A TRANSCRIPTIONAL ANALYSIS OF

CELL DIVISION GENES IN *ESCHERICHIA COLI*

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This Thesis is dedicated to the memory of my Grandfather,

Ernest George Gay, 1903-1982

DECLARATION

I hereby declare that I alone have composed this thesis, and that, except where stated, the work presented within it is entirely my own, both in conception and execution.

ABSTRACT

At 2 minutes on the genetic map of Escherichia coli there is a remarkable cluster of at least fourteen genes all concerned with cell envelope growth and division. The experiments presented in this thesis represent a detailed transcriptional study of five contiguous genes from within this cluster. Using a combination of gene fusion and in vitro transcription techniques it has been possible to precisely locate the ftsQ, ftsA, ftsZ and envA genes. The ftsZ gene has been shown to have three promoters, two of which are within the structural gene of ftsA. The upstream promoter (Pz_3) acts to enhance transcripton from the downstream promoters (Pz_2 and Pz_1) since total transcription is greater than the sum of their separate activities. This represents not only a novel form of transcriptional overlap, but also a novel biologically important form of promoter interaction.

A probable new gene (gene X) has been located between envA and secA although its function remains obscure. The directions of transcription of ftsZ, envA and gene X have been determined from direct gene fusion evidence whilst the directions of ftsA and ftsQ have been inferred from indirect evidence. All genes, including those bordering this region transcribe in the same direction, but have their own promoters and can be independently expressed.

By construction a novel variable copy number vector designed to demonstrate copy number effects in E.coli the DNA fragment containing the envA gene was shown to be not maintainable in multiple copies. Compilation of a Smith and Birnstiel restriction map facilitated

further cloning and the construction of two independent non overlapping deletions of the envA fragment demonstrated that overproduction of the envA gene product was lethal to the cell.

The factors responsible for regulating any division specific event are unknown, but two genes which might have an important role are cya and crp. The cAMP-CRP complex is shown to act either directly or indirectly to repress transcription from the ftsZ control region. Other cell division promoters tested are not affected by the cAMP-CRP complex. Preliminary evidence suggests that the ftsZ gene product may play a role in regulating transcription either of the ftsZ gene itself or of the ftsA gene.

A 500 bp DNA fragment mapping near the end of envA and the beginning of gene X appears to cause cell filamentation when present in multiple copies.

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Abbreviations used for enzymes:

A	AvaII
B	BamHI
Bg	BglII
C	ClaI
E or R	EcoRI
H	HindIII
Hc	HincII
K	KpnI
L	T4 DNA ligase
P	PvuII
Ps	Pst1
S	Sma1

NOTES

1. All references to the E.coli map are taken from:

   Bachmann, B. J. and Low, K. B., Microbiological Reviews, 44, p. 1-56, 1980.

2. Unless otherwise stated gel electrophoresis was carried out through 0.7% w/v agarose.

3. Unless otherwise stated DNA size standards are given in Kilobases (Kb).

C H A P T E R 1

INTRODUCTION

For Escherichia coli the process of growth and division requires the integration of many cellular activities such as chromosome replication, nuclear segregation, septum formation and the separation of daughter cells. The combined contributions of many investigators has indicated that these biochemically and morphologically distinct events are normally found in a predetermined sequence, the co-ordination between growth, DNA replication and cell division essentially maintaining accurate transfer of genetic material through the cell line.

In shape a typical Escherichia coli cell is approximately cylindrical with hemispherical poles. Under conditions of steady growth rate growth consists of elongation without much change in cell diameter (Marr et al., 1966; Donachie and Begg, 1970), nucleoid duplication precedes cell division (Zusman et al., 1973; Woldringh, 1976) and the septum always forms between two daughter nucleoids. After approximately doubling in length the cylindrical cell then separates by transverse fission into two cells of approximately equal length (Donachie et al., 1976). In order to do this 1) the assembly of cell surface polymers (peptidoglycan) must be highly regulated 2) the timing of initiation of septum formation must be controlled and 3) the location of the septum must be determined precisely.

1.1.1. On the timing of DNA replication and cell division

DNA replication in Escherichia coli consists of the bidirectional replication of the single, circular chromosome from a fixed starting point, to a fixed terminus (Masters and Broda, 1971;

Bird et al., 1972). In E.coli B/r this process takes 40 minutes (at 37°C) from initiation to completion (Helmstetter and Cooper, 1968), is dependent on temperature and the availability of DNA precursors but largely independent of growth rate (Cooper and Helmstetter, 1968).

Completion of chromosome replication is thought to be an essential prerequisite for cell division in wild type cells (Clark, 1968; Helmstetter and Pierucci, 1968; Jones and Donachie, 1973). Division takes place about 20 minutes after completion of chromosome replication (Cooper and Helmstetter, 1968; Helmstetter and Pierucci, 1976).

Control of the timing of division must allow for fast growth rates, when the time between successive divisions becomes shorter than the time taken to replicate the chromosome (Yoshikawa et al., 1964). Under these conditions initiation of one cycle of DNA replication occurs before termination of the previous cycle, resulting in segregation of more than one genome equivalent at division.

Protein synthesis is an essential prerequisite for cell division, and is specifically required at three stages:

1) Initiation of chromosome replication (Messer, 1972).
2) A period concurrent with chromosome replication (Pierucci and Helmstetter, 1969).
3) A short period of RNA and protein synthesis which occurs at or after completion of chromosome replication (Jones and Donachie, 1973).

This last period of protein synthesis led Jones and Donachie (1973) to suggest that synthesis of a 'termination' protein is signalled by the completion of chromosome replication. Synthesis of other required division proteins is initiated at the same time as chromosome replication but not dependent on it for completion. When the required amount of division protein has been synthesised and 'termination protein' has also built up to a sufficient level then the cell has all the necessary products and signals for division.

Alternatively, the periodic events of the cell cycle may be initiated as a consequence of the cell achieving certain critical dimensions, since key cell cycle events such as initiation of chromosome replication and cell division coincide with the times at which there is a doubling of basic unit volume or basic unit length. Such observed relationships have given rise to mathematical descriptions of the E.coli cell cycle (Donachie, 1968, 1981; Pritchard et al., 1969; Pritchard, 1974 (review); Helmstetter et al., 1979) and have been invaluable in providing a framework for experimental design.

1.1.2. On the coupling of DNA replication and cell division

Recent investigations of the response to DNA damage in E.coli K-12, the SOS response, has shown that highly specific mechanisms operate to allow repair to occur. These include inhibition of cell division (filamentation) which overrides normal division control (reviewed by Witkin, 1976) (Section 1.3.9). Many early experiments relating DNA replication to cell division

concerned blocking DNA replication with DNA damaging agents, and noting subsequent division inhibition. The effect observed was not a coupling between the two processes, but simply expression of the SOS response. Furthermore, many experiments were carried out on E.coli B/r, a strain now known to contain two mutations affecting division control, lon and lexA (W. Donachie, pers. comm.).

Reappraisal of the available data has made it clear that there is no strong evidence to suggest that cell division is triggered by DNA replication, and the actual septum initiating event remains unknown. An intriguing possibility is that division is a consequence of growth, and upon reaching a certain critical cell size division is initiated. That is, division reflects a change in the state of the cell. A new born cell has a minimum or unit length, L. Cell division is initiated when the cell length reaches 2L. This striking correlation is independent of growth rate (Donachie et al., 1976). At each doubling of the minimum length one potential division site is formed, and arises midway between a pair of pre-existing potential division sites. This includes the cell poles, which may be regarded as 'used' potential division sites. For each doubling in length, one quantum of 'division potential' is produced. This factor is used entirely in the formation of a single septum (Teather et al., 1974). With the exception of the cell poles, the probability of division occurring at any of the potential division sites is equal. Polar division can occur, as in the case of the minicell mutant, and it is here that use of division potential and probability of using any particular site is

best illustrated (Teather et al., 1974).

If the initiation of DNA replication is blocked, as happens in dnaA mutants then cell growth and division continue for a while. Potential division sites are laid down and when the cell reaches its critical size division occurs. However, anucleate progeny are produced (Hirota et al., 1968; W. Donachie and A. Jenkins, unpublished results).

Howe and Mount (1975) showed that thymine starvation (a DNA replication block) of a lexA$^-$ (blocked in derepression of SOS functions) strain produced an increase in the number of anucleate cells. Normally 0.1% of the divisions in an unstarved lexA$^+$ culture result in anucleate cells, but this could be increased to 20% in a lexA$^-$, thymine starved culture (see also Howe and Mount, 1978, 1979).

Similarly a recA$^-$ strain treated with nalidixic acid (to block DNA replication) can produce anucleate cells (Inouye, 1969; K. J. Begg, unpublished results).

Thus, when uncoupled from the SOS response, and blocked by any one of several means in DNA replication, E.coli cells carry on growing and dividing with normally timed and localized divisions. This suggests that there is no coupling between DNA replication and cell division.

It has been suggested (Hendrickson et al., 1982) that the origin of replication is bound to the outer membrane. Growth of the cell surface between the two points of attachment of sister DNA origins may account for the spatial separation of newly replicated chromosomes (Jacob et al., 1963; Liebowitz and Schaechter, 1975; Mendelson, 1982). Nucleoid segregation is always observed to

precede cell division except in a dnaA strain for example. An SOS type filament is straight sided with no indication of visible septa. Since DNA replication has been inhibited for repair, nucleoid segregation does not occur. Growth however, continues and potential division sites are formed as usual. When repair is complete the SOS system is turned off, replication proceeds, nucleoids are segregated and required division proteins can now be synthesised (see Section 1.3), allowing the cell to divide. Thus nucleoid segregation did not parallel growth of the envelope. This suggests either

(a) that growth of the envelope and nucleoid segregation are not coupled or

(b) SOS plays a role in uncoupling the two processes.

In either case, an alternative means for segregating nucleoids is required.

Cell division 'appears' to be triggered when cells reach a certain invariant size, and is not coupled to either the initiation of DNA replication, nor nucleoid segregation during unperturbed (steady state) growth. DNA replication and cell division have been reported to be independent in the gram positive organism, Bacillus subtilis (Donachie et al., 1971).

1.1.3 Growth of the cell surface; cell shape.

Assembly of cell surface polymers changes abruptly at the time of cell division when the peptidoglycan begins inward growth to form the septum and hence the cell poles. It is thus important to understand what determines assembly of peptidoglycan at different regions of the cell, and how this alters through the cell cycle, since this process is intimately connected with cell division.

The gram-negative (Escherichia coli) cell envelope consists of three layers (reviewed by Tipper and Wright, 1979)

1. The inner or cytoplasmic membrane is a phospholipid bilayer equivalent to the eukaryotic plasma membrane. It acts as the cells main permeability barrier and is responsible for active transport.

2. The peptidoglycan is a mono molecular layer of an alternating copolymer of N-acetylmuramic acid pentapeptide (NAMpp) and N-acetylglucosamine (NAG). The strength, rigidity and bacterial cell morphology can be largely attributed to the peptidoglycan, although there is some confusion as to whether peptidoglycan actually determines shape or merely maintains a shape determined by other factors (Henning, 1975; Helmstetter et al., 1979; Markiewicz et al., 1982; Rosenberger et al., 1978).

3. The outer membrane is an asymmetric bilayer containing lipopolysaccharide and protein (reviewed by Wright and Tipper, 1979). It provides a barrier to prevent loss of hydrolytic enzymes contained within the periplasmic space. Two of the outer membrane proteins, protein II* (coded for by the ompA gene) and lipoprotein (coded for by the lpp gene) are associated with the peptidoglycan. Wensink et al. (1982) showed that ten percent of the peptide side chains of the peptidoglycan were bound to lipoprotein, and that this percentage doubled in stationary phase. Neither a lpp or ompA single mutant has any morphological effect in E.coli, yet a lpp ompA double mutant grew as osmotically stable spheres (Sonntag et al., 1978). This implied a role for the outer membrane proteins in shape maintenance. Under some conditions

purified outer membranes can retain the rod morphology (Henning et al., 1973).

Whether or not the cell envelope extends from a small number of growth zones or by random insertion of new material (intercalated or dispersive growth) has exercised investigators for many years, with no clear result (Nanninga et al., 1982; Davison and Garland, 1983).

Overall cell morphology may be a balance between elongation, in which peptidoglycan is inserted in the lateral wall, and septation, where assembly is shifted towards construction of a hemispherical cell pole (Satta et al., 1979, 1980). These authors suggest that normal rods have a feedback control mechanism, which prevents septum formation if lateral wall elongation has not occurred.

1.1.4. Types of mutant

Defects in elongation, septation or eventual cell separation lead to altered cell morphology. The isolation of mutants defective in these processes has greatly facilitated studies on cell shape and cell division. Those mutants which form filaments are usually defective in septum initiation or formation. Most isolated so far are initiation mutants, which form nonseptate multinucleate filaments and are probably deficient in steps leading to preparation or triggering of the septum. Only two formation defective mutants which retain visible constrictions have been described, sep and ftsA (Walker et al., 1975). Chain forming mutants have been isolated eg, envA, envC, cha (see Section 1.2.2c).

Certain mutations cause E.coli to adopt a spherical morphology, for example, pbpA (Spratt, 1977; Iwaya et al., 1978),

rodA (Matsuzawa, 1973; Spratt, 1980) and a lpp ompA double mutant (Sonntag et al., 1978). The envB mutation gives rise to spherelike cells with asymmetric constrictions, which appear to be abnormal septa (Nomark, 1969). The crp and cya mutations (Kumar, 1976; Section 1.4.1) are often thought to confer a spherical phenotype, but are really shortened rods (coccobacillary).

1.1.5 An enzymatic model for cell division

A balance between two different enzymes, a transpeptidase and a D-alanine D-alanine carboxypeptidase provides a possible enzymatic route to septum formation. Mirelman et al. (1977) noted that filamentation could be associated with a decrease in the level of D-alanine carboxypeptidase. Cultures of PAT84 (ftsZ 84, ts; see section 1.2.2c) independently induced to form nonseptate filaments by three different means (nalidixic acid, cephalexin and a shift to the restrictive temperature) contained lower levels of D-alanine carboxypeptidase compared to the level in normally dividing cells. The newly synthesised murein had a higher degree of peptide side chain crosslinking than did the murein of normally dividing cells. Mirelman et al. (1977) suggested that the inability to form septa was due to an imbalance between the transpeptidase system which catalyses the incorporation and cross linkage of newly synthesised murein to the pre-existing wall and the D-alanine carboxypeptidase system. It was subsequently shown, by measuring both relative activities and murein synthesis through the cell cycle of E.coli B, that transpeptidase activity was maximal after division, whilst D-alanine carboxypeptidase activity was maximal

before division (Mirelman et al., 1978). Consistent with this, low doses of penicillin can partially inhibit the carboxypeptidase without affecting the transpeptidase. Under these conditions the amount of peptidoglycan inserted into the cell wall is increased (Mirelman et al., 1976). The D-alanine carboxypeptidase may act either as an endopeptidase to loosen pre-existing murein and hence initiate the necessary conversion from cylindrical wall to hemispherical septum or to remove the C-terminal D-alanine residues from murein strands thus creating extra acceptors for the transpeptidation reaction. Increased transpeptidation would then lead to septum formation. A shift in the carboxypeptidase:transpeptidase ratio towards the carboxypeptidase would ensure that a hemispherical septum is formed. The level of transpeptidase need not change, but the enzyme would be required until just before cell separation (Spratt, 1977). Inhibition of cell division could be achieved by inhibiting the activity of the carboxypeptidase, either directly or indirectly. This 'two enzyme' model does not preclude the involvement of many more genes and reactions involved in the full process of cell division. There must be a mechanism which determines precisely where a septum is initiated and also one ensuring that the carboxypeptidase acts only at the point of initiation and not along the length of the cylinder.

1:2.1 Gene Clusters in Escherichia coli

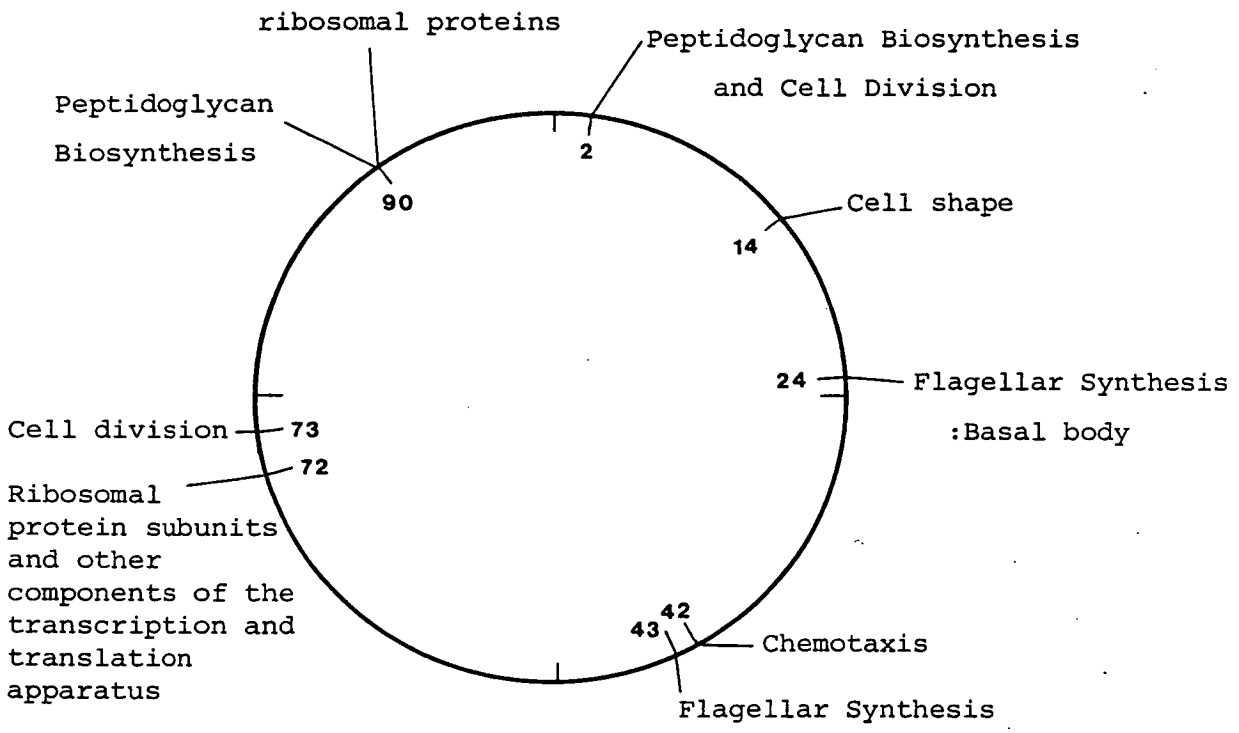
One particularly interesting aspect of genome organisation is the incidence of gene clusters. A gene cluster is a group of two or more genes of related function which constitute more than one independent operon. 'Independent' means that the constituents of an operon can be expressed in the absence of neighbouring operons. There are approximately 9 such clusters in Escherichia coli (Fig. 1).

The cluster of interest to us is a peptidoglycan biosynthesis and cell division cluster mapping at 2' on the E.coli map. Evidence is collecting for a second cell division cluster around 73'. At present this includes six genes: fic (Utsumi et al., 1981, 1982), cha (Begg and Donachie, 1983), envB (Normark, 1969), ftsE (Ricard and Hirota, 1973), Toe 32 (Salmond et al., 1983), and crp (Kumar, 1976). An alternative peptidoglycan biosynthesis cluster occurs at 90' (Miyakawa et al., 1972) and a cell shape cluster at 14' (Iwaya et al., 1978; Suzuki et al., 1978). Other clusters include genes involved with chemotaxis (42'), flagellar synthesis (24', 43') and ribosomal protein subunit and transcription-translation factor biosynthesis (72'; 90').

The 2' peptidoglycan biosynthesis/cell division cluster contains approximately one third of all genes known to be involved in the normal process of cell division (Fig. 1b). Apart from the 73 minute cluster all other cell division genes appear to be widely distributed.

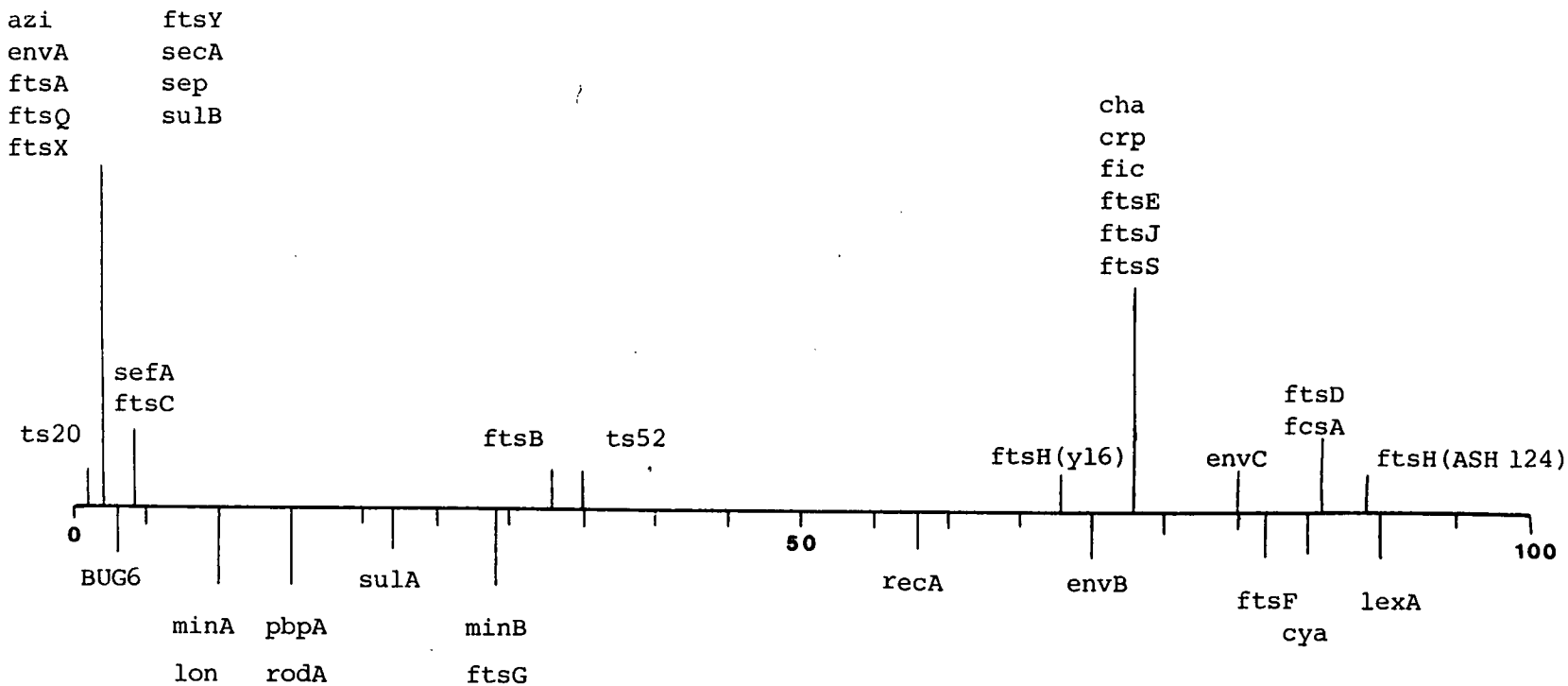
Gene clusters are by no means unique to E.coli and many

Fig. 1a Gene Clusters in Escherichia coli



(Taken from Bachmann and Low, 1980)

Fig 1b Distribution of Cell Division Genes in E.coli 20 cm = 100'



Compiled from 1) Bachmann and Low, 1980

2) Helmstetter et al., 1979

3) W. Donachie pers. comm.

examples exist both in procaryotes and eucaryotes. It is interesting to note that Bacillus subtilis may also have a cell division cluster (Mendelson, 1982).

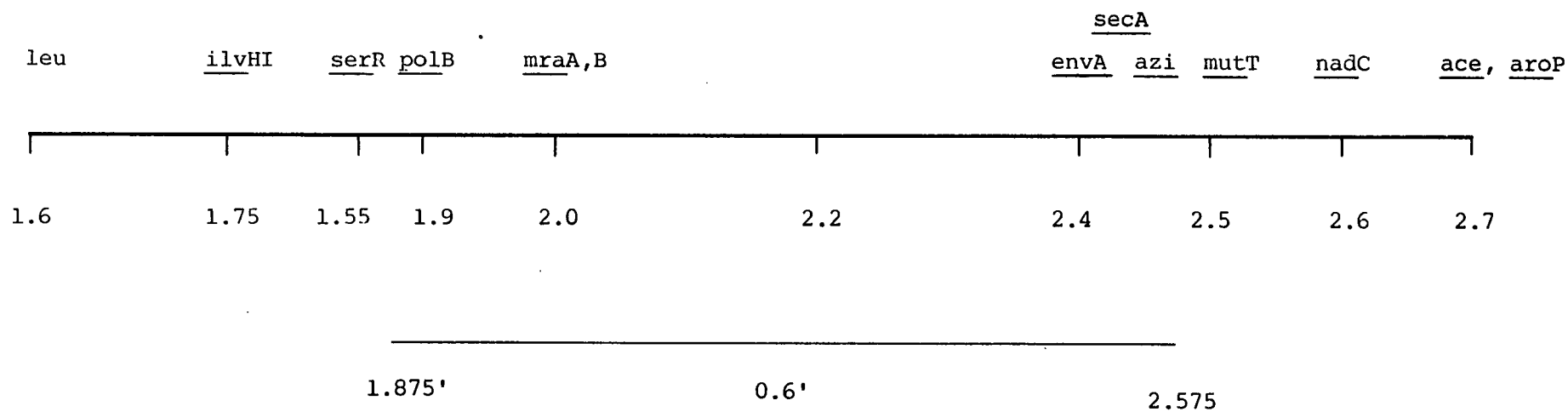
Since genes of similar function occur together it is interesting to speculate on the significance of such an arrangement. How is expression within a cluster controlled? Do neighbouring genes and operons interact?

1.2.2 The 2 minute cell division cluster

1.2.2a Boundaries. The two markers often used for co-transduction of genes within the cluster are leu (1.6') and azi (2.45'), although azi itself appears to be a cell division gene (Yura and Wada, 1968) (see Fig. 2). The leftward extent of the cell division cluster is probably between serR which controls the level of seryl tRNA synthetase and polB, the structural gene for DNA polymerase II. The polB gene may be analogous to dinA (Kenyon and Walker, 1980), an SOS associated gene (see 1.3.1). Two peptidoglycan biosynthesis genes, mraA, B, map to the right of polB (Miyakawa et al., 1972).

The rightward extent of the cluster is also open to discussion. An obvious cell division gene is envA (2.4') a gene involved in separation of daughter cells (Normark, 1970). The secA ts mutant forms filaments at the restrictive temperature (Oliver and Beckwith, 1981) and temperature sensitive azi mutants also filament at the restrictive temperature. However, the next gene, mutT (2.5') has no apparent cell division function. Mutations here cause AT to GC transversions perhaps functioning in the wild type to reduce the spontaneous mutation rate. Mapping next to mutT are nadC

Fig. 2. Extent of the 2 Minute Cluster



(2.6', Quinolinate phosphoribosyl transferase), aroP (2.7' aromatic amino acid transport) and ace (2.7' acetate usage). It is thus probable that the 2' cell division cluster ends between azi and mutT. The cluster is approximately 0.6 minutes long, which represents 29 Kb (Bachmann and Low, 1980).

1.2.2b Historical Introduction and Gene Order in the 2 minute cluster. Yura and Wada (1968) suggested that the azi gene was somehow related to the process of cell division or DNA replication. They showed that the gene was co-transducible with leu and that temperature dependent azide resistance was often associated with filamentation. Normark (1969, 1970, Ph.D. thesis 1971) isolated two chain forming mutants of an ampicillin resistant strain which were both sensitive to ampicillin and carried defects in the cell envelope. One of these was designated envA, shown to map between leu and azi and which forms chains during growth in rich media (Normark, 1970). The other, envB, is co-transducible with streptomycin resistance (rpsL, 73') (Normark, 1969).

Around this time various coworkers (van de Putte et al., 1964; Hirota et al., 1968) isolated mutants of E.coli with impaired cell division at elevated temperatures. Matsuzawa et al.(1969) isolated another class of mutant temperature sensitive for peptidoglycan biosynthesis, now known as ddl (Lugtenberg and van Schindel van dam, (1972c). Wijsman (1972) isolated three more mutants defective in peptidoglycan biosynthesis and named these murC, murE and murF. At the restrictive temperature these mutants form anomalous shapes and eventually lyse. Wijsman (1972) mapped some of the aforementioned

genes, including a mutation which form filaments at the restrictive temperature, PAT84 (Hirota et al., 1968). The order was:

leu murE murF murC PAT84 envA "mutT" azi

Wijsman (1972) first noted that this region contained a cluster of genes and that they all had particular importance to the process of cell division and peptidoglycan biosynthesis. Lugtenberg et al. (1972a, b) showed that cells with murE mutation had a low activity of meso-diamino pimelic acid adding enzyme, that L-alanine adding enzyme could be attributed to the murC gene and that D-alamine-D-alanine adding enzyme could be attributed to the murF gene. Miyakawa et al. (1972) reported the isolation of two clusters for peptidoglycan biosynthesis, mra between leu and azi, and mrb between argH and metA (90'). These authors noticed that there was no simple relation between gene location, the order of enzyme localisation, and the sequence of reactions involved in cell wall biosynthesis. Lugtenberg and Van Schindel Vandam (1972c)then showed that the mutation isolated by Matsusawa et al. (1969) had an impaired level of D-alanine:D-alanine ligase and hence called the gene ddl. Ricard and Hirota (1973) isolated loci temperature sensitive for cell division and classified them as ftsA through to ftsG. The PAT84 mutation was renamed ftsA, a locus previously mapped between ddl and azi. In a physiological study of the expression of the PAT84 mutation was shown to be dependent on salt concentration, being only expressed at low salt. In an electron microscopic study Burdett and Murray (1974a) noted that ftsA (PAT84) was defective in an early stage of division involving

cell wall modification before formation of the septum. Allen et al. (1974) isolated a mutation which they attributed to ftsA, ts 1882. In a later study it was noticed that this mutation conferred incomplete septa at the restrictive temperature, apparently representing many stages of septum formation. It was suggested that this product, the ftsA product was required during the process of septum assembly (Walker et al., 1975). The PAT84 mutation was again suggested to be a septum initiation mutant, perhaps in another fts allele closely linked to their own ftsA isolate (Walker et al., 1975). Wijsman and Koopman (1976) then investigated the relationship between envA and the new ftsA alleles deficient in septum completion. They showed the gene order to be: ftsA envA azi. Also an ftsA envA double mutant forms filaments at 42°C and not chains, but like its chain forming envA parent was sensitive to rifampicin. This suggested, as indeed the phenotype implied, that the ftsA product acts before the envA product in septation.

Spratt (1977) isolated a temperature sensitive cell division mutant with a thermolabile penicillin binding protein, which he called penicillin binding protein 3, PBP3. It was suggested that PBP3 activity is required up to at least 3 to 6 minutes before physical cell separation. The mutation conferred filament formation at the restrictive temperature and was attributed to the pbpB gene. Similarly Suzuki et al. (1978) found a series of penicillin binding protein mutants, one of which, the ftsI gene was mapped in the same region as pbpB at 1.8'. One of the alleles of Allen et al. (1974), ts 2158 was shown to belong to a new cell division gene, separate and distinct from ftsA, and was designated sep (Fletcher

et al., 1978). Using specialised lambda transducing phages these authors were able to prepare genetic and physical maps of the sep and ftsA genes in relation to murE, murF, murC, ddl and envA. The order was shown to be:

leu sep murE murF murC ddl ftsA envA

This confirms the sequence published by Wijsman (1972) and Wijsman and Koopman (1976) and extends it to include ddl and sep. The sep gene was subsequently cloned into λ Charon 10. Penicillin binding protein 3 was overproduced and incorporated into the E.coli inner membrane (Irwin et al., 1979). Nishimura et al. (1977) screened the Clarke-Carbon colony bank for strains that could transfer genes involved in cell division, thereby correcting the thermosensitivity of various fts mutants. A gene which corrected the ftsI mutation was shown to overproduce PBP3. Irwin et al. (1979) suggested that sep is probably identical to the pbpB and ftsI genes described independently by Spratt (1977) and Suzuki et al. (1978).

Many mutations which affect cell division had been isolated, but only in those which the mutation led to a loss of ability to bind penicillin was the gene product identified (Spratt, 1977). This assay is limited to only a few of the proteins involved in cell division. To circumvent this problem Lutkenhaus and Donachie (1979) isolated a nonsense mutation in ftsA. A lambda transducing phage (λ16-2) was subsequently constructed by in vivo extension of a transducing phage bearing envA. This could complement the ftsA (am) mutation. The mutation was transferred to the phage (to give λ 16-5) and used for detection of phage encoded proteins in UV irradiated cells. This approach enabled the ftsA product to be

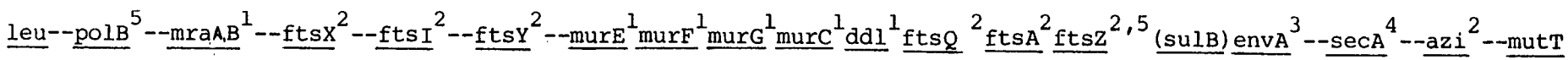
identified as a 50 Kd protein. Subsequent physiological and kinetic studies showed that synthesis of ftsA was required for division only during a specific part of the cell cycle (Donachie et al., 1979). Lutkenhaus et al. (1979) isolated deletion derivatives of the original λ16-2 and using these assigned the PAT84 allele to a new gene ftsZ which lies between ftsA and envA. Again using this series of transducing phages Lutkenhaus and Wu (1980) were able to assign products and directions of transcription to genes in this region (see Fig. 6).

The cell division gene ftsQ has been identified and mapped between ddl and ftsA (Begg et al., 1980) and Salmond et al. (1980) have reported another cell envelope gene, murG between murF and murC. A study of secretion led Oliver and Beckwith (1982a) to genetically map a thermosensitive lesion at 2.5' on the E.coli map. They attribute this to the secA gene, clockwise of envA (see next Section). Lutkenhaus (1983) has shown that ftsZ is allelic with sulB, a gene involved in inhibition of division after the SOS response (see 1.3.7). Hirota (C.S.H. 1983; personal communication to W. Donachie) has indicated that two other fts loci, ftsX and ftsY border ftsI.

The gene order of the 2' cell division cluster as of June 1983 is as in Fig. 3.

1.2.2c The individual genes: The work in this thesis concerns the region ftsQ to secA. This section deals with each gene in detail.

Fig. 3. Gene Order of the 2' Cell Division Cluster.



Function

1. Peptidoglycan Biosynthesis
2. Septation
3. Separation
4. Secretion
5. SOS

N.B. a dash indicates a space to which gene(s) have not yet been allocated.

ftsQ. A mutation, designated TOE-1 was isolated as a temperature sensitive lesion conferring defective cell division (Begg et al., 1980). It forms multinucleate straight sided filaments at the restrictive temperature and was shown to map within the 2' cluster by P_1 transduction. It was localised with the use of specialised lambda transducing phages to a 2.2 Kb EcoR1 fragment known to contain ftsA (Lutkenhaus and Wu, 1980). These authors had shown that ftsA probably mapped to the right of this fragment, that is, adjacent to ftsZ. Begg et al. (1980), having shown that the new mutation was not an allele of ftsA, inferred that ftsQ must lie to the left of ftsA and to the right of ddl.

ftsA. Several filament forming mutants had been isolated independently and shown to map in the 2' region. Wijsman (1972) mapped three alleles isolated by Van de Putte et al. (1964), fts10, fts12 and fts15 to the ftsA gene. Hirota et al. (1968) found that one of their filament forming mutants, PAT84 was linked to leu and this was also designated ftsA. Lutkenhaus and Donachie (1979) isolated an ftsA(am) and showed it to be allelic to the fts12 allele of van de Putte et al. (1964). Similarly, Allen et al. (1974) isolated a mutation, ts1882 which was shown to be co-transducible with leu at the same frequency as the PAT84 mutation (Walker et al., 1975). Lutkenhaus et al. (1980) demonstrated that the PAT84 mutation represented a different class of mutation and proposed a new gene, ftsZ (see next section). They suggested that all other alleles should retain their ftsA designation.

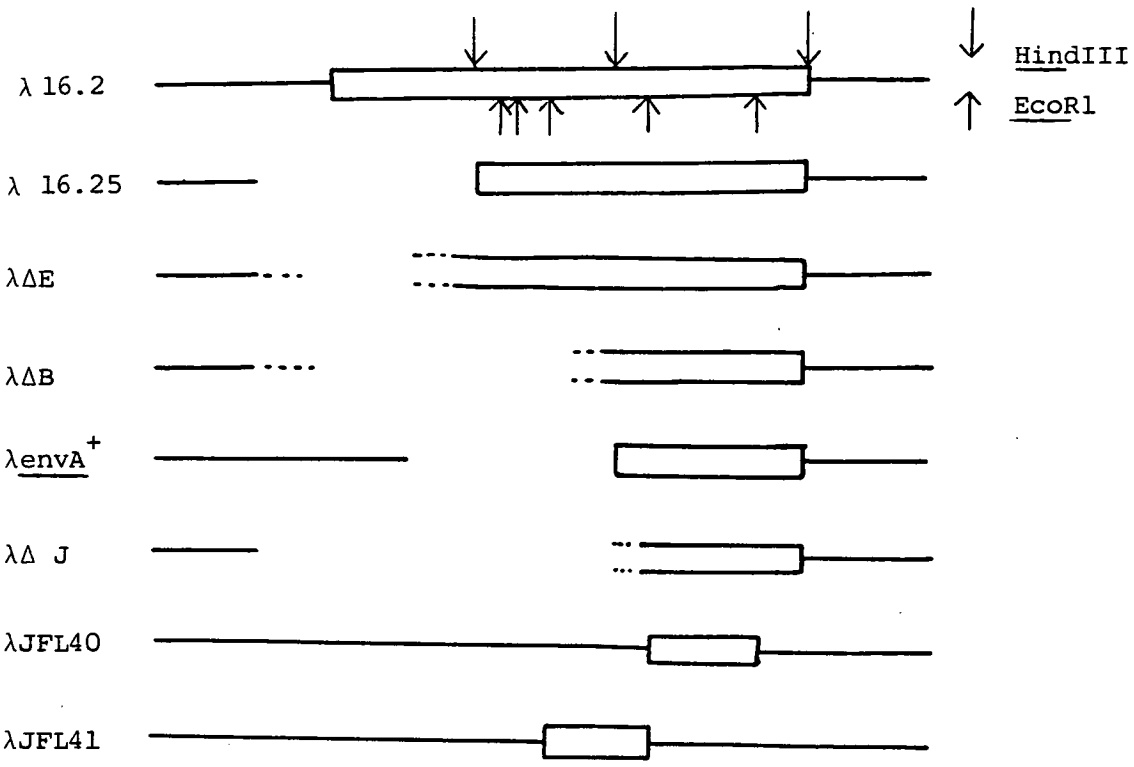
As previously mentioned, Lutkenhaus and Donachie (1979) identified the ftsA gene product as a 50 Kd polypeptide with the

use of a λ transducing phage carrying a ftsA nonsense mutation. Other workers, Allen et al. (1974) and Walker et al. (1975) had identified a missense mutation in ftsA (ts 1882) which resulted in synthesis of a temperature sensitive ftsA protein. When this mutant was shifted to the restrictive temperature, cell division was immediately arrested, even if the cells were in the process of forming a septum. This implies that the ftsA protein is required in an active form throughout the entire process of septation. Donachie et al. (1979) presented evidence to suggest that synthesis of ftsA protein is essential only during a short period immediately before the final stages of cell division (see also de Pedro et al. 1975 and Tormo et al., 1980).

ftsZ. As previously mentioned, Lutkenhaus et al. (1980) proposed that the PAT84 mutation of Hirota et al. (1968) be redesignated ftsZ 84 (ts). The location of this gene was determined by the isolation of deletion mutants of λ 16-2 and the subcloning of EcoR1 fragments from this phage (see Fig. 1.4).

Using the complementation analysis ftsZ was placed between ftsA and envA. Furthermore, ftsA and envA were shown to be functional units independent of neighbouring genes. Using these and other phages (Fig. 4) Lutkenhaus et al. (1980) were able to conclude that ftsA and ddl could be expressed independent of one another, and that murC and ddl probably constitute a single operon. The direction of transcription and gene product of ftsZ has been identified (Lutkenhaus and Wu, 1980; Section 1.2.2d) and ftsZ has been shown to be allelic with sulB (Lutkenhaus, 1983; Section 1.3.7).

Fig 4. λ16-2 and its deletion derivatives



Complementation analysis

	murC	ddl	ftsA	ftsZ	envA
λ16.2	+	+	+	+	+
λ16.25	-	-	+	+	+
λΔE	-	-	+	+	+
λΔB	-	-	-	+	+
λenvA+	-	-	-	-	+
λΔJ	-	-	-	-	-
λJFL40	-	-	-	-	+
λJFL41	-	-	+	-	-

(Taken from Lutkenhaus et al. 1979)

envA. Cell division is accomplished by ingrowth of the cytoplasmic membrane and peptidoglycan layer of the cell wall. Before cell separation these structures may form a complete or partial septum, and in E.coli B and B/r complete septa can be seen. These are rarely seen in E.coli K-12 and it is not clear whether K-12 divides by constriction or septation, i.e. whether or not cells forms distinct units prior to separation (Burdett and Murray, 1974 a,b; Normark et al., 1971). E.coli K-12 mutants form complete septa composed of cytoplasmic membrane and apparently two layers of fused peptidoglycan, giving the appearance of discreet, separated daughter cells. The electron microscope reveals that all layers of the envelope participate in invagination during cell division of the wild type (Normark et al., 1971) although it has been suggested that the outer membrane does not participate until the cells separate (Burdett and Murray, 1974a). It is thus possible that in the wild type peptidoglycan layers are fused, but cannot be demonstrated due to the activity of autolysins (Burdett and Murray, 1974 a,b). Normark and Wolf-Watz (1974) suggested that the envA defect was due to unbalanced growth between the envelope layers, since unlike the wild type the peptidoglycan was disassociated from the outer membrane. It is possible that autolysins normally present in the outer membrane cannot reach their site of action due to this defect. Normark et al. (1971) demonstrated that protein synthesis, but not DNA or murein synthesis is required for cell separation.

Other chain forming mutants have been isolated, for example,

the envC mutation of Rodolakis et al. (1973). This mutant forms sphere-like chains, with complete or partially complete septa. Interestingly, these septa either partial or complete, do not appear to correspond to potential division sites, that is, septa can be initiated at any point over the cell surface. Another chain forming mutant has recently been isolated in this laboratory (Begg and Donachie, 1983) and designated cha (~ 73'). This mutant forms chains of shortened rods, and appears to be defective in cell separation. Interestingly, two copies of the wild type envA gene, one in the chromosome, one in a λ , can suppress the cha mutation.

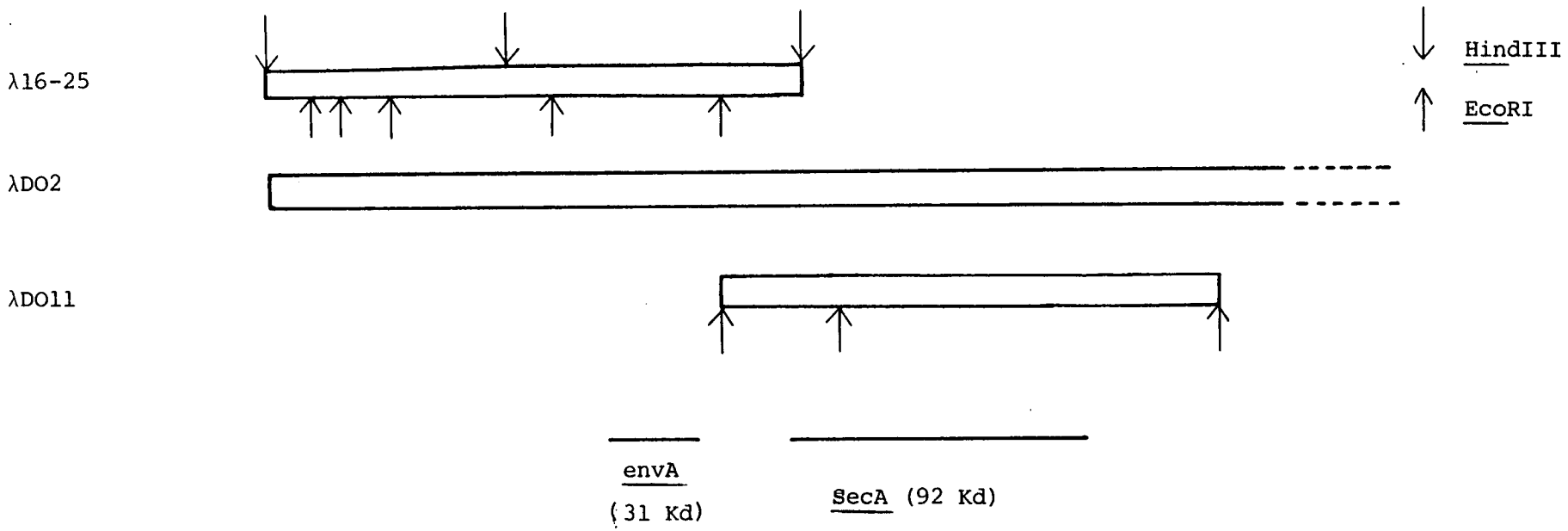
The envA mutation was originally isolated as a smooth ampicillin sensitive mutant of a rough ampicillin resistant strain, and was mapped between leu and proB (Normark et al., 1969). It was later shown, that besides the previously discussed abnormal cell division, the envA mutation conferred sensitivity to a number of antibiotics, both hydrophobic and hydrophilic (Normark 1970, 1971; Normark & Wolf-Watz, 197 Gustaffson, et al. 1973). Physiological induction of filamentation and suppression of septation reduces permeability, (Normark and Wolf - Watz, 1974) so on this basis Normark et al. (1976) were able to isolate antibiotic resistant revertants of an envA strain. Besides true intragenic revertants, mutations were obtained in another allele, sefA which mapped near proA. The mutant strain, envA sefA as well as the transductant sefA forms constrictions, but unlike envA, complete septa are not formed. Although the primary defect in sefA cells is unknown, the sefA allele appears to affect initiation of septation (Normark et al., 1976). The changes produced in the outer membrane of envA and sefA cells

have been studied. Normark (1971) noticed that phenethyl alcohol could suppress the envA phenotype. Low concentrations of this compound caused a transient inhibition of RNA synthesis which the author suggests may induce changes in the lipid moieties of the membrane. The envA strain also shows a modified protein profile and lower phosphatidylglycerol content when compared to the wild type (Wolf-Watz et al., 1973). No significant qualitative differences in outer membrane components were found either in the envA mutant or the sefA mutant. The sefA mutation was shown to suppress the envA phenotype by increasing the amount of protein in the outer membrane (Grundstrom et al., 1980). Thus the suppressing effect of sefA on envA may be quite indirect.

secA. A temperature sensitive mutant of E.coli (secA), that affects the cells ability to secrete certain proteins to the outer membrane and periplasmic space has been isolated by Oliver and Beckwith (1981), and mapped to 2.5'. It is reported to form filamentous multiseptate cells at the restrictive temperature and to accumulate the precursors of maltose binding protein, alkaline phosphatase, ompF protein and λ receptor. Ryter et al. (1975) have suggested that λ receptor is secreted at the cell septum and maltose binding protein may only be secreted at times of cell division (Dietzel et al., 1978). To show that the inhibition of secretion was not a secondary consequence of the cell division defect Oliver and Beckwith (1981) showed that ftsA, ftsZ and envA have no effect on the secretion of maltose binding protein. So secretion of this protein does not require cell division. In addition neither ompF protein nor alkaline phosphatase are

secreted preferentially at the cell septum (Smit and Nikaido, 1978; Begg, 1978) although they have been shown to be affected by the *secA* mutation (Oliver and Beckwith, 1981). The most likely cause therefore, for the cell division defect is inhibition of secretion of an as yet unidentified cell division protein. In a subsequent publication Oliver and Beckwith (1982a) isolated a λ transducing phage bearing *secA* (λ D 02) by *in vivo* extension of λ 16-25 (Lutkenhaus *et al.*, 1980). Neither λ16-25 nor λ16-2 could complement a *secA* t.s. but both could allow selection of wild type recombinants. Having shown the *secA* t.s. to be recessive this suggested that λ16-2 and λ16-25 contained a portion of the *secA* gene. The λ D 02 phage was reduced in size by restriction and the *secA* region was assigned to two *EcoR*1 fragments, 0.8 Kb and 2.8 Kb in length (see Fig. 5). The *secA* gene product was identified as a polypeptide of approximately 92 Kd and was shown to transcribe clockwise on the *E.coli* map, that is, in the same direction as *ftsA*. Oliver and Beckwith (1982b) suggest that secretion components, including *secA* are regulated according to the secretion needs of the cell. If secretion is inhibited by induction of a *MalE-lacZ* hybrid protein then the *secA* gene product is over-produced. Hence when the secretion capacity of the cell is reduced, the *secA* gene, and presumably others, are derepressed to compensate. Oliver and Beckwith (1982b) have shown that *secA*, which behaves as a membrane protein, is normally made in small amounts, and estimate 500-1000 copies per cell. This may well reflect the cellular requirement for *secA* at septation.

Fig. 5. Transducing Phages of the SecA Region



1.2.2d Overall organization of the ftsA region

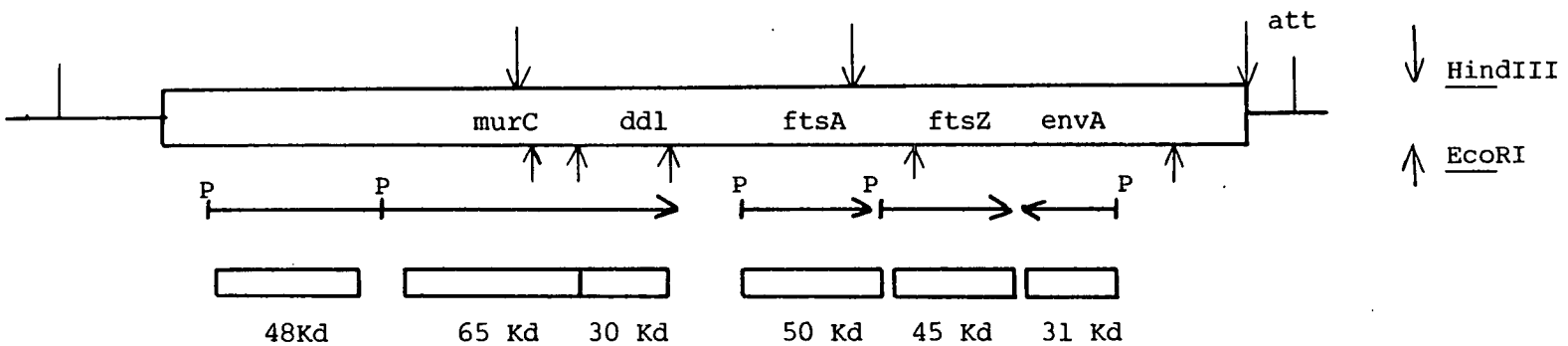
Following the isolation of λ 16-2 (Lutkenhaus and Donachie, 1979) and deletion derivatives (Lutkenhaus et al., 1980) it was feasible to examine protein synthesis directed by these transducing phages in UV irradiated cells (Lutkenhaus and Wu, 1980). The gene products of murC (65K), ddl (30K), ftsZ (45K) and envA (31K) were assigned and the ftsA product was verified as 50Kd. The approximate promoter locations and directions of transcription of these genes was determined. From genetic experiments (Lutkenhaus et al., 1980) it was concluded that ftsA and envA could be independently expressed and from the phage directed protein expression it was shown that ftsZ could be independently expressed (Lutkenhaus and Wu, 1980). In addition these authors isolated Tn5 insertions into envA and ftsZ and noted a corresponding loss of the 31 Kd and 45 Kd proteins respectively. The organization of genes present on λ 16-2 is as in Fig. 1.6.

1.2.2.e Enzymatic activities attributable to genes in this region

The enzymatic activities attributable to genes in the 2' region have largely been assigned to proteins involved in murein biosynthesis (see Table 1). One septation related protein, PBP3 has been identified, but specific septation enzymes have not been assigned to ftsX, Y, Q, A, Z, secA or azi. The sep gene product (sep, pbpB, ftsI) is particularly interesting: Olijhoek et al. (1982) showed that furazlocillin, which binds PBP3 did not inhibit the initiation of septation, but only its completion. This is consistent with the findings of Spratt (1977) (see Section 1.2.2.b). Olijhoek et al. (1982) found that the degree of

Fig. 6. The organization of genes present on λ16-2

(Taken from Lutkenhaus and Wu, 1980)



peptidoglycan cross linking in newly formed chains was at its greatest around division. Ishino and Matsuhashi (1981) purified PBP3 to homogeneity and showed that it was a bifunctional peptidoglycan synthetase. *In vitro* their enzyme catalysed the synthesis of cross linked peptidoglycan from its lipid precursor, N-acetylglucosaminyl-N-acetylmuramyl (pentapeptide)diphosphoryl undecaprenol. The reaction was in two steps, firstly a glycan chain extension (peptidoglycan transglycosylase) and secondly cross bridge formation (peptidoglycan transpeptidase). The transpeptidase reaction was found to be sensitive to the same β-lactam antibiotics that stopped septum formation *in vivo*. This strongly suggests that the role of PBP3 in cell division is as a transpeptidase, and possibly also a transglycosylase. As yet no D-D carboxypeptidase has been unambiguously assigned to a gene in *E.coli*.

The nature of the *envA* gene product is of interest, because as previously mentioned, the *envA* mutation confers a chain forming morphology in *E.coli*. This defect in cell separation is a later stage of cell division than septation, and different gene products are needed, although both processes are concerned with cell surface events and are under genetic control. The association of septal peptidoglycan between daughter cells appears covalent in nature, since separation is not achieved by boiling in 4% SDS for 1 hr. (Wolf-Watz *et al.*, 1973). Septation probably involves enzyme activities that fuse and separate septal peptidoglycan. The stable septa exhibited in the *envA* mutant could thereby be due to increased fusing activity or to a decreased septal peptidoglycan

splitting activity. Wolf-Watz and Normark (1976) have presented evidence favouring the latter alternative and suggest that the defect is due to N-acetyl muramyl L-alanine amidase, an enzyme known to cleave the amide bond between NAM and L-alanine in NAM L-alanyl-γ D-glutamyl-meso-DAP (Van Heijenoort et al., 1975)
Wolf-Watz and Normark (1976) demonstrated that 1) In rich media the envA mutant shows a sixfold reduction in amidase activity when compared to the wild type. 2) Both genetic and physiological suppression of chain formation was associated with increased amidase activity. 3) If the substrate N-acetylmuramyl L-alanyl D-glutamyl-meso-DAP was added to either wild type of mutant cells then a decrease in cell separation was observed. This was interpreted as competitive in vivo inhibition of the amidase.

However, these are all indirect lines of evidence and the determination of amidase activity was performed on crude cell extracts which may have contained other autolysis. E.coli has at least four other peptidoglycan hydrolases of unknown function and specificity (Van Heijenoort and Van Heijenoort, 1971). A study of the amidase by Van Heijenoort et al., 1975
suggested that the enzyme was either periplasmic or associated with the outer membrane, and that the MW of the homogenous enzyme was 39K. This does not fit with the 31K suggested for the envA gene product (Lutkenhaus and Wu, 1980). All physiological effects which suppress the low amidase activity also restore other defects of envA mutants, for example, resistance to antibiotics (see Section 1.2.2c). Leakage of amidase from the cells has not been detected (Wolf-Watz and Normark, 1976), so it is possible that the

Table 1. Enzymatic Activities

Gene	Enzyme	Reference
polB	DNA polymerase II	Knippers, 1970.
ftsI	PBP3; transglycosylase and transpeptidase	Spratt, 1977; Ishino and Matsuhashi, 1981.
murE	mDAP adding enzyme	Lugtenberg and van Schindel van dam, 1972b.
murF	D-ala, D-ala adding enzyme	Lugtenberg and van Schindel van dam, 1972a.
murC	D-ala adding enzyme	" " " " " " " "
ddl	D-ala D-ala ligase	Lugtenberg and van Schindel van dam, 1972c.

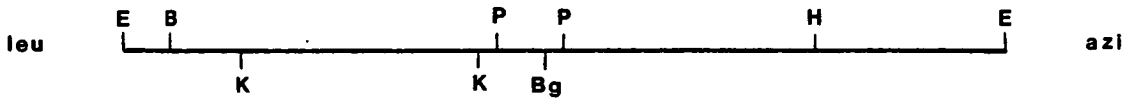
Enzymatic activities have not been unambiguously assigned to other genes of this cluster.

mDAP = meso-diaminopimelic acid

1.2.2f A restriction map of the 2.2Kb EcoR1 fragment

A restriction map of the 2.2Kb EcoR1 fragment bearing ftsA and containing sites of relevance to this thesis is as follows:

Fig. 1.7.



Distance from left hand EcoR1 sites in bp:

BamHl	110.
BglII	1040.
HincII	220, 300, 630, 1360, 1750.
HindIII	1780.
KpnI	290, 890.
PvuII	940, 1060.
EcoRl	0, 2270.

(Unpublished data of G. Hatfull and A. C. Robinson)

local environment, the outer membrane, could regulate the amidase activity. The low amidase activity in the mutant would then just be a secondary consequence of an alteration in the outer membrane structure.

The same enzyme activity has been identified in other bacteria, e.g. Bacillus subtilis, where a 50Kd N-acetylmuramyl L-alanine amidase has been purified to homogeneity (Van Heijenoort et al., 1975). The Bacillus subtilis enzyme is thought to be associated by ionic linkages to specific sites in the teichoic acid (Herbold and Glaser, 1975). Mixed autolysin preparations have been shown to affect cell separation in both Bacillus licheniformis (Forsberg and Rogers, 1971) and Bacillus subtilis (Fan, 1970). In Bacillus subtilis all autolysin mutants are leaky, which led Mendelson (1982) to suggest that a complete deficiency would be lethal. All autolysin defective mutants have been shown to have lost both amidase and glucosamidase activity, suggesting that these mutations lie in regulatory genes (Mendelson, 1982). Even so, there have been no firm conclusions concerning division specific autolysins.

1.3.1. The role of the SOS system in division inhibition in E.coli.

To combat damage to DNA E.coli has evolved an inducible repair system. This has been termed the SOS response (Radman, 1975) and operates after cells have been exposed to either ultraviolet light, various mutagens or have been blocked in DNA replication. The response includes inhibition of cell division, prophage induction and an enhanced capacity for DNA repair and mutagenesis (reviewed by Witkin, 1976; Gottesman, 1981; Little and Mount, 1982).

Upon induction of SOS, the process of septation is inhibited. However, growth continues resulting in the formation of straight sided filaments, i.e. filaments without visible signs of partially completed septa. This temporary block to division gives the cell time to repair its DNA and prevents the formation of anucleate cells. Once repair is complete, nucleoids are segregated and division then occurs at the pre-existing potential division sites (see Section 1.1.2).

The SOS functions are not expressed in E.coli carrying certain mutations in the recA or lexA genes. This first suggested a co-ordinately regulated set of functions (Witkin, 1976) and over the last few years a number of mutations affecting the SOS pathway have been isolated and analysed. The resulting model suggests that a signal generated by DNA damage activates the recA protein converting it to a protease form (Roberts et al., 1978; Craig and Roberts, 1980). The precise nature of the signal is unknown, but may involve one or more pathways depending on the nature of the initial damage (Gudas and Pardee, 1975; McPartland et al., 1980) DNA replication forks (Casaregola et al., 1982b and single stranded DNA and oligonucleotides (Craig and Roberts, 1980; Phizicky and Roberts, 1981) have all been implicated in activation of the SOS response.

Proteolytic cleavage of both cellular and prophage repressors causes induction of functions normally repressed in the absence of DNA damage. The host function lexA (Little et al., 1980) and viral repressors for λ (Roberts et al., 1978) and P22 (Phizicky and Roberts, 1980) have been shown to be substrates for the recA

protease. Mutations in the recA gene which lead to a loss of proteolytic activity, or mutations in lexA which cause lexA to be non-inducible or its product resistant to the protease, abolish induction of the SOS system. An important advance towards a molecular understanding of the SOS response has been the demonstration that the level of recA protein is greatly increased after treatment of cells with ultraviolet light or nalidixic acid (McEntee, 1977; Gudas and Mount, 1977). The level of recA mRNA is stimulated by DNA damaging agents (McPartland et al., 1980) and using a recA::lac fusion Casaregola et al. (1982a) have shown that recA expression increases 17 times within 10 minutes after UV irradiation or thymine starvation. A high level of recA production is by itself not sufficient to cause proteolytic activation. For example, in a lexA t.s. strain which codes for a thermolabile lexA protein, the recA gene is derepressed at the restrictive temperature, yet induction of lambda does not occur (see Section 1.3.2). Purified lexA protein has been shown to inhibit expression from both the recA and lexA genes (Brent and Ptashne, 1980; Little et al., 1981). Besides these two, lexA is thought to repress genes involved in DNA repair, cell division and site specific recombination (see Table 1).

1.3.2. Suppressors of tsl and tif filamentation

Two conditional lethal mutations, tif and tsl have proved useful for isolating mutations which suppress SOS induced filamentation. The tif allele is a mutation at the recA locus (Castellazzi et al., 1972, 1977) which causes the recA protein to

Table 2. Chromosomal lexA target genes

Gene	Location	Function	Reference
lexA	91	SOS Repressor	Brent and Ptashne, 1980, 1981. Little et al., 1981.
recA	58	General Recombination Protease	Radding, 1981. Roberts et al., 1978. Phizicky and Roberts, 1980. Little et al., 1980.
umuC	25	Mutagenesis	Bagg et al., 1981.
himA	38'	Site-specific recombination	Miller et al., 1981.
uvrA	92'	Excision Repair	Kenyon and Walker, 1981. Sancar et al., 1982a.
uvrB	17'	Excision Repair	Sancar et al., 1982b. Fogliano and Schendel, 1981.
sfiA	22'	Cell division Inhibitor	Huisman and D'Ari, 1981.
dinA	2'	Unknown	Kenyon and Walker, 1980. Kenyon et al., 1982.
dinB	8'	" "	As dinA.
dinD	80-85'	" "	As dinA.
dinF	91'	" "	As dinA.

assume its protease form at 42°C whilst remaining inactivated at 30°C. Thus by cleavage of lexA and subsequent derepression of SOS operons the tif mutation causes induction of the SOS response. For filamentation, protein synthesis must occur during the tif expression period (Huisman et al., 1980a). The tsl allele causes induction of filamentation (Mount et al., 1973) and recA protein synthesis (Gudas, 1976) at 42°C, but not of λ prophage development. As with tif, tsl induced filamentation requires protein synthesis (Satta and Pardee, 1978). The tsl mutation confers temperature sensitivity on the lexA protein, such that it can no longer act as a repressor. Hence the SOS operons are derepressed, including both lexA itself and recA.

It was noted by George et al. (1975) that tif mediated filamentation could be enhanced in the presence of the lon mutation (see Section 1.3.3), although neither prophage induction nor repair were affected by this mutation. Using a tif lon strain these authors were able to isolate suppressors of thermosensitivity and UV sensitivity. These were designated sfiA and sfiB. The sfiA locus has also been isolated as a suppressor of a tsl tif double mutant (Huisman et al., 1980b,c). The sfiA and sfiB mutations do not appear to suppress SOS functions other than filamentation (Huisman et al., 1980c).

1.3.3 The lon gene product

The lon gene product is one of a set of specific proteases found in E.coli (Goldberg et al., 1981). It has been identified as a 94Kd polypeptide (Zehnbauer et al., 1981) which may exist as

a tetramer (Chung and Goldberg, 1981). It is ATP dependent (Charette et al., 1981; Chung and Goldberg, 1981) and also binds DNA (Zehnbauer et al., 1981; Chung and Goldberg, 1982). Furthermore, the lon gene product hydrolyses ATP and protein in a stoichiometric fashion (Waxman and Goldberg, 1982). Charette et al. (1981) have noted that the ATP dependent protease might provide a timing mechanism for cell division, especially since Huzyk and Clark (1971) showed that there is a gradual increase in ATP concentration from zero age until the time of division. This is unlikely since in the absence of SOS induction, lon mutants grow quite normally (Leighton and Donachie, 1970).

Otherwise known as capR, deg or protease La the lon gene product affects a variety of physiological processes. The lon mutations overproduce capsular polysaccharide (Markovitz, 1977), are defective for lysogeny of λ (Walker et al., 1973) and P_1 (Takano, 1971) and have a decreased ability to degrade both abnormal proteins (Gottesman and Zipser, 1978; Shineberg and Zipser, 1973) and normal proteins (Mizusawa and Gottesman, 1983; Gottesman et al., 1981a). In addition lon mutants are sensitive to DNA damaging agents such as ultraviolet light or methylmethane sulphonate (MMS) (Howard-Flanders et al., 1964). This sensitivity is thought to result from an exaggeration of the normal inhibition of division after DNA damage (Witkin, 1967).

Leighton and Donachie (1970) showed that lon^- cells in liquid media can recover the ability to divide after a period of SOS induction but that the time required for recovery is much increased over that of lon^+ cells. The UV sensitivity of lon

mutants on solid media is probably due to the phenomenon of 'plating death' (Donachie and Hobbs, 1967; Donachie, 1969).

It was later suggested that UV induced filamentation is caused by a defect in protein degradation in *lon* mutants (George *et al.*, 1975). These authors proposed the existence of a division inhibitor which is produced in response to DNA damage (see also Witkin, 1967). This normally decays via the *lon* proteolytic pathway, but its persistence in *lon* mutants results in an inability to resume septation, and hence cell death (see Section 1.3.6). That the *lon* gene controls the stability of two proteins N and cII (Gottesman *et al.*, 1981a) and is involved in cell division illustrates the importance of protein degradation in the regulation of gene expression in *E.coli*. It is possible that other proteolytically regulated proteins exist. Of the *E.coli* proteases two are found in the periplasmic space (Sreedhara-Swamy and Goldberg, 1981), so these proteins could well affect steps in cell elongation or septation. The *recA* protein is even more specific than *lon* degrading only *lexA* protein and repressor whilst the cellular *hfl* function may regulate *cII* protein (Hersokwitz and Hagen, 1980). Thus *E.coli* may use a set of proteases for the regulation of cellular processes.

1.3.4. *Suppressors of lon*

Gayda *et al.* (1976) plated a *lon* mutant on to the DNA damaging agent nitrof urantoin, and in doing so isolated second site revertants in the *lon* gene. These were found to map at two loci; *sulA* near *pyrD* at 21' and *sulB* which co-transduced with *leu* at 2'. These mutations were shown to reverse the original UV sensitivity of the *lon* strain. Similarly Johnson (1977)

isolated sulA and sulB mutations as MMS resistant derivatives of an E.coli lon⁻ strain (see also Johnson and Greenberg, 1975; Donch et al., 1971). Under some growth conditions the sulB mutation causes cells to be temperature sensitive for the division process at 42°C, and only to exhibit this phenotype in low salt media. There was no decrease in viability at 42°C so Johnson (1977) suggested that sulB hinders, but does not block the cell division process at 42°C. Gottesman et al. (1981b) analysed the role of sulA and sulB in filamentation in more detail. Since a lon sulA double mutant has the wild type phenotype and a sulA strain has no detectable phenotype alone, these authors predicted, in agreement with George et al. (1975) that all lon effects on sul dependent filamentation are mediated through sulA. It is thought unlikely that sulA plays any role in the normal process of cell division (Gottesman et al., 1981b; Huisman et al., 1983). Mutations in sulB are about 10 fold less frequent than mutations in sulA (Johnson, 1977; Gottesman et al., 1981b) and are dominant (Gottesman et al., 1981; Lutkenhaus, 1983). The division defective phenotype of sulB strains is epistatic on both lon and sulA in the absence of an SOS inducing treatment, observations which led Gottesman et al. (1981b) to postulate that the sulB product is normally a part of the cell division apparatus and a target for sulA action.

1.3.5. Relationship between sfi and sul

The sfiA and sfiB mutations have map locations similar to sulA and sulB, that is, 22 and 2 minutes on the E.coli map. One sulA allele has been shown to suppress tif mediated filamentation without

affecting λ prophage induction (George et al., 1975). No other aspect of the SOS repair system is affected by sulA and sulB mutations. It has been suggested that sfiA and sulA may define a single locus and that sfiB and sulB may define a second locus (Gayda et al., 1976; Johnson, 1977). In addition, mutation to sulB or sfiB is much rarer than to sfiA or sulA in E.coli K-12, and has not been observed in E.coli B at all, whether one selects for suppression of tif and tsl (Huisman et al., 1980a) suppression of tif and lon (George et al., 1975) or suppression of lon (Gayda et al., 1976; Johnson, 1977). Huisman et al. (1980c) tested six sfiA alleles and found three to be recessive and three to be dominant. Gottesman et al. (1981b) did not find any dominant mutations in the five alleles they tested. Gottesman et al. (1981b) tested six sulB alleles and found them to be dominant, whilst Huisman et al. (1980c) tested two sfiB alleles and found them to be recessive. The experiments were performed in slightly different ways, Gottesman et al. (1981b) assayed filamentation and sensitivity to MMS in a lon strain whilst Huisman et al. (1980c) assayed UV sensitivity and temperature resistance in a tif lon strain. To attempt to resolve this dilemma Gottesman et al. (1981b) introduced tif to isogenic lon sulB and lon strains. The sulB mutation conferred resistance to MMS but did not completely block tif mediated filamentation. A sfiB mutation was subsequently shown to be dominant for MMS resistance. Therefore, it seems that both the dominance of sulB and the extent of suppression of tif filamentation is dependent on the strain background. Gottesman et al. (1981b) suggest that this may reflect a slight change in the balance of division associated components

between the two backgrounds, although it is possible that two classes of mutant exist, one recessive, one dominant.

1.3.6. Induction of *sfiA* by SOS

Witkin (1967) suggested that filamentation after SOS induction was due to synthesis of a division inhibitor. In accordance with this hypothesis, George *et al*. (1975) proposed that septation inhibition associated with the SOS response was due to a Repair Associated Division Inhibitor (RADI). Huisman and D'Ari (1981) have produced evidence strongly suggesting that induction of *sfiA* protein results in an inhibiton of cell division. By inserting the phage Mud (Ap, *lac*) (Casadaban and Cohen, 1979) into the *sfiA* gene Huisman and D'Ari (1981) were able to show that expression of *sfiA* is induced by thymine starvation, treatment with ultraviolet light, nalidixic acid and MMS. Since no induction of *lacZ* was observed on strains carrying Mud(Ap,*lac*) integrated in the *gal*-locus it was concluded that perturbations of DNA replication lead specifically to increased expression of the *sfiA* gene. In a *recA* or *lexA* strain, known to be completely suppressed in the SOS response, no induction of *sfiA* was observed after ultraviolet treatment. Similarly *tif* and *tsl* strains showed expression of *sfiA* at 42°C but not at 30°C. Furthermore, expression of *sfiA* via *tsl* did not depend on *recA*, so *sfiA-lacZ* operon fusions act as if repressed by *lexA*. Induction of *sfiA* was not affected by the *lon* mutation, so *lon* does not function at the transcriptional level. Huisman and D'Ari (1983) subsequently demonstrated that neither *sfiB* nor *sfiA* itself are transcriptional regulators of *sfiA* expression. Similarly McPartland *et al*. (1981) isolated Mud (Ap,*lac*)

insertions into the sulA (sfiA) gene and demonstrated that these could suppress the lon phenotype. Mizusawa and Gottesman (1983) cloned sulA on to a transducing phage and demonstrated that lon regulates sulA activity by degradation of the sulA protein. In wild type cells sulA is unstable, with a half life of 1.2 minutes. In the lon mutant the half-life is increased to 19 minutes. This is sufficient to explain the extended recovery period of lon mutants, since if sulA product is the division inhibitor synthesised after DNA damage, then its persistence in lon mutants would lead to extended septation inhibition. In the wild-type decay of sulA would ensure that normal septation was resumed when sulA was again repressed. If the cloned sulA gene is expressed in wild type cells in the absence of inducing treatment then filamentation occurs, and without affecting other SOS functions. This data suggests that DNA replication is coupled to cell division when SOS is induced. The sfiA dependent system of division inhibition is not involved in size adjustment after a nutritional shift up or in cell size determination or chromosome segregation. Thus this system is probably not involved in regulation of steadystate (unperturbed) cell division (Huisman et al., 1983). Furthermore, insertion of bacteriophage Mu into sfiA indicates that sfiA is non essential (Huisman and D'Ari, 1981).

1.3.7. FtsZ and SfiB are allelic

Using a recombination and deletion analysis of lambda transducing phages bearing sulB mutations Lutkenhaus (1983) has located sulB in the vicinity of ftsZ. These phages are impaired in their ability to complement the ftsZ mutation and produce an

ftsZ protein of slightly altered mobility. Lutkenhaus (1983) suggests that sulA inactivates or inhibits the product of ftsZ, blocking an initiation step in cell division.

1.3.8 The infA and infB genes

The infB mutation has been isolated as a suppressor of lethal filamentation amongst temperature resistant survivors of either tif or tif tsl strains (Huisman et al., 1980a). In addition, Bailone et al. (1975) isolated a mutation (infA) which suppressed tif mediated filamentation. Huisman et al. (1980a) reported that infB mutations not only suppressed filamentation but reduced tif mediated induction and mutagenesis in a tif tsl strain at 42°C. It was subsequently shown that both a tsl infB and a tif tsl infB strain showed reduced induction of sfiA at 42°C, accounting for the lack of filamentation (Huisman and D'Ari, 1983). However, in a tif infB background there was no suppression of filamentation, suggesting that the infB mutation causes an interaction with the lexA (tsl) protein allowing a degree of repression to occur. Suppression of tif by infA may similarly be a modification of the activity of recA protease, reducing the efficiency with which it can inactivate lexA and cI repressor. Thus there are other factors which appear to regulate SOS functions, and can, therefore, modulate sfiA expression.

1.3.9 Two pathways of division inhibition

Huisman et al. (1980 a,b) first suggested the possibility of two filamentation systems by observing filamentation after thymine starvation of sfi strains. They suggested that sfi mutations abolished rapid filamentation after induction of SOS but sometime

after a less efficient alternative pathway became operable. A similar observation was made by Gottesman et al. (1981b) who found that lon sul strains were blocked in the formation of abnormally long filaments, but not blocked in the transient filamentation observed 1 to 3 hours after UV irradiation. The precise nature of this sfi independent pathway is unclear, although Burton and Holland (1983) suggest that it only operates in cells active in DNA replication, requires protein synthesis and is dependent upon recA. Since the timing of division recovery in a sfi strain was indistinguishable from the wild type, Burton and Holland (1983) suggest that it may have a role in the timing of division recovery. These authors suggest that the need for DNA replication may reflect the normal cell cycle requirement for termination of chromosome replication.

D'Ari and Huisman (1982) have suggested two different modes of induction for the sfi dependent pathway. The first is induced by UV irradiated DNA in the absence of DNA replication. In support of this Little and Hanawalt (1977) demonstrated that DNA damage still induced recA protein in a dnaA t.s. strain, deficient in chromosome initiation. There may, however, be a requirement for the dnaC product (Casaregola et al., 1982b). In the second sfiA is induced by attempting to replicate damaged DNA. This agrees with Phizicky and Roberts (1981) who suggested that a replication complex encountering a lesion may generate a recA effector.

Thus, to summarise, there are at least two pathways for division inhibition after DNA damage, both dependent on recA. The first is sfi dependent and requires protein synthesis. It can be induced by ultraviolet irradiated DNA whether it is being replicated

or not. The second pathway is sfi independent, does not require protein synthesis and does not occur in cells lacking replication forks. After DNA damage DNA replication and cell division appear to be coupled. Either a recA or a dnaA t.s., sfiA strain will be uncoupled in both pathways, and assuming no others exist should reveal the coupling mechanism for unperturbed (steady state) division. As discussed in Section 1.1.2, there is no evidence for such a coupling.

1.3.10 Overall summary of Sfi dependent division inhibition:

Unperturbed growth: See Fig 8A

The lexA protein represses recA, sfiA, itself and other SOS genes. There is a basal level of recA and presumably sfiA. The ftsZ or sulB product functions in septation.

Induction of the SOS response: See Fig. 8B

In the presence of an effector, recA protein is converted to its protease form, and is then capable of cleaving lexA protein and the lambda repressor if present. Inactivation of lexA protein derepresses recA itself, sfiA, lexA and other SOS operons. The induced sfiA product is then free to inhibit the action of the ftsZ product, an essential part of the division machinery.

Recovery: See Fig. 8C

In the absence of an effector, recA protein is converted back to its non proteolytic form. As it does so, the derepressed lexA protein re-establishes repression. Autoregulation of lexA probably aids a rapid recovery from division inhibition. The sfiA protein which remains is degraded by the lon protease. The control

Fig. 1.8 The SOS Response

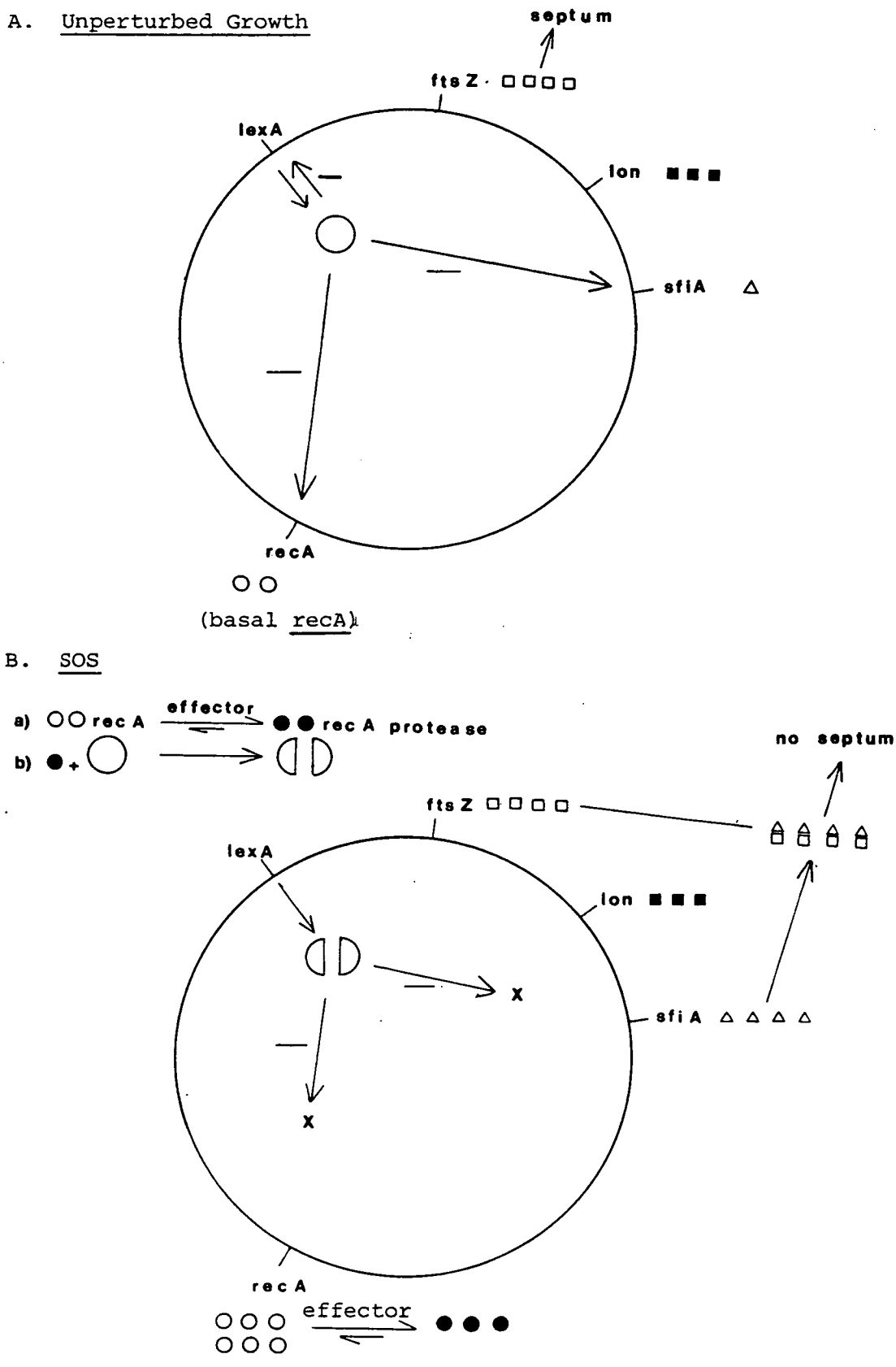
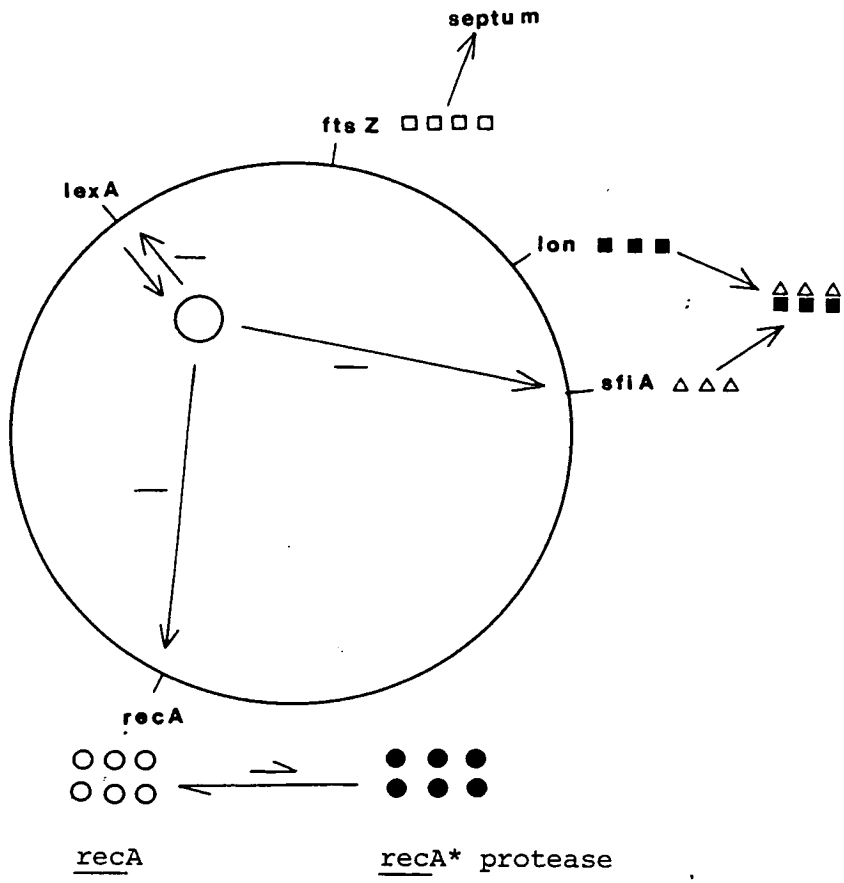


Fig. 1.8 (continued)

C. Recovery



of the lon gene in unclear. It is not known whether extra lon product is synthesised to cope with the extra sfiA substrate. In addition, it is not known whether the inactivation of the ftsZ product is reversible or whether cell division requires de novo synthesis of the ftsZ product.

1.4.1 Cyclic AMP and its role in cell division

Cyclic adenosine 3'-5' monophosphate (cAMP) in association with its receptor protein (CRP) influence a number of physiological processes in E.coli, primarily at the level of transcription (see Section 1.5.2). This includes repression of biosynthetic enzymes (Pastan and Adhya, 1976), autoregulation of CRP (Aiba, 1983), cAMP production (Majerfield et al., 1981), induction of some catabolic enzymes (Mitra et al., 1975; Wilcox et al., 1974), replication of certain plasmids (Katz and Helinski, 1974; Katz et al., 1973), lysogeny of bacteriophages, (Kumar, 1976; Yokota and Kasuga, 1972), synthesis of membrane proteins, (Movva et al., 1981, Mallick and Herrlich, 1979) and envelope proteins (Aono et al., 1978).

All mutations deficient in cAMP inactivate the cya gene which codes for adenylate cyclase (Brickman et al., 1973; Perlman and Pastan, 1969) and all cAMP receptor protein (CRP) mutants have lesions in the crp gene (Sabourim and Beckwith, 1975). Lesions in the cya or crp genes confer several characteristic properties, one of which has unusual morphology. Mutant cells are cocco bacillary approaching coccal (Spherical) in shape, in contrast to the rod shaped cells of the wild type (Harwood and Meynell, 1975; Kumar, 1976). If grown in the presence of exogenous cAMP for at least two cell

generations cya but not crp cells could be shown to assume wild type morphology (Kumar, 1976). In addition both cya and crp mutants were shown to grow with increased doubling times, to have increased resistance to antibiotics such as Nalidixic Acid and Ampicillin, and some mutagens. This, together with the close correspondence in characteristics to spherical E.coli mutants (Section 1.1.4) led Kumar (1976) to suggest that these unusual parameters were consequences of the defective envelope structure. Similarly, Scott and Harwood (1981) suggest that the increased resistance of cya strains to mecillinam is attributable to their slow growth rate, rather than an intrinsic property of the genetic lesion. It has been suggested that slow growth rates are a consequence of unusual morphologies (Wu and Pardee, 1973).

The morphological defect was further investigated by Aono et al. (1978) who searched for envelope proteins which determined cell shape and were regulated by cAMP. It was shown that the synthesis and not the integration of seven envelope proteins were regulated by cAMP. However, no morphological changes were observed and it was concluded that none of the proteins tested were related to cell shape. This is in contrast to the results of Aldea et al. (1980) who showed that the outer membrane proteins of Salmonella typhimurium were not affected by cAMP.

Changes in cAMP concentration have been correlated with morphogenesis of Arthrobacter crystallopoietes, in which a 30-fold increase in the internal concentration of cAMP precedes the observed change from spheres to rods (Hamilton et al., 1977).

In certain strains of E.coli cell division can be inhibited by the addition of cAMP (Utsumi et al., 1981) and cellular morphology is changed from rods to filaments. Septation is inhibited and nucleoids are regularly distributed. Filamentation produced by cAMP is particularly interesting, since it specifically implicates a role for cAMP in septation. The temperature sensitive lesion causing this effect has been located near rpsL (72.7') and has been called fic (filamentation induced by cyclic AMP) (Utsumi et al., 1982). Also in this region is crp (72.9') and ftsE (73.1') (Nishimura et al., 1977). The crp gene was shown to be distinct from fic by plasmid complementation, but Utsumi et al., (1982) do not state how close the two genes are, nor do they make any reference to the apparent dominance of the fic mutation. It must also be noted that the filamentation response was produced by physiologically abnormal cAMP levels, but the authors argue against a secondary effect of cAMP addition since filamentation was not observed at the non-restrictive temperature or in a crp mutant. Removing cAMP caused the cells to regain rod morphology. Utsumi et al. (1982) suggest that septum formation may be controlled by variations in the intracellular cAMP level, and that filamentation requires both the cAMP/CRP complex and a temperature sensitive factor, presumably the product of the fic gene.

The ftsE gene has since been mapped away from this region (G. Salmond, pers. comm.) making it unlikely that fic and ftsE are allelic.

1.4.2 cGMP as a cell cycle regulator

The inhibitory effect of cAMP on septation could be reversed if cGMP (cyclic guanosine 3'-5' monophosphate) was added (Utsumi et al., 1981). The authors suggest that this may reflect the role of cGMP in E.coli, and that cell division may be controlled by a relative change in the intracellular cAMP and cGMP concentrations. In this experiment cGMP acts as a competitive inhibitor of cAMP, perhaps suggesting a process in which cAMP binds to an effector in vivo. However, cGMP has been shown to antagonise the action of cAMP only in in vitro transcription experiments, there is little evidence for such a role in vivo (deCrombrugghe et al., 1971; Nissley et al., 1971; Emmer et al., 1970).

Transient increases in the intracellular cGMP concentration have been observed in synchronous cultures of E.coli bearing a mutation in spoT (Cook et al., 1980) a gene coding for guanosine 5' diphosphate 3' diphosphate pyrophosphatase (An et al., 1979). This led Cook et al. (1980) to suggest that cGMP may be involved in some aspect of the regulation of a cell cycle event and that this may be related to cellular guanosine polyphosphate (magic spot) metabolism.

It has been suggested (Bernlohr et al., 1974) that intracellular cAMP levels vary antagonistically to cGMP levels, however, Gonzalez and Peterkofsky (1975) indicate that cyclic nucleotide levels can vary co-ordinately, reciprocally or in an uncoupled fashion.

The relationship between intracellular levels of cGMP and cAMP levels is far from clear, and existing data relating cGMP to regulation of cell division is unconvincing.

1.4.3 cAMP and cell division in higher organisms

Cyclic AMP has been implicated in the cell cycles of higher organisms. Intracellular levels of cyclic nucleotides vary considerably during the cell cycle in a variety of cell types, and although only general trends can be extracted from the existing data, low internal concentrations of cyclic AMP can be associated with initiation of cell division, whereas the internal concentration of cGMP is at its highest during cell division (Charp and Whitson, 1980).

It is widely accepted that the primary mechanism of cyclic AMP action in eukaryotes is to affect the activity of cAMP dependent protein kinase, and hence to set the degree of phosphorylation of certain key proteins, whose functions and activities are regulated by this reversible covalent modification (Gottesman, 1980; Robison et al., 1971; Thorner, 1982). No phosphorylation event has been specifically associated with the cell cycle in mammalian cells. Mutants of the yeast Saccharomyces cerevisiae that require cAMP for growth (cyr-1, bcy-1) have been isolated, and it has been shown that cAMP-dependent protein kinase (and hence cAMP) has an essential function in allowing yeast cells to pass through G_1 to S phase of their cell cycle (Matsumoto et al., 1982). The relationship between cyr-1, bcy-1 and cell division cycle genes (Reed, 1980) is unknown.

It is clear that cAMP plays a fundamental role in the regulatory processes of many organisms. In yeast this acts through cAMP-dependent protein kinase and in E.coli through the CRP protein.

Although the evidence is circumstantial, cAMP may play a role in cell division of both procaryotic and eucaryotic cells. In both cases, a low internal concentration of cAMP is associated with cell division.

1.5.1 Gene Expression

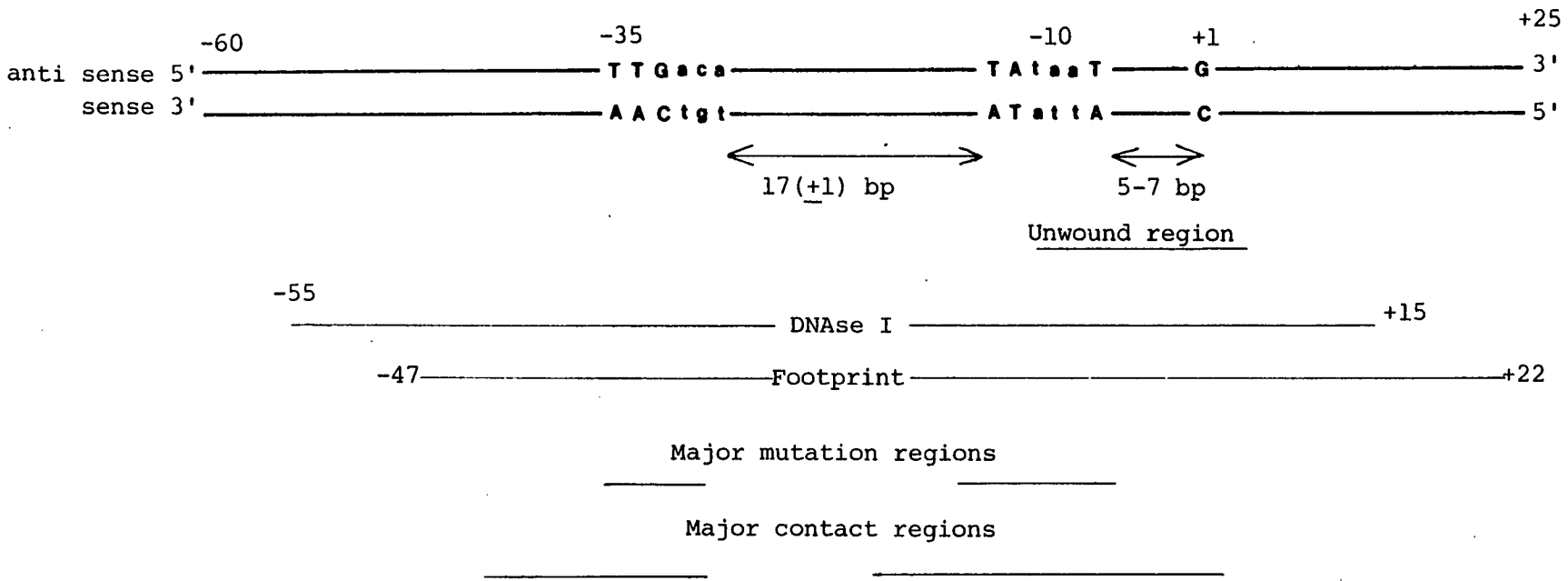
In Escherichia coli the level of gene expression is primarily determined by three factors; the rate of transcription, the stability of the RNA transcript and the efficiency of translation, each of which will now be considered.

1.5.1a The rate of transcription

The rate of transcription is controlled by the events leading to its initiation, that is, the interaction between RNA polymerase and promoter regions of DNA. A promoter contains all signals for proper binding and subsequent activation of RNA polymerase to a form capable of initiating an RNA chain. Within or immediately adjacent to a promoter there may be additional signals in the DNA for the specific binding of repressor or activator proteins that can modulate its activity. For convenience the overall polymerase promoter interaction can be divided into four steps 1) location of a promoter by the polymerase, 2) formation of the closed complex, 3) formation of the open complex, 4) mRNA initiation.

A statistical analysis of E.coli promoter sequence composition has resulted in the generation of model promoter sequences containing the most frequently found bases at given positions through the RNA polymerase recognition region (Rosenberg and Court, 1979; Siebenlist et al., 1980) (see Fig. 9). Two regions of general homology exist. These are the Pribnow box, or -10 region

Fig. 9. E.coli consensus promoter sequence (Taken from Von Hippel et al., 1982)



and the -35 region centered about 10 and 35 base pairs from the transcription initiation site, +1. The sequence is asymmetric and reflects the prime function of a promoter, to initiate mRNA synthesis in the correct direction and on the correct ("sense") template strand.

The polymerase is thought to locate a promoter by moving along the DNA in a one dimensional diffusion process, since the rate at which RNA polymerase can locate a promoter is greater than that expected by free diffusion (von Hippel et al., 1982). The -35 region is thought to be important for initial recognition whilst the -10 and start site are sufficient for maintenance of a stable complex and initiation (Simpson, 1982). The RNA polymerase can protect anything from -55 to +22 nucleotides from the initiation point (Simpson, 1982). The -10 region is AT rich and local melting here is thought to facilitate formation of the open complex. Initiation is usually with a purine, most often an A residue. Munson and Reznikoff (1981) have shown that for some promoters the first 6-9 nucleotide residues can be incorporated into the mRNA and the released, without dissociation of the open complex. After this, the initiation factor sigma is dissociated and elongation can proceed, catalysed by the core enzyme (Hansen and McClure, 1980). The role of sigma factor is far from clear, but is thought to alter the affinity of the polymerase for the promoter (von Hippel et al., 1982). Besides sigma other effectors can affect the rate of transcription. Two of these, cAMP-CRP and lexA repressor are considered in Section 1.5.2.

1.5.1b Stability of the mRNA

The factors which determine mRNA stability are not well understood and although a number of RNAses have been identified in E.coli (Apiron, 1974) little is known about the initiation sites for mRNA degradation nor which enzymes are responsible for determining the rate of mRNA decay in vivo. Recently a novel form of post-transcriptional regulation in which gene expression is regulated at a site which is promoter-distal to the functional unit (retroregulation) has been described (Guarneros et al., 1982; Saito and Richardson, 1981). The nucleotide sequence of the lambda sib region, 260 bp beyond the int gene displays hyphenated dyad symmetry allowing the RNA to adopt a structure characteristic of RNAse III substrates. Processing by RNAse III is thought to be followed by exonucleolytic degradation and hence the inability to translate a functional int (reviewed by Gottesman et al., 1982).

1.5.1c Translation efficiency

The factors influencing translation are a little more understood. The efficiency with which mRNA is translated is controlled at the level of ribosome recognition and translation initiation. The rates being determined by both specific sequence information and RNA conformation. The bacterial ribosome binding sites consists of a sequence preceding the AUG translation initiation codon (the Shine Dalgarno or SD sequence) complementary to the 3' end of 16-S RNA (Shine and Dalgarno, 1974). Both the position and extent of complementarity may influence the efficiency of ribosome interaction, whilst the secondary RNA structure

determines the accessibility of ribosomes to the SD sequence. Translation rates can differ by as much as 10 fold (Chang et al., 1980).

The galactose (gal) operon has two promoters P_1 and P_2. P_2 initiates transcription five nucleotides upstream from the transcription initiation site for promoter P_1 (Busby et al., 1982). These promoters regulate transcription of the polycistronic gal operon. Queen and Rosenberg (1981) have demonstrated that for the galE gene product, the 5' proximal gene is translated four times more efficiently from P_2 mRNA than P_1 mRNA. In contrast the 5' distal gene products galT and galK are translated with equal efficiency from P_1 and P_2. This difference is thought to result from a change in RNA conformation such that in the P_2 mRNA the SD sequence is more accessible to ribosomes and explains the discordinate expression of the gal operon under different metabolic conditions (Ullman et al., 1979). This assigns a physiological significance to the dual promoters and reveals a form of cellular regulation based on differential translation efficiency (see Section 1.5.3).

Post-transcriptional events also include the phenomenon of translational coupling. Whilst studying nonsense mutations of trpE Oppenheim and Yanofsky (1980) noticed that these mutations reduced expression of the adjacent trpD gene more strongly than that of other downstream genes; a result which could not be accounted for by transcriptional polarity (see 1.5.4). Genetic evidence suggested that this trpD specific polarity is entirely translational and that trpE and trpD are naturally coupled. This idea was enlarged by Schümperli et al. (1982) who noted that the galK gene was

translationally coupled to the gene immediately preceding it, galT and that this effect depends on the position at which upstream translation terminates relative to the galK start site. Coupling is most likely due to non release of upstream terminating ribosomes thus allowing reinitiation, or perhaps merely an increase in the local concentration of ribosomes around the SD sequence. In either case coordinate expression of galT and galK is ensured by translational coupling and may indeed enhance the coordination already achieved by simultaneous transcription.

An alternative mechanism for translational regulation of the synthesis of ribosomal protein has been reported (Johnson et al., 1982). Two ribosomal proteins, L10 and L12 form a complex and prevent translation by binding to the leader region of the L10 operon mRNA, perhaps implying that a specific mRNA secondary structure is required for translation.

These examples illustrate the importance of post-transcriptional regulation in the control of gene expression.

1.5.2 Regulation of Transcription at the binding stage

Certain promoters use auxillary proteins as a means of regulating their activity. Two such interactions will be discussed here, binding of the cAMP-CRP complex and binding of lexA protein.

1.5.2a Action of cAMP-CRP (see also 1.4)

In modulating transcription cAMP and CRP participate as positive control elements by both enhancing initiation and decreasing termination and as negative control elements by inhibiting initiation.

CRP and cAMP always act together, and no unique role for either has been found in E.coli. The CRP-cAMP complex affects transcription by binding to a site within or near the promoter (Taniguchi et al., 1979; Simpson, 1980; LeGrice and Matzura,1981). The CRP molecule is a dimer of two identical subunits each of which binds one cAMP molecule. Ebright and Wong (1981) suggest that the cAMP molecule has two structural domains. The first, the cyclic phosphate and furanose ring binds to the receptor, whilst the second, the exposed adenine moiety is free to interact with the DNA. Genetic analysis of mutants and DNA binding and protection studies have revealed the nature of the contacts between the CRP activity target (cat) on the DNA and the cAMP-CRP complex. A consensus sequence of 10 bp from 10 operons has been identified (Ebright, 1982) (see Fig. 10). The polarity of this consensus sequence is not fixed and also lacks any symmetry suggesting a single subunit contact. However, Aiba (1983) has pointed out that in all three genes (gal P_2, ompA, crp) where cAMP-CRP could act as a negative effector the consensus sequence is on the coding strand. In other genes (lac, araC, deo) where cAMP-CRP acts as a positive effector the sequence is found only on the non-coding strand, suggesting that there may be a relationship between CRP function and the orientation of the CRP binding sequence. In addition, Aiba (1983) noted that most direct and inverted repeats in the crp leader region exist around the cAMP-CRP (cat) binding site, and may thus play a role in recognition.

The cat site is located at significantly different positions upstream from the mRNA start site in different operons, for example, -92 in deo, -90 in ara, -62 in lac and -35 in gal. Indeed,

Fig. 10. Consensus sequence cAMP-CRP binding site

5' AA-TGTGA--T----CA 3'

(from Ebright, 1982)

Consensus sequence lexA binding site (SOS box)

taCTGTaTaTat-cat-CAG-ga

(from Little and Mount, 1982)

it has been reported that autoregulation of the crp gene is modulated by binding between +26 and +67. Thus the mechanism of regulation may vary between different operons. To achieve positive control cAMP-CRP may enhance closed complex formation perhaps improving the accessibility of RNA polymerase to the DNA. For negative control we might imagine a competition model in which CRP-cAMP prevents access of RNA polymerase to the promoter.

1.5.2b The action of lexA repressor

Purified lexA protein specifically inhibits in vitro transcription and binds to the control regions of the lexA, recA, uvrA and uvrB genes. Comparison of operator sequences has revealed considerable homology between them which has given rise to the term 'SOS' box (Little and Mount, 1982) (Fig. 10). Mizusawa and Gottesman (1983) noticed that upstream of the open reading frame for the sfiA gene are three potential promoter sequences. Overlapping the -10 regions of two of the three promoters is an SOS box which shares 18/20 base pairs of homology with the SOS box of the recA gene. This is consistent with the findings of Huisman and D'Ari (1981) (Section 1.3.6). The lexA repressor binds to different operators with different affinities, most strongly to the recA operator, followed by that of uvrB, unmethylated lexA and methylated lexA (Brent and Ptashne, 1981). Huisman et al. (1982c) suggest that differences in operator affinity do not affect inducibility of SOS operons, but merely serve to set the basal level of transcription (see Section 1.5.3).

1.5.3. Promoter interactions, internal and multiple promoters

1.5.3a Convergent transcription

Convergent transcription defines a situation where two promoters are transcribing towards one another in opposite directions. Expression of the trp operon was shown to be impaired in trp transcuding phages when transcription from the trp promoter was opposed by transcription from P_L (Hopkins et al., 1976). The possibility that lambda functions were inhibiting trp was eliminated by Ward and Murray (1979) who demonstrated that the effect was orientation specific and the consequence of convergent transcription. They suggested that inhibition was a dynamic process requiring continuous opposing transcription and that when two RNA polymerase molecules collide transcription ceases and the polymerases are released.

1.5.3b Promoter occlusion

Adhya and Gottesman (1982) demonstrated that activation of the lambda P_L promoter can reduce the activity of two nearby downstream promoters Pg1 and Pg2 of the gal operon, even though all promoters transcribe in the same direction. This is attributed to 'promoter occlusion' by which RNA polymerase molecules already transcribing can block the access of incoming RNA polymerases to downstream promoters, either by steric hindrance or distortion of the DNA. Adhya and Gottesman (1982) suggest that initiation from the upstream promoter must exceed that of the downstream promoter. However, by recombination Hausler and Somerville (1979) constructed a system where up to three trp promoters of similar strength could

ostensibly function simultaneously and independently to produce a mRNA which specifies tryptophan synthetase. In all cases the amount of enzyme produced was less than the levels predicted from summing the contributions of each promoter singly.

Such occlusion effects may exist widely in E.coli and play an important role in controlling the levels of gene products in temporally related sequences such as cell division and bacteriophage lambda development. Adhya and Gottesman (1982) suggest the possibilities for such a mechanism in production of the λ int protein.

1.5.3c Internal promoters

The E.coli tryptophan (trp) operon consists of five contiguous genes preceded by an operator and promoter region. However, there is a low level constitutive promoter located within the second structural gene, trpD. This promoter, trp-p2 was originally identified in Salmonella typhimurium by Bauerle and Margolin (1967) and subsequently identified in E.coli by Morse and Yanofsky (1968). Jackson and Yanofsky (1972) demonstrated that trp-p2 was within the trpD structural gene and was responsible for 80% of synthesis of enzymes from the three distal genes under conditions of full repression. Horowitz and Platt (1982) have precisely located trp-p2 and characterised its efficiency both in vivo and in vitro. The trp-p2 has extensive homology with the consensus sequence (for discussion see Chapter 3). The existence of the trp-p2 promoter within the translated region of trpD may illustrate a level of regulation not yet understood. Nichols et al. (1981) suggest that it ensures a basal level of trp expression to

permit recovery from extreme starvation, although more detailed physiological data is unavailable.

Besides promoters other control elements have been found within structural genes. Irani et al.(1983) have reported a second functional gal operator element within the structural gene of galE. Both operators appear to be involved in the repression of the galactose operon promoters, although the precise molecular mechanism of gal repressor action is unknown.

1.5.3d Multiple promoters

There are now many examples in procaryotes of genes which can be transcribed from more than one promoter. Why this is necessary is unclear, but the following cases may provide some clues.

In E.coli there are seven cistrons encoding ribosomal RNA sequences, and in all cases studied transcription is initiated from two tandem promoters (Glaser and Cashel, 1979). In the rrnA cistron both promoters are active in vivo, and under certain conditions the upstream promoter P1 is more active than P2. The structure containing two promoters separated by 120 base pairs may have a selective advantage since this arrangement is conserved in all six of the ribosomal genes so far sequenced. Glaser et al. (1983) suggest that this may enable the cell to manipulate the rate of transcription under different growth conditions. Both promoters seem to be transcribed independently. If the supply of RNA polymerase is low transcription occurs from the high affinity, slow transcribing P2 promoter, supporting maintenance synthesis. When polymerase is

abundant transcription is from the low affinity fast transcribing P1 promoter, allowing a greater rate of transcription and ensuring the maximal rate of rRNA synthesis during rapid cell growth. Under these conditions P1 is transcribed at maximum efficiency while that from P2 is inhibited, a situation similar to the 'promoter occlusion' previously discussed.

The ilv GEDA operon is also preceded by tandem promoters P1 and P2, this time 72 base pairs apart. Only the downstream ilv promoter is transcriptionally active in vivo (Adams et al., 1983) yet both the -10 and -35 region of the upstream promoter P1 is required for maximal transcription of the downstream promoter, P2. Thus it appears that unlike rrnA, activation of the downstream promoter requires the binding of RNA polymerase to the upstream promoter. Although the physiological significance of this enhancement of P2 transcription is unknown, various mechanisms have been proposed and will be discussed in Chapter 3.

The promoter-operator region of the uvrB gene has been sequenced and in vitro the gene is transcribed from two different promoters, designated P1 and P2. An SOS box is located at -40 to -63 (Fig. 11) and acts as the operator for P2. In the presence of lexA, transcription from P2 is inhibited while that from the overlapping P1 is unaffected. The significance of the lexA independent promoter P1 in uvrB is unknown, but it might serve to give a basal level of uvrB expression if the product is involved in other cellular processes requiring constitutive synthesis. In addition, a third promoter P3 is located 320 bp upstream of P2. Transcription of P3 is towards uvrB and terminates in vitro in the region of the SOS box whether lexA is bound or not

(Sancar et al., 1982). The effect which this promoter has on uvrB expression, if any, is unknown, but a similar three promoter organisation is seen in the galactose operon (Queen and Rosenberg, 1981) (Fig. 11) (Section 1.5.1c). The P1 and P2 promoters function according to the cellular cAMP level. P1 is activated by cAMP whilst P2 is inhibited by it. The levels of P1 and P2 initiated mRNAs appear to regulate cellular levels of gal enzyme expression under different metabolic conditions via cAMP-CRP (deCrombrugghe and Pastan, 1978). A third promoter, P3 has been observed 100 bp upstream of P1 and P2. Its role is unclear, but unlike the P3 mRNA of uvrB, its transcript traverses into the gal operon.

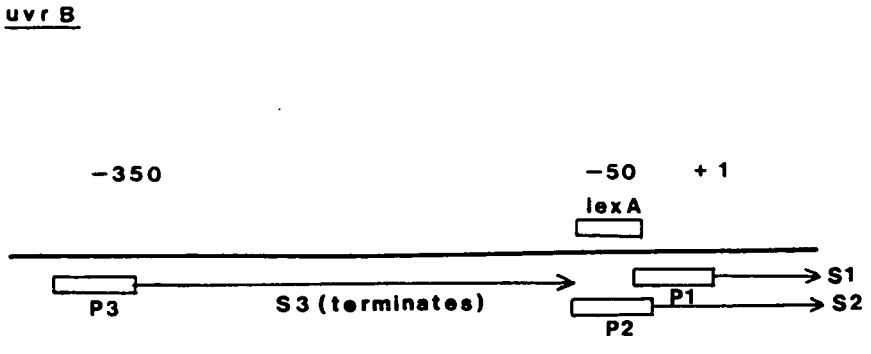
1.5.4 Termination as a regulator of gene expression

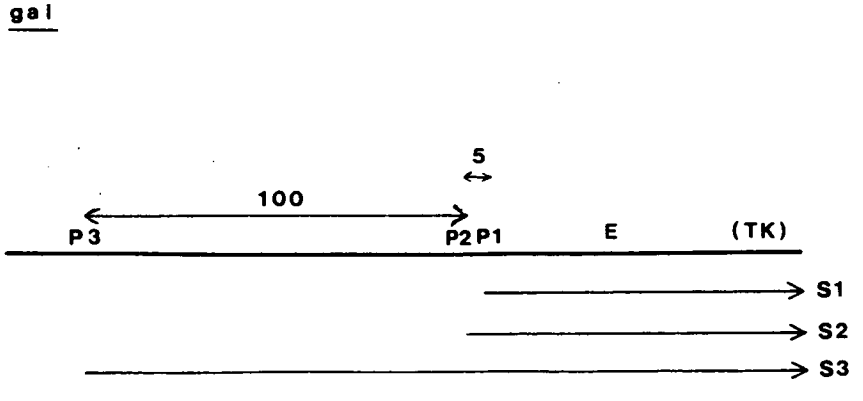
The termination of transcription is emerging as an important method for regulation of gene expression. Four aspects must be considered: 1) termination at the end of a gene, 2) attenuation, 3) pausing, 4) transcriptional polarity.

In the simplest case terminators at the end of a gene consists of a GC rich region of dyad symmetry followed by a stretch of U residues near the 3' terminus of the transcript and do not require additional factors. Other terminators depend on the termination factor rho and some on the elongation-termination factor nusA (reviewed by Platt, 1981).

As a general mechanism attenuation is the reduction of distal gene expression as a consequence of transcription termination. In the tryptophan and other biosynthetic operons attenuation is

Fig. 11 Three promoter operons





governed by the translation of codons specifying the amino acid and products of the enzymatic pathway (reviewed by Yanofsky, 1981).

RNA hairpins have been associated with pausing of the polymerase (Farnham and Platt, 1980, 1981), termination being prevented by the absence of a stretch of uridine residues. The rrnB operon is possibly regulated in vivo by "turnstile" attenuation (Kingston and Chamberlin, 1981). Separating the two rrnB promoters from a nusA dependent terminator is a 260 base pairs region containing pause sites, which may be involved in stalling the polymerase and regulating the rate of transcription in vivo.

Polarity in procaryotic gene expression is the reduced expression of promoter distal genes with respect to proximal genes, and is generally believed to be the result of premature termination of transcription. The cAMP-CRP complex is involved in suppression of polarity (Ullman et al., 1979) and may also play a role in the transcription termination process at the end of an operon (Guidi-Rontani et al., 1980).

1.5.5 The effect of supercoiling on transcription

In principle DNA supercoiling can affect various transcriptional steps from polymerase binding to termination and release of the transcript. Supercoiling can modulate the movement of the polymerase along the template (Wang, 1974) or any process which involves winding or unwinding of the DNA helix. The effects of supercoiling may be transmitted along the helix for a considerable distance (Burd et al., 1973; Snyder and Drlica, 1979; Smith, 1981).

A variety of studies have indicated that negative supercoiling can increase the amount of transcription both by raising the rate of transcription from existing initiation sites and by making new sites available. This stimulating effect is probably due to the reduced activation energy required to form the initiation complex in negatively supercoiled DNA. Hseih and Wang (1978) have shown that binding of RNA polymerase to a promoter causes a 10 bp disruption to the helix and has a co-operative effect on the binding of enzyme molecules to adjacent non promoter sites, which again causes a disruption of DNA base pairs. This co-operative effect may facilitate rapid transcription *in vivo*.

The level of DNA supercoiling is maintained by a dynamic balance of the levels of two topoisomerases; topoisomerase I which relaxes negative supercoils and DNA gyrase which introduces them (reviewed by Smith, 1981; and Wang, 1982). It is difficult to make a qualitative assessment of the effects of superhelicity on the expression of different genes *in vivo* since we cannot predict the effect of superhelicity on the rate limiting step in transcription due to different degrees of unwinding by RNA polymerase and the influence of regulatory factors. Promoters controlled by the CAP-cAMP complex may be particularly sensitive to supercoiling (Sanzey, 1979; Wei-Jue *et al.*, 1982).

1.5.6 Gene fusion systems

An approach which has proved useful in the study of operon control involves the technique of gene fusion. Such fusions place a known, assayable gene function *e.g.* *lac* or *gal* under the control

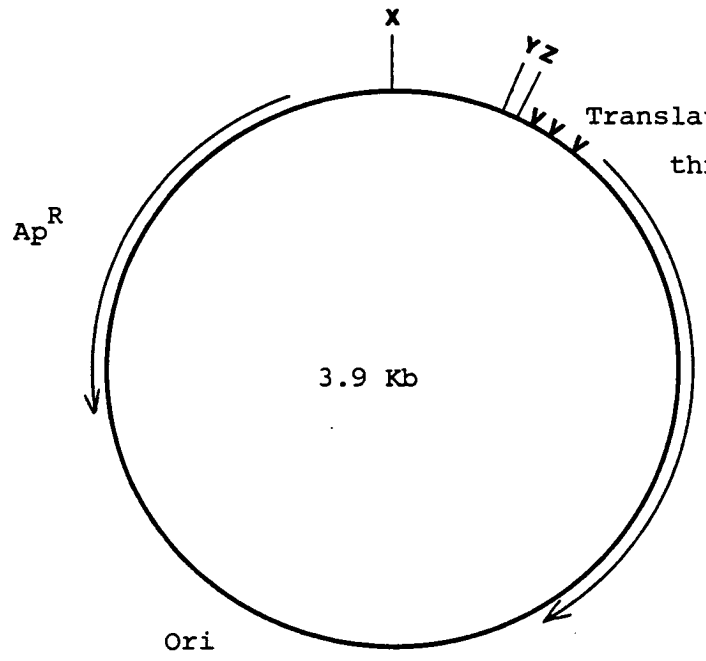
of the regulatory region of interest. The pKO system of McKenney et al. (1981) has circumvented many of the problems previously associated with gene fusions and has been used throughout this thesis. The galactokinase gene (galK) has been inserted into a pBR322 derivative such that only the replication origin and ampicillin resistance gene (bla) remain (Fig. 1.12). In addition, the natural galT-galK bonding has been left intact to ensure that the efficiency of galK translation is relatively independent of the RNA structure upstream of this region (Queen and Rosenberg, 1981; Schümperli et al., 1982). Convenient restriction sites were inserted upstream of the leader region.

Since many promoter containing DNA fragments also contain translation start sites, translation originating in the inserted DNA might traverse all or part of the leader region preceding galK and thereby exert unknown effects on galK translation. To circumvent this problem translation stop codons were inserted in all three reading frames bewteen the cloning sites and the initiation codon of galK.

A cell with a gal $E^+T^+K^-$ genotype does not metabolize galactose when carrying the pKO1 vector, that is, it grows as a white colony on MacConkey galactose indicator plates (Mac gal). If a promoter is inserted in to a pKO derivative in the correct orientation then galK is expressed and can complement the $galK^-$ host resulting in growth as a red colony on Mac gal plates.

An appraisal of this system can be found in Appendix 1.

Fig. 12. pKO Vectors



	X	Y	Z
pKO1	EcoR1	HindIII	Sma1
pKO4	EcoR1	HindIII	BamH1
pKO5	BamH1	HindIII	Sma1
pKO6	BamH1	HindIII	EcoR1

CHAPTER 2

MATERIALS AND METHODS

2.1 Media

LB (Luria Bertani) medium contained per litre; Difco Bacto-tryptone 10 g, Bacto yeast extract 5g, NaCl 10 g, pH to 7.5 with NaOH. LB agar was as above with the addition of Difco agar 15 g. NB (Nutrient Broth) contained per litre; Oxoid Nutrient Broth No. 2 25 g. NA (Nutrient Agar) was as NB but with the addition of Davis New Zealand agar (12.25 g.). MacConkey agar contained per litre; Difco MacConkey agar base 40 g. BBL top agar contained per litre Baltimore Biological Laboratories Trypticase 10 g, NaCl 5g, Difco agar 6.5 g. M9 salts (x4) contained per litre Na_2HPO_4 28 g, KH_2PO_4 12 g, NaCl 2 g, NH_4Cl 4 g, pH 7.0 with NaOH. For growth of E.coli to 1 x M9 salts was added per litre; $MgSO_4.7H_2O$ 1 g. For minimal agar to 1 litre 1 x M9 was added Davis New Zealand agar 25 g. Where required sugars were added to a final concentration of 0.2% w/v, amino acids to 20 µg/ml, biotin and vitamin B_1 to 2 µg/ml. Where required antibiotics were added to the following final concentrations: Ampicillin (Ap) 50 µg/ml, Chloramphenicol (Cm) 30 µg/ml, Kanamycin (Km) 50 µg/ml, Nalidixic acid (Nal) 20 µg/ml, Streptomycin (Sm) 25 µg/ml, Tetracycline (Tc) 15 µg/ml. Bacterial buffer contained per litre; KH_2PO_4 3 g, Na_2HPO_4 7 g, NaCl 4 g, $MgSO_4.7H_2O$ 0.2 g, Phage buffer contained per litre; KH_2PO_4 3 g, NaH_2PO_4 7 g, NaCl 5 g, $MgSO_4$ (0.1 M) 10 ml, $CaCl_2$ (0.01M) 10 ml, Gelatin (1%) 1 ml.

2.2 Bacterial and Phage strains

Bacteria were maintained on nutrient agar plates at 4°C or in stabs of nutrient agar at 4°C. Stocks were stored at -70°C as competent cells (See Section 2.4.8). Table 2.1 shows bacterial strains used. Table 2.2 shows phage strains used.

Table 2.1 Bacterial Strains

Strain	Genotype	Source
c600 K^-	thr leu thi galK lac tonA supE	R. Hayward
CA8306	Hfr H thi cya 854	M. Goman
D22	proA trp his rpsL ampA envA-1 F^-	Lab stock
ED5040	lac his trp lys gal rpsL F' lac::TnA	J. Maule
JC411polAt.s.	arg his lac leu mal met rps thy xyl polAt.s.	C. Henry
JC10-240	recA srl::Tn10 thr ilv rel spc Hfrpo45	C. Henry
Ken 2	As DM961 except leu^+ sulB25 gyrA	
Ken 10.5	c600K^- envA zad220::Tn10	K. Begg
Ken 11	c600K^- lexA t.s. mal E52::Tn10	"
Ken 12	c600K^- secA t.s. leu^+	"
Ken 15	c600K^- sep t.s. leu^+	
Ken 16	c600K^- sep t.s. leu^+	
Ken 17	PA3092 gal attλ bio, Tn10	"
Ken 18	c600K^- ftsE t.s. rpsL	"
Ken 90	ara lac galK galE trp leu ilv his thy ftsZ (84 t.s.)	"
N100	pro his galK recA	R. Hayward
NFS1	c600K^- gyrA	This thesis
NFS2	c600K^- gyrA ilv rbs::Tn10	" "
NFS3	c600K^- cya 854	" "
NFS4	c600K^- crp 868 rpsL	" "

Table 2 (continued)

Strain	Genotype	Source
NFS6	c600K$^-$ recA srl::Tn10	This thesis
NFS7	c600K$^-$ ftsZ 84 t.s., leu$^+$	" "
PA3090	thr leu thi arg his trp lac gal mtl xyl malA ara rpsL thyA tonA λ^R λ^- supE fic	R. Utsumi
TOE1	thyA argE leu his pro thr thi ftsQ t.s.	K. Begg
TOE13	thyA argE leu his pro thr thi ftsA t.s.	K. Begg
SJ2	panB zad220::Tn10 relA spoT thi	B. Bachmann
W3110	Prototroph	Laboratory stock
WP80	rbs::Tn10 arg pro glnS galE rpsL thi	B. Newman
WZ81	argG metB cya 855 crp 868 spc^R rpsL recA56 amp^R	M. Goman
3002	ilv$^-$ arg$^-$ met xyl mtl his lac tna λ^R rpsL trp$^-$ pyrE thi	B. Newman
3002T	3002 but rbs::Tn10	This thesis
TST1	flbB ptsF relA rpsL malE::Tn10 deoC	B. Bachmann
PAM161	sulB25 lon thr leu proA his met thi ara lac galK xyl mtl rpsL supE	B. Bachmann
DM961	thr leu pro thi his metA ara lac mtl xyl rpsL lexA3 (ts1-1)	P. Martin

Table 2.2 Phage strains

Strain	Comment	Source
P1Kc		Barbara Newman
λvir		Ken Begg
λwt		Barbara Newman
λNK55	b221, cIII 167::Tn10 c1857 ind$^+$ Oam29	Kleckner et al., (1978)
F'104		K. Begg

2.3 Growth of bacterial cultures, monitoring growth

Bacterial cultures were grown in LB at 37°C in a New Brunswick rotary shaker or as overnight cultures in LB incubated at 37°C without shaking.

Cell mass was measured in a Perkin-Elmer Coleman model 55 spectrophotometer at 540 nm (OD_{540}) unless otherwise stated.

2.4 Bacterial and phage techniques

For all phage work $CaCl_2$ (1 mM) was added to media (giving LBC and LAC, Section 2.1).

2.4.1 Growth of phage by liquid infection (λ or P_1).

A phage sensitive strain was grown with aeration at 30°C or 37°C in LBC to an OD_{540} of 0.5 and infected with phage at a multiplicity of infection of 1. Lysis occurred 1½ to 3 hours after infection and was completed with chloroform (1 ml/l). Lysates were clarified by centrifugation.

Phage stocks were titrated by mixing 0.1 ml phage diluted in phage buffer with 0.1 ml mid log phase W3110 and 2.5 ml Molten BBL top agar which was subsequently poured into LBC plates and incubated overnight 37°C. Titres for λ were usually 1×10^{10} pfu/ml and for P1 1×10^{11} pfu/ml (pfu; plaque forming unit).

2.4.2 Plate lysates

For small volumes 10^7 pfu/ml phage and 0.1 ml W3110 were mixed with 2.5 ml Molten BBL top agar and poured on to an LAC plate. After lysis the top agar was scraped off with a sterile spatula and vortexed with 2 mls phage buffer and 1 drop chloroform. This was clarified by centrifugation and titrated.

2.4.3. Pl Transduction

Recipient strains were grown to stationary phase in LBC ($1-5 \times 10^9$ cells/ml). Strains non lysogenic for Pl were concentrated 10 times to reduce phage killing. Pl lysates were diluted to 10^9 pfu/ml in phage buffer. 0.1 ml cells and 0.1 ml Pl were mixed and incubated at 37°C for 15 mins. prior to addition of 0.8 ml phage buffer and plating 0.1 ml of this mixture on selective media.

2.4.4 Induction of lambda

Phage were induced by either UV irradiation as with λ 16-2 and $\lambda\Delta E$ or by thermal induction as with λ $\underline{cI}_{857}$.

(i) UV induction

Lysogenic bacteria were grown to early log phage (OD_{540} of 0.5; 2×10^8 cells/ml) in LB at 37°C, harvested and resuspended in half the original volume of 10^{-2} M $MgSO_4$. The cells were irradiated in 10 cm diameter glass petri dishes with 400 ergs/mm^2 UV, then diluted four fold into fresh pre-warmed LB supplemented with 10^{-2}M $MgSO_4$ and grown for 2 hours until lysis occurred. Chloroform was added (1 ml/l) and the lysate clarified and titred.

(ii) Thermal induction

Lysogenic bacteria were grown as above except at 30°C. Cells were shifted to 42°C for 20 minutes and then to 37°C until lysis occurred.

2.4.5 Concentration and preparation of phage

Phage lysates were prepared and clarified as above. NaCl (40 g/l) and polyethylene glycol 6000 (PEG; 10% w/v) was added,

dissolved, and left overnight at 4°C. The precipitated PEG:phage complex was collected by centrifugation (10 K rpm; 15 mins.) and resuspended in phage buffer. DNase and RNase were added to 10 µg/ml and incubated at RT for 1 hour. Debris was removed by centrifugation (2 k rpm, 5 mins.). This phage suspension was loaded on to a CsCl step gradient composed of three 1 ml steps of 1.7, 1.42 and 1.3% w/v CsCl in phage buffer in a 17 ml polycarbonate tube. This was spun in the AH627 (swing out rotor) of a Sorvall OTD 50 centrifuge for 3 hours at 22K rpm. The phage band was removed through the side of the tube with a 23G needle and syringe. The phage were further purified in equilibrium gradients. Phage were mixed with 1.42 w/v CsCl in phage buffer loaded into 15 ml polyallomer heat seal tubes. Gradients were formed by spinning in the Ti50 rotor of a Sorvall OTD 50 centrifuge for 30 hours at 33K rpm. Phage bands were removed as above. Phage λ was stored in CsCl at 4°C.

2.4.6 Hfr mating

Hfr donors were diluted ten fold into pre-warmed LB from overnight cultures and grown in static culture at 37°C to a density of 4 x 10^8 cells/ml. Recipient cultures were grown with shaking at 37°C in LB to 4 x 10^8 cells/ml and equal volumes of donor and recipient were mixed and incubated without shaking at 37°C. Mating was terminated by vortexing after 0, 10, and 20 minutes and 0.1 ml of a 10 x dilution was plated on selective plates.

2.4.7 Selection of Tc^s from Tn10 containing strains

Approximately 10^6 cells were plated on the following selection medium. Only Tc^s derivatives can form colonies. Per

litre; agar 15 g, Bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, NaH_2PO_4 10 g, glucose 2 g, chlortetracycline hydrochloride 50 mg and quinaldic acid (10 mg/ml) 10 ml added after autoclaving (Bochner et al., 1980).

2.4.8 Preparation of competent cells

Cells competent for transformation were obtained by growing the required strain to an OD_{540} of 0.55 to 0.65 in 100 ml LB at a permissive temperature. Cells were chilled on ice for 30 minutes, pelleted and resuspended in 50 ml ice cold 0.1M $CaCl_2$, immediately repelleted and resuspended in 5 ml ice cold 0.1M $CaCl_2$/15% v/v glycerol. Aliquots of 200 µl were stored at -70°C.

For transformation the DNA and cells were mixed and left on ice for 30 minutes, heat shocked at 42°C for 2 minutes and returned to ice for 30 minutes. The mixture was diluted to 1 ml with LB and incubated at either 37°C or 30°C to allow expression of drug resistance. 0.1 ml was spread on selective plates.

2.4.9 Selection of Nalidixic acid resistant strains

The nalidixic acid sensitive (Nal^S) strain was grown overnight in LB and harvested. This was washed twice and resuspended in one tenth volume bacterial buffer. 0.1 ml. of these cells were plated on to an LB plate containing nalidixic acid. Resistant colonies were purified by restreaking three times on selective media.

2.4.10 Selection of galactokinase 'up' mutations

10 ml of $c600K^-$ cells containing plasmids which do not produce galactokinase were grown in LB to stationary phase. Cells were harvested and washed three times in bacterial buffer to remove

residual nutrients and finally resuspended in 1 ml ($^1/_{10}$ vol) bacterial buffer. 0.1 ml of these cells were plated on to M9 minimal agar containing galactose and incubated at 37°C. After 2 days *gal*$^+$ colonies appeared. These were picked and purified. To ensure that the desired mutations were in the plasmid and not simply chromosomal revertants minilysates were prepared (2.5.1d), the plasmids retransformed into c600K$^-$ and plated on Mac gal (MacConkey-galactose) media. Those which gave red colonies were selected for further study.

2.4.11 Centrifugation

Unless otherwise stated all centrifugations were carried out in a Sorvall OTD 50 or an MSE bench centrifuge. For centrifugations involving 1.5 ml snap cap tubes a Quickfit microcentrifuge was used.

2.5 DNA Techniques

2.5.1 Preparation of DNA

2.5.1a Buffers and Reagents

Tris-EDTA (TE) buffer was as follows: Tris-HCl pH 7.6 10 mM, EDTA pH 8.0 1 mM. Dialysis tubing was boiled for 10 minutes in TE and rinsed with distilled water prior to storage at 4°C. Phenol was distilled and stored at -20°C. Prior to use 8-hydroxyquinoline (0.1%) was added and the phenol equilibrated with TE. 'Chloroform' as used in DNA manipulation is a mixture of chloroform and isoamylalcohol (24:1 v/v). Ether was saturated with water prior to use.

2.5.1b Extraction of phage DNA

Phage which had been concentrated in CsCl was dialysed

against three changes of phage buffer over 12 hours and phenol extracted three times in a siliconised Corex centrifuge tube. The remaining aqueous layer was extracted with ether and dialysed for 15 hours against 4 changes of TE buffer. DNA was stored in TE at 4°C or -20°C.

2.5.1c Preparation of plasmid DNA

A 500 mls culture harbouring a Col E1 type plasmid was grown to stationary phase in selective media, harvested (5 Krpm for 15 min at 4°C) and resuspended in 6 ml 25% sucrose, 50 mM Tris-HCl pH 8.1, 40 mM EDTA. Lysozyme (10 mg) and EDTA (0.5 M pH 8.1 1 ml) were added and the mixture stood on ice for 5 mins. Cells were lysed with 13 ml triton lysis mix: per 200 ml; 10% triton X-100 2 ml, 0.5 M EDTA pH 8.1 25 ml and 1M Tris-HCl pH 8.1, 10 ml. The lysate was clarified by centrifugation (15 K rpm, 45 mins. 4°C) and to the supernatant, CsCl (0.95 g/ml) and ethidium bromide (5 mg/ml, 0.1 ml) were added. Plasmid DNA was banded by centrifugation (38 K rpm, 60 hours, 15°C) in a Sorvall OTD 50 Ti50 rotor. The closed circular plasmid DNA was visualised under long wave UV light and removed through the side of the tube with a syringe and 239 needle. Ethidium bromide was extracted with three changes of propan-2-ol (preequilibrated with water) and CsCl was removed by dialysis against TE for 2 hours at 4°C. The final DNA concentration was 1 µg/50 µl.

2.5.1d Small scale plasmid preparation

The method used was that of Birnboim and Doly (1979). The final pellet was suspended in 50 µl H_2O and 5 µl was RNase treated

and used for restriction analysis of putative recombinants.

2.5.1e DNA fragment purification

The following two methods have been used to produce fragments from agarose gels suitable for cloning.

(i) Electroelution into dialysis bags

The slice of gel containing the DNA is placed in a dialysis bag with 0.5 x TBE (see Section 2.6.1) and sealed. A current of 100 v for 2-3 hours elutes the DNA which is then purified by phenol extraction and ethanol precipitation. The percentage recovery depends upon fragment size.

(ii) Hydroxyapatite columns

A column of hydroxyapatite (HAP) in water was prepared in a blue Eppendorf tip plugged with glass wool. This is equilibrated with solution 1 (10 mM Na_2PO_4 pH 6.8, satd. with KI). The slice of gel containing the DNA is dissolved in solution 1 at 37°C, applied to the column, and followed by a thorough washing in solution 1 to remove agarose. KI is removed by washing with solution 2 (50 mM Na_2PO_4 pH 6.8) and DNA is eluted in 1 drop fractions with solution 3 (0.4 M Na_2PO_4 pH 6.8). 1 µl of each fraction was spotted on to an ethidium bromide (5 mg/ml) - agarose plate and visualised under UV light to identify DNA containing fractions. Required fractions were desalted in a Sephadex G-50 column equilibrated with water, and ethanol precipitated.

(iii) The following method was used to prepare DNA fragments from polyacrylamide gels and gives excellent yields (70-80%)

of DNA suitable for cloning or *in vitro* transcription. The desired band (~ 0.5 µg) is excised from a polyacrylamide gel and ground to fine particles with a siliconised glass rod in a siliconised corex tube. Gel particles are suspended in 1 ml gel elution buffer (0.5 NH_4OAc; 10 mM Tris-HCl pH 7.5; 1 mM EDTA) and left overnight at RT. The mixture is spun through siliconised glass wool (3 K rpm; 5 mins.) and the glass wool washed with a further 1 ml gel elution buffer. To the resultant clear liquor 3 volumes of ethanol were added, the DNA precipitated at -70°C for 15 mins. and pelleted for 25 mins. at 10 K rpm (0°C). The DNA was resuspended in 200 µl 0.3M NaOAc, phenol extracted twice, ether extracted twice, ethanol precipitated, washed with 70% (v/v) ethanol and dried. The pellet was resuspended in 15 µl H_2O.

2.5.2 Purification and Quantitation of DNA

All glassware used for DNA was siliconised before use. Glassware was washed thoroughly in detergent, dried and soaked (1 hour) in a 5% v/v solution of dichlorodimethyl silane in chloroform. Glassware was then rinsed thoroughly and baked for 2 hours at 180°C. Disposable plastic gloves were used for all manipulations involving DNA.

2.5.2a Purification by extraction

One of the key steps in purification of DNA is the removal of proteins. This is accomplished by extraction with organic solvents. Unless otherwise stated DNA was extracted once with phenol, once with 1:1 phenol-chloroform and once with chloroform (see Section 2.5.1a). Ether was used to remove traces of phenol or chloroform prior to ethanol precipitation.

2.5.2b Concentration of DNA

DNA was precipitated by the addition of 0.1 volumes of 3M NaOAc and 2 volumes of cold ethanol and incubating at -70°C for 15 mins. After centrifugation the pellet was washed with 70% v/v to remove salt. The pellet was dried in a vacuum dessicator and resuspended to the required volume. For small DNA molecules (< 200 bp) the original DNA solution was adjusted to 0.01 M $MgCl_2$ to improve the efficiency of precipitation.

Alternatively, the volume of a solution was reduced by extraction with butan-2-ol followed by ether extraction. This does not, however, remove salt.

2.5.2c Quantitation of DNA

(i) The absorbance of a DNA solution was measured at 260 and 280 nm using a Zeiss spectrophotometer. An OD_{260} of 1 corresponds to 50 µg/ml and an OD_{260}:OD_{280} ratio of 1.8 indicates a pure preparation. DNA used in this thesis had a ratio of 1.8 ± 0.1 and was, unless otherwise stated 1 µg/50 µl.

(ii) DNA samples were also quantitated using comparison with a known standard of ethidium bromide fluorescence on an agarose gel.

(iii) To measure incorporation of label into DNA TCA precipitation was used. A known volume of radioactive (^{32}P) DNA was diluted 10 x with salmon sperm DNA (0.5 mg/ml). The sample was divided and spotted on to two GF/C glass fibre discs. One was washed with 5 ml of ice cold 10% w/v trichloroacctic acid (TCA) and subsequently three times with 5 ml 5% w/v TCA and 5 ml 95% v/v ethanol. Both filters were thoroughly dried and counted in a toluene based scintillant (0.4% Butyl PBD in toluene). DNA greater than 20 nucleotides can be precipitated by this method. The ratio of unwashed:washed gives the incorporation.

2.5.3 DNA manipulation

2.5.3a Restriction of DNA

Restriction endonucleases and other enzymes were purchased from either Boehringer, New England Biolabs or Bethesda Research Laboratories and unless otherwise stated used at 1 unit/µg/hour. All restriction digests (except for EcoR1*; see Chapter 8) were carried out in universal buffer: 33 mM Tris-acetate pH 7.9,

10 mM MgOAc, 66 mM KOAc, 0.5 mM DTT. Reactions were carried out at 37°C except for TaqI (65°C) and terminated by either heating to 65°C for 10 mins. or by phenol extraction followed by ethanol precipitation. Partial digests were calibrated by sampling a reaction mix of 1 µg DNA:1 unit enzyme after 0, 2, 4, 10, 20 and 40 minutes and analysis by agarose gel electrophoresis.

2.5.3b Filling in 5' extensions

For cloning 5' extensions were filled in using the large fragment of DNA polymerase 1 (Klenow). Restricted or Bal 31 digested DNA (1 µg) was phenol extracted and ethanol precipitated. Reactions were performed in universal buffer (see Section 2.5.3a) and contained Klenow (1 unit), spermidine (to 1 mm), unlabelled dNTPs (as needed, 2 n moles) and [α-^{32}P]dNTP (2 p moles; spec. act > 400 Ci/mmol, Amersham) in a total volume of 10 µl. Prior to use the labelled nucleotide was dried, washed in 70% ethanol and redried. After reaction (37°C, 30 mins.) incorporation was measured (see Section 2.5.2$_c$,iii)) and unincorporated label removed either by (a) chromatography through Sephadex G-50 (equilibrated with TE) or (b) precipitation with ammonium acetate (200 µl, 2-5M NH_4OAc + 800 µl ethanol; -70°C, 15 mins) followed by ethanol precipitation.

2.5.3c Use of Bal 31 exonuclease

For Bal 31 digestion the enzyme was calibrated to remove 20 bp/min from each end of a linearised plasmid at 30°C. The plasmid DNA was restricted, phenol extracted, ethanol precipitated and resuspended in 50 µl of buffer containing 12 mM $CaCl_2$, 12 mM

$MgCl_2$, 600 mM NaCl, 20 mM Tris-HCl pH 7.9 and 1 mM EDTA. Bal 31 enzyme was added and 10 µl samples removed after 0, 2, 4, 10 and 20 minutes to a tube containing 2 µl 0.5M EGTA pH 8.0 to stop the reaction. Half of this material was checked for digestion by agarose gel electrophoresis. The time sample giving the required deletions was phenol/chloroform/ether extracted and ethanol precipitated prior to an 'end filling' reaction with Klenow (see Section 2.5.3b). Synthetic polynucleotide BamHI linkers were then attached (see Section 2.5.5v).

2.5.3d Use of Calf intestinal phosphatase (CIP)

Calf intestinal phosphatase was used to dephosphorylate the 5' ends of DNA. This is used in the kinase reaction (see Section 2.5.5) and to increase the frequency of recombinants in a cloning reaction. Since T_4 DNA ligase has an absolute requirement for a 5' phosphate residue, treatment of the vector can prevent recircularisation during ligation. CIP was used in universal buffer (2.5.3a) at 10 units/µg/hour.

2.5.3e Ligation of DNA

The DNA used for ligation was ethanol precipitated and dried prior to use. All ligations were performed in 66 mM Tris-HCl pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM DTT and 0.1 mM ATP. For clonings in which the restriction sites originally cut were not reconstructed the ligation was performed in the presence of the restriction enzyme to ensure a high frequency of recombinants. Where possible CIP was used on either vector or insert (see Section 2.5.3d).

(i) Sticky-end insertions

0.1 pmol of vector was mixed with 0.2 pmol insert and ligated in 20 µ for either 3 hours or overnight with 4 units of T4 DNA ligase at 14°C. The vector:insert ratio was always 1:2.

(ii) Blunt-end insertions

Here the reaction volume was reduced to 5 µl, the amount of ligase increased to 50 units and extra ATP (to 0.5 mM) were added. Reaction was overnight at room temperature.

(iii) Deletions

In order to delete a fragment internal to a plasmid 20 fmol was restricted and ligated as in 2.5.3e(i) except that the final reaction volume was 150 µl. This ensures re-circularisation of the plasmid.

(iv) Circularisation of a fragment

In order to ligate a DNA fragment on itself prior to recutting 0.1 pmol was ligated as in 2.5.3e(i).

(v) Use of linkers

Synthetic polynucleotide linkers were used to introduce a restriction site to the blunt ends produced from a Bal 31-Klenow treatment (see Section 2.5.3c). Since linkers are supplied without a 5' phosphate they must be phosphorylated prior to use. This is accomplished using polynucleotide kinase. To 60 µCi dried and washed [γ-^{32}P]ATP (spec. act. <3000 Ci/mmol; Amersham) was added BamHI linkers (6 µg), cold ATP and polynucleotide kinase (2 units) in a buffer consisting of 0.5M Tris-HCl pH 7.6, 0.1M M Cl_2, 50 mM DTT, 1 mM Spermidine, 1mM EDTA, total volume, 30 µl. Reaction

was for 1 hour at 37°C prior to termination by phenol extraction and ethanol precipitation. Phosphorylated linkers were stored at 4°C.

For ligation the Bal31-Klenow treated DNA was phenol extracted and ethanol precipitated. Ligation was as in Section 2.5.3e(ii) except that the reaction volume was 40 µl. Linkers were added in 50 fold excess.

2.6. Gel electrophoresis

2.6.1 Horizontal agarose gels

Gels (0.7-2% w/v) were prepared by boiling agarose in 200 ml TBE buffer in a microwave. (TBE gel buffer; 5 x stock, per litre; Tris base 54 g, Boric acid 27.5 g, 0.05M EDTA pH 8.0, 20 ml). The agarose solution was cooled to 50°C, ethidium bromide added to 5 µg/ml and poured into a perspex mould (30 cm x 14 cm x 0.5 cm) with a slot former 5 cm from one end. Samples (20 µl or less were mixed with TBE loading buffer (5 µl; 50% w/v glycerol, 50 v/v TBE, 0.1% Bromophenol Blue) and loaded by micropipette. The gel was placed in an electrophoresis tank, covered with buffer and electrophoresed for 16-20 hours at 30 V. Gels were visualised by transillumination with long wave UV light. Photography was with Ilford HP5 film using a 20 second exposure through a red filter.

2.6.2 Vertical agarose gels

These were used for Smith and Birnsteil mapping. Gels were prepared as above but without ethidium bromide and poured between two vertical plates (18 x 20 cm with a 3 mM spacing). A slot

forming comb was then inserted.

2.6.3 Vertical 5% polyacrylamide gels

Stock solutions were: 30% acrylamide (29% w/v acrylamide; 1% w/v bisacrylamide), 5 x TBE (see Section 2.6.1), 10% w/v ammonium persulphate (APS) stored frozen. A 40 ml solution containing 6.6 ml 30% acrylamide, 8 ml 5 x TBE, 0.4 ml APS and 25 ml H_2O was degassed (2 min swirling on a water pump). To this 25 µl TEMED (N,N,N',N'-tetramethylethylenediamine) was added and the solution poured between two vertical plates (18 x 20 mm with 1 mm spacing). A slot forming comb was inserted. DNA samples were loaded using a Hamilton microsyringe and gels run at 200 V for 2 hours in TBE. Gels were stained in ethidium bromide (5 µg/ml; 10 mins) and destained (H_2O, 10 mins) prior to visualization and photography as before (see Section 2.6.1).

2.6.4 Denaturing acrylamide gels for in vitro transcription

A 50 ml solution containing 6.25 mls 40% acrylamide (39% w/v acrylamide; 1% w/v bisacrylamide), 10 mls 5 x TBE, and 15 mls H_2O was added to 21 g urea and 0.5 ml 10% w/v APS. This was mixed until the urea dissolved when 20 µl TEMED was added and the solution poured between glass plates (40 cm x 20 cm x 1/3 mm) and a comb inserted. Prior to use the gel was pre-electrophoresed at 1200 V for 30 mins. Gels were run for 1-1½ hours at 1200 V or until the BPB marker had reached 3/4 of the length of the gel. Samples were loaded in SANGER dye, 80% formamide; 0.1% BPB and xylene cyanol (XC), 10 mM NaOH, 1 mM EDTA.

2.6.5 Size standards

For agarose gel electrophoresis λcI_{857} DNA was used as a size standard. A HindIII digest gives fragments of (in kb); 23.6, 9.64, 6.64, 4.34, 2.26, 1.98, 0.56, 0.14. An EcoR1 digest gives fragments of (in kb); 21.7, 7.52, 5.83, 5.64, 4.85 and 3.48. An EcoR1, HindIII double digest gives fragments of (in kb): 21.7, 5.24, 5.05, 4.21, 3.41, 1.98, 1.90, 1.57, 1.32, 0.93, 0.84, 0.58, 0.14. For polyacrylamide gel electrophoresis either on EcoR1, HindIII double digest of λcI_{857} or an AluI digest of pBR322 was used. An Alu digest of pBR322 (in bp) gives: 910, 659, 655, 520, 403, 281, 257, 226, 136, 100, 63, 57, 49, 19, 15, 11.

For in vitro transcription DNA size standards cannot be used. For this reason two transcription templates were used to produce mRNA of known size: (a) the spot 42 gene of E.coli cloned on to a pBR325 derivative (to give pCJ32). The gene is isolated as a 302 bp HindIII fragment which gives a 109 base transcript (Joyce and Grindley, 1982), and (b) the lac promoter operator region cloned into pBR325 (to give pGL101). The region is isolated as a 95 bp EcoR1-PvuII fragment and gives a 38 base mRNA (Backman and Ptashne, 1978).

Both plasmids were kindly provided by Dr. Barbara Newman.

2.6.6 Autoradiography

Polyacrylamide gels were fixed in 10% HOAc 1% glycerol (5 mins) and washed in H_2O (5 mins) prior to drying down on to Whatman 3MM paper. Autoradiography was at -70°C using flash-sensitized X-ray film (DuPont, Cronex 4) and an intensifying screen (Ilford, Fast Tungstate).

2.6.7 Determination of molecular weight

Unless otherwise stated all molecular weights were determined from a graph of log molecular weight versus mobility.

2.7 Enzyme assays

2.7.1. Galactokinase assays

There were performed as in McKenney et al. (1982).

2.7.2 Beta-lactamase assays

2 ml samples of log phase cultures (in LB) were frozen and kept overnight at -20°C. The samples were thawed and sonicated (in three 20s bursts with an MSE sonicator at 5 μm peak to peak amplitude). Cell debris was removed by centrifugation and supernatant fluids were assayed for β-lactamase by measuring the initial rate of substrate utilization in a Perkin-Elmer 320 recording spectrophotometer. To 3 ml of 0.1 M phosphate buffer pH 6.8 was added 10 μl of substrate; (87/312, 1 crystal in 0.1 ml DMSO) and the instrument zeroed before adding 10 μl of sample. From the initial rate of reaction activity was calculated as $\Delta OD_{500}/min/OD_{540}$.

2.8 Smith and Birnstiel mapping (Smith and Birnstiel, 1976)

The technique involves partial digestion of a singly end labelled DNA fragment. The plasmid containing the DNA to be mapped was restricted at a site close to the insert and treated with CIP. Following purification by phenol extraction and ethanol precipitation the DNA was end labelled using polynucleotide kinase (2:5.3 ev), again phenol extracted and ethanol precipitated and digested with a second enzyme to give a population of singly end labelled molecules.

In Chapter 8 pNS11 was cleaved with HindIII and CIP, kinase labelled and subsequently digested with AvaII, which allowed the isolation of a 3.3 Kb singly end labelled fragment containing the inverted 2.5 Kb PvuII fragment (see Chapter 7b).

The singly end labelled fragments were separated by agarose gel electrophoresis and the required band excised and purified by the HAP method (see Section 2.5.1e(ii)). A small aliquot was checked for both purity and quantity.

For partial digests the amount of label used is calculated on the expected number of bands, e.g. a four base specific enzyme should cut once every 254 nucleotides, that is, 12 times in 3.3 Kb. For good overnight exposure (with intensifier) 100 cpm/band was found to be suitable. The digestion is based on cutting λDNA (1 µg), since the concentration of labelled DNA is assumed to be negligible. 18 µl samples were taken from a total reaction volume of 90 µl after 2, 4, 10, 20, 40 minutes and stopped by the addition of 12 µlTE, 4 µl glycerol and 5 µl STOP (100 mM EDTA, 2% SDS, 0.5% BPB). Half of this material was loaded on to a 1% vertical agarose gel and ran until the dye reached 3/4 the length of the gel. The gel was dried and autoradiographed overnight with an intensifier. The best partial digest, i.e. that with most bands represented was loaded on to a 'master' agarose gel containing the best partials of all enzymes used. In this way the gel can be read in a stepwise manner to determine the orientation of one site relative to another. The following time sample is a more complete digestion and thus contains smaller fragments. This was loaded on to an 8% 'master' polyacrylamide gel, fixed, dried and autoradiographed as before.

2.9 In vitro transcrpition

RNA polymerase was purchased from New England Biolabs. [α^{32}P] UTP (spact> 400 Ci/mmol) was from Amersham. The 10 µl transcription reaction mixture contained 20 mM Tris-acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 4mM magnesium acetate, 200 µM each ATP, CTP and GTP, 5-10 µCi of [α^{32}P] UPT 0.1-0.2 pmol of DNA template, 0.1 pmol of RNA polymerase and KCl at either 10 mM, 50 mM, 100 mM 150 mM or 200 mM. After incubation for 20 minutes at 37°C the reaction was quenched with 100 µl of 0.3 M NaOAc, 1mM EDTA, carrier tRNA (0.5 mg/ml) and precipitated with 250 µl EtOH. RNA was dried and resuspended in 6 µl sanger dye. 3 µl of this was heat treated at 90°C for 5 min and loaded on to a 5% acrylamide/7 M urea gel. Gels were fixed, dried and set up for autoradiography as in Section 2.6.6.

C H A P T E R 3

THE PROMOTER REGION OF THE *ftsZ* GENE

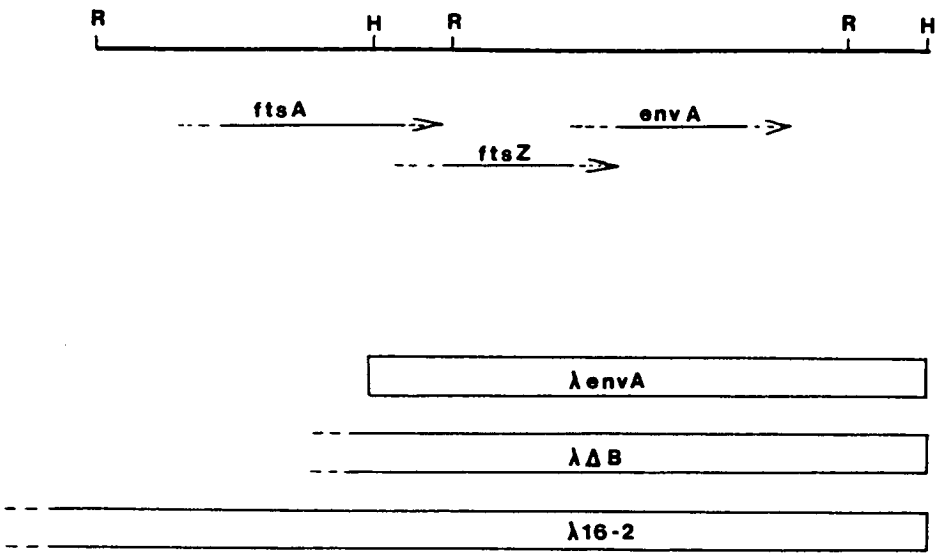
3.1 Introduction

Using λ transducing phages Lutkenhaus and Wu (1980) have identified the *ftsZ* gene product as a 45kd polypeptide and suggest that the gene transcribes clockwise on the *E. coli* map (Bachmann and Low, 1980). The *ftsZ* mutation, *ftsZ* 84t.s (Hirota *et al.*, 1968; Lutkenhaus *et al.*, 1980) can be weakly complemented with a λ transducing phage (λ*envA*$^+$) which contains a 3.5 kb *Hin*dIII fragment. If the size of the inserted fragment is increased to contain contiguous DNA from the structural gene of *ftsA* then good complementation of *ftsZ* is observed (λΔB; see Fig. 3.1). λΔB does not complement *ftsA* and gives more of the 45kd *ftsZ* gene product than λ*envA*$^+$ as determined by SDS-PAGE. Increasing the size of the inserted fragment still further (e.g. λ16-2), gives good complementation of the *ftsZ* mutation and no further increase in the amount of *ftsZ* product observed (Lutkenhaus and Wu, 1980). This suggests that some element required for full *ftsZ* expression is located within the *ftsA* structural gene. There are several possibilities (summarized in Fig. 3.2).

a) Juxtaposition of phage and bacterial DNA at the leftmost (*ftsA*) *Hin*dIII site in λ*envA*$^+$ has fortuitously created a weak promoter or that the new position has affected the activity of the normal promoter such that this activity is restored in λΔB.

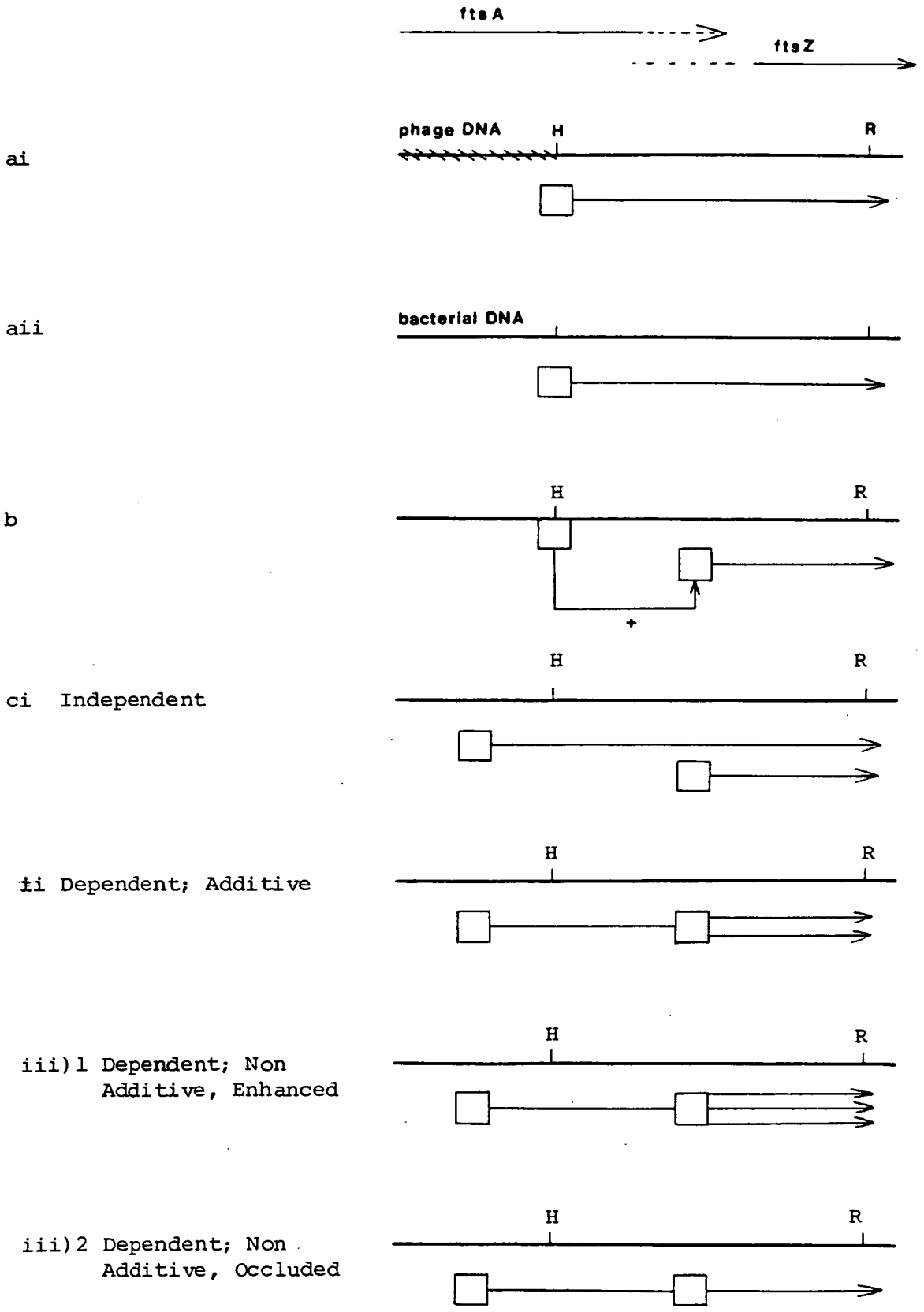
b) A positive (non-promoter) regulatory site is located at or near the *Hin*dIII site.

FIGURE 3.1 Enhancement of ftsZ



	Complementation of: ftsA	ftsZ	45Kd ftsZ pr.	33Kd envA pr.
λenvA	NO	WEAK	LITTLE	YES
λΔB	NO	YES	LOTS	YES
λ16-2	YES	YES	LOTS	YES

FIGURE 3.2


fts A
fts Z
ai
phage DNA
H
R
aii
bacterial DNA
b
H
R
+
ci Independent
H
R
ii Dependent; Additive
H
R
iii) 1 Dependent; Non Additive, Enhanced
H
R
iii) 2 Dependent; Non Additive, Occluded
H
R

c) There exists more than one promoter for ftsZ, located either side of the HindIII site. Lutkenhaus and Wu (1980) have calculated that ftsA must extend at least 135 nucleotides into the HindIII-EcoRI fragment, so at least one of the ftsZ promoters must be within the structural gene of ftsA. The action of two promoters in λΔB would enhance the expression of ftsZ giving better complementation and more 45kd protein. The two promoters could act either independently or together.

To investigate this interaction I constructed a series of pKO derivatives containing DNA from this region (Fig. 3.3). The 2.2kb EcoRI fragment bearing the ftsA gene was inserted into pKO6 (see 1.5.6) by ligating a mixture of EcoRI digested λ16-2 and EcoRI digested pKO6. One plasmid found to have the desired insert orientated such that the internal HindIII site was approximately 630bp from the AUG of galK was designated pNS27 (see Fig. 3.4). This plasmid was used for the construction of further derivatives. Firstly pNS28 was constructed by deleting the 400bp BamHI fragment. Secondly, pNS29 was constructed by deleting the 1.4kb BamHI-BglII fragment (containing within it a BamHI site). Since the two enzymes give the same 5' cohesive ends they can be religated although neither a BglII nor a BamHI site is reconstructed. pNS29 was used to construct pNS54. This has the 480bp HindIII-EcoRI fragment deleted, so that only DNA from the ftsA structural gene remains. HindIII and EcoRI do not produce the same 5' extensions so after restriction of pNS29 the ends were filled in using the large fragment of DNA polymerase I (Klenow) and the resulting linear, blunt ended plasmid was self ligated. This

FIGURE 3.3a. Construction of pNS27, pNS28 and pNS30

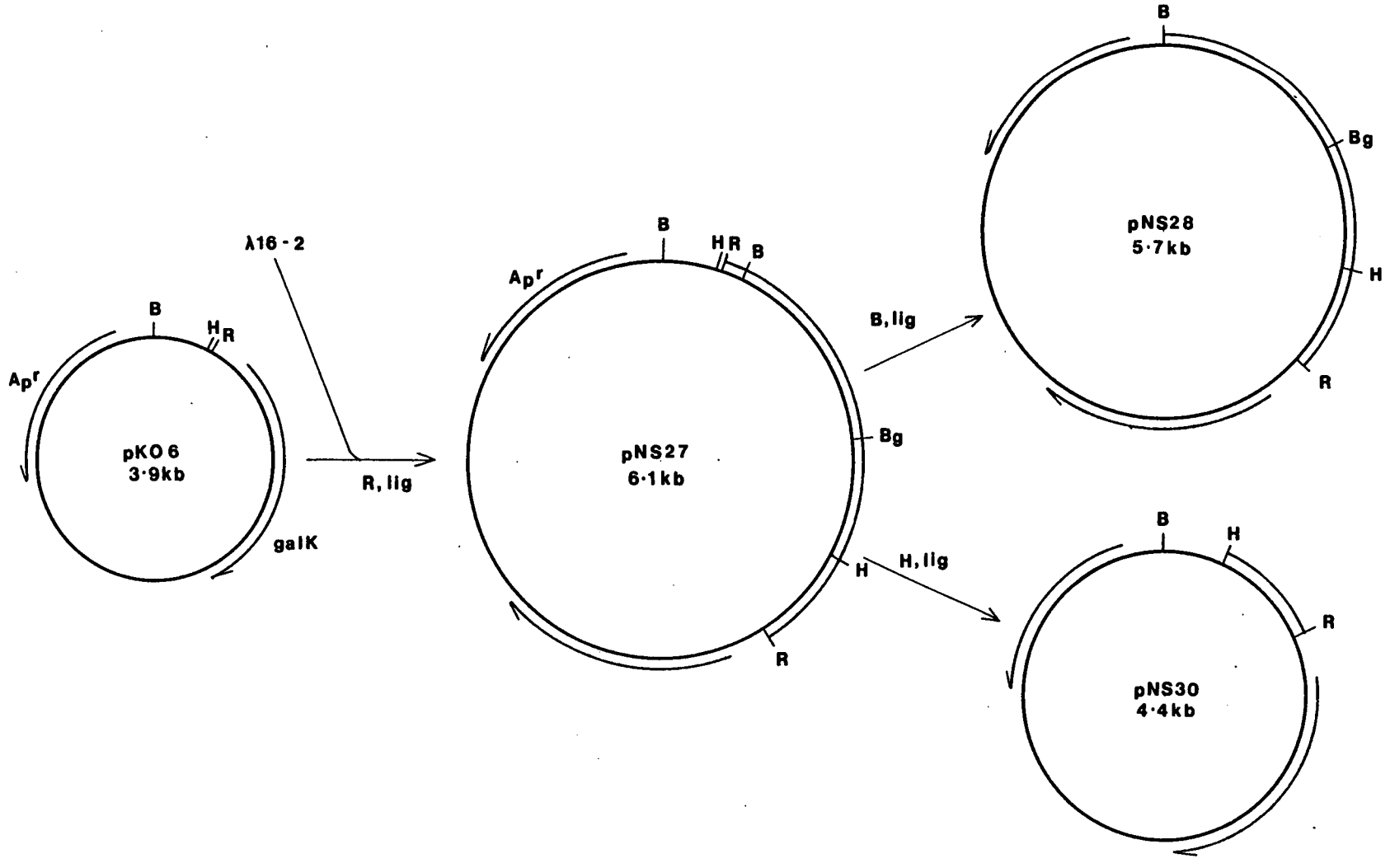
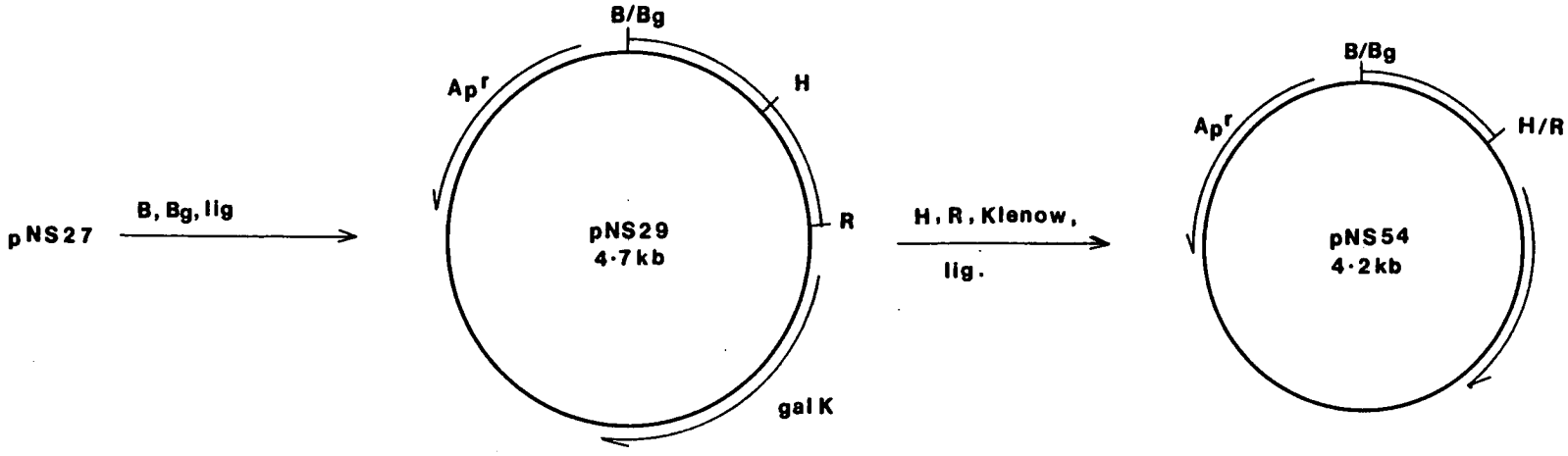


FIGURE 3.3b. Construction of pNS29 and pNS54.



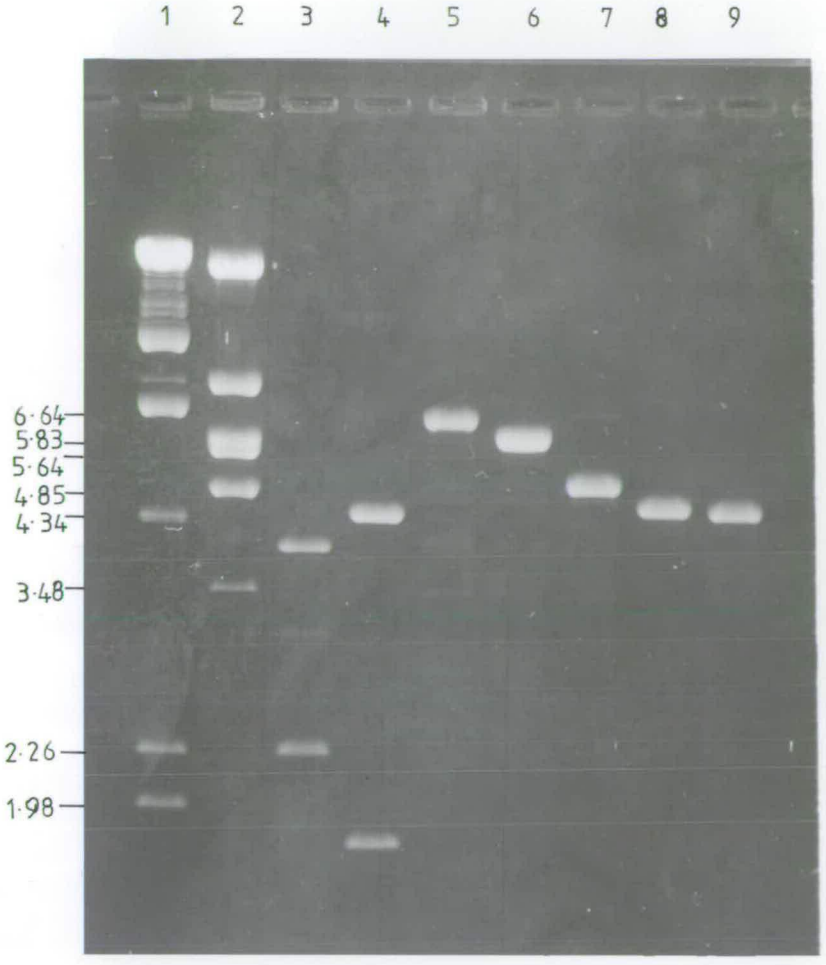

1
2
3
4
5
6
7
8
9
6·64
5·83
5·64
4·85
4·34
3·48
2·26
1·98

Fig. 3.4

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	λcI_{857}	EcoRl	-
3	pNS27	EcoRl	3.3a
4	pNS27	HindIII	"
5	pNS27	PstI	"
6	pNS28	PstI	"
7	pNS29	PstI	3.3b
8	pNS54	PstI	"
9	pNS30	PstI	3.3a

does not produce a functional restriction site. Thirdly, pNS30 was constructed from pNS27 by deleting the 1.7kb HindIII fragment. These constructions are summarized in Figure 3.5.

3.2 Results

The plasmid pNS30 shows promoter activity associated with the 480bp HindIII-EcoRI fragment reading left to right. This may be attributed to an ftsZ promoter (see Chapter 4.12) and is equivalent to 26% of the activity of the medium strength E. coli promoter pgal (see Table 3.1).

If the contiguous upstream DNA fragment is added as in pNS29 then the promoter activity increases 2.9 times to 75% of the strength of pgal. This plasmid contains only part of the ftsA structural gene since it cannot complement the ftsA mutation TOE13. If further contiguous upstream DNA is added as in pNS28, this time including the entire ftsA structural gene and promoter then transcription increases to 84% of pgal. The values for pNS29 and pNS28 are within 10% error but when assayed pNS28 always gave a higher value than pNS29, perhaps reflecting a contribution from the ftsA promoter. Since pNS28 begins within the ftsQ structural gene there can be no contribution from the ftsQ promoter (see Chapter 4).

Taken together with the improved ftsZ complementation and increased amount of ftsZ protein observed when upstream DNA is present, the gene fusion evidence presented above is consistent with the notion that there is a sequence within the structural gene of ftsA necessary for full expression of the ftsZ gene. Since pNS30, pNS29 and pNS28 all have the same downstream fusion junction any translation

TABLE 3.1

Plasmid	Complementation of *ftsA*[1a,b]	Galactokinase Specific Activity[2]	Control Plasmid[2]	Corrected galactokinase Specific Activity[2]
pNS28	+	3.01	pKOC1	2.55
pNS29	-	2.73	pKOC1	2.27
pNS54	-	1.17	pKOC1	0.71
pNS30	-	1.12	pKO1	0.78
pKG1800[3]	-	3.50	pKOC1	3.04
pKO1-lac[4]	-	3.02	pKOC1	2.56

1 a. None of these plasmids complement either *ftsQ* or *ftsZ*.

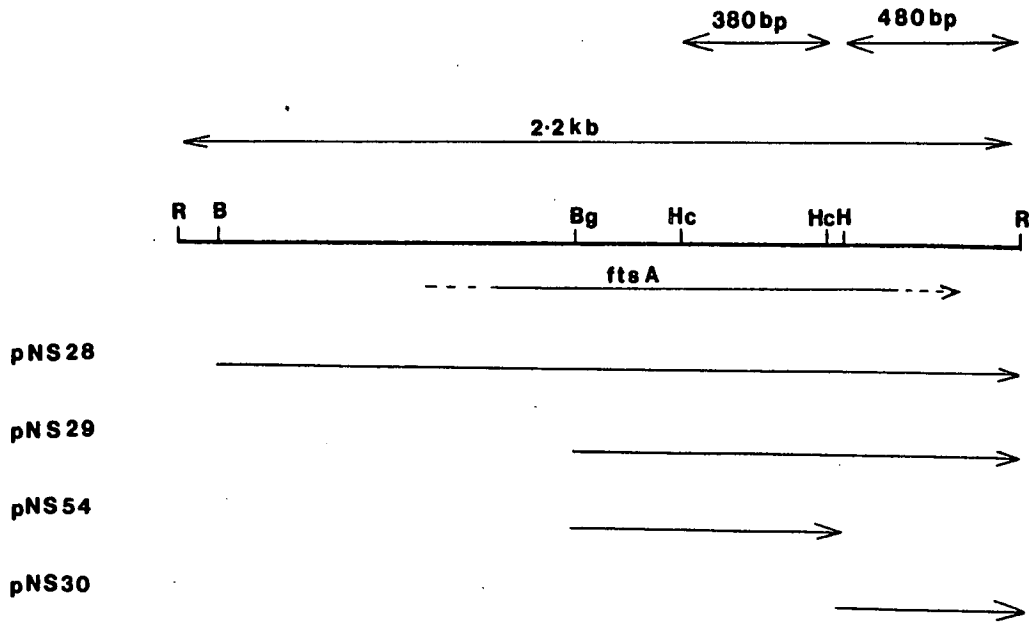
b. See Appendix II

2 See Appendix I

3 Contains the *E. coli* galactose operon promoter (p *gal*)

4. Contains the *E. coli* *lacZ* promoter (uninduced).

FIGURE 3.5. Summary of constructs and fragments used for *in vitro* transcription.



of ftsZ entering into the galK leader region (and hence into the galK gene) will be in the same frame, so that the observed enhancement of galK activity is unlikely to result from differential translation efficiency (Appendix 1.1a). No significant variation in copy number between pKO constructions was observed (Appendix 1.3).

When cloned separately as in pNS54 the DNA responsible for the enhancement of ftsZ transcription shows promoter activity equivalent to 23% of pgal. This is similar to the promoter activity of pNS30. These measurements indicate weak E. coli promoters, but are strong enough to be unlikely to result from a promoter split by HindIII restriction. The sum of these two promoter activities amounts to 49% of that of pgal, i.e. less than the value obtained for pNS29 (75% of pgal). Thus the enhancement of ftsZ transcription initiation arises from the tandem arrangement of two promoter containing fragments (see Fig. 3.2 ciii,l) and represents a novel form of genetic regulation whereby an upstream promoter can act to increase transcription from a downstream promoter or vice versa. This differs from the model proposed for eucaryotic enhancer elements (Banerji et al., 1981; Grosschedl and Birnsteil, 1982) whereby an upstream non promoter sequence mediates the frequency of transcription from a downstream promoter sequence and from the recently reported procaryotic 'enhancer' of the tyrT gene (Lamond and Travers, 1983).

3.3 Transcription in vitro (see also Appendix 3)

With the gene fusion system used here it is not possible to decide how many promoters are within a given fragment, since the

galactokinase activity reflects only total transcription from that fragment. To test the assignment of promoter position and locate likely start sites *in vitro* transcription experiments were performed, firstly from the 380bp HincII-HincII fragment (Fig. 3.6a) and secondly from the 480bp HindIII-EcoRI fragment (Fig. 3.6b), both indicated in Figure 3.5. The 380bp fragment shows non specific transcripts at 380, 285 and 214 bases, with that at 380 bases most likely to be an end-end transcript. A specific transcript with an optimum at 150mM KCl is observed at 211 bases. Comparison of this mRNA with the nucleotide sequence shows a possible -10 and -35 region upstream of the potential start of this mRNA (see Table 3.2, Fig. 3.8). No similar sequence is seen on the complementary strand. It is likely that this transcript is initiated from an *ftsZ* promoter (designated P_{z_3}) which lies within the *ftsA* structural gene and upstream of the HindIII site.

The analysis of *in vitro* transcription products from the 480bp HindIII-EcoRl fragment is more complex. There is a specific mRNA of 380 bases with an optimum of transcription of greater than 200mM KCl and a message of 176 bases with an optimum of 100mM KCl. Both start near possible -35 and -10 regions (Table 3.2, Fig. 3.8) and define two possible promoter sites within the 480bp HindIII-EcoRl fragment. The promoter initiating the 380 base transcript is designated P_{z_2} and that initiating the 176 bases mRNA is designated P_{z_1}. The 380 base mRNA is 6 times stronger than the 176 base mRNA at 200mM KCl, 4 times stronger at 150mM KCl and 2 times stronger at 100mM KCl *in vitro*. In order to decide how these promoters are

FIG 3·6a; 380 bp Hc - Hc

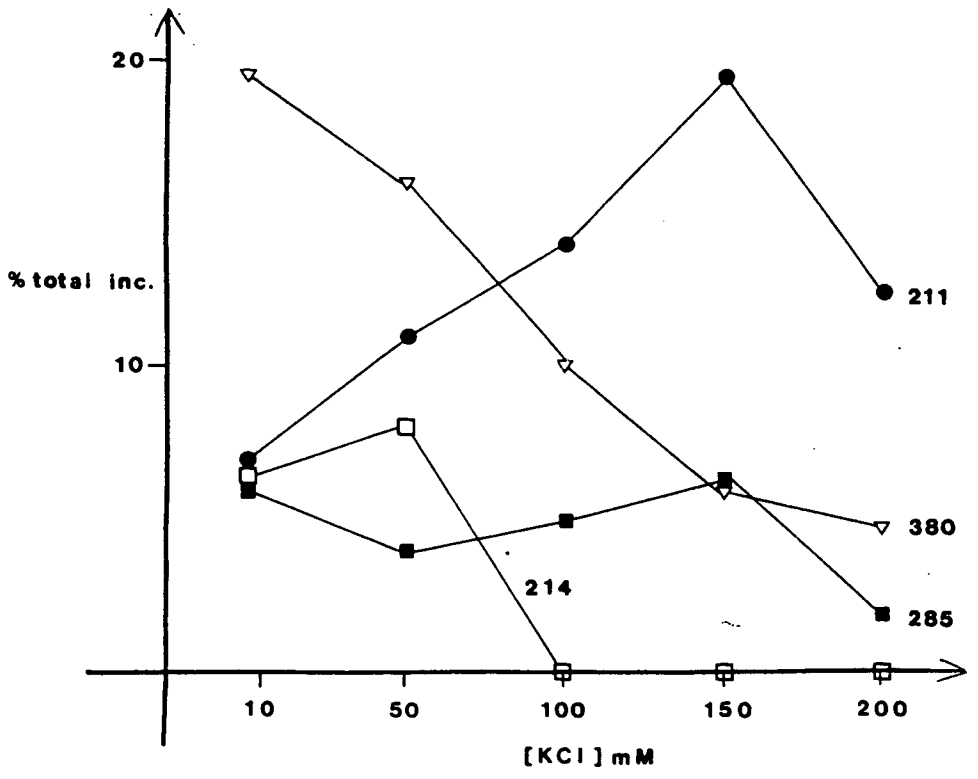
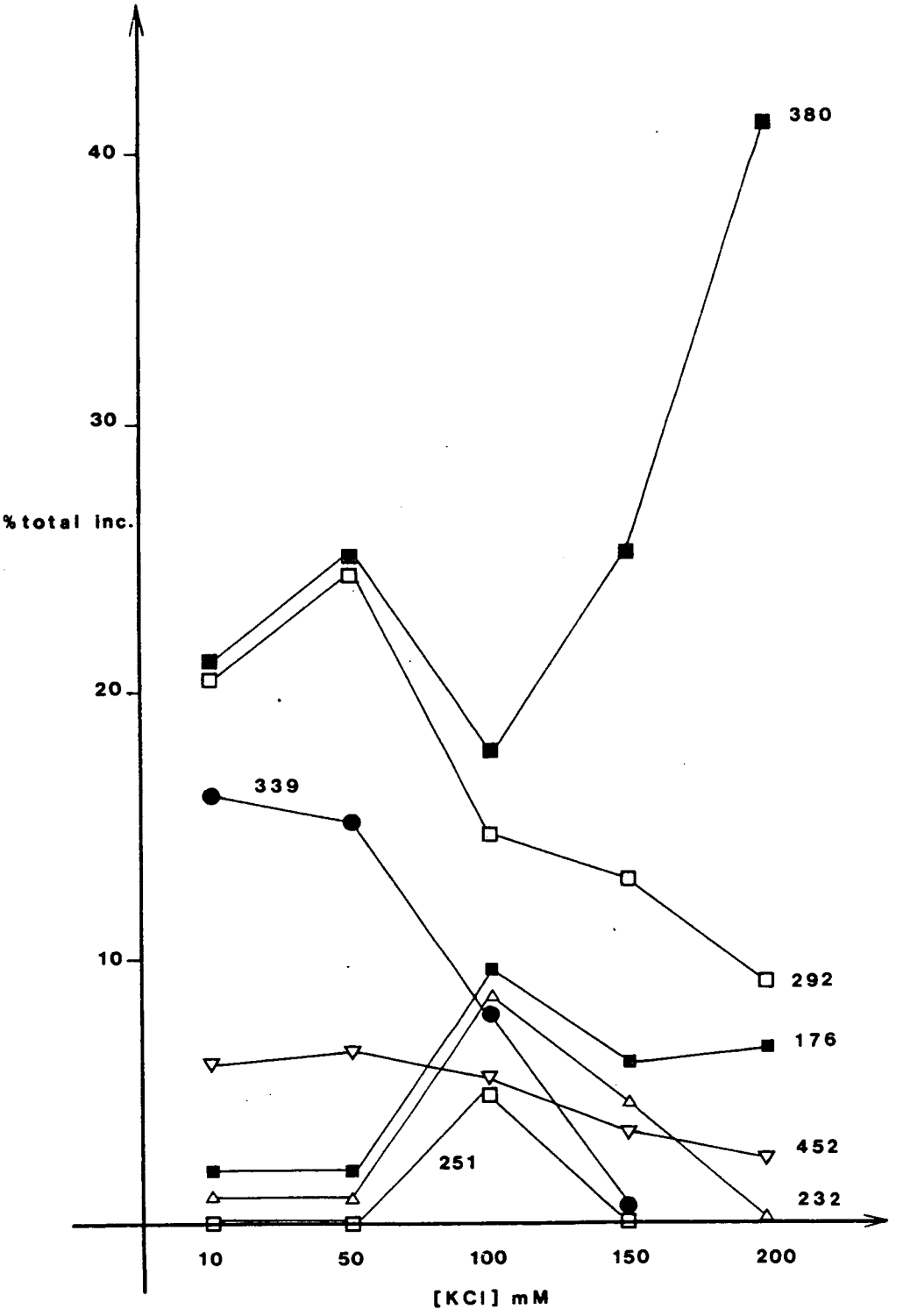


FIG 3·6 b ; 480 bp H-R



actually used *in vivo* awaits further experiments but the relative strengths of *in vitro* initiated transcripts argues that the 380 base mRNA may have a role to play. Since Lutkenhaus and Wu (1980) have calculated that *ftsA* must extend at least 135 nucleotides past the HindIII site and sequence analysis indicates an open reading frame for *ftsA* which extends 217 nucleotides past the HindIII site (Fig. 3.7 and Robinson *et al.*, 1983), then Pz_2 must be within the structural gene of *ftsA*. *In vitro* transcription of the 480bp HindIII-EcoR1 fragment shows non specific transcripts of 452, 339 and 292 bases, of which the 452 base mRNA is most likely to be an end-end transcript. The origin of the 251 and 232 base transcripts with optima at 100mM KCl is unknown, but neither correlate with promoter like sequences (Fig. 3.7). Reznikoff *et al.* (1982) have noticed that upon decreasing the KCl concentration in a reaction primed with *lac* promoter DNA, two additional specific transcripts of the same polarity as the normal transcript are synthesised, initiated either from their own or a shared set of sequence signals. Reznikoff *et al.* (1982) argue against any physiological significance for these transcripts and point out that initiation from multiple overlapping signals may reflect a previously existing functional state whereby a primordial *lac* promoter may have used alternative sites for transcription initiation. The 251 and 232 base transcripts or indeed even the 176 base transcript of Figure 3.6b may well be physiologically insignificant vestiges of a previously existing functional state.

3.4 Summary of results and *in vivo* significance of *ftsZ* promoters

The expression of the *ftsZ* gene is dependent on sequences which lie within the structural part of the *ftsA* gene. There are three

FIG 3·7

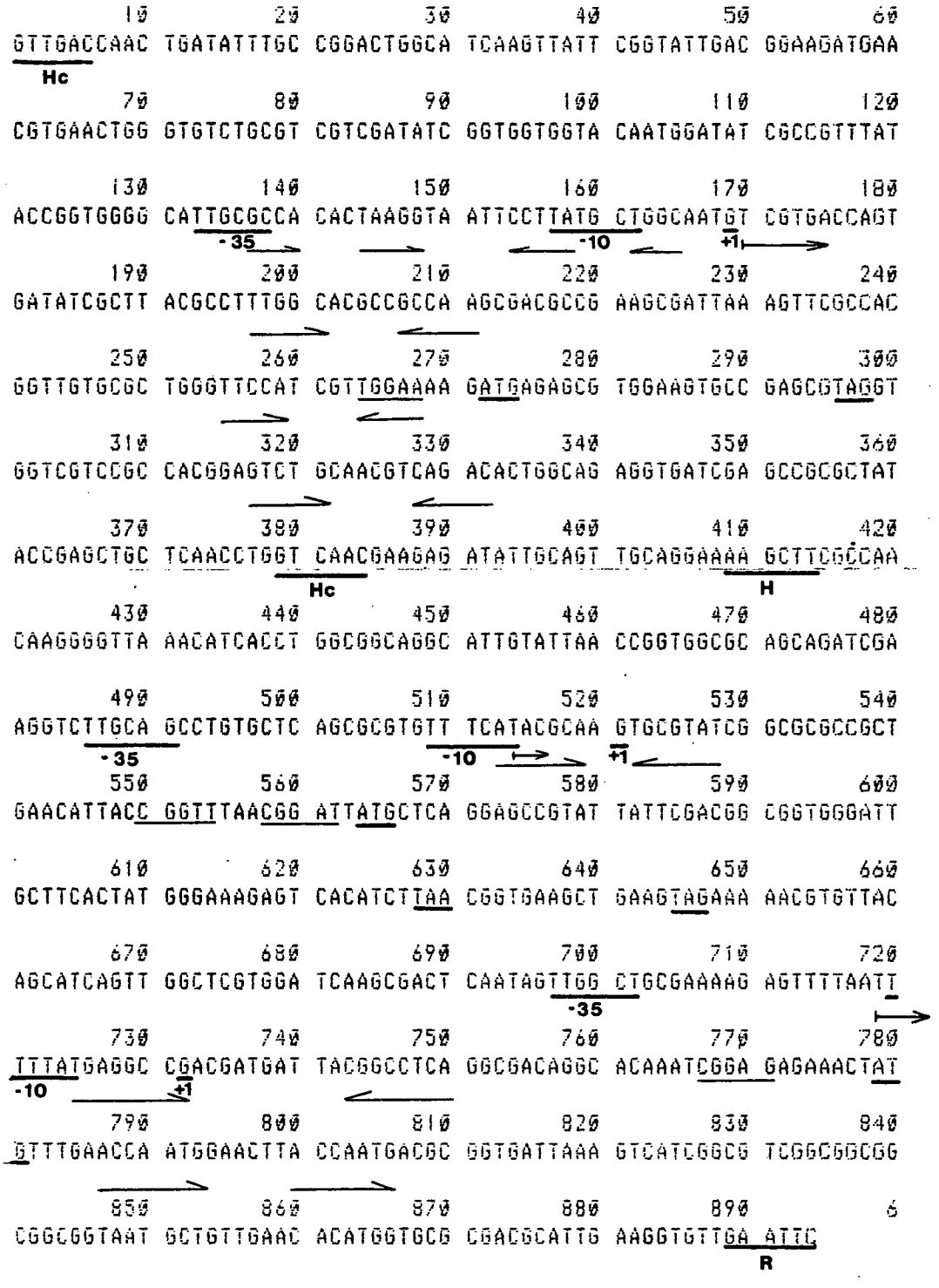

10 20 30 40 50 60
GTTGACCAAC TGATATTTGC CGGACTGGCA TCAAGTTATT CGGTATTGAC GGAAGATGAA
Hc
70 80 90 100 110 120
CGTGAACTGG GTGTCTGCGT CGTCGATATC GGTGGTGGTA CAATGGATAT CGCCGTTTAT
130 140 150 160 170 180
ACCGGTGGGG CATTGCGCCA CACTAAGGTA ATTCCTTATG CTGGCAATGT CGTGACCAGT
-35
-10
+1
190 200 210 220 230 240
GATATCGCTT ACGCCTTTGG CACGCCGCCA AGCGACGCCG AAGCGATTAA AGTTCGCCAC
250 260 270 280 290 300
GGTTGTGCGC TGGGTTCCAT CGTTGGAAAA GATGAGAGCG TGGAAGTGCC GAGCGTAGGT
310 320 330 340 350 360
GGTCGTCCGC CACGGAGTCT GCAACGTCAG ACACTGGCAG AGGTGATCGA GCCGCGCTAT
370 380 390 400 410 420
ACCGAGCTGC TCAACCTGGT CAACGAAGAG ATATTGCAGT TGCAGGAAAA GCTTCGCCAA
Hc
H
430 440 450 460 470 480
CAAGGGGTTA AACATCACCT GGCGGCAGGC ATTGTATTAA CCGGTGGCGC AGCAGATCGA
490 500 510 520 530 540
AGGTCTTGCA GCCTGTGCTC AGCGCGTGTT TCATACGCAA GTGCGTATCG GCGCGCCGCT
-35
-10
+1
550 560 570 580 590 600
GAACATTACC GGTTTAACGG ATTATGCTCA GGAGCCGTAT TATTCGACGG CGGTGGGATT
610 620 630 640 650 660
GCTTCACTAT GGGAAAGAGT CACATCTTAA CGGTGAAGCT GAAGTAGAAA AACGTGTTAC
670 680 690 700 710 720
AGCATCAGTT GGCTCGTGGA TCAAGCGACT CAATAGTTGG CTGCGAAAAG AGTTTTAATT
-35
730 740 750 760 770 780
TTTATGAGGC CGACGATGAT TACGGCCTCA GGCGACAGGC ACAAATCGGA GAGAAACTAT
-10
+1
790 800 810 820 830 840
GTTTGAACCA ATGGAACTTA CCAATGACGC GGTGATTAAA GTCATCGGCG TCGGCGGCGG
850 860 870 880 890 6
CGGCGGTAAT GCTGTTGAAC ACATGGTGCG CGACGCATTG AAGGTGTTGA ATTC
R

Fig. 3.7

The DNA sequence of the non-coding strand of the 3' end of the *ftsA* gens which includes the promoter region of *ftsZ* Taken from Robinson *et al.*, 1983 and A. Robinson, unpublished. Reproduced with the permission of W. D. Donachie.

Features of interest are indicated underneath the sequence, some of which are tabulated below. Due to the inherent variability of promoter sequences no satisfactory computer program designed to search for such sequences has been devised, so I have screened (by eye) this sequence for possible promoters (see Fig. 1.9). Those which correspond to *in vitro* trasncripts have been indicated (see also Table 3.2).

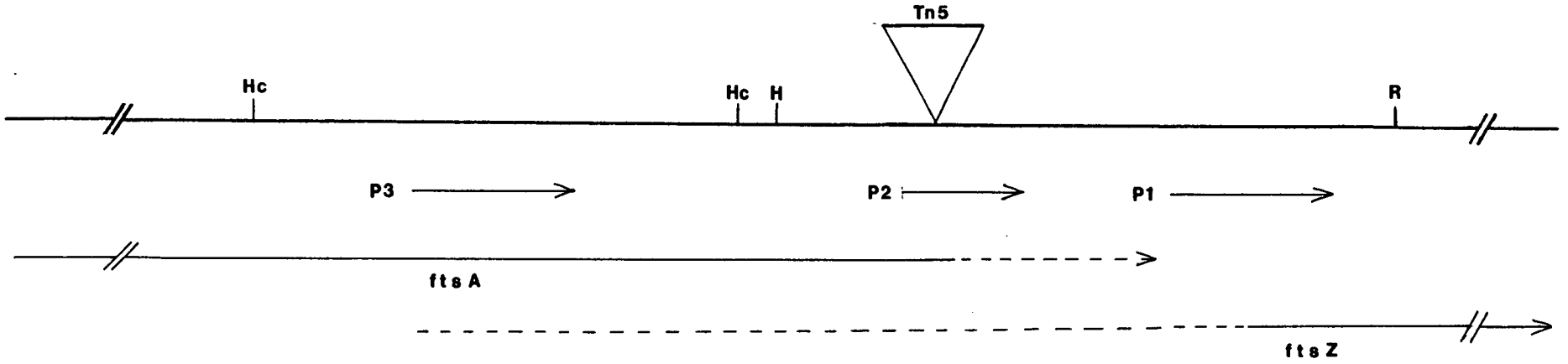
Coordinate	Feature
0-6	*Hinc*II site
133-168	Pz_3
169	*In vitro* mRNA
136-165	RNase III site
379-386	*Hinc*II site
409-414	*Hind*III site
486-521	Pz_2
514	*In vitro* mRNA
629	End of *ftsA*
697-732	Pz_1
719	*In vitro* mRNA
889-894	*Eco*R1

lines of evidence for this: (a) adding DNA from the coding region of *ftsA* improves complementation of the downstream *ftsZ* gene, (b) adding the same DNA increases the amount of *ftsZ* gene product observed by SDS-PAGE and (c) sequences within the structural gene of *ftsA* can initiate transcription which traverses into the *ftsZ* gene.

Using a combination of gene-fusion and *in vitro* transcription it has been possible to assign three promoters to the *ftsZ* gene (see Fig. 3.8). Pz_1 and Pz_2 initiate 176 and 380 bp from the right hand *Eco*R1 site of the 480bp *Hind*III-*Eco*R1 fragment. Pz_3 is located 344 bp upstream of Pz_2. Both Pz_2 and Pz_3 must be within the structural gene of *ftsA* and both are capable of initiating strong mRNA transcripts *in vitro*. Pz_3 in combination with either Pz_1 or Pz_2 or both is capable of raising the level of transcription above the level expected from addition of their separate promoter activities. Such enhancement of transcription represents a novel form of promoter interaction. Lutkenhaus (pers.comm.) has isolated a Tn5 insertion which simultaneously eliminates the *ftsA* gene and reduces *ftsZ* expression to 10% of the enhanced level. This implies that the transposon inactivates *ftsA* somewhere between Pz_1 and Pz_2 so Pz_3 and Pz_2 must be the *ftsZ* promoters used *in vivo*. Since Pz_2 and Pz_1 are only capable of mediating weak complementation (as in λ*envA*$^+$), Pz_3 must have *in vivo* significance. Assuming no transcription comes from the inserted transposon the residual *ftsZ* product may result from initiations at Pz_1.

In vitro transcription can only indicate sequences capable of

FIGURE 3.8. The location of ftsZ promoters relative to ftsA.



initiating mRNA, and does not provide information relating to the transcripts used *in vivo*. It is now most important to map the *ftsZ* *in vivo* transcripts and to perform fingerprint analyses to define precise start points and unequivocally determine the polarity of these transcripts.

3.5 A comparison between the *ftsZ* control region and other 3 promoter operons

Besides the *ftsZ* gene both the *uvrB* gene and the *gal* operon have three promoters, the upstream of which is capable of initiating mRNA. The P_3 mRNA of the *gal* operon has no known function but traverses the whole operon and can express *gal* enzymes. Neither *gal* P_2 nor *gal* P_1 appear to be dependent on P_3 for function (Queen and Rosenberg, 1981). In contrast, the P_3 mRNA of *uvrB* terminates *in vitro* in the neighbourhood of the *lexA* binding P_2 promoter whether *lexA* protein is bound or not, although this need not necessarily reflect the *in vivo* situation. P_3 does not seem to affect P_2 but *lexA* protein bound to P_2 inhibits transcription from P_3, possibly by jamming intervening RNA polymerase molecules. The function of P_3 is unknown (Sancar *et al.*, 1982). One of the functions of the *ftsZ* P_3 is to enhance or be enhanced by transcription from downstream promoters. Whether its mRNA is capable of expressing *ftsZ* (*cf. gal*) or terminates near P_2 (*cf. uvrB*) remains an interesting question.

3.6 A mechanism for enhancement

For each promoter on a given piece of DNA the rate of initiation (strength) is dependent both on the affinity of the RNA polymerase for its binding site and the rate of open complex formation. Any mechanism for the observed enhancement of *ftsZ* transcription must depend on these factors. Classically, the RNA polymerase molecules from Pz_3 act as positive activators of transcription from Pz_2 or *vice versa* and as such may influence gene expression in one of two ways, either via a conformational change in the DNA or via a protein-protein interaction.

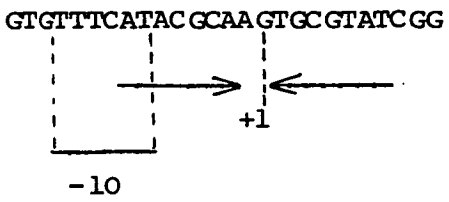
A plausible mechanism for the observed enhancement of *ftsZ* transcription may depend on a shift in the balance between the kinetic constants for binding and open complex formation for Pz_2 and Pz_3, assuming Pz_1 only plays a role in basal transcription (see Section 3.10). If Pz_3 has a high affinity for RNA polymerase but a low rate of open complex formation then only a small proportion of the RNA polymerase molecules which bind will be capable of productive initiation. The remainder will be free to slide along the DNA in a reaction dependent on ionic strength (Von Hippel *et al.*, 1982). On the other hand, Pz_2 may have a low affinity for RNA polymerase and a high rate of open complex formation. In this case a high proportion of the RNA polymerase molecules which bind can initiate but the rate of initiation is limited by the availability of RNA polymerase. Should these criteria apply then both of these promoters would appear weak when separated, as is indeed the case when the galactokinase levels are measured. In combination, however, the uninitiated polymerase molecules from Pz_3 can now initiate at Pz_2, so overcoming the poor

binding characteristic of this region. The net effect is a level of transcription over and above that expected for the sum of the separated promoters, i.e. the level of ftsZ transcription has been enhanced.

The architecture of this region also suggests a mechanism by which E. coli might regulate ftsZ expression at different growth rates. In fast growing cells where RNA polymerase is more abundant (Churchward et al., 1982; Enami and Ishimama, 1982) the low affinity promoter (Pz_2) can be used more efficiently, perhaps ensuring more ftsZ mRNA at increased growth rates. At slower growth rates when the availability of RNA polymerase is reduced, transcription may be more frequently initiated from the high affinity promoter Pz_3 thus ensuring the availability of ftsZ protein for division. A similar functional interrelationship is found between ribosomal RNA promoters where a low affinity - fast transcribable promoter upstream from a high affinity slow promoter with intervening spacer enables manipulation of the rate of transcription under different growth conditions (Glaser et al., 1983).

An alternative mechanism requires a conformational change induced by the passage of transcribing RNA polymerase molecules over a pause site. RNA polymerase hesitates or pauses at termination sites in the absence of the termination factor rho (Kassavetis and Chamberlin, 1981), an observation which led Farnham and Platt (1981) to suggest that pausing was due to an interaction between RNA polymerase and a hairpin structure within the mRNA. Within the 480bp HindIII-EcoR1 fragment we can identify two regions which may function either as

pause sites or rho dependent terminators in the mRNA, located adjacent to the -10 regions of Pz_1 and Pz_2. The stem loop structure associated with Pz_3 mRNA overlaps with the -10 region of the Pz_2 promoter:



A transcribing RNA polymerase core enzyme from Pz_3 will cause a local distortion of superhelicity due to unwinding of the helix (see 1.5.5). A pause at this site, due to the formation of a hairpin in the mRNA, may induce a conformational change in the DNA such that incoming RNA polymerase holoenzyme finds it easier to bind to the -35 region of Pz_2. Since the hairpin structure only occurs in Pz_3 mRNA, RNA polymerase molecules which bind at Pz_2 will not stall at this site and so will be unaffected by pausing. Thus Pz_3 and Pz_2 appear weak when separate but exhibit enhanced transcription when combined. A similar interaction is possible at Pz_1 where stem-loop structures can form in the mRNAs initiated from Pz_2 and Pz_3, but not in that initiated from Pz_1.

A cooperative enhancement of transcription initiation has been observed between tandem promoters of the ilv GEDA operon of E. coli (Adams and Hatfield, 1983). Based on the data of Chenchick et al. (1981) and Siebenlist et al. (1980) these authors suggest that the enhancement is mediated by a protein-protein interaction between two RNA polymerases bound to the same face of the helix at promoter-sites

separated by 72bp. As before this requires either an increase in the affinity of the RNA polymerase for the DNA or an increase in the rate of open complex formation. Such an interaction is unlikely for the ftsZ promoters due to the much greater distance separating pairs of promoters (see Table 3.2).

TABLE 3.2

Promoter	-35	Space	-10	+1	Pos^n of +1 in Fig.3.8	Pos^n of mRNA start in Fig. 3.8 as measured by IVT
Consensus	TTGaca	17 ± 1	TAtaaT	GorA	-	
Pz_1	TTGGCT	17	TTTTAT	G	732	718
Pz_2	TTGCAG	17	TTTCAT	G	521	514
Pz_3	TTGCGC	18	TATGCT	G	169	170

The dimerization of RNA polymerase may also have a role in promoter selectivity (Travers et al., 1982a, b). However, since dimerization only occurs at ionic strengths lower than 100mM KCl (Berg and Chamberlin, 1970) and the Pz_2 and Pz_3 transcripts have optima at high ionic strengths (200mM for Pz_2 and 150mM for Pz_3) such a protein-protein interaction is unlikely to be important in the mechanism of ftsZ enhancement.

3.7 Is ftsA transcription terminated?

The inverted repeat structure adjacent to the -10 region of Pz_1 (Fig. 3.7) could function either as a mRNA pause site for RNA polymerase molecules initiated at any of the three ftsZ promoters, to stabilize Pz_1 mRNA or to act as a rho dependent terminator for ftsA. Although the stem loop structure is larger than we might expect for a transcription terminator, there are no other candidates in this region. Limited evidence from multiple copy pKO gene fusions suggests that such a structure does not function to terminate ftsA transcription although the 480bp HindIII-EcoRI fragment can do so if in the opposite orientation (D. Kenan, unpublished results). Since these two inverted repeat structures are adjacent to the T rich -10 region of Pz_1 and Pz_2 they may function as rho independent terminators reading from right to left when transcribed into mRNA. This sequence alignment may have arisen by chance or be a remnant from a previous functional state. Alternatively, such a terminator may serve to dissociate RNA polymerase molecules transcribing in the opposite direction. This is deemed unlikely since there are no promoters reading right to left for at least 2.5Kb downstream (see Chapter 9).

If transcription from Pz_3 or Pz_2 is necessary for full ftsZ expression in vivo and since both are within the structural gene of ftsA then in the absence of an accessory factor or other modification transcription of ftsZ would be terminated by the ftsA transcription terminator. Thus the requirement for ftsZ transcription may preclude termination of the ftsA mRNA.

3.8 Possible translation products

1. Pz_1; RNA polymerase molecules bound at Pz_1 most likely initiate at the G residue at position 728 in Figure 3.7. There is a 50 base leader mRNA before the first AUG initiation codon preceded by a possible SD sequence (Table 3.3). Translation in this frame is open at least until the EcoR1 site providing a possible open reading frame for ftsZ. The other two reading frames are closed.

2. Pz_2; Transcription from this promoter may initiate at around position 521 (Fig. 3.7). There is a 43b mRNA leader before the first AUG, which is preceded by two possible SD sequences (Table 3.3). If translation is initiated at this AUG then it is possible to make a 27 amino acid protein of molecular weight 3.77Kd. Such a protein overlaps the ftsA coding region by 21 amino acids although the two proteins are coded in different frames (cf. ØX174, Smith et al., 1977). The protein sequence is:

(N terminus)

met	leu	arg	ser	arg	ileu	ileu	arg
arg	arg	try	asp	cys	phe	thr	met
gly	lys	ser	his	ileu	leu	thr	val
lys	leu	lys	STOP.				

An analysis of codon usage (Konigsberg and Godson, 1983) shows that 2 out of the 27 amino acids use unusual codons (underlined), although in such a small protein this is not statistically significant. At pH 6.0 the protein will have a net positive charge (+ 8). Such a small protein is unlikely to have tertiary structure but due to its net positive charge may function in DNA binding.

TABLE 3.3

	Position of +1 in Fig. 3.7	Length of Leader	Ribosome Binding site	Position of G prior to AUG	Position of AUG in Fig. 3.7	Position of in frame translational stop
Consensus	-	-	GGAG	11	-	-
Pz_1	728	51	CGGAG	11	779	undetermined
Pz_2	521	43	CGGTT; CGGAT	13, 5	564	645
Pz_3	169	103	TGGAA	7	272	296

3. Pz_3; Transcription from Pz_3 is most likely to initiate at position 169 (Fig. 3.7). After a 103 base leader mRNA there is an AUG translational start preceded by a possible SD sequence (Table 3.3). This can code for a 7 amino acid polypeptide of molecular weight 1.15Kd, contained entirely within the ftsA coding region. The sequence is:

(N Terminus)

met arg ala trp lys cys arg ala (STOP).

At pH 6.0 such a protein would have a net positive charge (+ 3). In the Pz_3 mRNA the SD sequence associated with this mini protein lies within a stem-loop structure (a 5 base stem, 4 base loop). Such a structure may prevent access to ribosomes and hence prevent translation as is observed for the 1.2 gene of phage T7 (Saito and Richardson, 1981).

All elements required to make the 3.77 kd and 1.15Kd proteins appear to be present. It is not known whether they are indeed made or whether they have in vivo significance.

3.9 The effect of ftsA transcription and translation on ftsZ expression

It is not possible to conclude whether or not the ftsA mRNA is terminated. Thus transcription of ftsA may affect one or all of the ftsZ promoters and proceed into the ftsZ coding region, perhaps producing a polycistronic mRNA. Such a molecule would be in addition to those initiated at the ftsZ promoters. It is unlikely that either transcription of ftsZ or the magnitude of the enhancement is dependent

ftsA transcription or translation since (a) the galactokinase levels of pNS28 and pNS29 are similar and (b) λΔB can complement the ftsZ 84 t.s. mutation well.

3.10 The molecular basis of periodic ftsA expression; significance of internal promoters

Perhaps one of the most striking features of the ftsZ control region is the incidence of promoters within the coding region of the gene preceding it. This may simply reflect the efficient use of sequence within this cluster but might be indicative of an additional level of regulation as well, i.e. transcription of ftsA may regulate ftsZ or vice versa.

The trpD gene has an internal promoter (trp-p2; Section 1.5) which Nichols et al. (1981) have suggested may allow tryptophan synthesis during conditions of extreme starvation. Horowitz and Platt (1981) have examined the departures of the trp-p2 sequence from the consensus and note that the requirement for a functional protein may impose limits on the trp-p2 sequence. Conversely, to maintain a reasonably good promoter sequence some of the amino acids of this region are coded for by rarely used codons. In contrast both Pz_3 and Pz_2 exhibit good promoter sequences neither of which cause unusual features in the ftsA protein.

Phenotypically the ftsZ mutation causes E. coli to form straight sided filaments at the restrictive temperature whilst an ftsA mutation causes filaments with invaginations. Thus the ftsA gene product may act at a later stage in septation than the ftsZ product. During the unperturbed cell cycle initiations from Pz_3 and Pz_2 may serve to prevent

ftsA translation. If Pz_3 is a high affinity promoter (see Section 3.6) then RNA polymerases which bind here will slow or prevent the completion of ftsA message. Since this is a dynamic process some ftsA will be made, but less than if Pz_3 and Pz_2 were non-functional. This model implies that for full ftsA expression Pz_3 and Pz_2 must be turned off. Thus the point in the cell cycle at which ftsA synthesis is required (Donachie et al., 1979) may be indirectly dependent upon the timed expression of ftsZ. Nothing is known of the factors which regulate ftsA expression.

The Pz_1 promoter may function either to provide a basal level of ftsZ mRNA throughout the cell cycle or to quicken recovery after a period of division inhibition.

3.11 Possible RNase III processing of ftsA mRNA

In E. coli mutations in rnc (RNase III), rne (RNase E) or other ribonuclease loci can affect the expression of a large number of genes presumably by post-transcriptional processing of the mRNA (Gitelman and Aprion, 1980). RNase III has been shown to be involved in the maturation of E. coli rRNA (Bram et al., 1980) whilst in phage T7 translation of the 0.3 gene mRNA is enhance by RNase III processing (Dunn and Studier, 1975). Again in T7 expression of the 1.1 gene is retroregulated via RNase III processing at a site distal to the 1.2 gene (Saito and Richardson, 1981). In phage λ RNase III processing controls expression of the int gene (Guarneros et al., 1981; section 1.5). An RNase III site usually consists of a stable hairpin with some central unpaired bases, seen as a region of hyphenated dyad symmetry in the sequence (see Saito and Richardson, 1981 for references).

Such a region occurs within the coding sequence of ftsA (Fig. 3.7) and suggests the possibility of RNase III processing of ftsA mRNA. Such processing is less likely if the mRNA is being translated since translating ribosomes can destabilize hairpin structures (Saito and Richardson, 1981). Thus in the absence of ftsA translation RNase III processing of its mRNA may occur. Factors controlling RNase III synthesis and hence the rate of RNase III processing are not understood, but changing the half life of a cell division associated mRNA molecules may provide yet another level of control for this process. Why this structure should overlap with the upstream ftsZ promoter (P3) is unclear.

CHAPTER 4

LOCATIONS AND DIRECTIONS OF TRANSCRIPTION OF *ftsQ*, *ftsA* and *ftsZ*

4.1 Plasmids used for the location of the ftsQ gene.

The extent of ftsQ can be deduced by complementation analysis of three plasmid constructions (Fig. 4.1). The plasmid pNS28 was constructed as described in Chapter 3. pNS36 was constructed by PvuII, SmaI digestion and religation in dilute solution of pDK302, a recombinant plasmid in which the 2.2Kb EcoR1 fragment had been inserted into the EcoR1 site of pKO1, in the same orientation as in pNS27 (Fig. 4.2, 4.10). A HindIII deletion of pDK302 gave pDK340 in which the HindIII-EcoR1 fragment bearing ftsZ promoters Pz_1 and Pz_2 had been removed. The plasmid pNS14 was a KpnI deletion of pDK340. (Fig. 4.2, 4.4). I am grateful to Dan Kenan for constructing pDK302 and pDK340.

4.2 The extent of ftsQ

The plasmid pNS36 will complement the ftsQ mutation TOE-1 (Begg et al., 1980). This indicates that the rightword extremity of ftsQ is to the left of the PvuII site. The plasmid pNS28 lacks the 109bp EcoR1-BamH1 fragment and does not complement ftsQ, implying that ftsQ must start or end within this region. The KpnI deletion, pNS14 also does not complement ftsQ suggesting that some of the structural gene of ftsQ lies within this fragment.

4.3 The direction of transcription of ftsQ

The plasmid pDK302 will complement ftsQ. If the 2.2Kb EcoR1 fragment is cloned into pKO1 in the reverse orientation, as in

FIGURE 4.1 Location of ftsQ.

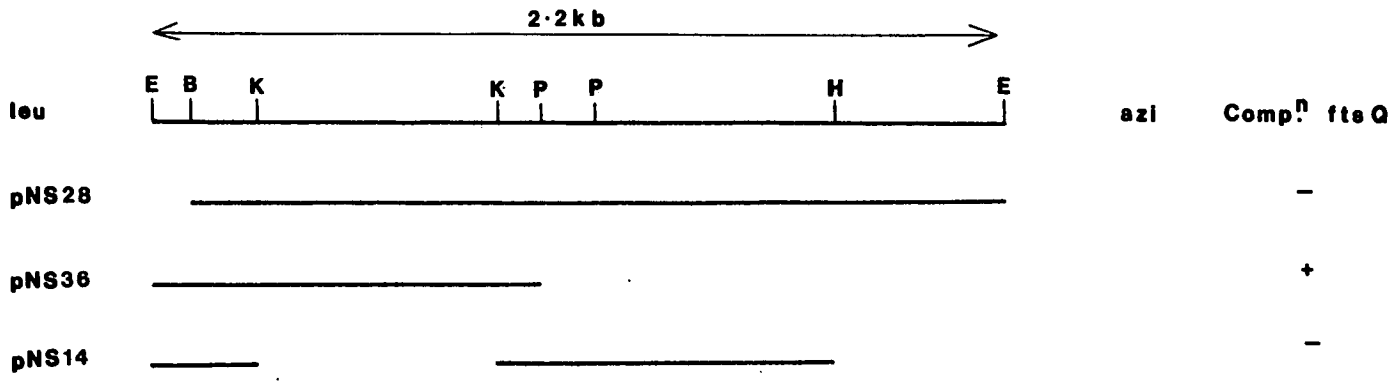


FIGURE 4.2a. Construction of pNS14.

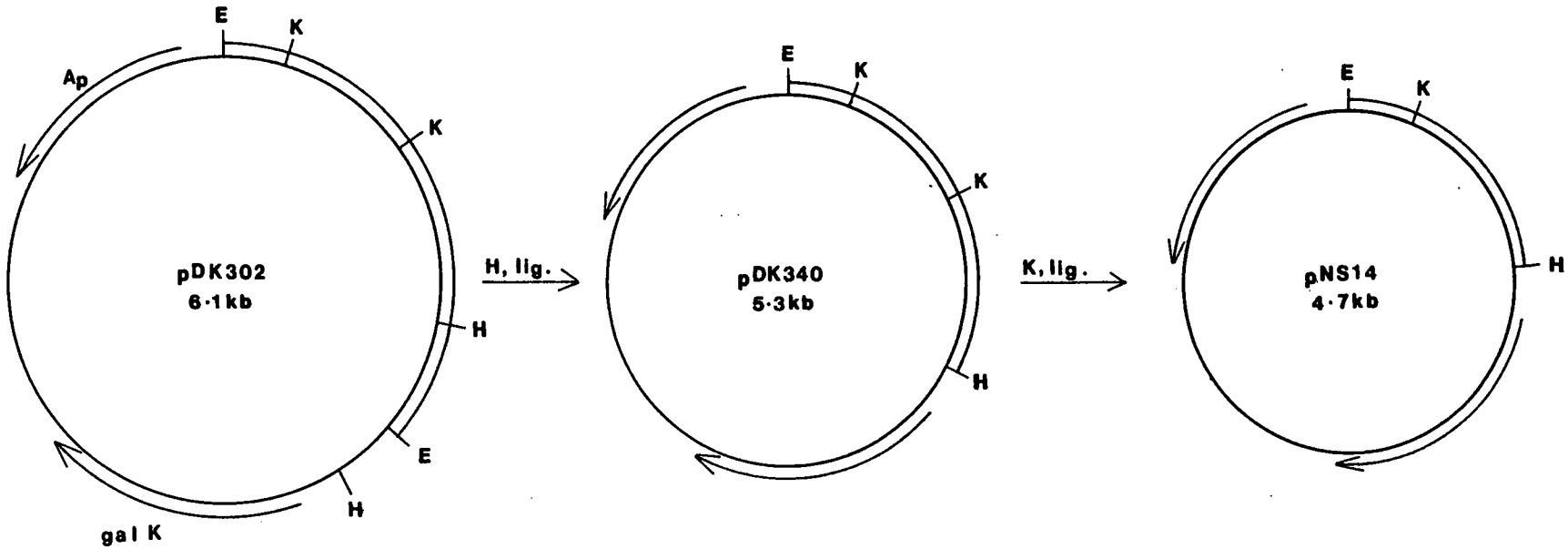
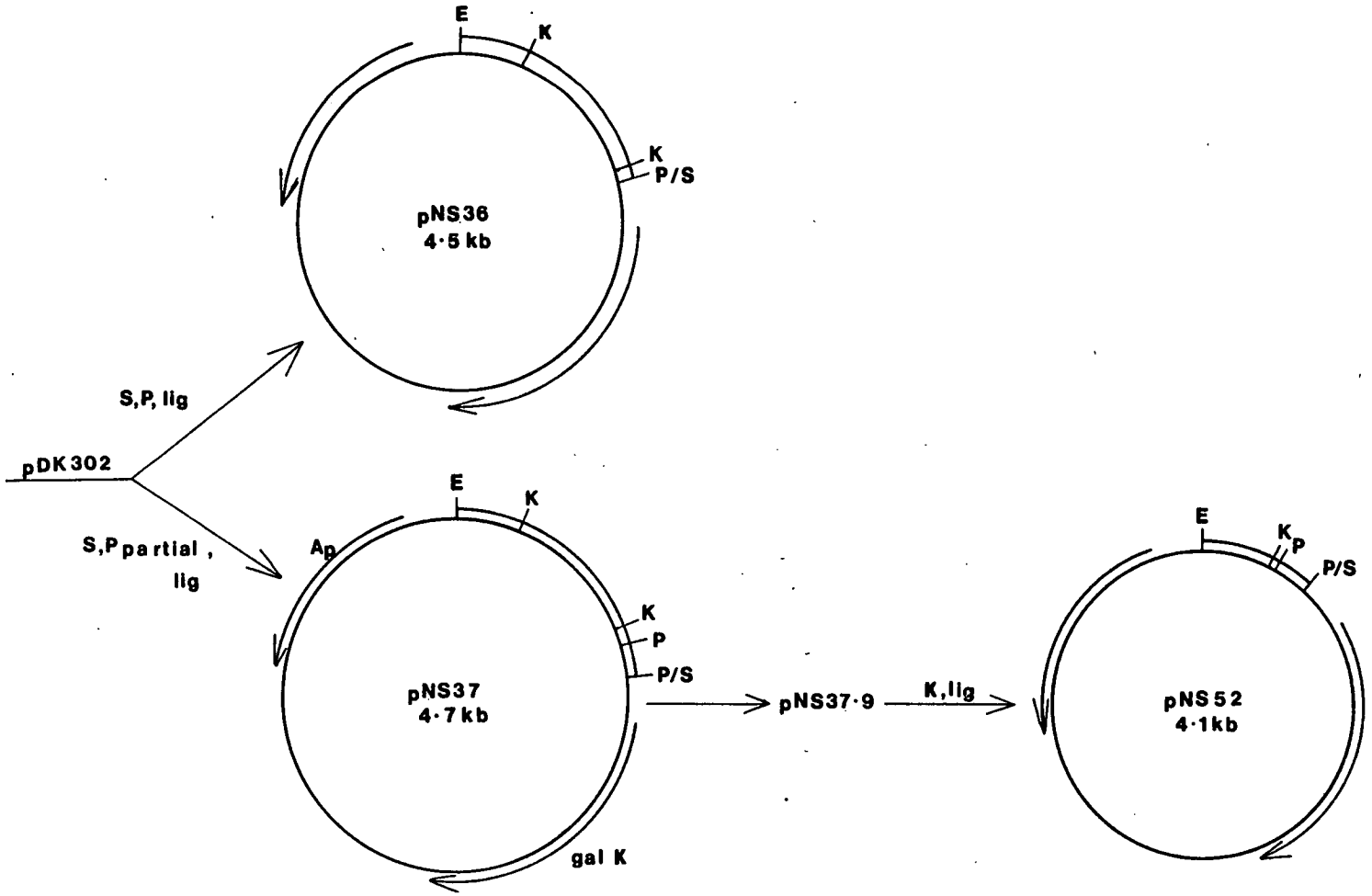


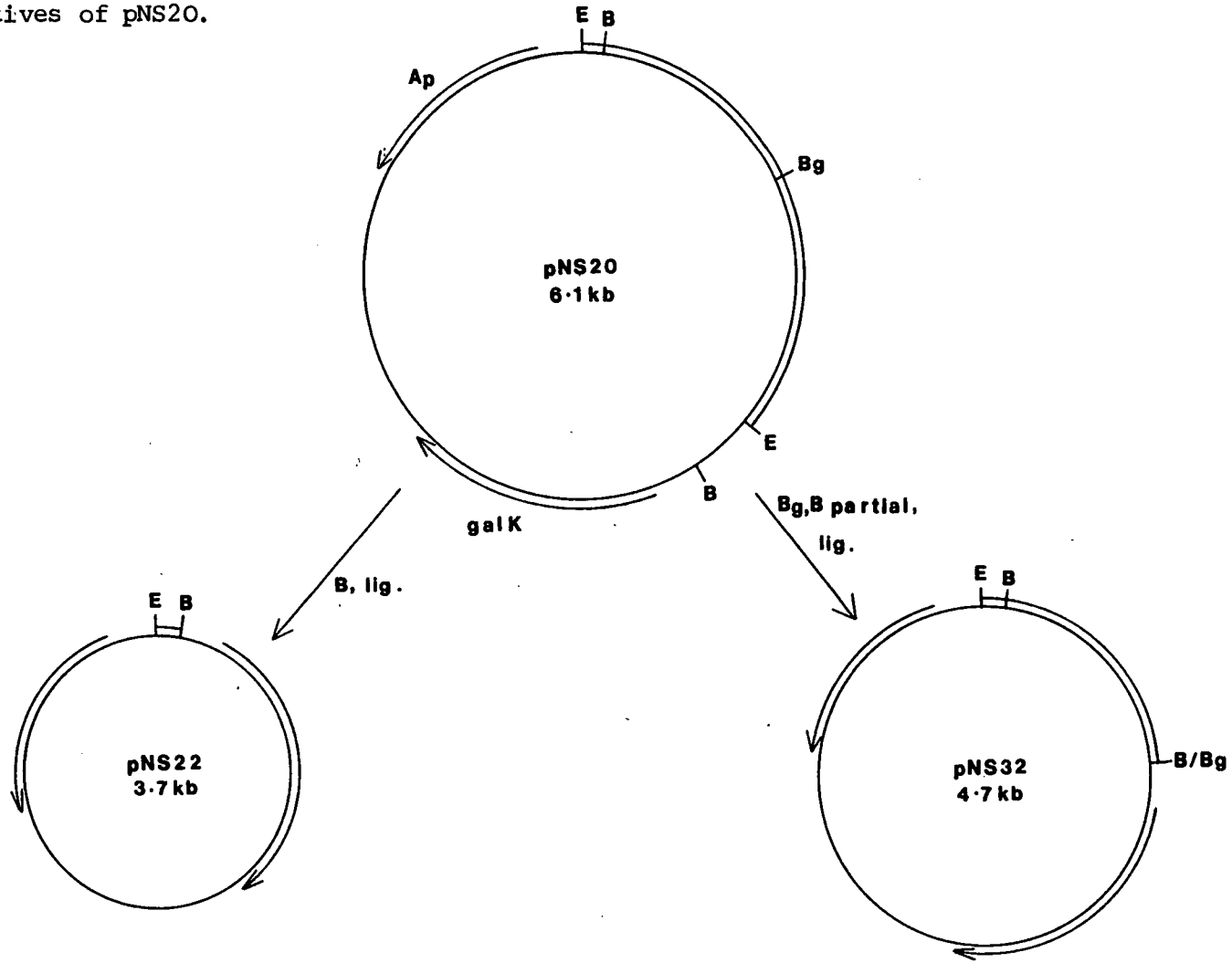
FIGURE 4.2b. Construction of pNS36, 37 and 52.



pGH300 (G. Hatfull, Ph.D. Thesis, 1981) then complementation of ftsQ is abolished. This makes it unlikely that the promoter of ftsQ is on the fragment and thus unlikely that ftsQ transcribes from right to left. Only part of the ftsQ promoter region may be at the lefthand end of the 2.2Kb EcoR1 fragment and transcription from the vector (see Appendix 1) allows complementation of ftsQ. Consistent with this pNS27 does not complement ftsQ. Here transcription from the vector is reduced by the transcription termination sequence which precedes the insert. Since the 109bp EcoR1-BamH1 fragment may contain part of the promoter of ftsQ the 2.2Kb EcoR1 fragment was inserted into pKO4 to give pNS20, with the insert orientated as in pNS27. Subsequent BamH1 digestion and religation gave the 109bp EcoR1-BamH1 fragment cloned next to galK (pNS22; Figs. 4.3, 4.4). This plasmid gave white colonies on Mac gal plates and no galactokinase activity. A sequence analysis has revealed a possible ribosome binding site on the EcoR1-BamH1 fragment but no promoter (G. Hatfull, Ph.D. Thesis, 1981; Robinson et al., 1983). Promoter activity reading rightward has been detected close to the EcoR1 site on the DNA to the left of the ftsQ structural gene (D. Kenan, unpublished results) and this region contains a reasonable consensus promoter sequence (Robinson et al., 1983).

Taken together these observation are consistent with the hypothesis that the promoter of ftsQ is upstream of the EcoR1 site and that ftsQ transcribes from left to right.

FIGURE 4.3. Derivatives of pNS20.



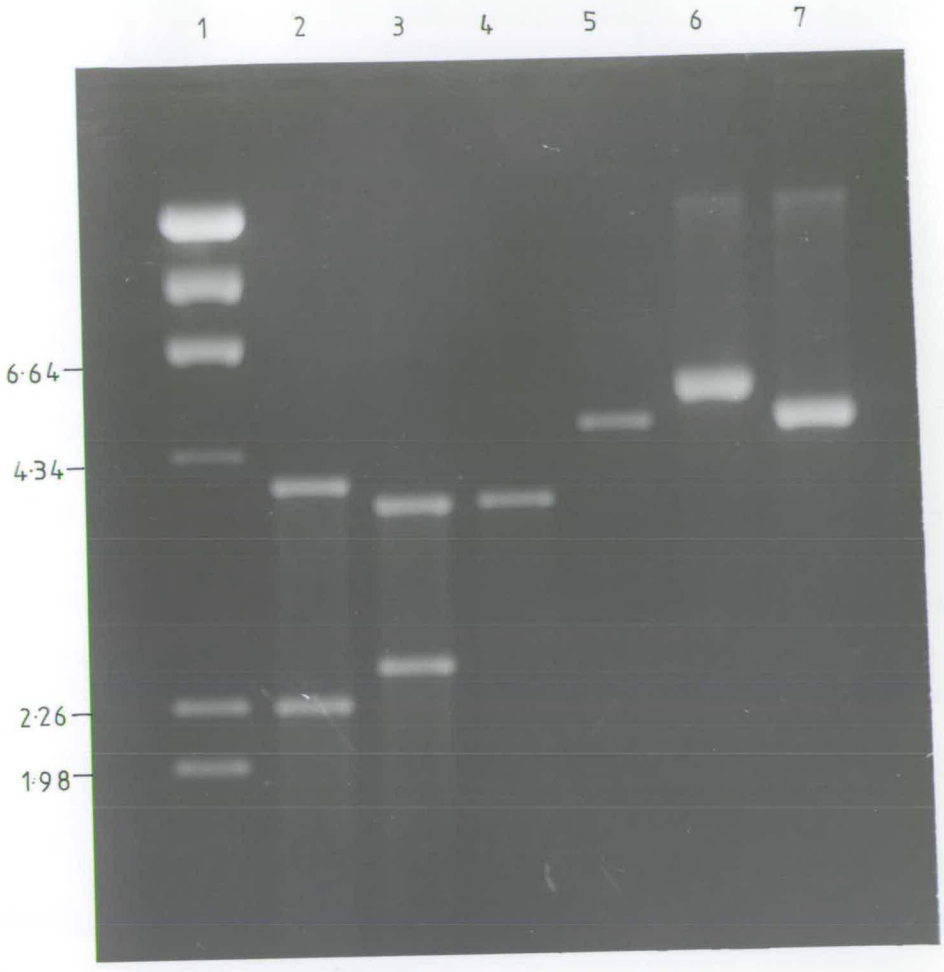

1
2
3
4
5
6
7
6·64
4·34
2·26
1·98

Fig. 4.4

Track	DNA	Enzyme	Refer to Fig
1	λcI_{857}	HindIII	-
2	pNS20	EcoR1	4.3
3	pNS20	BamH1	"
4	pNS22	BamH1	"
5	pNS32	BamH1	"
6	pDK340	EcoR1	4.2a
7	pNS14	EcoR1	"

Note: 1) In tracks 6 and 7 the DNA used is from a mini plasmid preparation (2.5.1d)

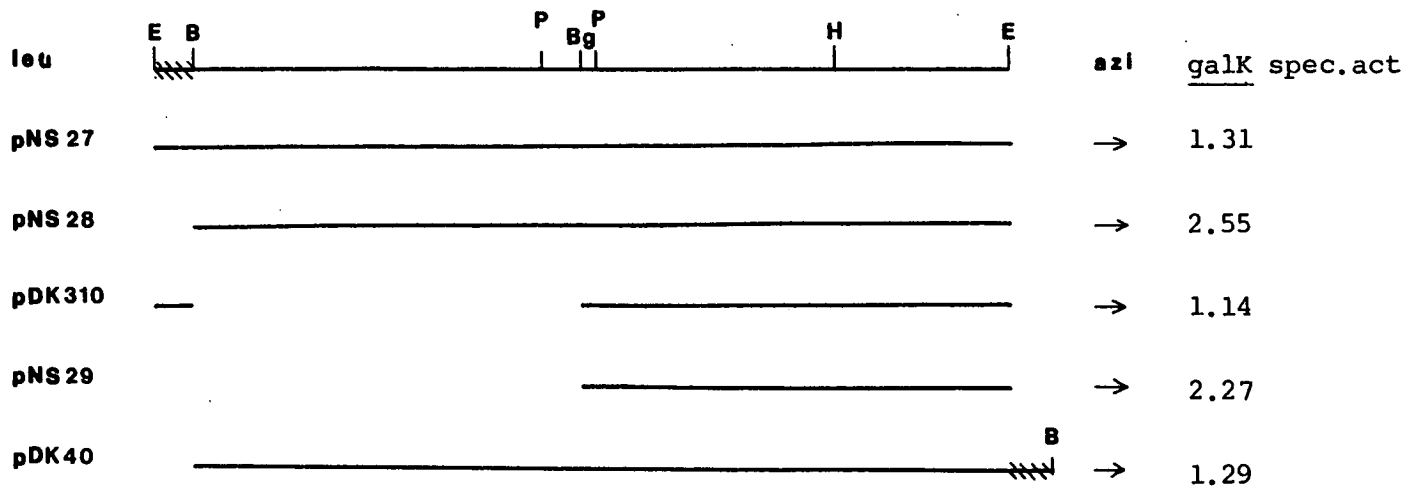
4.4 A transcriptional effect due to the 5' end of ftsQ

Extending the cloned fragment in pNS28 with 109bp of its upstream chromosomal DNA (pNS27) causes a 51% reduction in the galactokinase activity (Fig. 4.5). Both constructions complement ftsA, neither complement ftsQ and both have the same downstream fusion junction, eliminating any effect of differential galK translation. If we compare pNS29 with pDK310 (a BamHl-BglII deletion of pDK302; D. Kenan, unpublished) a similar effect is observed (50% reduction, Fig. 4.5). Neither of these constructions complement either ftsQ or ftsA. Taken together these results eliminate any effect due to ftsQ or ftsA transcription or translation. A common factor is the 5' end of the ftsQ mRNA, which in each case is fused to a different 3' end. Within this region is a 6bp stem with a 4 base loop (Robinson et al., 1983). Shifting the 109bp EcoRl-BamHl fragment to the downstream end of pSN28 (as in pDK40) still causes a reduction in galK expression although this may reflect termination or pausing of ftsZ transcription due to the now downstream stem-loop structure. Whilst the basis of this reduction remains unexplained it is perhaps conceivable that a secondary structure adopted by the 5' end of ftsQ mRNA might affect transcription from the ftsZ promoters.

4.5 The ftsQ protein

Experiments designed to identify proteins from this region did not reveal a candidate for the ftsQ protein (Lutkenhaus and Wu, 1980). The recent availability of plasmid vectors containing controllable promoters has made it possible to control the expression of

FIGURE 4.5. The EcoR1-Bam H1 effect.



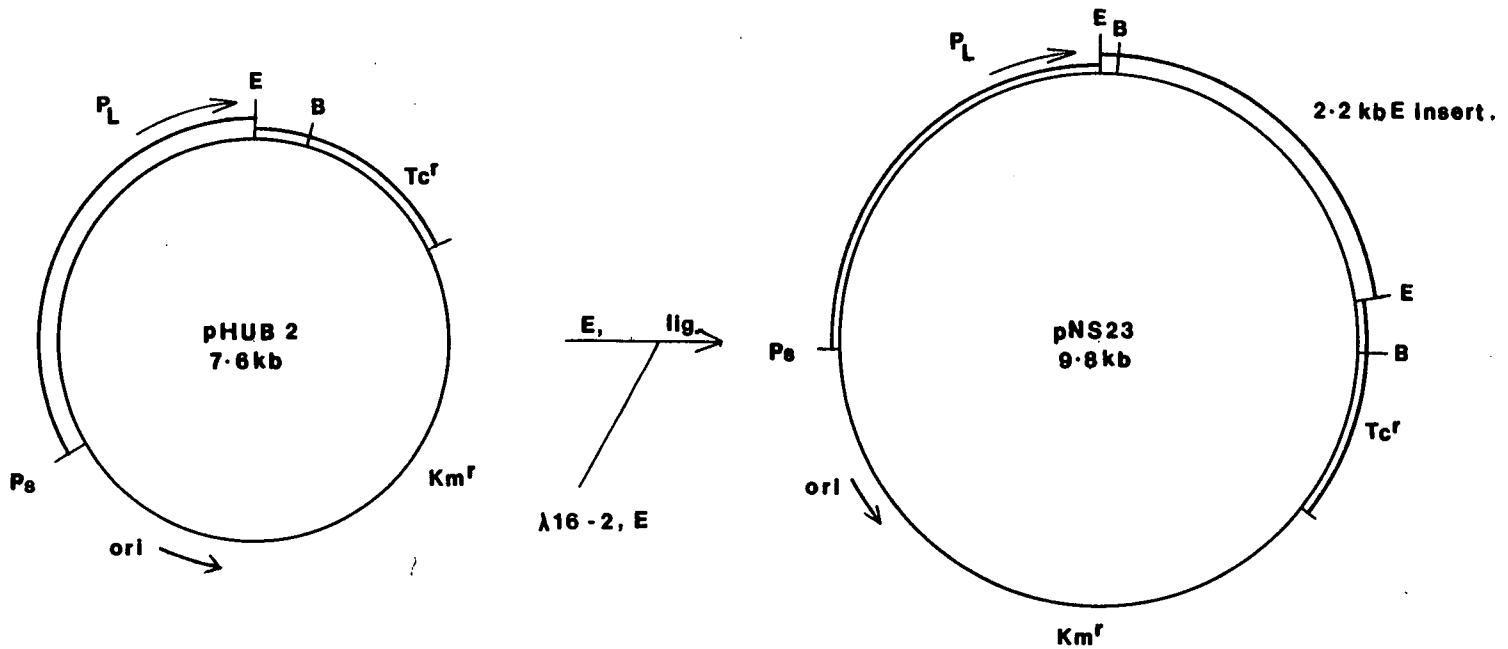
cloned genes. Cloning the 2.2Kb EcoR1 fragment in the correct orientation downstream of a controllable promoter would enable (a) identification of the ftsQ protein using the ftsA protein as an internal standard and (b) give the potential to turn cell division genes on and off to observe the effect of their expression on the cell. The system chosen was that of Bernard et al. (1979). This involves the use of a multiple copy ColE1 type cloning vehicle, pHUB2 (Km^R) which contains an EcoR1 cloning site downstream of the phage λ promoter P_L. The promotional activity of P_L is uninduced at low temperature (30°C) in the presence of a cI t.s. gene that specifies a t.s. λ repressor and can be activated by heat induction (42°C). The cI t.s. gene is maintained on a second plasmid pRK248 (Tc^R) which is compatible with the cloning vehicle.

4.6 Cloning the 2.2Kb EcoR1 fragment into pHUB2

EcoR1 digested λΔE was ligated with EcoR1 digest pHUB2 and transformed into a $c600K^-$ λ wt lysogen at 37°C. The use of the lysogen ensures repression of P_L. One recombinant, designated pNS23 had the 2.2Kb EcoR1 fragment inserted such that ftsZ and P_L transcribed in the same direction. A total of seven identical recombinants were obtained in separate experiments, but the insert was always found to be in the same orientation (Fig. 4.6, 4.7).

To check the complementation of ftsQ and ftsA by pNS23, λ wt lysogens of TOE1 and TOE13 were constructed. The plasmid was found to complement both mutations in the absence of P_L induction. Complementation of ftsQ is perhaps surprising, but may be due to slight leakage of transcription from P_L or transcription from the

FIGURE 4.6. Construction of pNS23.



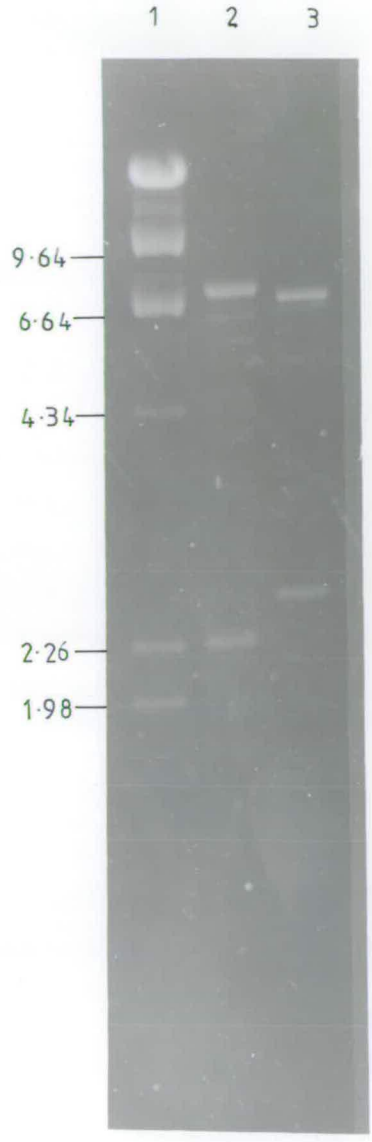

1
2
3
9·64
6·64
4·34
2·26
1·98

Fig. 4.7

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pNS23	EcoR1	4.6
3	pNS23	BamH1	"

plasmid origin as with the pKO vectors (Appendix 1).

4.7 Analysis of proteins encoded by pNS23

In the absence of an enzymatic assay for any of the gene products involved SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect plasmid encoded protein. In order to reduce the background of host proteins and eliminate possible disturbance of cell metabolism by overproduction of essential cell division proteins, minicells were used for this experiment. Minicells should contain cI t.s. repressor from pRK248, so incubation of minicells at 42°C during the labelling procedure will cause P_L induction.

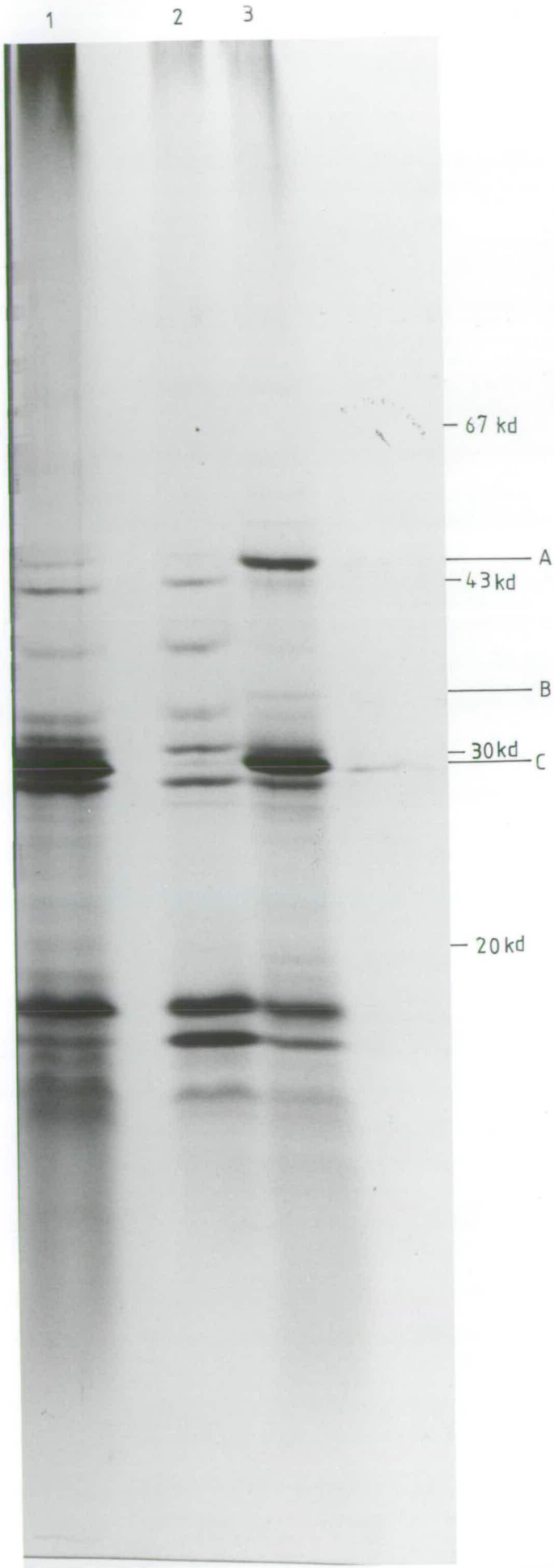
The minicell strain DS410 bearing (a) pRK248, (b) pRK248 and pHUB2 and (c) pRK248 and pNS23 was grown at 30°C and minicells prepared. Labelling (at 42°C) was carried out using ^{35}S methionine. Two proteins over and above those expressed from pHUB2 and pRK248 can be seen in Figure 4.8, one of 36Kd and one of 50Kd, the latter presumably being the ftsA protein (Lutkenhaus and Donachie, 1979). The 36Kd protein could either be the ftsQ gene product or a fusion product between the start of ftsZ and the vector. To distinguish between these possibilities a KpnI deletion was made on pNS23 to give pVD1. In minicells this plasmid shows the proteins encoded by pRK248 and pHUB2 but has lost both the 36Kd and the 50Kd protein. Loss of the 50Kd protein confirms the complementation data (Section 4.10) and indicates that ftsA extends to within the 600bp KpnI fragment. Loss of the 36Kd protein suggests that this is not an ftsZ-plasmid fusion product, but the product of the ftsQ gene.

Fig. 4.8

An autoradiogram of a 10-20% polyacrylamide gel showing proteins labelled in minicells containing various plasmids.

Track	Plasmid
1	pHUB2; pRK248
2	pRK248
3	pNS23; pRK248

Band	Product	Size (Kd)
A	*ftsA*	49
B	*ftsQ*	36
C	Km^R	29


1
2
3
67 kd
A
43kd
B
30kd
C
20kd

I am greatly indebted to Vicky Derbyshire for labelling of minicells, SDS PAGE and the pVD1 construction.

4.8 Is P_L functional?

The expression of the apparently P_L directed *ftsQ* and *ftsA* products is less than that of the non P_L directed Kanamycin gene (See Fig. 4.8). In addition expression of *ftsQ* is much lower than that of *ftsA*. From the available information all necessary signals should be present for *ftsQ* translation from P_L. The simplest explanation for these results is that P_L is not functional and that *ftsA* is transcribed from its own promoter. Lack of P_L expression may be attributed either to the creation of a transcription termination signal at the junction of P_L and *ftsQ* or a similar signal close to the N terminal end of *ftsQ*. Since pHUB2 is λN^-, P_L will be incapable of overcoming transcription termination signals. Alternatively, P_L may indeed be functional, and the low level of protein is due to poor translation of *ftsQ* and *ftsA* mRNA in minicells.

Whether P_L is functional or not this experiment has demonstrated that the product of the *ftsQ* gene is a polypeptide of 36Kd as measured by SDS-PAGE and has confirmed its location. The *ftsA* gene has been shown to traverse into the *KpnI* fragment.

4.9 The 'actual' size of *ftsQ*

Based on an average molecular weight for an amino acid of 110d in *E. coli* (R. Ambler, pers.comm.) the 36Kd *ftsQ* product would cover approximately 980bp. This would mean that the *ftsQ* gene extended

beyond the first PvuII site (Section 1.2.2.f), thus contradicting the complementation data. This also implies that the structural genes of ftsA and ftsQ must overlap since ftsA extends to within the KpnI fragment. Sequence analysis of this region indicates an open reading frame coding for either a 22Kd (J. Lutkenhaus, pers. comm.) or 32Kd (A. Robinson, pers.comm.) protein. A protein of either size is consistent with the complementation data and does not necessarily imply that ftsQ and ftsA overlap. The mobility of proteins in SDS gels is notoriously variable. For the majority of proteins the number of charged residues affects the ability to bind SDS and hence the mobility at a given pH (R. Ambler, pers.comm.; Jaurin and Grundstrom, 1981). In one particular case, that of ompA protein an increase in molecular weight due to heating has been observed. This is attributed to an increase in molecular asymmetry rather than to increased binding of SDS (Di Rienzo et al., 1978). It is likely that although the ftsQ protein is represented by a 36Kd protein on an SDS gel, this molecular weight is a consequence of either heating or binding of SDS, and that the actual size of the ftsQ product is somewhat less than this.

4.10 The location of ftsA

Lutkenhaus and Donachie (1979) demonstrated that the HindIII site within the 2.2Kb EcoR1 fragment cuts ftsA, and that ftsA must extend at least 135 nucleotides into the rightmost 480bp HindIII-EcoR1 fragment (Lutkenhaus and Wu, 1980). In addition, pDK340 does not complement ftsA (D. Kenan, unpublished results). To the left

pNS29 does not complement *ftsA* whilst pNS28 does complement. Thus the leftmost end of *ftsA* must be within the 930 bp BamH1-BglII fragment. This extremity of *ftsA* can be further localized with pDK320 (a KpnI deletion of pDK302) which does not complement *ftsA* (D. Kenan, unpublished result). It is thus likely that *ftsA* begins or ends within the 600bp KpnI fragment.

4.11 Location of the *ftsA* promoter; direction of transcription

There are several lines of evidence to suggest that the *ftsA* gene can form an independent functional unit within the 2.2Kb EcoR1 fragment. Firstly, a λ transducing phage bearing *ftsA* (λJFL40) can complement *ftsA* independent of neighbouring genes (Lutkenhaus *et al.*, 1980). Secondly, both pDK302 and pGH300 can complement *ftsA*, that is, *ftsA* is expressed independent of transcription from the pKO vector and so probably has its own promoter within the 2.2Kb EcoR1 fragment. Thirdly, pNS23 produces *ftsA* protein but the Kpn1 deletion plasmid pVD1 does not. If *ftsA* transcribed right to left we might expect to see either a truncated *ftsA* protein or fusion protein. That we do not suggests either that this new protein is unstable and rapidly degraded in minicells or more likely that the KpnI deletion removes the *ftsA* promoter and *ftsA* transcribes from left to right. Furthermore, Lutkenhaus and Wu (1980) assigned a direction of transcription of left to right for *ftsA*, albeit on the indirect evidence of reduced transcription from a λ transducing phage after P_L induction. Direct gene fusion evidence was required to unambiguously assign the direction of transcription of *ftsA*. Based on the above data and the

complementation tests the *ftsA* promoter must either be on the 600bp *Kpn*I fragment reading left to right, or on the 480bp *Hind*III-*Eco*R1 fragment reading right to left. The plasmid pNS36 contains within it the *Kpn*I fragment reading left to right but gives a white colony on Mac gal plates and when assayed shows no promoter activity (Figs. 4.9, 4.11). The *Hind*III-*Eco*R1 fragment was cloned reading right to left by making a *Hind*III deletion of pGH300 giving pNS45. This also gives a white colony on Mac gal plates and shows no promoter activity (Figs. 4.9, 4.10, 4.11). Where then is the *ftsA* promoter?

4.12 Isolation of 'up' mutations

pKO derivatives which do not contain a promoter and hence give white colonies on Mac gal plates cannot complement a *galK*$^-$ host. This also means that they cannot grow on minimal media containing galactose. It is possible therefore, to isolate spontaneous *gal*$^+$ revertants; clones which can grow on galactose and hence complement the *galK* mutation.

Since both pNS36 and pNS45 give white colonies these plasmids could be used for selection of 'up' mutations. In the interest of further characterization a slightly larger plasmid than pNS36 was used. This plasmid, pNS37 extends from the lefthand *Eco*R1 to the second *Pvu*II site and was constructed from pDK302 by *Sma*I digestion followed by a *Pvu*II partial digest and subsequent ligation in dilute solution. This plasmid was used to facilitate subcloning of potential mutant fragments.

FIGURE 4.9. Location of the ftsA promoter, Mutant isolation.

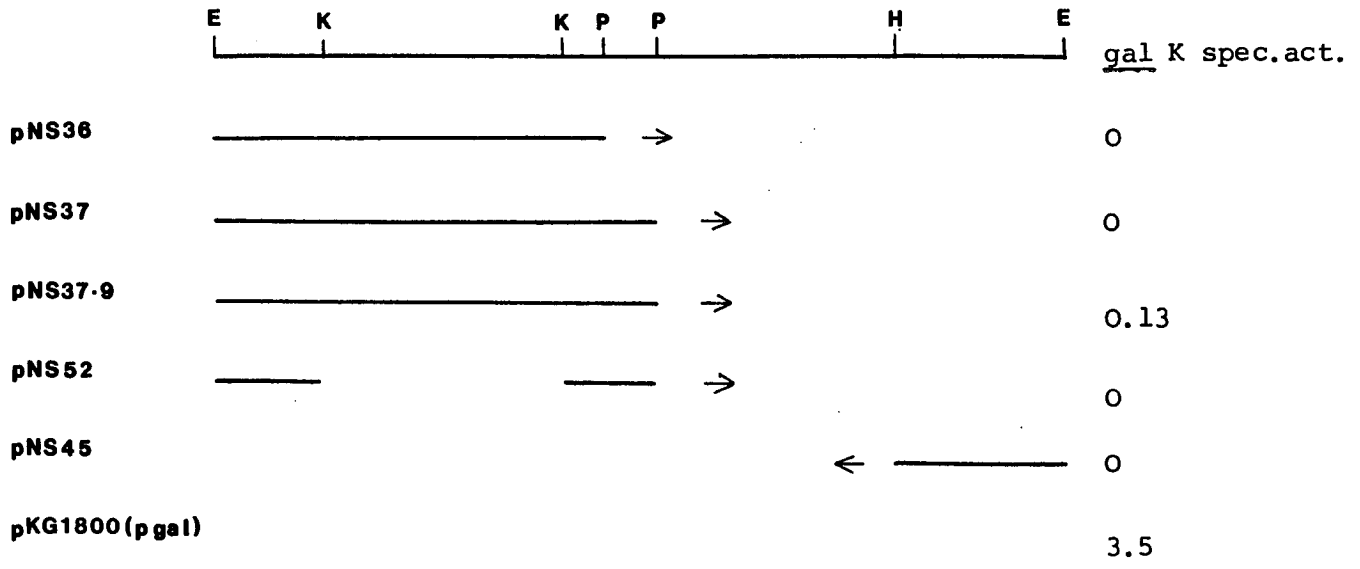
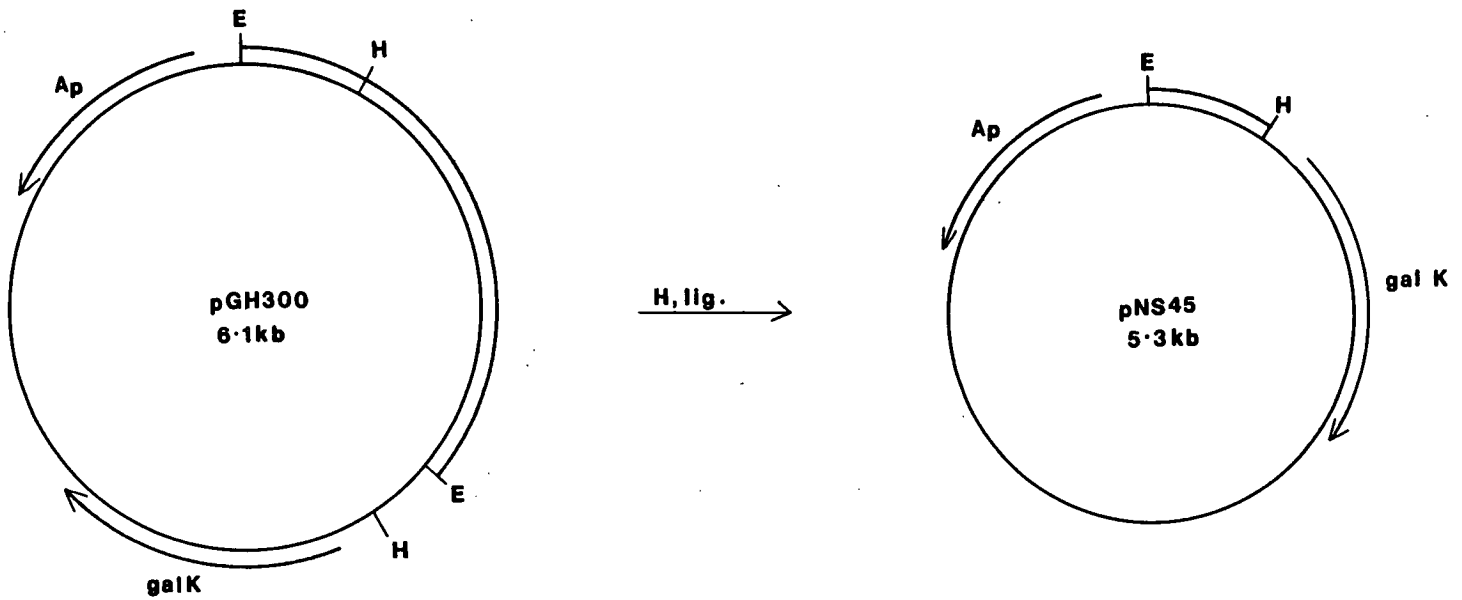
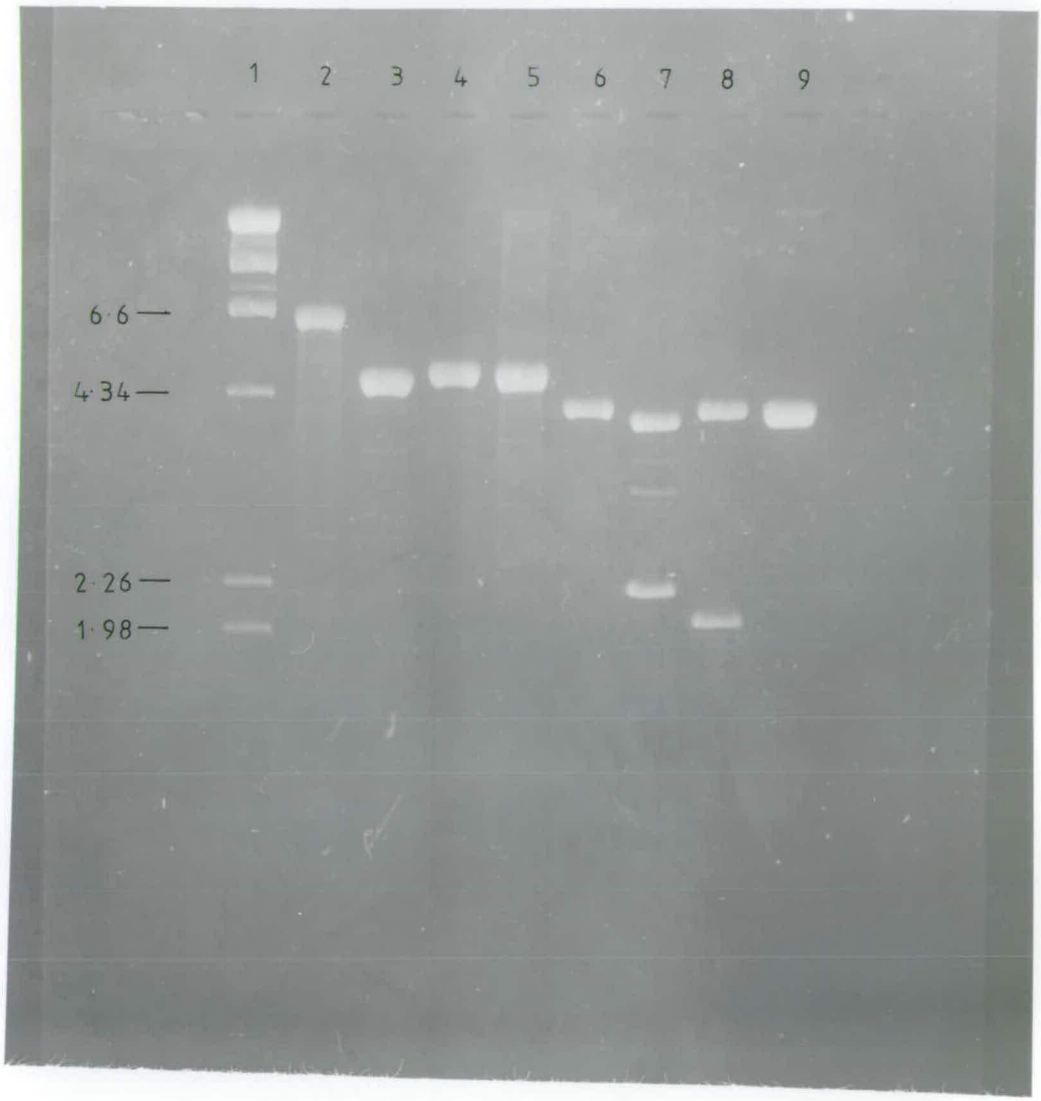


FIGURE 4.10. Construction of pNS45.

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6
7
8
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6·6
4·34
2·26
1·98

Fig. 4.11

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pDK302	PstI	4.2a
3	pNS36	EcoRl	4.2b
4	pNS37	EcoRl	"
5	pNS37.9	EcoRl	"
6	pNS52	EcoRl	"
7	pGH300	EcoRl	4.10
8	pGH300	HindIII	"
9	pNS45	HindIII	"

'Up' mutations were isolated and initially characterized as described in Chapter 2. Such mutations were only obtained with pNS37, none with pNS45. Neither pNS37 nor pNS45 complement *ftsA*. One mutation pNS37.9 which gave a red colony on Mac gal plates was selected for further study. The galactokinase level (See Fig. 4.9) indicated that the increase in transcription was small, only 4% of *pgal*. A *KpnI* deletion of pNS37.9 gave pNS52 (Fig. 4.2). This plasmid gave a white colony on Mac gal plates indicating that the mutation, whatever its nature must be in the 600bp *KpnI* fragment.

The increase in transcription in pNS37.9 could have a number of causes, either (a) a true promoter 'up' mutation, or (b) an *ftsA* 'attenuator' down mutation, or (c) an *ftsQ* terminator down mutation (if indeed a terminator exists and if *ftsA* and *ftsQ* overlap or (d) an up or down mutation in a binding site for a regulatory molecule. Isolation of more mutations, sequencing and *in vitro* transcription of these should reveal the nature of this region and hopefully lead to placement of the *ftsA* promoter.

There may be several reasons for a lack of assayable transcription from the region cloned on pNS36. (1) the promoter may be genuinely weak, (2) transcription may be masked by an *ftsQ* terminator or an *ftsA* attenuator (see above), (3) an accessory factor may be required, or (4) the promoter may be periodically expressed, i.e. only at a specific point in the cell cycle.

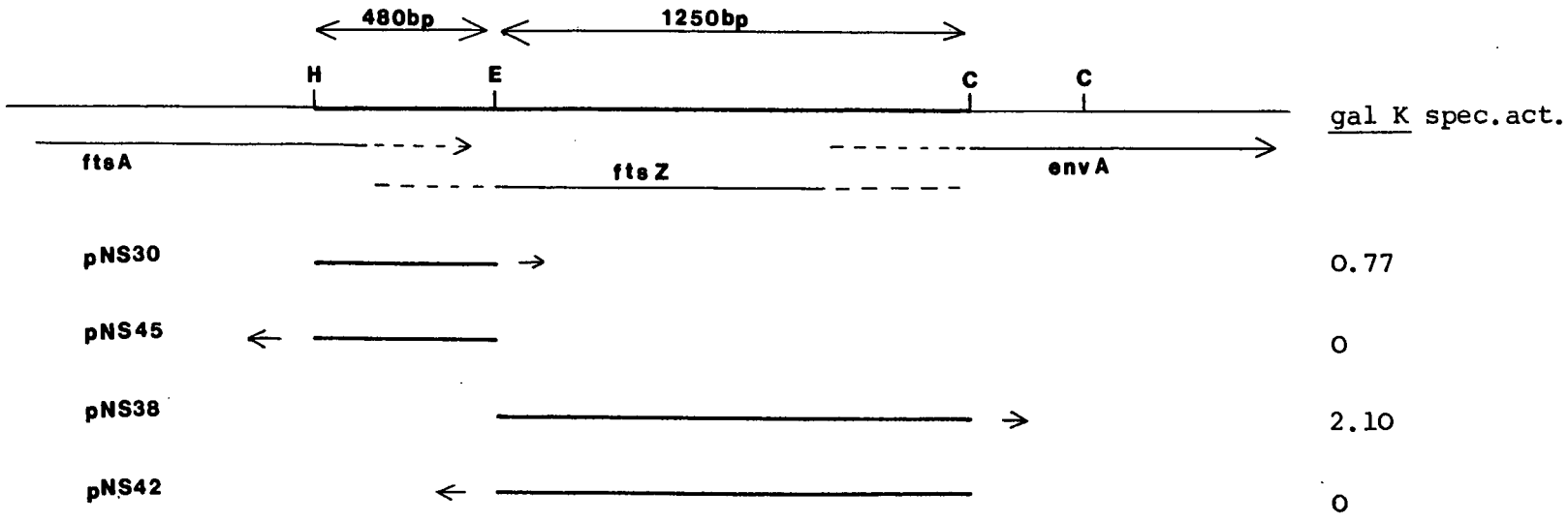
4.13 The direction of transcription of ftsZ

The ftsZ gene has been located between ftsA and envA by the use of λ transducing phages (Lutkenhaus et al., 1980). The direction of transcription has been inferred from phage encoded protein synthesis as clockwise on the E. coli map (Lutkenhaus and Wu, 1980). This has been confirmed using gene fusions (Fig. 4.12).

The 480bp HindIII-EcoR1 fragment has been cloned in both orientations. pNS30 shows a promoter reading left to right. In the opposite orientation there is zero promoter activity, less than the basal level of pKO transcription (0.34 units). This may reflect opposing transcription, i.e. RNA polymerases originating from the vector are stopped by RNA polymerase molecules transcribing in the opposite direction (Ward and Murray, 1979).

The EcoR1-ClaI fragment has also been cloned in both orientations (see Chapter 9). In pNS38 there is promoter activity attributable to the envA promoter whilst in the opposite orientation (pNS42) there is zero promoter activity. If ftsZ transcribed anticlockwise on the E. coli map then we might expect pNS42 to show promoter activity. Clearly it does not, suggesting that the promoter activity observed in pNS30 belongs to ftsZ and that this gene transcribes from left to right.

FIGURE 4.12. The Direction of ftsZ.



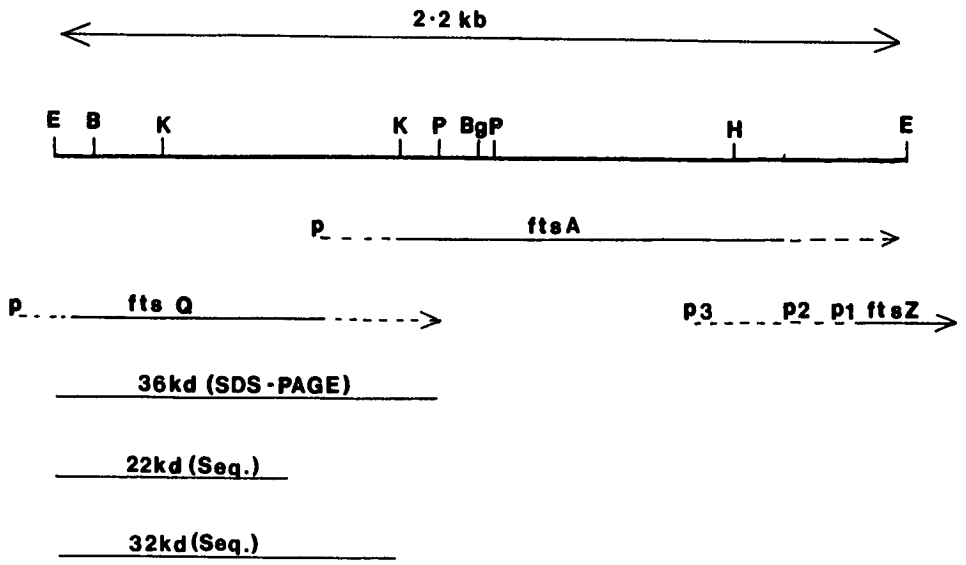
4.14 Summary (Fig. 4.13)

From the complementation data ftsQ extends from within the 109bp EcoR1-BamH1 fragment until before the first PvuII site (maximum 940bp). Both the -35 and -10 regions of ftsQ lie to the left of the EcoR1 site (approximately 120bp away) (Robinson et al., 1983) and the putative AUG start lies 20bp to the right of this site. The ftsQ gene transcribes left to right. The ftsQ gene product has been identified as a 36Kd protein by SDS-PAGE. Sequence data suggests that the protein is probably nearer either 22 or 32Kd.

For the ftsA gene the complementation data suggests that its leftward extent is within the 600bp KpnI fragment and its rightward extent within the 480bp HindIII-EcoR1 fragment. Complementation data suggests that ftsA must have a promoter since it can be expressed independent of neighbouring genes, although no promoter has been detected in pKO gene fusions. Lutkenhaus and Wu (1980) produced indirect evidence to suggest that ftsA transcribes left to right. Protein studies suggest that ftsA probably starts within the 600bp KpnI fragment and promoter up mutations have been obtained for this region. A promoter reading right to left from the 480bp HindIII-EcoR1 fragment has not been detected. Thus there is no direct evidence for location of the ftsA promoter and its direction of transcription although circumstantial data suggests that the promoter is on the 600bp KpnI fragment and that the ftsA gene reads left to right in common with its neighbours.

Consistent with the indirect evidence of Lutkenhaus and Wu (1980) gene fusion studies have confirmed the direction of ftsZ and suggest that its promoter reads left to right from the 480bp HindIII-EcoR1 fragment.

FIGURE 4.13. Gene Organization in the ftsA region.



CHAPTER 5

cAMP IN THE REGULATION OF CELL DIVISION

5.1 Introduction

The *cya* and *crp* loci may be regarded as cell division genes (see Section 1.4). Kumar (1976) noticed that *cya* or *crp* mutations conferred a cocco-bacillary shape on cells containing these lesions. Utsumi *et al*. (1981, 1982) reported that in strain PA3092 (fic) filamentation could be induced at 42°C with the addition of exogenous cAMP. These observations suggest that cAMP may act negatively to repress septation.

The cAMP-CRP complex can bind to DNA and exert either a positive or negative effect on transcription. Since cell division promoters have been cloned into the pKO system (Table 5.1) it seemed reasonable to screen these promoters for transcriptional effects via cAMP.

5.2 Strains used

For this experiment two strains isogenic with c600K$^-$ were constructed (see appendix 2) (a) NFS3; c600K$^-$ *cya*$^\Delta$ and (b) NFS4; c600K$^-$ *crp*$^-$

5.3 Treatment of results

Normalised galactokinase activities were determined as described in Appendix 1. The values for pKO1 and pKOC1 (0.34 and 0.46 respectively) were subtracted from the galactokinase activities obtained in c600K$^-$ to correct for transcription from the vector. In NFS3 slightly lower values were obtained for pKO1 (0.27) and pKOC1 (0.38). This may be due to an effect of cAMP on plasmid replication (Katz *et al*., 1973), although the β-lactamase assays performed in this thesis (Appendix 1) showed no difference in copy

Table 5.1

Cloned Promoter	Plasmid	Chapter
ddl/*ftsQ*	pDK30	D. Kenan, unpublished results
ftsA/*ftsZ*	pNS20	4
envA	pNS38	9
X	pNS41	9

number between pNS20 and pKO1 in isogenic *cya*$^{+}$ and *cya*$^{\Delta}$ strains. Within the 11% error obtained in β-lactamase assays very small changes in copy number may not be detected. The effect of growth rate on copy number is discussed in Appendix 1.3.

For pDK30, pNS20 and pNS30 the transcriptional correction factor for pKO1 was subtracted, for all other plasmids the value for pKOC1 was used.

A ratio of mutant:wild type indicates the effect on transcription from cloned fragments. A ratio of 1 indicates no effect whilst a ratio larger than this indicates an overall negative effect on transcription. A lower ratio may be due either to positive control or most likely an effect on plasmid copy number.

5.4 Results I

Table 5.2 shows that in an assay of cloned promoters transcription from the *ftsA*/*ftsZ* 2.2 Kb *Eco*R1 fragment is increased 2.4 times in the absence of cAMP. All other promoters tested show a slight decrease in the level of transcription. This result strongly suggests that in the wild type cell transcription from the *ftsA*/*ftsZ* region is repressed either directly or indirectly by cAMP, implicating cAMP as a negative effector in the control of normal cell division.

If exogenous cAMP is added to the medium then the effect can be reversed (Table 5.3) hence compensating the *cya* defect. The addition of cAMP to the c600K^{-} wild type reduced transcription to 80% of the level without cAMP.

The cAMP-CRP complex has different affinities for different promoters (Lis and Schleif, 1973; Piovant and Lazdunski, 1975).

Table 5.2 Assay of cloned promoters

Promoter	Strain	Plasmid	Units galK	cya$^{\Delta}$/cya^{+}
ddl/ftsQ	c600K^{-}	pDK30	1.45	0.77
	NFS3	pDK30	1.12	
ftsA/ftsZ	c600K^{-}	pNS20	1.43	2.39
	NFS3	pNS20	3.06	
envA	c600K^{-}	pNS38	2.19	0.77
	NFS3	pNS38	1.72	
x	c600K^{-}	pNS41	1.66	0.68
	NFS3	pNS41	1.20	

The induction of the lac, ara and tna operons responds differently to different concentrations of cAMP, which suggests that the cAMP-CRP complex is in equilibrium with dissociated cAMP and CRP. Increasing the concentration of cAMP would then increase the formation of active cAMP-CRP complex, permitting increased repression of transcription from this region.

In a crp$^-$ strain an increase in transcription is observed (Table 5.4). This suggests that the cAMP-CRP complex is involved in mediating this negative effect on transcription. The level of derepression observed does not reach that of the cya deletion, presumably because the crp allele used (a point mutation) is slightly leaky. Thus in a crp$^-$ strain (NFS4) there will be less CRP protein to complex with cAMP, less inhibition of division and therefore shorter cells.

Thus cAMP has been shown to affect transcription from a region of DNA containing the control elements of ftsA and ftsZ. Four neighbouring promoters in this cluster are not affected by cAMP (ddl, ftsQ, envA, X). The effect can be reversed with exogenous cAMP and is dependent on the CRP protein, implicating the cAMP-CRP complex.

The fic strain PA 3092 was obtained from R. Utsumi. Both this strain and a gal deletion derivative (Ken 17; constructed by Ken Begg) were unable to grow in the minimal medium necessary for galactokinase assays. Attempts to transduce the fic mutation to a different background (c600K$^-$) proved unsuccessful (K. Begg, pers. comm.).

Table 5.3 Reversal with exogenous cAMP[1]

Strain	Plasmid	Units galK	cya^{Δ}/cya^{+}
$c600K^{-}$	pNS20	1.33	1.37
NFS3	pNS20	1.55	

[1] Cells were grown overnight in fructose minimal media and then subcultured into the same media containing 2 mM cAMP (Sigma). Assays were performed as before.

Table 5.4 The effect of CRP

Strain	Plasmid	Units galK	crp^{-}/crp^{+}
$c600K^{-}$	pNS20	1.13	1.87
NFS4	pNS20	2.11	

5.5 Results II. Further localisation of the cAMP effect

There are at least four promoters known to be on this 2.2 Kb EcoR1 fragment, PA (the promoter of ftsA; see Chapter 4), Pz_1, Pz_2 and Pz_3, the promoters of ftsZ (see Chapter 3). Table 5.5 shows the effect of cAMP on three pairs of constructs from this region (summarised in Fig. 5.1).

The plasmid pNS22 shows no transcriptional effect of cAMP. This, together with the result obtained with pDK30 effectively eliminates the entire control region of ftsQ from participation in this effect. An increase in transcription is observed with pNS28, which contains the control regions of ftsA and ftsZ. No increase is observed with pNS32; thus eliminating the ftsA control region. The plasmid pNS32 is an EcoR1-BglII substitution into pKO4 and contains the 600 bp KpnI fragment within which ftsA starts (see Chapter 4). An increase in transcription is observed in pNS29 which contains all three ftsZ promoters, indicating that it is the control region of ftsZ which responds to cAMP. Neither pNS30 (Pz_1 and Pz_2) nor pDK340 (PA and Pz_3) show any transcriptional effect of cAMP, a result which perhaps implicates the cAMP-CRP complex in the mechanism of Pz_3.

5.6 Reversal with exogenous cAMP

In E.coli the intracellular concentration of cAMP varies according to growth conditions, for example at 37°C and during early exponential growth this varies from 2.4 μM for a cell growing on glucose to 7.3 μM for a cell using glycerol as a carbon source (Joseph et al., 1982).

Table 5.5 Localisation of the cAMP effect

Strain	Plasmid	Units galK	cya^{Δ}/cya^{+}
c600K^{-}	pNS22	0	-
NFS3	pNS22	0	
c600K^{-}	pNS28	2.60	2.28
NFS3	pNS28	5.93	
c600K^{-}	pNS32	0	-
NFS3	pNS32	0	
c600K^{-}	pNS29	2.34	1.80
NFS3	pNS29	4.21	
c600K^{-}	pDK340	0.54	1.07
NFS3	pDK340	0.58	
c600K^{-}	pNS30	0.80	1.00
NFS3	pNS30	0.80	

Fig. 5.1 Constructions used to localise the cAMP effect

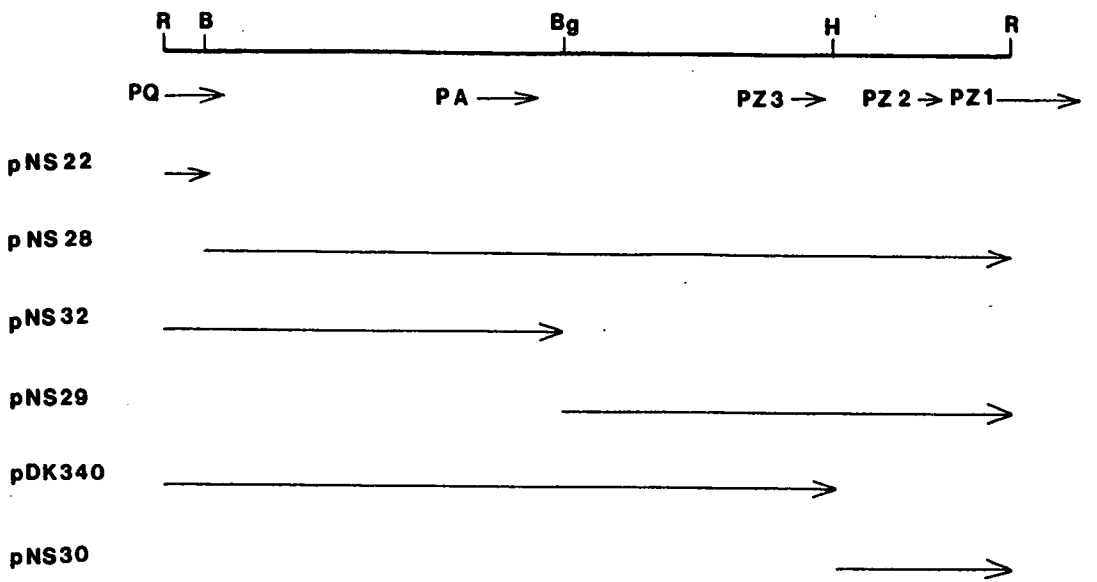


Table 5.3 shows that the *cya* deletion can be compensated by the addition of exogenous cAMP at a concentration of 2 mM. Utsumi *et al.* (1982) used 1.5 mM cAMP to induce filamentation in PA3092 (*fic*). Without measuring the intracellular cAMP concentrations it is difficult to predict how much extracellular cAMP enters the cell, although since cAMP and CRP are in equilibrium, at high intracellular cAMP concentrations the cellular pool of CRP will become saturated.

5.7 The level of derepression in other cAMP repressible operons

Random fusions of cAMP repressible operons to the coding region of *lacZ* (thus eliminating transcriptional effects) gives, on average a two fold increase (maximum five fold) in β-galactosidase activity in a cya^{Δ} strain when compared to the wild type. This occurs in both single and multiple copies (S. Adhya, pers. comm.). The 2.4 fold increase observed for the 2.2 Kb *EcoR1* fragment is thus well in agreement with this result.

5.8 Mechanistic considerations

The cAMP-CRP effect requires that the *ftsZ* promoters have their normal chromosomal arrangement and may be either a direct or indirect effect of the complex. If direct the cAMP-CRP complex may bind directly to the DNA to turn off transcription. Aiba (1983) has noted that in all three genes ($galP_2$, *ompA* and *crp*) in which CRP could act as a negative effector the consensus sequence (Ebright, 1982) is on the coding strand. Fig. 5.2 shows two such regions within the 890 bp fragment which contains the *ftsZ* promoters. Assuming this mechanism, we would expect one of these promoters to

Fig. 5.2

The DNA sequence of the non-coding strand of the 3' end of the ftsA gene, which includes the promoter region of ftsZ (as in Fig. 3.7). Restriction sites and ftsZ promoters (Pz_3, Pz_2 and Pz_1) are indicated by thick lines. Sequences which correspond to the concensus cAMP-CRP binding site (Ebright, 1982; Fig. 1.10) are indicated by thin lines. The upper lines indicate conserved nucleotides. Two consensus sequences on the non-coding strand (N) centered at positions 65 and 182 and two on the coding strand centered at positions 325 and 618 are indicated.

FIG 5·2

```
          10          20          30          40          50          60
GTTGACCAAC TGATATTTGC CGGACTGGCA TCAAGTTATT CGGTATTGAC GGAAGATGAA
Hc
          70          80          90         100         110         120
CGTGAACTGG GTGTCTGCGT CGTCGATATC GGTGGTGGTA CAATGGATAT CGCCGTTTAT
N
         130         140         150         160         170         180
ACCGGTGGGG CATTGCGCCA CACTAAGGTA ATTCCTTATG CTGGCAATGT CGTGACCAGT  PZ3
         190         200         210         220         230         240
GATATCGCTT ACGCCTTTGG CACGCCGCCA AGCGACGCCG AAGCGATTAA AGTTCGCCAC
N
         250         260         270         280         290         300
GGTTGTGCGC TGGGTTCCAT CGTTGGAAAA GATGAGAGCG TGGAAGTGCC GAGCGTAGGT
         310         320         330         340         350         360
GGTCGTCCGC CACGGAGTCT GCAACGTCAG ACACTGGCAG AGGTGATCGA GCCGCGCTAT
C
         370         380         390         400         410         420
ACCGAGCTGC TCAACCTGGT CAACGAAGAG ATATTGCAGT TGCAGGAAAA GCTTCGCCAA
                    Hc                                  H
         430         440         450         460         470         480
CAAGGGGTTA AACATCACCT GGCGGCAGGC ATTGTATTAA CCGGTGGCGC AGCAGATCGA
         490         500         510         520         530         540
AGGTCTTGCA GCCTGTGCTC AGCGCGTGTT TCATACGCAA GTGCGTATCG GCGCGCCGCT  PZ2
         550         560         570         580         590         600
GAACATTACC GGTTTAACGG ATTATGCTCA GGAGCCGTAT TATTCGACGG CGGTGGGATT
         610         620         630         640         650         660
GCTTCACTAT GGGAAAGAGT CACATCTTAA CGGTGAAGCT GAAGTAGAAA AACGTGTTAC
C
         670         680         690         700         710         720
AGCATCAGTT GGCTCGTGGA TCAAGCGACT CAATAGTTGG CTGCGAAAAG AGTTTTAATT  PZ1
         730         740         750         760         770         780
TTTATGAGGC CGACGATGAT TACGGCCTCA GGCGACAGGC ACAAATCGGA GAGAAACTAT
         790         800         810         820         830         840
GTTTGAACCA ATGGAACTTA CCAATGACGC GGTGATTAAA GTCATCGGCG TCGGCGGCGG
         850         860         870         880         890         6
CGGCGGTAAT GCTGTTGAAC ACATGGTGCG CGACGCATTG AAGGTGTTGA ATTC
                                                        R
```

respond to cAMP, but since both Pz_3 and Pz_2 and Pz_1 show promoter activity independent of the other and are not affected by cAMP-CRP such a direct effect is unlikely. This also eliminates any mechanism by which the cAMP-CRP complex blocks the passage of uninitiated RNA polymerase molecules from an upstream high affinity, low initiating promoter (see Chapter 3).

An alternative direct effect requires a modification of RNA polymerase. Ullman et al. (1979) have suggested that the cAMP-CRP complex may modulate transcription termination at rho dependent terminators either by directly interacting with the rho-RNA polymerase-ribosomal complex or by activation or derepression of an anti-terminator protein. By isolating carbohydrate positive pseudo-revertants of rho crp double mutants of E.coli these same authors subsequently proposed that the cAMP-CRP complex acts either directly or indirectly with RNA polymerase at sites located near the promoter and remains associated throughout the elongation phase of transcription. The action of the cAMP-CRP complex is thus to prevent the premature action of rho (Guidi-Rontani et al., 1980). One possible mechanism for the enhancement of transcription from the 480 bp HindIII-EcoR1 fragment by Pz_3 depends upon upstream transcription and the stem loop structure at Pz_2 (see Section 3.6). Such a structure resembles a rho dependent terminator (Platt, 1981). Thus the cAMP-CRP complex may affect the mechanism of enhancement by interacting with RNA polymerase during the elongation of Pz_3 mRNA. Modification of RNA polymerase by cAMP-CRP may prevent either the action of rho or indeed other accessory

transcription factors or prevent recognition of the stem loop in the mRNA, thus abolishing the enhancement of *ftsZ* transcription. The level of *ftsZ* transcription would thus be dependent upon the intracellular concentration of the cAMP-CRP complex.

The complex may act indirectly via another locus coding for a division specific protein. The complex may turn on transcription from this locus giving a protein which then represses division or *vice versa*. Despite a selection devised to isolate a temperature sensitive mutation in such a gene none was ever found (K. J. Begg; unpublished).

5.9 Conclusion and future prospects

The cAMP-CRP complex has been shown to either directly or indirectly repress transcription of the *ftsZ* gene implicating the complex as a negative effector in the control of *E.coli* cell division.

Experiments in progress include *in vitro* transcription of the *ftsZ* promoter region in the presence of cAMP-CRP in order to decide whether the 'cAMP effect' is direct or indirect.

CHAPTER 6

6.1 Transcriptional effects of cell division genes.

The interaction between the cAMP-CRP complex and the *ftsZ* gene prompted a search for *cis* and *trans* interactions between this region and other cell division genes. Alleles tested included four conditional lethal cell division genes, a cell separation gene and an 'SOS' associated gene (see Table 6.1).

Table 6.1

Strain	Gene/Allele	Function
Ken 15	*sep* t.s.	Septation
NFS7	*ftsZ* 84 t.s.	Septation
Ken 12	*secA* t.s.	Secretion/Septation
Ken 18	*ftsE* t.s. (TOE 22)	Septation
Ken 10.5	*envA*	Septation
Ken 11	*lexA* t.s.	SOS

For temperature sensitive alleles both parent and mutant (isogenic c600K$^-$) strains containing either pKO1 or pNS20 (see Chapter 4) were assayed for galactokinase at both 30°C and 42°C, whilst the *envA* strain and its parent were assayed at 37°C only. All strains were checked for the mutant phenotype prior to assay. The galactokinase specific activity depends upon the temperature at which the cells are grown and increases linearly from 30°C to 42°C (data not shown). At a given temperature the results of two independent assays have been normalised (see Appendix 1) and corrected for the basal transcription of pKO1. The ratio of mutant: wild type (M:WT) indicates the transcriptional effect of the mutation

on the cloned fragment (see Chapter 5).

6.2 Results and discussion

The mutant:wild type ratios indicate that neither *sep*, *envA*, *secA* nor *ftsE* mutations can affect transcription from the cloned 2.2 Kb *EcoR*I fragment either in *cis* (a direct transcriptional effect) or in *trans* (via the gene product) (see Tables 6.2 and 6.4).

Table 6.2 *Effect of sep t.s. at 42°C*[1]

Plasmid	Strain	Uncorrected *galK* specific activity	Corrected[2]	M:WT[3]
pKO1	c600K$^-$	0.20	-	-
pNS20	c600K$^-$	1.76	1.56	-
pKO1	Ken 15	0.23	-	-
pNS20	Ken 15	1.81	1.58	1.01

[1] Similar results were obtained with all other alleles except *ftsZ* (see Table 6.4).

[2] The corrected *galK* specific activity is obtained by subtracting the value for pKO1 from that of pNS20.

[3] The M:WT ratio is given for pNS20.

Table 6.3 Effect of *ftsZ* 84 t.s. at 42°C and 30°C

Plasmid	Strain	Uncorrected *galK* specific activity		Corrected		M:WT	
		42°	30°	42°	30°	42°	30°
pKO1	c600K$^-$	0.16	0.12				
pNS20	c600K$^-$	1.48	1.08	1.32	0.96		
pKO1	NFS7	0.24	0.08				
pNS20	NFS7	3.48	1.30	3.24	1.22	2.45	1.27

Table 6.4 M:WT ratios for pNS20

Gene	M:WT ratio		
	30°	37°C	42°
sep t.s.	0.95	-	1.01
ftsZ t.s.	1.27	-	2.45
secA t.s.	1.07	-	1.11
ftsE t.s.	0.98	-	1.05
lexA t.s.	1.18	-	1.06
envA t.s.	-	1.10	-

Table 6.3 shows that in the *ftsZ* mutant strain at the restrictive temperature transcription from the cloned fragment is increased 2.45 times. The fragment contains the control region, but not the structural gene of *ftsZ* itself, the complete *ftsA* gene and the SD sequence of *ftsQ* (see Chapters 3 and 4). This may

indicate that the ftsZ product functions in regulating either its own expression or that of the ftsA gene. Since transcription is higher in the mutant this suggests that the ftsZ product acts negatively to repress transcription from the cloned fragment. It must be noted that transcription from pKO1 increases slightly (0.24/0.16 = 1.5 times) so the ftsZ defect may mediate a general increase in transcription. At 30°C the ftsZ mutation is leaky and some short filaments can be observed. At this temperature there is an increase in transcription from pNS20 (1.27 times) but a decrease in transcription from pKO1 (0.08/0.12 = 0.67 times) arguing against a general stimulatory effect of the ftsZ mutation on plasmid transcription.

Whilst not conclusively indicating that the ftsZ product has a role in negatively regulating transcription from pNS20 there is clearly a difference between this mutation and the others tested. Experiments are in progress to investigate this further.

6.3 The effect of SOS induction on pNS20 transcription

At the time of this experiment the sulB gene, which plays a role in 'SOS' associated division inhibition had been approximately mapped to the ftsA-envA region (see Section 1.3.4). It seemed reasonable to search for SOS associated transcriptional effects on the cloned promoters of this region. Induction of the 'SOS' response in a lexA t.s. strain was found to produce no effect on transcription from the cloned fragment, indicating that neither ftsA nor ftsZ are damage inducible (din) via a sulA dependent pathway.

Consistent with this, Lutkenhaus (1983) has since shown that sulB and ftsZ are allelic and that division inhibition via the lexA-sulA pathway is probably due to inhibition of the ftsZ product.

6.4 Autoregulation of *ftsA*

Due to the influence of *ftsZ* promoters internal to the *ftsA* coding region it is not possible to decide whether or not the *ftsA* product is autoregulatory. This question could be resolved by measuring the galactokinase activity of pNS32 (see Chapter 5) at the restrictive and non-restrictive temperature in a *galK*$^-$ *ftsA* t.s. strain.

CHAPTER 7

7.1.1 Cloning the DNA fragment bearing envA

The DNA fragment bearing the envA gene has been cloned in single copy on lambda transducing phages (λenvA, λ16-2, λJFL40) (Lutkenhaus and Donachie, 1979; Lutkenhaus et al., 1980). This piece of DNA, a 2.5 Kb EcoR1 fragment, is interesting in several respects; (1) it contains the cell separation gene envA in its entirety (Lutkenhaus et al., 1980), (2) it contains part of the structural gene of the septation associated gene ftsZ (Lutkenhaus et al., 1980), (3) there is space for another gene between envA and secA (Oliver and Beckwith, 1982a) and (4) the septation inhibitor sulB has been mapped to this area (Johnson, 1977).

I decided to clone this fragment into multicopy plasmids in order to investigate the structural organisation of its genes, to obtain direct gene fusion evidence for the directions of transcription of the genes involved and to investigate the response of promoters on this fragment to SOS stimulation.

7.1.2 Cloning into pBR328

EcoR1 λ16-2 was ligated with EcoR1 digested pBR328 (Bolivar, 1976) and transformed into c600K$^-$ selecting for insertions into the chloramphenicol (Cm) gene (Cm^S, Ap^R, Tc^R). More than one representative of each class of possible recombinant was obtained, with the exception of the 2.5 Kb EcoR1 fragment. (Possible inserts (in Kb): 0.6, 0.9, 2.2, 2.5, arms of λ). Furthermore, repeating this experiment using the 2.5 Kb fragment purified from an agarose gel failed to produce any pBR328 derivatives with the desired insert. That other EcoR1 fragments within the same reaction

could be cloned whilst the envA fragment could not suggest that this fragment, when present in multicopy was lethal to the cell.

With an EcoR1, PvuII double digest of λ16-2 it was possible to show that the 2.5 Kb EcoR1 fragment was cut by PvuII (Fig. 7.1). This EcoR1 fragment was purified from an agarose gel using the hydroxyapatite method and 0.02 pmol was subsequently ligated on itself to form a circular molecule. This was then cut with PvuII to form an 'inverted' fragment (Fig 7.2a), which was blunt end ligated into the PvuII site of the pBR328 Cm gene (hence selecting for Cm^S, Ap^R, Tc^R). The resulting plasmid, pNS9 is shown in Figs. 7.1 and 7.2a. The EcoR1 digest orientates the fragment within the vector, and more importantly demonstrates that the PvuII site lies approximately 500 bp from one end. The 2.5 Kb EcoR1 fragment and the PvuII fragment are seen to be of equal size (±50 bp) (Fig. 7.1). When transformed into an envA mutant background (Ken 10) the pNS9 plasmid failed to complement the envA mutation implying that the PvuII site disrupts the envA gene (Appendix II). Part of the structural gene of ftsZ has been mapped to the left hand end of the fragment, so unless the envA and ftsZ structural genes overlap, this implies that PvuII cuts 500 bp from the right hand end.

7.1.3 Copy number effects in E.coli

It has been reported by Clarke and Carbon (1976) that certain hybrid plasmids carrying various segments of chromosomal DNA grow at different rates and that some segregate rapidly whilst others do not. It is thus possible that certain classes of plasmid do not

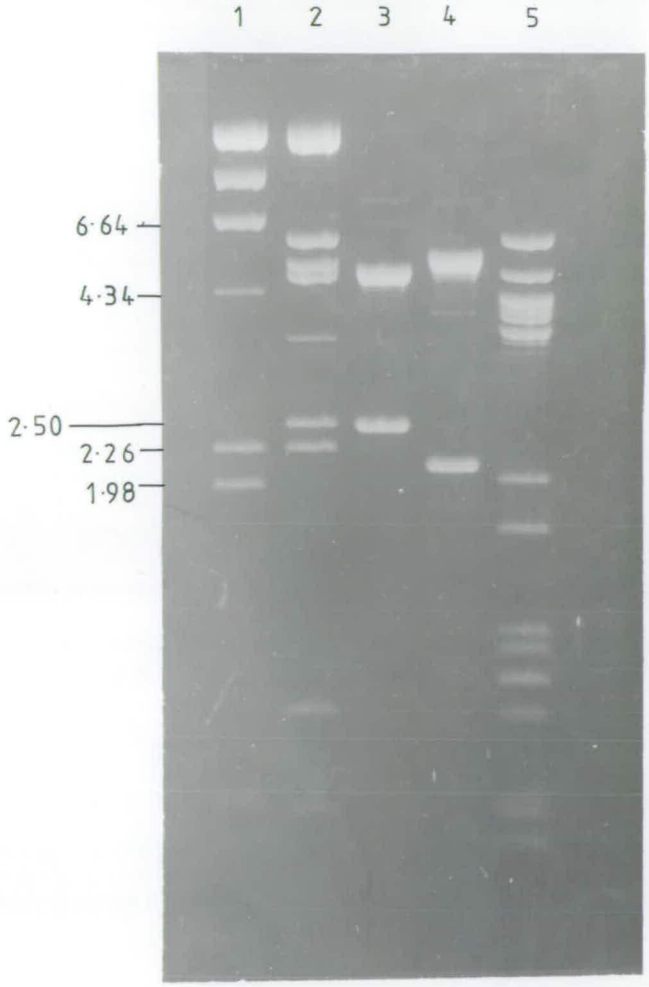
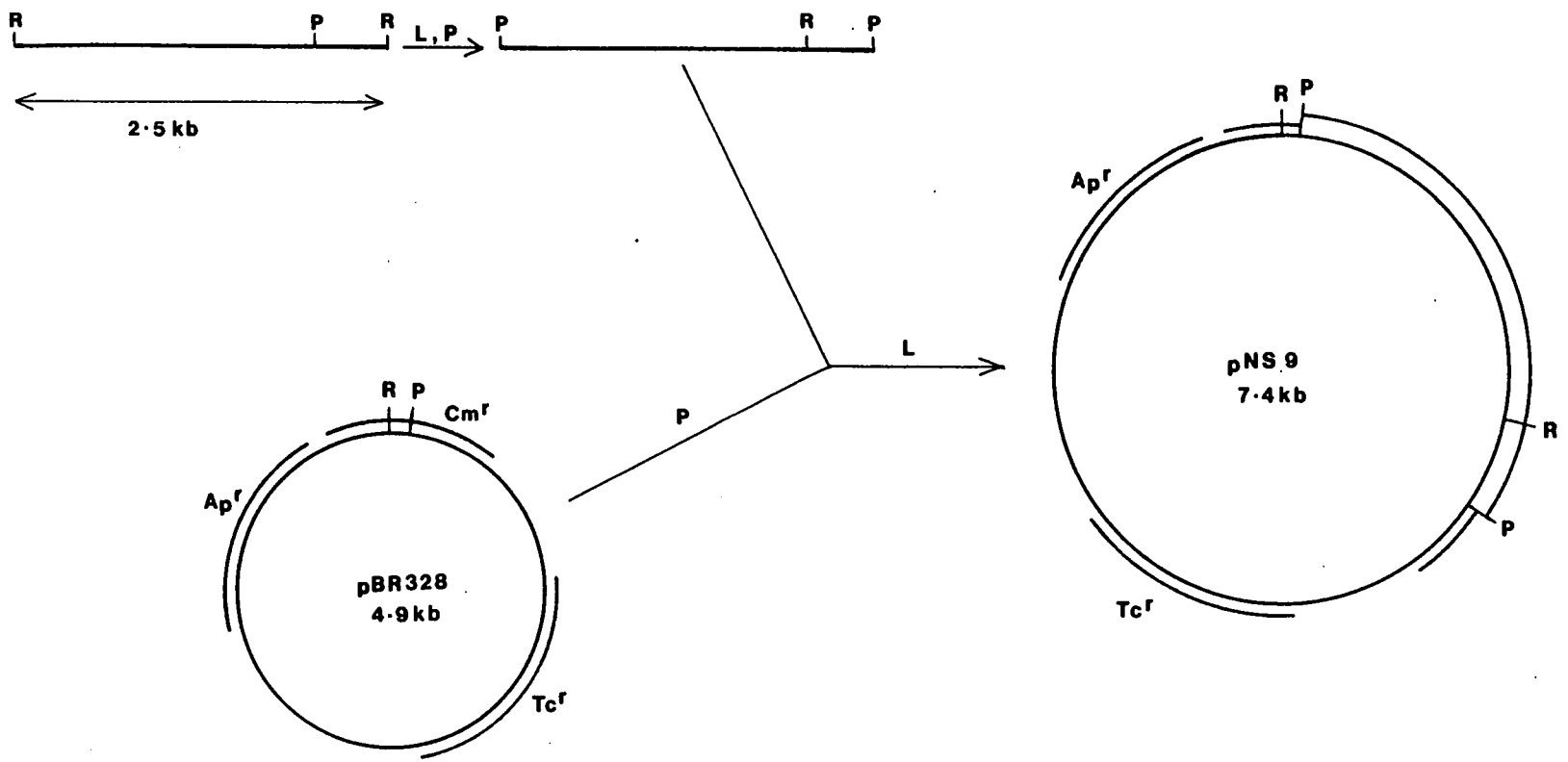

1
2
3
4
5
6·64
4·34
2·50
2·26
1·98

Fig. 7.1

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	$\lambda \Delta E$	EcoR1	-
3	pNS9	PvuII	7.2a
4	pNS9	EcoR1	"
5	$\lambda \Delta E$	EcoR1; PvuII	-

Fig. 7.2a Construction of pNS9



exist in this collection, i.e. those plasmids in which the presence of particular genes in multicopy may disturb normal cell metabolism. In a study designed to screen for Clarke and Carbon plasmids bearing cell division genes only certain of the then known division genes were shown to be present (Nishimura et al., 1977). The process of cell division is obviously complicated and the timing and triggering of such a coordinate and precise series of events almost certainly requires an equally complex regulatory system. Over-production of division products or titration of division factors by high copy number plasmids may disturb cellular regulation, resulting in a high rate of segregation or absence from the Clarke and Carbon collection.

Other E.coli genes have been shown to be not clonable in multicopy, for example ompA (Bremer et al., 1980; Henning et al., 1979) dnaA (Projan and Wechsler, 1981) and polA (Kelley et al., 1977).

The λcII protein has also been shown to have a deleterious effect on the cell (Shimatake and Rosenberg, 1981). Multiple copies of the E.coli spot 42 gene cause an increase in cellular generation time (Rice and Dahlberg, 1982).

The incidence of 'copy number' effects is by no means uncommon and overproduction of cellular proteins is potentially a means of elucidating their function. Markiewicz et al. (1982) have demonstrated that an increase in the level of PBP5, a D-alanine carboxypeptidase, can produce osmotically stable spheres in E.coli and that the gene coding for PBP5, dacA cannot be cloned into high copy number vectors. These authors suggest that high levels

of PBP5 remove the terminal D-alanine residues from murein precursors (see Section 1.1.5) and prevent further murein synthesis.

7.1.4 A novel vector system designed to study copy number effects in E.coli

A system in which one could vary the copy number of the plasmid and study the effect on the cell of high copy numbers of cloned genes was required. Such a system originating from the plasmid R1-drd-19 has been devised (Uhlin and Nordstrom, 1979). At 30°C the copy number is strictly controlled, but uncontrolled plasmid replication can be induced by shifting the temperature to 42°C. However, if runaway replication was allowed to take place for more than 120 minutes then the cells died (Uhlin and Nordstrom, 1979). Since a high copy number of the envA gene is a potentially lethal event, study of this phenomenon required a system whereby the copy number was variable yet controllable and did not result in a decrease in viability.

It has been shown that replication of ColE1 plasmids requires the product of the polA gene (Kingsbury and Helinski, 1973) whereas pSC101 derived plasmids are polA independent (Timmis et al., 1974). In a polA t.s. strain a ColE1 plasmid can replicate at 30°C but not at 42°C whereas pSC101 derived plasmids can replicate at either temperature. Initiations from the pSC101 replicon are inhibited at lower copy number than the ColE1 origin so that the 30°C the cointegrate should attain the copy number of ColE1 and be maintained as such (Cabello et al., 1976). At 42°C the cointegrate should be maintained at low copy number under the control of the pSC101 replicon.

The two plasmids chosen for construction of the cointegrate vector were the pSC101 derived replicon pPM30 (Meacock and Cohen, 1980) because of its small size and lack of tetracycline resistance and the ColE1 related plasmid pBR325 (Bolivar, 1976). Fig. 7.2b shows the construction of pNS10 and its *envA* bearing derivative pNS10-7. Fig. 7.3 shows a restriction analysis of the relevant plasmids. The vector pNS10 has cloning sites for *Eco*R1, *Hind*III and *Sal*I. β-lactamase assays were performed and the result showed that the copy number does indeed increase as the temperature is lowered (Table 7.1; Appendix I). The copy number of the cointegrate does not reach that of the parent pBR325 at 30°C possibly due to increased size or interference from the pPM30 replicon. The copy number of pBR325 itself does not reach the level normally expected of ColE1, presumably due to an incomplete return to the wild type *polA*$^+$ phenotype at 30°C. Indeed, a microscopic examination of JC411 *polA* t.s. at 30°C shows short filaments characteristic of partial SOS induction. Alternatively the low level may reflect the assay system. For example, when normalizing against the β-lactamase level of ED5040 it is assumed that there is only one copy of the *bla* gene. This does not account for multiple replication forks and hence gene dosage effects. However, using this system the copy number of the cointegrate does increase three times from 42°C to 30°C which was adequate for the experiment intended (7.1.6).

Fig. 7.2b Construction of the variable copy number vector pNS10 and its envA containing derivative pNS10-7

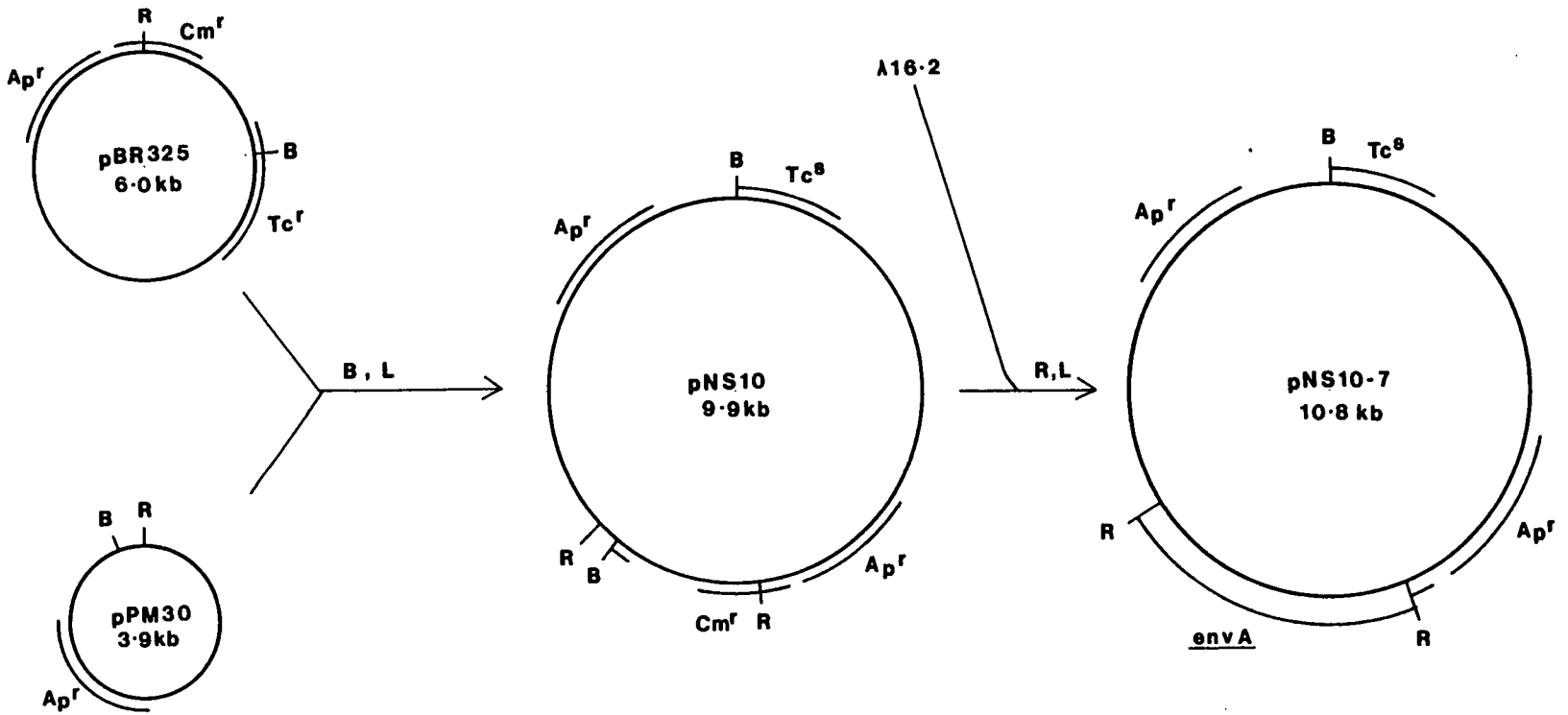
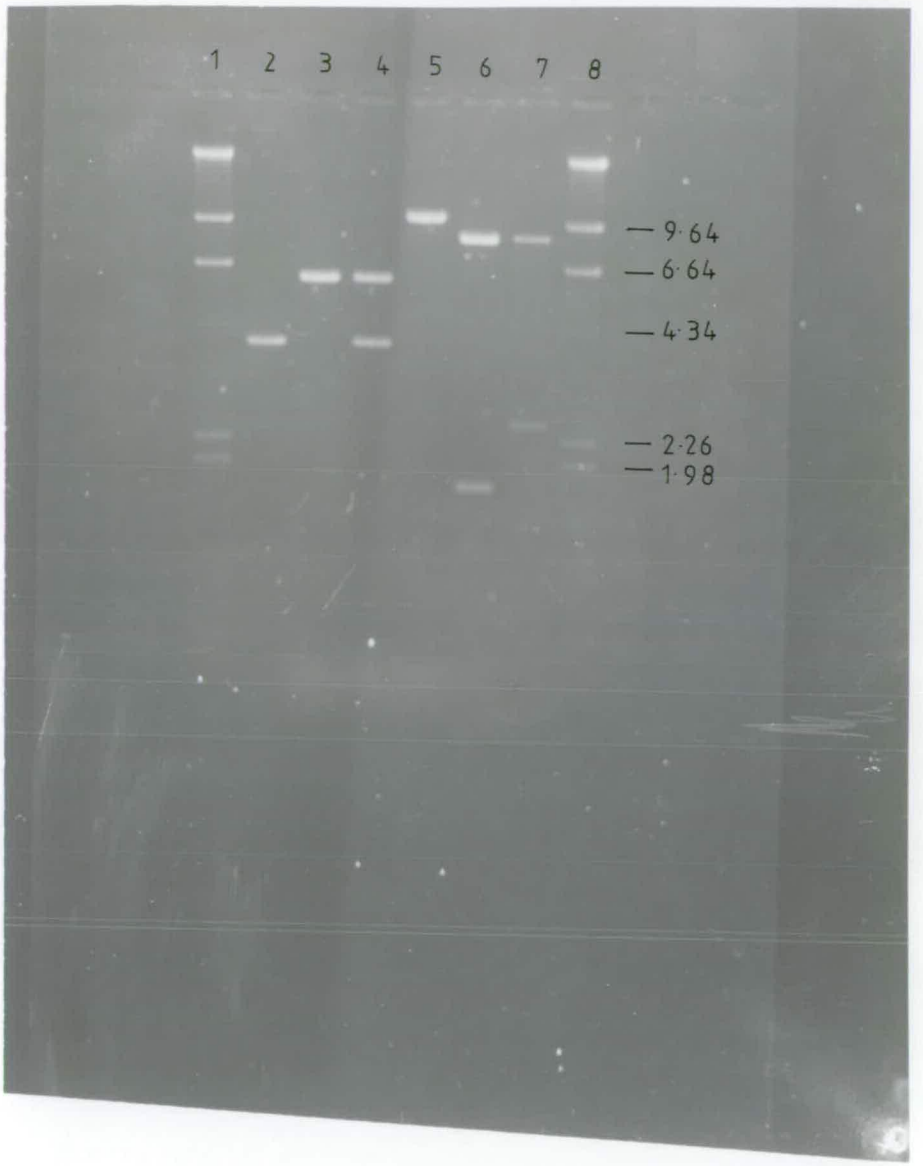


Fig. 7.3

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pPM30	BamHl	7.2b
3	pBR325	BamHl	"
4	pNS10	BamHl	"
5	pNS10	HindIII	"
6	pNS10	EcoRl	"
7	pNS10-7	EcoRl	"
8	λcI_{857}	HindIII	-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6
7
8
9·64
6·64
4·34
2·26
1·98

Table 7.1 Copy number determinations

Plasmid	$OD/min/OD_{540} \times 10^{-2}$ [1,2]		Estimated Relative Copy Number	
	30°C	42°C	30°C	42°C
pBR325	1.575	ND	21	ND
pPM30	0.31	0.365	4.1	4.9
pNS10[3]	1.81	0.65	12.1	4.3

[1] Error ± 10%

[2] Values are normalised against the bla activity of ED5040 grown at 30°C to give the relative copy number. $OD/min/OD_{540} = 0.075 \times 10^{-2}$; copy number = 1.

[3] Since pNS10 contains two copies of the bla gene the OD/min/OD540 values for this plasmid must be divided by two.

Cells were grown in LB plus ampicillin (50 μg/ml) at 42°C overnight (except pBR325, grown at 30°C) in the strain JC411 polA t.s. and subcultured 1:100 into non selective media. Growth was continued for at least 20 generations when samples were taken. β-lactamase assays were performed as described; Chapter 2, Appendix 1.

7.1.5 Complementation of envA by pNS10-7

In order to test complementation by pNS10-7 it is necessary to transfer the envA mutation to a polA background. Using the lysate described in Appendix II, JC411 polA t.s. was transduced to Tc^R at 30°C. However, out of 100 clones tested, none contained the envA mutation (approximately 6 were expected). It is likely therefore, that the polA and envA mutations in combination are not maintainable in the same cell, just as a polA recA double mutant cannot be constructed (Gross et al., 1971). Thus it has, unfortunately, not proved possible to test the complementation of envA by pNS10-7.

7.1.6 A copy number effect due to the cloned insert in pNS10-7

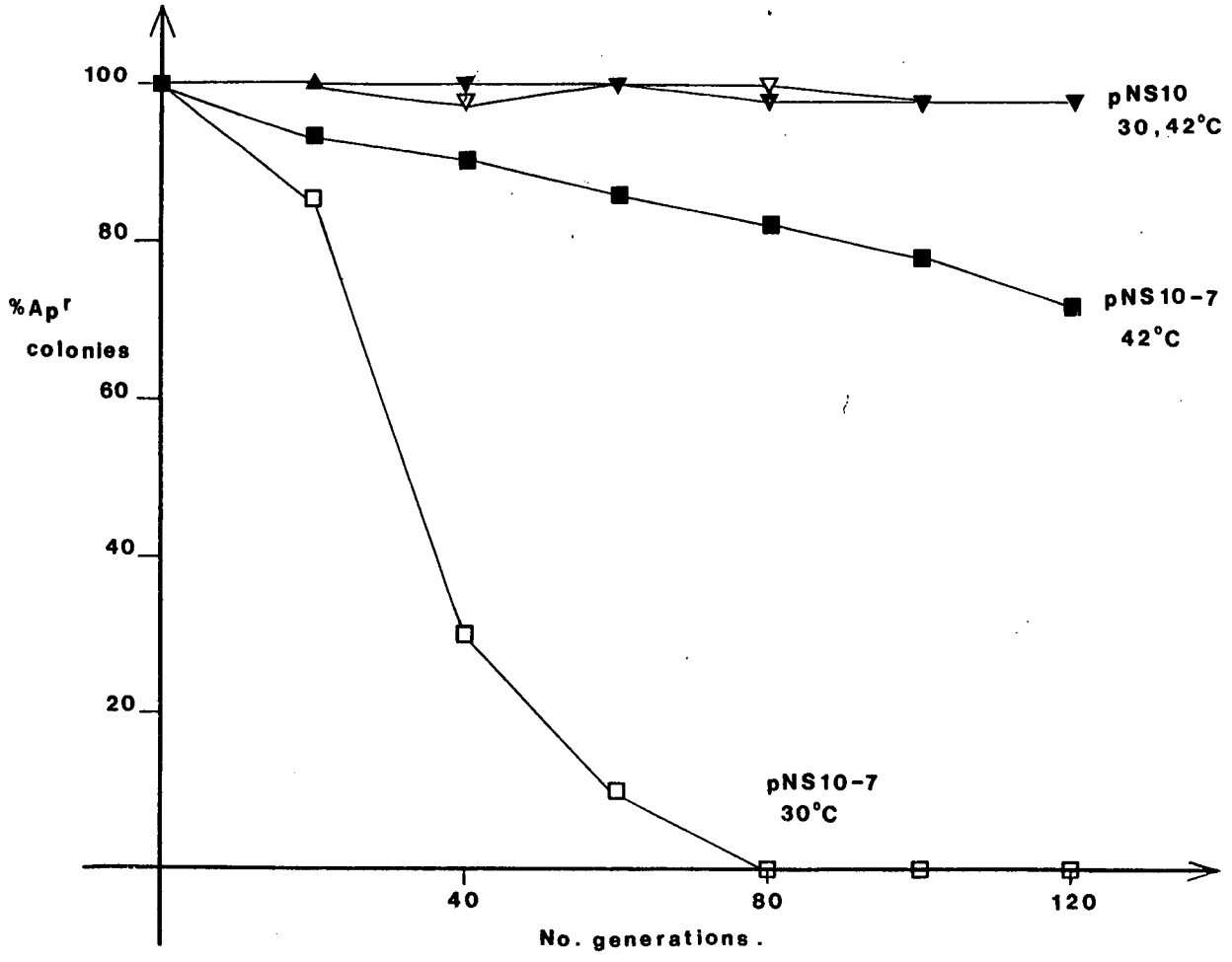
As can be seen from Fig. 7.4 the plasmid pNS10-7 containing the 2.5 Kb envA fragment segregates at both 30°C and 42°C, but at a much greater rate at the higher copy number (30°C). The control plasmid (pNS10) does not segregate appreciably at either temperature.

This effect may be attributed to either the product of the envA gene or to a titration effect of the DNA itself (see Section 7.2). Thus, using a novel plasmid designed to study copy number effects in E.coli lethality due to multiple copies of a 2.5 Kb EcoR1 fragment involved in cell division has been demonstrated.

7.1.7 Morphology

There was no observable morphological effect due to pNS10-7 at either 42°C or 30°C. However, one cannot be too dogmatic about this since the polA t.s. strain used showed filaments even at the non-restrictive temperature.

FIG 7·4


100
80
60
40
20
%Apr colonies
pNS10
30,42°C
pNS10-7
42°C
pNS10-7
30°C
40
80
120
No. generations.

7.2.1 The cause of the lethality

Having demonstrated lethality it was important to determine its cause. A Smith and Birnstiel map of the region (Chapter 8) revealed the positions of two ClaI sites and three PstI sites within the fragment (Fig. 7.5a). Given this it was possible to make two deletions within the fragment.

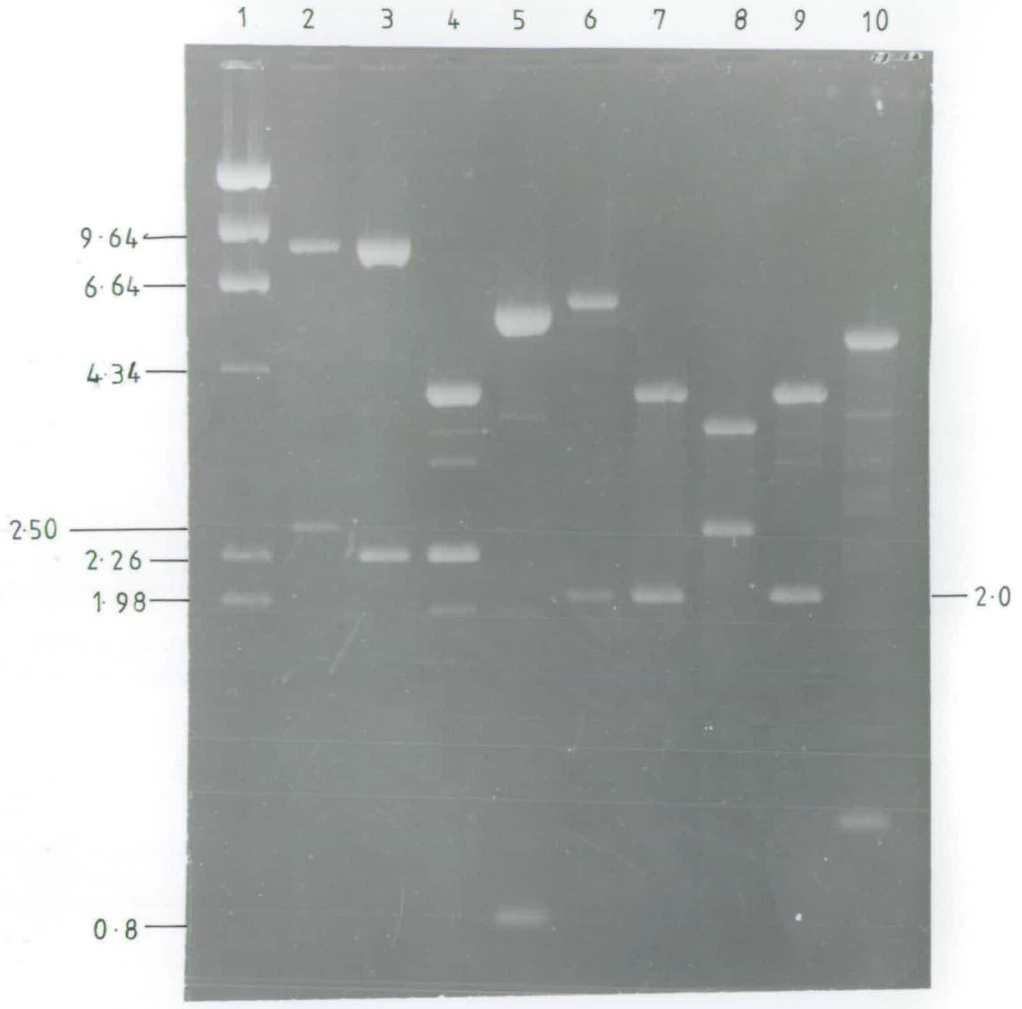
(1) A ClaI deletion

The plasmid pNS10-7 contains two ClaI sites, both within the 2.5 Kb EcoR1 insert. This plasmid was restricted with ClaI, ligated in dilute solution and transformed into JC411 polA t.s. at 42°C. One recombinant was designated pNS26 and was shown to have a 300 bp deletion within the EcoR1 insert (Fig. 7.5b and 7.6).

The plasmid pNS26 was maintainable in c600K$^-$ polA$^+$ and in addition did not complement the envA mutation in Ken 10. The 2.2 Kb EcoR1 fragment was transferred to the EcoR1 site of pKO1 to give pNS31 and orientated such that ftsZ and galK transcribed in the same direction (Fig. 7.5b and 7.6).

(2) A PstI deletion

Since pNS10-7 contained two PstI sites within the vector a similar strategy to that used for constructing pNS26 could not be used for the PstI deletion. Instead, the purified 2.5 Kb EcoR1 fragment was ligated on itself and subsequently cleaved with PstI. The inverted PstI fragment was cloned into the PstI site of the bla gene of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9·64
6·64
4·34
2·50
2·26
1·98
0·8
2·0

Fig. 7.6

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pNS10-7	EcoR1	7.2b
3	pNS26	EcoR1	7.5b
4	pNS31	EcoR1	"
5	pNS31	PvuII; HindIII	"
6	pNS4	PstI	7.2c
7	pNS24	EcoR1	7.2d
8	pNS24	PstI	"
9	pNS25	EcoR1	"
10	pNS25	PstI	"

Fig. 7.5a Restriction sites within the 2.5 Kb EcoR1 fragment (see Chapter 8)

(Scale 200 bp = 1 cm)

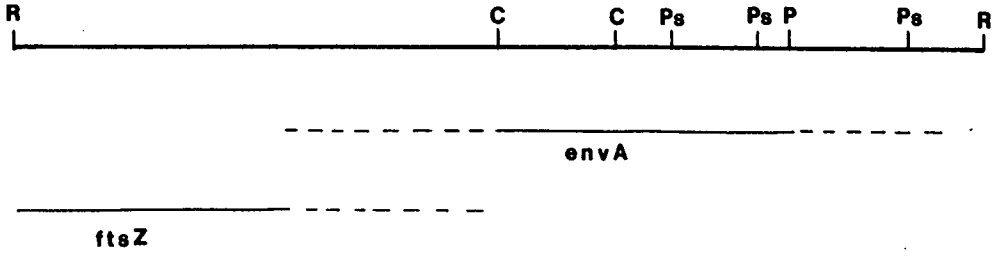
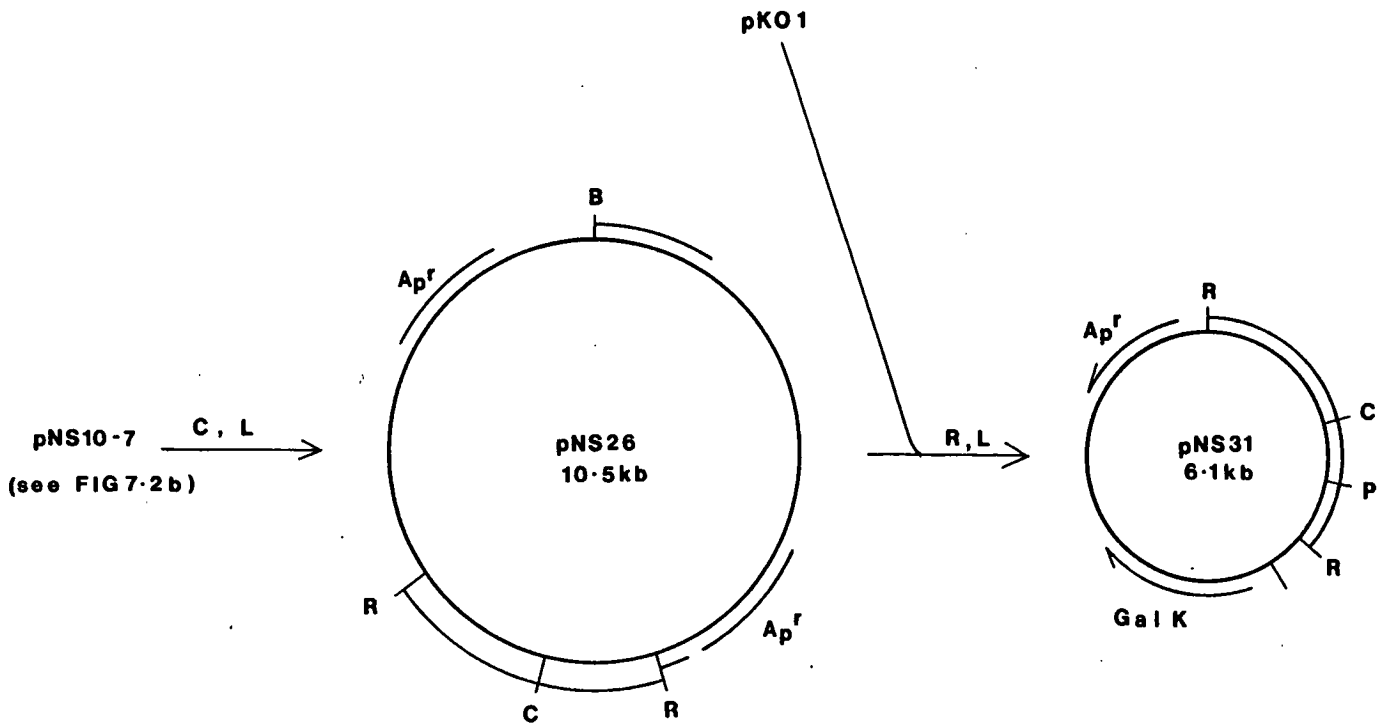


Fig. 7.5b Construction of pNS26 and pNS31



pBR325 (Bolivar, 1976) to give pNS4 (Fig. 7.5c). This fragment was shown to be 2 Kb in length, indicating the loss of 500 bp of the original fragment. The 2 Kb PstI fragment of pNS4 was then purified and ligated on itself as before. This was cleaved with EcoR1 to produce a 2 Kb EcoR1 fragment, the 2.5 Kb EcoR1 envA fragment with a 500 bp PstI deletion. This was inserted into the EcoR1 site of the pKO1 in both orientations to give pNS24 and pNS25 (Fig. 7.5d). pNS24 was orientated such that ftsZ and galK read in the same direction (Fig. 7.6). Both PstI deletions were maintainable in multicopy and did not complement the envA mutation in Ken 10.

7.2.2 The copy number effect

As already stated, the only complete gene known to exist on the 2.5 Kb EcoR1 fragment is the envA gene. That two, independent, non overlapping deletions, a 300 bp ClaI deletion and a 500 bp PstI deletion are both maintainable in multicopy and do not complement the envA mutation suggests very strongly that overproduction of the envA protein is lethal to the cell.

Fig. 7.5c Construction of pNS4

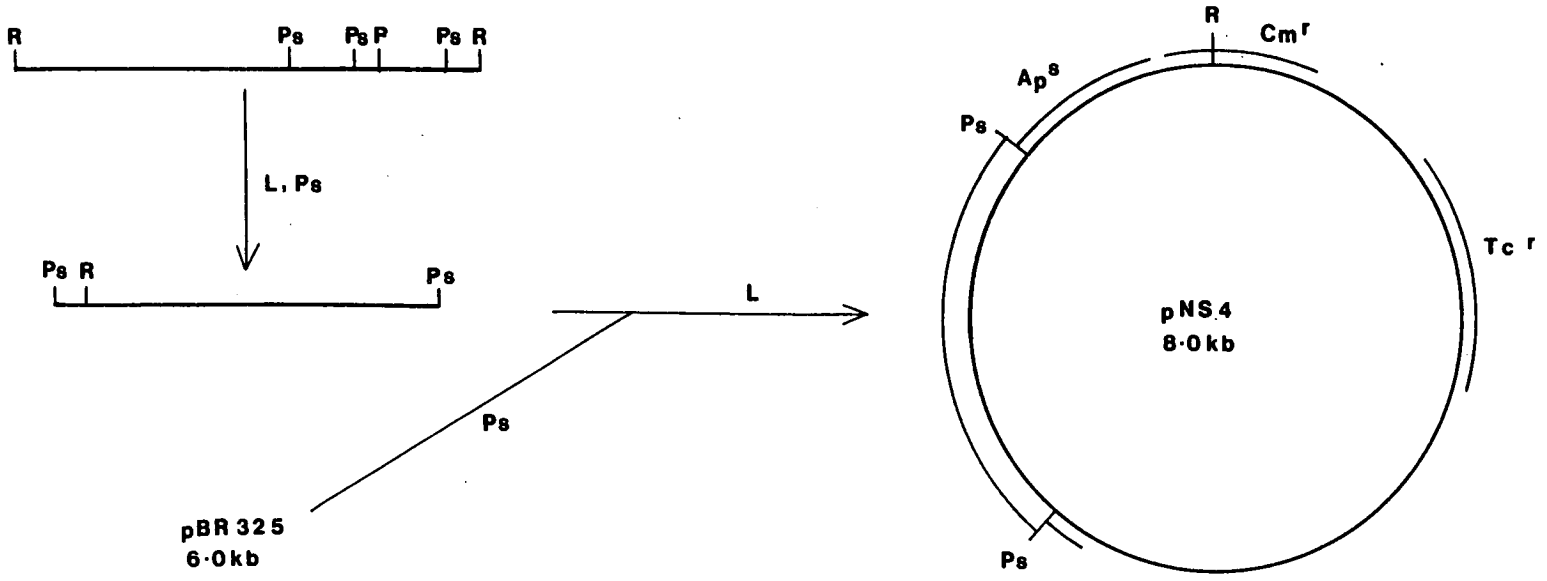
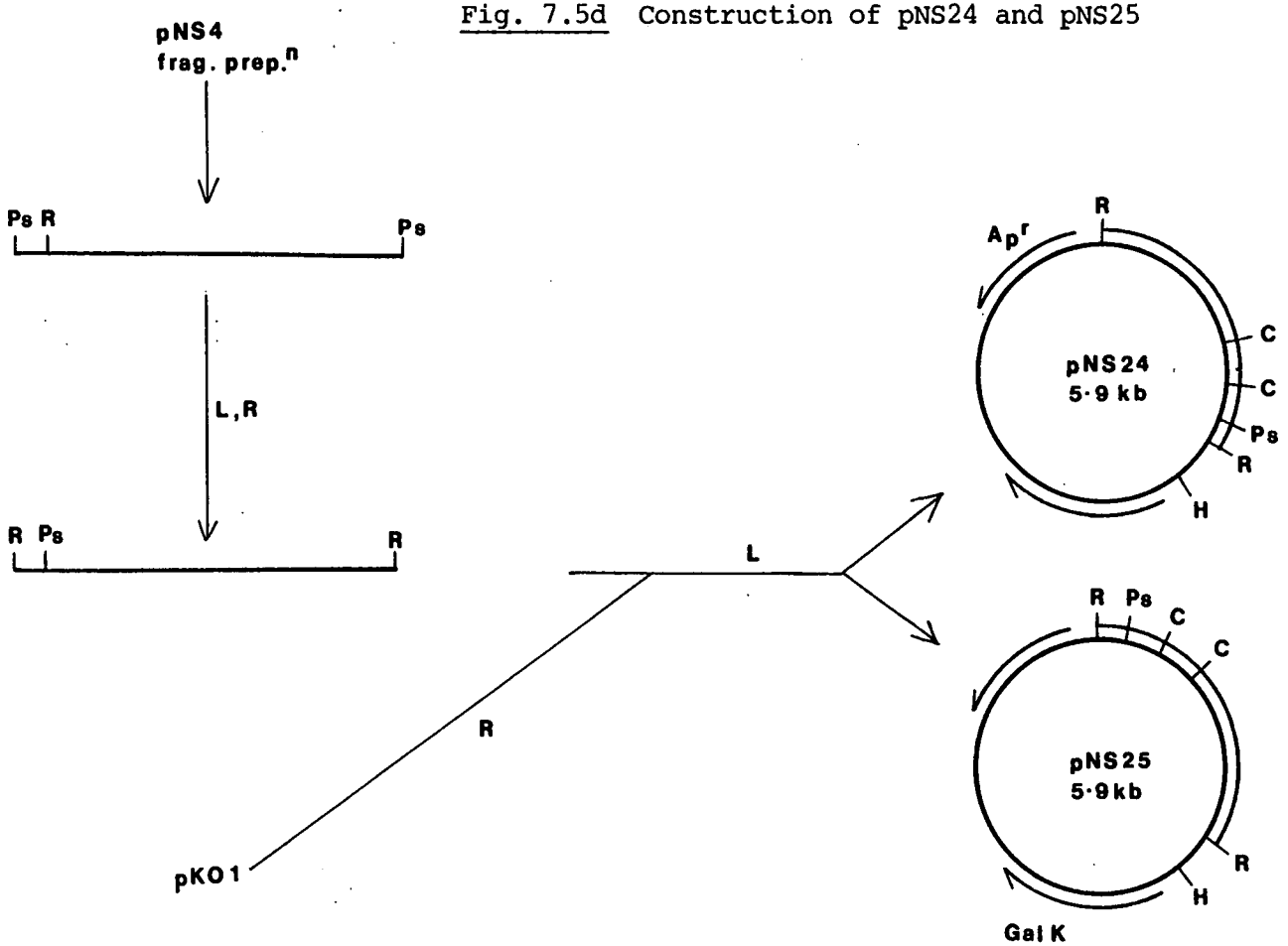


Fig. 7.5d Construction of pNS24 and pNS25



CHAPTER 8

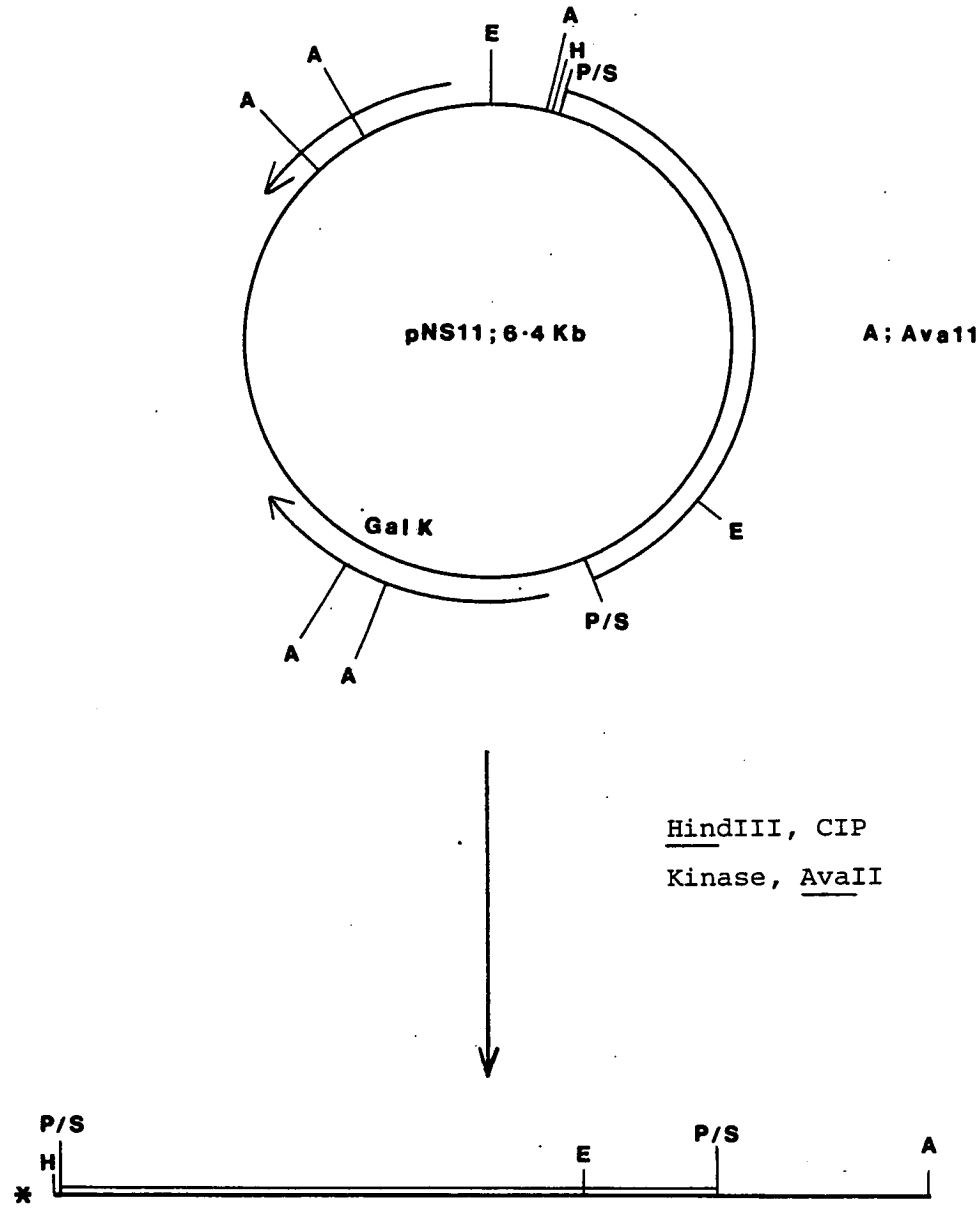
A restriction map of the 2.5 Kb EcoR1 fragment.

In order to facilitate further cloning and ultimately sequencing a restriction map of the 2.5 Kb EcoR1 fragment was prepared using the technique of Smith and Birnstiel (1976). This involves partial digestion of a singly end labelled DNA fragment with a variety of restriction endonucleases.

Since the 2.5 Kb EcoR1 fragment has been shown not to be clonable in multiple copy the inverted PvuII fragment was used for restriction mapping. This fragment, cloned into the PvuII site of pBR328 (pNS9) was transferred to the SmaI site of pK01 to give pNS11. The resulting plasmid gave white colonies on Mac gal plates and was orientated such that the EcoR1 site was 650 bp from the start of galK (Figs. 8.1, 8.2). Polynucleotide kinase labelling at the HindIII site (16 bp from the end of the insert) followed by AvaII digestion and subsequent purification of the 3.3 Kb fragment was deemed a suitable strategy. AvaII was shown not to cut the 2.5 Kb insert (Fig. 8.2). The 3.3 Kb singly and labelled HindIII-AvaII fragment was prepared and subjected to partial digestion and gel electrophoresis as described in Chapter 2.

Fig. 8.3 shows the 'master' agarose gel and Fig. 8.4 the 'master' acrylamide gel. Fragment sizes were determined from their mobility according to the method of Southern (1979) and represent the distance from the labelled HindIII site. End labelled AluI digested pBR322 and EcoR1, HindIII doubly digested $\lambda c_I 857$ (kindly provided by Dr. A. C. Robinson) were used as size standards. Above 900 bp values fragment sizes were taken from the agarose gel. Below this value from the acrylamide gel. Fragment sizes in regions

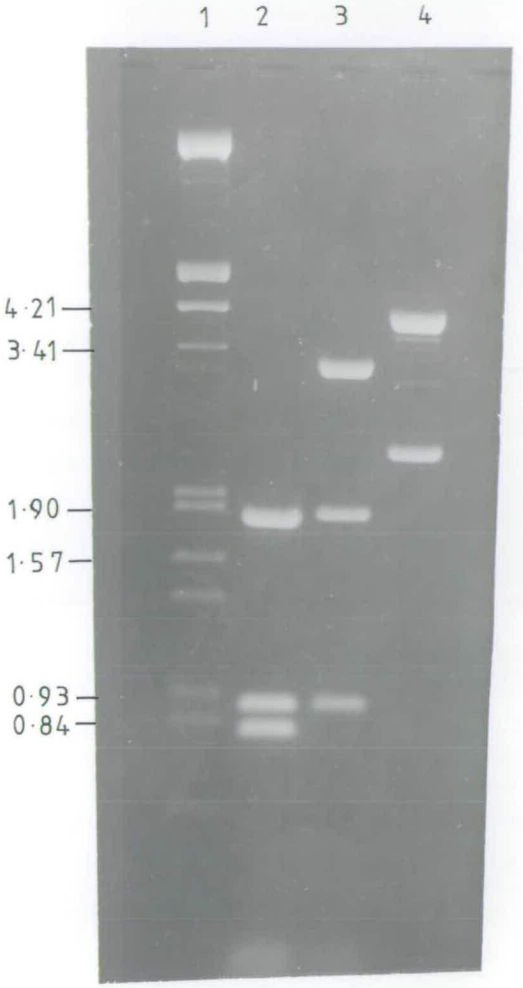
Fig. 8.1 Scheme of fragment isolation



The 3300 bp single end labelled fragment was separated from a 20 bp labelled fragment and 1861, 902, 222 and 167 bp unlabelled fragments.

Fig. 8.2

Track	DNA	Enzyme
1	λcI_{857}	EcoR1; HindIII
2	pKO1	AvaII
3	pNS11	AvaII
4	pNS11	EcoR1


1
2
3
4
4·21
3·41
1·90
1·57
0·93
0·84

of overlap (700-900 bp) were in good agreement (±30 bp) between the two gel systems.

Agarose master (in Kb)

AluI; 2.89, 2.37, 1.41, 1.25, 1.02.

EcoRl; 2.00.

EcoRl*; 2.03, 2.40.

HaeII; 2.74, 2.01, 1.93, 1.86, 1.57.

HincII; 2.06, 1.36.

KpnI; 1.79.

MspI; 3.09, 3.77, 1.79, 1.53, 1.26, 1.11, 1.03.

PstI; 2.31.

Sau3A; 3.00, 2.85, 2.25, 1.86, 1.63, 1.14.

TaqI; 2.93, 2.48, 1.38, 1.34, 1.02,

Acrylamide master (in bp)

AluI; 302.

ClaI; 769, 454.

EcoRI*; 528, 224.

HaeII; 769, 454.

HincII; 769, 289.

MspI; 910, 757, 676, 468, 134.

PstI; 336, 103.

Sau3A; 744, 528, 385, 202.

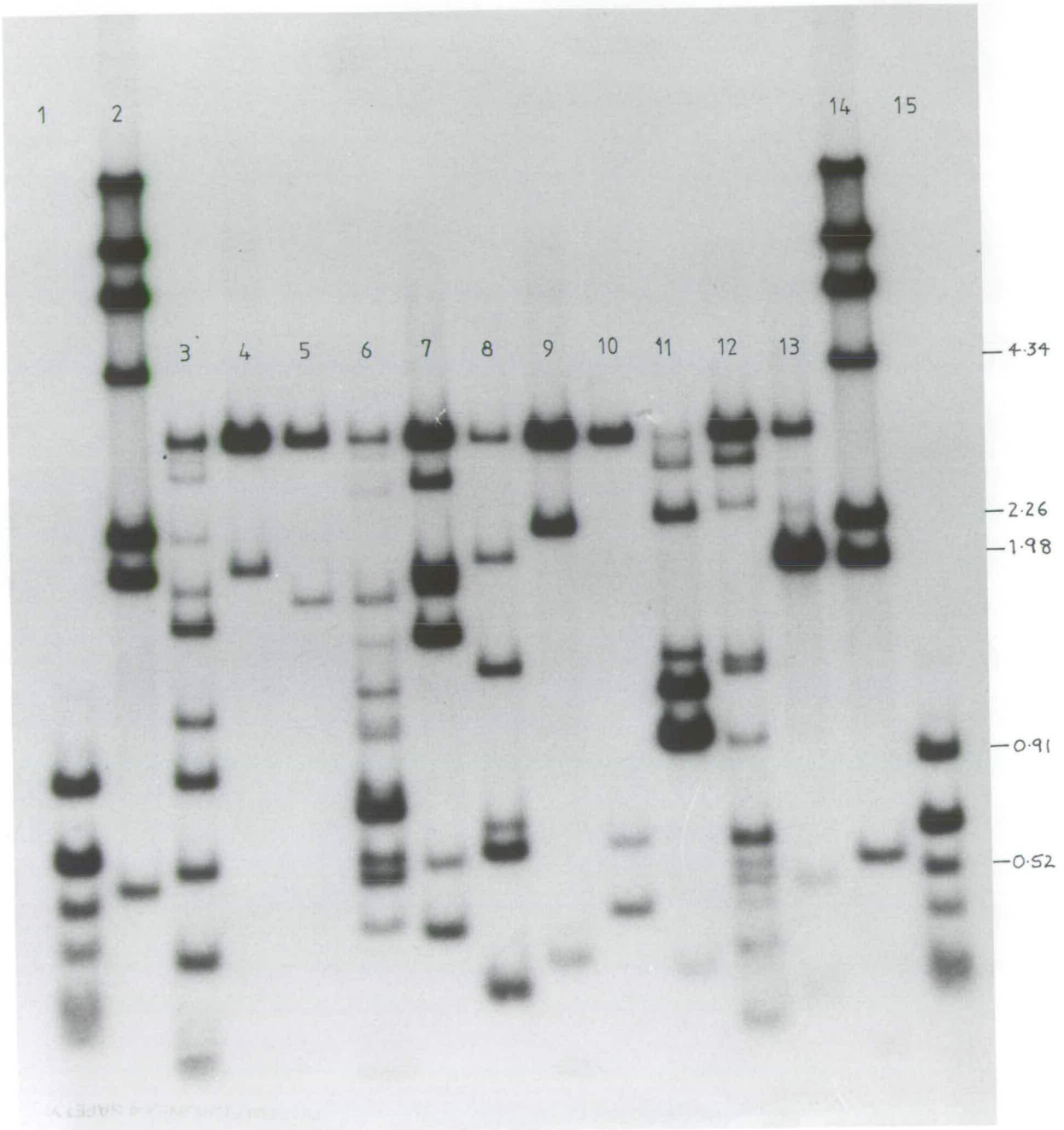
TaqI; 769, 625, 536, 454, 336, 298, 202.

The approximate positions of these restriction sites within the 3.3 Kb fragment can be seen in Fig. 8.5. In addition, the following enzymes were shown not to cut the inserted fragment: AvaI, BamHI,

Fig. 8.3

Autoradiogram of a 1% vertical agarose gel showing partial digests of the 3.3 Kb end labelled HindIII-AvaII fragment.

Track	DNA	Enzyme
1,15	pBR322	AluI
2,14	λcI_{857}	EcoRl, HindIII
3	3.3 Kb fragment	Sau3A
4	" "	EcoRl
5	" "	Kpnl
6	" "	Mspl
7	" "	HaeII
8	" "	HincII
9	" "	PstI
10	" "	ClaI
11	" "	AluI
12	" "	TaqI
13	" "	EcoRl*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7
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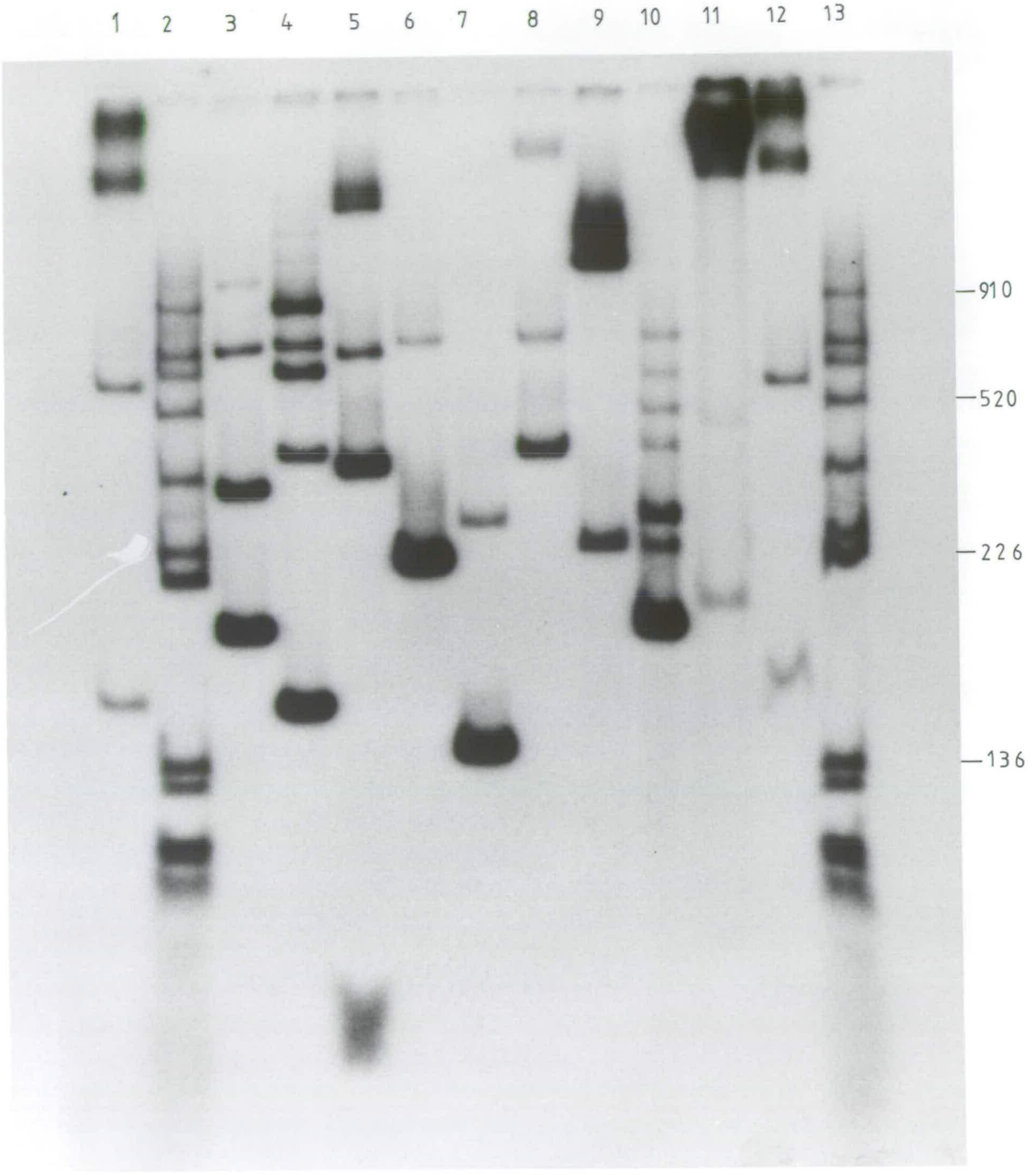
9
10
11
12
13
14
15
4·34
2·26
1·98
0·91
0·52

Fig. 8.4

Autoradiogram of an 8% polyacrylamide gel showing partial digests of the 3.3 Kb end labelled HindIII-AvaII fragment. Sizes are given in base pairs (bp).

Track	DNA	Enzyme
1,12	λcI_{857}	EcoRl; HindIII
2,13	pBR322	AluI
3	3.3 Kb fragment	Sau3A
4	" "	MspI
5	" "	HaeII
6	" "	HincII
7	" "	PstI
8	" "	ClaI
9	" "	AluI
10	" "	TaqI
11	" "	EcoRl*


1
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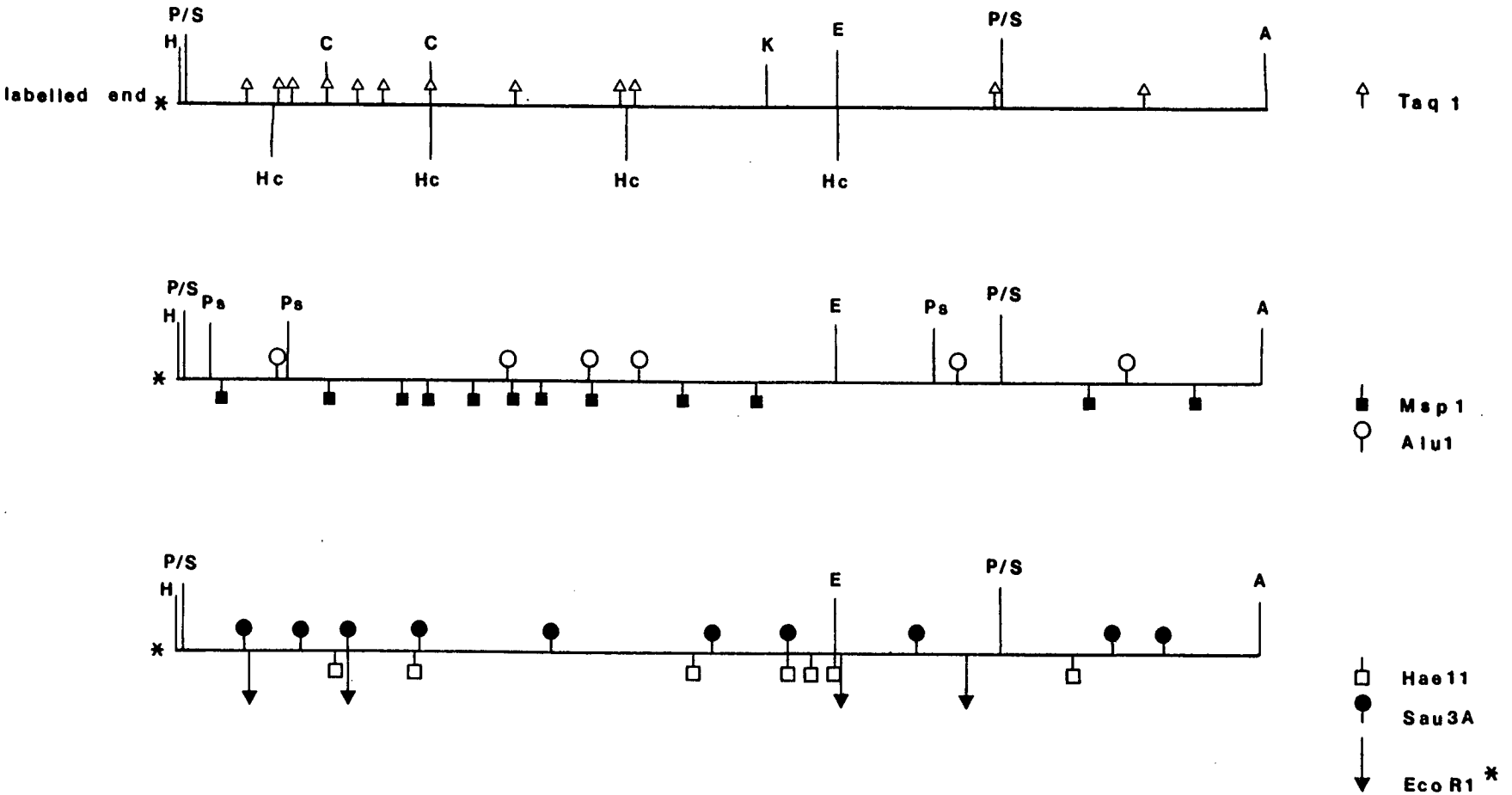
3
4
5
6
7
8
9
10
11
12
13
910
520
226
136

Fig. 8.5 Restriction map of 3.3 Kb fragment Scale 200 bp/cm



BglII, HindIII, SalI and XhoI. The labelled HindIII-AvaII fragment is 3.26 Kb in length which includes 780 bp of pKO1 DNA. Thus the size of the inserted fragment is 2.48 Kb. Since the distance between the HindIII and SmaI site in pKO1 is 16 bp, the inserted fragment ends 2.50 Kb from the labelled end.

Reorientation

In Fig. 8.6 the 2.5 Kb PvuII fragment and its internal restriction sites have been 'reinverted' to show the chromosomal orientation. What follows is the distance of each site from the left hand (ftsZ) EcoRI site (in bp):

AluI; 590, 750, 980, 1698, 2126.

ClaI; 1231, 1546.

KpnI; 210.

HaeII; 70, 140, 430, 1279, 1525, 2486.

HincII; 640, 1231, 1711, 2436.

MspI; 210, 470, 740, 890, 970, 1090, 1243, 1324, 1532, 1866.

PstI; 1664, 1897, 2186.

PvuII; 2016.

EcoRI*; 1472, 1776, 2096, 2466.

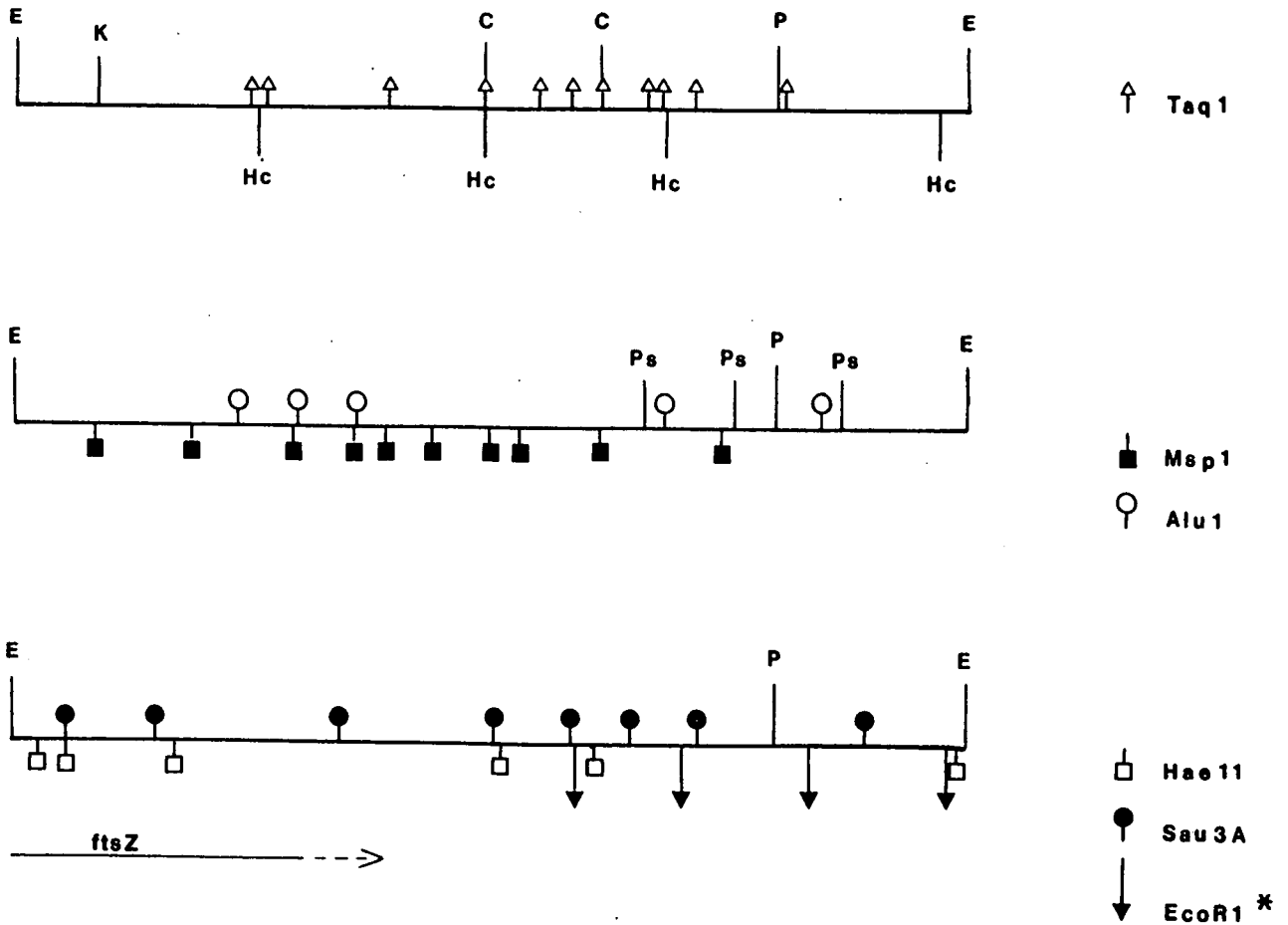
Sau3A; 140, 370, 860, 1256, 1472, 1615, 1798, 2246.

TaqI; 620, 660, 980, 1231, 1375, 1464, 1546, 1664, 1702, 1798, 2016.

Limitations and Accuracy of the technique

The Smith and Birnstiel technique as employed here makes it difficult to resolve restriction sites close to the end of the labelled fragment, and also two sites close to one another. However, the orientation of one site relative to another is clear, and 'master' gels can be read in a stepwise manner. The fragment employed here

Fig. 8.6 Restriction map of 2.5 Kb Fragment Scale 200 bp/cm



has vector DNA distal to the labelled end, facilitating an estimate of accuracy for the agarose gel. According to the sequence data of McKenney (1982) the two Sau3A sites between SmaI and AvaII in pKO1 should be 190 bp apart. In this experiment they were mapped to 150 bp apart. The AluI and TaqI site are both mapped as 400 bp from the SmaI site. Here they are mapped 390 bp and 430 bp away respectively. Hence the error is ± 40 base pairs. For the acrylamide gel no internal standards from the vector are available.

EcoRI* activity

By incubating the EcoRI enzyme in glycerol and/or low ionic strength buffers it is possible to relax the specificity of the enzyme from GAATTC through various intermediates to AATT (Woodbury et al., 1980). Thus it should be possible to reduce the specificity of EcoRI and clone EcoRI* fragments into an EcoRI site.

From 2.5 Kb one expects the sequence AATT to occur approximately ten times. Since only four EcoRI* sites have been found it is likely that the specificity has not relaxed completely, and the sites identified represent intermediates of the type GAATTN (where N is A,G or T). Such specificity was obtained in a buffer containing 33% glycerol and no NaCl: 20 mM Tris pH 8.0, 2mM $MgCl_2$, 33% glycerol.

CHAPTER 9

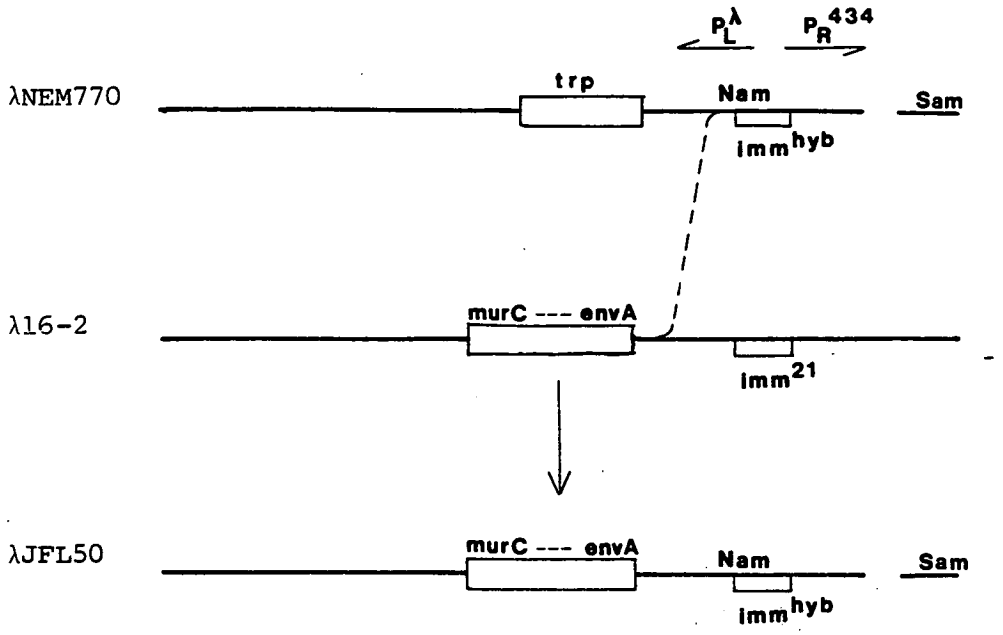
THE DIRECTION OF TRANSCRIPTION OF THE *envA* GENE AND IDENTIFICATION OF A PROBABLE NEW GENE BETWEEN *envA* AND *secA*

9.1 The direction of transcription of the envA gene

By using a hybrid immunity λ phage (λ JFL50, Fig. 9.1a) Lutkenhaus and Wu (1980) were able to assign directions of transcription to some of the bacterial genes cloned on to λ16-2. λJFL50 was constructed by crossing λ16-2 (imm^{21}) with λNEM770 (Wilson and Murray, 1979) a hybrid immunity phage with the genotype Nam7, 53; P_L^{λ}; cI^-; P_R^{434}; cro^{434}; nin 5; Sam7. Expression from rightward to leftward promoters is controlled by different repressors such that upon infection of a suppressing host lysogenic for λimm^{434} rightwards transcription from P_R^{434} is blocked by phage 434 repressor. Suppression of the N gene permits unmoderated transcription leftwards from P_L. In λJFL50 when transcription from P_L was allowed to proceed into the bacterial DNA the synthesis of both the 50 Kd ftsA protein and the 45 Kd ftsZ protein was reduced, presumably due to opposing transcription (Ward and Murray, 1979) whilst synthesis of the 31 Kd envA protein was enhanced. On this basis Lutkenhaus and Wu (1980) suggested that ftsA and ftsZ transcribed clockwise whilst envA transcribed anti-clockwise on the E.coli map. Although in other phage derivatives the 31 Kd protein can be ascribed to envA, in λJFL50 there are two other candidates, ddl (30 Kd) and gene X (~30 Kd, see Section 9.5). Furthermore, there are 7 known phage genes between P_L and the bacterial insert (Fig. 9.1b), all of which cotranscribe with P_L and might be expected to show enhanced expression of their respective products upon P_L induction. One of these, the red β product (28-30 Kd) may migrate similarly to the envA product in SDS gels and so complicate interpretation. It is not likely that P_R or P_R'

Fig. 9.1 Construction of λJFL50; λ Gene products between λP_L and the bacterial insert.

(a)



(b)

envA	int	xis	Ea 8.5	Ea 22	Ea9	red α	red β
31	36-44	7.9	8.5	19-22	9	25-26	28-30

gam	kil	cIII	Ea10	ral	N	P_L
16.5	16	ND	10-16	12	13.5	

Figures given are sizes of λ gene products (in Kd) between P_L and envA.

(a) Adapted from Wilson and Murray, 1979, Lutkenhaus and Wu, 1980.

(b) From Szybalski and Szybalski, 1979.

influence expression of the bacterial insert since these are specifically blocked by the phage 434 repressor. The data obtained by Lutkenhaus and Wu (1980) is indirect. In this chapter direct gene fusion evidence for the direction of transcription of envA is presented.

9.2 Initial gene fusions of the envA region.

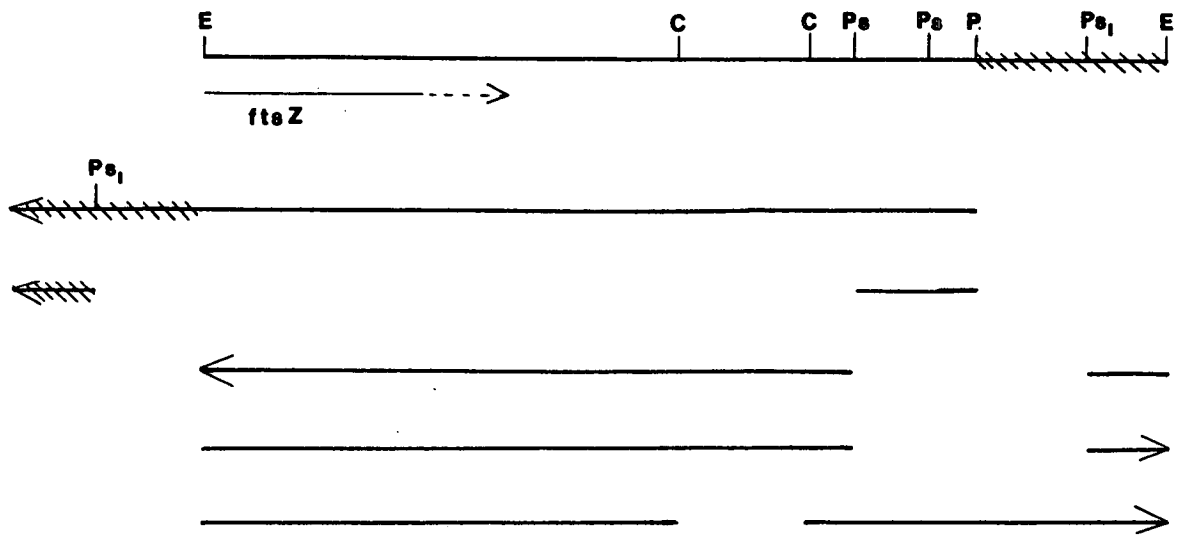
If the envA gene transcribed from right to left as suggested by Lutkenhaus and Wu (1980) then we might expect pNS11 to show promoter activity. None was detected (Fig. 9.2). A deletion derivative of pNS11 (pNS17; constructed via a PstI partial digest of pNS11) shows no promoter activity reading towards ftsZ and to the left of the PvuII site (Figs. 9.2, 9.3, 9.4). The two 500 bp PstI deletion derivatives pNS24 and pNS25 show a strong promoter in one orientation but not the other (Fig. 9.2). In pNS25 if envA reads from left to right then we might expect its promoter to be removed. The strong promoter(s) detected on pNS24 may be due either to envA reading left to right, or a new gene between envA and secA reading left to right or the sum total of both. A similar argument applies for pNS31.

9.3 Localisation of promoters within the 2.5 Kb EcoR1 fragment

As yet no pKO vectors containing either ClaI of PstI sites suitable for cloning are available. However, pBR322 has EcoR1, ClaI and HindIII sites close together:

```
   1       10                20                30                40
GAATTCTCATGTT    TGACAGCTTA    TCATCGATAA    GCTTTAATGC
 EcoR1                        ClaI    HindIII
```

Fig. 9.2 Initial gene fusions of the envA region



Plasmid	Corrected galK spec. act.
pNS11	0.08
pNS17	0.13
pNS25	0.03
pNS24	1.79
pNS31	2.42

Fig. 9.3 Construction of pNS12 and pNS17

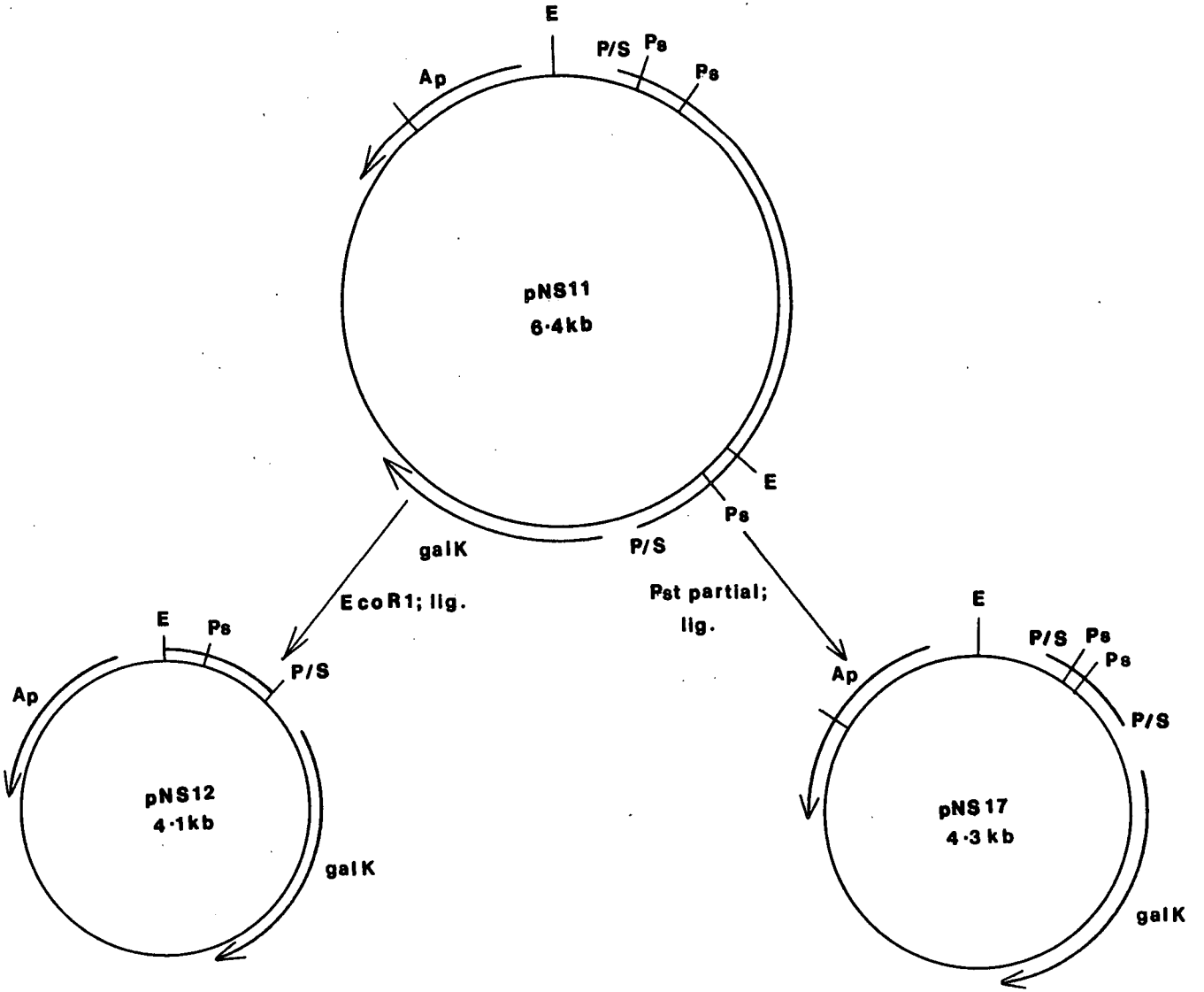
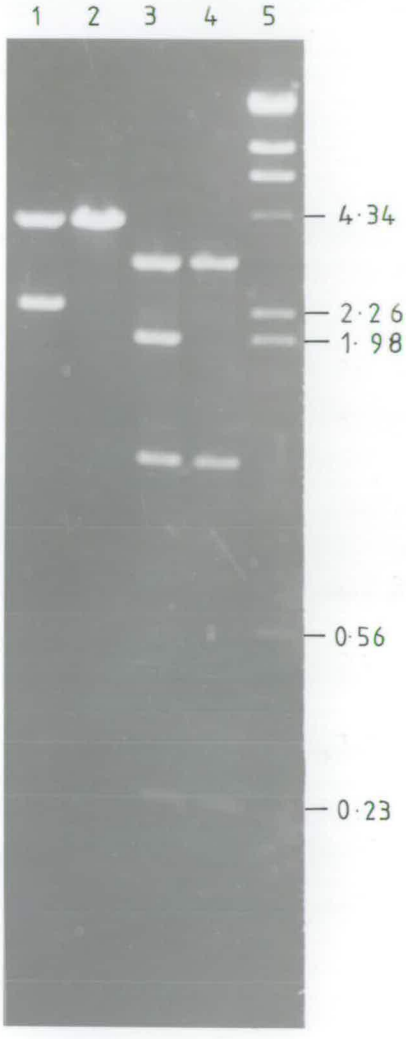


Fig. 9.4

Track	DNA	Enzyme	Refer to Fig.
1	pNS11	EcoR1	9.3
2	pNS12	EcoR1	"
3	pNS11	PstI	"
4	pNS12	PstI	"
5	λcI_{857}	HindIII	-


1
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4
5
4·34
2·26
1·98
0·56
0·23

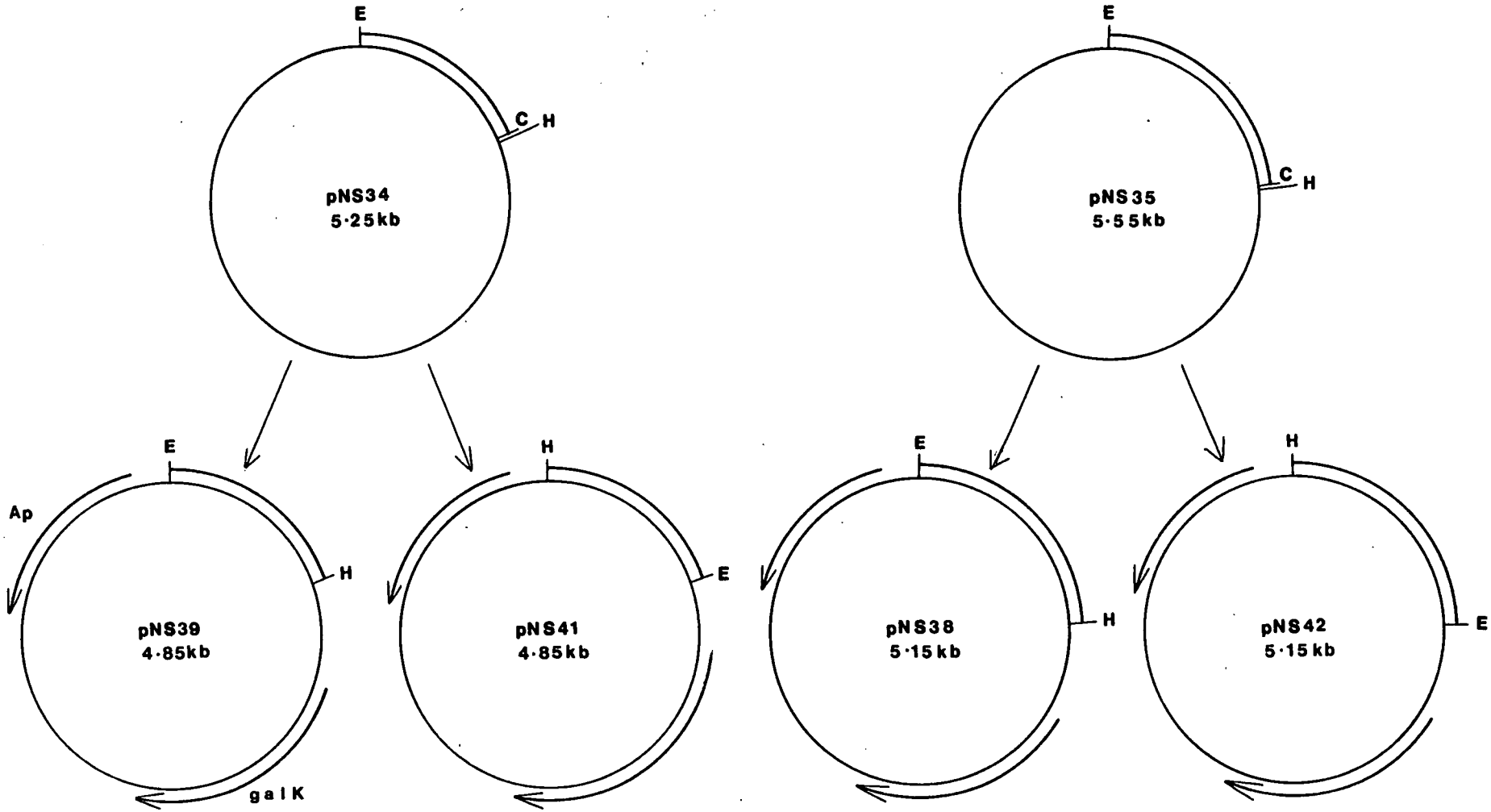
Thus an EcoR1-ClaI fragment can be inserted into pBR322 and excised again as an EcoR1-HindIII fragment suitable for cloning into pKO4 or pKO6. The net effect is the addition of a 6 bp linker to the ClaI site.

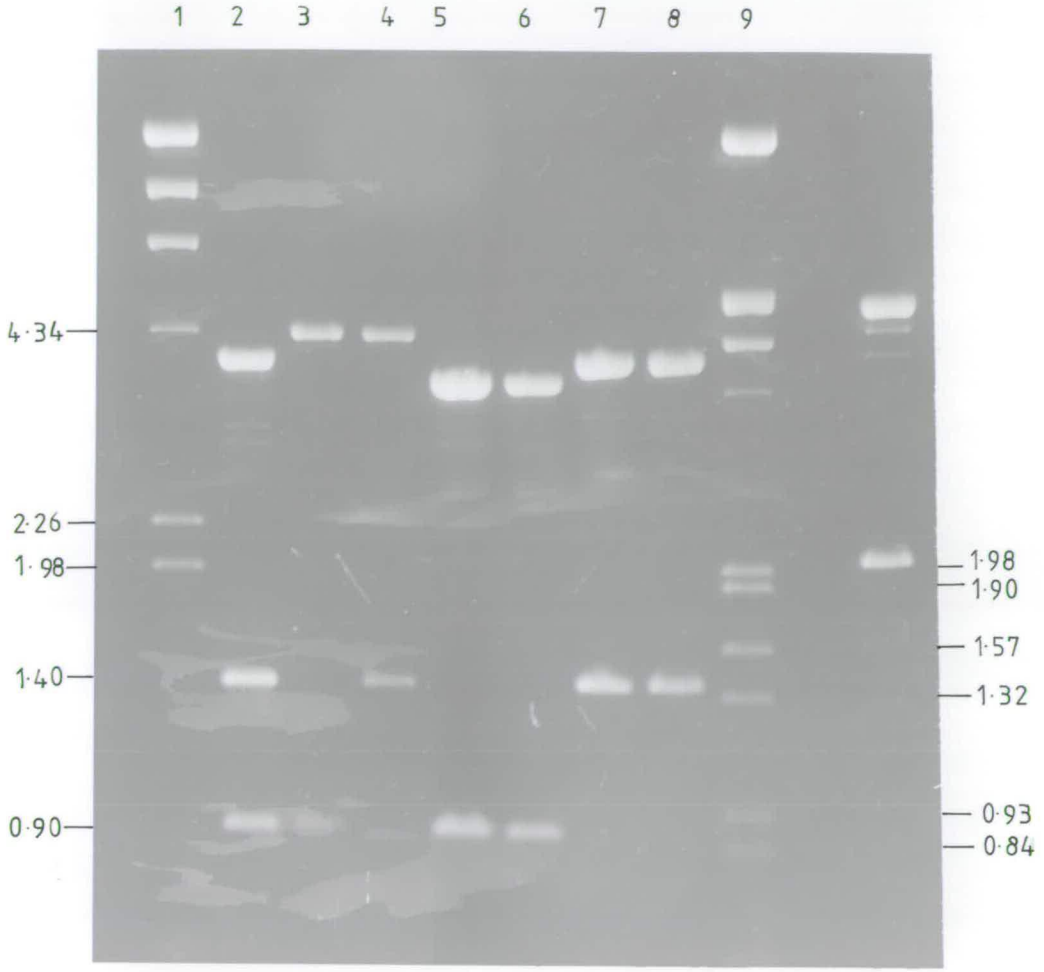
The 2.2 Kb EcoR1, ClaI Δ fragment from pNS31 was isolated from an agarose gel by the electroelution method and restricted with ClaI. This was ligated with EcoR1, ClaI and CIP digested pBR322. Two recombinants were found to have the 0.95 Kb and 1.25 Kb EcoR1-ClaI fragments separately inserted and were designated pNS34 and pNS35 respectively. The EcoR1-HindIII fragments were then subcloned into pKO4 and pKO6 in either orientation (Figs. 9.5, 9.6 and 9.7).

In pNS38, which contains the 1.25 Kb fragment inserted into pKO4, there is a promoter reading in the same direction as ftsZ most likely attributable to the envA gene reading left to right. In the opposite orientation (pNS42) no promoter is observed (see also Chapter 4). pNS41 which contains the 0.95 Kb fragment inserted into pKO6 shows promoter activity reading from left to right. This is most likely due to a new gene between envA and secA. In the opposite orientation (pNS39) no promoter is detected. This latter observation, taken together with the evidence presented for pNS11 and pNS17 (see Section 9.2) shows that the envA gene does not initiate within the 0.95 Kb fragment and does not transcribe from right to left as suggested by Lutkenhaus and Wu (1980).

To summarise (Fig. 9.8) the envA gene transcribes from left to right on the E.coli map in common with all other genes of the cluster. Another promoter has been detected reading left to right

Fig. 9.5 Construction of pNS38, 39, 41, 42

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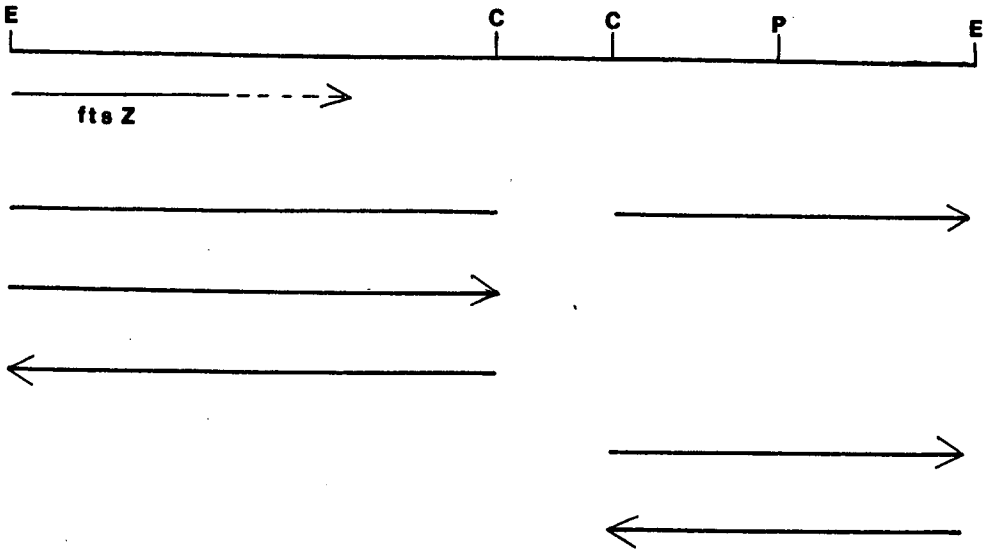
8
9
4·34
2·26
1·98
1·40
0·90
1·98
1·90
1·57
1·32
0·93
0·84

Fig. 9.6

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pNS31	EcoRl, ClaI	7.5b
3	pNS34	EcoRl, ClaI	9.5
4	pNS35	EcoRl, ClaI	"
5	pNS39	EcoRl, HindIII	"
6	pNS41	EcoRl, HindIII	"
7	pNS38	EcoRl, HindIII	"
8	pNS42	EcoRl, HindIII	"
9	λcI_{857}	EcoRl, HindIII	-

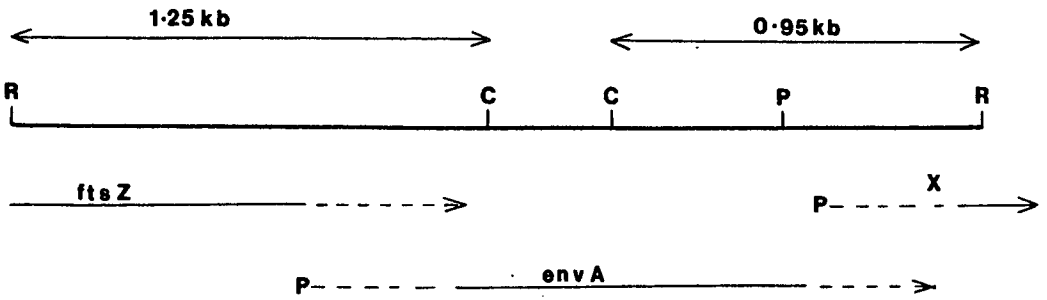
As measured by 0.7% agarose gel electrophoresis the two EcoRl, ClaI fragments generated from pNS31 are 1.40 and 0.90 Kb, whilst as measured by the Smith and Birnstiel technique their respective sizes are 1.25 and 0.95 Kb respectively. This discrepancy is probably a consequence of the different gel systems used.

Fig. 9.7 Localisation of the envA and 'X' promoters



Plasmid	Corrected galK spec. act.
pNS31	2.42
pNS38	2.19
pNS42	0.06
pNS41	1.66
pNS39	0.08
pHR1800 (pgal)	3.5

Fig. 9.8 Summary



between envA and secA and is most likely due to a new gene, henceforth designated gene X.

9.4 Further localisation of the gene 'X' promoter

Two deletion derivatives of pNS41 were constructed (Figs. 9.9, 9.11) Firstly, pNS41 was restricted with ClaI and the ends filled in using 'Klenow' enzyme. PvuII digestion followed by blunt end ligation deleted the 450 bp ClaI-PvuII fragment giving pNS47. This plasmid was shown to contain a promoter capable of expressing galactokinase (Fig. 9.10). Similarly pNS41 was restricted with EcoR1 and the ends filled in using 'Klenow' enzyme. PvuII digestion followed by blunt end ligation deleted the 510 bp PvuII-EcoR1 fragment to give pNS48. This plasmid did not contain a promoter capable of expressing galactokinase (Fig. 9.10). An EcoR1 deletion derivative of pNS11 (pNS12) in which the 510 bp PvuII-EcoR1 fragment was cloned in the opposite orientation compared to pNS47, did not show promoter activity (Figs. 9.3, 9.10). Thus the gene fusion evidence indicates that the 510 bp PvuII-EcoR1 fragment contains a promoter reading left to right.

In vitro transcription of the 510 bp PvuII-EcoR1 fragment shows a specific 'run off' mRNA of 295 bases with an optimum at 150 mM KCl (Fig. 9.12, 9.13). A weaker but possibly specific transcript is observed at 462 bases (optimum 100 mM KCl). With the 5% polyacrylamide gel used it is difficult to size such a large fragment accurately and this may well correspond to the end-end transcript.

The data presented in this and the preceding section strongly suggests that the 510 bp Pvu-II-EcoR1 fragment contains the promoter

Fig. 9.9 Construction of pNS47 and 48

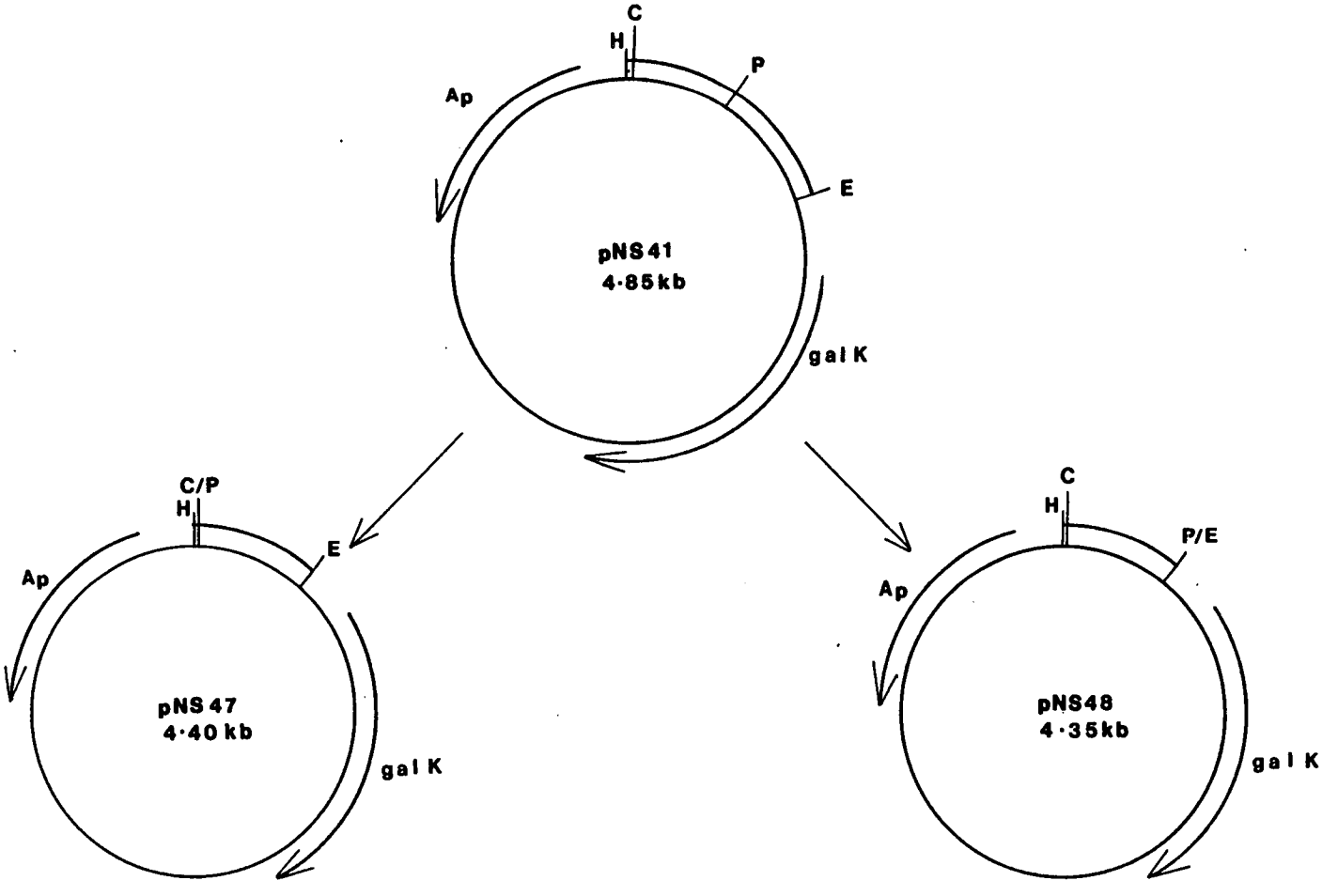
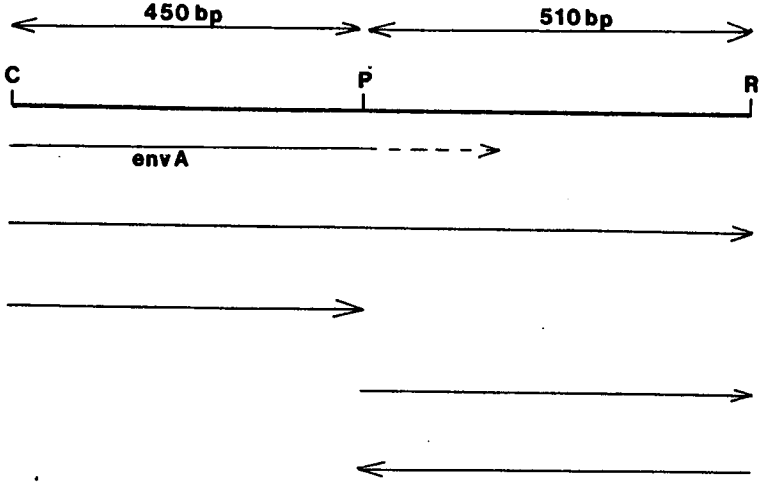
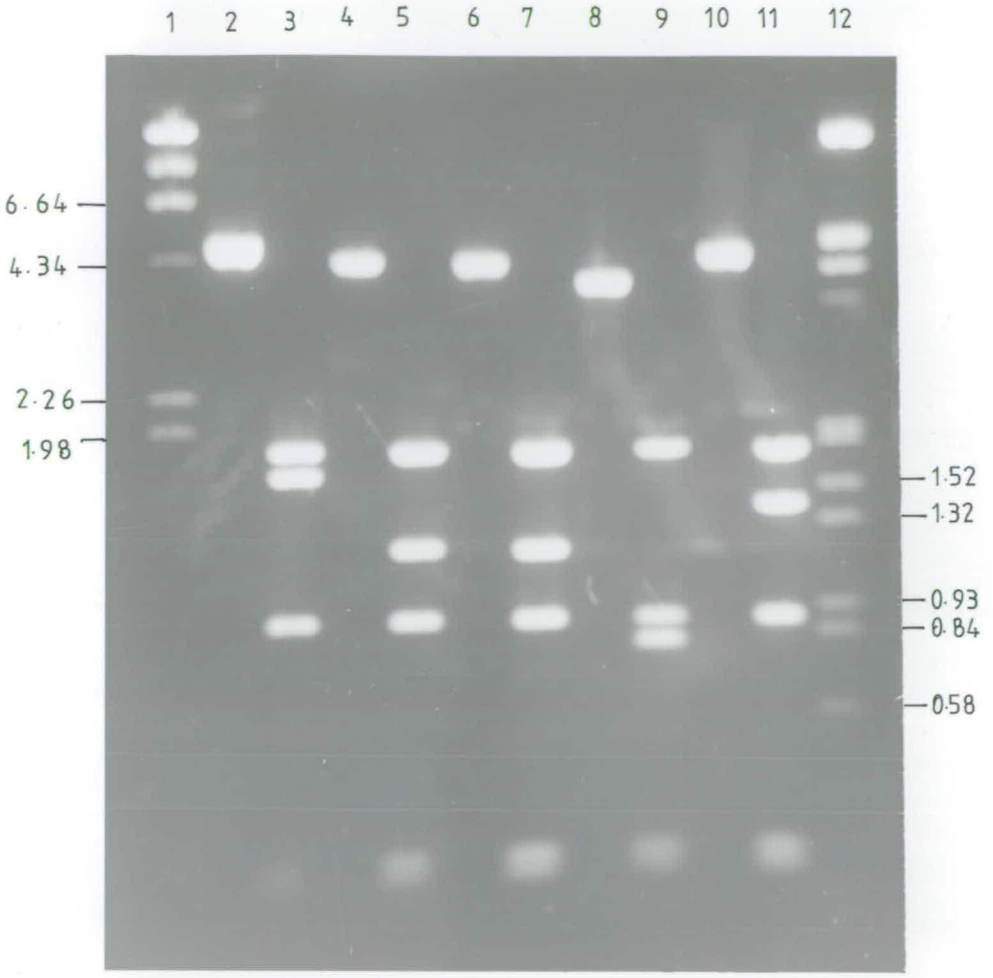


Fig. 9.10 Location of the gene 'X' promoter



Plasmid	Corrected galK spec act.
pNS41	1.66
pNS48	0
pNS47	1.43
pNS12	0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7
8
9
10
11
12
6.64
4.34
2.26
1.98
1.52
1.32
0.93
0.84
0.58

Fig. 9.11

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII	-
2	pNS41	HindIII	9.9
3	pNS41	AvaII	"
4	pNS47	EcoRl	"
5	pNS47	AvaII	"
6	pNS48	HindIII	"
7	pNS48	AvaII	"
8	pKOl	EcoRl	9.15a
9	pKOl	AvaII	"
10	pNS49	EcoRl	"
11	pNS49	AvaII	"
12	λcI_{857}	EcoRl-HindIII	-

Fig. 9.12

Autoradiogram of an *in vitro* transcription of the 510 bp *PvuII*, *EcoR1* fragment. Sizes are given in bases.

Track	Priming DNA	[KCl] mM
1	302 bp 'spot 42'	150
2	92 bp 'lac'	150
3	510 bp *PvuII*, *EcoR1*	200
4	"	150
5	"	100
6	"	50
7	"	10

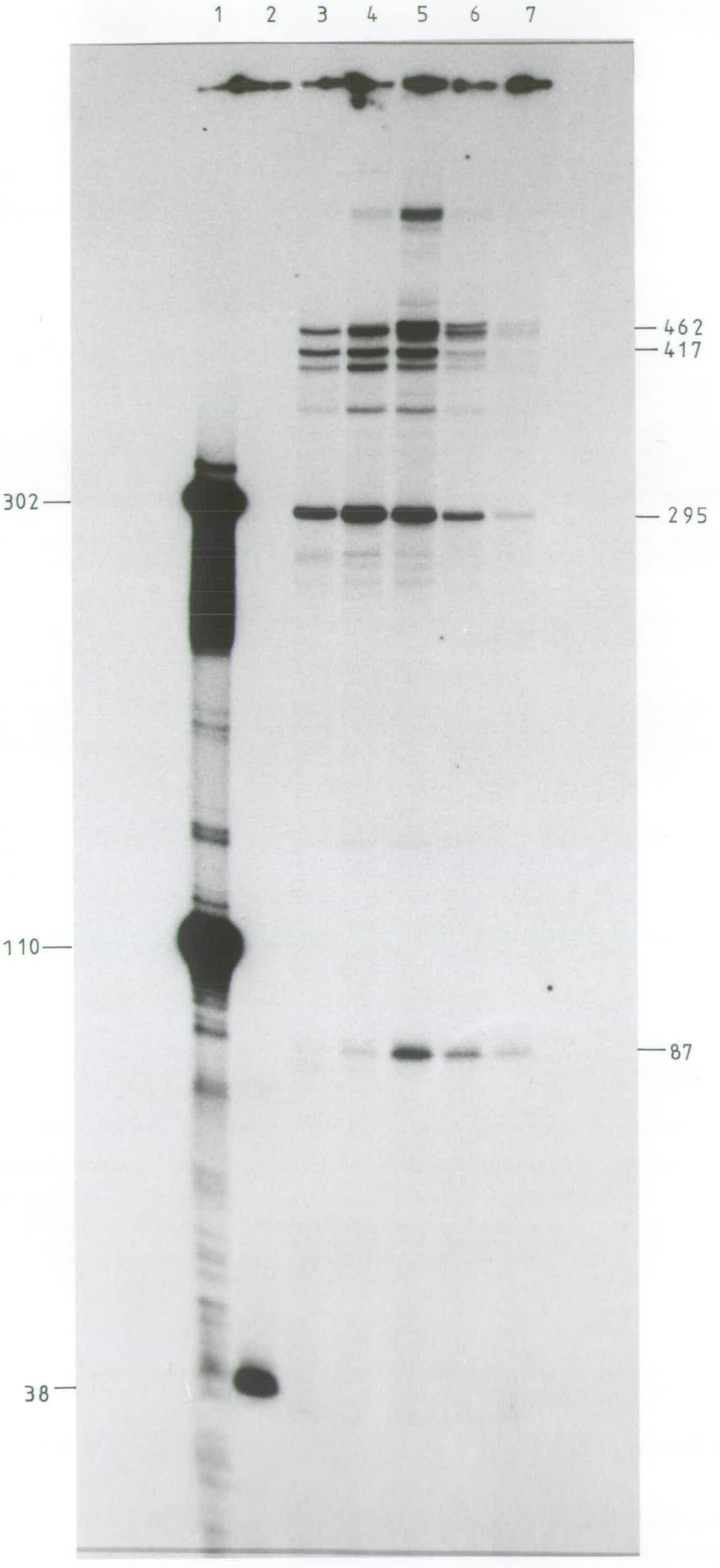
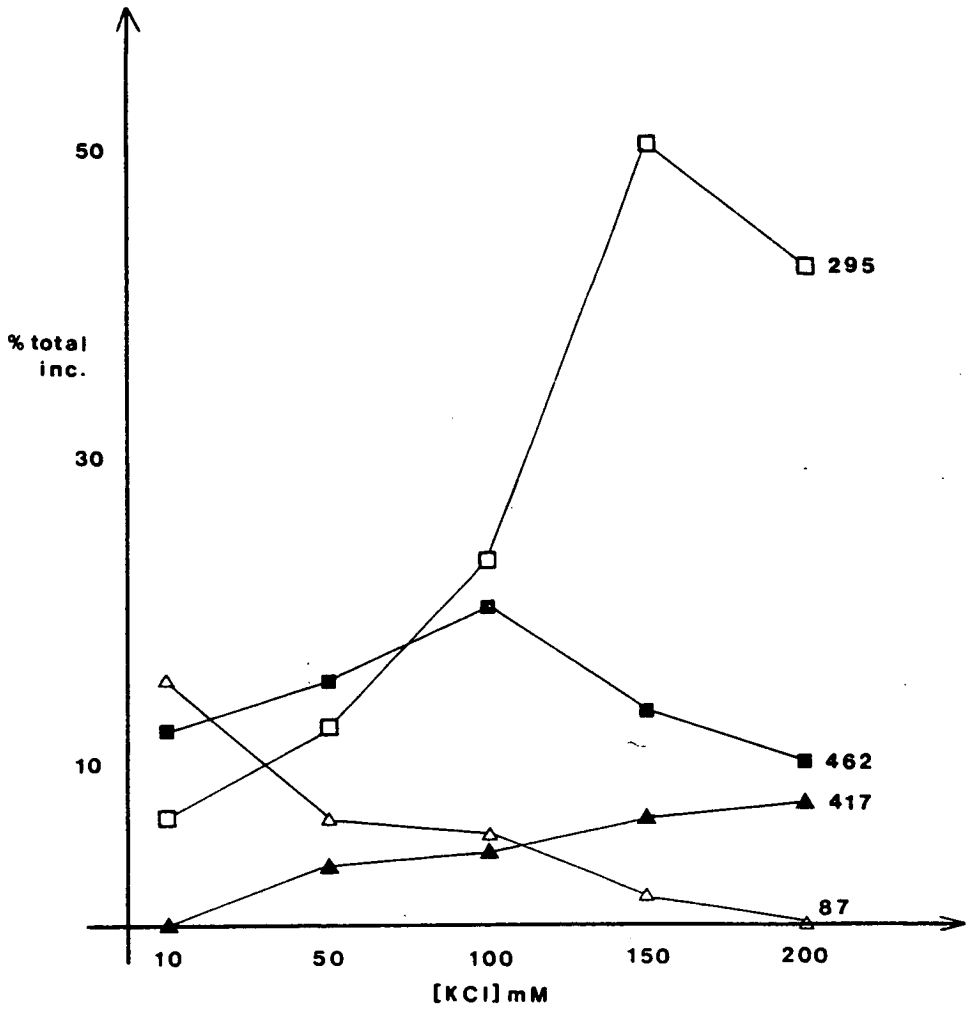

1
2
3
4
5
6
7
462
417
302
295
110
87
38

Fig. 9.13 In vitro transcription of the 510 bp PvuII-EcoRl fragment



for a new gene ('X') which lies between envA and secA and that this initiates 295 bases from the EcoR1 site and reads from left to right in common with all other genes in this cluster (Fig. 9.14).

9.5 Characteristics of the gene X

Gene X is located between a cell separation gene (envA) and a secretion gene (secA). No mutations attributable to this gene have been isolated and its function remains obscure. Evidence is presented in Chapter 11 which suggests that gene X may be a damage inducible gene (Kenyon and Walker, 1980). The control region of gene X may be associated with a cell size effect (see Chapter 10).

Assuming no overlap with secA, gene X contains enough DNA (approximately 800 bp) to code for a 30 Kd protein.

9.6a Localisation of the envA promoter

From the approximate size of the envA product and the probable extent of ftsZ the envA promoter was reasoned to be on a 590 bp HincII-HincII fragment internal to the insert in pNS38. This piece of DNA was cloned separately by ligating a mixture of HincII, CIP digested pNS38 with SmaI digested pKO1 in the presence of SmaI. In this way the donor vector cannot be recovered except as a cointegrate between pKO1 and the recipient vector. If self ligation occurs the donor vector is re-restricted. Ligation of a HincII site to a SmaI site does not produce a functional restriction site. A recombinant (pNS49) was obtained in which the 590 bp HincII fragment was inserted into pKO1 (Figs. 9.11, 9.15). pNS49 exhibited promoter activity (Fig. 9.16).

9.14 The location of gene 'X'

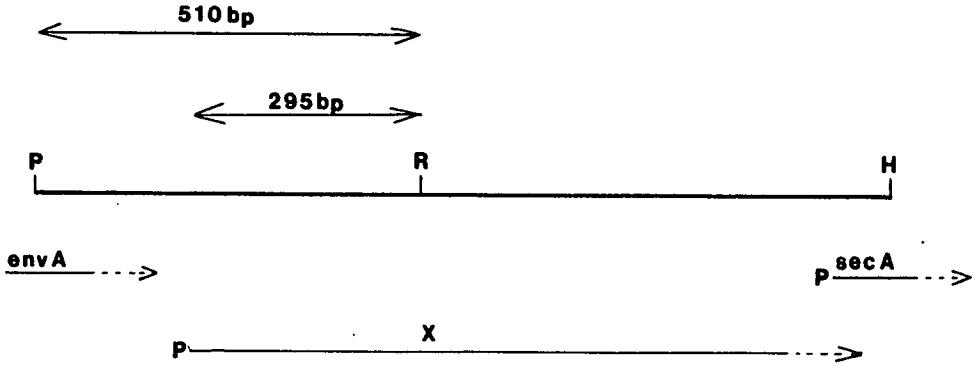


Fig. 9.15a Construction of pNS49

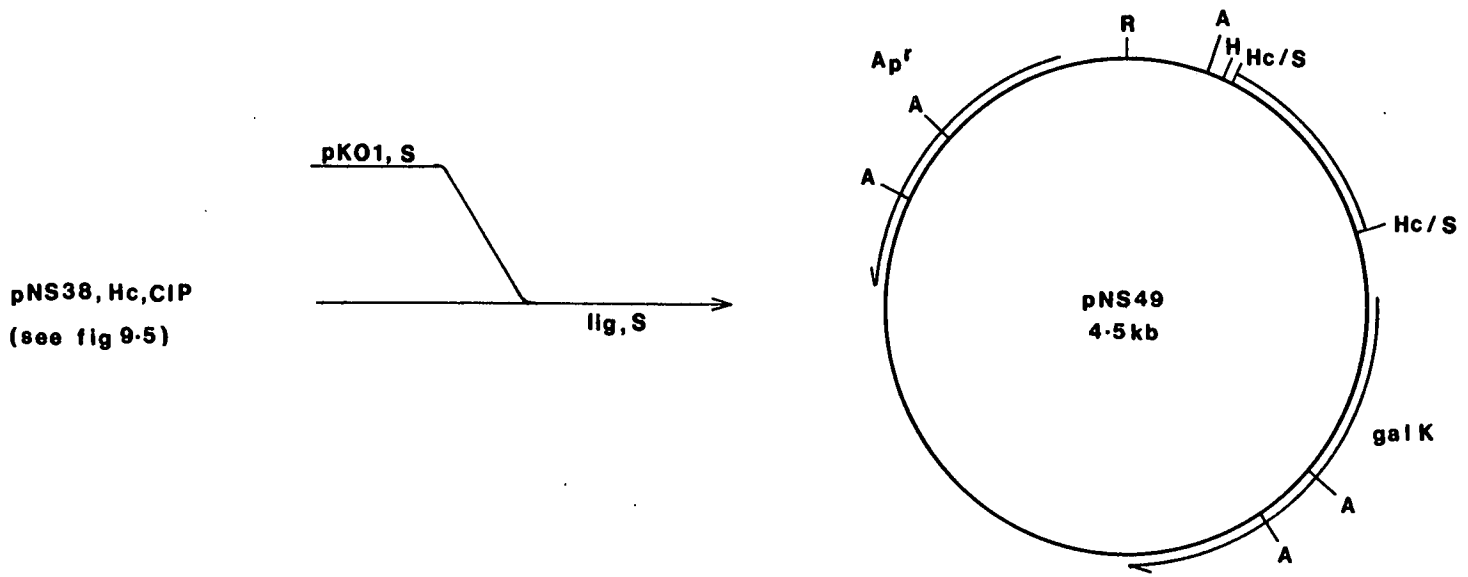


Fig. 9.15b Construction of pNS43 and pNS31-2

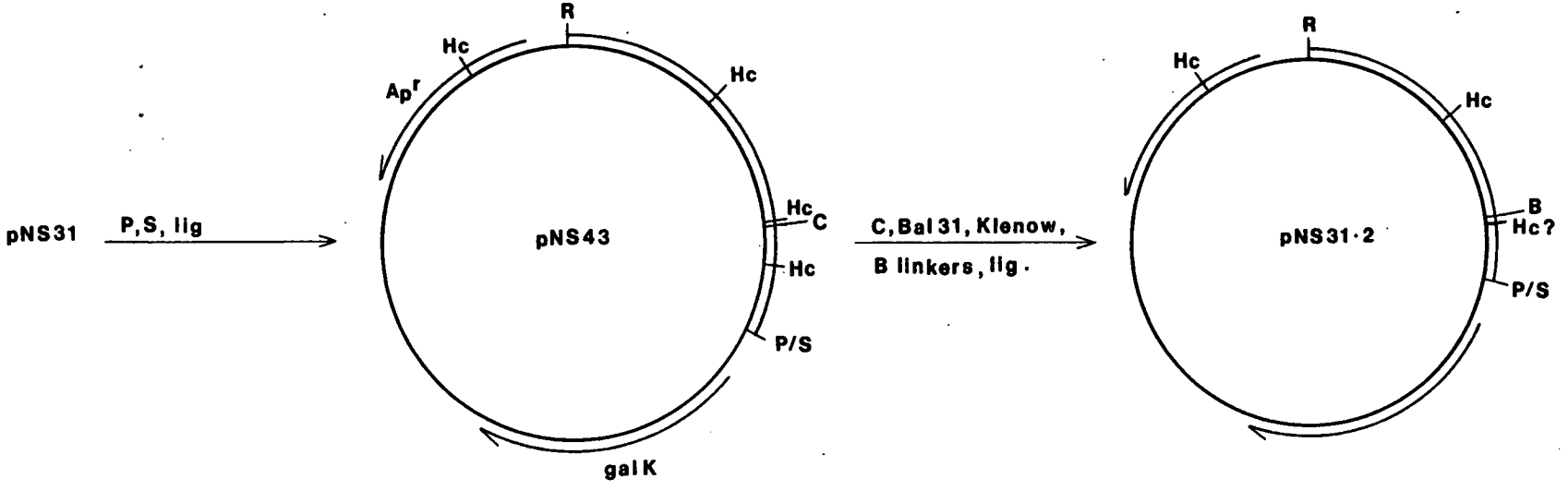
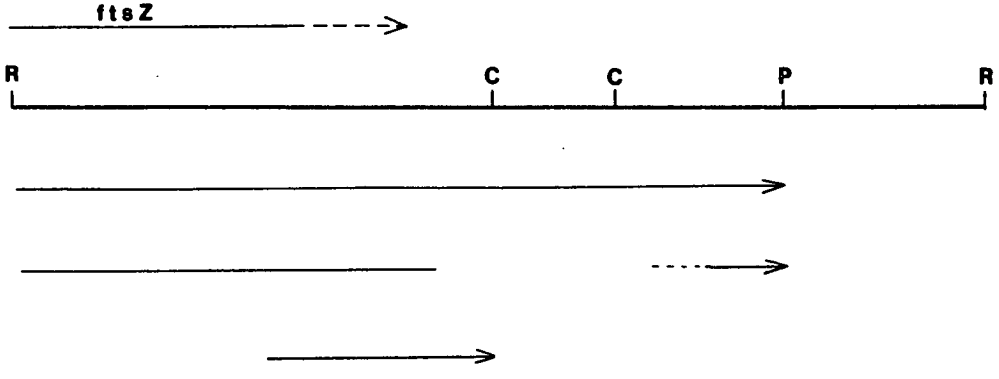


Fig. 9.16 Localisation of the envA promoter



Plasmid	Corrected galK specific act.
pNS43	2.38
pNS31-2	2.24
pNS49	2.34

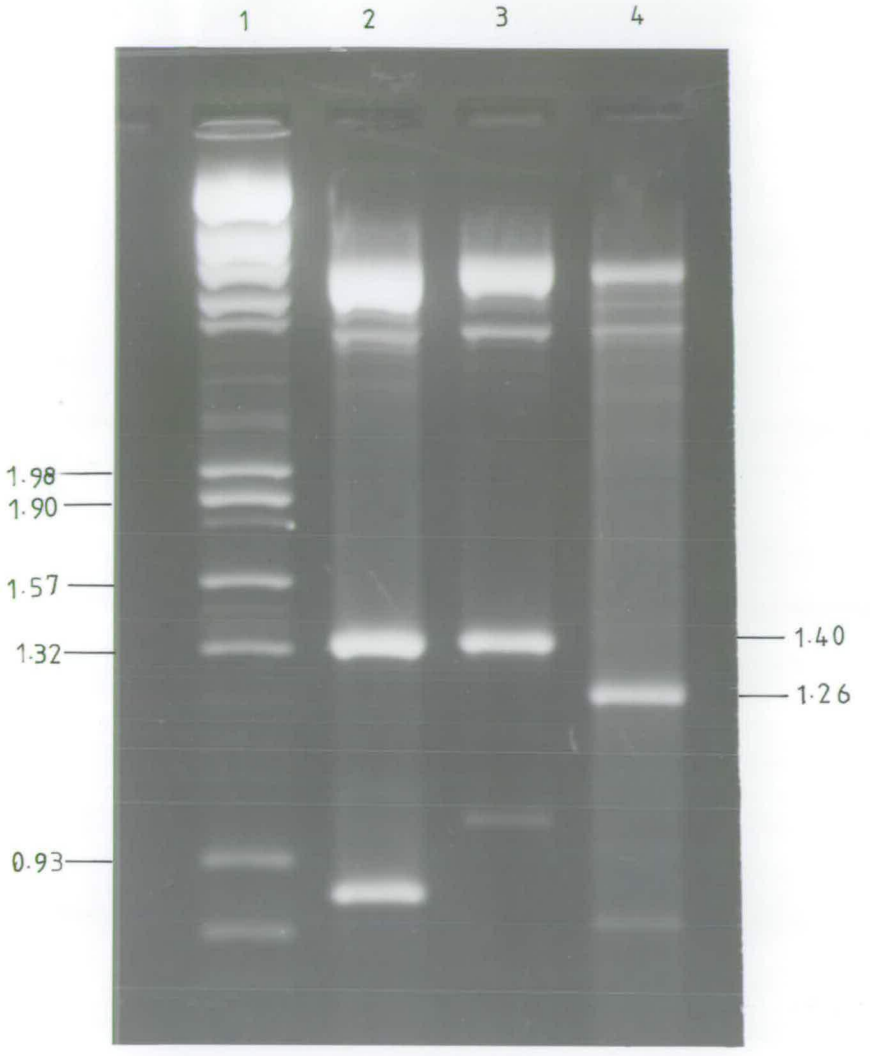

1
2
3
4
1·98
1·90
1·57
1·32
0·93
1·40
1·26

Fig. 9.17

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII, EcoR1	-
2	pNS31	EcoR1, ClaI	7.5b
3	pNS43	EcoR1, ClaI	9.15b
4	pNS31-2	EcoR1, ~~HindIII~~ BamHI	"

Notes: 1. Electrophoresis was carried out through 2% w/v agarose.

2. The DNA used in tracks 2, 3 and 4 is from a 'mini' plasmid preparation (2.5.1d).

In vitro transcription of the 590 bp HincII-HincII fragment showed the non specific run off transcripts of 130 and 437 bases and a specific mRNA of 323 bases, with an optimum of greater than 2M KCl (Figs. 9.18, 9.19à). The 323 base transcript appears as a doublet. Such multiple bands may indicate either heterogeneity at the 3' ends of run off transcripts (Reed *et al.*, 1982) or more than one point of initiation.

9.6b Construction of deletion derivatives

In order to confirm the location of the *envA* promoter the exonuclease Bal31 was used to generate deletions extending leftwards from the leftmost ClaI site. To prevent digestion of vector sequences and ensure that the inserted fragment only contained one promoter pNS43 was constructed from pNS31 by PvuII and SmaI digestion followed by ligation in dilute solution (Fig. 9.15b). This plasmid contains the *envA* promoter but not the promoter of gene X. The downstream sequence from the *envA* structural gene has previously been shown not to contain a promoter (pNS48, Fig. 9.10). The plasmid pNS43 was linearized with ClaI, digested with Bal31 and treated with Klenow to increase the proportion of blunt ends. Synthetic polynucleotide BamHI linkers were then attached and the DNA mixture transformed. The recombinant with the largest deletion (140 bp) was designated pNS31-2 and was used for further study (see Fig. 9.15, 9.17). The 140 bp deletion in pNS31-2 failed to remove the *envA* promoter (Fig. 9.16) which locates it at least 140 bp leftwards from the ClaI site in pNS43. A HincII-BamHI digest of pNS31-2 allowed the isolation of a 450 bp HincII-BamHI

Fig. 9.18

Autoradiogram of an *in vitro* transcription of the 590 bp *Hin*cII-*Hin*cII fragment. Sizes are given in bases.

Track	Priming DNA	[KCl] mM
1	302 bp 'spot 42'	150
2	590 bp *Hin*cII-*Hin*cII	200
3	" " "	150
4	" " "	100
5	" " "	50
6	" " "	10

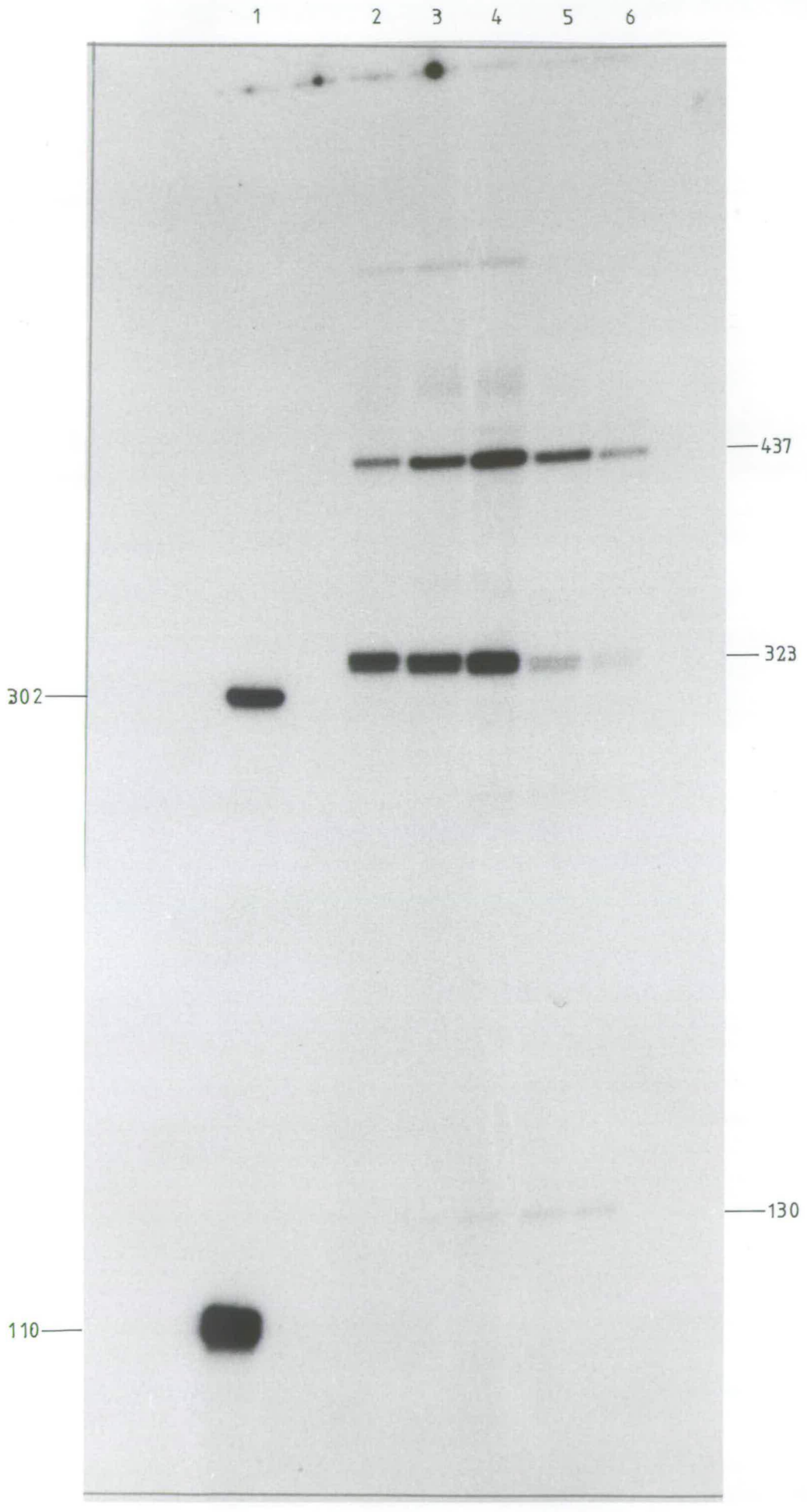

1
2
3
4
5
6
437
323
302
130
110

Fig. 9.19a In vitro transcription of 590 bp Hc Hc

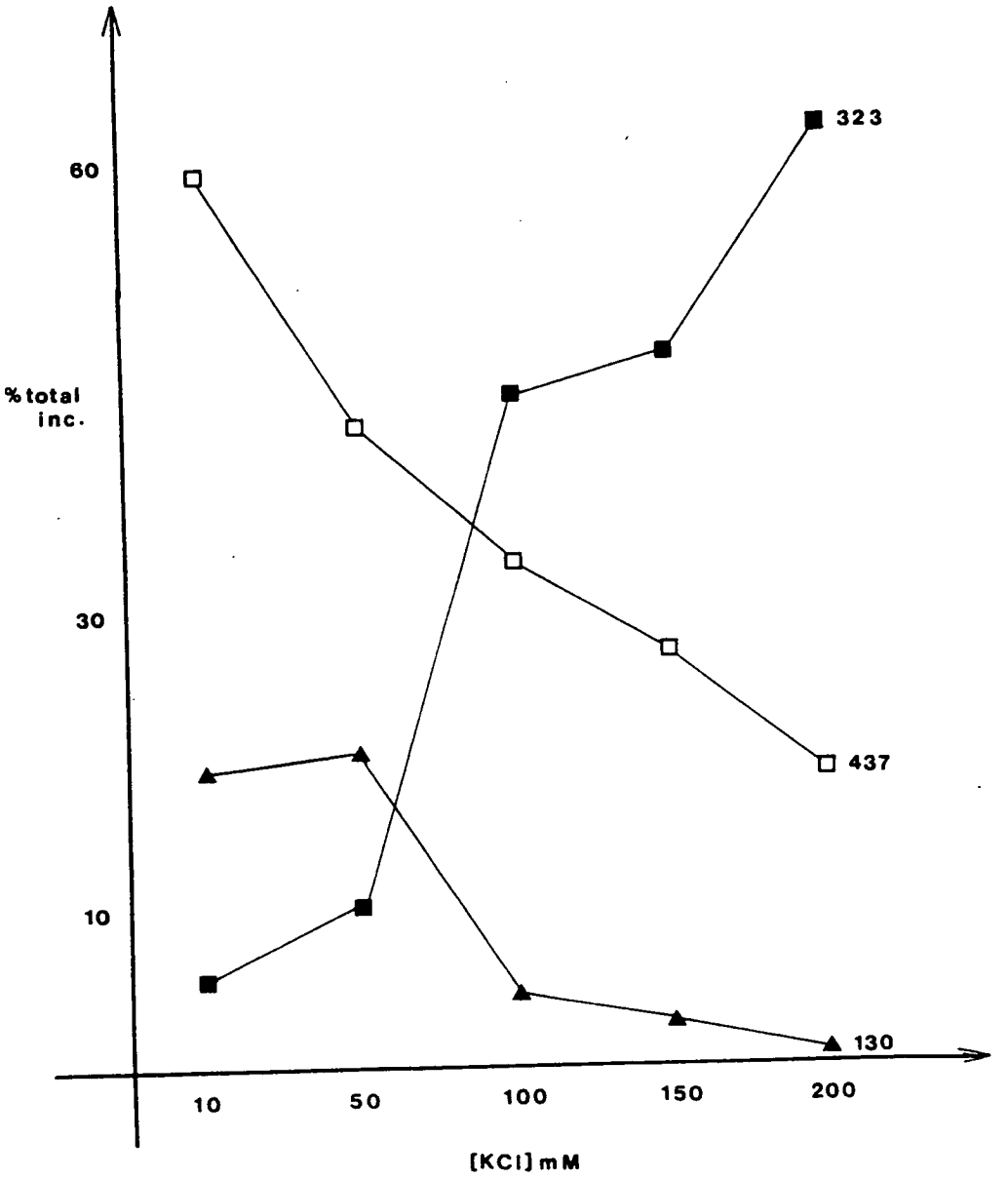
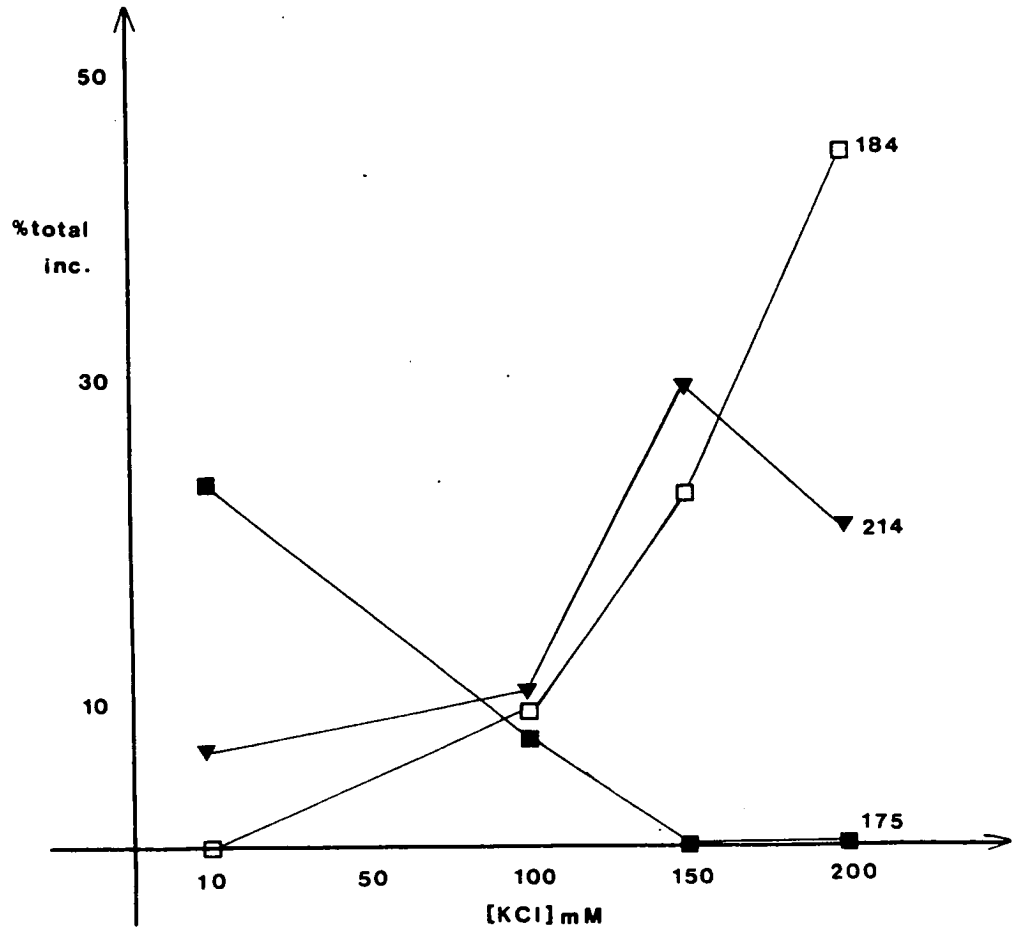


Fig. 9.19b IVT of 450 bp Hc-B

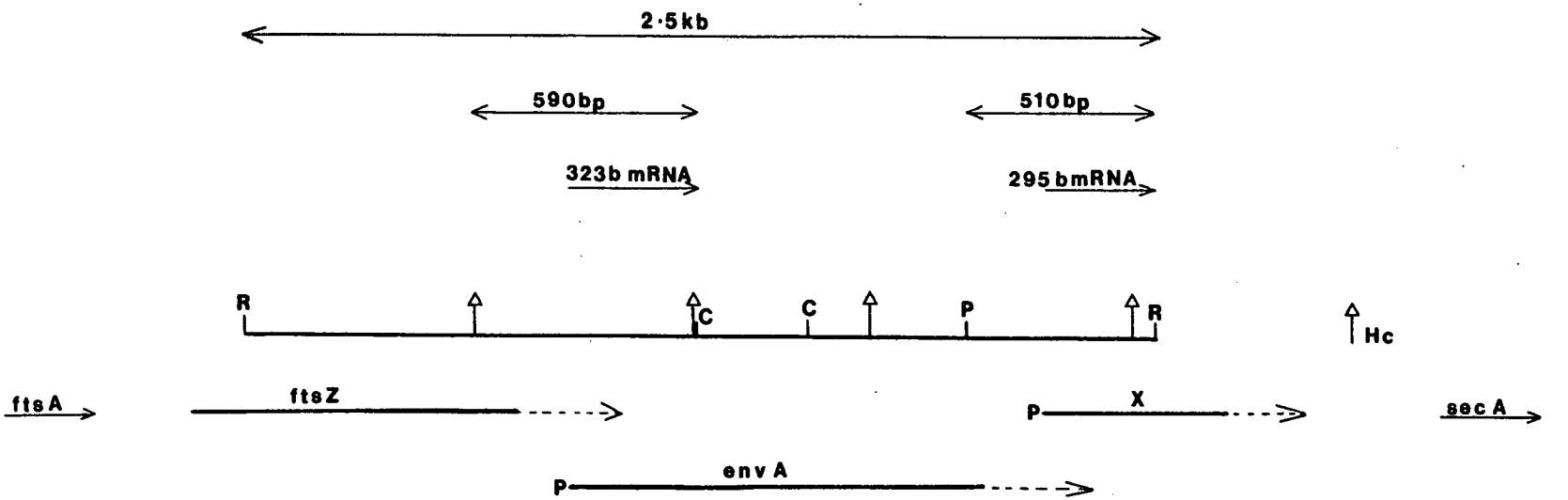


fragment which was subsequently used for *in vitro* transcription. This is equivalent to *in vitro* transcription of the 590 bp *Hin*cII-*Hin*cII fragment but with 140 bp removed from one end. Transcription of the 450 bp fragment shows a run off mRNA of 184 bp with a salt optimum of greater than 2M (Fig. 9.19b). This is consistent with the 323 base transcript obtained for the 590 bp *Hin*cII-*Hin*cII fragment (323b-184b = 139b) and suggests that the 323 base transcript runs from left to right. Since this mRNA is the only candidate for an *envA* transcript and promoter activity is associated with this region, the *envA* gene must read from left to right and start 323 bp leftwards from the leftmost *Cla*I site (Fig. 9.20).

Furthermore, the 184 base transcript from the *Hin*cII-*Bam*HI fragment does not exhibit multiple bands. Since the 323 and 184 base transcripts have different 3' ends then it is most likely that the doublet observed for the 323b mRNA is due to heterogeneity at the 3' end and not due to multiple initiation points for *envA*. Thus it is likely that the *envA* gene only has one promoter. The origin of the 214 base transcript from *in vitro* transcription of the 450 bp *Hin*cII-*Bam*HI fragment is unknown, but no counterpart is observed from *in vitro* transcription of the 590 bp fragment.

The position of *envA* can be seen in Fig. 9.20. The gene must cover at least 1070 bp giving a maximum coding capacity for a 39 Kd protein. This is not inconsistent with the 31 Kd suggested by Lutkenhaus and Wu (1980) after allowing for a possible leader mRNA or anomalous mobility in SDS gels.

Fig. 9.20 Summary



9.7 Comparison of promoter strengths

The envA promoter is approximately 78% of the strength of the medium strength E.coli promoter pgal (2.38/3.04 = 0.78), whilst the promoter of gene X is 55% of pgal (1.66/3.04 = 0.55).

9.8 Summary (Fig. 9.20)

By using a combination of gene fusion and in vitro transcription techniques it has been possible to precisely locate the envA gene and to locate a new gene between envA and secA. Both transcribe from left to right in common with all other genes of the cluster. The approximate start points of both genes have been located.

CHAPTER 10

FILAMENTATION DUE TO A CLONED 'envA' FRAGMENT

10.1 Results

During a microscopical examination of c600K$^-$ cells containing various plasmids it was noticed that some exhibited a filamentous and/or swollen phenotype. The plasmid pNS26 which contains a 2.2 Kb EcoR1 insert (the 2.5 Kb EcoR1 envA fragment with a 300 bp ClaI deletion, see Chapter 7b) causes filamentation (Fig. 10.1a) whilst the vector pNS10 shows normal rod morphology (Fig. 10.1b). The 2.0 Kb EcoR1, PstI deleted fragment was cloned into pBR328 to give pNS18 (Fig. 10.2, 10.3 c.f. pNS24 and pNS25). Both pNS18 and the parent pBR325 exhibited normal rod morphology. Thus a region within the 500 bp PstI fragment is in some was responsible for division inhibition. This was further investigated using pKO fusions (Fig. 10.4). All constructions in which galK was transcribed by moderate promoter activity caused a degree of cell swelling. Thus pKG1800 (Fig. 10.1c), pNS24, pNS31, pNS38, pNS41 and pNS20 all show swollen cells. In addition pNS31 exhibited filamentation. The plasmids pNS25, pNS11, pNS17 and pKO1 all show normal rod morphology.

A comparison of pNS31 and pNS24 again illustrates the division inhibition caused by the 500 bp PstI fragment. Furthermore, since pNS11 causes no effect on cell morphology PvuII restriction must disrupt the region responsible. PvuII restriction eliminates envA complementation implying that the right most end of the envA gene must be associated with this effect. In addition, pNS41 does not cause filamentation so either the envA promoter (or an equivalent promoter) is also required for division inhibition. Thus transcription of this 500 bp PstI fragment

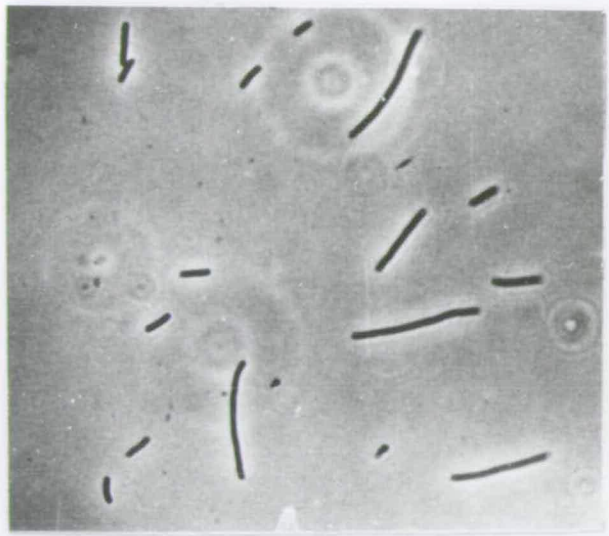
Fig. 10.1

Phase contrast micrographs of log phase

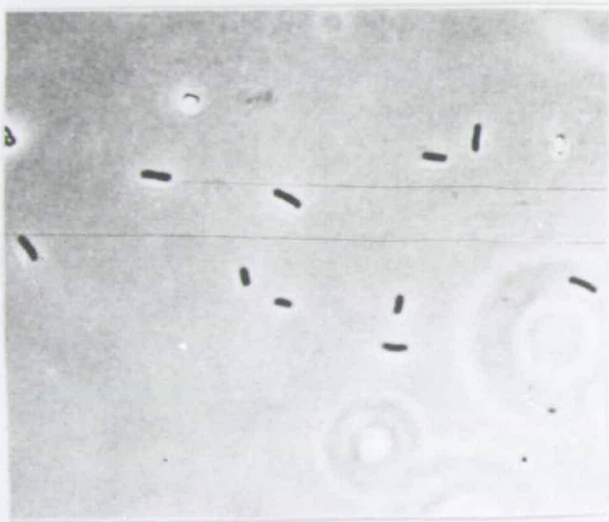
E.coli containing various plasmids. Magnification 500 times.

Fig 10·1

a) pNS 26



b) pNS 10



c) pKG 1800

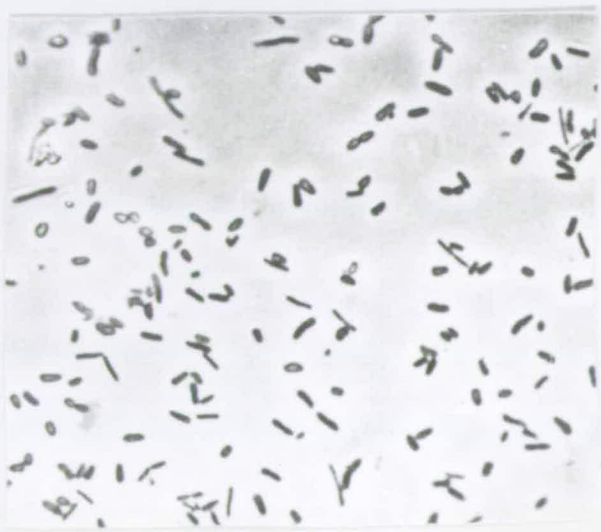
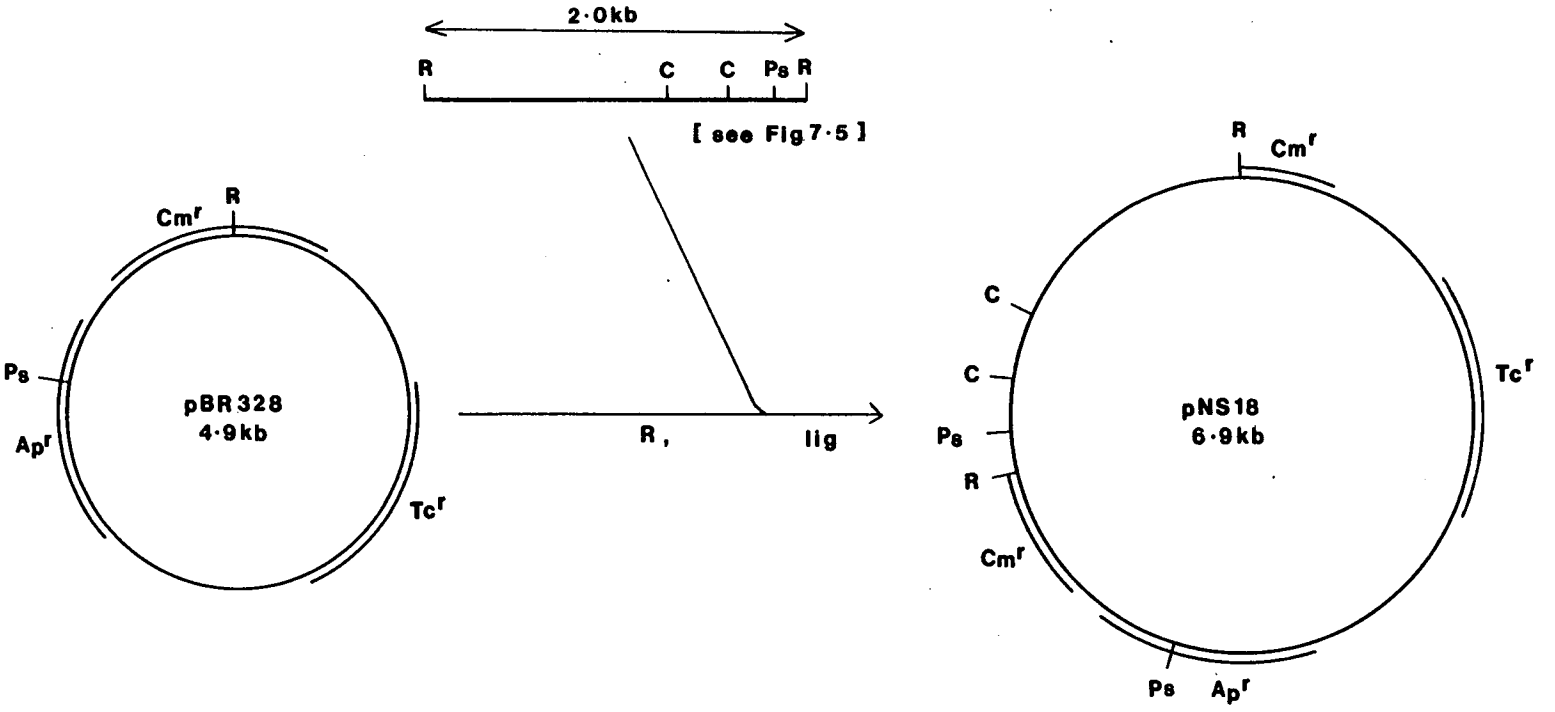


Fig. 10.2 Construction of pNS18



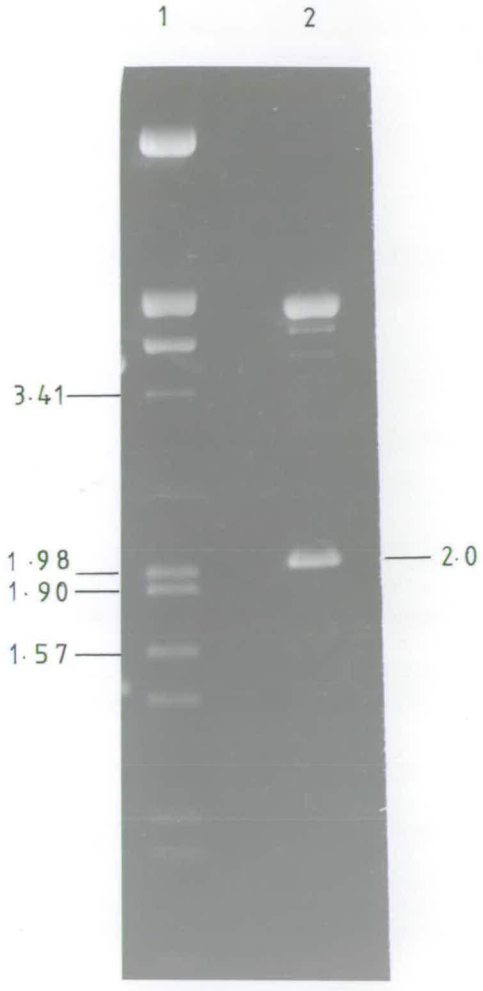
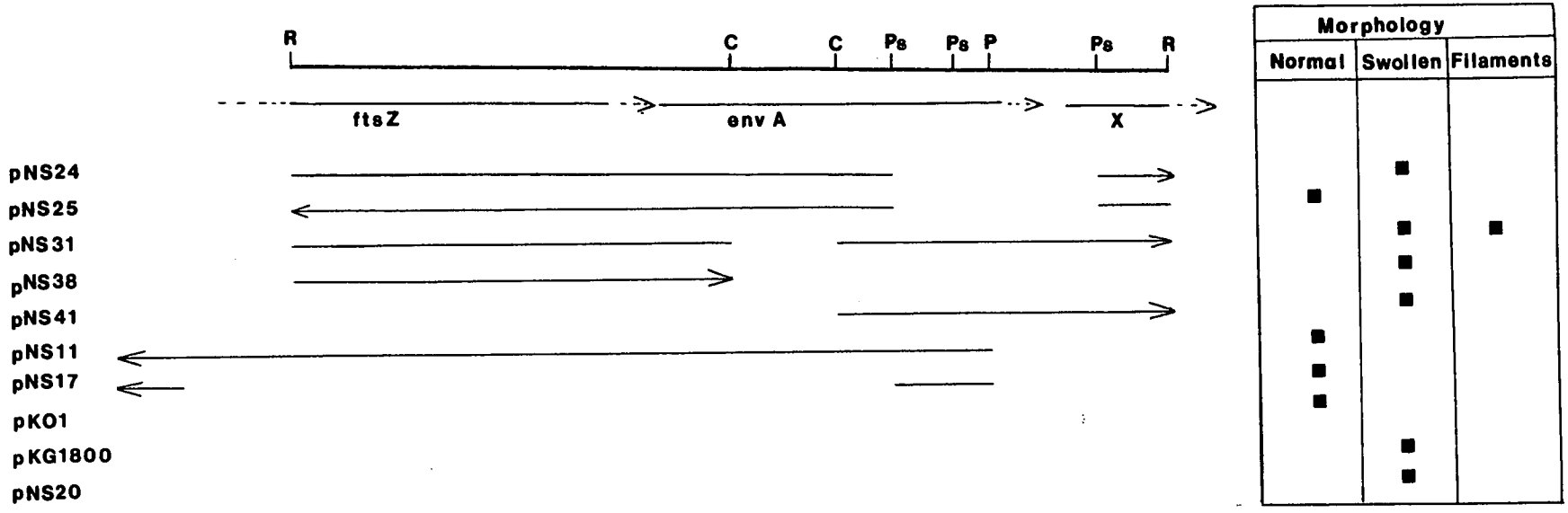

1
2
3·41
1·98
1·90
1·57
2·0

Fig. 10.3

Track	DNA	Enzyme	Refer to Fig.
1	λcI_{857}	HindIII, EcoR1	-
2	pNS18	EcoR1	10.2

Fig. 10.4 Morphological effect of cloned fragments



from left to right causes division inhibition.

The filaments observed in cultures of c600K$^-$ containing either pNS26 or pNS31 are straight sided, nucleate and are observed to divide as cells enter stationary phase.

10.2 Cell Swelling

Over production of galactokinase in c600K$^-$ can cause cell death either due to an excess of galactose-1-phosphate or UDP galactose (Fukasawa and Nikaido, 1961; Smith et al., 1970). The swollen phenotype observable due to pKG1800 and other galactokinase expressing plasmids probably reflects an accumulation of the cell wall precursor UDP-galactose. Even higher levels of galactokinase due to transcription from a stronger promoter than pgal may ultimately result in cell lysis.

10.3 Possible causes of division inhibition

(a) Multiple copies of this fragment may titrate a regulatory factor involved in cell division. Since septation is inhibited such a factor must normally act positively to induce division. This model does not satisfactorily account for the transcription requirement.

(b) The 500 bp PstI fragment may code for a protein (maximum 18 Kd). This must overlap with envA and possibly the control region of gene X, transcribe from left to right, not have its own promoter and act either directly or indirectly to repress cell division. Such a model explains the requirement for transcription and predicts that any promoter upstream of this region

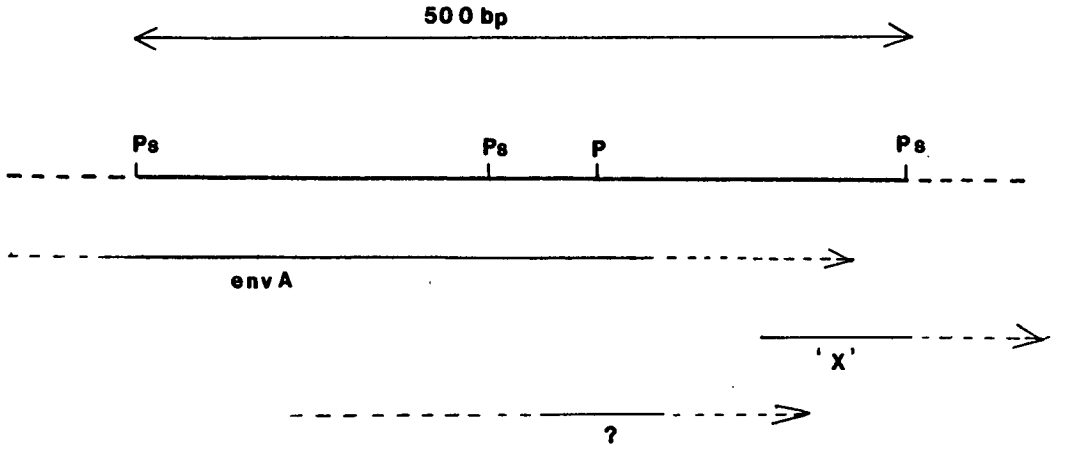
should be capable of causing filamentation (Fig. 10.5). *In vivo* such a gene must be transcribed either from the *envA* promoter or from read through from upstream, *e.g.* *ftsZ*.

(c) Filamentation is unlikely to be due either to SOS induction since cells are nucleated, or to a decrease in the rate of DNA replication (Donachie and Begg, 1978).

10.4 Summary

Plasmids in which galactokinase is overproduced have swollen morphology. A 500 bp region bounded by *Pst*-1 sites has been shown to inhibit cell division if transcribed from left to right (in its normal chromosomal orientation). This region may encode a protein which must overlap with *envA*.

Fig. 10.5 The region causing division inhibition; location of possible protein



CHAPTER 11

MISCELLANEOUS EXPERIMENTS

11(a) The effect of SOS on the envA and X genes

As with the promoters of the 2.2 Kb EcoR1 (ftsA) fragment the envA and gene X promoters were tested for possible regulation by SOS functions. Cultures of c600K$^-$ containing pNS43 (penvA) and pNS41 (pX) were separately given a UV dose sufficient to cause filamentation (10J/m^2). Galactokinase levels were measured until the cells began to recover from the division block (determined microscopically). Fig. 11.1a shows a time course of galactokinase specific activity for these plasmids, with and without UV treatment. Fig. 11.1b illustrates the induction kinetics of the three plasmids tested and shows that both penvA and pX are induced 40% by UV treatment. The gene X promoter is induced before penvA, whilst the vector pKO6 shows no induction. In all cases the galactokinase specific activity decreases with growth of the culture (Fig. 11.1a). This effect can be eliminated by dilution into fresh media (data not shown). In an effort to confirm this result galactokinase levels were measured in an isogenic tsl strain (see Chapter 6) at both the restrictive and non restrictive temperature. No induction was observed (data not shown). Thus it is not clear whether either of these promoters is indeed induced, and further experiments are needed to clarify this point. If the induction is real, then it is interesting to note that envA is expressed as the cells are recovering from the division block. Such delayed induction kinetics occur with other loci e.g. dinD (Kenyon and Walker, 1980) and uvrA (Kenyon and Walker, 1981).

Fig11·1a

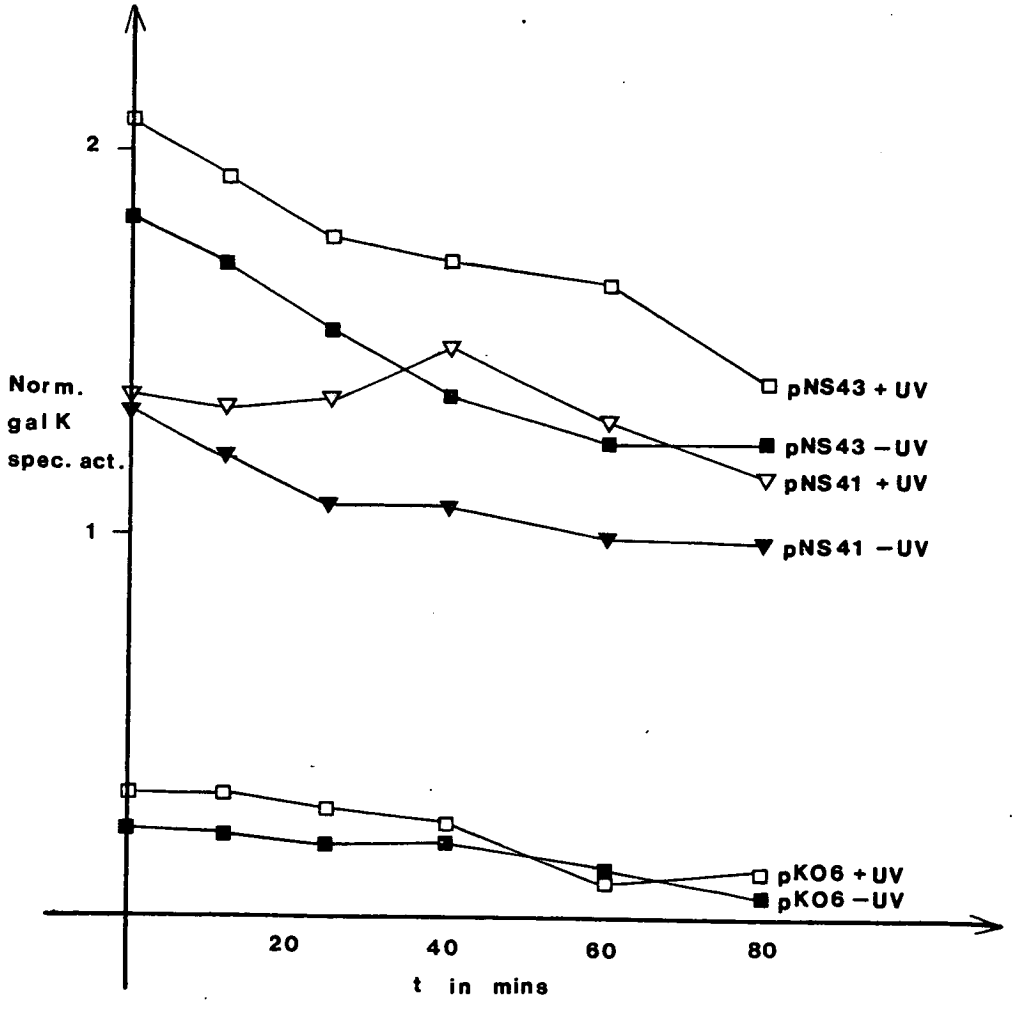

2
1
Norm.
gal K
spec. act.
pNS43 + UV
pNS43 −UV
pNS41 + UV
pNS41 −UV
pKO6 + UV
pKO6 −UV
20
40
60
80
t in mins

Fig 11·1b

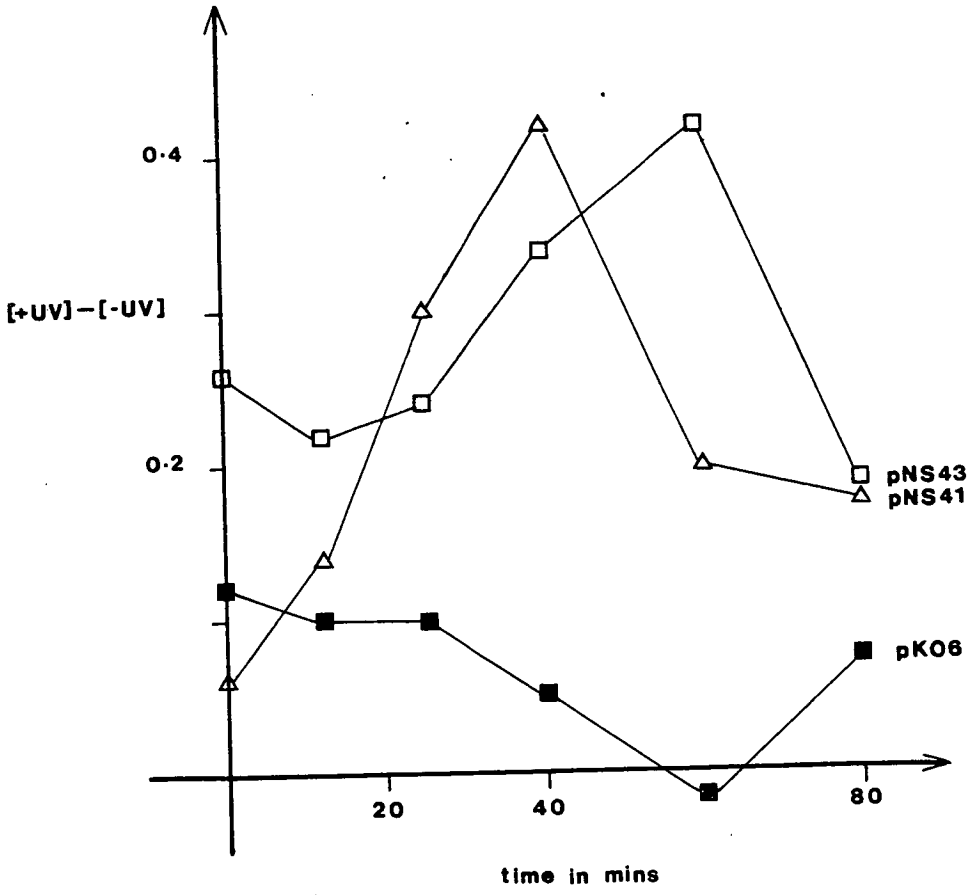
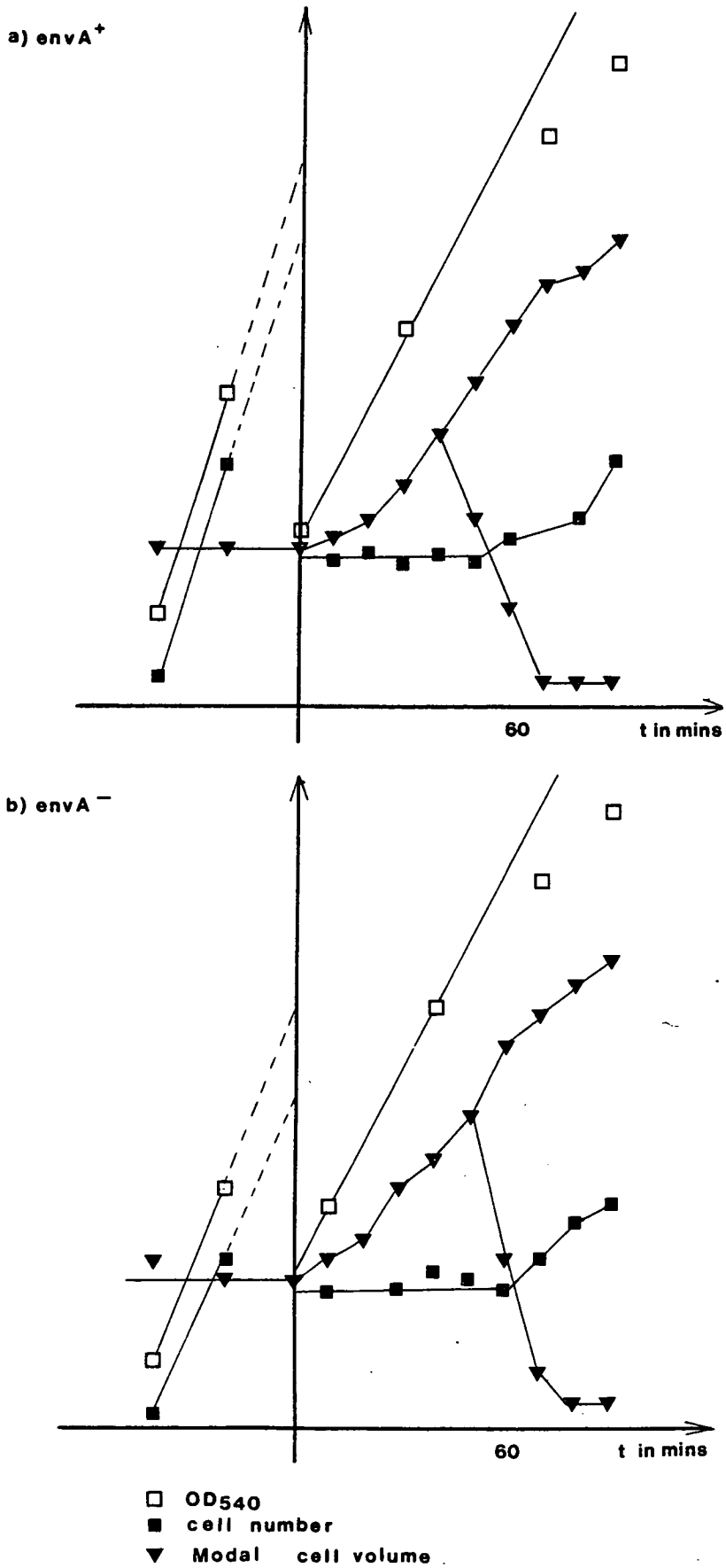


Fig 11·2


a) envA+
60
t in mins
b) envA−
60
t in mins
OD540
cell number
Modal cell volume

If the envA product was a non-induced target for division inhibition then the above experiment would not detect this. To investigate such a possibility steady state cultures of c600K$^-$ envA$^-$ (Ken 10.5) and its isogenic parent c600K$^-$ were treated with UV (10J/m^2) and their response monitored (Fig. 11.2). After UV treatment the cells continue to grow (OD_{540} increases) but do not divide (hence the cell number does not increase). The resultant filamentation is reflected in the modal cell volume (V mode) which increases until repair is complete. The two modes observed after 50 minutes reflect the increasing proportion of normal cells to filaments. The response of c600K$^-$ (Fig. 11.2a) and c600K$^-$ envA$^-$ (Fig. 11.2b) to UV irradiation is identical, suggesting that the envA product does not have a role to play in the SOS block to division. Since these experiments were performed Lutkenhaus (1983) has shown that the neighbouring gene ftsZ is an allele of sulB and is a probable non induced target for division inhibition.

A similar experiment was performed with c600K$^-$ containing either pNS10 or pNS26 to test whether or not the filamentation factor (see Chapter 10) was involved in the SOS response. In both cases cells respond normally to the UV induced division block (data not shown) supporting the contention that the cell size effect is not an SOS related phenomenon.

11(b) When in the same strain, the envA and tsl mutations are lethal.

The tsl strain DM961 was transduced to tetracycline resistance using the lysate described in Appendix 2 in an attempt to transfer the envA mutation. Since the tsl mutation was found to be salt

reversible nutrient agar was used for this experiment. All tetracycline resistant transductants were found to be temperature sensitive (i.e. tsl), but none were sensitive to rifampicin (5 µg/ml). Thus either the tsl and envA mutations are lethal in combination or the tsl mutation can suppress the envA phenotype. To elucidate this 20 independent transductants were made tetracycline sensitive (Bochner, et al., 1980) and transduced to tetracycline resistance using the strain TST1 (malE::Tn10). All of these clones were found to have lost the tsl mutation. None were rifampicin sensitive and hence did not contain the envA mutation. Thus it is likely that the tsl envA combination is lethal.

11(c) Transposon insertions into envA and sulB

A mixed Tn10 transducing lysate (Kleckner et al., 1978) grown on W3110 (leu^+) was used to transduce c600K$^-$ (leu^-, Tc^S) to leu^+ Tc^R. 100 Transductants were screened for sensitivity to 5 µg/ml rifampicin. None were obtained, perhaps indicating that envA is an essential gene. If the envA product is indeed associated with autolysin activity (Wolf-Watz and Normark, 1976) then complete inactivation by transposon insertion may well be lethal (Mendelson, 1982). Lutkenhaus (1983) has isolated a Tn5 insertion into the envA gene contained within λ16-25.

This same mixed Tn10 transducing lysate was used to transduce DM961 (tsl leu^-) to tetracycline resistance in an attempt to isolate insertions into sfiB, which could then be used to map this locus. The transduction was first plated on NA Tc (15 µg/ml) at 42°C. This was expected to select for either tsl or for tsl sfi::Tn10.

Of the 105 clones obtained none were linked to leu, so were probably either tsl revertants or transposon insertions into sfiA. Secondly, the transduction was plated on minimal agar at 42°C containing tetracycline (15 μg/ml) thus selecting directly for insertions into sfiB. No insertions were obtained. Thus sfiB may be an essential gene in accord with the suggestions of Johnson (1977) and Gottesman et al. (1981b). To circumvent this problem one possibility is to cover a sfiB mutation with F'104 (Bachmann and Low, 1976). One mutation available was sulB 25 of PAM161. This was transferred to a tsl background (DM961) and a nalidixic acid resistant derivative selected (Ken 2; constructed by Ken Begg). Ken 2 was mated with F'104 selecting for nalidixic acid resistance (the donor is nalidixic acid sensitive) and for leu^+ pro^+ thr^+ which selects for transfer of the F'104. Of 100 colonies so obtained all grew at 42°C indicating that the $sfiB^+$ of F'104 cannot complement the chromosomal sulB25 mutation, so sulB25 must be dominant. Lutkenhaus (1983) has since also demonstrated that sulB25 is dominant.

11(d) Evolutionary conservation of the 2.5 Kb EcoR1 restriction fragment

The single peptidoglycan layer of gram negative bacteria is likely to have evolved from the multi-layered peptidoglycan of a single ancestral gram positive strain (Nakamura et al., 1979). For survival this has required simultaneous evolution of genes capable of mediating cell division. In E.coli many of the loci for peptidoglycan biosynthesis and cell division are clustered

together at 2 minutes on the E.coli map (see Section 1.2.1). The following experiment describes the evolutionary conservation of a 2.5 Kb EcoR1 fragment from within this cluster. This region contains most of the structural gene of ftsZ (sulB), the entire envA gene and part of an unknown gene between envA and secA (Lutkenhaus and Wu, 1980; this thesis).

The bacterial species chosen represent both the Enterobacteriaceae and a range of phylogenetically divergent bacterial species. Amongst the tested Enterobacteriaceae were E.coli K-12, Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Serratia marcescens, Hafnia alvei, Proteus morganii and Proteus vulgaris. Besides the gram positive Bacillus subtilis were tested two gram negative organisms outside the Enterobacteriaceae, Pseudomonas putida and Azotobacter vinelandii.

E.coli chromosomal DNA was prepared by phenol extraction of a protease treated cleared lysate of c600K$^-$. All other chromosomal DNA was the generous gift of A. Jenkins. Chromosomal DNA (3 μg) was restricted with EcoR1 and electrophoresed in 0.7% w/v agarose prior to transfer to nitrocellulose (Southern, 1975) and hybridization to the nick translated (Rigby et al., 1977) 2.5 Kb EcoR1 fragment purified from λ16-2.

Results and Discussion

Fig. 11.3 shows that all eight species of Enterobacteriaceae have singly homology to the 2.5 Kb EcoR1 probe. The 2.5 Kb EcoR1 fragment is conserved in both E.coli and Enterobacter aerogenes whilst smaller fragments are observed in Citrobacter freundii,

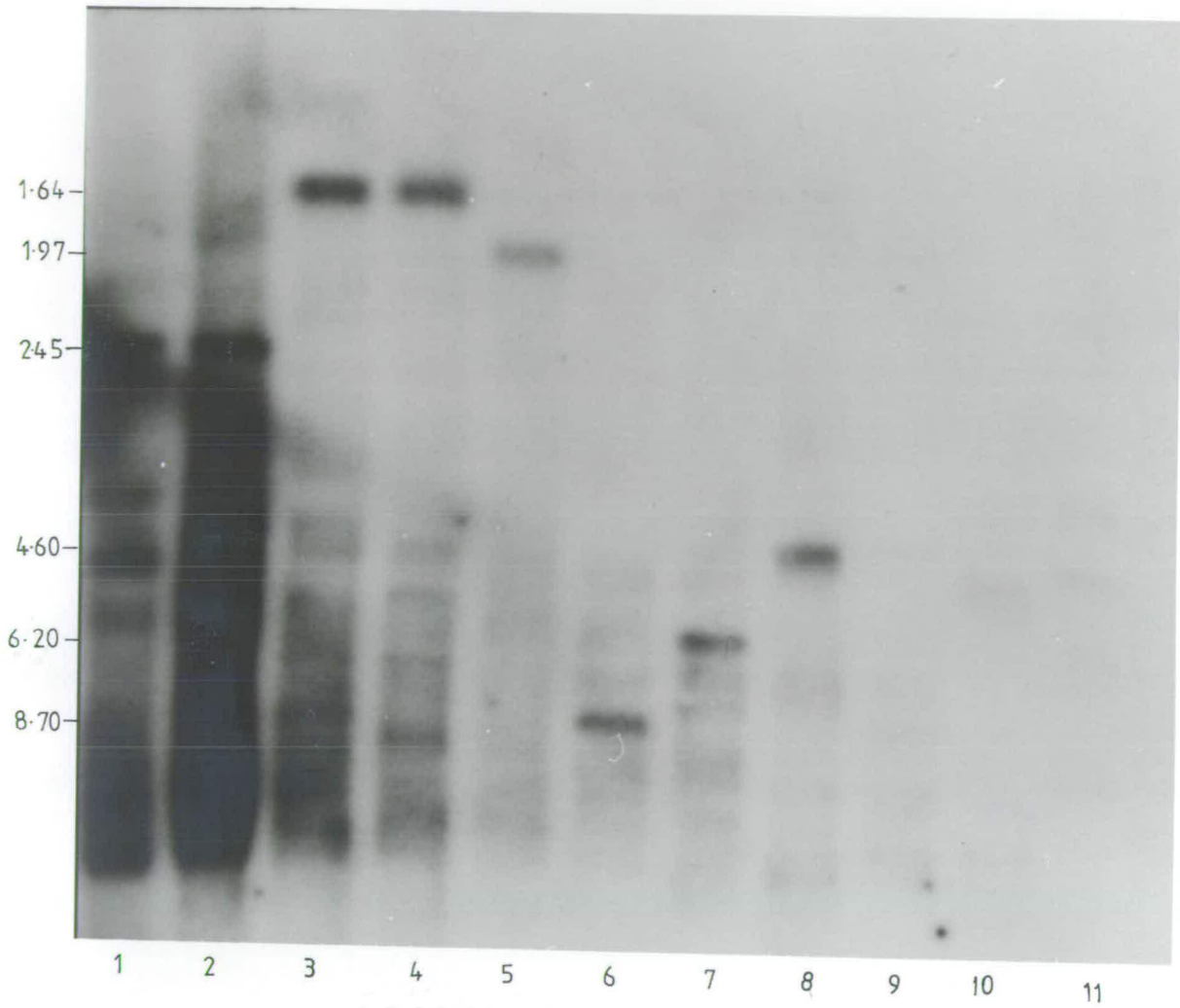

1·64
1·97
2·45
4·60
6·20
8·70
1
2
3
4
5
6
7
8
9
10
11

Fig. 11.3

Autoradiogram showing homologies of various bacteria to the 2.5 Kb EcoR1 fragment.

Track	Bacterium
1	Escherichia coli
2	Enterobacter aerogenes
3	Citrobacter freundii
4	Klebsiella pneumoniae
5	Serratia marcescens
6	Hafnia alvei
7	Proteus morganii
8	Proteus vulgaris
9	Pseudomonas putida
10	Azotobacter vinelandii
11	Bacillus subtilis

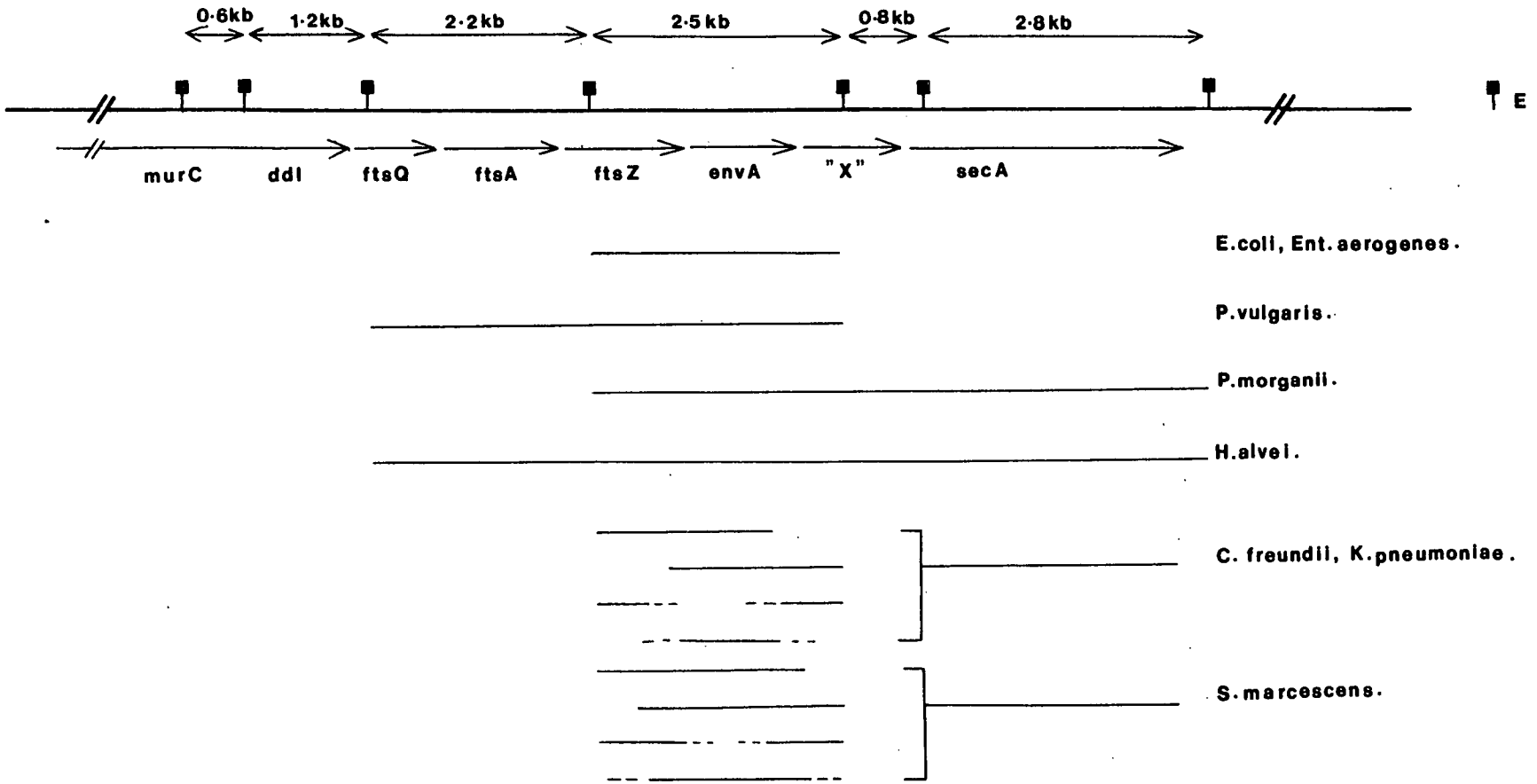
Klebsiella pneumoniae (both 1.7 Kb) and Serratia marcesens (2.0 Kb). Such fragments may arise either by generation of new restriction sites or a deletion within the envA region (Fig. 11.4). The envA fragment shows singly homology to larger regions in three species. Proteus vulgaris shows a 4.6 Kb homology, P.morganii a 6.2 Kb homology and Hafnia alvei an 8.7 Kb homology. Fig 11.4 correlates these homologies with the EcoR1 restriction map of the region (Lutkenhaus and Wu, 1980; Oliver and Beckwith, 1982a). For example, the 6.2 Kb homology observed in P.morganii is most likely to be the envA-secA region which has the 2.5 Kb envA region plus the adjacent 0.8 Kb and 2.8 Kb restriction fragments. Thus the two internal restriction sites have been lost, but the order envA,gene X,secA has been conserved. Similarly the order ftsQ, ftsA, ftsZ, envA appears to be conserved in P.vulgaris whilst in H.alvei the entire ftsQ-secA region appears to be conserved. Thus within the Enterobacteriaceae at least part of the 2' cell division cluster is conserved and the order of genes is maintained. No hybridisation was detected with two gram negative species outside the Enterobacteriaceae nor with the gram positive Bacillus subtilis. Further experiments using, for example, HindIII digested chromosomal DNA and/or alternative probes are required to confirm these conclusions, and may indeed reveal even greater conservation within this cluster.

The degree of sequence homology necessary for detection by this technique is unknown, since the intensity of hybridization depends upon the G plus C content, distribution and length of mismatched regions, length of homology and genome complexity of both probe

and chromosome (Britten et al., 1974).

In a similar study Nakamura et al. (1979) demonstrated conservation of the lpp gene within various Enterobacteriaceae except Proteus. Conservation within this genus has also been demonstrated for trp (Denney and Yanofsky, 1972), tuf (Filer et al., 1981), ilv (N. Sullivan and B. Newman, unpublished) and lon (Ruprecht and Markovitz, 1983). The presence of E.coli genes widely dispersed on the chromosome perhaps suggests that the presence of homologous DNA is due to selection pressure for these sequences rather than to transfer of DNA subsequent to the divergence of genera from their progenitors.

Fig. 11.4 Conservation within the Enterobacteriaceae



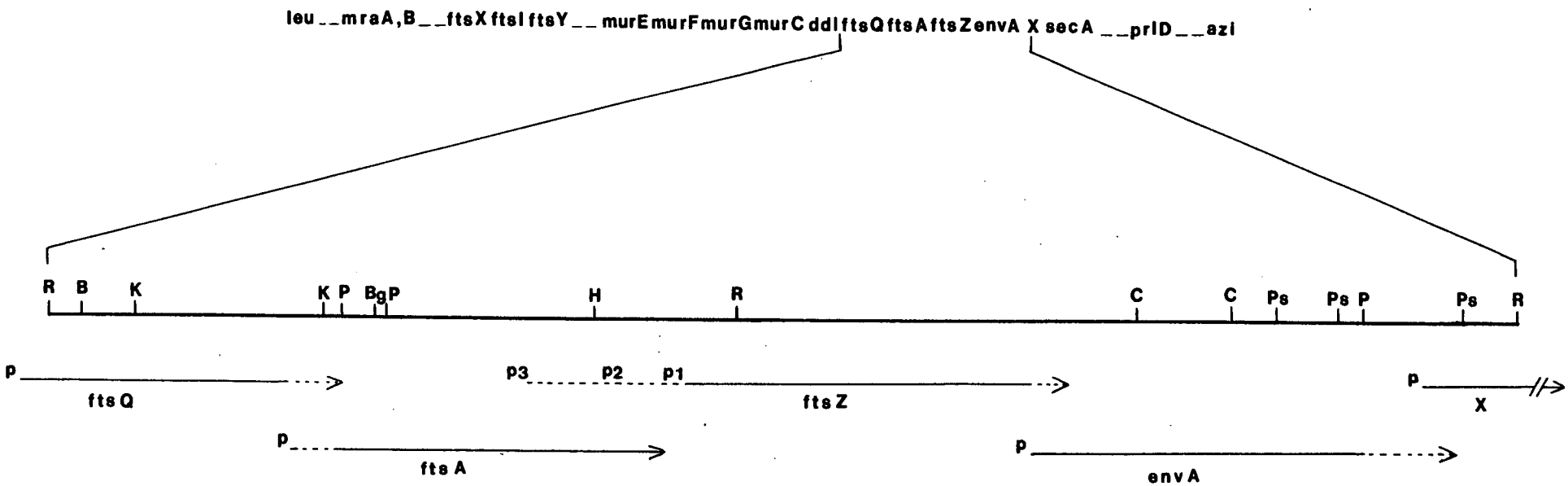
CHAPTER 12

DISCUSSION

The experiments presented in this thesis represent a detailed transcriptional study of a remarkable E.coli gene cluster. This cluster, which maps between leu and azi at 2' on the E.coli map contains a minimum of fourteen genes all concerned with cell envelope growth and division. I have concentrated on a subgroup of five contiguous genes, three of which are specifically involved in septation (ftsQ, ftsA and ftsZ), one in cell separation (envA) and one of unknown function (gene X). This has led to some interesting observations concerning the transcriptional organization of a group of genes which must surely play a central role in the morphogenesis of E.coli. Both the positions of these genes within the cluster and the precise locations of these genes within the subcluster are summarized in Fig. 12.1

Using a combination of gene fusion and in vitro transcription techniques it has been possible to precisely locate the promoters of the ftsZ and envA genes and also the promoter of a new gene which must lie between envA and secA, designated gene X. With the use of galactokinase expression vectors (McKenney et al., 1981) and contrary to previous evidence (Lutkenhaus and Wu, 1980) the envA gene has been shown to transcribe clockwise on the E.coli map. Using in vitro transcription the envA gene has been shown to have a single promoter located approximately 910 bp from the EcoR1 site within ftsZ (Lutkenhaus et al. 1979). A complementation analysis indicates that the envA gene must extend leftwards at least until a PvuII site located 1070 bp away. Assuming the average molecular weight of an amino acid in E.coli (110 d) then this is sufficient to code for a

Fig. 12.1 Summary of the transcriptional organisation of the ftsQ to X region



Scale 1 cm = 200 bp

40 Kd protein. This is larger than the molecular weight observed for envA (31 Kd; Lutkenhaus and Wu 1980), however, we may need to allow for a leader mRNA and the possibility of post-transcriptional processing.

A promoter reading left to right was detected within the 510 bp Pvu-II-EcoR1 fragment which overlaps with the end of envA. Between envA and secA there is approximately 900 bp of unassigned DNA, sufficient to code for an E.coli protein of average size. This promoter may belong to a hitherto unknown gene, designated gene X . As determined by in vitro transcription gene X has only one promoter which initiates 295 bases from the EcoR1 site.

Again using the galactokinase expression vectors of McKenney et al. (1981) the ftsZ gene has been shown to transcribe from left to right, in agreement with the indirect data of Lutkenhaus and Wu (1980). In vivo determination of galactokinase levels in two different gene fusions (pNS30 and pNS54) indicates that ftsZ must have at least two promoters. The cloned regions can direct the synthesis of three specific mRNA species as determined by in vitro transcription, all of which are preceded by concensus promoter sequences (Robinson et al., 1983). The ftsZ gene may thus have three promoters, Pz_3, Pz_2 and Pz_1, a similar situation to that found in the gal (Queen and Rosenberg, 1981) and uvrB (Sancar et al., 1982) operons. The transcription optima of Pz_3 and Pz_2 occur at high KCl concentrations (150 mM and 200 mM respectively) which indicates that these mRNA species are specifically initiated from a promoter. For Pz_1 the situation is less clear, and there

must be a question as to whether this is a specifically initiated mRNA species. S1 nuclease mapping of *ftsZ* transcripts initiated *in vivo* should resolve this problem.

The *ftsA* gene must extend into the 480 bp *Hin*dIII-*Eco*R1 fragment for at least 135 nucleotides (Lutkenhaus and Wu, 1980) and probably for 217 nucleotides (Robinson *et al.*, 1983). Thus both Pz_3 and Pz_2 must be within the coding sequence of the preceding gene *ftsA*. This represents a novel form of interaction between adjacent genes not previously described. The location of the promoters Pz_3 and Pz_2 is consistent with the phage complementation data and pattern of protein synthesis in uv irradiated cells superinfected with transducing phages. In λ*envA* weak complementation and reduced levels of *ftsZ* product are a consequence of *ftsZ* expression from Pz_2 and Pz_1. The Tn5 insertion which eliminates *ftsA* expression and reduces the amount of *ftsZ* product to 10% of the enhanced level (J. Lutkenhaus, pers. comm.) must therefore insert between Pz_2 and Pz_1. The residual 10% of *ftsZ* protein may be the result of either expression from the transposon or expression from Pz_1. The transposon Tn5 is capable of orientation independent activation of adjacent genes (J. Roth, pers. comm. to Simons *et al.*, 1983).

In λΔB complementation is improved specifically due to a contribution from Pz_3. Thus Pz_3 must play a role *in vivo* to enhance transcription from either or both of Pz_2 and Pz_1. The total galactokinase activity of the *ftsZ* promoters when cloned together as in pNS29 is 1.5 times greater than the sum of their separate activities (pNS54 plus pNS30; with Pz_2 and Pz_1 taken together). Thus there is cooperative enhancement between these

two promoter regions. Two possible mechanisms have been discussed in Chapter 3, both are consistent with the available evidence and the known interactions of RNA polymerase with DNA. The first requires that Pz_3 is a high affinity, low initiating promoter whilst the downstream promoter is a low affinity promoter capable of efficient initiation. In this case transcription is enhanced by an increased supply of correctly orientated RNA polymerases from the upstream promoter to the downstream promoter. The second mechanism takes account of a possible stem loop structure in the Pz_3 mRNA. Such a structure will allow the transcribing RNA polymerase to pause near Pz_2, perhaps inducing a conformational change which favours the binding of further polymerase molecules. This is consistent with the data of Hseih and Wang (1978) who suggested that a single bound RNA polymerase can exert a positive cooperative effect on the binding of incoming molecules.

Why should the ftsZ promoters Pz_3 and Pz_2 be within the coding sequence of ftsA? Complementation tests performed on fragments separately containing either ftsA or ftsZ indicate that these genes can be independently expressed; so a simple functional interaction seems unlikely.

Normal expression of the ftsZ gene requires the presence of a region of the neighbouring ftsA coding sequence but requires neither the transcription of that ftsA sequence nor the presence of an intact coding sequence. The expression of ftsZ is therefore dependent on a neighbouring gene in quite a novel way.

Both a complementation and protein analysis indicates that the leftward extent of ftsA must be within the KpnI fragment, so

ftsA must cover at least 995 bp. There is no direct evidence for the direction of transcription of ftsA although all circumstantial evidence is consistent with ftsA transcribing from left to right. It is clear that ftsA must have its own promoter but this is either very weak or non functional in the pKO system. Since the region which contains the ftsA promoter gives no galactokinase activity when cloned into the pKO system this allows the isolation of promoter 'up' mutations. Sequencing of these should facilitate identification of the sequences involved in promoting ftsA transcription.

The location of ftsQ has been determined by a complementation analysis. This gene almost certainly transcribes from left to right (D. Kenan, A. Robinson and N. Sullivan, unpublished) and its gene product has been identified as a 36 Kd polypeptide in minicells.

This cluster exhibits a number of interesting transciptional features. Firstly all genes from murC through to envA and probably secA are contiguous with no intervening 'spacer' DNA, i.e. this region (inclusive of the region covered by gene X) is greater than 90% coding. Neidhardt et al. (1983) calculated that on average 70% of the coding capacity was used in the inserted fragments in selected Clarke-Carbon plasmids, so by comparison the 2' region appears tightly packed. Secondly, all genes tested from murG down to secA transcribe in the same direction, i.e. from left to right on the E.coli map (collective data from 1) this thesis; 2) Lutkenhaus and Wu, 1980; 3) Oliver and Beckwith 1982a, D. Kenan, unpublished). Thirdly, all genes tested have their own promoters and can be independently expressed. As yet no strong transcriptional terminators

have been found between genes (D. Kenan, unpublished) and apart from the ftsA-ftsZ interaction there is no evidence that any of these genes overlap in any way. Thus the genes of this region do not conform to the classical definition of the operon, where genes coding for proteins of mutually dependent function are both co-transcribed and co-regulated by transcription from a single promoter. Therefore we cannot exclude the possibility of a more subtle form of regulation whereby the expression of a gene is mediated both by transcription from upstream genes and also from the promoter of the gene itself. An analysis of in vivo transcripts from this cluster will prove a most interesting study for the future. Excluding a regulatory interaction it is difficult to see why these genes should be clustered. As first noted by Miyakawa et al. (1972), there appears to be no simple relationship between the sequence of reactions involved in peptidoglycan biosynthesis and gene location. A similar argument applies to ftsQ, ftsA and ftsZ where two genes required throughout septation (ftsQ and ftsZ) sandwich a gene involved in late stages of septation (ftsA). The order of genes within this cluster appear to be conserved, at least within the Enterobacteriaceae. This indicates that these genes may have arisen by gene duplication and subsequent divergence of function in situ.

Conditional mutants in either ftsQ, ftsA or ftsZ cease dividing upon being shifted to the restrictive temperature and are thus required for septum formation. Two other genes within the 2 minute cluster, ftsI and secA also appear to have a specific effect on septation. All of the above grow as multinucleate filaments and with the exception of ftsA (and secA, for which no data is

available) are without visible signs of septa, suggesting that they are blocked in an early stage of septation. Upon being shifted to the restrictive temperature, temperature sensitive mutations in ftsQ, ftsZ and ftsI cause division to cease immediately, i.e. cells in the process of forming a septum do not complete it. The gene products involved are thus required throughout septation. At the restrictive temperature ftsA mutants have visible constrictions between nucleoids, and are therefore blocked at a later stage in sepatation (Donachie et al. 1979). None of these genes appear to be involved in elongation. Only the product of the ftsI gene, PBP3, has a known enzymatic activity. This has both transpeptidase and transglycosylase activities on peptidoglycan substrates in vitro (Ishino and Matsuhashi, 1981).

Periodic changes in D-alanine carboxypeptidase might initiate the change to synthesis of septal peptidoglycan (Mirelman et al., 1978). This model has been elaborated by Markiewicz et al. (1982) who suggested that a shifting balance between the relative activities of the septation specific PBP3 and the elongation specific PBP2 might alter the path of peptidoglycan biosynthesis. The D-alanine carboxypeptidase catalyses the removal of terminal D-alanine residues from NAM pentapeptide and in doing so may create a preferred substrate for PBP3. Via increased transpeptidation PBP3 can cross link nascent peptidoglycan chains to form the septum. Consistent with this, the level of D-alanine carboxypeptidase has been reported to be at its highest prior to division (Mirelman et al., 1978). Conversely, during elongation PBP2 (the product of the pbpA gene)

may use NAM penta peptide as its preferred substrate. Whilst providing an excellent working hypothesis this model does not assign a role to the products of other septation specific genes e.g. ftsQ, ftsA or ftsZ. Furthermore, the cause of the periodic changes in D-alanine carboxypeptidase remains obscure.

When construction of the hemispherical poles is completed at least two specific proteins, the products of the envA and cha genes are required to cause the separation of daughter cells. There exists only a single envA mutant which is non conditional and is thus only partially blocked in cell division. This mutant forms chains of cells during fast growth in rich media presumably because it requires longer than the wild type to complete a separation specific step. The mutation is associated with low levels of N-acetyl muramyl L-alanine amidase (Wolf-Watz and Normark, 1976), an enzyme involved in splitting peptidoglycan. Mutants in the cha gene (73 minutes) also form chains of unseparated cells but the mutation can be suppressed by a single extra copy of envA$^+$ cloned on a λ phage (Ken Begg, pers. comm.). Thus these two genes appear to share a common step in separation. If the envA gene regulates or codes for the N-acetyl muramyl L-alanine amidase enzyme then overactivity may well disrupt the biosynthesis of the hemispherical poles by cleaving the amide bond between NAM and the peptide side chain.

The 2.5 Kb EcoR1 fragment containing envA could only be cloned if the envA gene was disrupted (as in pNS9). To demonstrate this more clearly a variable copy number vector was constructed. In a polA t.s. strain a cointegrate formed between ColE1 and pSC101 derived replicons can replicate under ColE1 control at 30°C but not at 42°C.

Replication of pSC101 is unaffected by the polA mutation. Thus at 42°C the vector has a low copy number which rises as the temperature is lowered, reflecting replication from the ColE1 origin. Beta lactamase assays performed on cells containing the vector (pNS10) confirmed the predicted increase in copy number as the temperature of the culture was lowered (see Chapter 7.1). The envA fragment was subsequently inserted into pNS10 and shown to segregate rapidly at the high copy number (30°C) and less so at the low copy number (42°C). The vector did not segregate appreciably at either temperature. Thus the fragment of DNA containing envA is not stably maintained when cloned in multiple copy (see Chapter 7.1). Two independent non overlapping deletions of the 2.5 Kb EcoR1 fragment were constructed, firstly a 500 bp PstI deletion (pNS24) and secondly a 300 bp ClaI deletion (pNS31). Both plasmids were maintainable in multiple copy and neither complemented the envA mutation, inferring that it is the envA product and not a titration effect of the DNA that was responsible for this effect. Although the direct cause of cell lethality is unknown, it is tempting to speculate that death results from overactivity of the envA associated N-acetyl muramyl L-alanine amidase.

Also mapping within this cluster and close to envA is the secretion gene secA. Besides a deficiency in the secretion of certain proteins the secA t.s. mutant is blocked in septation at the restrictive temperature, possibly due to an inability to secrete septation specific proteins. Bankaitis and Bassford (pers. comm.) have mapped a secretion gene that is tightly linked to but distinct

from secA. This allele, prlD1 is located to the right of secA and in combination with the prlA104 allele causes a filamentous phenotype. Again this may reflect an inability to secrete division specific proteins.

The factors responsible for regulating any division specific event are unknown. However, two genes which may have an important role in the latter are cya and crp. Kumar (1976) first noticed that cya and crp cells have a cocco-bacillary morphology which in the case of cya cells can be reversed with the addition of exogenous cAMP. Utsumi et al. (1981, 1982) have identified a mutation (fic) which may be allelic to crp and causes filamentation in the presence of cAMP. Chapter 5 presents evidence which suggests that the cAMP-CRP complex may have a role in regulating transcription from ftsZ, either directly or indirectly. Firstly, using various cloned promoters from the subcluster it was demonstrated that neither the ddl, ftsQ, envA or gene X promoters were transcriptionally responsive to the cAMP-CRP complex, but that the transcription from a DNA fragment containing the promoter regions of ftsA and ftsZ was derepressed 2.4 times in the absence of the cAMP-CRP complex. The region responsible for this effect was further localized to the promoter region of the ftsZ gene, thus eliminating upstream effects from ftsA. All lines of evidence are consistent with the notion of the cAMP-CRP complex as an overall negative effector in the control of cell division, although precisely what role it plays in the morphogenetic cycle is unclear.

Besides cya and crp other cell division mutations were tested for transcriptional effects on the 2.2 Kb EcoR1 fragment containing

the control regions of ftsA and ftsZ. Neither the envA mutation, nor temperature sensitive mutations in sep, secA, ftsE or lexA affected transcript at the restrictive temperature but the ftsZ 84 t.s. mutant showed a 2.45 times increase in transcription, suggesting that the ftsZ gene product may exert a transcriptional effect on the promoters of either ftsA or ftsZ itself. Whether or not ftsA is autoregulatory remains to be determined, although it is clear that neither transcription nor translation of ftsA affects transcription of ftsZ in galactokinase expression vectors. Since there is no transcriptional effect in a lexA t.s. (tsl) background neither ftsA nor ftsZ are either damage inducible or repressible via a sfi dependent pathway.

An interesting observation relating to the control of cell division is described in Chapter 10. Certain plasmids containing a 500 bp PstI fragment from the end of envA and the start of gene X cause filamentation in E.coli, in an effect which appears to be dependent upon transcription of this region. Could this be due to overproduction of a protein (maximum 18 Kd) which acts to repress cell division?

The major pathway for division inhibition after SOS induction is the sfi dependent pathway in which the sfiA gene is derepressed after cleavage of the lexA repressor by the recA protease (see Section 1.3). The sfiA product is then thought to act via the sfiB locus to inhibit cell division. The sfiB (sulB) gene has been mapped close to envA (Johnson, 1977). The experiment presented in Chapter 11.1 shows that the envA product is probably not an uninduced target for division inhibition. The experiments designed to show possible transcriptional activation of both p envA

and pX after SOS (damage inducibility) indicates that there may be some induction of these loci. However, the kinetics of induction is not as rapid as we might expect if one of these loci was sulB and either (a) transcriptionally repressed by the sfiA product or (b) a sfiA induced division inhibitor. Instead the induction appears to occur during the recovery phase, with gene X induced after envA. On this basis then, envA would appear not to have the characteristics we might expect of sulB, although both it and gene X show some damage inducible character. Lutkenhaus (1983) has recently shown that sfiB is allelic to the septation specific ftsZ gene.

The understanding of the division cycle of E.coli requires that we answer several fundamental questions: (1) What factors time the initiation of cell division? (2) Are septum specific proteins synthesised continuously and used periodically or are they only synthesised at certain times in the cycle? (3) What are the enzymatic activities associated with the septation-separation specific genes? The technical difficulties associated with producing synchronous cultures of E.coli make it difficult to unambiguously follow cell cycle associated events in a metabolically unperturbed cell. The approaches adopted in this thesis have provided some interesting insights into gene organization within an E.coli gene cluster, but whether these characteristics are common to other clusters or only associated with temporally related sequences remains to be discovered. Study of the production of cell division specific mRNA in vivo will provide interesting elaborations to this model but in the absence of reliable synchronies

it will be difficult to relate mRNA production to a cell cycle specific event. The techniques of molecular biology have helped us identify and in some cases clone the genes responsible for cell morphogenesis, and we may confidently expect that someday we will understand the process which time and coordinate the morphogenetic cycle of Escherichia coli.

APPENDIX I

1. An appraisal of the pKO system

1(a) Translational coupling

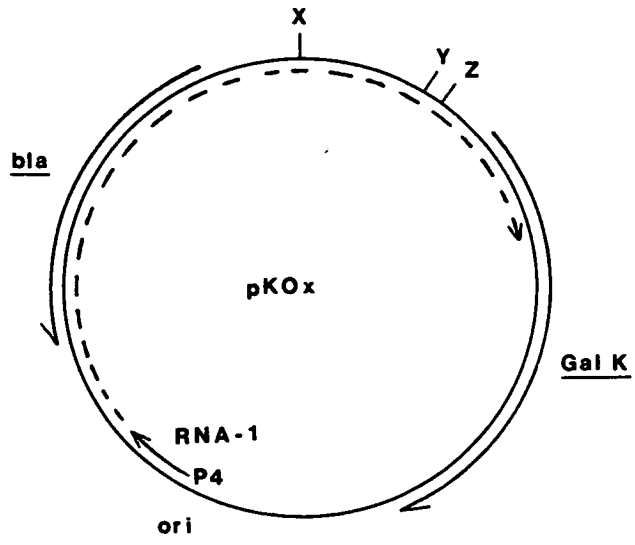
As mentioned in 1.5.5 translation stop codons have been inserted into the leader region of the galK gene to uncouple extraneous translation emanating from an inserted fragment. In two reading frames stop codons are positioned well ahead of the ATG galK start codon (103, 121 bp). In the third frame the TAA stop codon, the natural stop codon for galT is positioned only 6 bp from the ATG and within the galK Shine-Dalgarno sequence. Schümperli et al. (1982) have suggested that there is functional coupling of galT and galK translation (1.5.1c). It follows that any translation in this reading frame will be coupled to galK translation and hence to the level of galactokinase observed. Thus if translating ribosomes from the inserted fragment enter the galK leader region in the natural galT reading frame then translational coupling may occur. Comparison of constructs where translation is in different reading frames may be complicated by an unknown level of translational coupling.

1(b) Readthrough transcription from the vector

In the construction of pKO-1 a fragment of the λO gene was inserted between the EcoR1 and HindIII sites. This DNA contains a transcription termination signal and reduces transcription from the vector (M. Rosenberg, pers. comm., Fig. A1.1).

Transcription traversing site X is probably due to read-through from the P_4 promoter of pBR322, which normally functions to produce RNA-1, a molecule involved in control of replication (Morita and Oka, 1979).

Fig. A1.1



Consequently insertions into X and Y, or substititions in which the intervening 320 bp fragment is deleted cannot be quantitatively compared unless a correction is made.

Insertions

Insertions into sites Y and Z read directly into galK. The transcription from the vector is reduced by the 320 bp fragment, so a value for transcription emanating from the insert is obtained by subtracting the basal level for pKO1. With insertions into site X total transcriptions is the sum of P_4 readthrough and transcription from the insert. This will be reduced by the 310 bp fragment and although a value for transcription can be obtained by subtracting the basal level for pKO1, insertions here will not be comparable to insertions into sites Y and Z.

Substitutions

If as a consequence of the cloning strategy the piece of the λO gene is removed then the amount of galK transcription will be the sum of P_4 readthrough and transcription from the insert. A quantitative estimate of transcription from the insert can be obtained by subtracting the value for P_4 transcription. To obtain this value I constructed a control plasmid (pKOC1) in which the λO gene fragment was deleted. The pKO1 vector was restricted with EcoR1 and the ends filled in with Klenow. Subsequent SmaI digestion and blunt end ligation gave pKCC1. This plasmid was able to complement the galK mutation unlike pKO1 (see Table A1.1).

Table A1.1

Plasmid	Size (Kb)	Colour on Macgal	Assay
pKO1	3.9	White	0.34
pKOC1	3.6	Red	0.46

2. Treatment of results

Galactokinase enzyme is measured as n moles galactose phosphorylated/min/OD_{650} according to the method of KcKenney et al. (1981). Up to 16 assays were performed per experiment including duplications. Within each experiment relative differences between plasmid constructions were maintained although some daily variation was observed. To circumvent this problem and make possible the comparison of large numbers of constructs, the results from an experiment were normalised against one another

and these values averaged over at least four daily repetitions per plasmid construction. The values obtained were accurate to within 10%. Unless otherwise stated values quoted have been corrected for pKO1 or pKOC1 (see A1.1).

3. Effect of copy number

The experiments performed in this thesis were with multiple copy plasmid vectors in which the copy number could not be controlled. Ideally the galK transcription-fusion sequences of pKO plasmids should be transferred to single copy in the chromosome, but the genetic manipulations involved make the process long and involved.

Clewell and Helinski (1972) have shown that the copy number of ColE1 does not vary significantly with respect to growth rate with doubling times between 1 and 2 hours. Although possessing the same origin as ColE1, pKO vectors are considerably different and may not behave similarly. Thus it is important to have an estimate of the copy number at both different growth rates and with different constructions.

A number of different methods are available for determination of the intracellular copy number of plasmids. Centrifugation of cleared lysates through CsCl gradient containing an intercalating dye resolves chromosomal and plasmid DNA (Womble et al., 1977). However, for accurate determination of copy number the plasmid DNA must be in a supercoiled state so the method probably underestimates the amount of plasmid DNA. Using this technique Duester et al. (1982) were able to show that in a pKO derivative bearing pgal a

1.5 times decrease in doubling time resulted in an 11% increase in galactokinase activity.

Using DNA-DNA hybridization Adams and Hatfield (1983) demonstrated that *in vivo* copy number fluctuations can be correlated with both promoter strength and growth conditions, suggesting that there is an inverse relationship between the expression of galactokinase and the copy number of the transcription fusion plasmid. Wong *et al.* (1982) have reported that in pKO vectors bearing strong promoters the copy number decreases 3 to 5 times. This may be due to transcription reading through *galK* and reducing the amount of RNA primer or increasing the RNA repressor RNA-1, either of which will cause a decrease in copy number. Similarly Stueber and Bujard (1982) suggest that transcription through a ColE1 origin of replication can reduce copy number.

Alternatively high levels of the galactokinase enzyme may reduce copy number, since under some circumstances expression of this enzyme causes the accumulation of toxic intermediates (Fusikawa and Nikaido, 1961), a hypothesis supported by the data of Adams and Hatfield (1983). The physiological basis for this is unknown and it must be noted that so far 'galactose killing' has only been observed during growth on galactose based media.

Enzymatic assay of a product encoded by the plasmid of interest is another approach for determination of intracellular copy number and has been used in this thesis. Technically simple, it assumes a direct relationship between the amount of plasmid encoded product and the number of plasmids in the cell. For β-lactamase this is

this is linear up to 50 copies per cell (N. Willetts, pers. comm.). Assays were performed as described in Chapter 2.

Relative copy number (see Chapter 3 and 9)

The strongest promoter used in this study is the medium strength gal promoter (pgal; McKenney et al., 1981). β-Lactamase assays were performed on pKG1800 (containing pgal) pNS28, pNS30, pNS22, pDK302 and pKO1 all in c600K$^-$. Values quoted are for the substrate 87/312 and are the result of two independent assays. β-Lactamase specific activity is expressed as OD_{600}/min/OD_{540}.

In all cases error was ±0.4, that is 11%. As can be seen from Table A1.2, there is no relationship between either the amount of transcription or the size of the plasmid (pNS22 is the smallest) and there is no significant deviation in copy number. However, in a cell containing a ColE1 derivative of approximately 50 copies/cell on 11% error could result in a copy number variation of ±6 copies/cell. Small fluctuations in copy number may thus be undetected by this technique and the variation in galactokinase levels may in part reflect these fluctuations.

Growth rate dependent copy number (Chapter 5)

The cya derivative of c600K$^-$ (NFS3) was compared with its parent to see if the slower growth rate affected copy number. Plasmids used were pKG1800 and pKO1, assayed as before.

As can be seen from Table A1.3, within the 11% error there is no significant variation in copy number due to the cya defect.

Table A1.2

Plasmid	Uncorrected galK specific activity	Order of size	$\Delta OD_{600}/min/OD_{540}$ ($x10^{-2}$)
pKO1	0.34	2	3.13
pNS22	0.42	1	3.86
pNS30	1.12	4	3.25
pDK302	1.55	6	3.82
pNS28	3.01	5	3.69
pKG1800	3.84	3	3.24

Table A1.3

Plasmid	Strain	$\Delta OD_{600}/min/OD_{540} \times 10^{-2}$
pKO1	c600K$^-$	3.73
pKG1800	c600K$^-$	3.30
pKO1	NFS3	3.29
pKG1800	NFS3	3.21

Absolute copy number

To obtain an estimate of absolute copy number results (OD_{600}/min/OD_{540}) were nomalised to values obtained from a strain (ED5040) carrying a single chromosomal copy of TnA, and hence the bla gene (see Chapter 7a).

APPENDIX II

Construction of bacterial strains/P1 lysates and complementation tests.

1. Construction of NFS6

A spontaneous nalidixic acid resistant derivative of c600K$^-$ was selected (NFS1). This was subsequently mated with JC10-240, which transfers a recA mutation linked to a tetracycline transposon (Tn10). Nalidixic acid is used to select for the recipient and against the donor. Tetracycline selects for transfer. Five clones were then screened for UV sensitivity (killed by 10 J/m^2) and sensitivity to 2 ml/l 10% w/v MMS. All were found to be recA. One clone was designated NFS6.

2. Construction of NFS3

Phage P1kc grown on WP80 (rbs::Tn10) was used to transduce strain 3002 (ilv$^-$) to tetracycline resistance. Resistant clones were screened for an ilv$^-$ Tc^R transductant. Such colonies were obtained at a frequency of 50%. One clone, designated 3002T was purified and a P1 lysate made. This was used to transduce NFS1 to tetracycline resistance. As before clones were screened for an ilv$^-$ Tc^R colony. One such clone was designated NFS2. This was transduced back to ilv$^+$ with a P1 lysate grown on the cya strain CA8306. Transductants were screened for the cya defect by streaking on to LB plates containing XG (130 mg/l). One white colony (cya delete) was purified and made Tc^S by the method of Bochner et al., 1980 (Chapter 2). This clone was designated NFS3.

3. Construction of NFS4

c600K$^-$ was transduced to rpsL (streptomycin resistance) with a P1 lysate grown on WZ81. Transductants were screened for crp character by plating on XG as above. One clone was designated NFS4.

Both NFS3 and NFS4 were shown to have the characteristic coccal morphology (Kumar, 1976).

4. Construction of NFS7

c600K$^-$ was transduced to *leu*$^+$ at 30°C with a P1 lysate grown (at 30°C) on Ken 90 (*ftsZ* 84 t.s.). Transductants were screened for ability to form colonies at 30°C but not at 42°C, and the formation of non septate filaments at 42°C but not at 30°C. One clone, now c600K$^-$ *ftsZ* (84 t.s.) was designated NFS7.

5. Linking the *envA* mutation to a transposon.

Phage P1 grown on SJ2 was used to transduce D22 to tetracycline resistance. Transductants were screened for *envA* character by plating on nutrient agar containing 5 μg/ml rifampicin (see II.7). The *envA* gene and Tn10 were found to be 6% cotransducible. One clone, *envA*$^-$ *Tc*^{R} was purified and P1 grown on it to give a lysate capable of transducing Tn10 linked to the *envA* mutation. This was used in the construction of Ken 10 (by Ken Begg) and the attempted construction of a *polA envA* strain.

6. Complementation of *ftsQ* and *ftsA*

Plasmids to be tested were transformed into either TOE1 (*ftsQ* t.s.) or TOE13 (*ftsA* t.s.) at 30°C. Single colonies were either inoculated into LB at 30°C and grown to OD_{540} 0.2 before shifting to the restrictive temperature (42°C) or patched on to solid media at both the restrictive and non restrictive temperature. Complementation was judged in liquid by the presence or absence of filaments at 42°C after 2-3 cell generations or by colony forming ability at 42°C after overnight incubation on

solid media. Plasmids which complement either mutation do not produce filaments at 42°C and grow normally on solid media at this temperature.

7. Complementation of envA

As mentioned in 1.2.2c, the envA mutation mediates increased permeability to a number of antibiotics. One of these, rifampicin can be used as a basis for selection. At 37°C on solid media wild type cells are resistant to 5 μg/ml rifampicin, whilst envA mutant cells are not. Hence plasmids which complement the envA mutation allow growth on solid media containing 5 μg/ml rifampicin. The original envA mutant strain D22 (Normark, 1969) has a very poor transformation frequency for plasmid DNA, approximately 10^3-10^4 times lower than for a good transforming strain like c600K$^-$, and grows with an increased doubling time. The envA mutation was therefore shifted to a c600K$^-$ background (Ken 10). The mutation conferred sensitivity to rifampicin on c600K$^-$ and could be complemented with λ16-2 and λenvA (Ken Begg; pers. comm.). The strain retained the good transformability of c600K$^-$ but acquired the slow growth rate of D22. Thus the envA mutation affects growth rate but the poor transforming ability is a consequence of the D22 background.

APPENDIX III

In vitro transcription

In vitro transcription is the synthesis of mRNA from DNA in the presence of RNA polymerase, salts and nucleotides. There are two principal approaches; (a) transcription of whole plasmid or phage DNA and (b) 'run-off' transcription where synthesis is primed from a 50-800 bp DNA fragment. The run off method is used to locate promoters and complements both gene fusion and sequence studies.

1. RNA polymerase: DNA complexes

RNA polymerase is thought to locate its promoter in a one dimensional diffusion process. That is, the enzyme binds to DNA at random and slides up and down being released every 1000 bp (Von Hippel *et al.*, 1982). The interaction with DNA can be classified into two categories according to the mRNA initiated: (a) specific, where the polymerase locates a promoter and initiates a characteristic mRNA and (b) non-specific. Non-specific complexes vary enormously in structure and stability, from those that are weak to those that approach promoter site specificity (Hinkle and Chamberlin, 1972; Seeburg *et al.*, 1977; Williams and Chamberlin, 1977; Melancon *et al.* 1982). They differ from specific complexes in their thermodynamic and kinetic binding constants and their responses to temperature and salt (KCl) (Kadesch *et al.*, 1980). Experimentally the simplest way of differentiating between mRNAs initiated by specific and non-specific complexes is to vary the KCl concentration (von Gabain and Bujard, 1977). In general specific transcripts are favoured at high KCl

concentrations and non-specific transcripts by low KCl concentrations (Von Hippel et al., 1982; Küpper et al., 1975). For a given DNA fragment, RNA polymerase:DNA ratio and temperature optimum transcription is obtained at a characteristic KCl concentration, determined by titration against 10, 50, 100, 150 and 200 mM KCl.

Besides non-specific transcripts initiated from within a DNA fragment RNA polymerase can transcribe the entire fragment from a free end giving an end to end transcript equal in length to the DNA used. 'Poly' transcripts can be observed if sticky ended fragments associate in solution in an end to end manner. The polymerase is capable of transcribing the junction even if it is not ligated, resulting in the formation of transcripts of higher molecular weight than end to end trasncript (Küpper et al., 1975; Wu et al., 1978; Friden et al., 1982).

2. Limitations of 'run-off' transcription

This method is particularly useful for locating promoters. Due to the size of fragments used it is rarely possible to identify an mRNA expressed from a whole gene. The amount of specific mRNA synthesised from different DNA fragments does not give an accurate reflection of relative promoter strength since (a) transcription must be optimised with respect to DNA concentration. The amount of run off transcription may not be linearly related to the amount of DNA. (b) Different run off transcripts differ in stability (Wickens and Laskey, 1981). (c) the kinetics of incorporation depends upon the template used (Weil et al., 1979; Luse and Roeder, 1980).

3. Treatment of results

For any given DNA fragment both incorporation into a given mRNA and into total mRNA (both specific and non specific) varies with salt concentration. For this reason autoradiographs were scanned with a densitometer and incorporation expressed as a percentage of total mRNA synthesised. This percentage, plotted against salt concentration shows the relative salt dependence of any particular message.

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AB	Annals of Biochemistry
AIP	Annals of the Institute Pasteur (Paris)
AM	Archives Microb.
AMAC	Antimicrobial Agents and Chemotherapy
APMS	Acta Pathol. Microb. Scand. Sect.
ARB	Annual Reviews of Biochemistry
ARG	Annual Reviews of Genetics
ARM	Annual Reviews of Microbiology
ASM Abstract	Abstract from the American Society of Microbiology
B	Biochemistry
BBA	Biochim. Biophys. Acta.
BBRC	Biochem. Biophys. Research Communications
Biochimie:	Biochimie
BR	Bacteriological Reviews
Cell	Cell
CSH	Cold Spring Harbor
EJB	European Journal of Biochemistry
EMBO J	EMBO Journal
FL	Febs Letters
FP	Fed. Proc.
G	Genetics
Gene	Gene
GR	Genetics Research
IRC	Int. Rev. Cytology
JB	Journal of Bacteriology
JBC	Journal of Biochemistry

JGM	Journal of General Microbiology
JMB	Journal of Molecular Biology
JTB	Journal of Theoretical Biology
ME	Methods in Enzymology
MGG	Molecular and General Genetics
MR	Microbiological Reviews
MUR	Mutational Research
NAR	Nucleic Acid Research
Nature	Nature
Plasmid	Plasmid
PNAS	Proceedings of the National Academy of Sciences
PTRS	Philos. Trans. Royal Society
Science	Science

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