the 4.55 kb (Pv-II) fragment must carry the junction of the vector and chromosomal DNA,

Fig. 3.5.

C600 DNA fragments that hybridised to a pPM1011 plasmid DNA probe, the sizes of which are given in kb. Tracks; 1. Hind III, 2. EcoRI 3. Pst I, 4. Pvu II. Autoradiogram was exposed for 18 hours.

Fig. 3.6

(A) C600 DNA fragments that hybridise to a pPM1008 plasmid DNA probe. The autoradiogram was exposed overnight (16 hours). Tracks 1. Pvu II, 2. Pst I, 3. EcoRI, 4. Hind III.

(B) Restriction fragments of pPM1000 DNA that share homology with pPM1008 DNA. Tracks; 1. Sal I, 2. SalI/Pvu II, 3. Pvu II, 4. Hind III/Pst I . See Materials and Methods for experimental procedures. Autoradiogram exposed for 3 hours.

figure 3-5

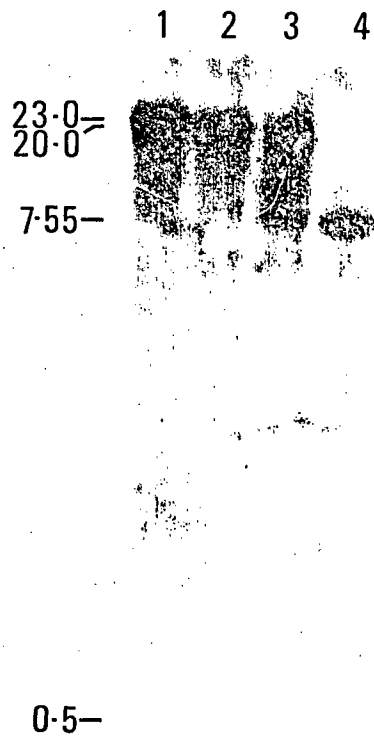
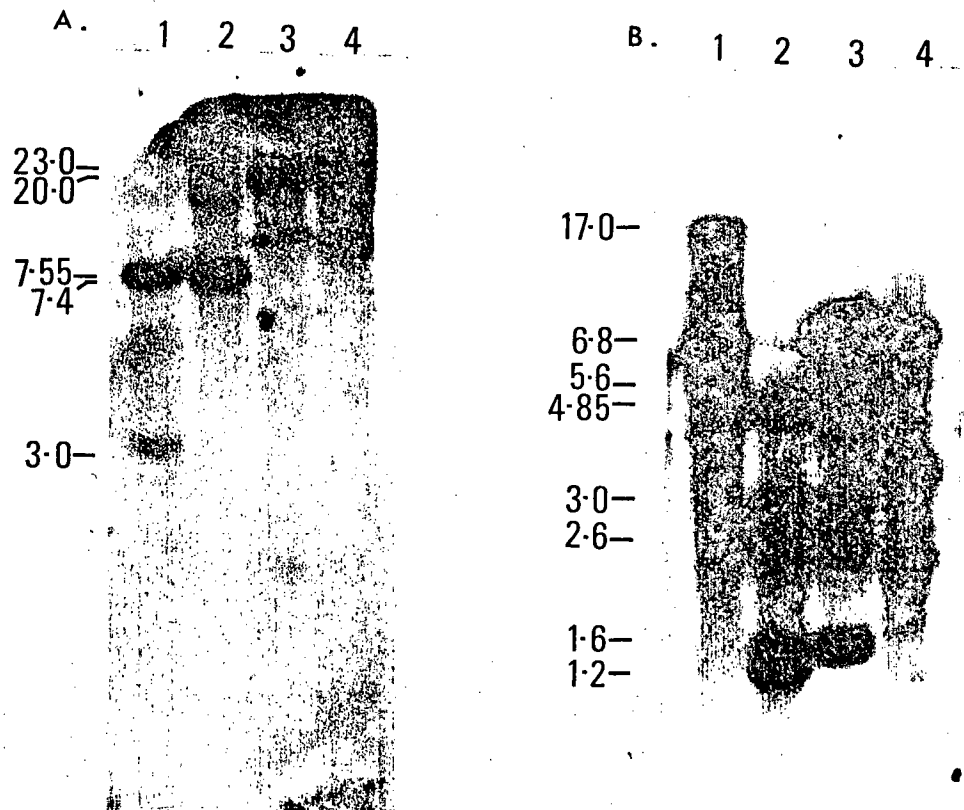


figure 3-6



with the 2.75 kb (Pv-V) and 1.75 kb (Pv-VIIa) fragments lying in between the 4.55 kb (Pv-II) and 7.5 kb (Pv-I) fragments. Positioning of the Pvu II site which generates the 2.75 kb (pv-V) and 1.75 kb (Pv-VIIa) fragments was determined from the above mapping of the Sal I sites (see Fig. 3.7); one of these cleaves the 2.75 kb (Pv-V) fragment to give 1.9 kb (Pv/S-VI) and 1.2 kb (Pv/S-VII) fragments, thus mapping the Pvu II site. Partial digests with Pvu II (data not shown) were used to map the four remaining Pvu II sites, in conjunction with the Pvu II/Sal I double digest data.

The completed restriction map of pPM1000 for the above four enzymes is shown in Fig. 3.7. Confirmation of the position of several Pvu II sites was obtained by hybridising Pvu II digests of pPM1000 DNA with pPM1008 DNA, a plasmid clone of pBR325 carrying the 7.4 kb (H/P-II) Pst I fragment of pPM1000. The expected Pvu II fragments in pPM1000 and C600 DNA, predicted from the restriction map, hybridised to a pPM1008 DNA probe, as well as those which carry vector DNA homology in pPM1000 Fig. 3.6.

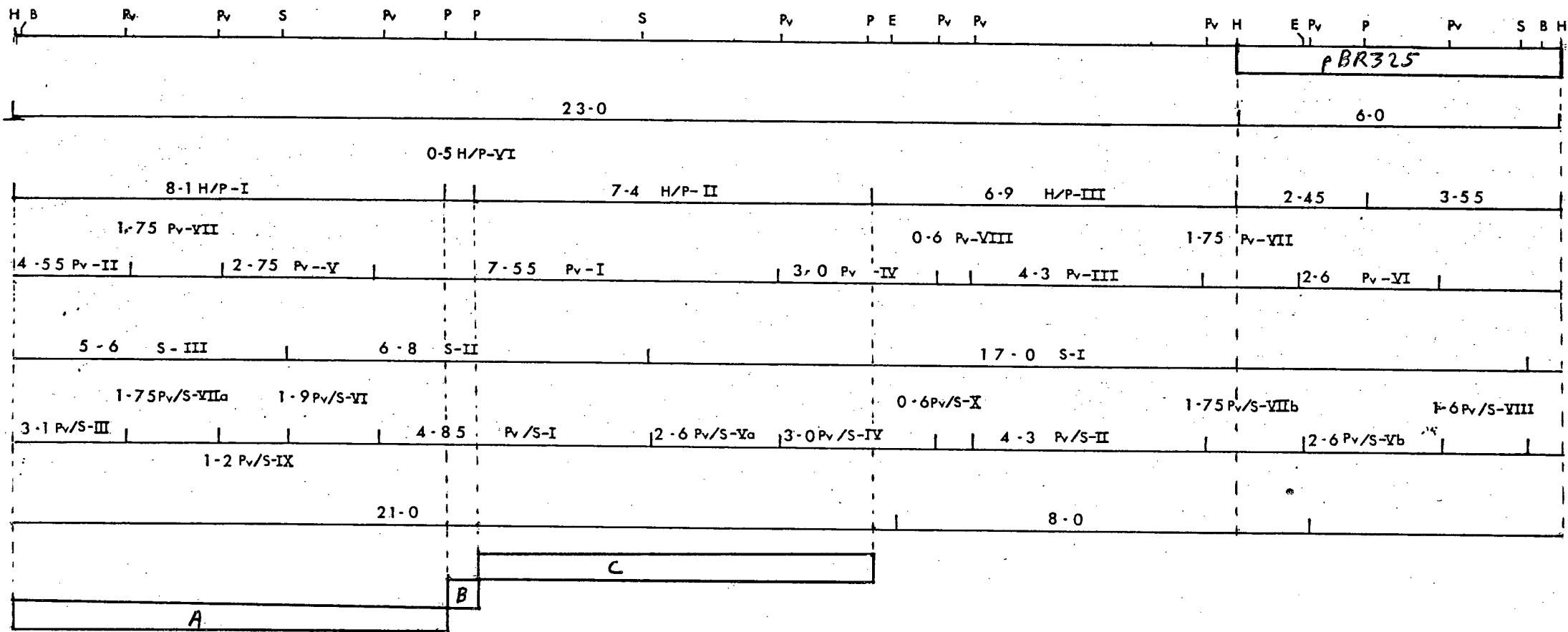
Hybridisation of the pPM1008 DNA probe to a single 7.4 kb Pst I fragment and a 23 kb Hind III fragment confirms the presence of these cloned DNA fragments in C600 DNA. Since pPM1008 hybridised to a single 20 kb EcoRI fragment the right hand EcoRI site of fragment(RI) in Fig. 3.13, probably lies to the right of the 7.4 kb Pst I fragment (PIIIa). Two Pvu II fragments in C600 DNA share homology with the 7.4 kb Pst I fragment (PIIIa). One of these, a 7.55 kb fragment hybridised much more strongly to the pPM1008 probe than a 3.0 kb Pvu II fragment, Fig. 3.6. Reference to the restriction map of pPM1000, Fig. 3.7, shows that a greater overlap exists between the 7.55 kb Pvu II

Fig. 3.7.

Restriction map of pPM1000. E, EcoRI; H, Hind III; P, Pst I; S, Sal I
Pv, Pvu II; B, BamHI. Sizes of DNA fragments are given in kb.

- A. REGION OF DNA ALSO CARRIED BY pTH51, pPM3000, pPM3001
B. " " " " " " pPM1011
C. " " " " " " pPM1008

figure 3-7



fragment (Pv-I) than the 3.0kb Pvu II fragment (Pv-IV). These overlaps are reflected in the extend of hybridisation of these two fragments in the C600 DNA Pvu II digest, Fig. 3.6.

As expected, the two Pvu II fragments (Pv-I) and (Pv-IV) in pPM1000, DNA, hybridised to the pPM1008 DNA probe, Fig. 3.6. The other three Pvu II fragments, which also hybridised to the DNA probe, carry pBR325 DNA homology with pPM1008. Respectively, these Pvu II fragments (Pv-II) (Pv-VIIIb) and (Pv-VI) are the two chromosomal/vector DNA junction fragments and an internal pBR325 Pvu II fragment. The presence of a Sal I site on 7.55 kb fragment (Pv-I) is confirmed, since a fragment of this size does not hybridise to the pPM1008 DNA probe in Pvu II/Sal I double digests of pPM1000 DNA, Fig. 3.6. It is predicted from the pPM1000 restriction map that the 7.55 kb Pvu II fragment is cleaved by Sal I to give 4.85 kb and 2.6 kb Pvu II/Sal I fragments, Fig. 3.7. The 4.85 kb (Pv/S-I) fragment shows homology with the pPM1008 probe, as expected, however the homology with the 2.6 kb (Pv/S-Va) fragment is not resolved from a similarly sized vector fragment (Pv/S-Vb). All of the other Pvu II/Sal I fragments shown in Fig. 3.7 carry vector DNA homology with the DNA probe, these are fragments (Pv/S-III), (Pv/S-VIIb) and (Pv/S-VIII). The 1.75 kb Pvu II junction fragment does not possess a Sal I site. The 3.1 kb left hand junction fragment (Pv/S-III) is not resolved as a separate band from the 3.0kb Pvu II fragment (Pv-IV), (Pv/S-IV) in Fig. 3.7.

All of the Sal I fragments hybridised to the pPM1008 DNA probe as shown in Fig. 3.6. Two of the three Sal I fragments of pPM1000 contain pBR325 DNA homology with the DNA probe. One of these fragments (S-I) in pPM1000, Fig. 3.7, overlaps with the right hand end of the

7.4 kb Pvu I fragment (H/P-II) carried by the DNA probe, and the 6.8 kb (S-II) fragment overlaps the left end.

pPM2000

The DNA homology shared by pPM2000 with the 9.6 kb Pst I trg fragment (PII) is contained within a 1.4 kb Hind III/Pst I fragment (H/P-IV; Table 3.1; Fig. 3.3). The Sal I fragments (S-I, S-II) and one Pvu II fragment (Pv-II) also have homology with the trg DNA probe. The very much weaker hybridisation to the 5.45 kb Sal I fragment (S-II) than the 6.1 kb (S-I) fragment, can be explained if most of the chromosomal DNA homology lies in the 6.1 kb (S-I) fragment of the plasmid. In one direction there is 5.4 kb of DNA between the Sal I and Hind III restriction sites of the vector pBR325, thus, the 5.45 kb (S-II) fragment must only extend about 50 base-pairs to the left of the Hind III site which forms the junction of the chromosomal and vector DNA (Fig. 3.8). Apparently, from the hybridisation result shown in Fig. 3.3, this is sufficient homology to give a weak hybridisation signal after an overnight exposure of the autoradiogram.

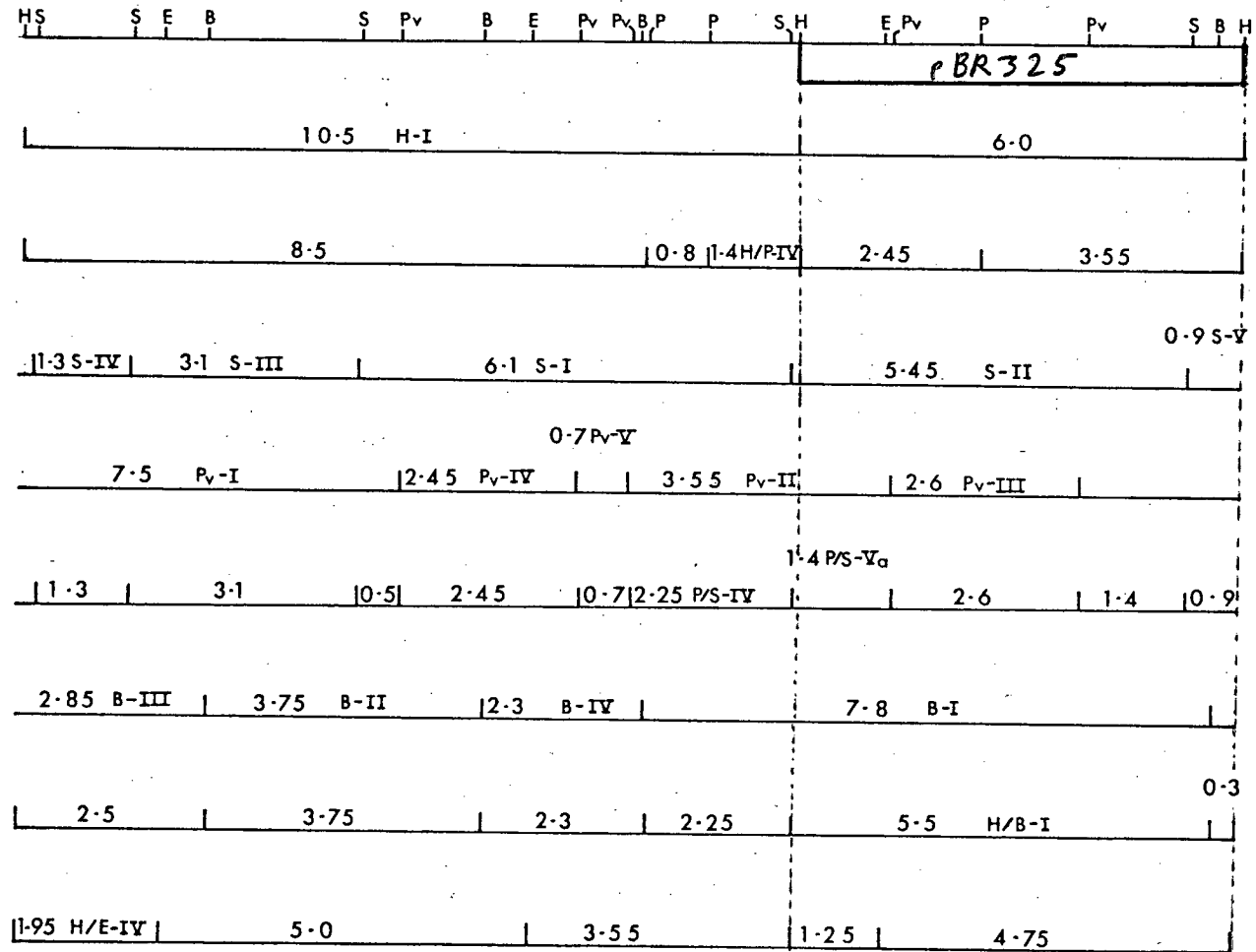
The 1.4 kb of homology with the trg DNA probe is contained within a 3.55 kb Pvu II fragment (Pv-II). This fragment is cleaved by Sal I to give a 2.25 kb Pvu II/Sal I fragment (Pv/S-IV) which hybridises strongly with the trg DNA probe and a 1.4 kb Pvu II/Sal I fragment (Pv/S-Va) showing only very weak hybridisation, due to precisely the same short DNA sequence carried by the 5.45 kb (S-II) fragment.

By combining the above hybridisation results of pPM2000 with the known restriction map of pBR325 the relative orientation of the 10.5 kb

Fig. 3.8.

Restriction map of pPM2000, E, EcoRI; H, Hind III; P, Pst I;
S, Sal I; Pv, Pvu II; B BamHI. The order of the closely
spaced Pvu II, BamHI and Pst I sites at the centre of the map have
not been confirmed by restriction digest analysis. Sizes of DNA
fragments are given in kb.

figure 3-8



Hind III insert (H-I) was determined with respect to the Sal I and Pvu II sites. This information only allowed 2 kb of the chromosomal DNA to be mapped. Construction of a unique restriction map of pPM2000 was greatly facilitated by hybridisation of the pPM2000 digests shown in Fig. 3.3 with pPM4000 (see Fig. 3.9), a plasmid clone carrying a 6 kb insert of chromosomal DNA which overlaps the 10.5 kb (H-I) fragment of pPM2000 by a common 1.95 kb Hind III/EcoRI fragment (H/E-IV).

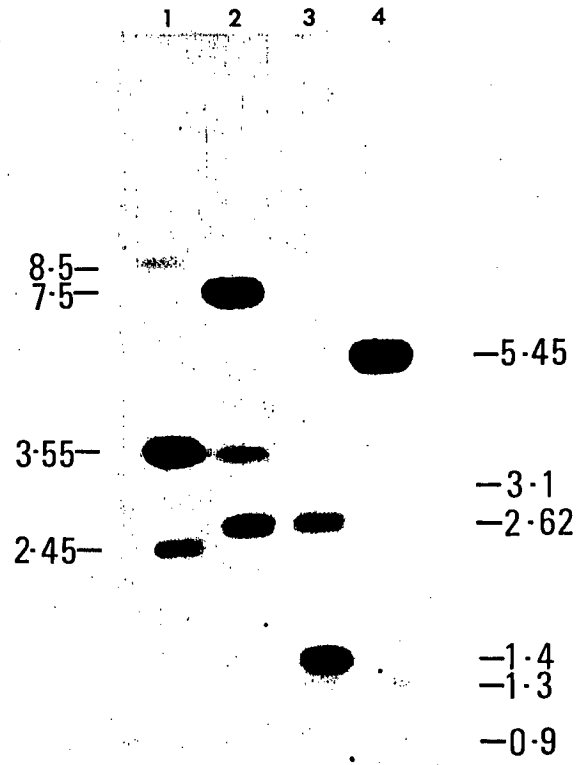
Four of the five Sal I fragments (S-II, II, IV and V) hybridised to a pPM4000 DNA probe (Fig. 3.9). Only one of these, the 5.45 kb (S-II) fragment, showed homology to the trg DNA probe and consists mostly of vector DNA. A 3.1 kb Sal I fragment (S-III) hybridises very weakly and is calculated to possess only 0.375 kb of DNA homology with pPM4000 and overlaps the EcoRI end of the 1.95 kb (H/E-IV) fragment. Two Sal I sites which generate 1.3 kb (S-IV) and 0.9 kb (S-V) fragments that hybridise to pPM4000 DNA probably lie within the homologous chromosomal DNA carried by both plasmids. However, the positions of these two sites cannot be determined from the hybridisation result. A 1.25 kb fragment (S-III) is present in Sal I digests of pPM4000 DNA (Table 3.2) and maps to within the 1.95 kb Hind III/EcoRI fragment (H/E-III in Fig. 3.12; H/E-IV in Fig. 3.8); therefore, the 0.9 kb (S-V) fragment of pPM2000 most likely carries the right hand Hind III site in Fig. 3.8 at the junction of the chromosomal and vector DNA in this plasmid.

Hybridisation of three Pvu II fragments (Pv-I, II and III), of pPM2000 with the pPM4000 probe is easily explained; homology of the 2.62 kb Pvu II fragment (Pv-III in Fig. 3.8) of PBR325 with the vector DNA of pPM4000; similarly, vector DNA homology carried by 3.55 kb (Pv-II) fragment (which also hybridised with the trg DNA probe as described

Fig. 3.9.

Restriction fragments of pPM2000 DNA that hybridise to a DNA probe prepared from pPM4000 plasmid DNA. Tracks; 1. Hind III/Pst I ; 2. Pvu II; 3. PvuII/Sal I, 4. Sal I. DNA fragment sizes are in kb. Experimental procedures are given in the Materials and Methods. Autoradiogram was exposed for 3 hours.

figure 3-9



earlier); homology with a 7.5 kb Pvu II fragment (Pv-I) which due to its size, must span the left hand junction of vector and chromosomal DNA in Fig. 3.8. Since both 2.45 kb (Pv-IV) and 0.7 kb (Pv-V) fragments do not show homology with any of the two DNA probes, an unaccounted for Pvu II site must be bounded by the 7.5 kb (Pv-I) and 3.55 kb (Pv-II) fragments. This Pvu II site could not be mapped from the above results. To locate this site, firstly a BamHI restriction map of pPM2000 was constructed from the data given in Table 3.1. Two BamHI fragments, 7.8 kb (B-I) and 2.85 kb (B-III) are cleaved by Hind III. Only one of these two fragments (B-I), is large enough to accommodate the 5.65 kb Hind III/BamHI fragment (H/B-I) of the pBR325 vector DNA, and is easily located on the pPM2000 map (Fig. 3.8). Fragment (B-III) therefore, carries the junction of vector and chromosomal DNA at the left end of Fig. 3.8. But it is not possible to order the two remaining BamHI fragments (B-II and B-IV) from the restriction data given in Table 3.1. A BamHI/EcoRI double digest shows one of the BamHI fragments (B-II) not to possess an EcoRI site (Howard Pringle, this Lab.). This information resolves the order of the (B-II) and (B-IV) fragments, since two of the three EcoRI sites were expected to cut the mapped BamHI fragments (B-I) and (B-II) leaving the right hand EcoRI site of the 10.5 kb Hind III fragment, Fig. 3.8, which has now been shown to cleave the 3.75 kb (B-IV) fragment.

A small fragment is seen in Hind III/BamHI digests of pPM2000 (Fig. 3.3). This was presumed to be the small Hind III/BamHI fragment of the pBR325 vector, but this is not always easily observed in 0.7% agarose gels. However, if a 0.35 kb BamHI fragment is carried by the chromosomal DNA of pPM2000, then in Hind III/BamHI double digests, the presence of a

0.35 kb doublet will aid the observation of the DNA in this region of the gel. By consideration of Pvu II/BamHI double digest data, only one of two possible positions for the unmapped Pvu II site produced a 0.9 kb Pvu II/BamHI fragment (data not shown). The completed pPM2000 restriction map is given in Fig. 3.8.

3.6 EXTENDING THE CLONED REGION.

A strategy analogous to that used to isolate pPM1000 and pPM2000 enabled the 6 kb EcoRI fragment (RIII in Fig. 3.13) which hybridised to a pPM2000 DNA probe (Fig. 3.3) to be isolated upon a pBR325 hybrid plasmid. This was made possible by having available the 10.5 kb Hind III fragment of pPM2000 to use as a hybridisation probe to screen a library of C600 DNA EcoRI fragments that had been cloned into pBR325 (Materials and Methods).

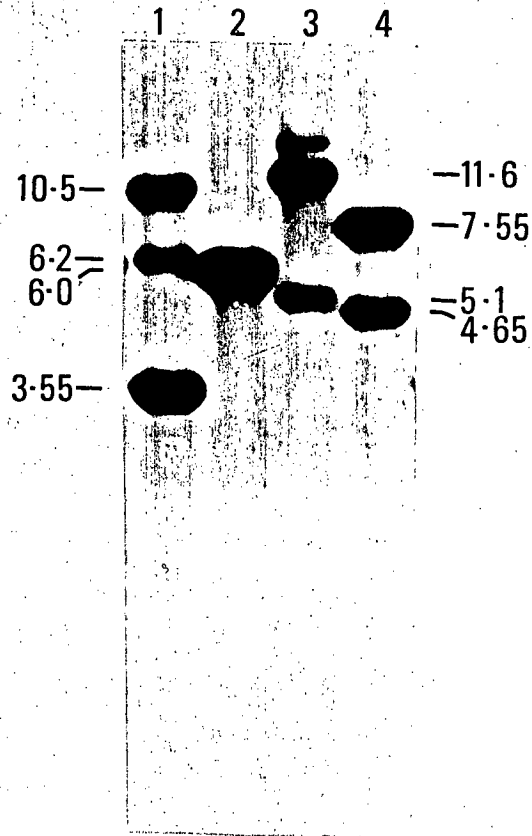
It can be seen from Fig. 3.13 and the restriction map of pPM2000 (Fig. 3.8) that there is 1.95 kb of DNA between the EcoRI site of the overlapping 6 kb fragment to the Hind III site at the left end of the 10.5 kb Hind III fragment. Therefore about 4 kb of additional DNA sequence was isolated by this 'step' of the 'walking' strategy. The plasmid carrying the 6 kb EcoRI chromosomal DNA fragment (RIII) inserted into the unique EcoRI site of pBR325 was designated pPM4000.

A ³²P-labelled DNA probe was made with purified pPM4000 DNA and used for hybridisation with EcoRI, Hind III, Pst I and Pvu II digests of C600 DNA (Fig. 3.10). As expected a single EcoRI fragment of 6 kb (RIII) and a 10.5 kb Hind III fragment (HII) hybridised to the pPM4000 DNA probe. Two other Hind III fragments contained homology to the DNA

Fig. 3.10.

C600 DNA restriction fragments (given in kb) which hybridise to a pPM4000 DNA probe. Tracks; 1. Hind III; 2. EcoRI; 3. Pst I; 4. Pvu II. See Materials and Methods for experimental procedures. Overnight exposure of the autoradiogram.

figure 3-10



probe. A 3.55 kb fragment (HIV) hybridised strongly compared to a 6.2 kb fragment (HIII) which showed significantly weaker hybridisation. This result suggests that the 3.55 kb Hind III fragment (HIV) lies internal to the 6 kb of chromosomal DNA carried by pPM4000 and the 6.2 kb Hind III fragment (HIII) overlaps a small region at one end of the EcoRI fragment (EIII), Fig. 3.13.

One of the two Pst I fragments which hybridised to the pPM4000 DNA probe is similar in size (11.6 kb) to a 12 kb Pst I fragment which showed homology to a pPM2000 DNA probe (Fig. 3.3). This Pst I fragment (PI) presumably extends from within the 10.5 kb Hind III fragment (HII) carried by pPM2000 to within the 6 kb (EIII) insert of pPM4000. Since only two Pst I fragments (PI and PIV) show homology with the pPM4000 DNA probe, then the Pst I 5.1 kb fragment (PIV) probably also overlaps the left hand end of the 6 kb EcoRI fragment (Fig. 3.13).

Interpretation of the result obtained from hybridisation of the pPM4000 DNA probe with Pvu II digests of C600 DNA required construction of a Pvu II map of pPM4000, from which the extent of overlap between the 6 kb EcoRI fragment (EII) with the 7.55 kb (PvI) and 4.65 kb (PvIII) Pvu II fragments, which hybridised to the pPM4000 DNA probe (Fig. 3.10) could be calculated. It can be predicted from the completed restriction map of pPM4000 (Fig. 3.12) that the 6 kb EcoRI fragment (E-I) is cut by Pvu II to give 1.6 kb and 4.4 kb Pvu II/EcoRI fragments and that the latter fragment carries the common Hind III/EcoRI region of pPM4000 and pPM2000. From the restriction map of pPM2000 it can be calculated that the distance from the EcoRI site of the 4.4 kb Pvu II/EcoRI fragment to the next Pvu II site within the 10.5 kb Hind III insert of pPM2000 is about 3.5 kb. Therefore, the 7.55 kb Pvu II fragment (PvI) rather

than the 4.65 kb (PvIII) Pvu II fragment, which hybridised to the pPM4000 DNA probe, overlaps the chromosomal DNA inserts of both pPM2000 and pPM4000. Consequently the 4.65 kb Pvu II fragment (PvIII) on the chromosome must extend about 3.05 kb beyond the 1.6 kb Pvu II/EcoRI fragment of pPM4000.

3.7 RESTRICTION MAPPING OF pPM4000.

Most of the restriction endonuclease map of pPM4000 can be deduced from the data given in Table 3.2 and Fig. 3.11. The chromosomal DNA insertion carried by pPM4000 does not possess a BamHI site but the digested DNA gives a single 12 kb fragment by cleavage at the unique BamHI site within the vector pBR325 DNA. In Fig. 3.11 cleavage of pPM4000 by EcoRI is seen to produce only one fragment, but, this has increased Ethidium bromide fluorescence compared to bands of similar size in the same gel and is therefore probably an unresolved doublet. Thus the cloned chromosomal EcoRI fragment is similar in size to the 6 kb EcoRI cut vector DNA and these two fragments (E-I, E-II) co-migrate under the gel electrophoresis conditions used.

Two Hind III sites are located on the 6 kb chromosomal DNA insert. These lie 3.35 kb apart, since a fragment of this size is found in both Hind III (H-II) and Hind III/EcoRI (H/E-II) double digests (Table 3.2, Fig. 3.11), a 1.95 kb Hind III/EcoRI fragment (H/E-III) common to both pPM4000 and pPM2000 defines the extent of homologous chromosomal DNA between these two plasmids. In pPM2000, a 1.3 kb Sal I fragment was mapped within the common 1.95 kb Hind III/EcoRI DNA (see Fig. 3.8) and a fragment of similar size (S-III) is present

Fig. 3.11.

Agarose gel electrophoresis of restriction digests of pPM4000

DNA. Tracks; 2. EcoRI; 3. EcoRI/Hind III; 4. Hind III; 5. EcoRI/BamHI
6. BamHI; 7. Hind III/Pst I; 8. Hind III/Pvu II; 9. Pvu II; 10. SalI/
Pvu II; 11. Sal I; 12. Hind III/Sal I. Tracks 1 and 13. Hind III

digests of Lambda CI857 DNA. Fragment sizes are in kb. Conditions
for electrophoresis and enzyme digests are given in the Materials
and Methods.

figure 3-11

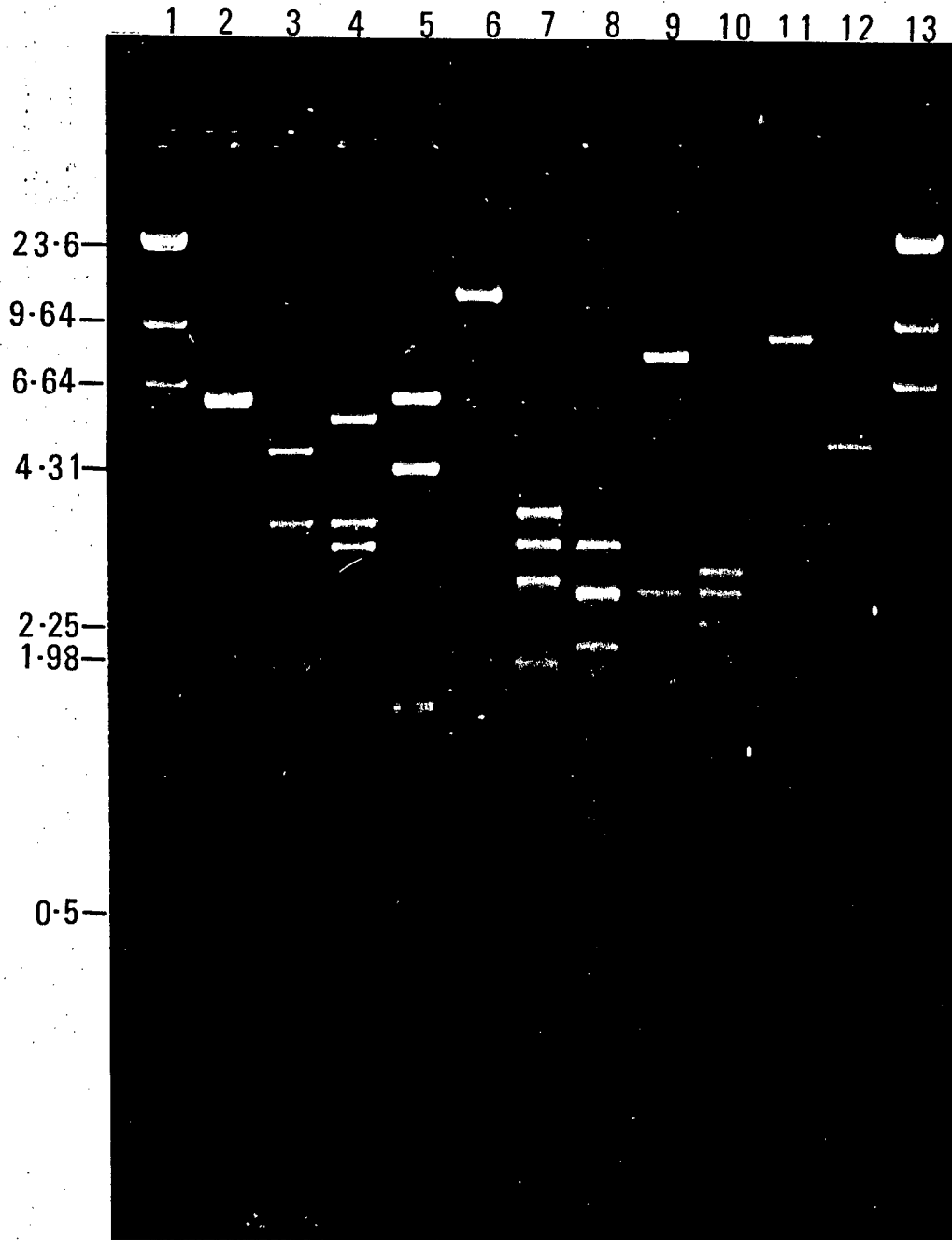


TABLE 3.2.

pPM 4000.									
E	E/H	H	E/B	B	E/P	E/Pv	Pv	Pv/S	S
6.0	4.75	5.65	5.9	12	3.5	3.05	7.9	2.8	9.0
	3.35	3.35	4.25		3.05	2.55	2.55	2.55	2.25
	1.95	3.0	1.7		2.65	2.5	1.75	2.25	1.25
	1.25				2.0	2.1		1.75	
	0.9				0.8	1.05		1.4	
						0.9		1.25	

Sizes of DNA restriction fragments, given in kb, of the plasmid pPM4000, obtained from analysis of the agarose gel shown in Fig. 3.11.(d) doublet of unresolved fragments.

Enzyme abbreviations given in text.

in pPM4000 Sal I digests. The remaining Sal I site of pPM4000 is located within the vector DNA and only one orientation of the chromosomal DNA insert into pBR325 gives 9.0 kb (S-I) and 2.25 kb (S-II) fragments upon cleavage with Sal I (Fig. 3.12). Two of the three Pvu II sites in pPM4000 are carried by the vector DNA, and by knowing the orientation of the chromosomal DNA insert, the position of the third Pvu II site is easily located upon the restriction digest map.

The Hind III, Sal I and Pvu II restriction map of pPM4000 given in Fig. 3.12 was confirmed by Hind III/Pvu II and Sal I/Pvu II double digests. The single Pst I site, carried by the chromosomal DNA insert of pPM4000 cleaves the 3.35 kb Hind III fragment (H-II) which lies internal to the 6.0 kb EcoRI fragment (R-I) and only the one position of this Pst I site, shown in Fig. 3.12 generates the Hind III/Pst I fragments of 2.65 kb (H/P-III) and 0.86 kb (H/P-V) and a 2.1 kb Pst I fragment (P-IV).

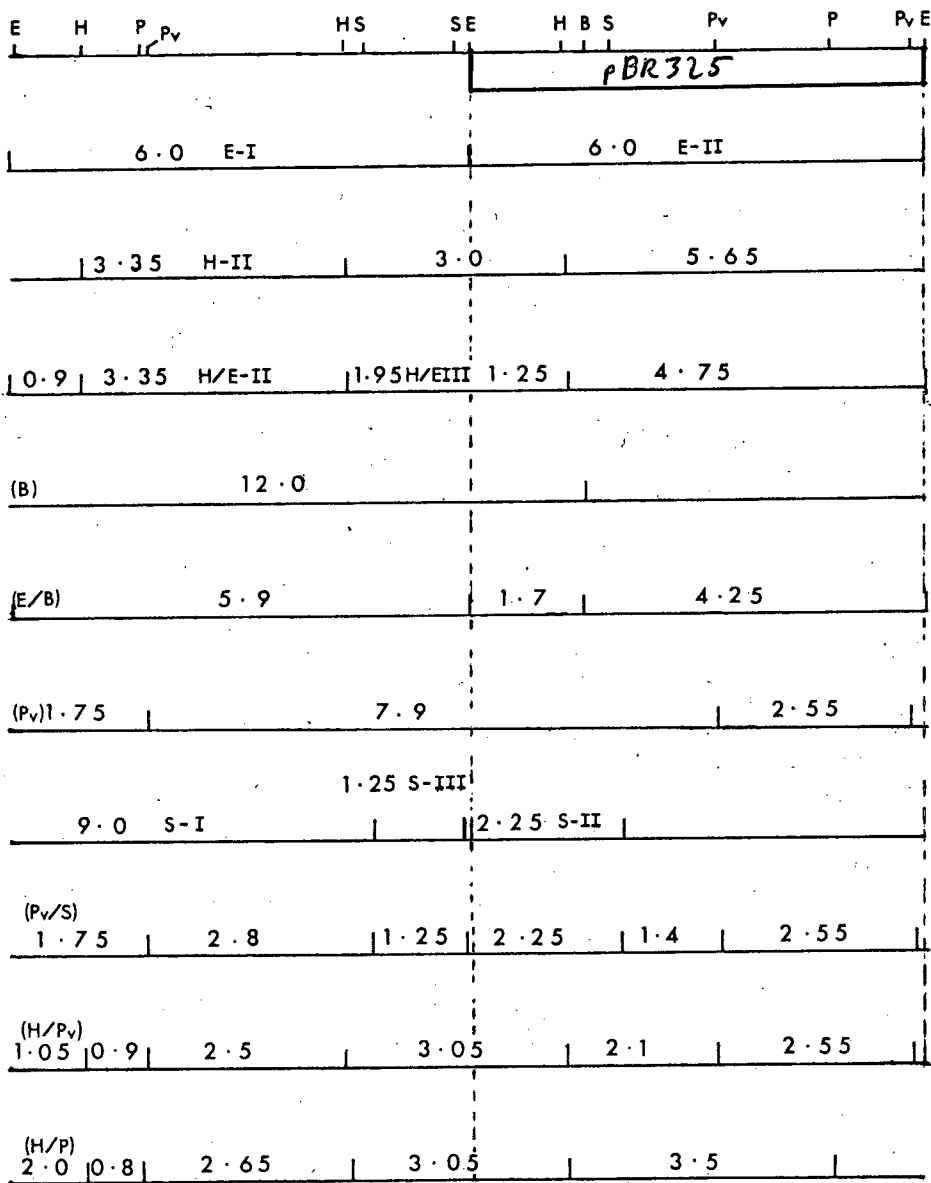
3.8 PHYSICAL MAP OF THE ter C REGION.

A physical map of the ter C region (Fig. 3.13), was constructed primarily from the restriction endonuclease mapping results obtained for the plasmids pPM1000, pPM2000 and pPM4000. The chromosomal DNA Hind III inserts of pPM1000 and pPM2000 are probably adjacent on the chromosome since only 23 kb (HI) and 10.5 kb (HII) Hind III fragments hybridised to the 8.7 kb trg Pst I DNA probe (Fig. 3.1). Also the restriction maps of pPM1000 and pPM2000 (Fig. 3.7 and Fig. 3.8), the map of 8.7 kb trg Pst I fragment (Harayama et al, 1980) and the DNA/DNA hybridisation of digests of pPM1000 and pPM2000 (Fig. 3.4) all suggest

Fig. 3.12.

Restriction map of pPM4000. E, EcoRI; H, Hind III; P, Pst I; S, Sal I;
Pv, Pvu II; B, BamHI. Sizes of DNA fragments are given in kb.

figure 3-12



that there is only one Hind III site present on the 9.6 kb Pst I fragment (PII). Assuming that no small chromosomal Hind III fragment remains undetected in this data, then abutment of the restriction map of the 23 kb Hind III insert of pPM1000 and the 10.5 kb Hind III insert of pPM2000 allows construction of a physical map spanning 33.5 kb. To this map can be added the restriction map of the chromosomal DNA carried by pPM4000 which overlaps the 10.5 kb insert of pPM2000.

To align these three restriction maps such that a continuous stretch of chromosomal DNA is represented, requires knowledge of the sizes of DNA fragments spanning the restriction sites which were used for cloning the isolated regions into the vector molecule. This data is obtained from the hybridisation of C600 DNA fragments with each of the plasmid DNA probes (Figs. 3.1, 3.3 and 3.9). For instance, the relative orientation of the 23 kb Hind III (HI) insert of pPM1000 with to the 10.5 kb Hind III (HII) insert of pPM2000 can be determined from the region of homology with the 9.6 kb trg Pst I fragment (PII) and one end of each Hind III fragment. The 1.95 kb of overlap of the chromosomal DNA of pPM2000 and pPM4000 simplifies the alignment of the DNA fragments in this region.

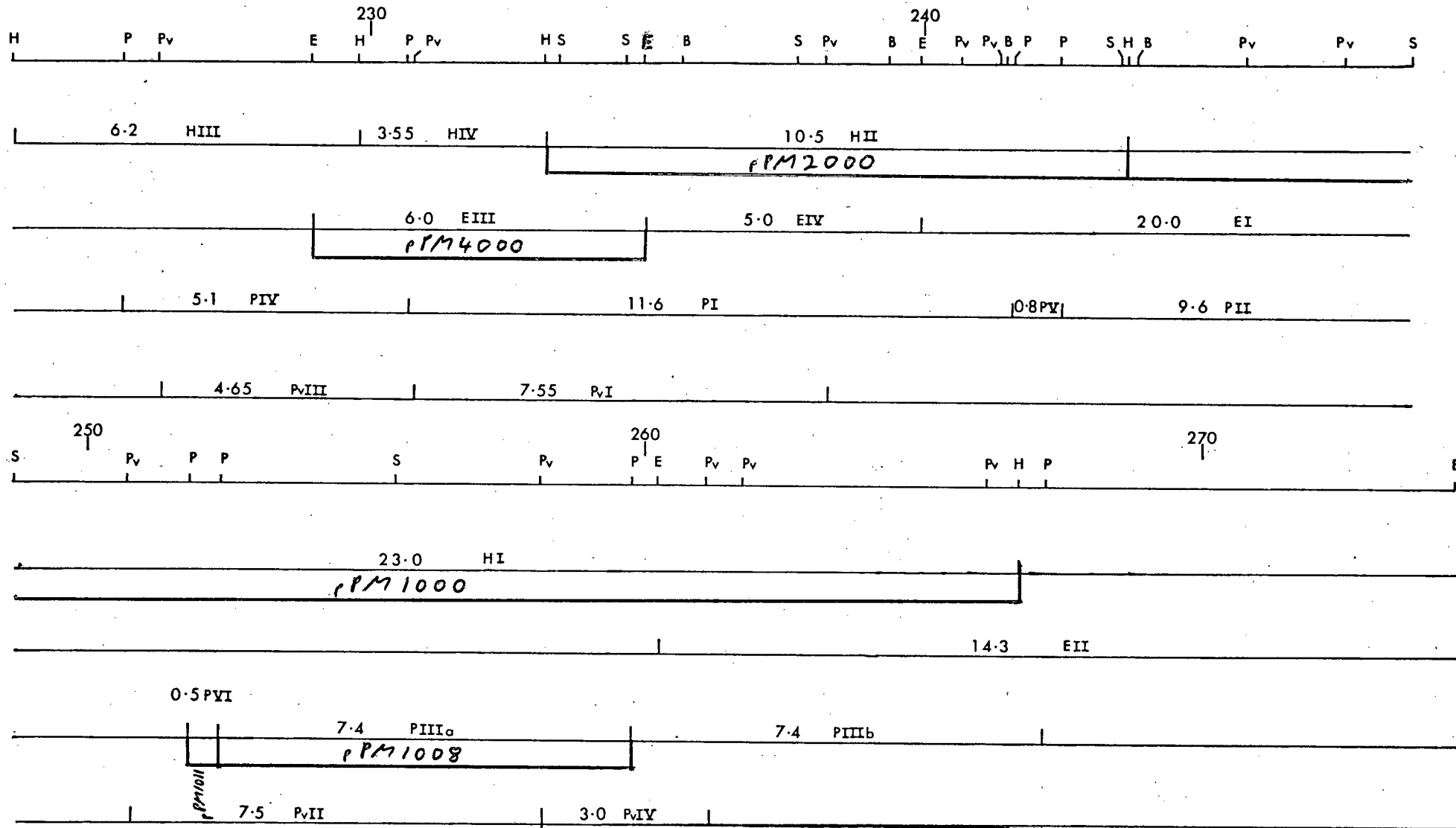
Furthermore, it is possible to calculate the amount of DNA with which some of the homologous C600 DNA fragments extend beyond the cloned regions. Thus the map shown in Fig. 3.13 has restriction sites lying outwith the total cloned region that were mapped at the right hand end by calculating the extent of overlap of C600 DNA EcoRI and Pst I fragments with the 23 kb Hind III fragment of pPM1000. At the other end Pvu II, Pst I and Hind III fragments, which overlapped the left hand end of the 6 kb insert of pPM4000, were used. The result of these calculations is described earlier in each of the respective sections.

Fig. 3.13.

Physical map of the terminus region constructed from restriction analysis of pPM1000, pPM2000, pPM4000 and DNA hybridisation results, as described in the text. Coordinates have been assigned (expressed in kb) according to the Bouche (1982) map of the terminus region.

DNA fragment sizes are in kb. E, EcoRI; H, Hind III; P, Pst I; S, Sal I; Pv Pvu II, B, BamHI.

figure 3-13



3.9 DISCUSSION.

These results show that the molecular cloning of a genetically 'silent' region by the chromosome 'walking' strategy has proved to be very successful. Approximately 38 kb of DNA from the ter C region of the E.coli K12 chromosome was isolated as three separate DNA fragments carried by recombinant, pBR325 molecules.

Physical mapping techniques were used to construct restriction endonuclease maps of the recombinant plasmid clones. These restriction maps were combined by recognising the DNA restriction fragments in C600 DNA which not only hybridised with the cloned fragment, but also overlapped the ends of the cloned region. Thus, a physical map of the entire cloned region was produced, including locations of cleavage sites (for some of the six enzymes) lying outside the region of the chromosome covered by the cloned DNA.

Particularly useful is the information that a 14.3 kb EcoRI fragment and a 6.2 kb Hind III fragment, overlap the 23 kb Hind III fragment and 6.0 kb EcoRI fragment at either end of the 38 kb cloned region respectively. These two fragments would be those sought after by probing an EcoRI library of recombinant pBR325 molecules with ³²P-labelled 23 kb Hind III fragment from pPM1000 DNA, or a Hind III library with a DNA probe of the EcoRI chromosomal DNA fragment of pPM4000. If the 14.3 kb EcoRI fragment was isolated this 'step' in the 'walking' strategy would extend the total cloned region by about 8 kb. In the opposite direction a further 5.3 kb of DNA would be obtained as the 6.2 kb Hind III fragment shares 0.9 kb of homology with the 6 kb insert of pPM4000.

Comparison of my physical map of the ter C region with the map of Bouche (1982) reveals that, barring two exceptions, the number and positions of EcoRI, Hind III and Pst I cleavage sites are very similar between the E. coli K12 strains. Only in two instances do the two physical maps differ. An additional Pst I site was located 0.5 kb to the right of the 9.6 kb trg fragment. Another Pst I site has been found in the 6 kb chromosomal DNA insert of pPM4000 (Howard Pringle, this Lab.), this generates a small Pst I fragment which is not seen in Fig. 3.9. Bejar and Bouche (1983) report having cloned 86% of the DNA sequences from 30.5 to 34 minutes on the genetic map. Three regions were not completely cloned, leaving gaps at positions 214.8-225 kb, 242.5-243.3 kb and 253-264 kb on the Bouche (1982) physical map. The latter two gaps are bridged by the chromosomal DNA inserts of pPM2000 and pPM1000 respectively.

In the following chapter, it is seen that restriction maps of pPM1000, pPM2000 and pPM4000 were necessary to plan the construction, and characterisation, of plasmid derivatives of the above three plasmids. Also the restriction maps were essential in the interpretation of some of the protein synthesis results, where suspected gene fusions were thought to have been created in the plasmids.

Construction of a physical map of the cloned ter C region is required for mapping genes that may code for any proteins synthesised by sections of chromosomal DNA, carried by the ter C region plasmids.

CHAPTER 4.

4.1 INTRODUCTION.

This chapter describes the characterization of proteins that were found to be synthesised by cloned ter C region DNA. Additional plasmids, to those described in the previous chapter, were constructed by cloning the chromosomal DNA carried by these plasmids, to obtain DNA fragments inserted in both orientations into the pBR325 vector DNA, and to generate sub-clones of the large Hind III fragment of pPM1000.

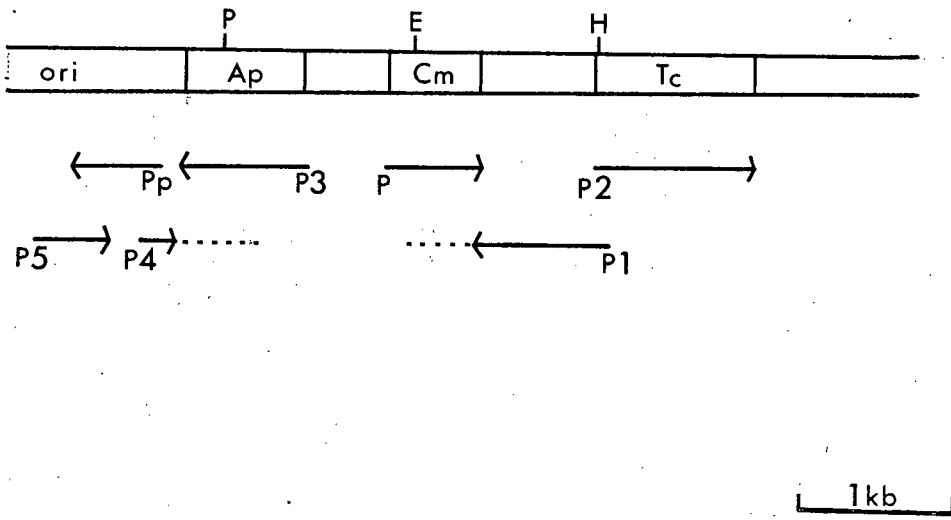
Both orientations of the DNA fragments were desired in order to identify any accretion of coding regions created by in-frame fusions. Fig. 4.1 shows that two of the unique restriction sites in pBR325, that are useful for cloning, lie in the structural genes for chloramphenicol acetyl transferase (chloramphenicol resistance) and B-lactamase (ampicillin resistance). Failure to recognise a polypeptide as being a fused gene product, may complicate the analysis of protein expression data and also result in an overestimation of the amount of coding DNA of that region. On the other hand, having identified a fused gene product, by subtracting the contribution of the antibiotic resistance gene from the size of the polypeptide, an estimate of the size of an open reading frame on the chromosomal DNA can be obtained.

By assigning gene products to specific regions of DNA carried by recombinant plasmids, I hoped to locate genes upon the physical map of the cloned region given in Chapter 3. This mapping may be achieved in at least three ways ; characterisation of polypeptides coded by a section chromosomal DNA, thus locating the structural gene to that cloned DNA fragment; identification of common polypeptides coded by overlapping DNA fragments; loss of polypeptides from plasmids that have had DNA deleted.

Fig. 4.1.

Physical map of the cloning vehicle pBR325, showing unique sites for the restriction enzymes EcoRI (E), Hind III (H) and Pst I (P) within the antibiotic resistance genes; chloramphenicol acetyl transferase (Cm), tetracycline resistance (Tc) and B-lactamase (Ap). Transcriptional organisation of known promoters is shown. The plasmid molecule, as drawn, is opened up at the Pvu II site which is also common to the progenitor molecule pBR322 (adapted from Stuber and Bujard, 1981).

figure 4-1



A list of the proteins synthesised by the ter C region plasmids and a map of the gene distribution is given in section 4.9.

4.2 SUB-CLONING THE ter C REGION.

Plasmids pPM1000, pPM2000, pPM4000 and pTH51 were used to construct additional plasmids with a view to dissecting the cloned chromosomal DNA for genetic analysis in protein synthesising systems. Fig. 4.2 shows the sections of ter C region DNA which have been inserted into the cloning vehicle pBR325.

Pst I fragments were sub-cloned from pPM1000 to give pPM1008 and pPM1009 which have a 7.4 kb insert, and pPM1011 carrying a 0.5 kb fragment. An 8.7 kb Pst I fragment from pTH51 was cloned in both possible orientations, resulting in pPM3000 and pPM3001. All these plasmids were picked as ampicillin sensitive, putatively recombinant molecules and were confirmed to be single insertions into the unique Pst I site of pBR325.

pPM2001 and pPM4001 were constructed by cutting and religating the parent molecules, pPM2000 and pPM4000, using the appropriate restriction enzyme and screening phenotypically for recombinants. Two plasmids were chosen on the basis of restriction digests showing them to have insertions of the same chromosomal DNA into pBR325, but in opposite orientation to the parent plasmids.

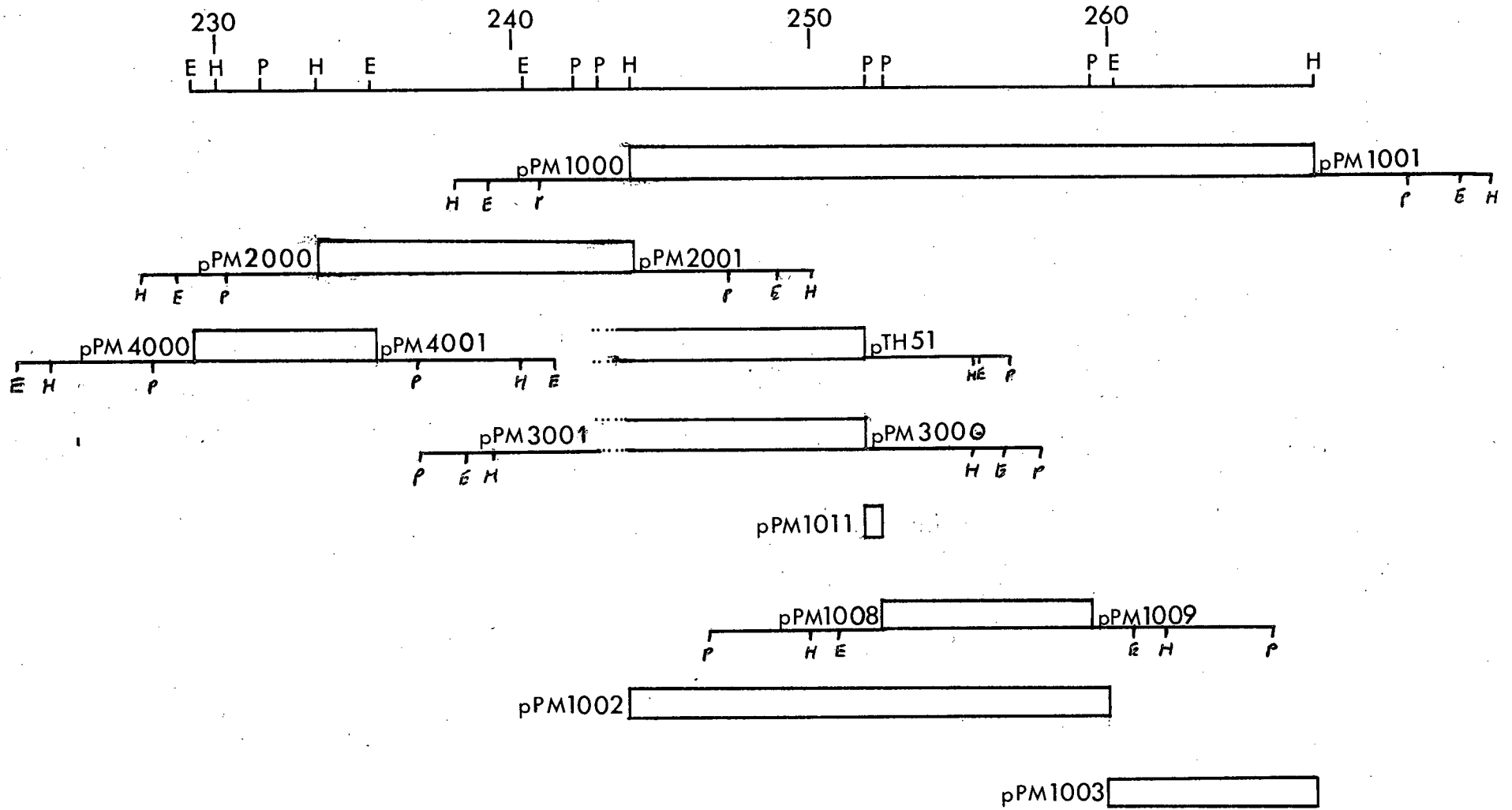
Attempts at using the latter approach to insert the 23 kb Hind III fragment of pPM1000 were unsuccessful. This was eventually achieved

Fig. 4.2.

Sections of the chromosome carried by the ter C region plasmids described in the text. Where two designations are given, means that the DNA fragment has been cloned in both orientations with respect to the pBR325 vector molecule. The co-ordinates, given in kb, are according to the Bouche (1982) physical map of the terminus region. EcoRI (E); Hind III (H); Pst I (P).

..... INDICATES END POINT OF CHROMOSOMAL DNA UNKNOWN.
ORIENTATION OF THE FRAGMENTS RELATIVE TO THE
VECTOR DNA IS SHOWN.

figure 4-2



by firstly, isolating a large amount of 23 kb Hind III fragment from an agarose gel, via elution from Whatman 3mm paper. An excess of 23 kb fragment over Hind III digested pBR325 DNA in a ligation reaction enabled the desired clone to be constructed and was designated pPM1001 (Howard Pringle, this Lab.).

An EcoRI digest of pPM1000 was used to obtain pPM1002. In this case the cut DNA (0.25 ug) was diluted to 50ul before ligation, to encourage self-ligation (circularisation) of their linear molecules. pPM1000 possesses two EcoRI sites, one within the C600 DNA insert dividing the 23 kb Hind III fragment into 16.5 kb and 6.5 kb Hind III/EcoRI fragments. The other EcoRI site lies within the chloramphenicol resistance gene of the pBR325 DNA. Circularisation of EcoRI cut pPM1000 DNA and recovery by transformation upon ampicillin plates, yields the fragment which carries both the Ap^R gene and replication origin of pBR325, and results in deletion of the 6.5 kb Hind III/EcoRI C600 DNA fragment as well as about 1.25 kb of vector DNA, which encodes a large proportion of the chloramphenicol resistance gene.

Finally, deletion of 16.5 kb from the 23 kb Hind III fragment was achieved by using pPM1001 as the parent molecule and treated in an analogous manner as that described for generating pPM1002 above. Interestingly, this derivative, pPM1003, exists at much lower copy number than either pPM1000 or pPM1001. The reason for this is not known and occurs in both C600 and DS410 strains.

4.3 PROTEIN SYNTHESIS IN MINICELLS, DIRECTED BY

pPM's 1000, 1008, 1011, 3000, 3001 AND pTH51.

Purified minicells from strain DS410 harbouring plasmids carrying up to 23 kb of ter C region DNA were labelled with ^{35}S -methionine and the proteins expressed were analysed on gradient P.A.G.E. gels.

Polypeptides of 77K, 66K, 64K, 62K, 61K, 38K, 35K, 31K, 29K, 26K and 20K are always present in the autoradiograms of the labelled proteins expressed by pPM1000 Fig. 4.3. Only pPM1000 synthesised the 77K and 38K polypeptides. The two genes which code for those two polypeptides may be located on the Pst I/Hind III fragment at the right hand end of the 23 kb Hind III fragment of pPM1000, Fig. 4.2. This may be deduced from the absence of 77K and 38K polypeptides from the proteins expressed by the 17.5 kb of chromosomal DNA extending up to the right hand Pst I site of the 23 kb insert of pPM1000. The region to the left of this Pst I site is carried as individual Pst I fragments by plasmids pPM3000, pPM3001, pPM1011 and pPM1008. None of these plasmids synthesise a 77K or 38K polypeptide, Fig. 4.3, therefore, the two genes coding for these proteins possibly lie to the right of the cloned region carried by these plasmids.

Comparison of the gene products of pTH51, a plasmid carrying the trg gene and a gene encoding a periplasmic protein (Harayama et al, 1982; Harayama & Hazelbauer, 1982), with those of pPM1000, identifies the 66K and 64K polypeptides as unprocessed and mature trg proteins, and the 62K and 61K polypeptides as the periplasmic protein with and without its signal peptide. The ratio of labelled proteins within each pair of gene products sometimes differed, presumably due to varying extents of post-translational processing in any particular labelling experiment.

Fig. 4.3.

Polypeptides expressed from plasmids in minicells and labelled with ^{35}S -methionine. Apparent molecular weight sizes are in kilodaltons. Tracks; 1.pPM3001, 2.pPM3000, 3.pPM2001, 4.pPM2000, 5.pPM1000, 6.pTH51, 7.pBR325, 8.pPM1011, 9.pPM1008. Electrophoresis was carried out on SDS-polyacrylamide gradient gels, 10-20% tracks 1-7; 7-20% tracks 8 and 9. Conditions for radiolabelling and other experimental procedures are described in the Materials and Methods. Autoradiograms were exposed for 3 days. (Minicells prepared from DS410 not transformed with a plasmid did not produce any labelled polypeptides, not shown).

The trg and periplasmic protein genes have been located on an 8.7 kb Pst I fragment of pTH51 (Harayama et al, 1982; Harayama & Hazelbauer, 1982). Plasmids pPM3000 and pPM3001 carry this Pst I fragment, both these plasmids synthesised the periplasmic protein, but only pPM3001 synthesised the trg gene, Fig. 4.3. An explanation for this result is put forward in section 4.4.

The 8.7 kb Pst I fragment of pTH51 hybridised to a 9.6 kb Pst I fragment in C600 DNA and to a 8.1 kb Hind III/Pst I fragment which mapped at the left end of the 23 kb Hind III insert of pPM1000 (Chapter 3). This result in conjunction with the mapping data of pTH51 reported by Harayama and co-workers, allowed the trg and periplasmic protein genes to be placed on the restriction map of this region, Fig. 4.7.

Fig. 4.3 shows the presence of a 21K polypeptide apparently expressed by pPM1000. However, this polypeptide is not always seen as one of the gene products of pPM1000. The conditions governing the appearance of the 21K polypeptides are not known. Harayama et al (1982) found a polypeptide of similar size in this region of the chromosome, which appeared as a poorly labelled gene product in minicells. Plasmid pPM1008 synthesises a 48K and a 21K polypeptide, Fig. 4.3, the 21K polypeptide was always seen as a gene product. The 7.4 Pst I fragment of pPM1008, which was sub-cloned from pPM1000, probably overlaps 2.3 kb at one end of the Harayama et al (1982) plasmid that was found by these workers to produce a 2K polypeptide. Possibly a gene coding for the 21K polypeptide is located in the region of DNA common to these two plasmids, as indicated in Fig. 4.7.

The 48K polypeptide synthesised by pPM1008 is not represented in the gene products of pPM1000, Fig. 4.3. This polypeptide may be the product of a truncated gene resulting from cleavage by Pst I. Alternatively, this polypeptide may be a fused gene product, carrying part of the B-lactamase gene of the vector. The latter possibility can be ruled out because of the plasmid pPM1009, which carried the 7.4 Pst I fragment in the opposite orientation to pPM1008, also synthesises a 48K as well as a 20K polypeptide (Renate Fingerhut, this Lab.).

It is possible that the 48K polypeptide is the N-terminal end of the 77K polypeptide expressed by pPM1000. The 77K polypeptide is coded by a roughly mapped gene in this region, as described above, and is large enough to accommodate a N-terminal fragment of 48K.

Harayama et al (1982) also reported the synthesis in minicells of 20K and 30K proteins dissected by a plasmid carrying a section of DNA that overlaps with the chromosomal DNA of pPM1000. The 30K protein, if expressed by pPM1000, would probably be obscured by the ampicillin resistance gene products of the pBR325 vector DNA. A 20K polypeptide synthesised by pPM1000, pPM3000, pPM3001 and pTH51, Fig. 4.3, may be coded by a gene located within the $\frac{8.4}{\text{Hind III}}$ kb/Pst I fragment of pPM1000.

Three gene products are clearly expressed by pPM1000 that are common to the polypeptides produced by the cloning vehicle pBR325. The chloramphenicol acetyl-transferase gene originally derived from Tn 9 (Alton & Vapnek, 1979), is most strongly labelled and evident at 26K in this gel system Fig. 4.3. The B-lactamase and the more abundant mature enzymes are seen in Fig. 4.3 to be at 31K and 29K. Dougan and et al. (1979), found similar apparent molecular weights for the ampicillin resistance gene of pBR322, which is the progenitor molecule

of pBR325 (Bolivar et al, 1977). Both the 31K and 29K proteins are absent from all the ampicillin sensitive pBR325 recombinants carrying Pst I insertions, for example, see tracks 1 and 2 in Fig. 4.3. A third polypeptide of 35K apparent molecular weight is synthesised by the ampicillin resistant plasmids, this also disappears in the Pst I recombinant plasmids. Dougan et al. (1979), did not report synthesis of a 35K protein by pBR322, indeed the 8610 base-pair sequence of the pBR322 ampicillin resistance gene (Sutcliffe, 1972), which should be homologous with the same resistance gene of pBR325, is not sufficient to code for a protein of this size. I do not as yet have an explanation for the expression of this protein apparently associated with the ampicillin resistance gene of pBR325.

Comparison of similar sized proteins between different tracks in Fig. 4.3, shows the 35K polypeptide to be more intensely labelled in the gene products of pPM1000 than that of pPM2000 and pPM2001. Similarly, this is also observed for the 20K polypeptide synthesised by pPM1000 when compared with a polypeptide of this size coded by pPM3000, pPM3001 and pTH51. One explanation may be that more than one gene carried by pPM1000 codes for proteins of 35K and 20K, thus the relative increased intensity of the 35K and 20K bands on the autoradiogram is due to the presence of two (or more) labelled proteins in these two regions of the polyacrylamide gel.

None of the plasmids that share chromosomal DNA homology with pPM1000, Fig. 4.2, code for a 35K protein Fig. 4.3. If the above result with pPM1000 is not an artifact, a gene encoding the 35K protein may be located at the right hand end of the 23 kb Hind III fragment carried

by pPM1000, (not shown) in the region not covered by these other plasmids. A 20K polypeptide is synthesised by pPM1011, Fig. 4.3. This plasmid carries a 0.5 kb Pst I fragment sub-cloned from pPM1000. Perhaps this piece of chromosomal DNA also codes for a 20K protein and may explain the relatively stronger autoradiogram signal obtained from the 20K band of pPM1000, described above.

4.4 THE Pst I trg FRAGMENT DOES NOT EXPRESS

THE trg GENE IN pPM3000.

As already described in section 4.2, the 8.7 kb Pst I Trg fragment from pTH51 was cloned into pBR325 to give pPM3000 and pPM3001. One of these plasmids, pPM3000, did not produce any detectable amount of trg gene product in minicells, whereas protein bands of 66K and 64K were synthesised by both pPM3001, which carries the opposite orientation of the 8.7 kb Pst I fragment, and the Trg plasmid pTH51, Fig. 4.3.

Plasmids, pPM3000 and pTH51 are equivalent with respect to their 8.7 kb inserts, the fragment is inserted in the same relative orientation within the B-lactamase gene of pBR325 and pBR322, respectively. pTH51 carries an additional 0.5 kb Pst I fragment located at the trg proximal end of the 8.7 kb fragment. One possibility is that expression of the trg gene in pPM3000 is inhibited by transcription from a vector DNA promoter converging with the trg gene. Perhaps the 0.5 kb Pst I fragment in pTH51 blocks, or opposes, transcription from the vector molecule and allows expression of the trg gene.

A promoter, designated P4, is known in pBR322 to transcribe into the C-terminal end of the B-lactamase gene Fig. 4.1, and is strong enough to express genes carried on DNA fragments inserted at the Pst I site.

(Stuber & Bujard, 1981). The orientation of the 8.7 kb Pst I fragment of pPM3000 is such that P4 does lie proximal to the trg gene. Convergent transcription has been shown to mutually impair both promoters and can lead to one of the promoters being totally blocked (Ward & Murray, 1979).

If transcriptional activity does converge with that of the trg gene, then presumably, no other active genes lie between trg and the end of the 8.7 kb Pst I fragment.

4.5 EXPRESSION IN MINICELLS OF POLYPEPTIDES

CODED BY, pPM2000 and pPM2001.

The 10.5 kb Hind III fragment of pPM2000 and pPM2001 codes for four polypeptides of apparent molecular weight 12K, 37K, 28.5K and 24K. These polypeptides must be coded by genes that map within this Hind III fragment, since either orientation of this DNA insert into pBR325 appears not to affect their expression, Fig. 4.3. No additional information is as yet available to narrow down the position of these genes.

As with all the ampicillin resistant plasmid studied in minicells, both pPM2000 and pPM2001 synthesised the 31K B-lactamase gene product. But, with pPM2000 a much stronger 31K band is seen in autoradiograms of labelled proteins produced by this plasmid than that of pPM2001, compare tracks 3 and 4 of Fig. 4.3. Since these two plasmids only differ in the orientation of a 10.5 kb Hind III fragment relative to the vector DNA, and since the Hind III site, into which this fragment is inserted, is separated from the ampicillin resistance gene by the chloramphenicol resistance gene in pBR325, Fig. 4.1, then it is unlikely

that the B-lactamase protein is more strongly expressed in one of the two plasmids due to the presence of the 10.5 kb Hind III fragment. A plausible explanation may be that a gene coding for a 31K protein is situated at one end of the 10.5 kb Hind III fragment and is expressed from a promoter in the adjacent vector DNA. In support of this suggestion, a promoter is known to direct transcription from within the tetracycline resistance gene through the Hind III site in pBR322 (Stuber & Bujard, 1981) see Fig. 4.1. This DNA region of pBR322 is also carried by pBR325 (Bolivar et al, 1977).

The reverse situation may be true for pPM2001, where a promoter, located at the other end of the 10.5 kb Hind III fragment, directs transcription out of the fragment into the vector DNA expressing the tetracycline resistance gene of pBR325 and results in a low level of tetracycline resistance of this plasmid, see Table 4.1.

If this promoter exists on the chromosome, that is, if it has not been fortuitously created in the construction of pPM2001, then it may be part of a gene located at the left hand end of the 9.6 kb Pst I fragment in the chromosome where this fragment and the 10.5 kb Hind III fragment overlap, Fig. 4.1. The trg gene maps at this end of the 9.6 kb Pst I fragment Fig. 4.7. Additional evidence is required to determine whether this promoter is the trg gene promoter, although the results described in section 4.4 suggests that the trg gene is transcribed in the opposite direction, or if it is the promoter of an unidentified gene, or even a remnant of a once functional gene.

4.6 DIFFERENTIAL EXPRESSION IN MINICELLS AND
MAXICELLS OF THREE POLYPEPTIDES SYNTHESISED BY pPM2000 and pPM2001.

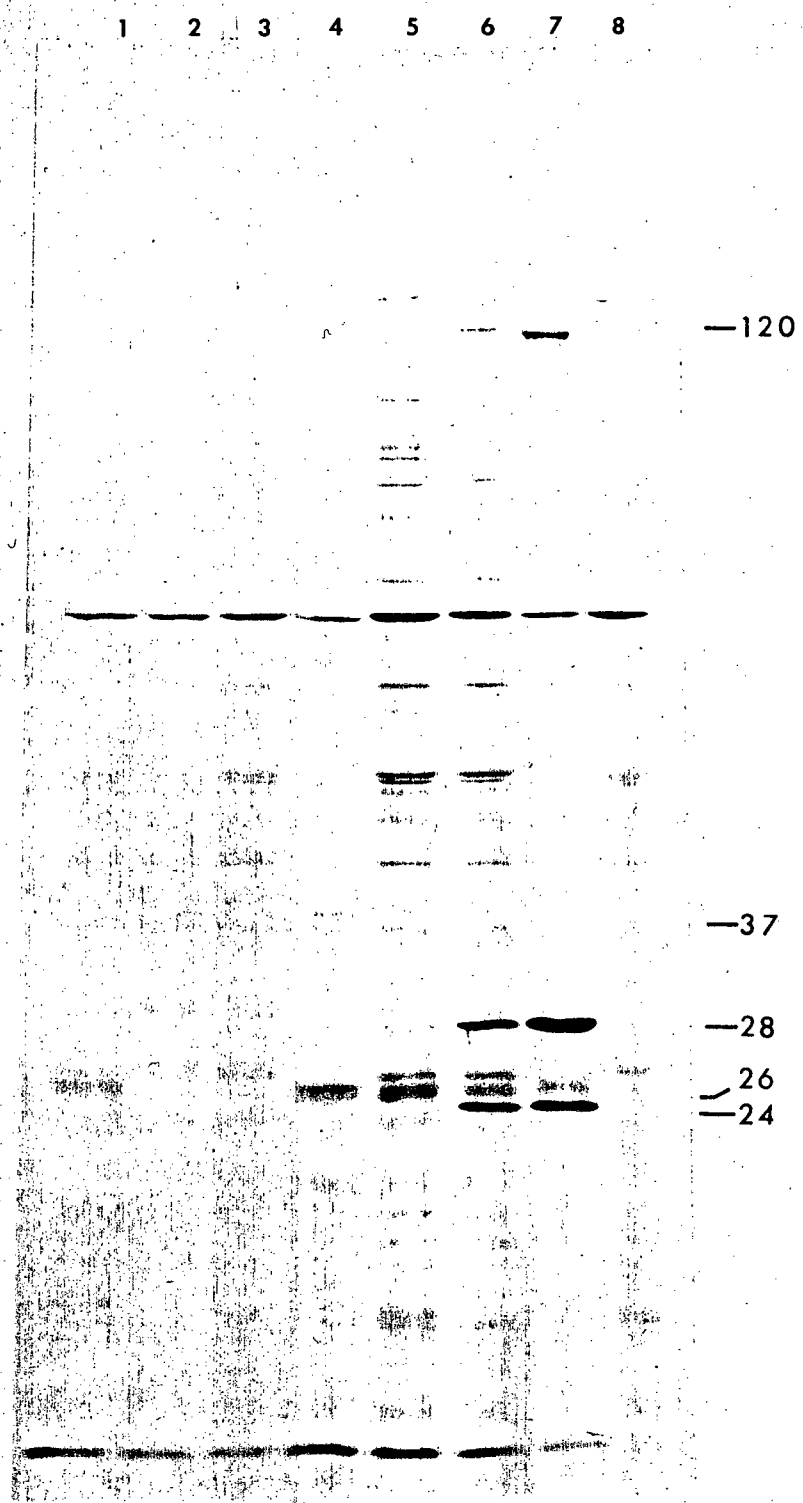
As an alternative to minicells for studying in vivo protein synthesis, the 'maxicell' system of Sancar and Rupp (1979) was employed using most of the ter C region plasmids. Only limited success was achieved with maxicells due to a large background of labelled polypeptides on the P.A.G.E. gels, presumably as a result of gene expression from a number of cell survivors. However, an interesting discovery ensued involving two plasmids, pPM2000 and pPM2001. Both these plasmids carry a 10.5 kb Hind III fragment from C600 DNA, see Fig. 4.1, and differ only in the orientation of their Hind III inserts.

Fig. 4.4 clearly shows that in maxicells the 10.5 kb Hind III fragment directs the synthesis of three polypeptides, 12K, 28K and 24K. Polypeptides of similar apparent molecular weights are also synthesised in minicells harbouring, pPM2000 and pPM2001, Fig. 4.3, (although often the 24K polypeptide is masked by the strongly labelled chloramphenicol acetyl-transferase). But, there is a marked difference in expression of these three polypeptides between the two in vivo protein synthesising systems. All three polypeptides are labelled strongly in maxicells and are most likely being expressed at a high level such that they are easily distinguished above the background of many labelled proteins, Fig. 4.4. For comparison, the 26K chloramphenicol acetyl-transferase of pBR325 is consistently the most highly expressed gene in the minicell labelling experiment, Fig. 4.3, but it is only just visible in these 'maxicell' results.

Fig. 4.4.

Polypeptides labelled with ^{35}S -methionine by the 'maxicell' method and separated on a 7-20% SDS-polyacrylamide gel. Tracks; 1.pBR325, 2.pPM4001, 3.pPM4000, 4.pPM3001, 5.pPM3000, 6.pPM2001, 7.pPM2000, 8.pPM1000. The 37K tetracycline resistance gene product is readily detected in maxicells, see track 4 for best example, compare with tracks 6 and 7, showing the Tc^{S} plasmids pPM2001 and pPM2000. No expression of the 26K Cm^{R} gene product, or any other plasmid encoded polypeptides carried by pPM1000 (track 8), is seen. This plasmid because of its large size may be degraded along with the chromosomal DNA during incubation of the 'maxicells'. preparation of maxicells and other procedures are described in the Materials and Methods.

figure 4-4



It is reasonable to suggest that expression of the 120K, 28K and 24K polypeptides is under common control and possibly organised in an operon, thus accounting for the observed co-ordinate expression of these three genes. If a repressor molecule is the controlling element then titration of this molecule by the high copy number plasmid may be ~~raising the~~ ^{raising the} ~~basal level~~ ^{basal level} of expression in minicells. In maxicells this hypothetical repressor is in some way inactivated. Positive control by an effector molecule is also possible as this type of molecule may only be present at low concentration in minicells, but its synthesis is stimulated in maxicells, for instance, by the ultra-violet irradiation treatment of the host cells.

It is hoped that molecular dissection and localisation of the individual genes upon the 10.5 kb Hind III fragment will help provide an explanation of these minicell versus maxicell results.

4.7 PLASMIDS, pPM2000 AND pPM2001 CARRY A GENE WHICH MAY BE CONTROLLED BY CATABOLITE REPRESSION.

The expression of the 57K polypeptide coded by pPM2000 and pPM2001 is not enhanced in 'maxicells', as was observed for three other proteins carried by the 10.5 kb Hind III fragment (previous section) and is only weakly labelled in minicells, Fig. 4.3. However, a large amount of 57K protein is observed in commasie blue stained P.A.G.E. gels of minicells carrying either pPM2000 or pPM2001 and tends to overload the 55-60k region (data not shown).

That a lot of 57k protein was present and this was not reflected in labelling of the minicells, was puzzling. But when cyclic adenosine

monophosphate (cAMP), the activator for the positive effector molecule CAP (Ullman & Monod, 1968), was added to the minicell labelling reaction this resulted in strongly labelled 57K protein, Fig. 4.5. None of the other proteins coded by the 10.5kb Hind III fragment were affected.

It is known that CAP in the presence of cAMP stimulates transcription initiation at the promoter sites of many genes under catabolite repression (de Crombrughe et al, 1969). Enhanced expression in the presence of cAMP of the gene encoding the 57K polypeptide implies involvement of the type of positive control mechanism exerted by CAP.

In the absence of cAMP some expression of catabolite controlled operons can occur, for example, the lac operon (Ullmann & Monod, 1968) and the gal operon (de Crombrughe et al. 1969). This may account for the small amount of 57K polypeptide that is labelled in minicells without added cAMP. Possibly the 57K polypeptide is quite stable and low level expression of this polypeptide results in its accumulation, within minicells, during several hours incubation of the parental minicell culture. Thus explaining the relatively large amount of the 57K protein in purified minicells.

4.8. AN EXCESSIVE NUMBER OF POLYPEPTIDES ARE PRODUCED

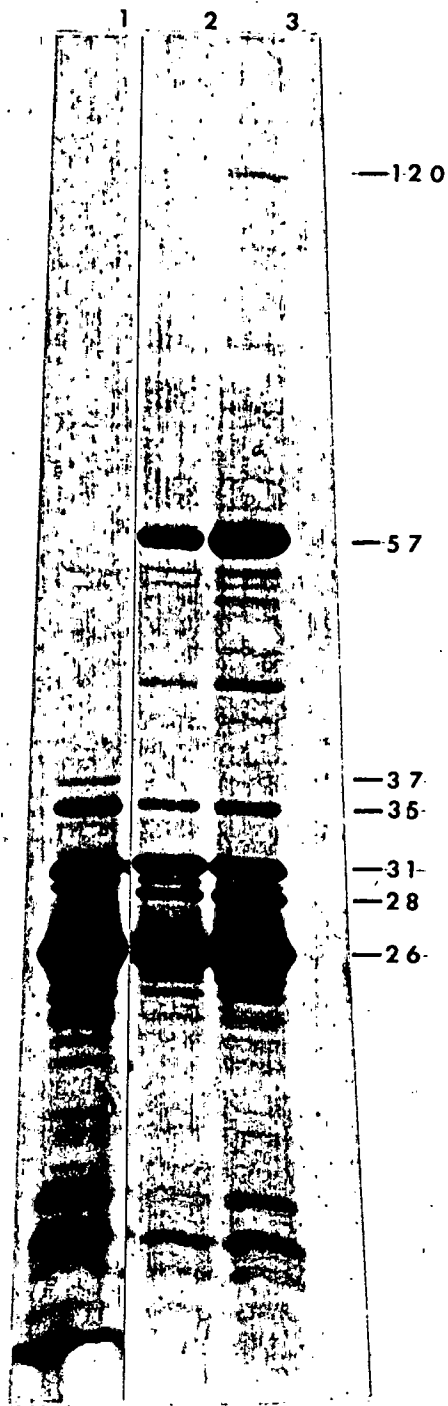
BY PLASMIDS pPM4000 and pPM4001 IN MINICELLS.

Detailed analysis, and a catalogue of the polypeptides synthesised by pPM4000 and pPM4001 in minicells, is difficult at this preliminary stage. As can be seen in Fig. 4.6, numerous bands appear in autoradiograms of labelled proteins synthesised by both these plasmids. To estimate the

Fig. 4.5.

³⁵S-labelling of minicells showing increased expression in the presence of cAMP of the 57K polypeptide coded by pPM2000 and pPM2001. All tracks are from the same gel; 1. pBR325, 2. pPM2000, 3. pPM2001. 10 μ l of 100mM cAMP in H₂O was mixed with the minicells before radiolabelling. Electrophoresis was carried out on a 7-20% SDS-polyacrylamide gel. The autoradiogram was exposed for 3 days. See materials and methods for experimental procedures.

figure 4-5



number of genes responsible for the synthesis of all the polypeptides that can be detected is impossible from this data. Furthermore, there is insufficient coding capacity upon the 6.0 kb of chromosomal DNA common to these two plasmids for each polypeptide, or even each pair of closely spaced polypeptides, to be the product of a single gene. Considerable post-translational modification, degradation or gene overlap must be occurring.

Every polypeptide that is a product of pPM4001 also appears, as far as resolution allows, to be represented in the range of gene products from pPM4000. These proteins, common to both plasmids, are probably synthesised by genes wholly contained within the 6.0 kb of chromosomal DNA. However, a strongly labelled 79K polypeptide and many other smaller polypeptides appear to be uniquely associated with pPM4000 Fig. 4.6. One plausible explanation for the additional gene products expressed by pPM4000, may be a result of a gene fusion of the N-terminal region of the pBR325 CAT¹ gene and a large, approximately 71K, section of coding DNA. Hence the strong expression of the 79K protein which is also easily observed in commassie blue stained P.A.G.E. gels (data not shown).

Restriction site mapping of pPM4000 was useful in identifying the end of the 6.0 kb EcoRI fragment in which the 71K of open reading frame must be located in order to fuse with the CAT gene of the pBR325 DNA. Transcription from this resistance gene promoter reads into the left end of the 6.0 kb EcoRI fragment in the orientation shown in Fig. 4.1. Therefore the N-terminal end from which the 71K coding sequence is initiated must lie to the left of this EcoRI fragment, in a region of the chromosome that was not isolated. Since almost 2kb of DNA sequence

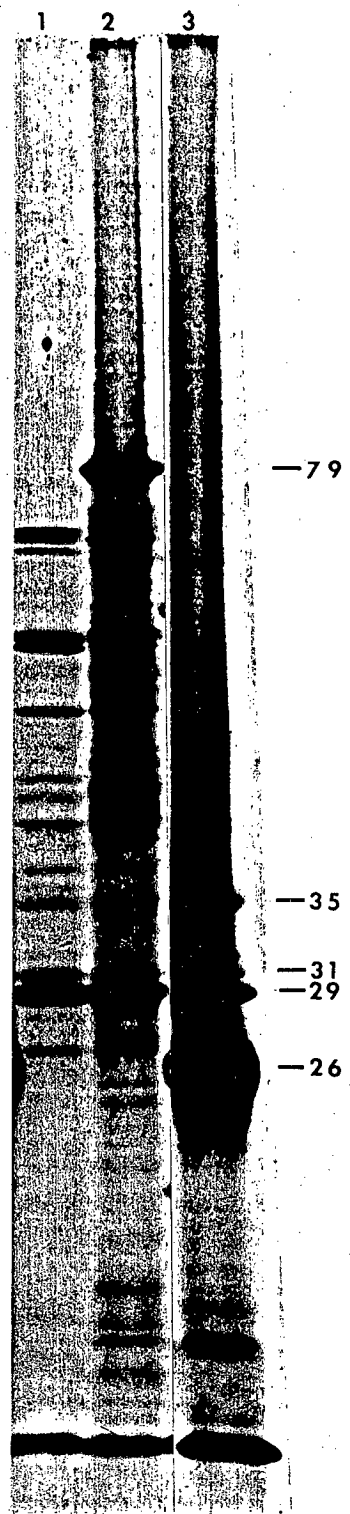
1. Chloramphenicol acetyl transferase.

Fig. 4.6.

Polypeptides expressed from pPM4001 (track 1.), pPM4000 (track 2) and pBR325 (track 3) labelled with ^{35}S -methionine in minicells.

The three tracks were ran on a single 7-20%SDS-polyacrylamide gel. Apparent molecular weights are in kilodaltons. Experimental procedures are described in the Material and Methods.

figure 4.6



is required to code for a polypeptide of 71K, this restricts even further the amount of DNA available to code for the many polypeptides synthesised by pPM4000 and pPM4001. Proteolytic degradation of the 79K fused protein, resulting in a series of small molecular weight fragments may explain the many additional polypeptides seen to be labelled in minicells harbouring pPM4000 that are clearly not expressed by pPM4001.

4.9 DISCUSSION.

The results presented in this chapter are summarised in Table 4.1 and Fig. 4.7. Table 4.1 lists all the polypeptides that were seen to be synthesised in minicells and maxicells harbouring ter C region plasmids. One omission is pPM4000, since the 79K suspected fusion protein and its many supposedly degradation products make the autoradiogram pattern too complicated for a reasonable interpretation.

What is evident, is that the genetically 'silent' ter C region of E.coli is not gene sparse. This result answers the simple question of whether this particularly large genetically 'silent' region is actually devoid of genes or if it possesses as yet undiscovered genetic loci.

Possibly over half the coding capacity of this cloned section of ter C region was found to code for 15-20 genes, assuming that the several chromosomal gene products of pPM4001 are coded by at least four or five genes. The coding capacity of this approximately 38 kb of DNA would be about 35 averaged sized proteins of 40K. A discussion of how the amount of coding DNA compares with selected, physically characterised gene dense regions, is given in Chapter 6.

TABLE 4.1.

PLASMID.	POLYPEPTIDES SYNTHESISED (kilodaltons).
pPM1000	77, 66, 64, 62, 61, 38, 35 ^{*1} , 31 [*] , 29 [*] , 26 [*] , (21), 20 ¹
pPM3000	62, 61, 26 [*] , 20
pPM3001	66, 64, 62, 61, 26 [*] , 20
pTH51	66, 64, 62, 61, 20
pPM1008	48, 26 [*] , 21
pPM1011	26 [*] , 20
pPM2000	120, 57, 35 [*] , 31 ^{*1} , 29 [*] , 28, 26 [*] , 24
pPM2001	120, 57, 35 [*] , 31 [*] , 29 [*] , 28, 26 [*] , 24
pPM4000	79, SEE pPM4001, and others.
pPM4001	71, 69, 58.5, 50, 44, 42.5, 40.5, 36.5, 34.5, 31 [*] , 29 [*]
pBR325	35, 31, 29, 26

Polypeptides expressed in minicells carrying ter C region plasmids.

¹ Possibly more than one gene product of this size. The polypeptide in parenthesis only occasionally appeared as a gene product.

* POLYPEPTIDES EXPRESSED BY THE VECTOR DNA.

Fig. 4.7.

Physical map of the cloned ter C region DNA showing positions of the polypeptides described in the text that were mapped to particular sections of DNA. Precise positions for the trg and periplasmic proteins (Harayama et al. 1982) are shown. Apparent molecular weights, given in kilodaltons, in parentheses denotes the exact position of the gene encoding the polypeptide; are not known. Dotted lines indicate suspected coding regions. The boxed-in question mark is a region where the actual number of polypeptides is uncertain. The open-ended box at the left hand end is a coding region of the C-terminal end of a chromosomal gene that was discovered as a 79K CAT-fusion polypeptide coded by pPM4000. Coordinates, in kb, are according to the Bouche (1982) map of the terminus region.

figure 4-7

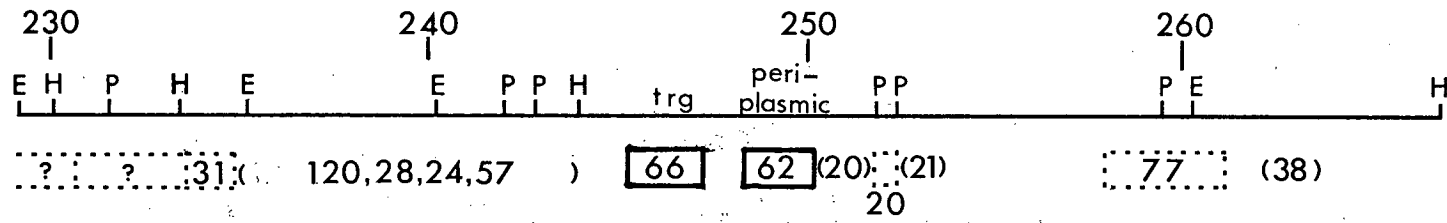


Fig. 4.7 summarises the mapping data of genes that were identified as gene products coded by the chromosomal DNA inserts of the ter C region plasmids described in individual sections of this chapter. Plasmids carrying overlapping sections of chromosomal DNA served to localise some of the gene products and eliminate others as being coded within those regions. Other gene products were mapped to within a particular restriction fragment if both orientations of the DNA fragment, inserted into the pBR325 vector, coded for similar sized polypeptides.

The 77K polypeptide coded by pPM1000 has been tentatively placed in Fig. 4.7 spanning the Pst I site at 259kb . This mapping information was based on a suspected 48K truncated gene product, expressed by pPM1008 and pPM1009, which both carry the 7.4 kb Pst I fragment of pPM1000. Only the 77K polypeptide is large enough to accommodate a truncated gene product of this size, and was thought to map at the right hand end of the 23 kb Hind III fragment.

Two suspected gene fusions, the 79K polypeptide of pPM4000 and a 31K polypeptide from pPM2000, have led to identification of the possible location of chromosomal DNA coding sequences at the left end of the 6 kb EcoRI fragment at around 230kb , and the left end of the 10.5 kb Hind III fragment at about 234kb , Fig. 4.7. The 31K polypeptide may be a complete structural gene fused to a promoter sequence directing transcription out of the tetracycline resistance gene of pBR325.

In Fig. 4.7, the coding sequence represented as an open dotted box is the amount of DNA required to code for the 79K polypeptide less the approximately 8k contribution from the N-terminal end of the CAT gene product. The size of the N-terminal region up to the EcoRI site within

the CAT gene was calculated from DNA sequence data (Alton and Vapnek, 1979).

A third fused gene may have been created that results in expression of the tetracycline resistance gene of pBR325 by a promoter located on the 10.5 kb Hind III fragment, in pPM2001. The Hind III target within the tetracycline resistance gene of pBR325 lies in the promoter region. Thus a DNA fragment carrying a suitably placed promoter and cloned into the Hind III site, may result in expression of the tetracycline resistance gene. In this way the plasmid vector can be used as a promoter expression cloning vehicle (See An & Friesen, 1979). Other promoters within the cloned ter C region may be located in this way.

In this respect work is underway to isolate promoter sequences from the cloned 38 kb ter C region, using specially constructed promoter expression vehicles (McKenney et al, 1981). This cloning system allows one to screen or select for promoter sequences through fusion with the gal K gene of E.coli.

DNA fragments have been sub-cloned from pPM2000, using Hind III and BamHI single and double digests, into a promoter expression plasmid, pkO-4 (McKenney et al, 1981). Transformants of a gal K mutant strain, C600K⁻, were selected on ampicillin and screened for gal K expression on galactose plates. The location of the cloned DNA fragments upon the pPM2000 restriction map and the characterisation of gene products coded by these plasmid clones is currently being studied.

Another approach is being considered to map the genes carried by the ter C region plasmids. This involves generating insertions of a transposon into several different sites within the cloned chromosomal DNA. A system of transposon mutagenesis is now available where mutagenised plasmids can be isolated carrying a number of insertions of a chloramphenicol resistance transposon, Tn1725 (Altenbuchner, et al, 1983.)

This requires only standard genetic and DNA manipulation techniques. 'Knock-out' of any of the gene products normally coded by the plasmids can be identified in a protein synthesising system, such as minicells.

This approach would be particularly useful in studying the apparent co-ordinate expression of the 12CK, 28K and 24K polypeptides synthesised by pPM2000 and pPM2001. If genes coding these three polypeptides are organised into an operon, for instance, polar mutations would be expected to be created by insertion of Tn1725 upstream of the promoter distal genes.

CHAPTER 5.

5.1 INTRODUCTION.

To extend the 38kb of terminus region DNA, isolated by the method of chromosome walking (Chapter 3), sufficient homology is required from overlapping DNA fragments extending beyond the cloned region. It was foreseen, from the outset of this work, that at some point in using the walking strategy, progression would be most likely slowed down by closely spaced restriction sites and halted completely by nearly coincident sites. Consequently, unless libraries were made of recombinant molecules carrying chromosomal DNA cleaved with restriction enzymes other than Hind III or EcoRI, another approach to cloning was needed.

The data given in the Bouche (1981) physical map of the terminus region, suggests that the cloned DNA described in chapter 3, may only be extended a further 6.9kb clockwise and 8.25kb anticlockwise by chromosome walking. Respectively, two positions, 221kb and 274kb, on the Bouche (1981) physical map, see fig. 5.1, would be arrived at by two steps to the left, a 6.2 kb Hind III fragment followed by a 3.15kb EcoRI fragment, and one step to the left, a 14.3kb EcoRI fragment, whereupon insufficient homology may exist to continue the walking process.

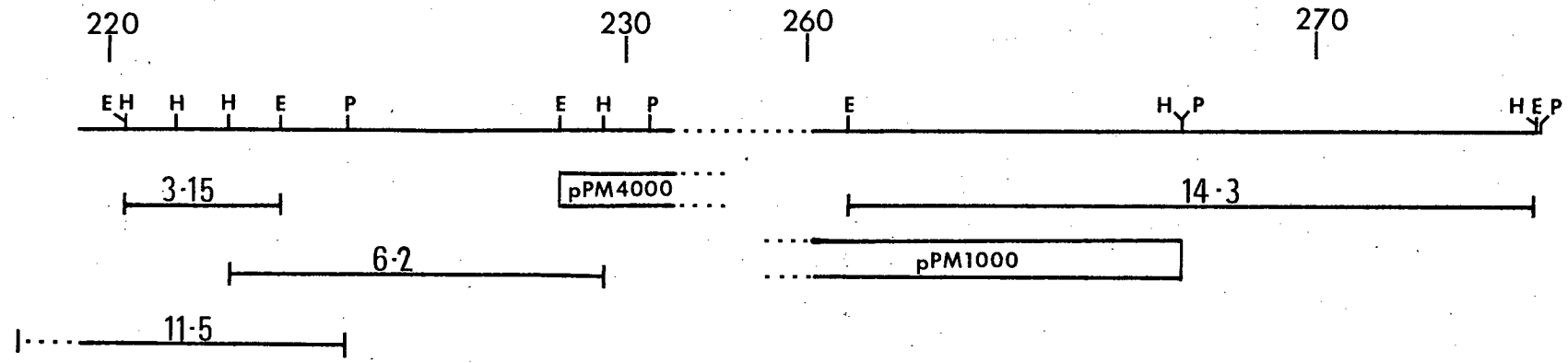
Even if the restriction enzyme Pst I had been used to set up a third library, for instance, by exploiting the unique Pst I cleavage site within the B-lactarase gene of pBR325 (Bolivar, 1978), a block to walking may still have been encountered at the Bouche, map reference 274kb, where sites for Hind III, EcoRI and Pst I are all coincident.

If DNA was available to use as a specific hybridisation probe for the region of the chromosome immediately beyond the position of coincident

Fig. 5.1.

Physical map of the ter C region extending beyond the cloned sections carried by pPM1000 and pPM4000. Coincident EcoRI and Hind III sites are encountered in both directions. Sizes of the overlapping fragments (given in kb) were determined from DNA hybridisation experiments (see Chapter 3), or taken from the Bouche (1982) physical map of this region. EcoRI (E); Hind III (H); Pst I(P).

figure 5-1



restriction sites, then DNA fragments could be isolated to bridge the section of DNA lying in between.

The following approach was adopted to overcome, or 'leap past', possible blocks to chromosome walking caused by coincident restriction sites. A cloning strategy was developed by which DNA from E.coli K12 strains carrying the transposon Tn 10 inserted at suitable map locations is used as the target DNA. Two Hind III cleavage sites are present in Tn 10 (Jorgensen & Reznikoff, 1979), one of which cuts within the region conferring tetracycline resistance, fig. 5.2. By cloning DNA fragments partially digested with Hind III, it was hoped that DNA fragments possessing intact tetracycline genes and an amount of flanking chromosomal DNA would be isolated.

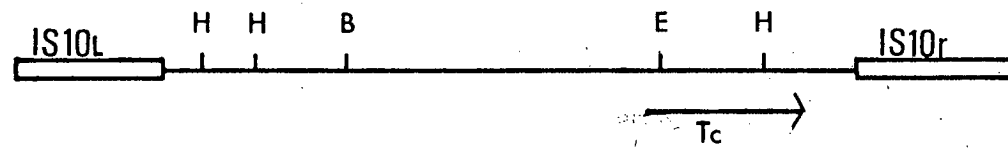
Cloning of terminus region DNA was attempted using the above strategy. Two strains were employed, PLK1291 and PLK1110 (Bitner and Kuempel, 1981) which Tn 10 inserted into the chromosome at 32 minutes and 34 minutes respectively. Recently, Henson and Kuempel (1983) have successfully cloned 14.2kb of chromosomal DNA flanking the left hand IS10 of Tn 10, fig. 5.2, from a strain related to PLK1291 and similarly carrying the transposon at 32 minutes. Conceptually their cloning strategy was analogous to that described above, but initially they isolated the DNA using a Lambda phage BamHI cloning vehicle and recombinant phage were screened by plaque hybridisation using a plasmid carrying Tn 10 as a 32p labelled DNA probe.

Experiments are described in this chapter which failed to result in isolation of trg DNA by transposon cloning strategies. Results are presented of a limited characterisation of two plasmid clones that were obtained by employing this approach. All techniques are described in Chapter 2.

Fig. 5.2.

Simplified restriction map of transposon Tn10 (adapted from Jorgensen and Reznikoff, 1979) showing that both EcoRI (E) and Hind III (H) enzymes cleave within the tetracycline resistance genes, but not Bam HI (B).

figure 5-2



1kb

5.2 ATTEMPTED CLONING OF *trg* DNA USING Tn 10 AND Tn 5 INSERTIONS.

Chromosomal DNA was prepared from two strains, TH1013 and TH1024, these are derivatives from strains originally isolated after transposon matagenesis, and shown to carry Tn 10 and Tn 5 inserted into the *trg* gene (Harayama et al, 1979). Limit digests of TH1013 DNA with Bam HI and TH1024 with EcoRI, were ligated with suitably digested pKO-4 DNA, a small multicopy plasmid cloning vehicle (McKenney et al, 1981). After transformation of competent C600 cells, selection was applied for the tetracycline resistance genes derived from Tn 10 and kanamycin was used to select for the resistance genes of Tn 5 (Berg et al, 1978). All attempts to obtain tetracycline or kanamycin resistant clones, whether in solid or in liquid medium, failed.

Tn 5 does not possess an EcoRI cleavage site (Berg et al, 1975; Jorgensen et al, 1979) and therefore chromosomal DNA from both sides flanking the transposon would have been expected to be cloned. Hybridisation of a *trg* carrying Pst I fragment to EcoRI digested chromosomal DNA, Fig.3.1 shows the *trg* gene to be contained within an approximately 20kb EcoRI fragment, which will presumably increase to about 25.4kb in strain TH1024 due to the size of Tn 5 (Berg et al, 1978). Fragments of this size are not too difficult to clone in small plasmids, for example, the plasmid pPM1000 Fig.3.7. Also, Tn 5 has been cloned upon the high copy number plasmid ColeI (Jorgensen et al, 1979) with no particular problems in obtaining the construction. It is not clear why the cloning experiments involving *trg* :: Tn 5 were not successful.

Fig. 5.2 shows a physical map of the transposon Tn 10 and the position of the tetracycline resistance genes. In the initial cloning experiments

using strain TH1013 DNA and the restriction enzyme Bam HI, it was expected that any tetracycline resistant recombinant pkO-4 molecules would possess transposon DNA extending from the single Bam HI cleavage site within Tn 10 through the right hand IS₁₀ and into the flanking chromosomal DNA.

No tetracycline resistant plasmid molecules were found, possibly because of the following reason. Although the mechanism of control of Tn 10 tetracycline resistance is not fully understood, it is evident that when the tetracycline resistance genes are maintained in a multicopy state a phenotypic effect, resulting in lowered levels of resistance, is found. (Jorgensen & Reznikoff, 1979; Coleman & Foster, 1981). Throughout the transposon cloning attempts with a Tn 10 insertion of trg, a concentration of 15µg/ml of tetracycline was routinely used. Inhibition of cells harbouring tetracycline genes, derived from Tn 10, in a multicopy state, may be expected to occur at 15µg/ml of tetracycline as described below.

5.3 USE OF HIND III PARTIAL DIGESTS TO CLONE THE TETRACYCLINE GENES AND FLANKING DNA FROM TWO Tn 10 INSERTIONS.

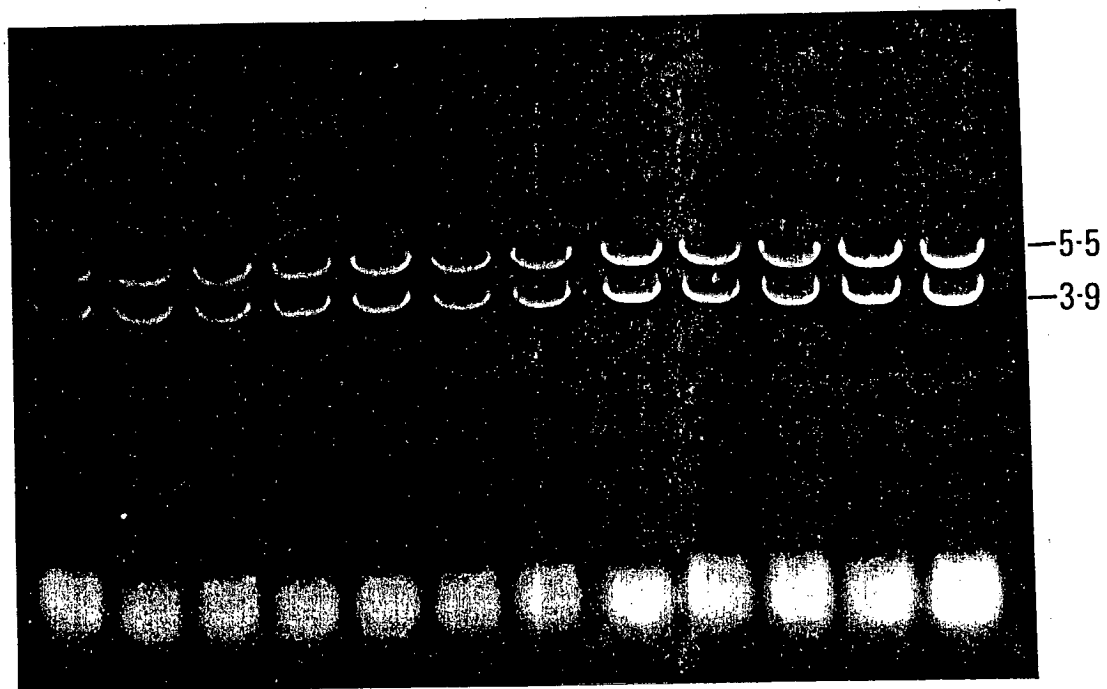
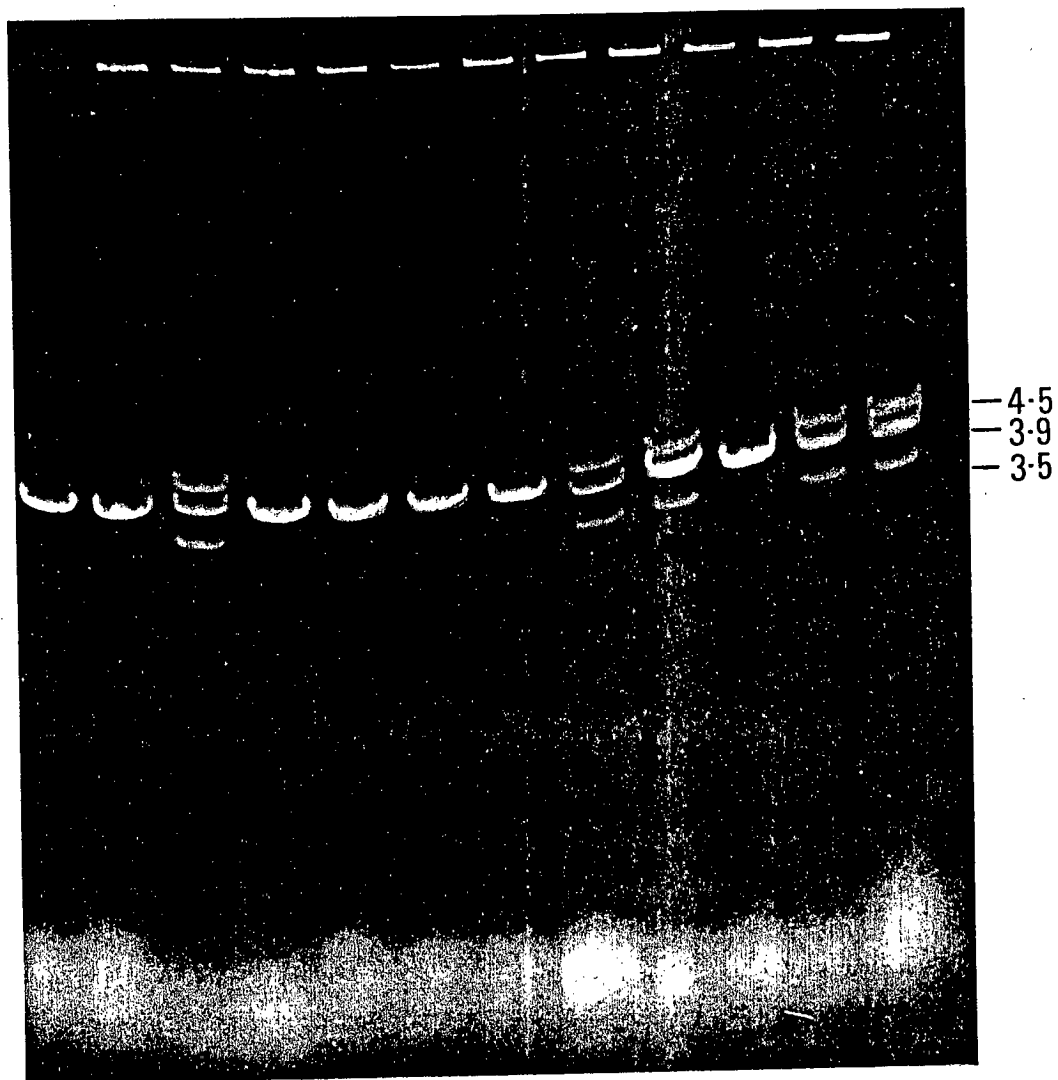
The antibiotic tetracycline has the property of inhibiting the growth of sensitive bacterial cells without resulting in cell death. This bacteriostatic mode of action was relied upon in the following cloning experiments.

Partially Hind III digested PLK1110 (Tn 10 at 34 minutes) and PLK1291 (Tn 10 at 32 minutes) DNA was ligated with Hind III cut pkO-4 DNA. Competent C600 cells were transformed with the ligated molecules and

Fig. 5.3.

Agarose gel electrophoresis of Hind III digested DNA mini-preps performed upon overnight cultures set up from Ap^R Tc^R transformants obtained from the Tn 10 cloning experiments described in the text. The source chromosomal DNA was from strain PLK 1110 in the upper photograph and strain PLK 1291 in the lower. The upper photograph shows that combinations of three Hind III fragments have resulted from the cloning experiment; the plasmid in the third track from the left was kept for further analysis (pTN 1034). All plasmids in the lower photograph appear to be identical and may have arisen from a single transformant; one of these, designated pTN 1032, was retained for analysis. DNA fragment sizes are in kb. Experimental procedures are given in the Materials and Methods.

figure 5-3



used to inoculate LB liquid cultures containing 15µg/ml of tetracycline. After overnight incubation at 37°C, one culture, in which strain PLK1291 had been used for the source DNA, showed slight growth, other cultures did not. All cultures were diluted one half with fresh LB without added tetracycline. Further incubation resulted in a dense culture, from the low growth culture seen earlier, after about 3-4 hours. A dense growth was obtained after about 24 hours further incubation of a PLK1110 derived culture. Putative tetracycline transformants were serially diluted and plated to achieve isolated colonies on ampicillin plates containing 7.5µg/ml tetracycline. Several doubly resistant colonies were picked from each cloning and their plasmids were analysed by agarose gel electrophoresis after digestion with Hind III, fig. 5.3.

One plasmid, derived from PLK1110 DNA, was retained for further study and designated pTN1034, this had the following expected features; at least three Hind III fragments, that is, one derived from an internal Hind III fragment of Tn 10 measuring about 4.5kb in size, 3.9kb Hind III cut, μ KO-4 DNA, and a Hind III fragment of at least 2.27kb to span the DNA from the Hind III site within the Tn 10 tetracycline resistance genes to the right hand IS10, Fig. 5.2. None of the plasmids in fig. 5.3 derived from PLK1291 DNA, displayed all of these features, however, one plasmid, pTN1032, was kept for analysis of any chromosomal DNA homology.

5.4 PHYSICAL CHARACTERISATION OF pTN1032 AND pTN1034.

A unique physical map of pTN1034 Fig. 5.5, was constructed from data generated by restriction enzyme single and double digestions using Hind III, EcoRI and Pst I. Table 5.1 gives the sizes of the restriction fragments calculated from the gel shown in fig. 5.4.

Fig. 5.4.

Agarose gel electrophoresis of restriction digests of plasmids pTN1032 and pTN1034, and an autoradiogram of this gel transferred to nitrocellulose filter and probed with the 1.06kb Hind III/Pst I fragment of pTN1034. Tracks; 1 and 13, lambda CI857 DNA digested with Hind III; 7. Lambda CI857 DNA digest with EcoRI; 2-6 and A-E pTN1032 DNA; 8-12 and F-J, pTN1034 DNA; from left to right both DNAs are digested with, EcoRI, EcoRI/Hind III, Hind III, Hind III/Pst I, Pst I. The autoradiogram was exposed for 1 hour. Experimental procedures are given in the Material and Methods.

figure 5-4

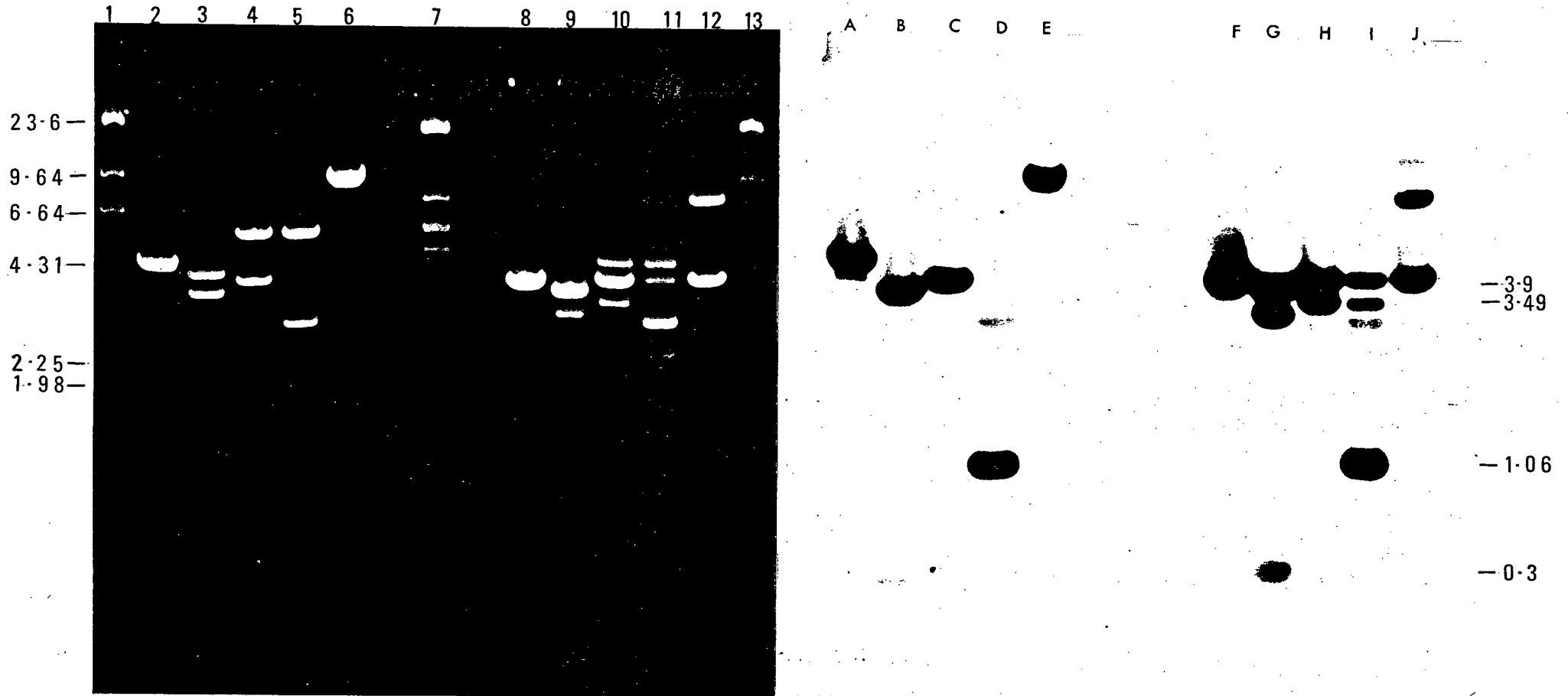


TABLE 5.1.

pTN 1034.					pTN 1032.				
H	H/E	E	P	H/P	H	H/E	E	P	H/P
4.46 I	3.60tI a,b,c,	4.10 I	7.70 I	4.46 I	5.50	3.95	4.25d	9.30	5.50
3.90d II a,b	3.25 II	3.90t II,a,b,c	4.15 II	2.85 d II, a,b	4.4w	3.60	3.8w	8.60w	4.4w
3.50 III	0.3t III a,b,c		3.90 III	2.20 III	3.90	0.86	0.68		2.85
				1.29 IV		0.68			1.06
				1.06d V,a,b		0.3			

Sizes of DNA restriction fragments, given in kb, of the plasmids pTN 1034 and pTN1032, obtained from analysis of the agarose gel shown in Fig. 5.4. The weak DNA bands of digest pTN 1032 DNA are identified (w). (d) and (t), doublets and triplets, respectively, of unresolved DNA fragments.

Enzyme abbreviations given in text.

It can be seen in Fig. 5.5, that fragments (H/P-IVa, b, c) share homology with many of the DNA fragments produced by the restriction digests given in Table 5.1. Indeed, this is in accordance with the hybridisation result shown in Fig. 5.4, where a ^{32}P -DNA probe was made from the 1.06 kb Hind III/Pst I fragment, isolated by the 'freeze-squeeze' technique, and hybridised to a southern blot of the DNA digests shown in Fig. 5.4.

Only fragments (H/R-III), (H-I) and (H/P-I) did not show any homology with the DNA probe. This was expected, since the size of these fragments are consistent with them being derived from the tetracycline resistance region of Tn10. It is not clear why the 2.85 kb Hind III/Pst I fragment of pK0-1 (H/P-IIa, b) and the 2.2 kb chromosomal DNA fragment (H/P-III), weakly hybridised to the DNA probe.

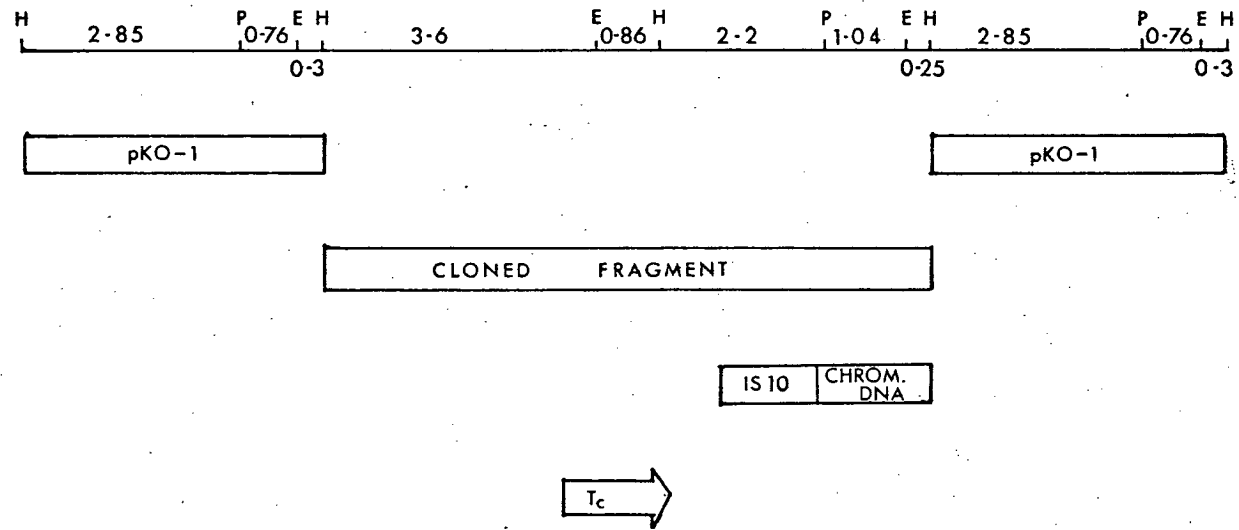
The latter result may be due to the presence of a phage lambda homologous sequence, upon the chromosome that has hybridised with lambda DNA carried by the pK0-1 vector. DNA from the lambda O gene region is present on the 1.06 kb fragment of pK0-1 (McKenny et al, 1981), this is one of the fragments used for making the DNA probe, as described above. The lambda O gene is located at about 81% on the linear lambda molecule (Maniatis et al. 1982). a position that is present on the 5.83 kb EcoRI and 6.64 kb Hind III fragments of lambda CI857 DNA. Hence, the observed homology of the DNA probe with the EcoRI and Hind III lambda CI857 molecular weight standard fragments in Fig. 5.4

Analysis of the restriction data of pTN1032 initially appeared complicated due to the presence of weakly ethidium bromide stained bands, which could not be explained as partially digested fragments. However, if the weak bands seen in Fig. 5.4 are ignored then an unambiguous restriction map

Fig. 5.5.

Restriction map of pTN1034. The cloned DNA consists of a partially digested Hind III fragment carry the tetracycline resistance genes and the right hand IS10 of transposon Tn10 (see Fig. 5.2), also, a section of adjacent chromosomal DNA. Fragment sizes are in kb. EcoRI (E); Hind III (H); Pst I (P).

figure 5-5



can easily be constructed from the simple restriction fragment pattern, Fig. 5.6. Unfortunately there are no common restriction fragments between this plasmid and the physical map of Tn10, Fig. 5.1, therefore, it is unlikely that this plasmid carried the tetracycline resistance genes of Tn10, unless extensive molecular rearrangement has occurred.

The hybridisation result shown in Fig. 5.4, confirms that pTN1032 DNA is derived from pKO-1 DNA. Two Hind III/EcoRI fragments of 3.6 kb and approximately 0.3 kb, a 3.9 kb Hind III fragment and a 1.05 kb Hind III/Pst I fragment, all strongly hybridised to the 1.06 kb (H/P-IVa,b,c) DNA from pTN1034. Respectively, these pTN1032 restriction fragments are equivalent to the (H/R-II), (H/R-IVa,b,c), (H-IIa,b) and (H/P-IVa,b,c) fragments of pTN1034, Table 5.1. Weak hybridisation to the 2.85 kb Hind III/Pst I fragment of pKO-1, which was seen with pTN1034 DNA, was also found with pTN1032 DNA.

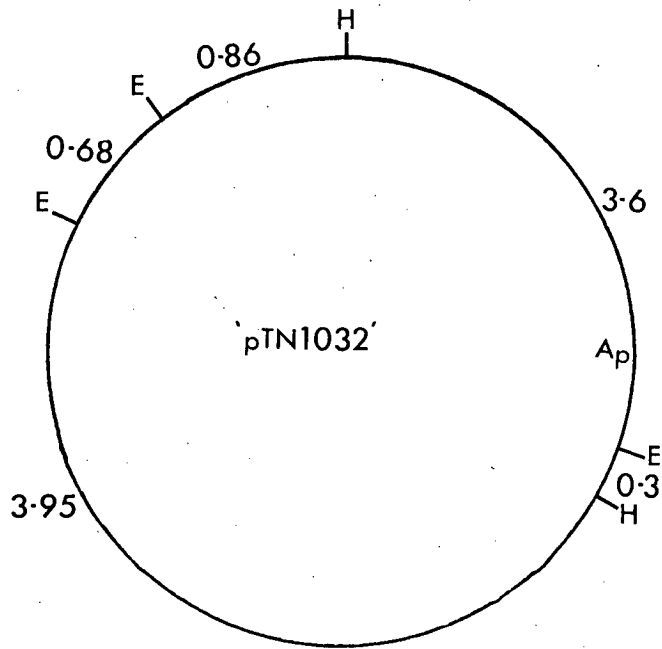
Two of the faint bands on the agarose gel of Hind III and Hind III/Pst I digests of pTN1032 DNA, Fig. 5.4, measure 4.4 kb in size. This is the size of one of the Hind III fragments in pTN1034, and this fragment is the one that is expected to be cloned from Tn10, carrying part of the tetracycline resistance region. Also, a faint band of 8.6 kb is seen a Pst I digest of pTN1032 DNA.

It is possible that the strain from which the pTN1032 DNA was prepared, carried two plasmids, one at high copy number and derived from pKO-1, another existing in a much lower number of copies, conferring tetracycline resistance. Both ampicillin and tetracycline were present in the growth media for the strain carrying pTN1032, therefore, selection for both plasmids would have been maintained.

Fig. 5.6.

Physical map of 'pTN 1032' constructed from the restriction data given in Table 5.1, ignoring the weak DNA bands observed in some of the tracks in Fig. 5.4.

figure 5-6



The level of resistance to tetracycline conferred upon C600 by pTN1034 and pTN1032 was tested. A minimum inhibitory concentration of tetracycline, above which a strain will not grow, cannot be determined for the tetracycline resistance coded by Tn10 (Jorgensen & Reznikoff, 1979). Instead, differences of the level of tetracycline resistance, between strains, is determined by observing the efficiency of plating (E.O.P.) of cells on agar plates containing a range of tetracycline concentrations.

Table 5.2 shows the result of such an E.O.P. test on: the host strain C600; three plasmids that are pBR325 recombinant molecules, two have Hind III inserts and the other, a Pst I insert; the two Tn10 derived plasmids to be compared, pTN1032 and pTN1034. Strain C600 and C600 carrying pPM2000 are sensitive to 5µg/ml of tetracycline, the lowest concentration employed in this experiment. pPM2000 and pPM2001 both have an insertion of a 10.5 kb Hind III fragment into the tetracycline resistance gene of pBR325 and only differ in the orientation of their Hind III fragments (chapter 4). The tetracycline resistance observed with pPM2001 may be due to a fusion of the pBR325 tetracycline structural gene and a promoter on the cloned Hind III fragment, this is discussed in Chapter 4. An intact tetracycline resistance gene of pBR325, carried by pPM3001, confers resistance across the range of tetracycline concentration used in table 5.2, and serves as a positive control in this experiment.

Clearly, there is a difference between the E.O.P. of C600 carrying pTN1034 on tetracycline concentrations above 5µg/ml compared with pTN1032. A useful measure of levels of tetracycline resistance, is the E.O.P. at which 50% cell survival is observed quoted as E.O.P.50 (Jorgensen & Reznikoff, 1979). Reference to Table 5.2 shows the E.O.P.50

TABLE 5.2.

TETRACYCLINE CONCENTRATION $\mu\text{g/ml.}$							
STRAIN	0	5	7.5	10	12.5	15	30
C600	173	0	0	0	0	0	0
pPM2000	147	0	0	0	0	0	0
pPM2001	127	89	0	0	0	0	0
pPM3001	77	57	68	59	58	53	50
pTN1034	174	92	59	19	12	8	10
pTN1032	137	106	103	88	87	80	81

C600 and C600 transformed with the named plasmids, challenged on a range of tetracycline concentrations. Overnight cultures grown on L-Broth were diluted to achieve isolated colonies and spread on the test plates. All plates were poured at the same time and treated alike.

to lie at 5-7.5 µg/ml tetracycline, whereas, the E.O.P.50 of pTN1032 is not achieved up to 30 µg/ml tetracycline, the highest concentration employed in this experiment.

Coleman & Foster(1981) have shown that the Tn10 tetracycline resistance gene in a multicopy state, that is, when cloned onto a high copy number plasmid, confer a lower level of tetracycline resistance to the host strain than when cloned on a low copy number plasmid or a single insertion of Tn10 into the chromosome. Hence, it is reasonable to suggest that the E.O.P.50 of pTN1032 is higher than that of pTN1034, because, there are fewer copies of the cloned Tn10 tetracycline resistance region in the strain carrying pTN1032.

The above genetic evidence in conjunction with the DNA analysis in the previous section, suggests that the pTN1032 DNA consists of two plasmids. One carries an ampicillin resistance gene and the other, the Tn10 tetracycline resistance region at a low copy number within the cell. Either growth of the strain carrying pTN1032 on a single antibiotic, rather than both ampicillin and tetracycline, or better, transformation of a competent strain and single selection of one of these two antibiotics, should resolve two individual plasmids and possibly provide an answer to whether pTN1032 DNA does consist of two plasmids or not.

5.5 DISCUSSION.

The success of the 'leap' to other parts of the ter C region will not be known until the cloned chromosomal DNA, thought to be carried by the plasmids described in this chapter, has been used as a DNA probe to

identify restriction fragments in E.coli chromosomal DNA digests. From the sizes of DNA fragments in Hind III, EcoRI and Pst I restriction digests that share homology with the DNA probes, it may be possible to locate the cloned regions on the Bouche (1982) physical map of the terminus region.

One of the plasmids that was obtained, pTN1034, was shown by restriction analysis to carry a Hind III fragment from the tetracycline resistance region of the transposon Tn 10 and probably some flanking chromosomal DNA of an E.coli strain which has Tn 10 inserted at 34 minutes on the genetic map. An insufficient number of appropriate restriction sites are present on the small section of chromosomal DNA carried by pTN1034 to identify this piece of DNA in the 34 minute region on the Bouche map. Thus, it is not known if the region of the chromosome carried by pTN1034 has also been cloned by other workers who have isolated DNA from the 34 minute region (Kaiser, 1979; Bejar & Bouche, 1983).

The DNA of the other plasmid described in this chapter, pTN1032, may consist of two individual plasmids, see section 5.4.

If this is indeed the case, then, the plasmid conferring tetracycline resistance is particularly interesting.

In order for this plasmid to be maintained within the bacterial cell, it must possess an origin of replication. It is unlikely that the replicon for this plasmid is carried by a pKO-1 vector fragment, for two reasons. Firstly, only one DNA fragment hybridises to the DNA probe carrying pKO-1 DNA in EcoRI and Pst I digests of pTN1032 DNA, Fig. 5.4. Even the weak bands seen in the agarose gel of these digests, if they contained pKO-1 derived DNA, would still be expected

to show fairly strong hybridisation with the DNA probe. Second, the 'other' plasmid, which was shown by restriction analysis and DNA hybridisation to be a pKO-1 recombinant molecule, would be quickly lost from the transformed bacterial cell due to incompatibility, if this pKO-1 derived plasmid had the same replicon as the tetracycline resistant 'Tn 10 derived', plasmid. Plasmids with the same replicon are known to be 'incompatible' and cannot be maintained inside the same cell unless selection is applied for all the plasmids. (Timmis, 1979).

How is the TN1032 'tetracycline resistance plasmid' replicated?

Recently, a DNA replication origin, ori X, has been located around the 32 minute region of the chromosome (de Massay et al. 1984)

Ori X is the second origin to be found in the ter C region, the other ori J, is associated with the rac prophage at about 31 minutes

(Diaz et al. 1979) and is distinct from ori X (de Massay et al, 1979)

Perhaps, ori X, or a third origin, is located close to the Tn 10 inserted at 32 minutes in strain PLK1291 and this origin has been isolated upon a partially digested Hind III fragment, along with the tetracycline resistance genes of Tn 10, resulting in an autonomously replicating DNA molecule that carries tetracycline resistance genes.

To test this hypothesis, one could simply ligate partially Hind III digested PLK1291 DNA (or use another suitable restriction enzyme) transform a competent E.coli strain and select for tetracycline resistant colonies at an appropriate tetracycline concentration (Table 5.2). This suggests a novel method for possibly isolating large sections of ter C region DNA, the size of which will be initially dependent upon the extent of digestion by the restriction enzyme. Or, as a tool to search

for other replication origins in the ter C region, using a number of transposon insertions across the ter C region that have now been constructed (Bitner & Kuempel, 1981; Fouts & Barbour, 1982).

DISCUSSION.

CHAPTER 6.

DISCUSSION.6.1 CODING DNA IN THE TERMINUS REGION.

The main objectives of the work presented in this thesis have been achieved. A region of the E.coli K12 chromosome, which is still almost devoid of genetic markers, has been isolated through the techniques of molecular cloning and gene products encoded by the cloned DNA were found to be expressed in vivo. From physical mapping studies (see Chapter 3) it can be deduced that a continuous stretch of DNA spanning the genetic map distance between position 31.0 and 31.8 minutes is entirely represented in the bacterial DNA inserts of the plasmids described in Chapters 3 and 4.

In total about 38 kb of DNA was cloned from the ter C region. The coding capacity of this amount of DNA, if fully transcribed and translated, is almost 140K, assuming an average amino acid molecular weight of 110 daltons. Possibly, over half of this amount of coding, 742K, was found to be expressed from the cloned ter C region DNA fragments. An estimate of 15-20 genes may code for the proteins that were found to be expressed in a minicell protein synthesising system and identified as ³⁵S-methionine labelled polypeptides in SDS-polyacrylamide gradient gels, see figures and table in Chapter 4.

The estimate of the extent of coding and the number of genes within the cloned section of ter C region DNA may be affected by various factors that influence the expression of proteins in protein synthesising systems. Attempts were made to identify, and where possible, eliminate factors which could lead to either an over-estimation of the amount of coding

carried by a DNA fragment, or an under-estimation. Below, factors that were recognised as probably leading to:

- a) a high estimate and,
- b) a low estimate,

are discussed.

a) Over-estimation:

Are the number of polypeptides seen to be expressed from a cloned fragment a true reflection of the minimum number of genes carried upon the DNA? In many cases this has been shown not to be so. Dougan et al. (1979) reported that the expression of the ampicillin resistance gene of the plasmid, pBR322 results in three polypeptides that are identified in polyacrylamide gels; the 31K unprocessed pre- β -lactamase, the 29K mature β -lactamase and a 27K presumed degradation product. At least two of these gene products, 31K, and 29K, were found to be coded by pBR325 and its ampicillin resistant derivatives. They were easily identified as two polypeptides that were absent in ampicillin sensitive Pst I recombinants of pBR325, see figures in Chapter 4.

66K and 64K trg gene products (Harayama et al, 1982), and the 62K and 61K gene products a gene coding for a periplasmic protein (Harayama & Hazelbauer, 1982) coded by some of the ter C region plasmids, fig. 4.3 were shown by Harayama and co-workers, to be the products of single genes. Harayama et al, (1982) demonstrated that the trg polypeptide doublet was inactivated by an insertion of the transposon, Tn 5, into the trg gene, also their expression was dependent upon a chemotaxis and motility control gene, flbB (fla I). Harayama and Hazelbauer (1982) showed the 62K polypeptide to be the unprocessed precursor of a 61K

periplasmic protein, which was also located as the mature protein in the cell membrane of minicells.

None of the other gene products coded by pPM1000, plasmid sub-clones of pPM1000, or pPM2000, gave close banding patterns 'characteristic' of processed molecules. That is, regions in the autoradiograms of the polyacrylamide gels that contained closely spaced labelled proteins and similar in appearance to the doublets observed for the trg and periplasmic protein gene products. It was thus assumed that single polypeptides were the products of individual genes.

One very good example of the problem of multiple protein bands on gels leading to over-estimation of coding DNA is that of the proteins synthesised in minicells by plasmids pPM4000 and pPM4001 (see Fig. 4.6). An estimate of the amount of coding required for the polypeptides synthesised by pPM4001, that can be clearly observed, is in excess of 300%. The synthesis of at least 6 or 7 polypeptide doublets are seen to be directed by pPM4001. Perhaps this region of the chromosome contains a cluster of membrane proteins, since, the appearance of these doublets is very similar to 62K and 61K doublets of the periplasmic protein discussed above. Even if each doublet was the product of a single gene, these genes would have to overlap to be accommodated on the 6 kb insert of pPM4001. Extensive processing or degradation of a large polypeptide is possibly an alternative explanation, as evidenced by the 79 K fused chloramphenicol gene product of pPM4000. This produces many degradation products that appear as bands in the autoradiogram of the polyacrylamide gel.

The complex gel pattern produced by the labelled proteins of pPM4000 and pPM4001 would be most easily resolved by reducing the number of gene products through deletion or subcloning of the bacterial DNA inserts. Neidhardt et al (1983) found that individual plasmids from the Clarke and Carbon library had a range of coding from 36% to 123%. No explanation of % coding values higher than 100% was offered by these workers.

Problems associated with identifying multiple products of a single gene are not easily overcome and require much additional work before individual polypeptides can be shown to be alike or otherwise. This may involve a biochemical assay of the proteins extracted from a gel, tedious peptide mapping, insertions into or deletions of the DNA encoding the gene; by in vitro or in vivo manipulations to completely remove or alter the family of gene products synthesised by the cloned DNA fragment.

Another complicating factor is the plasmid vector itself, as well as the promoters normally active in expressing the three antibiotic resistances, pBR325, has other promoters directing transcription in both directions from various locations around the plasmid. These have been characterised for the pBR325 progenitor molecule pBR322 (see Fig. 4.2). Interference from these promoters is thought to be occurring in at least two instances described in Chapter 4; a fusion involving two structural genes, the N-terminal end of chloramphenicol acetyl transferase (CAT) and an extensive region of chromosomal coding DNA carried by pPM4000; the other involves convergent transcription with the trg gene and is discussed in Section 4.4.

In the case of pPM4000, the precaution of inverting the chromosomal DNA insert proved to be very worthwhile. A 79K polypeptide expressed by pPM4000 see Fig. 4. is suspected to be a fused gene product consisting of about 8K of the N-terminal end of the CAT protein fused to the C-terminal end of a structural gene encoded by the chromosomal DNA. If the 79K polypeptide was not the product of a fused gene, but instead, coded by a gene in the chromosomal DNA insert of pPM4000, then it would also be expected to be expressed by pPM4001, which carried the same DNA fragment. A 79K protein was not found to be one of the gene products of pPM4001. Thus, this large protein was not falsely attributed to a gene in this region of the chromosome, but it can be said that a large coding region probably exists at one end of the cloned DNA fragment.

It is easily imagined that a gene fusion could occur involving the C-terminal end of the CAT gene, adding about 18K of protein, and a gene that is transcribed out of the end of a DNA fragment. Similarly fusions could be created by insertion of Pst I fragments into the B-lactamase gene of pBR325.

Inversion of the cloned DNA fragments, where possible, should guard against over-estimation of the amount of coding sequence upon a DNA fragment, due to gene fusions, as shown above.

b) Under-estimation.

Interference by transcription from the vector molecule could lead to a low estimate of the extent of coding of a cloned DNA fragment. Section 4.4, of Chapter 4, describes the failure of the plasmid pPM3000 to express the trg gene product, possibly due to transcription from a vector promoter converging with the trg gene promoter. Transcription from this vector promoter was separated from the trg gene allowing its expression, by having the chromosomal DNA fragment cloned in the opposite orientation in the plasmid pPM3001.

DNA fragments inserted into the antibiotic resistance structural genes of the plasmid vector, must have the promoter of the antibiotic resistance gene directing transcription into one end of the cloned DNA. Chromosomal genes that are located at the antibiotic gene promoter-proximal end of the inserted DNA, and are transcribed towards the end of this DNA fragment, may have their expression reduced, or completely inhibited, by convergent transcription with the antibiotic gene promoter. This interference of chromosomal gene expression can be eliminated from affecting a particular gene by having the cloned DNA fragment inserted in the opposite orientation.

Another source of under-estimation could arise from not visualising, by autoradiography, very rare E.coli proteins. Rare abundance proteins are difficult to detect in labelled whole cell lysates on two-dimensional polyacrylamide gels (O'Farrell, 1975). However, the minicell and maxicell systems, used to study protein synthesis from the ter C region plasmids, have the advantage over whole cell labelling methods of having cloned genes in a multicopy state.

High specific labelling of some proteins and the increased gene dosage, may assist in their identification in the autoradiograms.

Since, only ^{35}S -methionine was used as label in this study, proteins that do not contain methionine will not have been detected.

^{14}C -labelled amino acid mixes and ^3H -leucine are commonly employed for radiolabelling proteins. Protein synthesis studies of the ter C region plasmids, using these radiolabels, could be undertaken to discover any methionine-minus gene products.

In one instance, mentioned in Chapter 4, the amount of coding DNA may have been under-estimated due to co-migration in the polyacrylamide gels of similar sized proteins. A polypeptide of 30K was reported by Harayama et al (1982) to be synthesised by a plasmid carrying a section of chromosomal DNA which is also present on pPM1000. This polypeptide was not observed as a pPM1000 gene product, and may be obscured by the 31K or 29K ampicillin gene products. It is not known how many other gene products may have been missed through co-migration with other polypeptides.

Gene expression problems were found by Neidhardt et al (1983) who have attempted to identify the gene products of about 275 plasmids from the Clarke and Carbon library. They comment that there is no certainty that genes, although in multicopy state, need necessarily be expressed. Special physiological conditions may be required for gene expression which are not supplied by the protein synthesising system. Also protein or non-protein regulatory or effector molecules active in positive or negative control, may be involved.

For example, two different types of factor influencing the expression of cloned chromosomal genes were discovered during this study. Both involved genes carried by the plasmids pPM2000 and pPM2001. A most striking and unexpected result involving the 120, 28 and 24 K polypeptides, shown to be expressed in minicells, was achieved with the maxicell system. Whenever these proteins were labelled by the maxicell method their expression increased dramatically, as judged by the strength of the autoradiogram signal. In fact these were the only plasmid gene products clearly discernible above the background of labelled proteins, presumably synthesised from surviving chromosomal DNA (see Fig. 4.4). Whatever peculiarity of maxicells stimulated the expression of 28K, 24K and the normally very weakly expressed 120 K polypeptides can only be guessed at until further investigation. A regulatory system immediately suggests itself by analogy, except perhaps, the u.v. induction of phage lambda. The presence of two cryptic prophages within 1 and 3.5 mins either side of the pPM2000 insert, one of which rac is capable of excision, prompts the suggestion that either the direct effect of u.v. irradiation or the resulting moribund physiological state of the maxicells induces a third cryptic prophage in this region of the chromosome.

Studies of the effects upon the host plasmid DNA integrity within treated maxicells would show if any deletions or re-arrangements had occurred, which may have influenced the expression of the cloned fragment. Recovery of transformable plasmids with the correct restriction digest pattern of pPM2000, was achieved from purified minicells using the Birnboim and Doly (1979) method, but this was not tried with maxicells.

Increased expression of the 50K polypeptide encoded upon the pPM2000 and pPM2001 inserts was brought about through addition to the mini-cell system of the well documented effector molecule cyclic adenosine monophosphate (C-AMP). The crp gene of E.coli K12 codes for a catabolic activator protein (CAP), which in conjunction with C-AMP is capable of binding to a specific DNA sequence (CAP site) present in some promoter regions, and stimulates initiation of transcription of the downstream gene. This positive control mechanism is exerted on the expression of many genes, mainly on those involved in catabolism (Ullmann & Monod, 1968; de Crombrughe et al., 1969).

Thus the promoter of the gene encoding the 57K polypeptide of pPM2000 and pPM2001 most likely has a CAP binding site and the much stronger signal observed in the autoradiogram in Fig. 4.4 is probably a direct result of increased amounts of 57K polypeptide on RNA within the minicells due to a higher level of transcription induced by C-AMP-CAP complex. It is encouraging that expression of the four polypeptides described in the two examples above, did not require special conditions to be observed by the standard minicell labelling method. Without the maxicell or C-AMP experiments the amount of coding DNA carried by pPM2000 and pPM2001 would not have been calculated to be any less.

6.2. COMPARISON OF THE CLONED ter C REGION DNA WITH OTHER PARTS OF THE CHROMOSOME.

Reference to the genetic map (Bachmann, 1983) strongly indicates that the E.coli chromosome possesses gene dense regions interspaced by more sparsely populated regions. The section of the terminus region

that was studied is not 'silent' and would not even be defined as genetically sparse. Numerically, there are more genes coding for polypeptides within the approximately 38 kb of cloned ter C region DNA than there are loci contained within an equivalent amount of DNA in over 50% of the chromosome.

Having found that the ter C region was not genetically 'silent' and did in fact contain active genes, it was of interest to determine how much of the DNA in this region of the chromosome carries coding sequences. Also, how typical was this compared to well genetically and physically characterised regions of high gene density. Three gene dense regions were chosen see Fig. 6.1, as a frame of reference for the maximum amount of coding sequence one could expect from a section of E.coli K12 DNA. First, a 14 kb section of DNA extending from the ATP synthase genes, formerly unc now atp, to asn A and includes the DNA replication origin, ori C. Second, a region that lies approximately 36-48 kb anti-clockwise to ori C, and includes genes involved in DNA synthesis and recombination, dna A, dna N, gyr B, rec F, a ribosomal gene rpm H (rim A) and the gene that codes for tryptophanase, tna A. Third, the 2 minute region on the E.coli genetic map contains a cluster of cell envelope genes, a stretch of about 12 kb of DNA from this region carries 9 genes from mur G to sec A.

The DNA of these three regions was in each case isolated by selecting for genes carried on specialised lambda transducing phages; lambda asn (Von Meyenburg et al, 1978), lambda tna (Hansen et al, 1979) and lambda env A (Lutkenhaus & Donachie, 1979) which complements env A, one of the genes in the 2 minute region. The chromosomal DNA carried by lambda env A was extended in vivo to give a lambda transducing

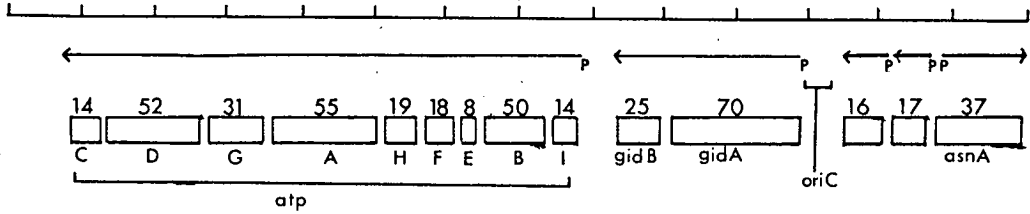
phage that was shown to carry the genes from env A to mur C (Lutkenhaus & Donachie, 1979). By genetic and physical analysis the order of the genes on the chromosome and their gene products were determined. The method of phage infection of irradiated cells, described in Chapter 1, was used by the above workers to characterise the gene products and investigate the organisation of transcription units in these regions. Data contained in the maps of these three regions, Fig. 6.1, includes additional and up-dated information to the phage infection experiments.

The atp-operon has been thoroughly characterised genetically and the complete nucleotide sequence is known, cited in Nielsen et al (1984). This operon covers about half of the DNA region shown in Fig. 6.1 a). The eight sub-units of ATP synthase and another gene, atp I, of unknown and dispensable function (Von Meyenburg et al, 1982), are transcribed from the major promoter atp Ip, see Fig. 6.1, and transcription is terminated about 50bp downstream from the atp C gene (Nielsen et al, 1984). The role of two weaker promoters, atp B1p and atp B2p, which lie in the atp I structural gene, is not known. Very little spacer DNA sequence separates the nine structural genes of the atp-operon and consists of non-coding sequences of less than 20 bp to under 100 bp. Between the ends of the atp-operon and the next genes either side on the chromosome, there are 606 bp to the right, separating the structural genes of atp I and gid B (Nielsen et al, 1984) and 400 bp to the left, from atp C to glm S (Saraste et al, 1981). These two spacer regions do not contain any coding sequences. The atp I - gid B region is extensively AT-rich and stop codons (TAA, TGA and TAG) are encountered frequently.

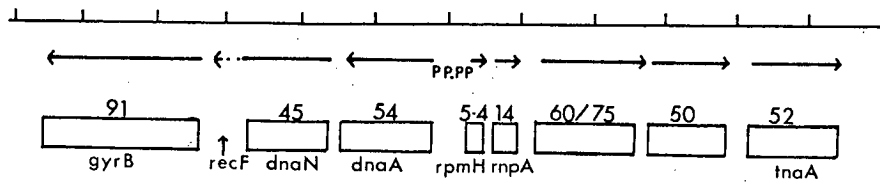
Fig. 6.1.

Example of gene dense regions of E.coli showing known genetic loci, the sizes of their gene products in kilodaltons, other polypeptides identified in the region and the transcriptional organisation. A. section of the 83 minute region that includes the replication origin. The DNA sequence of ori C is known and does not contain coding regions but does possess promoters (Sugimoto et al, 1979) these are not shown here. B. The gyrB-tga A region at 82 minutes, after Von Meyenburg (1984). C. A cluster of cell envelope/division genes located at 2 minutes, showing overlapping transcriptional units.

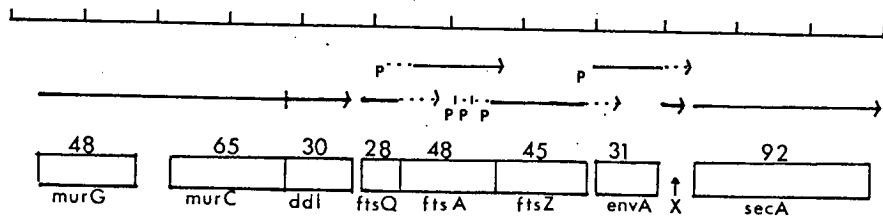
figure 6-1



B.



C.



1kb

It may be expected that genes organised in an operon would be found to have an economical usage of DNA sequence and the atp-operon illustrates this very well. At least two other genes in this gene dense region are probably co-transcribed, gid A and gid B (Hansen et al, 1981). That the DNA surrounding the atp-operon consists of short spacer sequences and the possibility that the neighbouring genes are organised into transcriptional units, suggests that this region of high gene density is highly coding. Overall the atp to asn A section of DNA shown in Fig. 6.1 a) is at least 85% coding, see Table 6.1 .

Similarities are found between the atp - asn A region and the gyr B - tna region. The amount of coding DNA from gyr B to tna is at least 87%, this does not include the rec F gene which has been characterised genetically, but no gene product has yet been identified (Ream et al, 1980). Short spacer sequences separate the structural genes in the section of DNA from this region that has been sequenced; about 30 or 50 bp separate the dna A and dna N genes depending on the start site of the dna N gene (Hansen et al, 1982). These two genes are probably co-transcribed. Hansen et al (1982) suggest they may be part of a dna A-operon that may include rec F and gyr B, which may also be co-transcribed (Sako & Sakakibara, 1980). A sequence of about 500 bp separates the dna A and rpm H genes (Hansen et al, 1982) a size comparable with the distance between the atp-operon and its neighbouring genes. (This region is shown in Fig. 6.1b).

The gene dense cell envelope cluster, Fig. 6.1c) has features in common with the above two regions; highly coding at over 88%; organisation of genes into transcriptional units, mur C and ddl are

co-transcribed with the possible inclusion of mur G (Lutkenhaus & Donachie, 1979); grouping of functionally related genes. One interesting feature of this region is the overlap of the fts A and fts Z genes. Sullivan & Donachie (1984) have shown that the fts Z gene has two promoters, one of which lies in the coding sequence of the neighbouring fts A gene. Normal levels of fts Z gene product is dependent upon the region of overlap between the two genes. These authors suggest that transcriptional regulation of fts Z has evolved in this gene dense region such that expression of fts Z is regulated in some novel way that depends on the neighbouring genes. Greater economy of DNA sequence is certainly achieved by an overlapping arrangement of genes in this particularly highly coding region.

How does the genetic organisation and the amount of coding DNA in the cloned section of ter C region compare with the gene dense regions above? To make this comparison the ter C region gene distribution map, see Fig. 4. 7, was divided into three sections. Each section contains 9-15 kb of DNA, Table 6. 1, an amount similar to the selected gene regions and thus directly comparable. These sections of ter C region DNA are carried by the plasmids indicated in Table 6. 1. The sections are contiguous on the chromosome in the order pPM4000/pPM2000-pPM1000₁-pPM1000₂, see Fig. 4. 7.

Due to insufficient data on the chromosomal locations of the genes that code for the polypeptides described in Chapter 4, the main comparison that can be made between the ter C region and gene dense regions is the % coding of each section of DNA, see Table 6. 1.

TABLE 6.1

REGION	SIZE (kb)	NO. OF GENES	% CODING.
pPM4000/pPM2000	15	6 - 10	84
pPM1000 ₁	9	4	53
pPM1000 ₂	14	3	26
atp-asn A	14	14	85
gyrB-trnA	12	9	87
murG-secA	12	8	88

Amount of coding DNA within the cloned ter C region versus three genetically dense regions. An average amino acid molecular weight of 110 daltons was assumed. Region pPM4000/pPM2000 is the chromosomal DNA inserts of these two plasmids, combined. pPM1000₁ is the region extending from the left hand Hind III site of the 23kb Hind III fragment carried by pPM1000 to the right hand Pst I site of the 0.5kb Pst I carried by this Hind III fragment (see Figure 4.1). pPM1000₂ is the region covered by adjacent 7.4kb Pst I and 6.9kb Pst I/Hind III fragments of pPM1000₁ at the right hand end of the 23kb Hind III fragment.

Section pPM4000/pPM2000 at over 84% coding the most coding of the three sections. This amount of coding sequence is fairly close to that of the atp-asnA region. Two other features of section pPM4000/pPM2000 are in common with the gene dense regions; an operon is possibly carried by pPM2000, where 120K, 28K and 24K gene products may be organised into a transcription unit, since, under some condition found in maxicells these three polypeptides are co-ordinately expressed, see Section 4.4, in Chapter 4. Also, either the excessive number of gene products produced by the chromosomal DNA, carried by pPM4000, is artifactual, or several closely spaced, if not overlapping, genes are present in this region.

In section pPM1000₂ over half the DNA is coding. There is no evidence for an operon in this region and the genes are probably spaced fairly evenly along the chromosome, see Fig. 4.7. This section carries the only known genetic locus in the stretch of ter C region DNA that was cloned in this work. The identified gene, trg (Harayama et al 1979; Harayama et al, 1982) is involved in chemotaxis. Other chemotaxis genes have been found in a cluster at 42 minutes on the genetic map (Bachmann, 1983). Harayama et al (1982) showed using a Trg⁺ plasmid that expression of the trg gene on the plasmid was dependent upon flb B, a gene known to be essential for the expression of chemotaxis genes (Koiwai et al., 1980). None of the other polypeptides synthesised by the Trg chromosomal DNA fragment, were dependent upon flb B and Harayama et al (1982) concluded that it is unlikely that trg lies in a cluster of chemotaxis genes in the ter C region.

Genetically 'silent' DNA was found in section pPM100Q₂. Only three polypeptides were coded by this 14.3 kb piece of DNA. A gene coding for a 21K polypeptide may lie at the left hand end of this section of DNA and genes encoding 38K and 77K polypeptides are probably located at the right hand end, see Fig. 4.7. In between these genes lies an approximately 6 kb region that is apparently devoid of genes.

From the above analysis it was concluded that the 31.1 min - 31.8 min region of the genetic map is not genetically 'silent' and contains many genes of which only one has a known biochemical role within the cell.

Reference to the literature cited in the text for the three gene dense regions shown in Fig. 6.1, shows that both genetic loci and coding sequences, have been added to the physical maps over the past five or six years. For example; a protein of 30K was attributed to the ddl gene by phage infection experiments (Lutkenhaus & Donachie, 1979), but, a cell division gene, ftsQ, with a 30K gene product was later isolated and found to lie between ddl and ftsA (Begg et al, 1980). The similarity in size of the ddl and fts gene products resulted in failure of an additional gene, in 2 minute region, to be identified by the initial protein synthesis experiments.

Another example of proteins that probably co-migrate in polyacrylamide gels is found in the atp-operon. In this case, nucleotide sequence data revealed the presence of a coding sequence, gene I, at the beginning of the operon, which should produce a 14K gene product (Gay & Walker, 1981). The epsilon subunit of ATP synthase is also

about 14K (Saraste et al, 1981). Failure to resolve the labelled gene products of the two genes, in phage infection experiments, probably led Hansen et al (1981) to erroneously place the atp C gene, which codes for the epsilon subunit, between the atp D and atp G genes of the atp-operon. Figure 6.1 a. shows the proper gene order of this operon.

Thus, it is likely that further investigation of the cloned ter C region DNA will reveal additional genes which have not been observed so far.

6.3 OBTAINING MORE INTERPRETABLE DATA.

Some suggestions can be made in order to add to the information that was collected about the proteins synthesised by the cloned ter C region DNA.

To improve upon the resolution of proteins synthesised by the ter C region plasmids, polyacrylamide slab gels of a range of concentrations could be used to separate proteins migrating close to each other in a particular molecular weight range. Although more technically difficult to set up, two-dimensional polyacrylamide gel electrophoresis gives far superior resolution over SDS-P.A.G.E. gels which are run in one dimension (O'Farrell, 1975). Any doubt about a protein spot on a two dimensional gel being a single gene product can be virtually eliminated, see section 1. of Chapter 1.

The problem of when more than one gene product is suspected to be derived from a single gene, for example, the many proteins synthesised

by pPM4000 as seen in Fig. 4. 6, may be resolved in two dimensional gels by an experienced worker (Neidhardt et al, 1983). Otherwise, this problem may be best solved by subcloning the chromosomal DNA insert, preferably to generate a series of overlapping DNA fragments cloned into plasmid vectors with some carrying just one gene. Thus the physical position of the gene on the cloned DNA and its gene product(s) could be characterised. However, one must then be aware of the interference from genetic fusions, as discussed earlier.

Alternatively, an in vitro protein synthesising system (Zubay, 1973; Pratt et al., 1981) would allow purified DNA fragments from the problem region to be investigated without interference from plasmid DNA gene expression. In vitro protein synthesis has the advantage of producing greater quantities of high specific activity labelled proteins than in vivo systems thus making detection of weakly expressed gene products easier. Also, post-translational modification and protein degradation may be more limited in the in vitro system and possibly result in less complicated gel autoradiograms.

6.4 OTHER APPROACHES FOR STUDYING GENETICALLY SILENT REGIONS.

The work of Neidhardt, co-workers and other collaborating groups, will eventually, it is hoped, lead to the complete characterisation of the proteins synthesised by the chromosomal DNA carried by the Clarke and Carbon collection of plasmids (Neidhardt et al, 1983). A number of these plasmids have been mapped by genetic complementation and synthesis of common proteins. Thus it is only a matter of time before all of the genetically 'silent' regions on the chromosome are physically characterised by these workers.

None of the mapped loci and gene products catalogued in the most recent edition of the gene-protein index of E.coli (Neidhardt et al, 1983) are located in the ter C region. The gene products described in Chapter 4 should be analysed by two dimensional gel electrophoresis and assigned co-ordinates according to the gene-protein index of Neidhardt et al (1983). It would be of interest to see how many gene products of the ter C region plasmids were present in whole cell lysates and, therefore, normally expressed by E.coli. Double isotope labelling (McConkey, 1979) employed by Neidhardt and co-workers to identify in whole cell lysates proteins synthesised by the Clarke and Carbon plasmids, appears to work very well.

Another approach for investigating the transcriptional activity of genetically 'silent' regions, is to search for promoters. A promoter fusion system that results in expression of the E.coli gal K gene carried by a plasmid (McKenney et al, 1981) was briefly described in chapters 4 and 5. An investigation into the transcriptional organisation of the ter C region DNA carried by pPM2000 was started, employing the McKenney et al system, as described in Chapter 4.

Application of this promoter fusion system to the region that was found to be the most genetically 'silent' of the cloned ter C region DNA, may locate transcriptional activity that was not observed by protein synthesis in minicells. This approach may detect DNA sequences that are capable of promoting transcription that are not actually genes. For instance, remnants of transcriptional units once part of ancestral E.coli genes, the remains of plasmids or bacteriophages associated with E.coli during its evolution. Isolation of RNA molecules showing homology with this region may then be required to determine if active transcription normally occurs from this region.

Whatever the function of the genes that code for the proteins that were identified in this study, it is known that they are non-essential, since, mutants have been constructed carrying deletions of the entire region that was cloned (Henson et al, 1984). The method used to create the deletion mutants involves using transposons (Kleckner et al., 1979) and should be applicable to other genetically 'silent' regions of the chromosome. It would be of interest to discover if they are also non-essential and how many genetically 'silent' region deletions could be tolerated by the cell.

How would the integrity of chromosome structure be affected in multiple 'silent' region deletion strains? In Chapter 1, reference was made a number of times to the suggestion of Bachmann et al (1976) that genetically 'silent' regions may be buried inside the bacterial nucleoid and play a structural role in maintaining the condensed form of the chromosome. By investigating folded chromosome isolated from 'silent' region deletion strains, this hypothesis could be tested.

6.5 LOCATING ter C, THE TERMINUS OF DNA REPLICATION.

An interesting aspect of this work and an added bonus to the molecular cloning of the ter C region; is the possibility of having cloned the E.coli K12 replication terminus. According to Bouche et al (1982) the point where two replication forks, from initiation at ori C and moving away from each other, will converge is of a map location of 31.2 ± 0.2 minutes. This locus is present on the cloned region described in this study. Preliminary studies were initiated using the plasmids pPM1000 pPM2001 and pPM4000 and the methodology which proved so effective in

locating or eliminating the presence of the R ϕ K terminus sequence upon ColeEI type plasmid vectors. It should be noted that the effect of the E.coli K12 terminus upon ColeEI type replication is not known and if no inhibition of DNA replication was found with any terminus region plasmid clones it would not necessarily rule out the presence of a functional terminus. However, since a specific DNA sequence of R ϕ K blocks replication in heterologous replicons with both ColeEI and pSC101 hybrid plasmids (Kolter & Helinski, 1978; Crosa et al, 1978) there is no prima facie reason why a similar type of DNA sequence in the E.coli K12 terminus region should not inhibit replication forks initiated at a ColeEI type origin. Particularly when inhibition by the E.coli K12 terminus has been demonstrated using strains integratively suppressed by the heterologous origins of bacteriophage P2 (Kuempel et al, 1977) and the resistance plasmid R100 (Lourarn et al, 1979).

A slightly modified method of Bastia et al (1981) was used to attempt to isolate replicative forms (crushed 10mM KCN/TRIS ice cubes were used to rapidly stop DNA replication). Unfortunately several attempts with C600 harbouring pPM1000, pPM2000 and pPM4000 failed to yield any replicative forms on CsCl/ethidium bromide gradients when examined by electron microscopy. Purified minicells (see Methods) isolated from an exponentially growing culture of DS410 transformed with pPM1000 were shown to take up ³H-thymidine (data not shown) but again no replicative forms were obtained.

Electron microscopy confirmed the presence of plasmids, in the region of the CsCl gradient about halfway between the covalently closed circular DNA band and open circular DNA band, that had taken up the ³H-thymidine pulse label. It is not known why replicative plasmid forms were not detected amongst a large population of plasmids.

This approach could also detect DNA replication origins carried by the ter C plasmids and care would have to be taken to distinguish between a replication origin, for example ori X (de Massay et al. 1984) which lies in the ter C region, and a DNA replication terminus.

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