The Regulation of *rpoBC* in *Escherichia coli*

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To My Parents

ABSTRACT

The genes rpoB and rpoC of E. coli, encoding the RNA polymerase subunits β and β', are co-transcribed with four 50S ribosomal protein genes rplK, -A, -J and L. It is known that under certain conditions such as challenge with the antibiotic rifampicin, or amino acyl tRNA limitation, a partial uncoupling of rpoBC from rplKAJL transcription occurs.

The rpoBC operon is transcribed in the order rplKAJLrpoBC from a strong promoter, P_{L11}, upstream of rplK. Some other interesting signals include a strong promoter P_{L10}, between rplA and rplJ, which is normally occluded by P_{L11}; and an RNaseIII mRNA-processing site downstream of a partial terminator of transcription, t_{L7} (normally 80% efficient), both present in the 319bp intercistronic space between rplL and rpoB.

I have been investigating the roles played in uncoupling by P_{L10} and t_{L7}, by applying SI-nuclease mapping to examine directly transcription in vivo through the DNA regions carrying the above signals. I have demonstrated that P_{L10} is not detectably stimulated after challenge with rifampicin, or during partial amino acid starvation. However, I have shown that a 2-fold stimulation of transcriptional readthrough of t_{L7} occurs after treatment with rifampicin.

I provide preliminary evidence that RNaseIII processing is involved in the post-transcriptional regulation of β and β' syntheses.

I have also examined the possibility that the dominance of the rpoB3(rif^d18) allele has a regulatory basis, by DNA sequencing and protein analyses. The main conclusion from this study is that a second mutation, distinct from rpoB3(Rif-R), is probably required to explain the dominance of rif^d18, although no evidence was obtained to suggest that this second mutation has a regulatory effect.

Transcriptional and post-transcriptional regulation of β and β' syntheses are discussed.

DECLARATION

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

MAY 1986

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ABBREVIATIONS

Amp^R	-	Ampicillin resistant
bp	-	base pair
BSA	-	Bovine Serum Albumim
Cm^R	-	Chloramphenicol resistant
CRP	-	cAMP receptor protein
DTT	-	Dithiothreitol
kb	-	kilobase pair
kd	-	kilodalton
m.o.i.	-	multiplicity of infection
pfu	-	plaque forming unit
P_x	-	Promoter (where x is a number or appropriate letters)
rif	-	rifampicin
Rif-R	-	Rifampicin resistant
Rif-S	-	Rifampicin sensitive
R-pol	-	RNA polymerase
ts	-	temperature sensitive
t	-	terminator of transcription
$Tet^{R/S}$	-	Tetracycline resistant (R) or sensitive (S)

CHAPTER 1

Introduction

The RNA polymerase of *Escherichia coli* is a complex enzyme consisting of four non-identical subunits α, β, β' and σ (Burgess, 1969) encoded by the genes *rpoA*, *B*, *C* and *D* respectively. The minimal form of the enzyme, known as the "core enzyme", carries out RNA chain elongation and comprises three of the subunits in the molar ratio $\alpha_2\beta\beta'$ (Burgess, 1969). The active form of the core *in vivo* has been reported to be monomeric (Zarudnaya *et al*., 1976). The core enzyme when complexed with σ factor is known as the "holoenzyme" and has the structure $\alpha_2\beta\beta'\sigma$. *In vitro* core enzyme will initiate synthesis of RNA chains very poorly from random sites on an *E. coli* DNA template; however, the addition of σ to the core enzyme restores controlled activity by allowing selective initiation of RNA synthesis from promoter regions (Burgess, 1971). Hence the dissociable sigma factor is responsible for specific initiation of transcription at promoters.

The transcription process in *E. coli*, which involves three distinct stages, initiation, elongation and termination, is regulated in a variety of ways. RNA polymerase, responsible for the synthesis of all cellular RNA species,has a central role in the regulation of transcription. Therefore, the elucidation of the mechanism of regulation of the synthesis of the enzyme's own individual subunits, and of the interaction of these subunits with other cellular factors, is of crucial importance for our understanding of cellular metabolism and gene expression in *E. coli*.

The next section deals with a few of the ways in which gene

expression can be regulated in E. coli, chosen because of their possible relevance to the regulation of RNA polymerase synthesis. For positive and negative regulation of initiation, see the reviews by de Crombrugghe et al. (1984), Pabo and Sauer (1984), Ptashne (1984) and von Hippel et al. (1984).

1.1 Some mechanisms by which gene expression is regulated in E. coli

i) Antisense RNA and steric hindrance.

The binding of two complementary RNA species to form a stable duplex is a way of negatively regulating gene expression at the translational level. There are a number of examples of this type of regulation; however in some cases (see below) the part played by antisense RNA is not properly distinguished from the possible effects of convergent transcription; the "anti-sense" transcription complex might sterically hinder progress of that which is copying "sense RNA" from the other DNA strand (Ward and Murray, 1979).

One of the best characterised examples includes the multicopy inhibition of transposition of Tn10 and IS10 (Simons and Kleckner, 1983). It was originally observed that at high copy numbers of the IS10 element the transposition of a single chromosomal Tn10 element was inhibited in trans. The authors could delete all but 75bp of the 5' end of the transposase coding region without affecting inhibition. The 75bp region contains two opposing promoters, P_{IN} and P_{OUT}, such that the respective RNA molecules from P_{IN}, the promoter for the transposase gene, and P_{OUT}, an opposing promoter internal to the transposase gene on the non-coding strand, have 36bp of complementarity including the start signals for the transposase gene. Studies of gene fusions of this region to lacZ suggested that multicopy inhibition is

exerted at the translational level, possibly because the two complementary RNA molecules from P_{IN} and P_{OUT} bind together, preventing translation. Indeed it has now been shown that RNA_{OUT} and RNA_{IN} can pair *in vitro* (Kittle and Kleckner, 1985), with a second order rate constant similar to that observed for the binding between RNA-I and primer RNA which regulates the replication of ColE1 (Tomizawa, 1984). P_{OUT} also regulates IS*10* transposition *in cis* although whether this is due solely to antisense RNA regulation or to steric hindrance is unclear (Simons *et al*., 1983).

Translational regulation by a complementary RNA has been proposed to play a role in the expression of two genes *ompC* and *ompF* which encode *E. coli* outer membrane proteins (Mizuno *et al*., 1984). The expression of these two proteins has been found to be regulated in response to different environmental conditions. A 174bp mRNA transcribed from the *ompC* promoter region was found *in vivo* to inhibit *ompF* expression *in trans*. Sequence analysis of this mRNA showed that there were no open reading frames for translation, but that there was extensive homology with the 5' end of the *ompF* transcript, including the ribosome binding site (Mizuno *et al*., 1984). This RNA species, termed *micF* (mRNA - interfering complementary RNA), is transcribed in the opposite direction from, but not overlapping, *ompC* (Fig. 1.1).

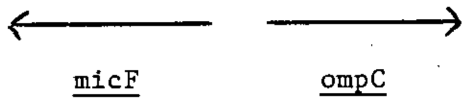


Figure 1.1 The transcription units of *ompC* and *micF*.

Another example which has been highlighted recently plays a role in the regulation of bacteriophage lambda gene expression. In the

course of λ development the decision between the lytic cycle and lysogeny is a complex process with a number of phage and host proteins involved. cII, a phage-encoded protein, has a major role in directing the phage towards lysogeny (for a review see Herskowitz and Hagen, 1980). It achieves this by activating specific λ promoters, P_{RE} and P_I, directing the expression of the cI repressor and integrase protein genes respectively. Work by various groups has suggested that cII activates these promoters by binding TTGC sequences on either side of the -35 region of the promoter, thereby allowing RNA polymerase to bind (to the opposite side of the double helix) despite the very poor approximation to "consensus" of the -35 sequences present in these promoters (Schmeissner *et al*., 1980; 1981; Shimatake and Rosenberg, 1981; Ho and Rosenberg, 1982; Wulff and Rosenberg, 1983). Interestingly a promoter, which is activated by cII and shares sequence homology with P_{RE} and P_I, has been discovered within the coding region of the λ*Q* gene (Hoopes and McClure, 1985; Stephenson, 1985). The latter encodes the Q antitermination protein required for the activation of late gene expression. Since this promoter, P_{aQ} (anti-Q), is orientated in the opposite direction to *Q* transcription, it would generate a mRNA which is complementary to the *Q*mRNA. A mutation in one of the TTGC sequences surrounding the -35 region of P_{aQ} displayed altered cII binding properties *in vitro*, and when crossed into a λ*cro* mutant it resulted in the relief of cII dependent growth inhibition *in vivo* (Hoopes and McClure, 1985). It is clear that cII activates P_{aQ}, and could thereby block *Q* gene expression either by interfering with *Q* translation (through an mRNA interaction) or by interfering with *Q* transcription, through steric hindrance.

ii) Promoter occlusion.

In *E. coli* a number of examples exist of "promoter occlusion", where

the initiation of transcription at a promoter X is blocked by transcription proceeding through that promoter, initiated from another promoter Y upstream on the same DNA strand. In the best characterised example of this phenomenon the strong promoter, P_L, of λ has been shown to interfere with the activity of two nearby downstream promoters, P_1 and P_2, of the E. coli gal operon (Adhya and Gottesman, 1982). The authors speculated that promoter occlusion may play a role in the regulation of the λ life cycle, perhaps by ensuring the sequential appearance of viral functions. For example P_L activation could prevent initiation at P_I. This would serve to stop the premature synthesis of Int protein because the P_L initiated transcript yields very little Int protein due to post-transcriptional regulation (see Echols and Guarneros, 1983, for a review).

It is clear that occlusion does occur, and models to explain the mechanism will be discussed in Chapter 6.

The rpoA, rpoBC and rpoD operons all contain occluded promoters. These include the P_α promoter of the rpoA operon, occluded by P_{spc} (Cerretti et al., 1983), the P_{L10} promoter of the rpoBC operon, occluded by P_{L11} (Brückner and Matzura, 1981; Morgan and Hayward, 1985; C. Squires, pers. comm.) and a number of minor promoters in the rpoD operon, occluded by the main operon transcription (Burton et al., 1983; Lupski and Godson, 1984). The relevance of these promoters to the regulation of the synthesis of RNA polymerase will be discussed later in this chapter and in Chapter 6.

iii) Antitermination.

In E. coli the modulation of readthrough of transcriptional terminators between structural genes is a means by which gene expression can be regulated. Two mechanisms by which this is achieved have been identified:

attenuation and antitermination. Antitermination is the most important regulatory mechanism by which the transcription of rpoBC is controlled and consequently it is discussed in depth below. For attenuation see the review by Platt and Bear (1983). Also see Kotter and Yanofsky (1984) for a good example of the work.

One well characterised system displaying regulation by antitermination is that mediated by the N-protein of bacteriophage λ, which regulates the switch from early gene expression to the next stage of expression (delayed early) in the lytic cycle. Here the N protein allows RNA polymerase to overcome termination signals downstream of the early promoters, P_L and P_R (Roberts 1969; Fig. 1.2). Mutational and sequence analysis identified a *cis*-acting site downstream of P_L, called *nutL*, which is essential for *N* to make transcription initiating at P_L terminator resistant (Rosenberg *et al*., 1978; Salstrom and Szybalski, 1978). More specifically mutations conferring an *N*-resistant phenotype lay within a 17bp region of potential hyphenated dyad symmetry (Rosenberg *et al*., 1978). Analysis of sequences downstream of P_R revealed a striking (16bp out of 17bp) homology with the dyad symmetry region of *nutL* (Rosenberg *et al*., 1978), in a region downstream of *cro* known to be essential for N action (de Crombrugghe *et al*., 1979). Further sequence comparisons, between the *nutL* and *nutR* sites of λ and of a group of closely related lambdoid phages, revealed considerable homologies outwith the dyad symmetry region, but immediately adjacent to it (Olson *et al*., 1982 ; Fig. 1.3). Based on these data three distinct sites have been identified, termed *BoxA*, *BoxB* and *BoxC*, where *BoxB* is the dyad symmetry in which the original *nut* mutations were found. The *nut* sites of the different lambdoid phages share much homology, yet each phage has specificity for its own particular N protein.

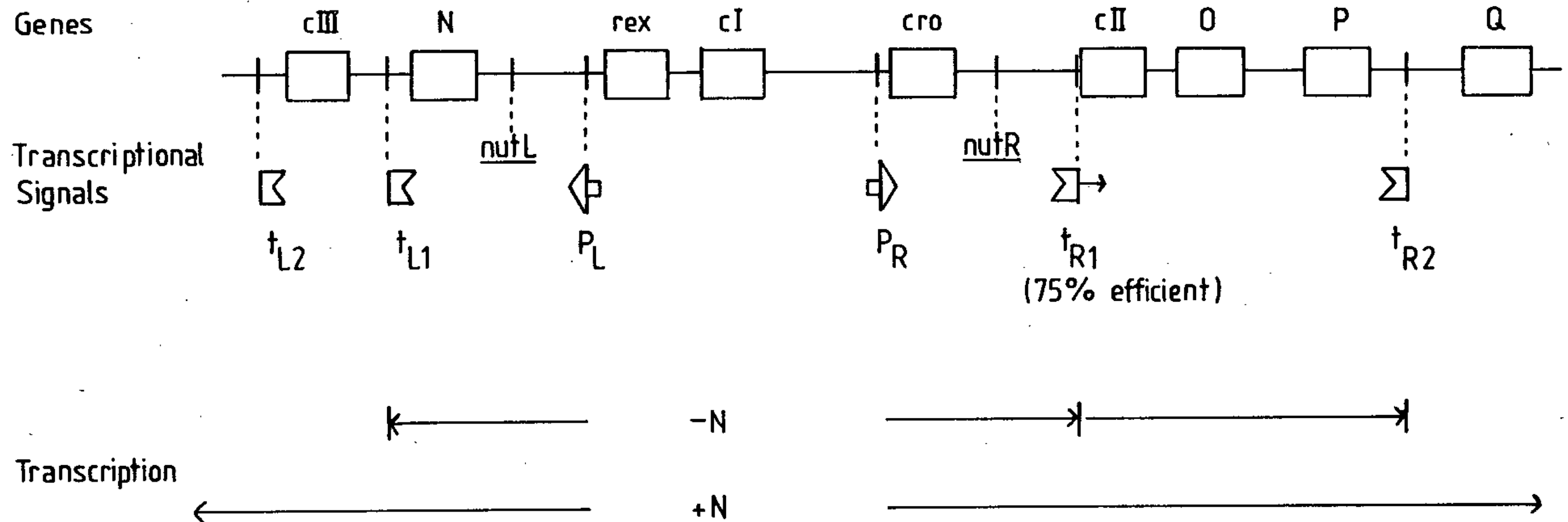


Fig.1.2

Fig. 1.2 A partial genetic map of bacteriophage λ, showing the major transcripts produced in the presence or absence of the N protein, the antitermination factor of phage λ required for late gene expression. Also shown are the positions of the sites nutL and nutR, required for N action on the terminators downstream.

	Box A	Box B	Box C

λnutL ATGAAGGTGACGCTCTTAAAAATTAAGCCCTGAAGAAGGGCAGCATTCAAAGCAGAAGGCTTTGGGGTGTGTGATAC

λnutR TAAATAACCCCGCTCTTACACATTCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACCTATGGTGTATGCATTTAT

21nutR TAAGCAAATTGCTCTTTAACAGTTCTGGCCTTTCACCTCTAACCGGGTGAGCAAACATCAGCGGCAAATCCATTGGGTGTGCGCT

P22 nutL AACGCTCTTTAACTTCGATGATGCGCTGACAAAGCGCGAACAAATACCAAACGAGATTGGTTTGGACTGGCGTGTGGT

Fig.1.3

Fig. 1.3 Comparison of DNA sequences in the nut regions of lambda and some closely related phages. The converging arrows indicate the regions of hypenated dyad symmetry (BoxB) specific for the N protein. Underlining indicates the BoxA and BoxC sequences postulated to interact with ancillary proteins important in the N-antitermination mechanism.

+ - Altered to T in boxA1 (Friedman and Olson, 1983), giving CTTTA (cf. BoxA of 21 nutR).

This led to the proposal that BoxA and BoxC, which show more homology between the four related phages, recognise protein factors common to all four interactions, while BoxB, which has a more variable sequence, is involved in N recognition (Olson et al., 1982). Other host "Nus" proteins, apart from RNA polymerase and Rho, have been shown genetically to be involved in the N antitermination mechanism. These include the S10 ribosomal protein, nusE (rpsJ), and two other proteins, NusA and NusB (for a review see Friedman and Gottesman, 1983). Greenblatt has shown that NusA binds to core RNA polymerase in vitro and that σ is able to displace it from the complex (Greenblatt and Li, 1981a). Greenblatt has also demonstrated the binding of NusA to N protein in vitro, but has been unable to show binding of N protein to RNA polymerase in vitro (Greenblatt and Li, 1981b). Moreover, the isolation of mutations mapping in N (punA) that permit λ to grow at 42°C in a nusA1 host (NusA1 protein is ts, and does not support normal N-dependent λ growth at 42°C) or that allow λ to use the nusA gene of Salmonella typhimurium, suggests an in vivo interaction between the two proteins (Friedman et al., 1981; Friedman and Olson, 1983). Therefore it has been suggested that NusA bound to RNA polymerase acts as an adapter to couple N to RNA polymerase (Greenblatt and Li, 1981a; 1981b). The role of NusB is unclear, although it is required in vitro and in vivo for N-dependent antitermination (Friedman and Gottesman, 1983; Greenblatt et al., 1985). Mutational analysis has provided direct evidence that BoxA is the site of interaction of the NusA protein in the N-mediated antitermination reaction (Friedman and Olson, 1983). In particular, a mutation BoxA1, which changes TTA⟶ TTT at the 3' end of the BoxA of λnutR (Fig. 1.3), allows λ to use the NusA protein of Salmonella typhimurium. The latter is not normally active with the N product of λ.

No direct evidence for the role of BoxC exists. However, a suggestion has been made that perhaps NusB and/or S10 interact at this site. The evidence for this is indirect and comes from an analysis of the λQ protein antitermination system. The Q protein, required for late gene expression in λ, antiterminates transcription initiating at $P_{R'}$ such that it can read through the terminator $t_{R'}$ into the late genes. The proposed site of action of Q protein (qut), adjacent to the Q gene, has features in common with nut sites including a BoxA and BoxB, but no identifiable BoxC (Friedman and Gottesman, 1983). An in vitro system, prepared with well-purified components, requires only Rpol, NusA, DNA containing the qut site, and Q in order to allow read-through of $\lambda t_{R'}$ (Grayhack and Roberts, 1982; Friedman and Gottesman, 1983; Somasekhar and Szybalski, 1983; Roberts et al., 1985). This implies either that BoxC is required for the N-system, perhaps for a NusB and/or NusE interaction, or that BoxC is redundant in both cases.

The identification of the nusE71 mutation in the rpsJ gene (encoding ribosomal protein S10) led to the suggestion that the ribosome could be involved in the N antitermination reaction, although it was also noted that S10 protein might play a role independent of the ribosome (Friedman et al., 1981). In vitro experiments confirmed that S10 or the 30s ribosomal subunit was required for the N antitermination reaction (Das and Wolska, 1984; M. Chamberlin, pers. comm.). Interestingly the nusE71 mutation was found to be suppressed by a mutation in rplP, which encodes the ribosomal protein L16 (Friedman et al., 1985; Schauer and Friedman, 1985). This enhances the argument that the ribosome plays a role, because the likeliest way by which an alteration of one ribosomal protein suppresses the effect of a mutation in another is through direct protein-protein interaction. In this respect note that S10 is a component of the small ribosomal subunit,

while L16 is a component of the large subunit.

How then might the ribosome by involved? Two pieces of evidence suggest that translation *per se* is not involved. Firstly an analysis of the DNA sequence upstream of the *nutL* of λ indicates that there are no translation initiation signals which could lead to the binding of a ribosome to the mRNA if that were necessary for it to participate in *N* action at *nutL* (Franklin and Bennett, 1979). Secondly, various studies have shown that termination of translation within *cro*, upstream of the normal stop codon (which lies immediately upstream of *nutR*), does not interfere with the effect of N at *nutR* (Olson *et al*., 1984; Warren and Das, 1984).

The mechanism of antitermination remains unresolved. However, the most obvious hypothesis is that RNA polymerase is modified to an "antitermination state" by N at the *nut* site. The *nus* factors are either essential for normal termination, with *N* acting to prevent their function; or else they are not essential for termination *per se*, but simply as components of an "antitermination complex" formed with *N*. The *nut* site in the antitermination reaction is made at least in part of RNA (Olson *et al*., 1982) and could be carried along as part of an antitermination complex (Greenblatt, 1984). If the ribosome is involved then it must be introduced to the *nut* site not by translation but in some unusual way. However, the roles of S10 and L16 as proteins free of the ribosome have not been ruled out.

Apart from the regulation of λ gene expression, antitermination mechanisms have been implicated in the control of the *E. coli* *rpoBC*, *rpoD* (Howe *et al.*, 1982; Lupski *et al.*, 1983) and ribosomal RNA operons (Morgan, 1980; Brewster and Morgan, 1981; Siehnel and Morgan, 1983; Aksoy *et al.*, 1984; Li *et al.*, 1984; Holben *et al.*, 1985). Recent

analysis of the rrnG operon, one of the ribosomal RNA operons, revealed a DNA fragment immediately preceding the P_2 promoter which had antitermination activity in a plasmid screening system (Li et al., 1984). Interestingly BoxA BoxB BoxC - like sequences could be identified which had strong sequence similarities with the λnut loci although the order of the boxes were different in the rrnG operon, i.e. BoxB BoxA BoxC (Li et al., 1984). The BoxA and BoxC - like sequences are precisely conserved in all six previously sequenced rrn operons, but, the BoxB - like sequences are more diverse. Recently Gourse and colleagues (1986) have shown that the region upstream of BoxA, including BoxB, in the rrnB operon is not required for antitermination while the region containing BoxA and BoxC is required. Further more they demonstrate that antitermination determinants apparently do not play a role in growth rate dependent regulation since rrnB promoter-lacZ fusions which do not contain any rRNA sequences, including the BoxB BoxA BoxC, are still regulated in a growth rate dependent manner. The authors therefore propose that an antitermination mechanism is probably important in rrn operons in vivo as a way of ensuring that RNA polymerase does not terminate transcription prematurely because of the high degree of secondary structure in the RNA and the lack of translation, but not for regulatory purposes. A more detailed mutational analysis is required to define precisely the sequences important for the antitermination described above (Li et al., 1984; Gourse et al., 1986).

To summarise, published observations suggest the presence of antitermination mechanisms in E. coli, responsible at least for vigorous synthesis of rRNA and possibly involved in the regulation of rpo gene expression (see later in this chapter and Chapter 6) which have features in common with the N antitermination system of λ.

1.2 The RNA polymerase subunit genes

The RNA polymerase molecule interacts with a wide variety of cellular proteins. This, combined with the fact that the subunits of RNA polymerase have no individual enzyme activities, has meant that the isolation conditional lethal and antibiotic resistance mutations has been necessary to map the chromosomal loci of the structural genes of RNA polymerase.

i) The sigma subunit (σ).

Sigma is a polypeptide of molecular weight approximately 70kd, involved in specific initiation of transcription. Its structural gene, rpoD, has been mapped to 66 min. on the E. coli chromosome in the metC-argG region (Gross et al., 1978; Harris et al., 1978; Travers et al., 1978).

The σ subunits of E. coli C and K12 have different mobilities on an SDS polyacrylamide gel, and this has been exploited to show that rpoD is co-transduced at a high frequency with dnaG. The latter gene codes for primase, a protein involved in the initiation of DNA replication (Harris et al., 1977). Analysis of various plasmids and λ transducing phages carrying the dnaGrpoD region indicated that dnaG and rpoD are transcribed clockwise, in that order, and that a promoter for rpoD lies between dnaG and rpoD (Gross et al., 1979; Scaife et al., 1979; Nakamura, 1980). Subsequently it became clear that rpoD shares an operon not only with dnaG, but also with rpsU. This gene encodes ribosomal protein S21, which is involved in the initiation of translation (Burton et al., 1983; Lupski et al., 1983; Lupski and Godson, 1984). The structure of the operon, and its various transcriptional features, are shown in Fig. 1.4 (for an interesting, up-to-date review see Lupski and Godson, 1984).

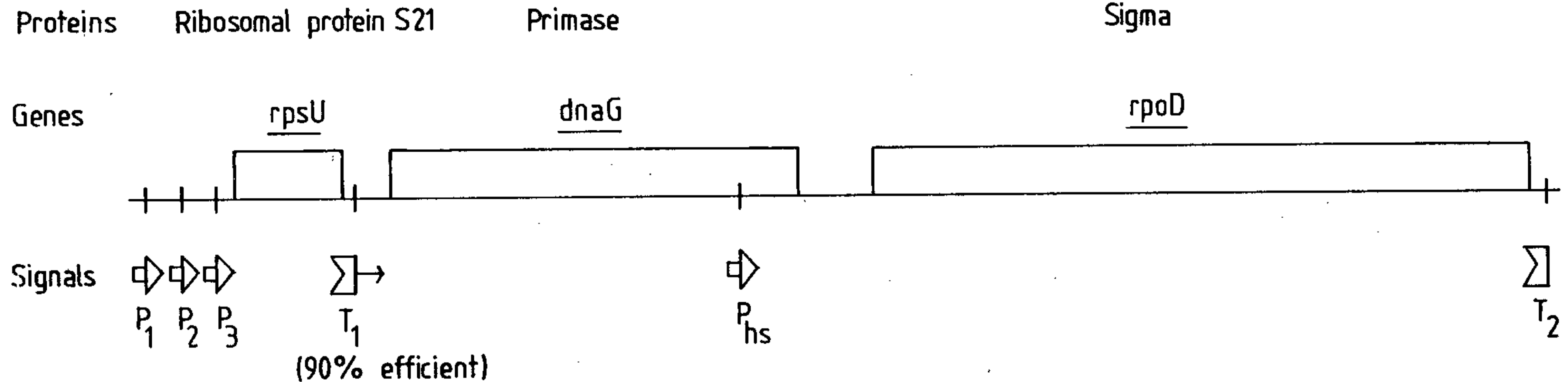


Fig.1.4

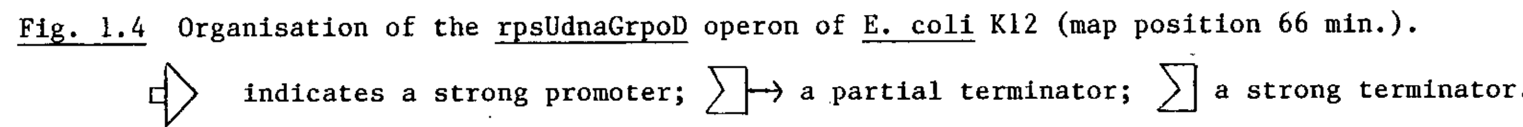


Fig. 1.4 Organisation of the rpsUdnaGrpoD operon of E. coli K12 (map position 66 min.). [symbol] indicates a strong promoter; [symbol] a partial terminator; [symbol] a strong terminator.

A number of mutations exist in *rpoD*, whose properties confirm the biochemical evidence that sigma is involved in the primary recognition event at promoters. One particularly interesting example, called *alt*, was selected as a revertant of *E. coli* mutants lacking the CRP-cAMP system (necessary for the full expression of the catabolite sensitive arabinose operon: Silverstone *et al.*, 1972; Travers *et al.*, 1978). Scaife, Travers and colleagues discovered that sigma isolated from *alt* mutants had an altered activity in an *in vitro* transcription system. Genetic mapping placed *alt* very close to the *dnaG* locus, leading to the conclusion that *alt* is a sigma mutation with altered promoter specificity.

The regulation of *rpoD* is in the early stages of characterisation; however, a number of interesting features have already come to light. It seems clear, for example, that induction of a lysogenic λ in strains harbouring plasmids carrying the *rpoD* region stimulates σ synthesis 9- to 10-fold relative to wild-type levels (Nakamura, 1980). An earlier study had demonstrated that a selective stimulation of chromosomally-encoded σ synthesis occurred during λ infection and after induction of a λ lysogen, and that this effect was attributable to the N protein of λ (Nakamura and Yura, 1976b). This led to the proposal that the observed stimulation of σ synthesis was the direct result of an antitermination mechanism. The proposal was strengthened when a nucleotide sequence, in many respects similar to the λ*nut* site, was discovered within the *rpsU* gene, upstream of the partial terminator T1 (Lupski *et al.*, 1983; see Fig. 1.4). This led to the further suggestion that an *E. coli* factor analagous to the N protein of λ might be involved in the regulation of this operon (*ibid.*). As discussed previously, the NusA protein is intimately involved with the N-system of λ, and recent *in vitro* experiments suggest that NusA increases the synthesis of σ (Peacock *et al.*, 1985).

Exposure of E. coli to a sudden temperature increase activates the expression of a number of genes encoding the so-called "heat-shock proteins" (Ashburner and Bonner, 1979). The production of these proteins, of which 17 have been identified to date in E. coli K12, is presumably correlated with an increased tolerance of the cell to the higher temperature. Stimulation of heat-shock protein synthesis in E. coli is transient, peaking around 5 min. after the temperature induction, and then setting back to an intermediate steady-state level typical of the higher growth temperature (Lemaux et al., 1978; Yamamori et al., 1978). The positive activator of the heat-shock response in E. coli has been shown to be a 32kd protein, encoded by the htpR gene, which functions as an alternative sigma factor recognising, and stimulating transcription from, heat-shock promoters (Grossman et al., 1984). One of the genes whose transcription is stimulated under these conditions is rpoD, although rpsU is not affected (Gross et al., 1982; Gross et al., 1984). This uncoupling of rpoD from rpsU transcription is due to the transient induction, after temperature up-shift, of a minor promoter, Phs (see Fig. 1.4), located within dnaG 360bp upstream of rpoD (Lupski et al., 1984; Taylor et al., 1984). In vitro analyses have shown that the stimulation of Phs is due to the interaction of the HtpR protein and core RNA polymerase with Phs (Grossman et al., 1984). Although the Phs promoter shows quite a close match with the normal E. coli promoter consensus in the -35 region, the -10 region shows little resemblance to the usual Pribnow box. This may explain why Phs is so weak a promoter, in plasmid fusions, at 30°C (Taylor et al., 1984). Interestingly, SI-nuclease mapping and Bal31 deletion studies of Phs revealed the importance of a DNA sequence in the -44 to -36 region for the heat-shock response (Taylor et al., 1984).

Sigma is a typical heat-shock protein in terms of the kinetics of activation and re-adjustment of its synthesis. Perhaps sigma -70 protein synthesis is required for the latter stage of the heat-shock response, when the initial stimulation of heat-shock gene expression is reduced to an intermediate steady state level, by the excess σ synthesised competing with HtpR for RNA polymerase binding.

Other facets of sigma regulation will be discussed later in this chapter.

ii) The alpha subunit (α).

The rpoA gene, encoding the 40kd alpha subunit of RNA polymerase, proved difficult to locate because, to date, no known antibiotic resistance mutations are found in the protein (Matzura, 1980). The position of rpoA was first identified by studying specialised λ transducing phages carrying the str-spc region from 72 min. on the E. coli map (Jaskunas et al., 1975a; 1975b). When one of these phages, λfus2, infected UV-irradiated bacteria a protein of similar tryptic fingerprint and mobility to bona fide α was synthesised and as expected this product was also precipitated with antiserum directed against alpha.

rpoA lies in a region containing a cluster of 27 ribosomal protein genes, and genes for a protein secretion catalyst (prlA) and elongation factors EF-Tu and EF-G (Jaskunas et al., 1975b; Shultz et al., 1982). This region is organised into 4 main regulatory units termed the str, S10, spc and α operons (Jaskunas et al., 1977; Jaskunas and Nomura, 1977; Lindahl et al., 1977a; see Fig. 1.5). Extensive polar insertion and deletion analysis of the specialised λ transducing phages demonstrated that rpoA is co-transcribed with the rpsM, K, D and rplQ genes encoding the ribosomal proteins S13, S11, S4 and L17 respectively

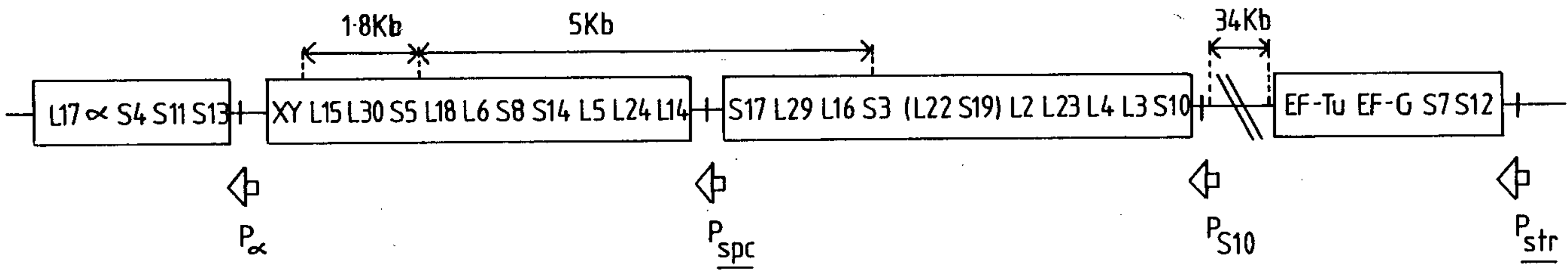


Fig.1.5

Fig. 1.5 Organisation of the *str-spc* ribosomal gene cluster of *E. coli* K12 (map position 72 min.). The 3 main transcription units encoding the proteins are shown, including the major promoters necessary for their expression. The position of the occluded P_{α} promoter is also shown.

+ - Y has recently been identified as *prlA* encoding a protein secretion catalyst (Shultz *et al.*, 1982).

(Jaskunas *et al.*, 1975b; 1975c; 1977; Jaskunas and Nomura, 1977). A functional promoter was found between the α and *spc* operons, called P_{α} (see Fig. 1.5), leading to the proposal that these two operons were independently transcribed (Jaskunas *et al.*, 1975c; Post *et al.*, 1980; Miura *et al.*, 1981). However, SI-nuclease analysis of transcripts from exponentially growing cells has recently demonstrated that *in vivo* transcription extends from the *spc* operon into the α operon (Cerretti *et al.*, 1983). Although under these conditions P_{α} is inactive, it is possible that it may have an important role to play in the regulation of α synthesis.

A number of mutations of *rpoA* have been isolated which provide clues to the protein's role in the transcription process. The first such mutation discovered (in *rpoA* of *E. coli* C, and called *gro*109) interestingly reduced late gene expression of bacteriophage P2 (Sunshine and Sauer, 1975). The α subunit purified from a *gro*109 mutant was shown not only to have an altered mobility on a urea-polyacrylamide gel, but also (by tryptic mapping) to have a leu→his substitution (Fujiki *et al.*, 1976).

More recently temperature sensitive mutations of α have been isolated (Ishihama *et al.*, 1980). The corresponding RNA polymerase, isolated from strains screened for defectiveness of RNA synthesis at 43°C *in vivo*, displayed erroneous transcription *in vitro* even at the permissive temperature of 30°C; although attempts to show this *in vivo* proved unsuccessful.

iii) The beta and beta-prime subunits (ββ').

The beta and beta-prime subunits of RNA polymerase, encoded by *rpoB* and *rpoC* respectively, are the two largest individual polypeptides in the polymerase molecule. Their molecular weights are 154kd and 161kd

respectively. Their genes are co-transcribed.

a) The beta subunit (β). All known resistance to the antibiotic rifampicin, a drug which blocks transcriptional initiation but not elongation, is due to mutations which map between the *purD* and *argH* loci at 88.5 min. on the *E. coli* chromosome (Matzura, 1980). They were identified as *rpoB* mutations in a series of RNA polymerase reconstitution experiments *in vitro* (Heil and Zillig, 1970). These authors observed that when the subunits were mixed in all possible combinations, only when β from a rifampicin resistant strain was present was resistance restored to RNA polymerase. Their findings were corroborated in a later study applying a similar approach; interestingly, in this latter case the resistant β had a CNBr cleavage pattern differing from that of wild-type (Boyd *et al.*, 1974).

b) The beta-prime subunit (β'). Unlike *rpoB* no known antibiotic resistance mutations lie in *rpoC*. Hence *rpoC* was more difficult to locate. However, various lines of evidence suggested that it might lie in close proximity to *rpoB*. For example β and β' protein synthesis were followed in an *E. coli* K12 strain partially resistant to rifampicin, a phenotype which allows synchronous restoration of initiation of transcription following removal of the drug (Matzura *et al.*, 1971). β synthesis was detected 2 min. after rifampicin removal, whereas β' synthesis lagged by a further 90 sec. The authors interpreted this difference to suggest that *rpoB* and *rpoC* are transcribed in a single polycistronic message (although other interpretations of the data were possible). In corroboration, strains merodiploid for the *rif* region (*rpoB*) overproduced both β and β' *in vivo* (Hayward *et al.*, 1974). In addition an amber mutation of *rpoB* was described which had a polar

effect on β' synthesis, implying that *rpoB* and *rpoC* are co-transcribed. Studies of deletions extending from beyond the *bfe* gene (encoding the receptors for the virulent phage BF23) to *rpoB* strengthened the co-transcription proposal for *rpoBC* (Errington *et al.*, 1974). One such deletion, ∇18, which extends from *argB* at 88.5 min. to *rpoB* at 89, placed β' synthesis under the control of the *arg* repressor. This indicated that *rpoC* was transcribed clockwise (like *argB*) and additionally, because the deletion end point in *rpoB* was mapped between a polar (*rpoB*38) and a non-polar (*rpoB*52) amber mutation of *rpoB*, suggested that the *rpoC* promoter lay upstream of *rpoB*. Final proof of this location of *rpoC* came from the analysis of proteins synthesised from purified restriction fragments, derived from a specialised λ transducing phage (λ*rif*^{d}18) carrying the *rpoB* region (Kirschbaum and Konrad, 1973), in an *in vitro* DNA dependent protein synthesising system (Lindahl *et al.*, 1977b).

c) The *rpoBC* operon. *rpoB* and *rpoC* share an operon with four 50s ribosomal protein genes, *rplK*, *A*, *J* and *L* encoding L11, L1, L10 and L7/12 proteins respectively. The basic structure and the main transcriptional signals of the operon are shown in Fig. 1.6.

The possibility that all these genes share one operon was first recognised through *in vitro* translation analysis of restriction fragments of λ*rif*^{d}18, described above (Lindahl *et al.*, 1977b). The four ribosomal protein genes, in this study, mapped immediately upstream of *rpoBC*. Previously it had been demonstrated that all the bacterial genes carried by λ*rif*^{d}18 were transcribed in the same direction (Jaskunas *et al.*, 1976); hence it was reasonable to speculate that *rplKAJL* and *rpoBC* were transcribed in a single operon.

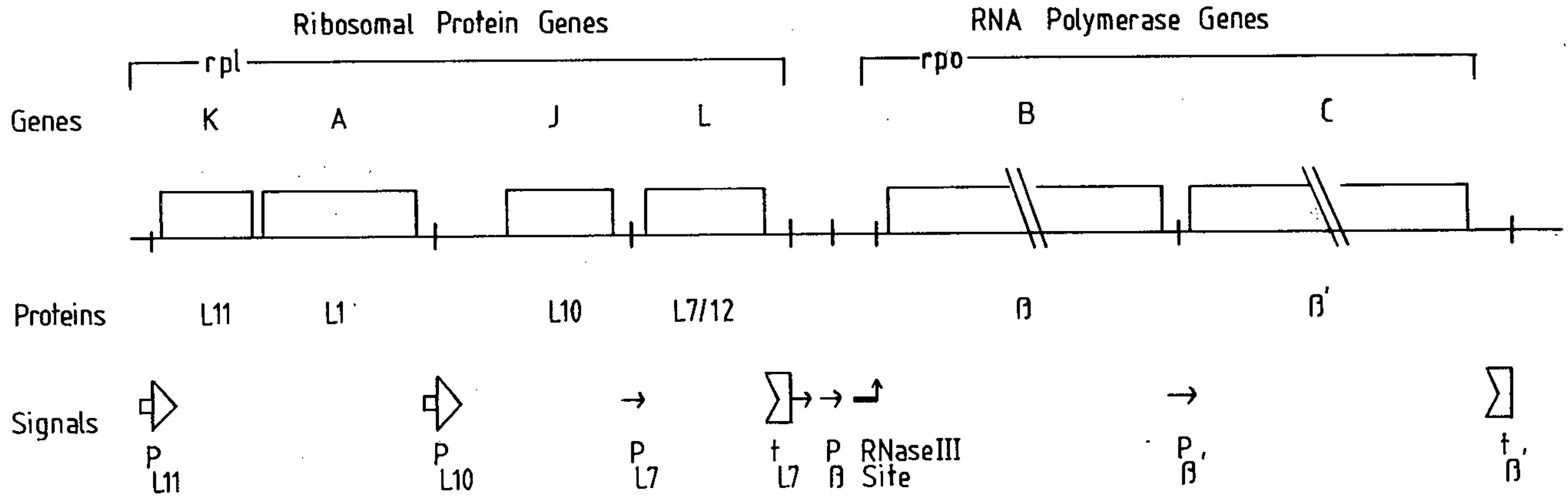


Fig.1.6

Fig. 1.6 Organisation of the *rplKAJLrpoBC* operon of *E. coli* K12 (map position 90 min.), based on Newman *et al.* (1979), Post *et al.*(1979), An and Friesen (1980), Barry *et al.* (1980) and Ma *et al.* (1981). ⊏▷ indicates a strong promoter; ⟶ a weak promoter; ∑⊢→ a partial terminator; ∑] a strong terminator and ▬↑ an RNaseIII processing site. Note that the precise locations of the weak promoters P_{L7}, P_{β} and $P_{\beta'}$ are unknown.

Studies in three laboratories suggested that the major transcription of rpoBC initiated at the promoter P_{L10} (Fig. 1.6), forming a single transcription unit containing rplJL and rpoBC (Linn and Scaife, 1978; Yamamoto and Nomura, 1978; Newman et al., 1979). All these groups, however, analysed deletions which extended from upstream of rplKA towards P_{L10}; therefore the contribution of the promoter P_{L11} (Yamamoto and Nomura, 1979) to operon transcription was difficult to estimate. Indeed SI-nuclease mapping of in vivo transcripts has indicated that the major transcript in the P_{L10} region is initiated at P_{L11}; thus P_{L10} is normally occluded by P_{L11} (Brückner and Matzura, 1981; C. Squires, pers. comm.). Note the similarity of this to the occlusion of P_{α} (α operon) by the P_{spc} promoter of the spc operon (Cerretti et al., 1983). The possible regulatory significance of occlusion in both these cases will be discussed in the next section and in Chapter 6.

As indicated in Fig. 1.6 a number of weak promoters have been discovered in the rpoBC operon, mainly through the analysis of a series of partial plasmid and λ clones of the operon (Newman et al., 1979; Barry et al., 1979; Ma et al., 1981). All these weak promoters have estimated strengths which are less than 1% of those of P_{L11} and P_{L10}; consequently the relative contribution to operon expression is insignificant. However, they may have a role under extreme environmental conditions. A putative promoter, possibly P_{β}, has been mapped by an "up-mutation" in the intercistronic space between rplL and rpoB (An and Friesen, 1980).

The 319bp intercistronic DNA between rplL and rpoB contains a number of features in addition to the minor promoter, P_{β}. Measurements of relative transcription frequencies of rplKAJL and rpoBC from $\lambda rif^{d}18$ fragments cloned in ColE1 plasmids, revealed that rpoBC was transcribed

at about one quarter to one fifth the frequencies of rplKAJL (Dennis, 1977b). The author proposed that a transcriptional terminator, between rplL and rpoB was the cause of this discrepancy. This observation is reflected at the translational level; i.e. the molar yield of ββ' protein was about one fifth that of the ribosomal proteins L11, L1, L10 and L7/12 (Lindahl et al., 1977b). Final confirmation of the presence of a terminator between rplL and rpoB arose from SI-mapping of in vivo transcripts from this region (Barry et al., 1980). The major transcript was seen to terminate at the first base in a run of 4 thymines, preceded by a sequence with potential hyphenated dyad symmetry (see Fig. 1.7). An RNaseIII processing site, fully functional in vivo, was also highlighted in this study (Fig. 1.7). A region of DNA in the vicinity of the RNaseIII processing site has been shown, by deletion analysis of plasmid fusions of the rplL-rpoB intercistronic DNA, to be required for the efficient translation of rpoB (Dennis, 1984). This region, distinct from the translational start signals of rpoB, has been consequently proposed to be involved in the post-transcriptional regulation of β synthesis.

The contributions of these various regulatory elements to the regulation of ββ' synthesis will be discussed in the next section.

1.3 Regulation of RNA polymerase synthesis

The structural genes of RNA polymerase, rpoA, B, C and D are located at three separate loci on the E. coli chromosome, with only rpoB and rpoC being co-transcribed. In addition all these genes share operons with others encoding ribosomal proteins. Interestingly, under certain conditions the regulation of the synthesis of the RNA polymerase subunits can be partially uncoupled from that of its ribosomal protein

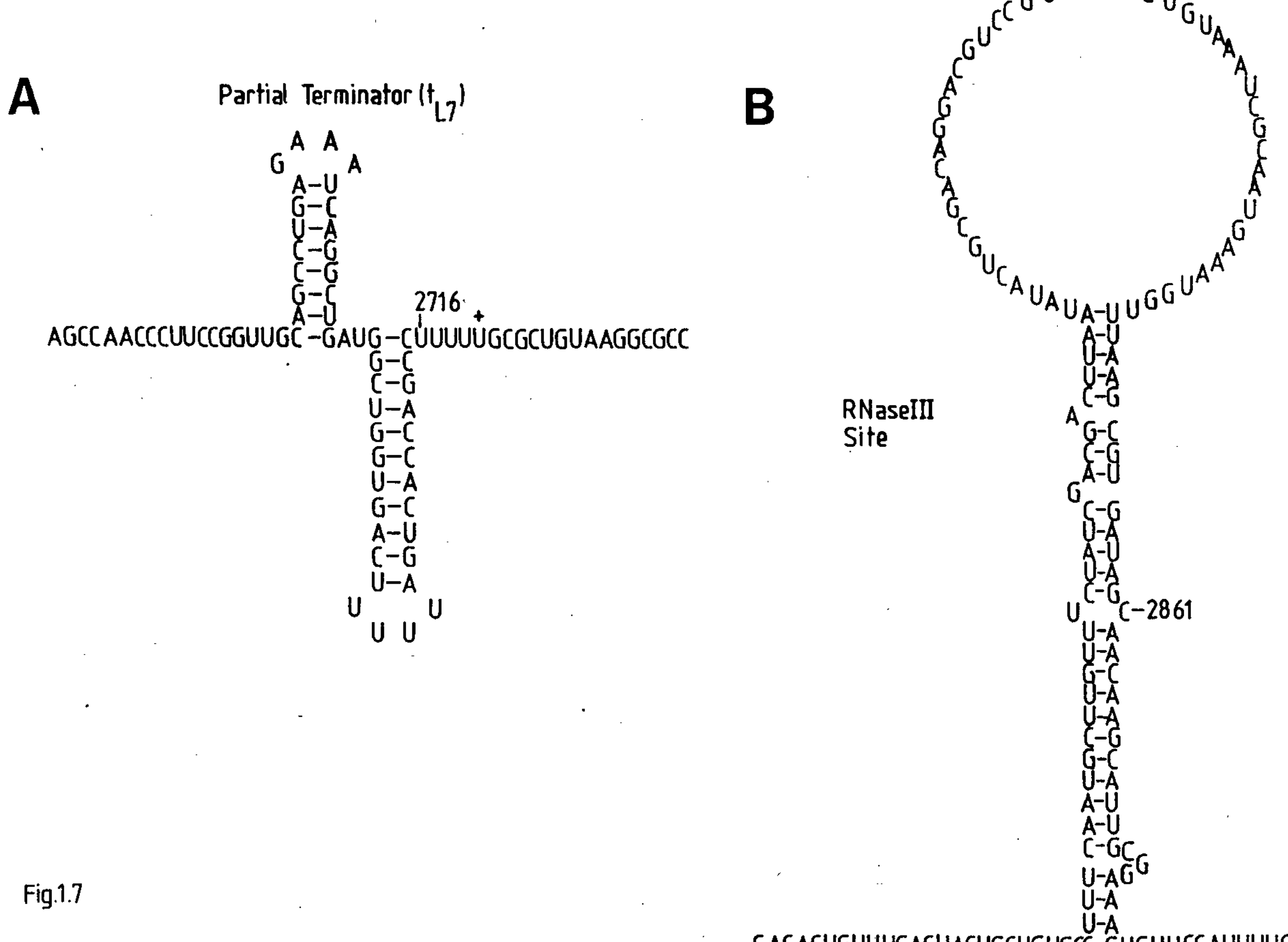


Fig.1.7

Fig. 1.7 Possible secondary structures of the mRNA in the region of A) the partial terminator t_{L7} (termination occurs in the run of U's at 2716) and B) the RNaseIII processing site at approximately 2861.

The nucleotide sequence is from Post *et al.* (1979), with a correction in t_{L7} (+) by Morgan *et al.* (1984).

Termination and RNaseIII-processing data are from Barry *et al.* (1980) and Fukuda and Nagasawa-Fujimori (1983).

neighbours, yet polymerase subunits are expressed co-ordinately. In both slow and fast growing cells, protein turnover does not appear to be a major element in this regulation (Matzura _et al._, 1973), although such turnover is seen when _rpo_ merodiploid strains are studied (Hayward _et al._, 1974; Hayward and Fyfe, 1978b). However, it is clear that the synthesis of the RNA polymerase subunits is regulated autogenously at both the transcriptional and post-transcriptional levels, utilising antitermination, translational regulation, and mRNA processing. The evidence for autogenous regulation and the role of the elements involved will be discussed below.

i) Transcriptional regulation.

In an attempt to study the regulation of RNA polymerase synthesis several growth constraints have been placed on _E. coli_. The earlier experiments included conditions in which RNA synthesis was partially inhibited either by the antibiotic rifampicin, or by growing _rpo_^{ts} and ts-suppressed _rpo_ amber mutants of the enzyme subunits at partially restrictive temperatures. (For reviews see Yura and Ishihama, 1979; Matzura,1980; Lindahl and Zengel, 1982.)

Hayward and co-workers observed that when a heterodiploid strain, containing a rifampicin sensitive and a recessive drug resistant RNA polymerase, was challenged with rifampicin an immediate reduction in total RNA and protein synthesis occurred (Hayward _et al._, 1973). In contrast, β and β' protein syntheses were stimulated transiently 3- to 4-fold relative to total protein, peaking 15-20 minutes after challenge with the drug. In contrast the antibiotic streptolydigin, a transcriptional inhibitor which blocks _elongation_, although causing the same reduction in total RNA and protein syntheses, showed no stimulation of ββ' synthesis in the analagous _stl_ merodiploid (Tittawella and

Hayward, 1974). Interestingly, in similar experiments to those described previously, rifampicin challenge of heterodiploids was shown to have little effect on the synthesis of ribosomal protein L7/12 (Hayward and Fyfe, 1978a). In contrast, sigma protein synthesis was stimulated faster and to a greater extent than β and β' in this study, and a less striking stimulation of alpha synthesis was also observed. Hence production of all the RNA polymerase subunits is stimulated to some extent by rifampicin. The extra ββ' synthesised was shown to be unstable, presumably because the extra protein is not assembled into complete enzyme (Hayward and Fyfe, 1978a). Estimates of the relative contributions of transcription and translation to the observed stimulation were not available; no specific mRNA analysis was attempted in these studies. However, an earlier study concluded that the excess β and β' synthesised following drug treatment reflected a transcriptional rather than a translational stimulation (Tittawella, 1976b).

Treatment of haploid E. coli with low concentrations of rifampicin induced stimulation of α, β, β' and σ syntheses similar to that observed for the heterodiploids (Nakamura and Yura, 1976a). Transcription of rplKAJL and rpoBC has been directly estimated under these conditions; rpoBC shows a 150% stimulation of mRNA synthesis, whereas rplKAJL transcription was only raised by 30% (Blumenthal and Dennis, 1978). This confirms Tittawella's conclusion that the rifampicin stimulation of ββ' synthesis seen in the heterodiploid work was transcriptional (Tittawella, 1976b).

Temperature sensitive mutations of the RNA polymerase subunits have played an important role in studies of the regulation of RNA polymerase synthesis. One such mutation, rpoD285, which inhibits transcriptional initiation at the restrictive temperature of 42°C, stimulates

rpoBC transcription and ββ' synthesis in a manner reminiscent of rifampicin (Blumenthal and Dennis, 1980b). In contrast rplL transcription was stimulated to a lesser extent. rpoA transcription was also stimulated, to an extent similar to rpoBC, but the specificity of this effect was unclear because transcription of the neighbouring ribosomal protein genes showed comparable stimulation.

Two further mutant polymerase alleles, one of rpoB (rpoB7rpoB2(Ts)) and one of rpoC (rpoC4(Ts)), cause a defect in RNA polymerase assembly when cells are exposed to 42°C (Little and Dennis, 1979). These have been reported to show an increase in ββ' protein synthesis at 42°C, relative to 30°C. The rate of synthesis of the β and β' subunits was elevated about 2-fold (relative to total protein) in the rpoC4(Ts) strain, after a prolonged period at the restrictive temperature; in contrast the rpoB7rpoB2(Ts) mutant under the same conditions exhibited an initial, transient decrease before reaching a 150% stimulation in the relative synthesis rates of ββ'. The cause of the transient decrease in rpoB7rpoB2(Ts) is unknown. Transcriptional measurements indicated qualitatively similar alterations of rpoBC mRNA and ββ' expression in these two mutants, at the restrictive temperature. In these mutants RNA polymerase assembled prior to the temperature shift is active at the non-permissive temperature; however, as cell division proceeds and cellular mass increases there is a gradual decrease of the intracellular, preassembled pool of RNA polymerase, leading to a slow inhibition of cellular transcription. Therefore the stimulation of β and β' syntheses observed in these two strains after a prolonged period at the restrictive temperature could be due to an inhibition of the initiation of transcription (cf. rifampicin treatment and the rpoD285 case), rather than a direct result of defective assembly. The rpoC4(Ts) allele was

later shown to be recessive to the wild-type rpoC^{+}, in terms of the transcriptional response at the restrictive temperature, when a KLF10 episome carrying the wild-type rpoBC operon was introduced into a Rec^{-} derivative of the rpoC4(Ts) strain (Taketo et al., 1976).

A partial inhibition of transcription was induced by shifting another β' mutant (rpoC56(Ts)), totally defective in RNA synthesis at 42°C, from 30°C to the intermediate temperatures of 38.5 and 39°C (Dennis, 1977a; Little and Dennis, 1979; Little et al., 1981). At these temperatures the strain displayed a rapid increase of rpoBC transcription relative to total cellular transcription, with a concomitant stimulation of β and β' relative to total protein synthesis. Comparisons between these studies suggest that apart from a strong stimulation of rpoBC transcription, there may be a lesser but significant increase in rplJL transcription accompanied by an unexpectedly unchanged transcription rate for rplKA (Dennis, 1977a; Little and Dennis, 1979; Little et al., 1981).

The simplest hypothesis to explain the above results is that there is autogenous regulation of β and β' syntheses at the transcriptional level, which is sensitive to the level of free active RNA polymerase in the cell. Studies on a series of temperature sensitive-suppressed amber mutations of rpoB provide further support for this model. One such mutant strain, carrying in the chromosome an unsuppressed amber mutation in rpoB, and a wild-type rpoB^{+} on the episome KLF10, synthesised wild-type β protein at the normal rate (Hayward et al., 1974). This was an interesting finding because the relative copy number of the amber rpoB gene to the wild-type rpoB^{+} is 2:1 under the exponential growth conditions employed; hence the production of normal levels of wild-type β protein implied a compensatory mechanism. Another non-polar amber

mutation in rpoB, rif^{o}_{amD12} , when suppressed by only 20% led to synthesis of normal levels of wild-type β protein, and showed a 2- to 3-fold increase in β' synthesis (Glass et al., 1975). Again this implies that a compensatory mechanism, sensitive to the level of functional β, is responsible for the induction of β (and β') synthesis. Attempts to obtain a 100-fold stimulation of β and β' syntheses in these strains (by use of very weak suppression) failed, suggesting that although β and β' are not normally synthesised at their maximum rates, there is a finite limit to their induction (Glass et al., 1975). Transcription of rplJL and rpoBC was estimated for strains carrying ts-suppressed polar and ts-suppressed non-polar amber rpoB mutations rpoB38 and rpoB-C1 (Little and Dennis, 1980). A comparison of the behaviour of these two strains at 25°C indicates that rplJL transcription is stimulated by 40% in the polar strain, while that of rpoBC is stimulated by a further 40% (to nearly 2-fold). The above transcriptional and translational data, derived from studies of amber rpoB mutants, are consistent with the proposed model that an autogenous mechanism sensitive to free RNA polymerase concentration is active at the transcriptional level.

The partial uncoupling of rplKAJL and rpoBC transcription observed above also occurs when the bacterium is exposed to partial amino acyl tRNA limitation, clearly a more normal, physiological constraint than those discussed above. A response is invoked, called the stringent response, one of the effects of which is to cause a strong reduction in RNA accumulation as a consequence of highly specific inhibition of rRNA, ribosomal protein-mRNA, and tRNA transcription. (Reviewed by Gallant, 1979.) The molecular mechanisms underlying the stringent response are not well understood. However, it is known that, when starved

for amino acids, E. coli rapidly synthesises guanosine tetraphosphate (ppGpp), whose production is strongly correlated with metabolic changes including the reduced RNA synthesis characteristic of the stringent response (Cashel and Gallant, 1969; Ryals et al., 1982). Under partial amino acid starvation conditions rplKAJL transcription is curtailed, whereas rpoBC transcription remains relatively unaltered. ββ' protein synthesis closely parallels the rpoBC mRNA levels (Blumenthal et al., 1976; Reeh et al., 1976; Maher and Dennis, 1977; Blumenthal and Dennis, 1980a). Nothing has been published concerning sigma synthesis after application of this constraint; however, rpoA mRNA synthesis has been shown to decrease to a greater extent that rplKAJL transcription after partial amino acid starvation (Blumenthal and Dennis, 1980a). In contrast alpha protein synthesis remained relatively constant, implying translational regulation of alpha synthesis under these conditions.

What is the locus or loci of the transcriptional regulation? Perhaps the two most obvious candidates to mediate this control mechanism are the partial terminator, t_{L7}, and the "silent" promoter, P_{L10} upstream of rplJ (see Fig. 1.6).

In an attempt to define the transcriptional signals involved in the rifampicin effect, segments of the rpoBC operon derived from λrif^{d}18, and carrying the relevant signals were fused in vitro upstream of either galK or lacZ in plasmid expression vectors (Howe et al., 1982; Newman et al., 1982). Low levels of rifampicin were found to increase readthrough of the partial terminator, t_{L7}, located between rplL and rpoB. However, it was also shown that rifampicin stimulates readthrough of two bacterophage T7 rho-independent terminators (Howe et al., 1982; Newman et al., 1982), and the classic rho-dependent terminator of bacteriophage λ, t_{RI} (Cromie and Hayward, 1984). This strongly suggests

that rifampicin has a general, non-specific effect on termination. The mechanism of uncoupling by rifampicin clearly includes increased read-through of t_{L7}; however, our unpublished observations of fusions suggest that rifampicin might also activate P_{L10}, whether passively (by reducing initiation at P_{L11}), or by some more active mechanism.

There is no direct evidence as to whether t_{L7} and/or P_{L10} are involved in "uncoupling" rpoBC transcription during the stringent response. As previously described rpoBC transcription is stimulated when certain rpo^{ts} mutants or ts-suppressed rpo amber mutants are exposed to partially restrictive temperatures. In some of these cases rplJL transcription was also raised, though to a lesser extent than that of rpoBC, suggesting that, apart from decreasing termination at t_{L7}, P_{L10} activation may well have an important role in regulation.

The mechanism(s) of transcriptional regulation of rpoBC remains obscure. The rifampicin effect on termination, being general, may be explained simply by proposing that the drug binds directly to RNA polymerase in the elongation phase of transcription (Yarbrough et al., 1976), and interferes with the ability of the enzyme to recognise terminators, perhaps by induction of a conformational change. Three points are of relevance here: 1) all mutations conferring Rif-R map in rpoB (Matzura, 1980); 2) certain rif mutations in rpoB reduce the ability of RNA polymerase to recognise terminators (Lecocq and Dambly, 1976; Yanofsky and Horn, 1981) and 3) the locus of certain mutants, whose phenotype is characterised by a dependence on rifampicin for growth, map very close to rho, a gene encoding a termination factor which interacts with RNA polymerase (Dabbs, 1982). However, an alternative, specific regulatory mechanism which might explain the rifampicin stimulation of ββ' synthesis has been postulated, based on the observation that

the presence of amino acid analogues which alter the fidelity of protein synthesis in the first 4 min. (but not later) following drug addition, eliminated the rifampicin effect on β and β' syntheses (Tittawella, 1976b). The author proposed that the increase in *rpoBC* transcription in response to rifampicin was a direct consequence of the expression of a short-lived regulatory protein, termed π, whose expression was normally repressed by free core polymerase. No mRNA studies were carried out; as a result there was no evidence as to whether π (if it exists) acts at the transcriptional or post-transcriptional level.

Earlier, one group reported the isolation of a conditionally lethal amber mutation, am100, which when temporarily unsuppressed led to a gradual decline in the rate of β and β' syntheses (Nakamura and Yura, 1975). However, the authors did not verify that the mutation responsible for the effect on *rpoBC* expression mapped to am100.

As can be seen, a number of constraints affecting the synthesis or activity of RNA polymerase yield a similar response in terms of the partial uncoupling of *rpoBC* transcription from *rplKAJL* transcription. It seems unlikely that a non-specific mechanism can be invoked to explain all these effects. A model for transcriptional regulation of *rpoBC* will be discussed in Chapter 6.

ii) Post-transcriptional regulation.

The complex regulation of β and β' syntheses includes, in addition to a transcriptional aspect, a post-transcriptional component (Lindahl and Zengel, 1982). A post-transcriptional mechanism also plays a role in the regulation of α synthesis (Blumenthal *et al.*, 1976; Reeh *et al.*, 1976; Blumenthal and Dennis, 1980a). Translational regulation of *rpoBC* includes some element of autogenous regulation (cf. transcriptional

control), although a complete understanding of the mechanism still eludes us.

Gene dosage experiments have provided evidence for post-transcriptional regulation. For example, a 7- to 12-fold increase in rpoBC dosage resulted in a 6-fold increase in rpoBC mRNA production, yet only a 2-fold increase in β and β' syntheses was observed (Dennis and Fiil, 1979). Induction of a λrif^{d}18 lysogen should presumably lead to rapid production of several hundred copies of the rpoBC genes, yet the rates of synthesis of β and β' increased by only 2- to 3-fold (Kirschbaum, 1973b: note that no transcriptional studies were performed). Again, it has recently been shown that induction of a strain lysogenic for a different rpoBC recombinant λ phage caused only a 2- to 3-fold increase in overall ββ' synthesis, whereas rpoBC mRNA production increased by factors of 15- to 20-fold (Meek and Hayward, in press).

Studies of certain rpo^{ts} and ts-suppressed rpo amber mutants have also provided evidence for post-transcriptional regulation. For example, a strain carrying a temperature sensitive mutation of β', rpoC110, displayed after 5 min. at 42°C a 5- to 10-fold increase in the absolute rate of ββ' synthesis, whereas rpoBC mRNA production was raised by only 2- to 3-fold relative to total cellular RNA synthesis (Kirschbaum, 1978). In a more recent study a deficiency in β subunit synthesis was created by the weak suppression of two amber mutations of rpoB: rpoB1603 and rpoB12 (Dennis et al., 1985). Earlier work on similar mutations had suggested that the stimulation of β and β' syntheses produced by growth at a partially permissive temperature was closely paralleled by the stimulation of rpoBC transcription (Little and Dennis, 1980). In contrast when the mutant strains, rpoB1603 and rpoB12, are poorly suppressed a much less significant stimulation of

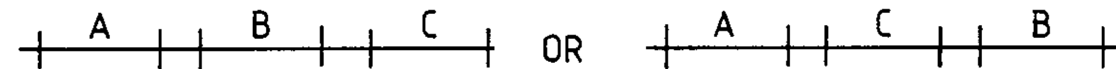
rpoBC transcription occurs. Transcription was increased 1.5-fold relative to a wild-type control; whereas translation was stimulated 5- and 2.3-fold in the respective mutants. In view of the earlier observations, it seems clear that the stimulation of β and β' synthesis can be solely a transcriptional effect, or can also involve a post-transcriptional mechanism, conceivably depending on the detailed phenotype/amino acid sequence of the "suppressed amber" β-polypeptide.

Evidently the artificial overproduction of rpoBC mRNA, in several examples cited above, leads to a decrease in its translational efficiency, and consequently only modest increase in the rate of ββ' synthesis. On the other hand the studies with the rpoB1603 and rpoB12 mutant strains indicate that translational activation of ββ' synthesis can occur (Dennis et al., 1985).

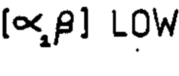
What is the mechanism(s) of this post-transcriptional regulatory system? Theoretically a number of possibilities exist by which the regulation of β and β' translation could be achieved, such as altered mRNA stability, or direct alteration of the efficiency of translational initiation. The gene dosage experiments are best explained in terms of autogenous regulation at the level of mRNA translation (Kirschbaum, 1973b; Dennis and Fiil, 1979). In vitro studies have suggested that the cellular effector of this regulation is either the $\alpha_2\beta$ subassembly or complete holoenzyme (Ikeuchi et al., 1975; Fukuda et al., 1978; Kajitani et al., 1980; Lang-Yang and Zubay, 1981; Peacock et al., 1982). Later, rpoBC plasmids were constructed with various deletions upstream of rpoB (i.e. in the intercistronic space between rplL and rpoB) to assess the relative contributions of different sequences to rpoB translation (Dennis, 1984). Sequences in the vicinity of the RNaseIII site, well upstream of the translational start signals, were found to be important

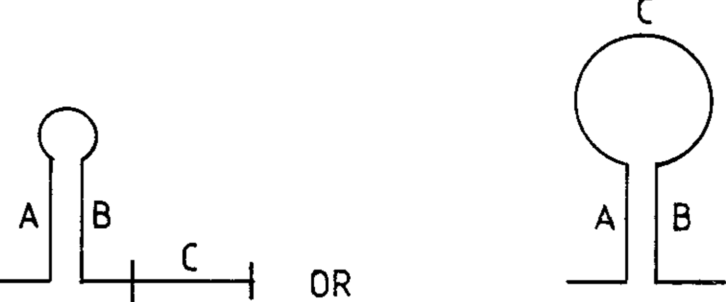
for efficient translation of rpoB. RNaseIII processing is already known to have a role in post-transcriptional regulation of gene expression in some systems, e.g. bacteriophage λ and T7 (Saito and Richardson, 1981; Gottesman et al., 1982). However, Dennis found that processing at the RNaseIII site upstream of rpoB had, per se, little or no effect on rpoBC transcription, or translation of the mRNA. This confirms earlier results showing that lack of RNaseIII processing has no apparent effect on the transcription of rpoB in exponentially growing cells, although this latter study did not include data on ββ' protein synthesis (Barry et al., 1980). The role of RNaseIII processing remains unclear.

How then are β and β' translation controlled? Dennis provided evidence that the efficiency of translation of rpoB mRNA is related in an inverse manner to the synthesis rate of β protein. Hence it can be imagined that as the concentration of β rises in the cell, either free β or an assembly intermediate such as $\alpha_2\beta$ (see above) binds to rpoB mRNA, preventing translation. This could in principle be due to direct binding (with exclusion of ribosomes) at the rpoB start site. However, an alternative model should be considered, to accommodate Dennis' findings that deletions of the upstream RNaseIII target inhibit rpoB mRNA translation, although RNaseIII processing per se seems irrelevant. Suppose that there is a mRNA segment (B) which is complementary both to the β-start region (C) and the RNaseIII target (A: see Fig. 1.8), and that $\alpha_2\beta$ can bind to the latter. Then in the presence of plentiful β (and since α is normally in excess), A would be sequestered by $\alpha_2\beta$, and B would be free to pair with C, blocking translation of rpoB mRNA. Deletion of A would mimic $\alpha_2\beta$-binding, explaining Dennis' finding. In low concentrations of β, however, A would pair with B, leaving C free and hence allowing β-synthesis. Interestingly, recent results from our laboratory favour



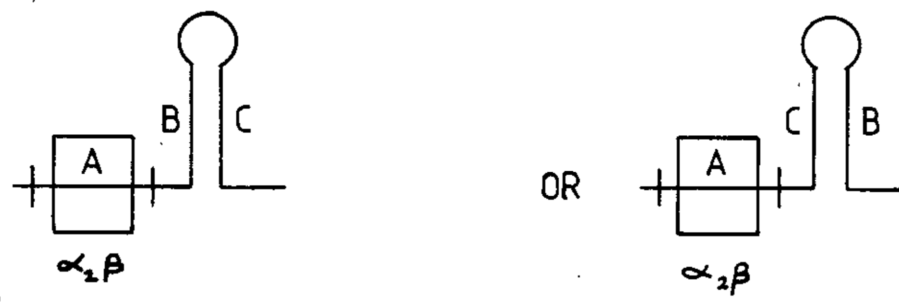
Region
A Sequences around the RNase III site.
B Sequences which can pair with regions A and C.
C The translational initiation sequences of rpoB.





Region C "free" ∴ rpoB translation can occur.

[$\alpha_2\beta$] HIGH



Region C "sequestered" ∴ rpoB translation blocked.

Fig.1.8

Fig. 1.8 The proposed secondary structures in the *rpoBC* mRNA in the vicinity of the RNaseIII processing site and translational initiation signals of *rpoB* which could explain the post-transcriptional regulation of *rpoB*.

this model and, further, suggest that the regulations of β and β' translation may be independent (Meek and Hayward, in press). Specifically, when a λ vector expressing rpoB but not rpoC is induced from the lysogenic state, a cut down in chromosomally-encoded β translation is observed, but β' synthesis from the same (chromosomally-encoded) mRNA remains at normal levels. Although no transcriptional measurements were performed it is tempting to suggest that β and β' syntheses are regulated independently at the translational level, perhaps via different regulatory molecules: e.g. $\alpha_2\beta$ may regulate rpoB, while $\alpha_2\beta\beta'\sigma$ regulates rpoC.

The gene dosage studies make it clear that repression of β and β' syntheses occurs at the post-transcriptional level. A positive activation mechanism has also been inferred to operate at this level, for example when rpoC110(Ts) is grown at restrictive temperatures or when the rpo amber mutations rpoB1603 and rpoB12 are poorly suppressed. However, these data could also be interpreted in terms of a repressor model, in which the rpo mutation indirectly prevents the autogenous inhibitor(s) (thought to include β and/or β') from binding. Thus the effects on β and β' translation may only reflect the inability of the inhibitory molecule to bind and regulate translation, rather than a mechanism which detects the actual concentrations of the individual subunits or of active R-pol, and compensates for deficiencies by activating translation or by lifting repression; on the assumption that, normally, translation is not at maximum efficiency (cf. ribosomal protein genes).

iii) The role of regulation.

Both transcriptional and post-transcriptional regulation of RNA polymerase occur, although the characterisation of the mechanism is

incomplete at present. What then is the physiological role of these mechanisms?

The synthesis of β and β' has been studied under various conditions in _E. coli_ B/r and _E. coli_ K12 strains, and found to remain largely unchanged relative to total protein synthesis at different growth rates (Matzura _et al._, 1973). It was also apparent that ββ' synthesis was rather insensitive to the sudden gene doubling effect of DNA replication in synchronised cells. In contrast a gene dosage effect was observed when heterodiploids of the _rif_ region were constructed by introducing an additional copy of the _rpoBC_ operon on a KLF10 episome (Hayward _et al._, 1974). Normally about 1.5 copies of _rpoBC_ per genome would be expected in these heterodiploids, because the KLF10 is replicated late in the cell cycle (as judged by rif^s/rif^r RNA polymerase ratios). Measurements of β and β' syntheses in fact revealed a rate of 1.44 relative to total protein, to be compared with a haploid rate of 1.04. These results echo the predicted gene dosages of _rpoBC_ in the two strains. The extra β and β' was degraded quite rapidly, presumably because the excess protein is not assembled into complete enzyme. The seeming contradiction between Hayward and Matzura's results can be resolved because in the experiments of Matzura, the _rpoA_ gene would be duplicated at about the same time as _rpoBC_; thus the failure to observe significant gene dosage effects in the latter case can be understood if one postulates that the autogenous regulation of _rpoBC_ gene expression involves complex(es) which contain alpha (R. Hayward, pers. comm.). Hence perhaps post-transcriptional repression of _rpoBC_ translation is utilised to maintain a steady expression of these genes at times when overproduction, due to gene dosage effects during the cell cycle (which would differ as between slow and fast growing cells),

could be expected. In contrast transcriptional regulation of rpoBC, seen to occur in response to numerous constraints, may be important in increasing the synthesis of RNA polymerase at times when the pool of active enzyme is depleted. This could be due to a number of reasons. However, perhaps the most important arises because ribosomal protein - and RNA polymerase - gene transcription are normally co-ordinated via shared promoters. Hence anything reducing ribosomal protein transcription, e.g. the stringent response, would be predicted to reduce RNA polymerase synthesis. This may be undesirable to the cell under particular environmental conditions. Therefore a regulatory mechanism able partially to uncouple ribosomal protein from RNA polymerase transcription could be an important way to ensure that the level of active polymerase can be maintained despite changes in ribosomal protein synthesis.

The alpha and sigma operons have been less well characterised than the rpoBC operon. However, it is likely that both operons are regulated at the transcriptional and post-transcriptional levels: preliminary evidence for this has already been discussed.

The experiments described in the following chapters are an attempt to analyse and define features relevant to the regulation of rpoBC. The approach used was as follows:

1) To characterise the nature of the $rif^{d}18$ mutation of rpoBC, because preliminary evidence suggests that a regulatory alteration may be involved. This is also relevant because DNA from $\lambda rif^{d}18$ has been used by most groups for regulatory studies of the operon.
2) To perform SI-nuclease studies of *in vivo* transcripts isolated from *E. coli* subjected to either of two constraints: rifampicin treatment, or partial amino acid starvation, both known to uncouple rplKAJL from rpoBC transcription: in order to identify directly the transcriptional signals important for these responses.

CHAPTER 2

Materials and Methods

2.1 Growth media

All quantities listed below, unless otherwise stated, refer to 1 litre of solution.

L-broth contained: Difco Bacto Tryptone, 10g; Bacto Yeast Extract, 5g; NaCl, 5g; adjusted to pH7.2. L-agar contained, in addition: Difco agar, 15g.

Oxoid broth contained: Lab-Lemco Powder (Oxoid L29), 10g; Peptone (Oxoid L37), 10g; NaCl, 5g. Oxoid agar contained, in addition: N.Z. agar, 12.5g.

BBL-agar contained: Baltimore Biological Laboratories trypticase, 10g; NaCl, 5g; Difco agar, 10g.

BBL-top-agar is BBL-agar with only 6.5g Difco agar.

Phage buffer contained: KH_2PO_4, 3g; Na_2HPO_4, 7g; NaCl, 5g; 0.1M $MgSO_4$, 10ml; 0.01M $CaCl_2$, 10ml; 1% (w/v) gelatin, 1ml.

Bacterial buffer contained: KH_2PO_4, 3g; Na_2HPO_4, 7g; NaCl, 4g; $MgSO_4 \cdot 7H_2O$, 0.2g.

Galactose MacConkey agar contained: Bacto-Peptone, 17g; Difco Protease Peptone, 3g; Bacto-Bile Salts No. 3, 1.5g; NaCl, 5g; Bacto-Agar, 13.5g; Bacto-Neutral Red, 0.03g; Bacto-Crystal Violet, 0.001g; Galactose, 10g.

Spizizen minimal medium contained: $(NH_4)_2SO_4$, 2g; K_2HPO_4, 14g; KH_2PO_4, 6g; tri-sodium citrate dihydrate, 1g; $MgSO_4 \cdot 7H_2O$, 0.2g. Final concentrations of supplements were: sugars or glycerol, 0.2% (w/v); amino acids, 20μg/ml; vitamin B1, 2μg/ml. Spizizen minimal agar

contained in addition: Difco agar, 15g.

Antibiotics were used at the following concentrations:

Ampicillin	50μg/ml
Chloramphenicol	50μg/ml
Rifampicin	100μg/ml
Spectinomycin	100μg/ml
Streptomycin	100μg/ml
Tetracycline	25μg/ml

2.2 Bacterial strains

All strains were derivatives of *Escherichia coli* K12 (see Table 2.1).

2.3 Bacteriophages

See Table 2.2 for details.

2.4 Phage techniques (λ, P1 and M13)

(i) Titrations (λ and P1).

Phage were serially diluted in phage buffer. 0.1ml of the dilutions were mixed with a suitable indicator strain, prepared as follows:

a) For λ titrations a fresh overnight culture, grown in L-broth at 37°C, was diluted 10-fold and grown at 37°C to a concentration of 2-5 x 10^8 cells/ml. Cells were then pelleted at 6,000 rpm in a bench centrifuge (MSE) for 5 minutes and resuspended in 0.5 volumes of 10mM $MgSO_4$.

b) For P1 titrations a fresh overnight culture, grown at 37°C in L-broth supplemented with 5mM $CaCl_2$ and enriched with 1% glucose, was diluted 10-fold in the same medium and grown at 37°C for 3 hours.

Table 2.1 Bacterial strains

Strain	Genotype	Donor
JM101	Δ(lac pro) thi strA endA sbcB115 supE/F' traD36 $lacI^{q}$Δ(lacZ)M15 pro^{+}	K. Murray
C600	lac leu thi thr tonA supE	N.E. Murray
H105 lysogen	recA1 HfrKL16 thi ($\lambda cI^{ts}857$ Sam7)($\lambda rif^{d}18$)	Kirschbaum and Konrad, 1973
H105	recA1 HfrKL16 thi	This work*
CR63	Hfr supD λ^{R}	Appleyard, 1954
BM1	Hfr supD λ^{R} thy	Constructed from CR63*
BM3	Hfr supD λ^{R} thy rpsL(Str-R)	Constructed from BM1*
BM6	Hfr supD λ^{S}-malE::Tn10 thy rpsL(Str-R)	Constructed from BM3*
BM5	Hfr supD λ^{S}-malE::Tn10 recA56 rpsL(Str-R)	Constructed from BM6*
W3110	sup^{oc}	J. Scaife
BM25	sup^{oc} thy	Constructed from W3110*
BM26	sup^{oc} recA56	Constructed from BM25*
W3110-del1	sup^{oc} metE::Tn10 rpoB1554-1	R.E. Glass and V. Nene
BM7	sup^{oc} metE::Tn10 rpoB1554-1 thy	Constructed from W3110-del1*
BM9	sup^{oc} metE::Tn10 rpoB1554-1 recA56	Constructed from BM7*
ED3867	thi trp lys galK Δ(lac)X74 malA rpsL (Str-R) tsx	J. Maule

Table 2.1 (contin.)

AB4141(valS^{ts})	metC56 thi-1 valS7(Ts) lacY1 galK2 rpsL69(Str-R) tfrS tsx57 supE44	A. Jenkins
EMR3(valS^{+})	metC56 thi-1 valS^{+} lacY1 galK2 rpsL69(Str-R) tfrS tsx57 supE44	E.A. Marson
AJN10	recA56 metB str thi	Newman and Hayward, 1980
AJN1	recA56 metB rpo-rcs40 str thi	Newman et al., 1979
JC5088(JG47)	Hfr KL16 recA56 thr ilv spc	R.S. Hayward
MM223	str thr leu thi thy	M. Masters
Ymel	supF	N.E. Murray
S159	gal strA uvrA	Jaskunas et al., 1975b
S159(λimm21)	gal strA uvrA (λimm21)	Linn and Scaife, 1978
AB1886	thr leu proA his arg lac gal ara xyl mtl thi tsx str uvrA6 P1^{s}	H. McQueen
CGSC6137	araD139Δ(argF-lac)205 flbB5301 ptsF25 relA1 rpsL150 malE52::Tn10 deoC1	A. Jenkins
A19	HfrPØ21 rna19 metB1 rel-1 his lac lmb	Kindler et al., 1973
AB301-105	HfrPØ21 rna19 metB1 rel-1 his lac lmb rnc bio suc ranA2074^{ts} (and/or other ts mutations)	Kindler et al., 1973; Silengo et al., 1974 and Apirion and Watson, 1975

All BM strains were constructed during the course of this work.

* see text for details of these constructions.

Table 2.2 Bacteriophages

Phage	Genotype	Donor
λNM1	$cI857^{ts}$ ind	N.E. Murray
λNM14	b2 immλ cI	N.E. Murray
λNM54	imm21	N.E. Murray
λNM144	h^{82}b522 immλ cI sk-1°	N.E. Murray
λNM243	λvir	N.E. Murray
λNM244	h^{80} immλ vir	N.E. Murray
λNM507	b^{2} imm21 cI	N.E. Murray
λNM508	h^{80} imm21 cI	N.E. Murray
λNM549	h^{80} immλ cI	N.E. Murray
λAJN63	srIλ(1-2)$^{\nabla}$ rplLrpoBC$_{L}$ (att-red)$^{\nabla}$ imm21 nin5$^{\nabla}$	A.J. Newman
λAJN81	b538 imm434 cI::(t$_{L7}$) shnλ6°	A.J. Newman
λAJN261	chiA131 srIλ(1-2)$^{\nabla}$ rpl'AJLrpoBC$_{L}$ (att-gam)$^{\nabla}$ cI857 nin5$^{\nabla}$	A.J. Newman
λAJN321	sbλ1° sbλ(2-3)$^{\nabla}$ rpl'AJLrpoBC'$_{R}$ imm21 nin5$^{\nabla}$	A.J. Newman
λAJN363	sbλ1° sbλ(2-3)$^{\nabla}$ rpl'AJLrpoBC'$_{L}$ imm21 nin5$^{\nabla}$	A.J. Newman

Phage and cells (for both λ and P1) were added to 3ml of BBL-top-agar (46°C) and poured onto a fresh BBL plate (both the top agar and BBL plate being supplemented with 5mM $CaCl_2$ in the case of P1). Plaques were scored after 12-18 hours at 37°C.

ii) Plate lysates (λ and P1).

Hosts for both λ and P1 were prepared as for titrations.

a) For λ: 0.2ml of cells, 5×10^5 pfu of λ (from a fresh plaque) and 3ml of BBL-top-agar were mixed and poured onto a fresh, moist L-broth plate. The plates were incubated at 37°C, usually for 5-8 hours, to achieve confluent lysis. The agar was then covered with 3-4mls of L-broth and refrigerated overnight. The next day the broth was harvested, mixed with a drop of chloroform, clarified by centrifugation (8,000g at 4°C for 10 minutes) and titrated on a suitable indicator strain.

b) For P1: 0.2ml of cells, 10^6 pfu of P1 (from a fresh plaque) and 3ml of BBL-top-agar (supplemented with 5mM $CaCl_2$) were mixed and poured onto a fresh, moist L-broth plate (supplemented with 5mM $CaCl_2$). After incubation at 37°C overnight the top layer was transferred with a sterile spatula into a sterile Corex tube. 1.5ml of phage buffer and 0.15ml of chloroform were added, and mixed by repeated aspiration with a 10ml pipette and bulb. The tube was swirled at 37°C for 1 hour, cell debris pelleted (16,000g at 2°C for 10 minutes) and the clear supernatant collected and stored at 2°C with 1-2 drops of chloroform.

iii) Liquid lysates (λ).

A fresh overnight culture was diluted 20-fold in L-broth supplemented with 10mM $MgSO_4$ and grown at 37°C with aeration to an O.D. 650nm of

0.45-0.6. Phage were then added to give a m.o.i. of 1. When the O.D. 650nm reached a minimum (due to cell lysis), chloroform was added (1ml/500ml lysate), and the lysate shaken for a further 10 minutes. The lysate was clarified by centrifugation (8,000g at 4°C for 10 minutes) and stored at 4°C with 0.02ml of chloroform/ml. The lysate was titrated at this stage. The phage were concentrated by ultracentr-fugation (40,000g at 4°C for 3 hours) and the pelleted phage resuspended in 0.05 volumes of phage buffer by shaking gently at 4°C. After further clarification (10,000g at 4°C for 15 minutes) the supernatant was treated at 20°C for 1-3 hours with RNaseA and DNaseI (both at a final concentration of 10μg/ml). Phage were concentrated and purified as described in 2.4 (v).

(iv) Preparation of λ*rif*^{d}18.

A fresh 25ml oxoid overnight culture of H105 (λ*c*I^{ts}857 *S*am7) (λ*rif*^{d}18) grown at 30°C in the presence of rifampicin was subcultured 20-fold in oxoid broth, and grown to an O.D. 650nm of 0.45. Following induction at 42°C (5 minutes) incubation was continued for 2 hours at 39°C. The cells were then spun down (10,000g at 4°C for 10 minutes) and resus-pended in 0.02 volumes of phage buffer. 1ml of chloroform was added per 50ml of suspension, and the cells shaken at 37°C for 10 minutes. DNaseI (10μg/ml final concentration)was added, and after a further 10 minutes at 37°C the cell debris was pelleted (10,000g at 4°C for 10 minutes). The phage were further concentrated and purified using a CsCl step gradient, followed by a CsCl equilibrium gradient (see 2.4 (v)). λ*rif*^{d}18 were separated from the λ*c*I^{ts}857*S*am7 helper phage either by removing the appropriate band through the side of the tube, or by collect-ing 0.5ml fractions from the bottom. The fractions were then titrated on the strain *Ymel*, to locate those containing helper phage (λ*rif*^{d}18

is defective, giving no plaques).

(v) Phage purification (λ).

Phage were purified by banding in a CsCl step gradient. Three steps of CsCl were routinely used, with densities of 1.3, 1.5 and 1.7g/ml in phage buffer (31%, 45% and 56% (w/w) CsCl). The 1.3g/ml CsCl solution was added to the centrifuge tube first, and the denser solutions gently underlaid with a syringe. After overlaying the gradient with the phage sample, the tubes were placed in a MSE 6 x 14 Ti swing-out rotor, and spun at 33,000 rpm at 15°C for 2 hours. Phage bands were collected through the side of the tube with a syringe and 19G needle. If further purification was necessary the pooled phage bands were mixed with 41.5% (w/w) preclarified CsCl solution and heat-sealed into a Beckman 16 x 76mm tube. Centrifugation in a Spinco 50 Ti rotor occupied 48 hours at 90,000g and 18°C. The phage band was collected through the side of the tube with a syringe and 19G needle.

(vi) DNA preparation (λ).

Phage bands, collected from CsCl step gradients or equilibrium gradients (see 2.4(v)), were dialysed against TE buffer (10mM Tris/HCl pH8.0, 1mM EDTA) for 1 hour at 4°C, to remove the CsCl. After three phenol extractions, carried out by rolling the phage solution with an equal volume of phenol (pre-equilibrated with TE), the final aqueous layer was dialysed against TE, with several changes over 24 hours at 4°C to remove phenol. DNA concentration and purity were estimated by UV spectrophotometry at 260nm and 280nm, and by minigel analysis (see 2.7(i)).

(vii) Bacteriophage M13 single-stranded DNA preparations.

A fresh M13 plaque was inoculated into 1ml of a JM101 overnight culture,

diluted 100-fold in L-broth. Following growth at 37°C with slow shaking for 5-6 hours, the culture was spun in a microfuge for 3 minutes. The supernatant was carefully decanted into a fresh Eppendorf tube, and 200μl of 20% polyethylene glycol 6000 in 2.5M NaCl were added. After mixing by inversion and Vortex, and standing at 0°C for 20 minutes, the tube was spun in the microfuge for 5 minutes, and the supernatant removed carefully and discarded. 100μl of TE and 100μl phenol (equilibrated with TE) were added, the tubes vortexed thoroughly several times over a 5 minute period, and then spun for 1 minute in a microfuge. The aqueous layer was removed and the DNA precipitated with ethanol at -70°C (see 2.8(i)). 1ml preparations of single-stranded DNA were dissolved in 25μl TE, providing sufficient template for sequence analysis (see 2.11). 100ml preparations were made as above (but using Corex tubes), and the DNA was dissolved in 1ml TE, and its concentration estimated by UV spectophotometry (O.D. 260nm and 280nm).

(viii) Construction of λ lysogens.

Cells were prepared as for titrations. After shaking for 30 minutes at 20°C a suitable volume of cells (e.g. 0.5ml) were infected with the phage at a m.o.i. of 5 (preferably: but 2 may be adequate), plus a heteroimmune helper phage (m.o.i. 5) if required. The mixture was shaken at 20°C for 20 minutes to allow phage adsorption, then diluted 2-fold with L-broth and incubated at 30°C for 1 hour. At this stage, if a heteroimmune helper had been used, selection for lysogens could be achieved by applying homoimmune <u>c</u>I$^-$ "killer" phages (λNM14 and λNM144 for selection of <u>imm</u>λ; λNM507 and λNM508 for selection of <u>imm</u>21). This involves incubating 5 x 10^8 cells with 3 x 10^8 pfu of each killer phage at 20°C for 20 minutes. 0.2ml aliquots of undiluted and 10-fold dilutions (in cell buffer) mixtures were then plated, in 2.5ml of BBL-top-agar,

on L-broth plates. (Each top layer (46°C) was poured immediately after adding the mixture, to avoid induction.) After incubation at 32°C possible lysogens were picked, and tested for the appropriate phage immunities and sensitivities.

(ix) P1 transduction.

A fresh overnight culture of the recipient strain was subcultured 5-fold in L-broth, and grown to an O.D. 650nm of 0.45-0.6. The cells were pelleted (6,000 rpm at 20°C for 5 minutes) and resuspended in 0.5 volumes 0.01M $MgSO_4$, 5mM $CaCl_2$. After shaking at 37°C for 15 minutes 0.1ml aliquots of cells were mixed with 5 x 10^6 pfu of P1v grown on the appropriate donor: the m.o.i. is important. Further incubation at 37°C for 15 minutes was followed by the addition of 0.2ml of 1M tri-sodium citrate dihydrate, to chelate Ca^{2+} and so prevent reinfection of transductants with virulent P1. Each mixture was then added to 2.5ml of Difco-top-agar and plated on the appropriate selective medium. In certain cases selection cannot be applied immediately, e.g. if phage- or drug-resistance requires time to be expressed. Candidate transductants were purified and characterised.

(x) In vitro packaging of λ DNA (D. Ish-Horowitz, pers. comm.).

All solutions and packaging mixtures were kindly prepared and donated by Dr D.W. Meek; therefore only a brief description of each will be included. Solutions and mixtures included the following: Buffer A (20mM Tris/HCl pH7.5, 3mM $MgCl_2$, 0.05% (w/v) β-mercaptoethanol, 1mM EDTA pH7.0): Buffer M1 (consisting of 6μl 0.5M Tris/HCl pH7.5; 300μl 50mM spermidine, 100mM putrescine neutralised with tris base; 9μl 1mM $MgCl_2$; 75μl 0.1M ATP neutralised with NH_4OH; 1μl β-mercaptoethanol; and dH_2O to 500μl): SE - sonicated extract of BHB2690 met$^-$ (λimm434 cI^{ts}62 red3 Dam15 Sam7)λ^R: and FTL - freeze/thaw lysate of BHB2688 met$^-$

(λ<u>imm</u>434 <u>c</u>I^{ts}62 <u>red</u>3 <u>E</u>am4 <u>S</u>am7)λ^{R}.

For the packaging reaction 7μl Buffer A, 5μl DNA (>200ng), 1μl Buffer M1, 6μl <u>SE</u> and 10μl <u>FTL</u> were mixed in that order, and incubated at 25°C for 1 hour. Following addition of 0.5ml phage buffer, 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated on the appropriate cells (prepared as in 2.4(i)a) to obtain single plaques.

2.5 Bacterial techniques

(i) Thymine^{-} selection (Miller, 1972).

0.02ml of an overnight culture was incubated at 37°C with 1ml of minimal medium containing trimethoprim at 200μg/ml. After 1-2 days at 37°C drug-resistant candidates were streaked on a nutrient plate containing thymine, and then screened on minimal medium plates for a Thymine^{-} phenotype.

(ii) Interrupted mating.

The donor culture was grown overnight in oxoid broth (an antibiotic can be included here, but not in the following subcultures), diluted 20-fold the next day in prewarmed oxoid broth, and grown to an O.D. 650nm of 0.3 to ensure optimal fertility. The recipient strain was prepared in the same way as the donor, unless a homosexual cross was intended. In this case an overnight culture, grown at 37°C in 10ml of oxoid broth with good aeration, was pelleted at 6,000 rpm at 20°C for 10 minutes and the pellet resuspended in 1 ml of cell buffer. After at least 1 hour at 20°C the recipient was ready. To mate (for both homosexual and normal crosses) 0.5ml of the donor and 0.5ml of the recipient were mixed and incubated at 37°C without shaking. At the required interruption time, 0.2ml of the mixture was diluted into

1.8ml of cell buffer. 0.2ml of the undiluted, and 0.1ml of the diluted mixture were then each mixed vigorously for 15 seconds with 3ml of Difco-top-agar to separate the mating pairs, and poured onto selective minimal plates for growth of recombinants. Positive and negative controls, plated in the same way, were also included.

(iii) Competent cells and transformaton.

a) $CaCl_2$ procedure (suitable for plasmid DNA). An overnight culture grown at 37°C in L-broth was diluted 50-fold in L-broth and grown to an O.D. 650nm of 0.6. After standing in ice for 20 minutes the cells were pelleted at 6,000 rpm for 10 minutes at 20°C. The pellet was washed with 100mM $MgCl_2$, and then resuspended in 0.3 volumes of 100mM $CaCl_2$. After 30 minutes on ice, the cells were pelleted as before and finally resuspended in 0.05 volumes of 100mM $CaCl_2$. Following 30 minutes on ice the cells were ready for transformation.

In the transformation step up to 40ng of plasmid DNA were mixed with 200μl of competent cells, left on ice for 30 minutes to allow non-specific DNA adsorption, then heat-shocked at 42°C for 2-5 minutes (to promote DNA uptake). The tubes were left on ice briefly. 1ml of L-broth was then added, and the tubes incubated at 37°C for 1 hour to allow expression of antibiotic resistance, and recovery of the bacteria. 200μl aliquots were then plated on selective medium. This technique routinely gave 10^5 transformants/μg of plasmid DNA.

b) $CaCl_2$ procedure (suitable for M13 DNA transformation). JM101 was grown from a single colony at 37°C, with gentle agitation (to avoid loss of pili) to an O.D. 650nm of 0.5. The cells were then pelleted at 4,000 rpm at 20°C for 10 minutes and resuspended carefully, to protect the pili, in 0.5 volumes of 100mM $MgCl_2$. After repelleting immediately,

the cells were resuspended in 0.5 volumes of 100mM $CaCl_2$ and left on ice for 20 minutes. The cells were again pelleted, resuspended in 0.05 volumes of 100mM $CaCl_2$, and after 1 hour on ice were ready for transformation.

Transformation: ≤100ng of M13 DNA were mixed with 200µl competent cells and incubated at 0°C for 30 minutes. After heat-shock for 2-5 minutes at 42°C the mixture was left at 0°C for a further 30 minutes. To plate, 0.5ml of a JM101 culture grown to an O.D. 650nm of 0.5, to provide a lawn, and 3ml BBL-top-agar were added, mixed, and poured into a tube containing 20µl IPTG (Isopropylthiogalactoside, 25mg/ml in dH_2O) and 30µl X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside, 25mg/ml in dimethylformamide). This mixture was poured onto a BBL plate and incubated at 37°C for 12-16 hours. Vector DNA alone gave blue coloured plaques, while recombinants gave colourless plaques. By this method 10^6 plaques/µg of unrestricted vector, and 10^4 plaques/µg of restricted/ligated vector, could reliably be expected.

2.6 Plasmid techniques

(i) Birnboim preparation of small amounts of plasmid DNA (Birnboim and Doly, 1979).

A single colony was picked and grown overnight in L-broth plus an antibiotic (where relevant) at 37°C. 1.5ml of this culture, in an Eppendorf tube, was spun for 2 minutes in a microfuge. The pellet was resuspended by vortex-mixing in 100µl of lysis solution (25mM Tris/HCl pH8.0, 10mM EDTA pH8.0, 50mM glucose, lysozyme 2mg/ml) and left on ice for 10 minutes. 200µl of alkaline SDS solution (0.2M NaOH, 1% (w/v) SDS) were then added, the tube inverted twice, and incubated on ice for a further 5 minutes to selectively denature the chromosomal DNA.

After the addition of 150μl of "high salt" solution (3M CH_3COONa pH4.8) the tube was mixed thoroughly, and left on ice for 60 minutes with occasional shaking. Most ribosomal RNA and protein was pelleted, together with the aggregated, denatured chromosomal DNA, by centrifugation for 5 minutes. 400μl of the supernatant were then decanted into a fresh tube, 1ml of ethanol was added, and after inverting 3 times the mixture was left at -70°C for 20 minutes. After centrifugation for 2 minutes, nucleic acids were resuspended in 100μl 0.1M CH_3COONa pH6.0 by brief vortex-mixing. Following ethanol addition, nucleic acids were precipitated at -70°C and pelleted as before. The final pellet was dried in a vacuum desiccator for 20 minutes, and resuspended in 25μl TE. 3-5μg of DNA were routinely recovered, and could be used for restriction analysis (see 2.8(ii)) and cloning (see 2.8(iii)). This technique was also applied to isolate small amounts of M13 RF (double-stranded replicative form) DNA essentially as described above, except that the overnight culture contained a fresh plaque of M13 and a single colony of JM101 picked into 5ml of L-broth.

(ii) Midi preparations of plasmid DNA (Birnboim and Doly, 1979).
This is essentially a modified Birnboim small-scale preparation (see 2.6(i)). An overnight culture in 10-40ml L-broth was harvested by sedimentation at 10,000 rpm for 5 minutes at 20°C in Corex tubes. The supernatant was discarded and the cells resuspended in 1ml of lysis solution (see 2.6(i)) and left on ice for 5 minutes. 1.5ml of high salt solution (see 2.6(i)) was added, mixed gently by inversion, and left for a further 60 minutes on ice. The tube was spun at 10,000 rpm for 10 minutes at 4°C, and the supernatant transferred to a fresh 15ml Corex tube. 8ml of cold ethanol were added and the tube placed at -70°C for 15 minutes. After pelleting (10,000 rpm for 5 minutes at 4°C) the

supernatant was discarded and the pellet resuspended in 2ml TE. 2ml of phenol (equilibrated with TE) were added, vortex-mixed, and then spun at 10,000 rpm for 2 minutes at room temperature. The aqueous phase was removed into a fresh Corex tube, 2 volumes of cold ethanol were added, and the tube left at -20°C for 15 minutes. After centrifugation (10,000 rpm for 5 minutes at 4°C) the pellet was redissolved in 0.4ml of TE, transferred to an Eppendorf tube, 60µl of 1M CH_3COONa pH8.0 and 1ml cold ethanol added, and left at -20°C for 10 minutes. After centrifugation for 2 minutes the pellet was redissolved in 0.2ml of TE, 30µl 1M CH_3COONa pH8.0 and 0.5ml cold ethanol added, and the tube again incubated at -20°C as before. After centrifugation as before the pellet was redissolved in 0.2ml TE and 5µl of 10mg/ml RNaseA (heat-treated at 95°C for 15 minutes) added to destroy RNA. Following incubation at 37°C for 1 hour 7.5µl of 4M CH_3COONa pH6.0 and 0.3ml of ethanol were added and the tube left at room temperature for 10 minutes. Finally the pellet, after centrifugation, was dissolved in TE. Yields of up to 100µg plasmid DNA/10ml culture could be obtained.

(iii) Large scale preparation of plasmid DNA.

A single colony was inoculated into 200ml L-broth (plus an antibiotic where relevant) and grown at 37°C overnight with aeration. Cells were harvested (6,000 rpm at 4°C for 10 minutes) and washed in 0.5 volumes TE. The pellet was resuspended in 3ml of sucrose solution (25% (w/v) sucrose, 50mM Tris/HCl pH8.0) prior to the addition of 1 ml of lysozyme solution (lysozyme 10mg/ml in dH_2O). After 5 minutes on ice with frequent mixing, 1ml of 0.5M EDTA pH8.5 and 0.4ml RNaseA (5mg/ml in dH_2O, heat-treated as described in 2.6(ii)) were added, mixed, and left on ice for a further 5 minutes. Following the addition of 5ml triton mixture (0.1% (w/v) Triton X-100, 64.5mM EDTA pH8.5, 50mM Tris/HCl pH8.0), and subsequent

incubation for 10 minutes on ice, the mixture was cleared by centrifugation at 27,000g at 4°C for 30 minutes. 9g of CsCl and 0.9ml of ethidium bromide (EtBr:5mg/ml) were added to the supernatant. After mixing, this solution was transferred to a Beckman 16 x 76mm tube and spun at 90,000g for ≥48 hours at 18°C. The tube was viewed under long wave ultraviolet light and the plasmid band (the lower band) removed through the side of the tube with a syringe and 19G needle. The solution was dialysed against TE at 4°C for 1 hour to remove CsCl, phenol extracted 3 times in Eppendorf tubes to remove residual protein and EtBr, and dialysed further against TE at 4°C, with several changes of TE over a 24 hour period, to remove all traces of phenol. The DNA was ethanol precipitated (see 2.8(i)) and finally dissolved in TE. DNA purity and concentration were checked by UV 260/280nm absorbance and/or minigel analysis.

The technique described above was adapted, with a few modifications, to the isolation of large scale preparations of M13 RF (double-stranded DNA). The day before this procedure a fresh M13 plaque (full of infected viable cells) was inoculated into 1ml of L-broth and grown with slow shaking at 37°C overnight. A 5ml L-broth culture of JM101 was also grown in the same way. The next day the JM101 culture was diluted 20-fold in L-broth and grown with slow aeration at 37°C to an O.D. 650nm of 0.5. The dilution step was repeated in 200ml of L-broth and when the correct O.D. was reached 200µl of the overnight plaque growth was inoculated and the flask left at 37°C with slow aeration for a further 4 hours. The M13 DNA was then isolated as described above.

2.7 Gel electrophoresis

(i) Agarose minigels.

1% (w/v) agarose solutions were prepared by dissolving the appropriate

weight of agarose in TAE buffer (40mM Tris-acetate pH8.3, 33mM CH_3COONa, 1mM EDTA pH8.0) and heating for 10 minutes in a Sharp Carousel microwave oven. When cool (60°C) the solution was poured onto a 5 x 6.5 x 0.2cm perspex plate with a 6-tooth slot-former, and allowed to set. 1µl of loading buffer (0.5 x TAE, 0.125M EDTA, 0.1% (w/v) Bromophenol Blue, 50% (w/v) glycerol) was added to each 10µl DNA sample, mixed, and then transferred to the gel slots using a Gilson micropipette. The gel was supported between two reservoirs containing 1 x TAE. Wicks of Jay cloth were hung at either end of the gel, forming electrical contacts between the reservoirs and the gel. 50V was applied to the gel, and the samples allowed to "run in" for 5 minutes. The gel was then soaked with TAE, covered with Saran wrap, and run (50V) until the Bromophenol Blue dye had reached the other (anode) end (usually 1 hour). The gel was stained in 5µg/ml ethidium bromide for a few minutes, washed briefly with water, and the DNA bands visualised by long wave UV (Ultraviolet Products Inc., Chromatovue C-62, 365nm peak transmission). Photography was on Ilford HP5 film using a 25 second exposure through a red filter. This technique is useful for checking that DNA digestions are complete, testing the quality of DNA, and estimating its concentration.

(ii) Horizontal agarose gels (McDonnell _et al._, 1977).

Agarose gels (0.8 - 2% (w/v) agarose, depending on the size range of fragments to be analysed) were prepared by heating 200ml of TAE buffer and the correct weight of agarose in the microwave oven as above, but for 20 minutes. After cooling to 60°C the gel solution was poured into a 30 x 14 x 0.5cm perspex mould. A 13- to 25-tooth slot-former was set in position, and the gel allowed to set. The gel was placed between reservoirs as described previously (see 2.7(i)). The DNA

samples (<30µl, mixed with 5µl of agarose loading buffer, see 2.7(i)) were loaded into the gel slots with a micropipette. The gel was run for 10 minutes at 120V, then wetted thoroughly with TAE buffer, and covered with Saran wrap. After running at 100V for 12-16 hours the gel was stained in 5µg/ml ethidium bromide for 30 minutes, destained in water for 10 minutes, and the DNA bands visualised under UV as above (see 2.7(i)).

(iii) Vertical gel electrophoresis.

a) Polyacrylamide gel electrophoresis of DNA. The stock solutions used were the following: 1) 10 x TBE buffer (0.89M Tris-borate pH8.3, 0.89M boric acid, 0.02M EDTA pH8.0); 2) 30% acrylamide (29% (w/v) acrylamide, 1% (w/v) bisacrylamide); 3) 10% (w/v) ammonium persulphate (AMPS) in dH_2O.

These solutions can be used to prepare acrylamide gels of various percentages, suitable for different DNA fragment size ranges. The quantities for the preparation of a 5% polyacrylamide gel are given below, as this was most commonly used. A 60ml solution containing 9.94ml 30% acrylamide, 6ml 10 x TBE, 43.32ml dH_2O and 750µl AMPS was mixed in a conical flask and degassed using a vacuum pump. 37.5µl of TEMED (N,N,N',N'-tetramethylethylenediamine, Serva) were added, the flask swirled gently, and the gel solution pipetted into a 20 x 14 x 0.1cm glass sandwich, separated by perspex spacers, which had been sealed around the edges with molten water agar. Water saturated butan-2-ol was overlaid and the gel left to polymerise at room temperature over 1-2 hours. When set the butan-2-ol was removed and the gel surface rinsed with 1 x TBE. Then a 3½% acrylamide stacking gel solution (containing 1.67ml 30% acrylamide, 2ml 10 x TBE, 16ml dH_2O and 330µl AMPS, which had been degassed and 10µl TEMED added) was overlaid.

A 13- or 15-tooth slot-former was inserted, and the gel left to polymerise (usually 30 minutes). The bottom spacer and the slot-former were removed, the sandwich attached to a perspex gel kit, and the bottom and top reservoirs filled with 1 x TBE. 20µl DNA samples containing 5µl TBE loading dye (0.5 x TBE, 0.125M EDTA pH8.0, 0.1% (w/v) Bromophenol Blue, 50% (v/v) glycerol) were loaded into the preformed slots with either a micropipette or a Hamilton microsyringe. The gel was run at 80V for 12-16 hours, until the Bromophenol Blue dye had reached the bottom (the dye runs like DNA of ≅ 65bp on a 5% gel). The stacking gel was discarded, and the gel stained in 5µg/ml ethidium bromide for 30 minutes, destained in water for 10 minutes, and the DNA bands visualised from overhead with a shortwave UV lamp (Mineral-light C5, 254nm peak transmission), against a black background. Photography was as described in 2.7(i), except that 1 minute exposures were preferred.

b) SDS polyacrylamide gel electrophoresis (Laemmli, 1970). Stock solutions used were as follows: 1) A + BA (29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide); 2) 4 x Lower Tris (1.5M Tris/HCl pH8.8, 0.4% (w/v) SDS); 3) 10% (w/v) SDS; 4) 4 x Upper Tris (0.5M Tris/HCl pH6.8, 0.4% (w/v) SDS); 5) 4 x Tris-glycine reservoir buffer (12% (w/v) Tris base, 57.6% (w/v) glycine, brought to pH8.6 with concentrated NH_4OH); 6) 10% (w/v) AMPS in dH_2O.

For 5% SDS polyacrylamide gels; 22.4ml dH_2O, 10ml 4 x Lower Tris, 6.8ml A + BA and 60µl AMPS were mixed in a conical flask and degassed with a vacuum pump. 20µl TEMED were added, the flask swirled gently, and the mixture pipetted into a 23 x 14 x 0.08cm glass sandwich, with perspex spacers, and the edges sealed with molten water agar. After polymerisation for 1-3 hours at room temperature under a butan-2-ol

overlay (water saturated) the butan-2-ol was removed and the gel surface washed with 1 x Upper Tris buffer, several times. (The gel may alternatively be left overnight at this point, if overlaid with 1 x Lower Tris buffer.) 3% stacking gel mixture (12.92ml dH_2O, 5ml 4 x Upper Tris, 2ml A + BA, 80µl AMPS, degassed, then 40µl TEMED added) was pipetted into the sandwich, and a 13-tooth slot-former inserted. When polymerisation was complete (about 1 hour) both the bottom spacer and the slot-former were removed, and the sandwich attached to a perspex vertical gel kit. The upper and lower reservoirs were filled with 1 x running buffer (250ml 4 x Tris-glycine buffer, 10ml 10% (w/v) SDS, and dH_2O to 1 litre). Protein samples (see 2.10) were loaded in 20-30µl with a Hamilton microsyringe, and the gel run at 80V until stacking was completed. Gels were then run at 100V until the Bromophenol Blue dye reached the bottom (12-16 hours). The gel was separated from the plates, the stacking gel discarded and the gel fixed in 45% (v/v) methanol, 9% (v/v) acetic acid for 15 minutes at 37°C. After staining in the same fixative plus 0.1% (v/v) Coomassie blue for 15 minutes at 37°C, the gel was destained in 7% (v/v) acetic acid, 5% (v/v) methanol at 37°C, with several changes over 1-2 hours. Following a brief wash the gel was transferred to blotting paper, covered on top with Saran wrap, and dried for 1½ hours on a Biorad gel drier, with heating and under vacuum. The dried gel was autoradiographed at room temperature with no intensifier screen, using X-ray film (DuPont, Cronex 4). [^{35}S]-labelled protein bands were visualised after 12-60 hours exposure.

For 5-15% SDS polyacrylamide gradient gels the same stock solutions were used. Two 15ml solutions, 5% and 15% in acrylamide, contained the following: 2.5ml or 7.5ml A + BA, 3.75ml 4 x Lower Tris, 10µl AMPS and dH_2O to 15ml. Both solutions were degassed in a conical

flask, and then 10μl TEMED added. The solutions were added into a 2-chamber linear gradient maker, which was then used to pour a 5-15% gradient into a glass sandwich of the same dimensions, and prepared in the same way as above. The gradient was overlaid with butan-2-ol and allowed to polymerise (usually 2 hours). The butan-2-ol was removed and the gel surface washed with 1 x Upper Tris buffer. A 3% stacking gel and 13-tooth slot-former were placed in the sandwich as described previously. Protein samples were then loaded, electrophoresed and visualised by autoradiography as described for 5% gels.

c) Buffer-gradient denaturing polyacrylamide gel electrophoresis (Biggin *et al.*, 1983). This gel system was routinely used in DNA sequence analysis. Stock solutions included: 1) 40% acrylamide (380g acrylamide, 20g bisacrylamide and dH_2O to 1 litre); 2) 10 x TBE (see 2.7(iii)); 3) 0.5 x TBE gel mix (150ml 40% acrylamide, 50ml 10 x TBE, 460g urea and dH_2O to 1 litre); 4) 2.5 x TBE gel mix (150ml 40% acrylamide, 250ml 10 x TBE, 460g urea, 50g sucrose, 50mg Bromophenol Blue and dH_2O to 1 litre); 5) 25% (w/v) AMPS in dH_2O.

For each gel two beakers were prepared containing 7ml 2.5 x TBE gel mix, 14μl AMPS and 14μl TEMED in one; and 35ml 0.5 x TBE gel mix, 70μl AMPS and 70μl TEMED in the other. First, using a 10ml pipette provided with a Pumpette, 4ml of 0.5 x TBE gel solution were taken up, followed by 6ml of 2.5 x TBE gel solution in the same pipette. The buffer gradient was set up by passing 3-4 air bubbles up the pipette through the interface. The mixture was then poured carefully down one edge of a 40 x 20 x 0.04cm glass sandwich, with Plastikard spacers and sealed with Sellotape, lowering the sandwich to the horizontal when necessary to stop the flow. The rest of the gel space was topped up with 0.5 x TBE gel solution, quickly added with a syringe across the

top of the gel. A 32-tooth slot-former was inserted, and the gel allowed to polymerise for 30 minutes. When polymerisation was complete the bottom tape and slot-former were removed, and the gel attached to a perspex vertical gel kit. 1 x TBE was added to the top and bottom reservoirs, and the slots thoroughly washed out with a pasteur pipette. DNA sequencing reaction products (see 2.11) were then loaded into the slots with a drawn out glass capillary, and the gel run at a constant 40 watts until the Bromophenol Blue dye reached the bottom (≅ 2 hours). In order to obtain sequence information >250bp from the primer initiation site, the gels (or some earlier-loaded samples) were run for longer than 2 hours. The gel was then removed from the glass plates, fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 15 minutes, drained well, placed on blotting paper, and covered on top with Saran wrap. The gel was then dried on a Biorad gel drier with heating, under vacuum for 45 minutes, and autoradiographed at room temperature with no intensifier screen, using X-ray film (DuPont, Cronex 4). Sequences could normally be read after 12-60 hours exposure.

This rapid method of generating a gradient in a pipette is rough, but perfectly adequate. The system has the advantage, over normal sequencing gels, that 250bp of sequence can be analysed in a single 2 hour run because of fractionation through a greater length of gel.

2.8 DNA techniques

(i) Ethanol precipitation.

DNA or RNA was precipitated, in Eppendorf or Corex tubes, by adding 0.1 volumes of 3M sodium acetate (pH4.8) and 2 volumes of ethanol and leaving at -70°C for ≥30 minutes. The nucleic acid was pelleted by centrifugation (5 minutes in a microfuge at 4°C; or, if Corex tubes

were used, 10 minutes at 12,000g at 4°C). The pellet was dried by vacuum desiccation, and redissolved in TE buffer.

(ii) Restriction endonuclease digestion.

A list of restriction enzymes used in the course of this work is given in Table 2.3. The reaction conditions used for most of these type II restriction endonucleases were identical. Normally DNA was digested in a total volume of 15-50μl, containing 0.1 volumes of 10 x "Universal" restriction buffer (33mM Tris-acetate pH7.9, 10mM Mg-acetate, 66mM K-acetate, 5mM DTT, 1mg/ml BSA (high quality from BCL: Boehringer Corporation Ltd)), and 0.9 volumes of DNA dissolved in TE. The reactions were incubated at 37°C for the required time, then stopped by heating the digest at 70°C for 10 minutes, followed by rapid cooling on ice. The two exceptions, during this work, were *Sma*I digestions, which contained 0.1 volumes of special 10 x *Sma*I buffer (200mM KCl, 10mM Tris/HCl pH8.0, 10mM $MgCl_2$, 1mM DTT); and *Tha*I digestions, which were performed at 60°C with 0.1 volumes of special 10 x *Tha*I buffer (100mM Tris/HCl pH7.4, 100mM $MgCl_2$, 1mM DTT). In the latter case the reaction was stopped by phenol extraction.

(iii) DNA ligation.

For ligation of cohesive-ended restriction endonuclease fragments, a typical reaction contained: DNA, 10-25μg/ml (final concentration); 2μl 10 x ligation buffer (660mM Tris/HCl pH7.2, 10mM EDTA pH8.0, 100mM $MgCl_2$, 10mM ATP, 100mM DTT); 2μl T4 DNA ligase (8 Weiss units in 1 x ligation buffer); and dH_2O to a final volume of 20μl. The reaction was incubated at 12°C for 4-16 hours. Blunt-ended restriction endonuclease fragments were ligated in the same way, except that 80 Weiss units of T4 DNA ligase were used and DNA concentrations were 100-200μg/ml.

Table 2.3 Restriction endonucleases

Endonuclease	Bacterium of Origin	Sequence Specificity	Source
AccI	*Acinetobacter calcoaceticus*	GT↓(AT/CG)AC	P.L. Biochemicals
BamHI	*Bacillus amyloliquefaciens* H	G↓GATCC	BCL (Boehringer Corporation Ltd)
BglII	*Bacillus globigii*	A↓GATCT	BCL (Boehringer Corporation Ltd)
EcoRI	*Escherichia coli* RY13	G↓AATTC	BCL (Boehringer Corporation Ltd)
HaeIII	*Haemophilus aegyptius*	GG↓CC	BRL (Bethesda Research Laboratories)
HindIII	*Haemophilus influenzae* Rd	A↓AGCTT	BCL (Boehringer Corporation Ltd)
NarI	*Norcardia argentinensis*	GG↓CGCC	New England Biolabs
PstI	*Providencia stuartii* 164	CTGCA↓G	BCL (Boehringer Corporation Ltd)
PvuII	*Proteus vulgaris*	CAG↓CTG	BCL (Boehringer Corporation Ltd)
SalI	*Streptomyces albus* G	G↓TCGAC	Amersham or BCL (Boehringer Corp. Ltd)
SmaI	*Serratia marcescens* S_b	CCC↓GGG	BCL (Boehringer Corporation Ltd)
ThaI	*Thermoplasma acidophilum*	CG↓CG	BRL (Bethesda Research Laboratories)

(iv) [^{32}P]-labelling of DNA by nick translation (Maniatis et al., 1975; Rigby et al., 1977).

10μCi of α-^{32}P-dCTP were dried by vacuum desiccation, washed once with 20μl dH_2O and redried (to remove ethanol), then resuspended in 20μl of a solution containing 52.5mM Tris/HCl pH7.5, 5.25mM $MgCl_2$, 20μMdATP, 20μMdGTP, 20μMdTTP, 5μg/ml gelatin and 10mM β-mercaptoethanol (stored at -20°C). To start the reaction 1μl of DNaseI (0.01μg/ml), 1μl of E. coli DNA polymerase I (10 units/μl, supplied by Northumbrian Biochemicals Ltd) and 1μg of DNA were added, and incubated at 15°C for 1½-2 hours. The reaction was stopped by adding 200μl TE. The labelled DNA was separated from the unincorporated [^{32}P]-dCTP by passing the products through a Sephadex G-50 column (15 x 0.75cm). 0.5ml fractions were collected, and the first peak (labelled DNA) identified by Cerenkov counting in a scintillation counter. Labelled DNA was stored frozen, at -20°C.

(v) Plaque detection by blotting (Benton and Davis, 1977).

"Plaques" were arranged in a grid formation on a BBL plate (with a preformed top-layer carrying the indicator strain) and allowed to grow overnight. (The plates should be very dry, so that the top-layer adheres well to the bottom agar.) The next day a nitrocellulose disc (82mm x 0.2μM Membranfilter, from Shleicher and Schüll) was placed carefully over the top-layer, and allowed to become wet. The filter was removed, placed on a pad of blotting paper soaked in 0.5M NaOH (to denature the DNA), and left for 5 minutes (the surface that had been in contact with the plaques being uppermost). The filter was transferred successively to petri dishes containing the following solutions, and left for 20 seconds in each: 1) 0.1M NaOH, 1.5M NaCl; 2) 0.5M Tris/HCl pH7.5, 1.5M NaCl; 3) 0.5M Tris/HCl pH7.5, 1.5M NaCl;

4) 2 x SSC (0.3M NaCl, 30mM tri-sodium citrate dihydrate). The filter was then air-dried at 37°C for 20-30 minutes, and baked at 80°C under vacuum for 2 hours. The filter was now ready for hybridisation. The filter was prehybridised by sealing it in a plastic bag containing 20ml of hybridisation buffer (4 x SSC, 50% (v/v) formamide, 0.02% (w/v) gelatin, 0.1% (w/v) SDS, 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinyl-pynrolidone) and 50μg/ml denatured Salmon Sperm DNA, and swirled at 37°C for 1 hour. [^{32}P]-labelled DNA probe (see 2.8(iv)), denatured by heating at 100°C for 2-3 minutes, was added and the filter left to hybridise at 37°C as before, but for 12-16 hours. The filter was next washed with 2 x SSC, 0.1% (w/v) SDS over 1-2 hours at 37°C, changing the wash several times. The filters were dried at 37°C, placed on blotting paper, covered in Saran wrap and autoradiographed at -70°C using flash-sensitised X-ray film (DuPont, Cronex 4) and an intensifier screen. 12-48 hour exposures were usually sufficient.

(vi) Southern transfer for detection of DNA or DNA/RNA hybrids (Smith and Summers, 1980).

The agarose gel (see 2.7(ii)) was shaken very slowly for 15 minutes in 0.25M HCl. After a 10 minute wash in dH_2O the gel was placed in denaturation solution (0.5M NaOH, 1.5M NaCl) and left for 30 minutes. Further washing, as before, was followed by gentle agitation of the gel in 1M NH_4CH_3COO, 0.02M NaOH for 1 hour. The gel was then laid on a glass plate and a sheet of nitrocellulose, cut to the correct dimensions and pre-soaked in the ammonium acetate/NaOH solution for a few minutes, was placed on top of it. 30-40 layers of blotting paper were next placed on top of the nitrocellulose (the first few sheets being soaked in the same ammonium acetate/NaOH solution). A glass plate and heavy weight completed the sandwich. After leaving for a few hours, or overnight, the

nitrocellulose was removed, rinsed briefly in 2 x SSC, dried at 37°C and finally baked at 80°C for 2 hours under vacuum. To detect bands the filter was treated as described in 2.8(v).

2.9 RNA techniques

(i) RNA isolation (Shaw and Guest, 1982).

Typically, 500ml cultures were grown to an O.D. 650nm of 0.5 in L-broth. For rifampicin treatment (described in Chapter 5) the cultures were grown for a further 10 or 25 minutes, at 30°C or 37°C, in the presence or absence of rifampicin (10µg/ml). For the valyl-tRNA limitation experiments the isogenic $valS^{+}$ and valS(Ts) strains, grown at 30°C, were shifted to 38°C or 42°C and grown for a further 10 or 25 minutes. The cells were then harvested by centrifugation (6,000 rpm for 10 minutes at 4°C) and washed in 0.1 volumes of TE. After resuspension in 6.5ml 10mM KCl, 5mM $MgCl_2$, 10mM Tris/HCl pH7.3, 2mg of lysozyme was added, and the cells frozen at -70°C for 30 minutes, then thawed. Following the addition of 0.9ml 10% (w/v) SDS, and incubation at 64°C until the turbidity dropped (usually 5-10 minutes), 0.33ml of 2.5M sodium acetate, pH5.2 was added (the solution goes cloudy). The solution was then extracted with 1 volume of H_2O-saturated phenol, by shaking at 64°C for 4 minutes. After 10 minutes centrifugation at full speed in a bench centrifuge (MSE) the aqueous layer was removed and phenol-extracted again, as above. The aqueous layer was then added to a sterile bottle contining 1g of heat-sterilised solid NaCl, mixed, and spun for 10 minutes in a bench centrifuge. The supernatant was pipetted into Corex tubes, 1.5 volumes of ethanol added, and left at -20°C for 1 hour. The nucleic acid precipitate was collected by centrifugation (10,000 rpm for 10 minutes at 4°C), and the pellet washed

3 times with 70% (v/v) ethanol, 10mM Tris/HCl pH7.5, 10mM NaCl. It was then dried under vacuum, and finally redissolved in 0.5ml of dH_2O. Routinely 500ml of cells yielded 20-30mg of RNA (as judged by UV spectrophotometry, O.D. 260/280nm).

(ii) SI mapping of transcripts (Shaw and Guest, 1982).

1.5mg of total cellular RNA was hybridised with excess (25µg) M13 single-stranded DNA (see 2.4(vii)) by mixing with 10µl hybridisation buffer (0.1M Tris/HCl pH8.0, 1.5M NaCl, 0.1M $MgCl_2$, 1mM EDTA pH8.0), and dH_2O to 0.1ml, in Eppendorf tubes. Following incubation at 68°C for 1 hour hybrids were allowed to form by leaving the tubes to cool down to room temperature in the same waterbath, over 2-3 hours. 1ml of ethanol was added, and the hybrids precipitated at -70°C overnight. Following centrifugation in a microfuge for 10 minutes, the pellet was dried in a vacuum desiccator and finally dissolved in 10µl hybridisation buffer, 1µl 3M sodium acetate pH4.5, 1µl 0.1M $ZnSO_4$ and 88µl dH_2O. 300 units of SI-nuclease (3µl of Boehringer Corporation Ltd enzyme) were added, and digestion carried out at 37°C for the appropriate incubation time. 1ml of ethanol was then added, and the SI-resistant hybrids were precipitated at -70°C for 1 hour. They were then pelleted for 10 minutes in a microfuge, and the pellet was washed twice with 80% (v/v) ethanol, 1mM EDTA pH8.0 and finally dried by vacuum desiccation. After dissolving in 25-30µl TE and 3µl agarose loading dye (see 2.7(i)) the hybrids were analysed on 1.5-2.0% (w/v) agarose gels (see 2.7(ii)). Following ethidium bromide staining and UV-visualisation, hybrids were transferred to nitrocellulose (see 2.8(vi)), probed with [^{32}P]-labelled DNA (see 2.8(v)) and autoradiographed at -70°C with flash-sensitised X-ray film (Du-Pont, Cronex 4) and an intensifier screen. Protected transcripts could be visualised after 16-72 hour exposure.

2.10 Protein techniques (protein labelling)

Bacterial cultures to be labelled with L-[^{35}S]-methionine (Amersham) were grown overnight in Spizizen minimal medium, supplemented with the required amino acids, yeast extract (for unknown, unk, requirements) and antibiotics where relevant, at 30°C (if lysogens or ts-strains used) or 37°C with aeration. The following day the culture was diluted 50-fold in the same medium (prewarmed), and grown to an O.D. 650nm of 0.5 (approx. 3 x 10^8 cells/ml). 5 x 10^7 bacteria (166µl) were dispensed into an Eppendorf tube, 20µCi of [^{35}S]-methionine added, and the cells incubated at 30°C or 37°C for 4 minutes. After "chasing" with 17µl of unlabelled methionine (2mg/ml), the tube was chilled in ice and 0.02 volumes of 1M sodium azide added. The sample was mixed (then could be kept in ice until all samples were ready to spin), and next pelleted in a microfuge for 6 minutes at 4°C. The supernatant was decanted and the pellet washed in 1ml of Matzura buffer (0.0625M Tris/HCl pH6.8-7.4 (not critical), 0.1mM DTT, 200µg/ml methionine - stored frozen at -20°C). The pellet was resuspended in 10µl of ice-cold Matzura buffer and then 50µl SDS sample buffer added (per 100ml: 58ml 5%(w/v) SDS, 20ml glycerol, 5ml β-mercaptoethanol, 12.5ml 4 x Upper Tris buffer (see 2.7(iii)b), dH_2O to 100ml) by vortexing. After heating for 3 minutes at 100°C samples could be stored for some weeks at -70°C, or analysed directly by SDS polyacrylamide gel electrophoresis (see 2.7(iii)b). 1µl aliquots of lysates were counted in 3ml solubilising scintillant (Petri, 1972: 1.44g PPO, 40.4mg dimethyl POPOP, 3.6ml 1% (w/v) SDS, 40ml Soluene (Packard), 360ml toluene), in a Packard scintillation counter, to allow loading of a known and equal number of "total protein" counts from each sample on the gel.

2.11 M13 ddNTP-based DNA sequencing

All sequencing was carried out on DNA cloned in M13-mp10 and -mp11 (Messing and Vieira, 1982) using a recent modification of Sanger's dideoxynucleotide approach (Biggin et al., 1983).

Necessary solutions included: 1) TM (100mM Tris/HCl pH8.5, 50mM $MgCl_2$); 2) Primer (17-mer, 500pM/ml, from New England Biolabs); 3) dNTP chase (0.25M each of dTTP, dCTP, dGTP, dATP); 4) Formamide dyes (100ml deionised formamide, 0.1g Xylene cyanol FF, 0.1g Bromophenol Blue, 2ml 0.5M EDTA pH8.5); 5) T,C,G and A sequencing reaction mixtures (see Table 2.4); 6) "Klenow mix" (10mM Tris/HCl pH8.5, 10mM DTT, 4μCi $[^{35}S]$-dATP (New England Nuclear), 1.5 units of DNA polymerase I-Klenow large fragment (Boehringer, Amersham, or P.L. Biochemicals), dH_2O to 8μl). 8μl of Klenow mix is enough for 4 sequence reactions.

5μl of M13 single-stranded template (see 2.4(vii)) was mixed with 5μl of primer mix (3μl 17-mer primer, 1μl TM, 1μl dH_2O) in an Eppendorf tube and left at 60°C to hybridise for 1 hour. After cooling (and a quick microfuge spin) sequencing reactions were set up in Eppendorf tubes, as described in Table 2.5. After incubation of the sequencing reactions at room temperature for 20 minutes, 2μl of the "dNTP chase" were added and the reactions incubated at room temperature for a further 20 minutes. Products could be stored overnight at 0°C at this point. When ready to analyse 2μl of the formamide dyes were added, the tubes heated at 100°C for 5 minutes, and the sequencing reactions loaded on a buffer-gradient denaturing polyacrylamide gel as described in 2.7(iii)c.

Table 2.4 T,C,G and A sequencing reaction mixes

	T*	C*	G*	A*
0.5mM dTTP	25µl	500µl	500µl	500µl
0.5mM dCTP	500µl	25µl	500µl	500µl
0.5mM dGTP	500µl	500µl	25µl	500µl
10mM ddTTP	50µl	-	-	-
10mM ddCTP	-	8µl	-	-
10mM ddGTP	-	-	16µl	-
10mM ddATP	-	-	-	1µl
TE	1000µl	1000µl	1000µl	500µl

Table 2.5 Sequencing reactions

	T	C	G	A
†Template/Primer mix	2μl	2μl	2μl	2μl
#T*	2μl	-	-	-
#C*	-	2μl	-	-
#G*	-	-	2μl	-
#A*	-	-	-	2μl
†Klenow mix	2μl	2μl	2μl	2μl

† - see text for details

- see Table 2.4

Footnote: All 2μl aliquots were dispensed onto the side of an Eppendorf tube, the "Klenow mix" being kept on ice and dispensed last. The reactions were started by spinning briefly in a microfuge, to bring all additives to the bottom of the tube.

CHAPTER 3

Sequence analysis of a DNA fragment carrying the rplLrpoB intercistronic region and portions of the flanking genes: implications for the nature of the rif^d18 mutation

3.1 Introduction

In *Escherichia coli* all mutations producing resistance to the antibiotic rifampicin map within the *rpoB* gene (Matzura, 1980). It has been observed that resistant alleles can be either dominant (rif^d) or recessive (rif^r) to the wild-type (rifampicin sensitive: rif^s): i.e. strains heterodiploid for wild-type and resistant alleles will grow in the presence of the drug only if the resistance character is dominant. Spontaneous rif^r mutations occur at a frequency of $10^{-7}/10^{-8}$, whereas rif^d occur at a 100-fold lower frequency (Kirschbaum and Konrad, 1973). To explain the basis of the recessive nature of rif^r it has been proposed that, in the heterodiploid, the drug-inactivated sensitive RNA polymerase molecules bind unproductively to promoters sufficiently long enough to severely limit access by the drug-resistant (rif^r) polymerase (Ilyina *et al.*, 1971; Bordier, 1974). The evidence for this model came from *in vitro* studies of transcription and of the binding of RNA polymerase to DNA templates. Hayward later corroborated this model by exploiting a mutation ($rif^{s-rcs40}$) which produces a *recessive* sensitive phenotype: $rif^{s-rcs40}/rif^r$ heterodiploids are drug-resistant. He showed that the RNA polymerase extracted from the haploid rcs-40 strain produced a less severe drug-dependent blockade of promoters *in vitro*, in experiments similar to the above (Hayward, 1976). This effect was associated with the core polymerase; and the rcs-40 mutation mapped in or near *rpoBC*.

As a consequence of this model it can be predicted that dominant-resistance mutations (rif^{d}) produce a resistant R-pol better able than "rif^{r}" enzyme to compete for promoters in the presence of the drug-inactivated sensitive R-pol. However, this appears not to be the case for at least one example of dominance mutations (rif^{d}18: Kirschbaum and Konrad, 1973) as judged by *in vitro* studies using rif^{r}, rif^{d}18, and wild-type enzymes (Smith, 1982). If this finding can be extrapolated to the *in vivo* situation (which may not be possible since, for example, a protein necessary to allow "rif^{d}18" enzyme to overcome blockade could be missing from the *in vitro* system) then how does "rif^{d}18" R-pol overcome sensitivity? One possibility is that more than one mutation is involved in the basis of the phenotype, with one mutation conferring rifampicin resistance and a second, regulatory mutation increasing the proportion of rifampicin resistant versus sensitive R-pol in heterodiploids (without necessarily affecting the total number of enzyme molecules). Since rif^{d} alleles appear only 100-fold less frequently than rif^{r}, it is unlikely that *all* are produced by double mutations; but this does not exclude the possibility for rif^{d}18. However, Kirschbaum observed that extracts of rif^{d}18/rif^{s} heterodiploids appeared to contain comparable levels of the sensitive and resistant R-pol (Kirschbaum and Konrad, 1973). In contrast, when 50% pure R-pol from a λrif^{d}18 lysogen of a rif^{s} strain was examined, it appeared to consist mainly of resistant enzyme (Schweitzer and Matzura, 1977). A possible resolution of this paradox is suggested by the observation that purified rif^{d}18 R-pol was more sensitive to rifampicin *in vitro* than a rif^{r} (rpoB70) R-pol (Boyd *et al.*, 1974; Smith, 1982). Hence perhaps it is difficult to estimate the ratio of rif^{d}18 to rif^{s} enzyme, in crude extracts of the heterodiploid strain, by the method used by Kirschbaum; an overestimation

of the rif^{s} R-pol would be likely. Tittawella has shown that rif^{r}/rif^{s} heterodiploids which contain equal levels of the 2 R-pols can give rise to mutants which have a higher ratio of the rif^{r} enzyme, as judged by RNA pulse labelling and studies on crude extracts (Khesin et al., 1971; Tittawella, 1981; 1985). The fact that such mutations can arise lends weight to the hypothesis that, at least in the case of certain rif^{d} alleles, dominance can be explained in terms of two mutations; one conferring rif^{r}, and the second increasing the proportion of the rif^{r} enzyme in the R-pol pool of the heterodiploid. It seems important to establish whether or not this is true for rif^{d}18, since λrif^{d}18 has been the source of DNA for many of the regulatory studies of the rplKAJLrpoBC operon.

Attempts to map the rif^{d} mutation(s) carried by λrif^{d}18 (the "rif^{d}18 genotype) and in another specialised transducing phage, λrif^{d}47 (Mindlin et al., 1976) have placed the dominance mutation within the 2.87kb EcoRI fragment completely internal to rpoB (Yamamoto and Nomura, 1978; Collins, 1979; Ovchinnikov et al., 1981a; 1983: see Fig. 3.1). The only difference discovered between λrif^{d}18 and wild-type DNA in this 2.87kb fragment is the rpoB3 mutation (TTC encoding phe; replacing TCC encoding ser) at nucleotide 4561; similarly the only reported difference between λrif^{d}47 and wild-type DNA is the rpoB255 mutation (GTC encodes val, replacing GAC for asp) at 4516 (Ovchinnikov et al., 1983: and see Fig. 3.1). Ovchinnikov et al. suggest that the single rpoB3 mutation is responsible for the rif^{d}18 genotype, and similarly that rpoB255 is solely responsible for the dominant resistance carried by λrif^{d}47. However, genetic evidence suggests that dominance and resistance of the rif^{d}18 mutation can be separated (Newman and Hayward, 1980). Specifically, Rec-mediated integration of a λ-derivative, λAJN172 (carrying the HindIII 'rplJLrpoBC fragment of λrif^{d}18) into the chromosomal rpoBC

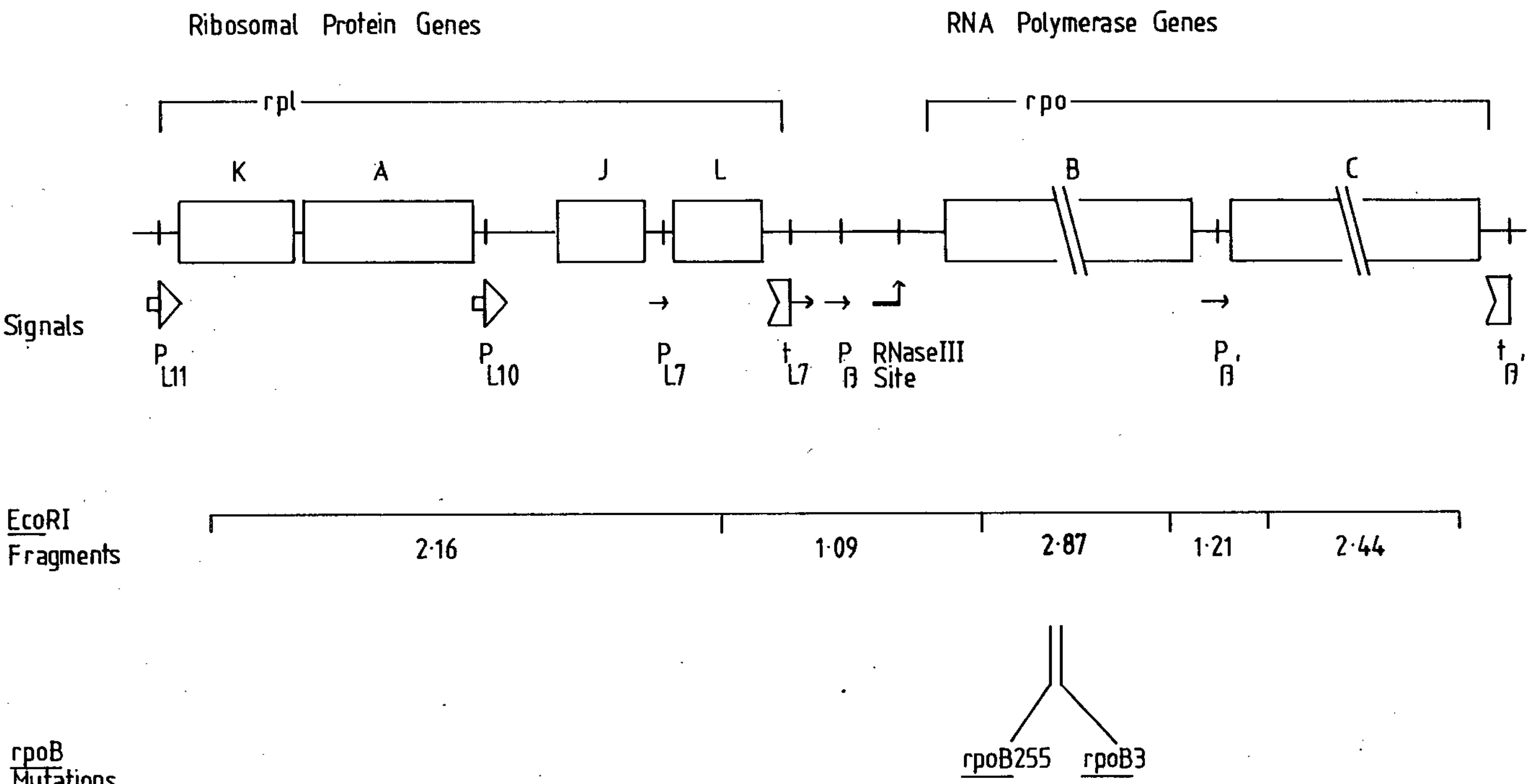


Fig.3.1

Fig. 3.1 A map of the *rplKAJLrpoBC* operon of *E. coli* K12. The diagram indicates the position of the genes, signals (strong promoter, ⟶ weak promoter, partial terminator, strong terminator, RNaseIII processing site), *Eco*RI restriction targets, and the map positions of two *rif*^{d} mutations, *rpoB*255 (*rif*^{d}47) and *rpoB*3 (*rif*^{d}18), on the scale of Post *et al.* (1979) as corrected by Delcuve *et al.* (1980) and the present work. This is an adaption of the scale of Gurevich *et al.* (1980) and Ovchinnikov *et al.* (1981b; 1982) to include the whole operon.

operon of a wild-type (rif^{s}) strain, followed by illegitimate excision, yielded a phage, λAJN261, which had inherited the P_{L10} promoter from the chromosomal operon (see Fig. 3.2). Interestingly λAJN261 had retained the ability to confer resistance to rifampicin, but had lost the dominance attribute associated with λrif^{d}18 (see Table 3.1). This suggests that the initial recombination of λAJN172 into the rpoB^{+} chromosome, at a crossover point which could lie anywhere between the HindIII target in rplJ and the rpoB3 mutation, had separated rpoB3 from a mutation(s) upstream necessary for dominance. Admittedly the alternative hypothesis, that expression of rpoB3 from λAJN261 is anomalous (e.g. because it depends on the normally occluded P_{L10}) cannot yet be excluded. Assuming that there is a second mutation, candidates include a structural mutation in the N-terminus of rpoB, or more intriguingly a regulatory mutation which increases the relative expression of the rif^{r} enzyme in heterodiploids: e.g. a mutation in the transcriptional terminator t_{L7} might increase readthrough of transcription. Other explanations are possible: for example, in the genetic manipulations giving rise to λAJN261 it might have lost the rpoB3 mutation and acquired a different rif^{r} mutation. Alternatively, the crossover that generated λAJN261 removed rpoB3, but left a second structural mutation of rpoB (present in λrif^{d}18, but in the distal end of rpoB) which confers a rif^{r} genotype. These points will be discussed later in this chapter, and in Chapter 4.

The nucleotide sequence of wild-type DNA, as previously mentioned, has been reported only for the 2.87kb EcoRI fragment of rpoB (Ovchinnikov et al., 1983: see Fig. 3.1). However, extensive sequencing studies have been performed on DNA of the entire operon, derived from λrif^{d}18 (Post et al., 1979; Delcuve et al., 1980; Squires et al., 1981) and λrif^{d}47

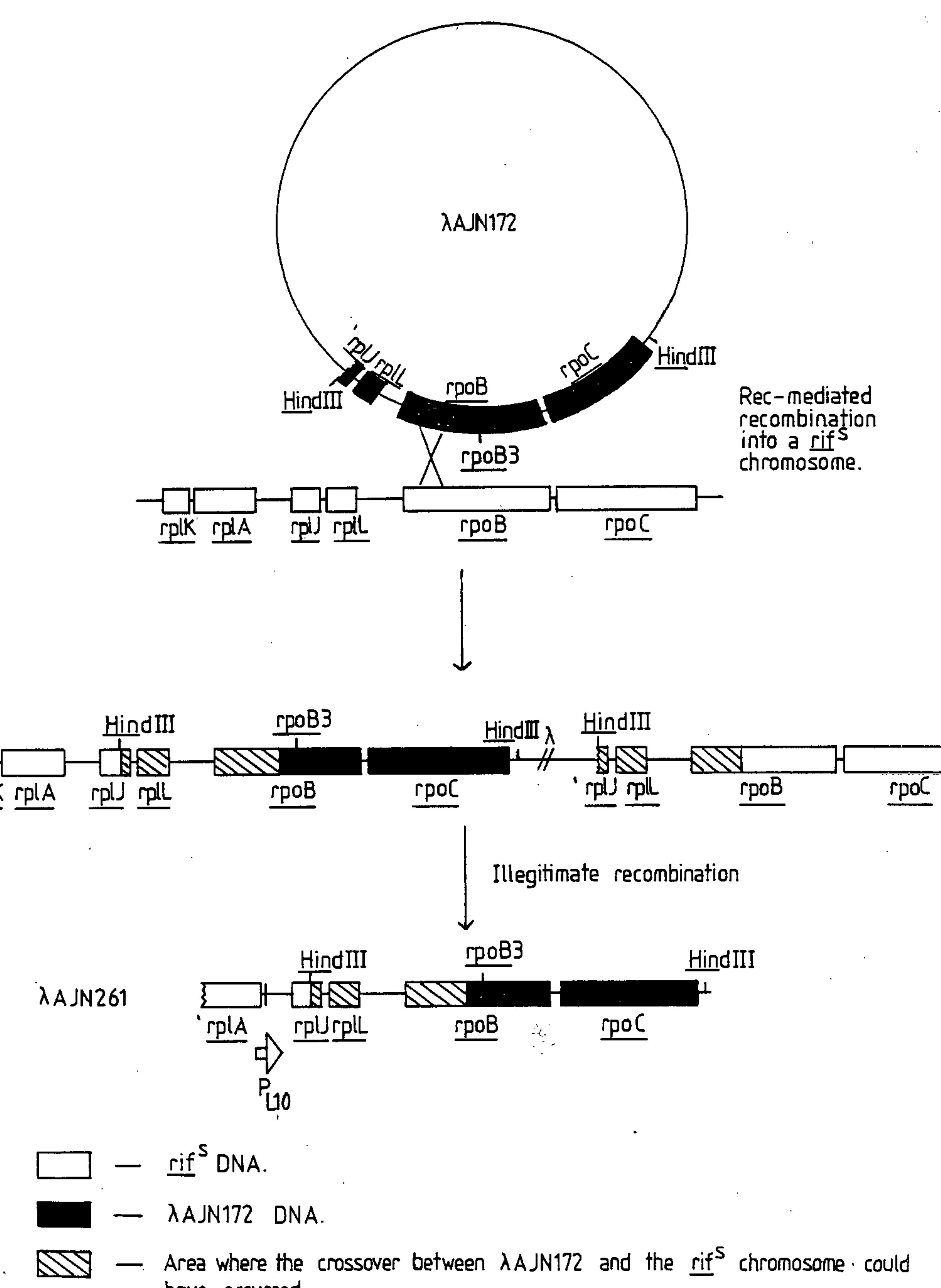


Fig. 3.2

Fig. 3.2 This diagram represents the most likely series of events which occurred to generate λAJN261, which contains the functional promoter P_{L10} and carries the *rpoB*3 mutation. The original phage λAJN172 carried the 10.14 kb *Hind*III fragment of λ*rif*^{d}18, which contains most of the operon but no strong promoter.

Table 3.1

Host / Phage	AJN10	AJN1
λ*rif*^{d}18	Rif-R	Rif-R
λAJN261	Rif-S	Rif-R

This table shows the phenotypes of lysogenic derivatives, either λ*rif*^{d}18 or λAJN261, of *E. coli* K12 strains AJN10 (*rif*^{s}) amd AJN1 (*rif*$^{s\text{-}rcs40}$) in response to the antibiotic rifampicin. Rif-R denotes resistance, Rif-S sensitivity.

(Gurevich et al., 1980; Ovchinnikov et al., 1981b; 1982). Interestingly, comparisons between the reported λ*rif*^{d}18 and λ*rif*^{d}47 DNA sequences in the 1.09kb *Eco*RI fragment, carrying the '*rplL* - t_{L7} - *rpoB*' region (Fig. 3.1), reveals a number of conflicts, including a surprising deletion of AGC in the 5' coding region of *rpoB* from λ*rif*^{d}18, as compared with λ*rif*^{d}47. More interestingly, the "tail" sequences of the t_{L7} terminator differ between these two alleles (Fig. 3.3). This latter finding lends credibility to the suggestion that a mutation in the terminator could be the second regulatory mutation conferring dominance to *rif*^{d}18 (see earlier).

To clear up these conflicts I have sequenced the 1.09kb *Eco*RI DNA fragments derived from λ*rif*^{d}18 and λ*rif*^{d}47. In addition I have sequenced the 1.09kb fragment of wild-type DNA, not only to extend the limits of wild-type nucleotide sequence already reported, but also to allow comparison of the wild-type sequence with those of λ*rif*^{d}18 and λ*rif*^{d}47 in this region. The 1.09kb fragment of λAJN261 was also sequenced, in the hope of identifying the putative second mutation of λ*rif*^{d}18. Additionally, the appropriate region of the 2.87kb *Eco*RI fragment was sequenced to confirm or deny the presence of the "ser → phe" *rpoB*3 mutation.

3.2 Determination of the nucleotide sequence of the wild-type 1.09kb *Eco*RI fragment

The source of wild-type DNA for this analysis, λAJN63, was isolated by Dr A.J. Newman from a library of *Hin*dIII fragments of *E. coli* K12 strain CR63, cloned in NMλ761 (kindly provided by Dr N.E. Murray). Restriction analysis by Newman confirmed that the 1.09kb *Eco*RI fragment carrying '*rplL* - t_{L7} - *rpoB*' was present on this phage (Newman, 1980).

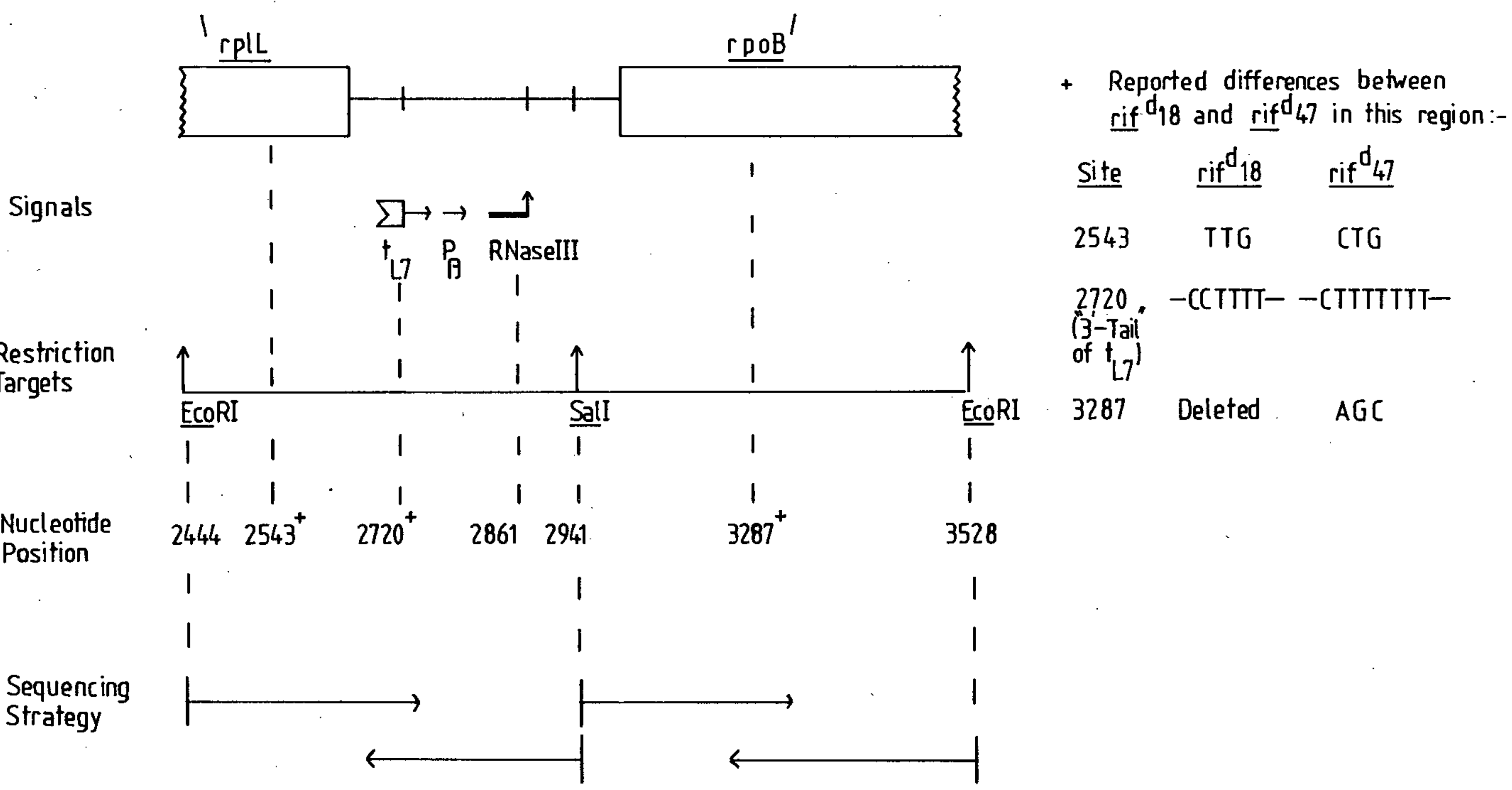


Fig.3.3

Fig. 3.3 A map of the intercistronic region between *rplL* and *rpoB*. Shown are most of the relevant features, including conflicts in the published sequencing analyses (Post *et al.*, 1979; Delcuve *et al.*, 1980; Gurevich *et al.*, 1980; Ovchinnikov *et al.* 1981); and the sequencing strategy adopted for my work.

In order to sequence (on both strands) the regions of the 1.09kb EcoRI fragment reported to differ between λ*rif*^{d}18 and λ*rif*^{d}47, λAJN63 was completely digested with the restriction endonucleases EcoRI and SalI, and the resultant restriction fragments were sub-cloned into the M13 sequencing vectors mp10 and mp11 (Messing and Vieira, 1982), digested with the same two enzymes. (The sequencing strategy for the 1.09kb EcoRI fragment is outlined in Fig. 3.3.) Following transformation into JM101, potential recombinants were identified by their colourless plaque phenotype (see 2.5(iii)b). These were picked and purified, then replated in an ordered array on a lawn of JM101. Screening by plaque hybridisation was then required to identify the appropriate recombinants, partly because when M13 preparations are self-ligated deletions are generated which give rise to colourless plaques, and partly because digestion of λAJN63 with EcoRI and SalI generates a number of fragments irrelevant to the sequencing study. Further, to distinguish between the two EcoRI/SalI fragments required the plasmids outlined in Fig. 3.4 were used as probes. M13 recombinants carrying the "t_{L7}" EcoRI/SalI insert hybridised to both [^{32}P]-pHR11 and [^{32}P]-pNA26, while M13 recombinants carrying the "*rpoB*" SalI/EcoRI insert hybridised *only* to [^{32}P]-pNA26. pHR11 is a derivative of pHR1800 (Newman *et al.*, 1982) carrying a 336bp AluI fragment (with HindIII linkers) which includes the 3' end of *rplL*, t_{L7}, the RNaseIII processing site, and P_{β} (see Fig. 3.4). It was constructed and kindly donated by Dr J-C. Ma. pNA26 is a derivative of pSF2124 (So *et al.*, 1976) carrying the 1.09kb EcoRI fragment of λ*rif*^{d}18 (see Fig. 3.4); it was constructed and kindly provided by Dr A. Nicolaidis.

All the DNA sequencing data presented below were obtained using a recent modification of Sanger's dideoxynucleotide approach (Biggin *et al.*,

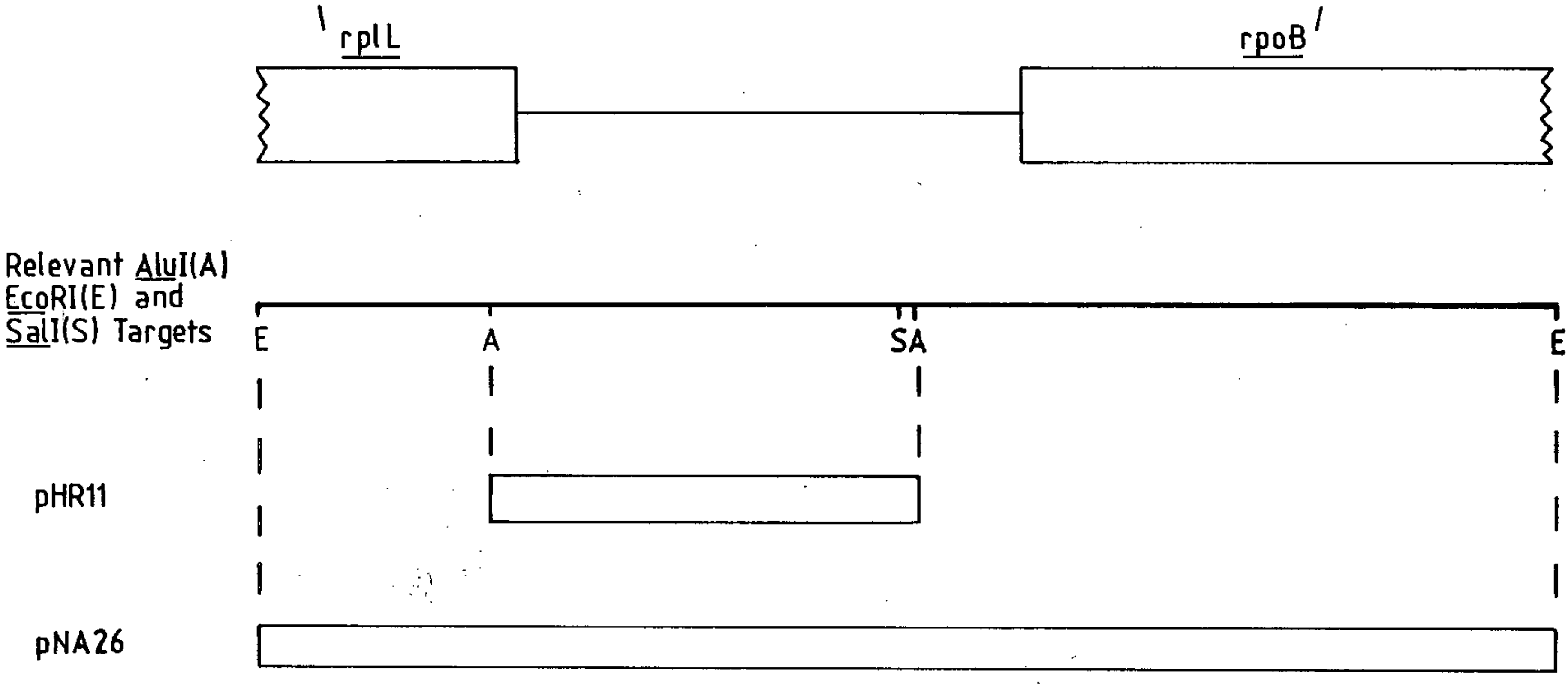


Fig. 3.4

Fig. 3.4 The extent of operon DNA contained in the plasmids pNA26 and pHR11 (Newman *et al.*, 1982).

1983; see 2.11). To confirm that the correct M13 recombinants had been identified by the hybridisation strategy single-stranded DNA preparations (see 2.4(vii)) from the "positives" were first used as templates in a "T-screen" sequencing reaction. This entails using each template in a single base-specific reaction (in this case ddTTP) and then size-fractionating the product by buffer-gradient polyacrylamide gel electrophoresis (see 2.7(iii)c). In this way a pattern characteristic of each template, representing the position of every T in the sequence, is obtained. Comparisons of the T-patterns of the DNA inserts in the selected M13 with those of the published $rif^{d}18$ nucleotide sequence (Post et al., 1979; Delcuve et al., 1980) confirmed that the hybridisation strategy had been successful. The correct clones were then fully analysed to generate a 1.09kb wild-type DNA sequence (determined on at least one strand) extending from the EcoRI site at 2444 to the EcoRI site at 3533 (Fig. 3.5). (For all nucleotide positions, from this point onwards, I use the nucleotide scale of Post et al. (1979), as corrected by Delcuve et al. (1980) and adjusted for two other discrepancies discussed below.) As shown in Fig. 3.5 the sequence was analysed on both strands between 2645 and 2731, and from 3175 to 3318. My main conclusion is that wild-type and $\lambda rif^{d}18$ sequences are identical throughout this 1.09kb region, except for three discrepancies demonstrated in Fig. 3.6: 1) As can be seen, and as confirmed on both strands, the wild-type terminator t_{L7} sequence is identical to that of $\lambda rif^{d}18$, except that 5 rather than 4 successive T residues are present in the DNA corresponding to the "3'-tail" of the mRNA terminated at t_{L7}, specifically at nucleotide 2716 (Barry et al., 1980; see Fig. 3.6A). 2) Wild-type DNA does not have a deletion of AGC at nucleotides 3286-3288 (see Fig. 3.6B). 3) C, not T occurs at position 2543; thus the leucine residue determined here is encoded by CUG rather than UUG (see Fig. 3.6C).

```
2460 2480 2500 2520 2540 (i) 2560
GAATTCGACGTAATTCTGAAAGCTGCTGGCGCTAACAAAGTTGCTGTTATCAAAGCAGTACGTGGCGCAACTGGCCTGGGTCTGAAAGAAGCTAAAGACCTGGTAGAATCTGCACCGGCT

2580 2600 2620 2640 (ii) 2660 2680
GCTCTGAAAGAAGGCGTGAGCAAAGACGACGCAGAAGCACTGAAAAAAGCTCTGGAAGAAGCTGGCGCTGAAGTTGAAGTTAAATAAGCCAACCCTTCCGGTTGCAGCCTGAGAAATCAG
TTTATTCGGTTGGGAAGGCCAACGTCGGACTCTTTAGTC

2700 (iii) 2720 2740 2760 2780 2800
GCTGATGGCTGGTGACTTTTTAGTCACCAGCCTTTTTGCGCTGTAAGGCGCCAGTAGCGTTTCACACTGTTTGACTACTGCTGTGCCTTTCAATGCTTGTTTCTATCGACGACTTAATAT
CGACTACCGACCACTGAAAAATCAGTGGTCGGAAAAACGCGACATTCC

2820 2840 (iv) 2860 2880 2900 2920
ACTGCGACAGGACGTCCGTTCTGTGTAAATCGCAATGAAATGGTTTAAGCGTGATAGCAACAGGCATTGCGGAAAGTGTTCCATTTTCCGGTCAACAAAATAGTGTTGCACAAACTGTCC

2940 (v) 2960 (vi) 2980 3000 3020 3040
GCTCAATGGACAGATGGGTCGACTTGTCAGCGAGCTGAGGAACCCTATGGTTTACTCCTATACCGAGAAAAAACGTATTCGTAAGGATTTTGGTAAACGTCCACAAGTTCTGGATGTACC

3060 3080 3100 3120 3140 3160
TTATCTCCTTTCTATCCAGCTTGACTCGTTTCAGAAATTTATCGAGCAAGATCCTGAAGGGCAGTATGGTCTGGAAGCTGCTTTCCGTTCCGTATTCCCGATTCAGAGCTACAGCGGTAA

3180 3200 3220 3240 3260 3280
TTCCGAGCTGCAATACGTCAGCTACCGCCTTGGCGAACCGGTGTTTGACGTCCAGGAATGTCAAATCCGTGGCGTGACCTATTCCGCACCGCTGCGCGTTAAACTGCGTCTGGTGATCTA
TTATGCAGTCGATGGCGGAACCGCTTGGCCACAAACTGCAGGTCCTTACAGTTTAGGCACCGCACTGGATAAGGCGTGGCGACGCGCAATTTGACGCAGACCACTAGAT

(vii) 3300 3320 3340 3360 3380 3400
TGAGCGCGAAGCGCCGGAAGGCACCGTAAAAGACATTAAAGAACAAGAAGTCTACATGGGCGAAATTCCGCTCATGACAGACAACGGTACCTTTGTTATCAACGGTACTGAGCGTGTTAT
ACTCGCGCTTCGCGGCCTTCCGTGGCATTTTCTGT

3420 3440 3460 3480 3500 3520
CGTTTCCCAGCTGCACCGTAGTCCGGGCGTCTTCTTTGACTCCGACAAAGGTAAAACCCACTCTTCGGGTAAAGTGCTGTATAACGCGCGTATCATCCCTTACCGTGGTTCCTGGCTGGA

CTTCGAATTC
```

Fig. 3.5

Fig. 3.5 The nucleotide sequence of 'rplL-rpoB' of the E. coli K12 derivative CR63. The sequence was determined on at least one strand (presented here as the coding strand) between the EcoRI site at 2444 (rplL) and the EcoRI site at 3533 (rpoB); and on both strands from 2645 to 2731, and from 3175 to 3318. The scale adopted is that of Post et al. (1979) as corrected by Delcuve et al. (1980) and the present study. Features indicated are as follows:

(i) It is reported that TTG encodes leucine in $\lambda rif^{d}18$.

(ii) The translational stop codon of rplL.

(iii) The "3'-tail" of t_{L7}.

(iv) The approximate position of RNaseIII processing.

(v) The SalI restriction endonuclease site.

(vi) The translational start codon of rpoB.

(vii) The reported AGC deletion in $\lambda rif^{d}18$.

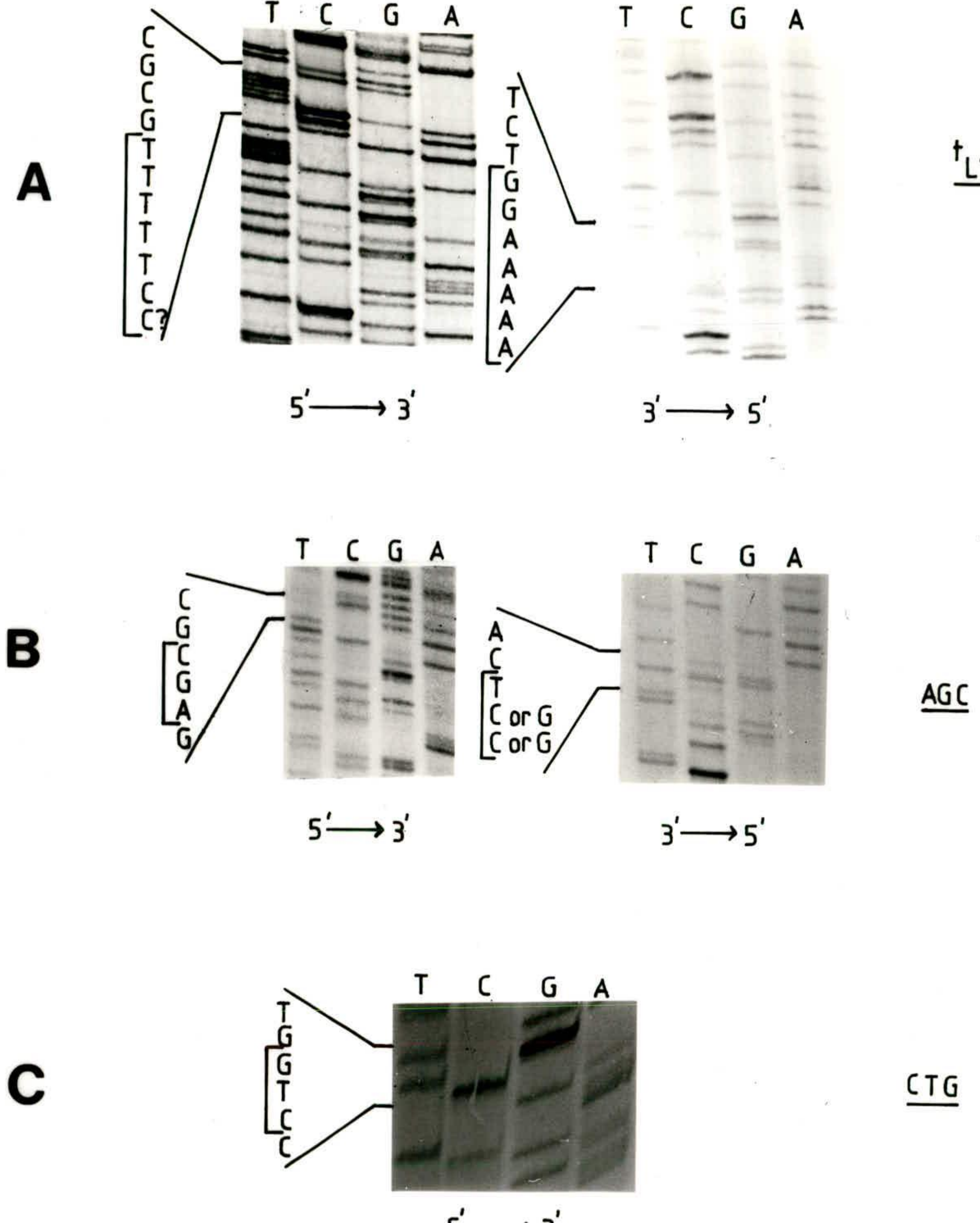


Fig. 3.6

Fig. 3.6 A sequencing analysis of DNA derived from the *E. coli* K12 strain CR63 at the following sites within the *rplKAJLrpoBC* operon: A) The "3'-tail" region of t_{L7}. B) The reported AGC deletion in *rpoB* of λ*rif*^{d}18 (Delcuve *et al.*, 1980). C) The TTG encoding leucine in *rplL* of λ*rif*^{d}18 (Post *et al.*, 1979).

Notes:

1 The direction of the sequencing analysis with respect to the sense strand of the operon is indicated. Hence the regions described in A and B are presented on both DNA strands while C is only on the sense strand.

2 The regions of interest in A, B and C are indicated by a vertical bar.

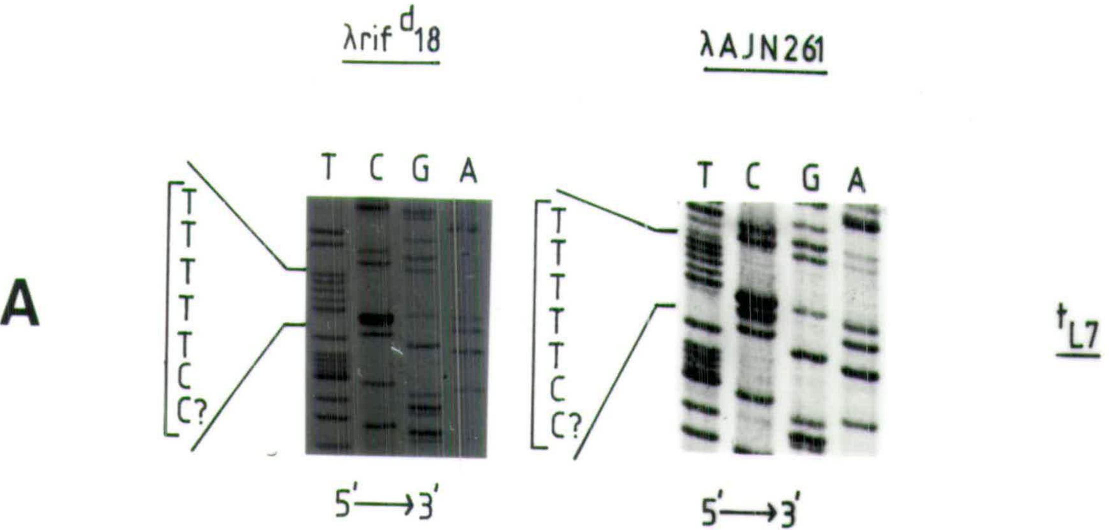
3 Ambiguities in the sequence are indicated by a question mark. Note that where these occur (in A and B) the problem is resolved by analysis of the opposite DNA strand.

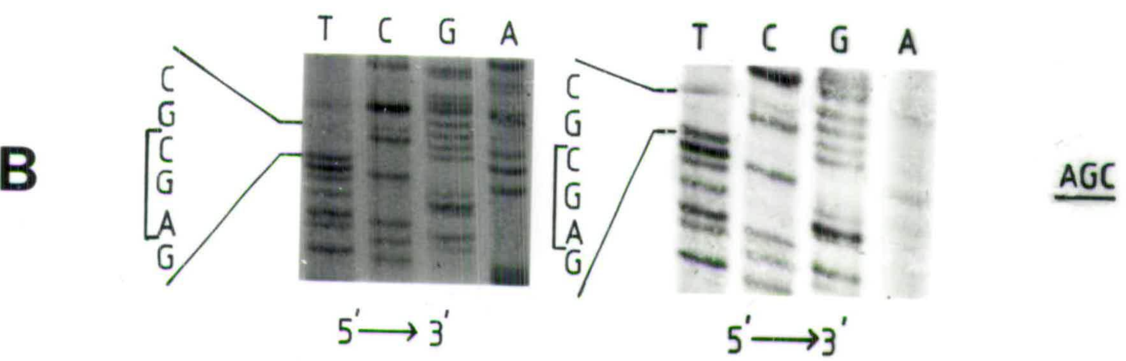
3.3 Determination of the nucleotide sequence of the 1.09kb *EcoRI* fragments derived from λ*rif*^{d}18 and λAJN261

The differences highlighted above, between the wild-type sequence and that reported for λ*rif*^{d}18, were intriguing. Hence a sequencing analysis similar to that described above was performed on λ*rif*^{d}18, to confirm the reported sequence; and on λAJN261, hopefully to demonstrate the presence of some wild-type sequence absent from λ*rif*^{d}18.

An M13 cloning strategy similar to that adopted for the wild-type DNA analysis was used. After extensive analysis the DNA sequence of the whole 1.09kb *EcoRI* fragment was obtained on at least one strand for both λ*rif*^{d}18 and λAJN261; and for both strands between the following limits: λ*rif*^{d}18 DNA, 2534 to 2836 and 3200 to 3348; λAJN261 DNA, 2659 to 2785 and 3188 to 3291. The results demonstrated that the λ*rif*^{d}18 and λAJN261 sequences are identical to wild-type throughout the 1.09kb *EcoRI* fragment, *including* the sites of the three reported discrepancies presented above (see Fig. 3.7). Even in regions where "compressions" or "pile-ups" engender possible ambiguities in reading the sequence, the band patterns from all three sources are so identical as to convince me that there could be no hidden differences. Note also that two independent preparations of λ*rif*^{d}18 were sub-cloned and sequenced in the critical regions; and that the batch of λAJN261 DNA used was repackaged *in vitro* (see 2.4(x)) and shown to produce the same rifampicin phenotype as bona fide λAJN261, when lysogenised in the strains AJN10 and AJN1 (cf. Table 3.1).

In order to explain the difference between the Rif phenotypes of λ*rif*^{d}18 and λAJN261 lysogens, one possibility raised in the introduction to this chapter proposed that the *rif*^{r} mutation present in λAJN261 is not *rpoB*3. This seemed unlikely, but had not been rigorously disproved.





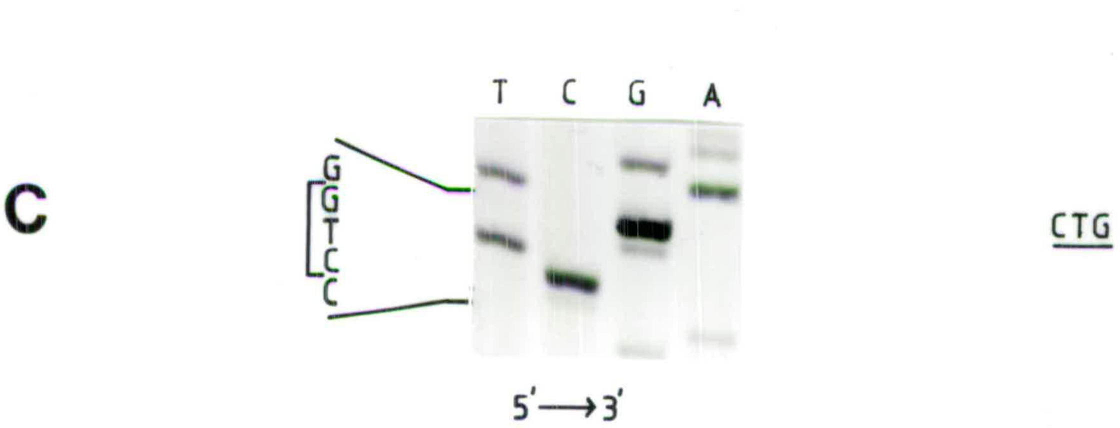


Fig. 3.7

Fig. 3.7 A sequencing analysis of DNA derived from λ*rif*^{d}18 and λAJN261 at the following sites within the *rplKAJLrpoBC* operon: A) The "3'-tail" region of t_{L7}. B) The reported AGC deletion in *rpoB* of λ*rif*^{d}18 (Delcuve *et al.*, 1980). C) The TTG encoding leucine in *rplL* of λ*rif*^{d}18 (Post *et al.*, 1979).

Notes:

1 The direction of the sequencing analysis with respect to the sense strand of the operon is indicated.

2 The regions of interest in A, B and C are indicated by a vertical bar.

3 Ambiguities in the sequence are indicated by a question mark. Note that analysis of the opposite DNA strand (data not shown) has resolved these difficulties and indicated that the interpretation presented is correct.

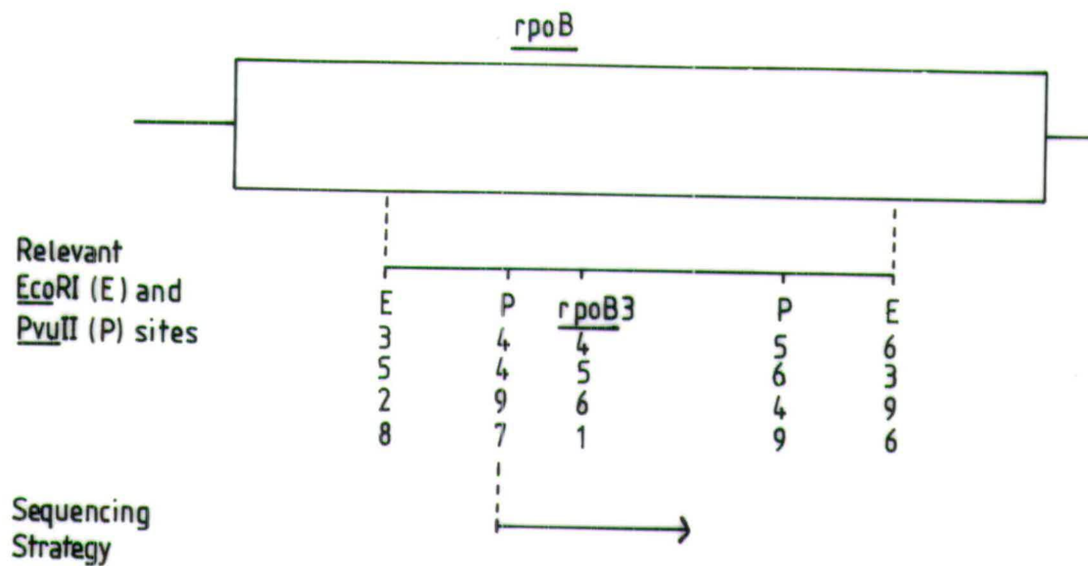
4 The C-track pile-up in the t_{L7} region of λ*rif*^{d}18 and λAJN261 (see A) immediately preceding the "3'-tail" appears different in the two cases. However, analysis of the opposite DNA strand in both cases (data not shown) has indicated that both sequences are identical to each other and the published DNA sequence of λ*rif*^{d}18 (Post *et al.*, 1979).

5 The DNA sequence of the leucine codon in *rplL* (see C) is presented only for λ*rif*^{d}18 and on one DNA strand. However the presence of CTG has been confirmed on the opposite DNA strand for λ*rif*^{d}18 (data not shown) and on one strand (the sense strand) for λAJN261 (data not shown).

To investigate this proposal I decided to sequence the appropriate region of λAJN261. To achieve this λAJN261 was completely digested with the restriction endonuclease PvuII, and the resultant restriction fragments were ligated with the M13 vector mp10 after it had been digested with the restriction enzyme SmaI. Clones carrying PvuII inserts internal to the 2.87kb EcoRI fragment (which includes the site of rpoB3) were identified by plaque hybridisation with [^{32}P]-pNA60. pNA60 is a derivative of pSF2124 (So et al., 1976) carrying the 2.87kb EcoRI fragment (internal to rpoB, see Fig. 3.1) of λrif^{d}18; it was constructed and kindly donated by Dr A. Nicolaidis. The required PvuII insert spanning rpoB3 was further identified by "T-screening" (see 3.2). Sequence analysis of this clone proved that λAJN261 is identical in sequence to wild-type DNA (Ovchinnikov et al., 1983) between nucleotides 4497 and 4824 (as determined on one strand, only); except that, as expected, it retains the rpoB3 mutation of λrif^{d}18 at position 4560-4562 (TCC → TTC substitution: see Fig. 3.8).

3.4 Determination of the nucleotide sequence of the terminator, t_{L7}, of λrif^{d}47

λrif^{d}47, the specialised transducing phage carrying another dominant rifampicin resistance mutation (rpoB255), has been reported to have the nucleotide sequence C_1T_7 in the "3'-tail" of the t_{L7} terminator (Gurevich et al., 1980). This sequence clearly differs from that determined above for wild-type DNA (C_2T_5). Hence I decided to resequence the relevant region of λrif^{d}47, with the thought that this difference might be real, and perhaps represents a regulatory mutation forming the basis of dominance of rif^{d}47. The source of DNA for this analysis was a plasmid sub-clone of λrif^{d}47 DNA, pIB52, kindly provided by



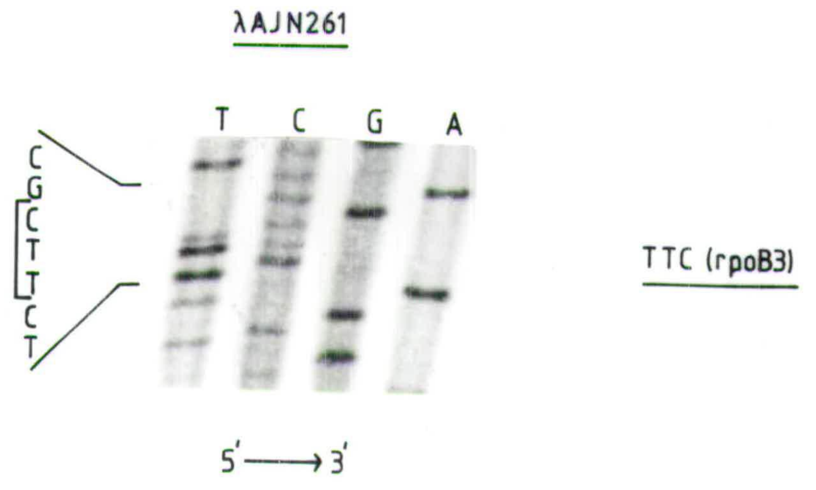


Fig. 3.8

Fig. 3.8 A sequencing analysis of the site of the *rpoB3* mutation in λAJN261. The sequencing strategy adopted and the results obtained for λAJN261 are shown.

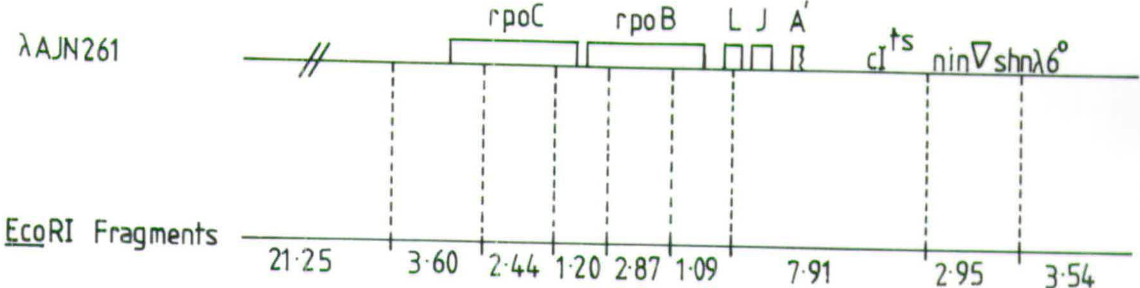
Notes:

1. The direction of the sequencing analysis with respect to the *rpoB* coding strand is 5' → 3'.
2. The *rpoB3* mutation, TTC, is indicated by a vertical bar on the sequencing gel.

Professor R.B. Khesin. The plasmid was checked for the presence of the 1.09kb EcoRI fragment by restriction analysis (see Fig. 3.9). λAJN261 was digested with EcoRI and loaded in track 1 as a control. In track 2 it can be clearly seen that the plasmid sub-clone does contain the 1.09Kb EcoRI fragment. In addition the 1.20kb, 2.44kb and 2.87kb fragments found in the λAJN161 track can also be seen in the plasmid. The DNA of the λrif^d47 clone was therefore completely digested with EcoRI and SalI, and the resultant fragments ligated with the M13 vector mp9 (Messing and Vieira, 1982) digested with the same two enzymes. M13 clones carrying the EcoRI/SalI insert containing t_{L7} were identified by plaque hybridisation with [^{32}P]-λAJN81 DNA. λAJN81 is the λimm434-HindIII "immunity-insertion" vector, λNM590, carrying the same 336bp AluI fragment (with HindIII linkers) as pHR11 (see 3.2, and Fig. 3.4); it was constructed and kindly donated by Dr A.J. Newman. This particular DNA was used instead of pHR11 for the plaque hybridisation step, because pHR11 might highlight false positives due to homology of pHR11 vector sequences with sequences of the vector used to construct pIB52 and unrelated to the t_{L7} EcoRI/SalI fragment of λrif^d47. The "positive" M13 plaques hybridising with [^{32}P]-λAJN81 DNA were further T-screened to confirm their identity, and then used to sequence the λrif^d47 DNA (on one strand only) between base pairs 2444 and 2729. I observed that λrif^d47 DNA was identical to wild-type DNA throughout the sequenced region, including the sites of the two reported discrepancies: there is a C instead of a T at position 2543, and C_2T_5 is the true "3'-tail" of the terminator t_{L7}, in both cases (Fig. 3.10).

3.5 Comparisons of all the available sequence data

The main points of the sequencing data presented in this chapter along with all other available reported sequences for the relevant regions



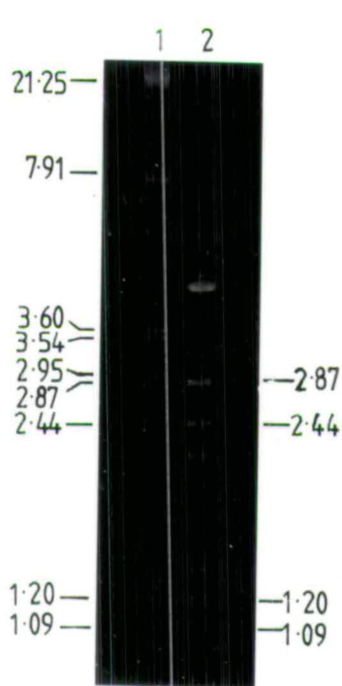


Fig. 3.9

Fig. 3.9 1% agarose gel electrophoresis

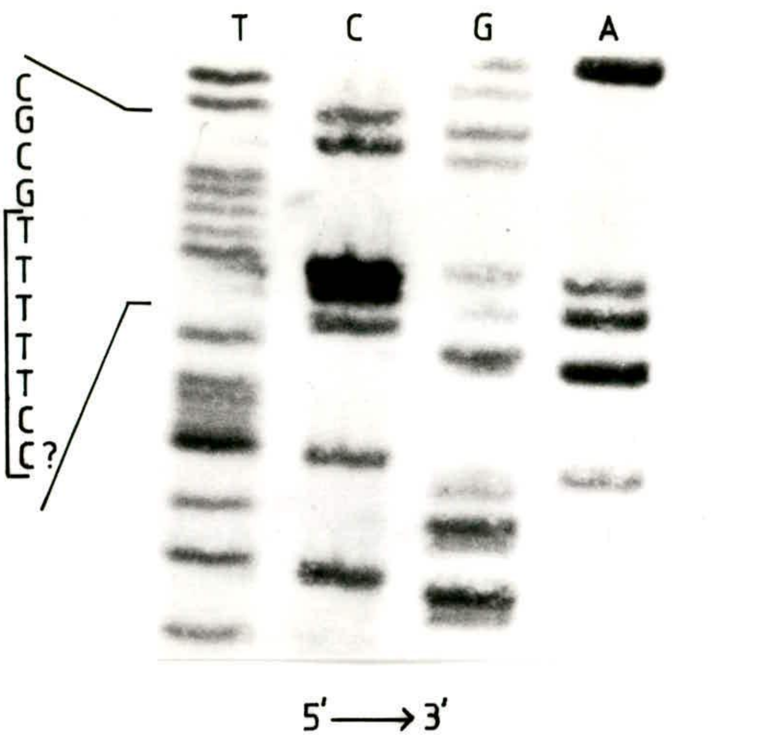
Track number	DNA	Restriction endonuclease
1	λAJN261	*Eco*RI
2	pIB52	*Eco*RI

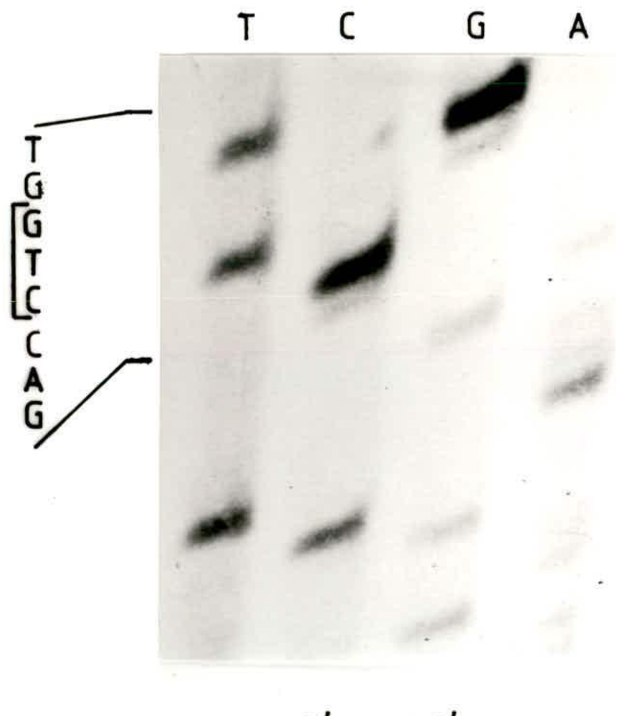
Notes:

1 The marker track is λAJN261/*Eco*RI.

2 At the top of the figure is the *Eco*RI restriction map of λAJN261.

3 The sizes of the marker digest and of selected fragments from track 2 are given in kb.





5'→3'

Fig. 3.10

Fig. 3.10 A sequencing analysis of DNA derived from λ*rif*^{d}47 in the "3'-tail" region of t_{L7} and at the site where CTG encodes leucine within *rplL* of wild-type, λ*rif*^{d}18 and λAJN261 DNA.

Notes:

1 The direction of the sequencing analysis with respect to the sense strand of the operon is indicated.

2 The regions of interest are indicated by a vertical bar.

3 An ambiguity is indicated by a question mark. Although the sequencing analysis was carried out only on one strand at the site of the "3'-tail" of t_{L7} and the CTG within *rplL* the band patterns presented here are so identical to those obtained for wild-type, λ*rif*^{d}18 and λAJN261 DNA that I am convinced there can be no hidden differences.

are shown in Table 3.2. My main conclusion is that wild-type DNA, λrif^{d}18 and λAJN261 sequences are identical throughout the 1.09kb EcoRI fragment; and further, that λrif^{d}47 DNA is also identical to wild-type in this region (on the assumption that where the early sequence analysis by Gurevich et al. (1980) disagrees with our data (2444-2729) or those of Ovchinnikov et al. (1981b), it is the Gurevich et al. results which are in error).

Two points concerning the 1.09Kb EcoRI fragment remain to be discussed. Firstly, C not T occurs at position 2543: thus the leucine residue of rplL is encoded by CUG rather than UUG. This is perhaps not unexpected, as codon usage analysis of the rplKAJL genes has shown that 43 of the other 46 leucine residues in their four protein products are also encoded by CUG, rather than the rare UUG (Post et al., 1979). Secondly, my results indicate that λrif^{d}18 does not have a deletion of AGC at nucleotides 3286-3288, contrary to the report by Delcuve et al. (1980). The latter authors reported restriction analyses which appeared to confirm their sequencing data; viz. they reported the failure of the restriction endonuclease FnuDII to cut λrif^{d}18 DNA at this point. This would be expected if AGC were absent. However, I have found that the isochizomer ThaI does cut at this site, although prolonged digestion was required. As shown in Fig. 3.11, if the AGC were absent from the sequence, and/or ThaI had some difficulty in digesting at this site, then a 230bp fragment would be predicted. However, if the AGC is present and the site is cut by the enzyme, then a 200bp and a 30bp fragment would be produced. In order to test this, DNA of the M13 mp11 vector and of M13 mp11 carrying the EcoRI/SalI rpo fragment (nucleotides 2941 to 3528) were digested to completion with ThaI. The marker tracks shown (Fig. 3.11), tracks 2 and 3, represent pHR35 cut with the restriction endonuclease HaeIII. pHR35 is a derivative of pK04 (Newman et al.,

Table 3.2 Differences between the wild-type and other DNA sequences in the 'rplL-rpoB' region of E. coli DNA, base pairs 2444 to 6401 (scale of Post et al. (1979) as corrected by Delcuve et al. (1980) and by the present work).

Nucleotide position	Locus	Sequence observed: Wild-type	λAJN261	λrif^d18	λrif^d47	Ref.
2543	rplL	-	-	T^a	C^b	a,b
		C	C	C^*	C	This work
2714-2720	t_{L7} terminator	-	-	$C_2T_4{}^a$	$C_1T_7{}^b$	a,b
	(3'-tail)	$C_2T_5{}^*$	$C_2T_5{}^*$	$C_2T_5{}^*$	C_2T_5	This work
3286-3288	rpoB	-	-	deleted[c]	AGC[d]	c,d
		AGC*	AGC*	AGC*	-	This work
4515-4517	rpoB255	GAC[e]	-	GAC[e]	GTC[e]	e
		-	GAC	-	-	This work
4560-4562	rpoB3	TCC[e]	-	TTC[e]	TCC[e]	e
		-	TTC	-	-	This work

Notes:

- a Post et al., 1979
- b Gurevich et al., 1980
- c Delcuve et al., 1980
- d Ovchinnikov et al., 1981b
- e Ovchinnikov et al., 1983
- * Determined on both strands of the DNA in this work.

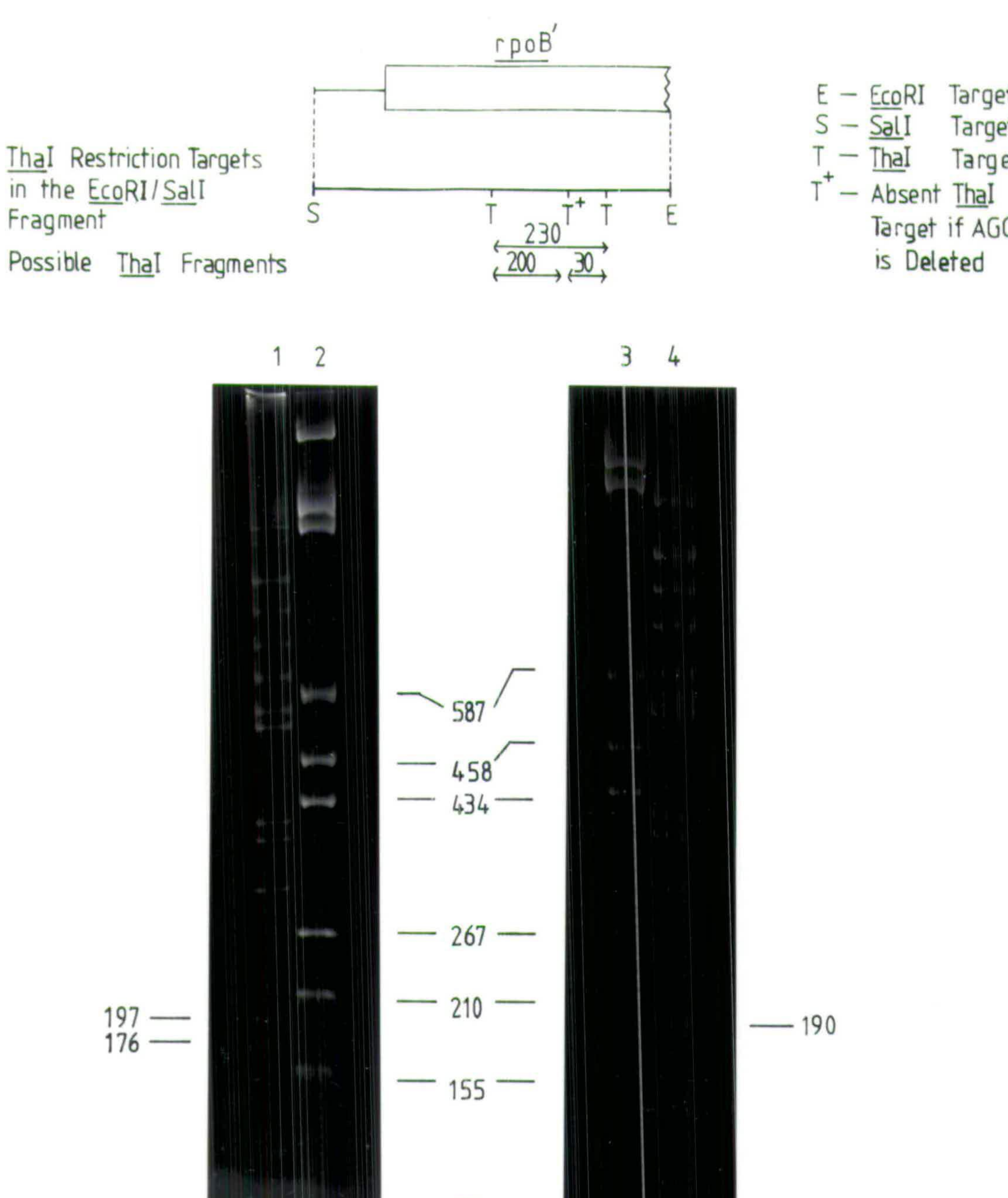


Fig.3.11

Fig. 3.11 5% polyacrylamide gel electrophoresis.

Track number	DNA	Restriction endonuclease
1	EcoRI/SalI insert in mp11	ThaI
2	pHR35	HaeIII
3	pHR35	HaeIII
4	mp11	ThaI

Notes:

1. The marker tracks are pHR35/HaeIII.
2. At the top of the figure is the predicted ThaI restriction digest of the SalI/EcoRI fragment cloned into mp11 (track 1).
3. The sizes of the marker digest and of selected fragments from tracks 1 and 4 are given in bp.

1982) which, when digested with HaeIII, gives a marker digest in the appropriate size range for this particular study. It was constructed and kindly donated by Elizabeth Marson. As can be seen in Fig. 3.11 track 1, when the M13 clone containing the EcoRI/SalI fragment is digested with ThaI, no 230bp band appears between 210bp and 267bp, as would be expected either if the AGC were not present, or if the enzyme were unable to cut. However, two bands do appear between 155bp and 210bp in the same digest (track 1), representing fragments whose calculated sizes are 176bp and 197bp. The known sequence of M13 mp11 predicts that its ThaI digest should contain only one of these two fragments: a 190bp fragment derived from a region of the vector remote from the cloning sites. Track 4 in Fig. 3.11 confirms this prediction. I conclude that ThaI does indeed cut at the AGC site, and that the earlier report must have reflected a misleading negative result. It may be relevant that I found the AGC region tricky to sequence, because of "compression" tendencies. This problem may have led to the seeming AGC-deletion in the sequence determined by Delcuve *et al.* (1980).

3.6 Discussion

The main conclusion of this chapter is that the sequence of wild-type, λAJN261, λ*rif*^{d}18 and λ*rif*^{d}47 DNA is identical throughout the 1.09kb EcoRI fragment. Further, the sequence of *E. coli* CR63 DNA obtained, in conjunction with the work of Ovchinnikov *et al.* (1983), extends the known wild-type nucleotide sequence from position 2444 (near the middle of *rplL*) to 6401 (near the distal end of *rpoB*). The only difference between *rif*^{d}18 and wild-type DNA in this region is the *rpoB*3 mutation, a nucleotide substitution changing the "codon" TCC to TTC at position 4561 (Ovchinnikov *et al.*, 1983; Morgan *et al.*, 1984). This finding supports the argument of Ovchinnikov *et al.* (1983) that this

single mutation may cause both the rifampicin resistance of rif^d18, and its dominance. Although I have not accomplished an extensive characterisation of the 1.09kb EcoRI fragment of λrif^d47, my preliminary results, combined with the previously reported data (Ovchinnikov et al., 1981b) suggests a similar conclusion for the rif^d47 mutation; i.e. only one difference, the mutation rpoB255, a GAC → GTC "codon" substitution at position 4516, exists between rif^d47 and wild-type DNA between nucleotides 2444 and 6401. Therefore the sequencing observations for both rif^d mutations, rif^d18 and rif^d47, are consistent with the hypothesis that such mutations form a special class of single rpoB mutation, occurring at a one hundred-fold lower frequency than normal rif^r. If so their dominance over rif^s may reflect production of a drug-resistant RNA polymerase which is better able to compete for promoters than normal "rif^r" enzyme, in the presence of drug-inactivated sensitive R-pol (Kirschbaum and Konrad, 1973; J.B. Kirschbaum, pers. comm.). However, as mentioned in 3.1, this hypothesis does not seem to be true, at least in vitro, for the "rif^d18" enzyme (Smith, 1982). In addition, the difference in dominance properties between λAJN261 and λrif^d18 when lysogenised in E. coli strains AJN10 and AJN1 (Newman and Hayward, 1980; Table 3.1) remain to be explained. Four main possibilities could be considered to explain the latter result: 1) The rif^r mutation present in λAJN261 is not rpoB3. However, sequence analysis of the appropriate region, as reported in this chapter (see 3.3), has demonstrated that λAJN261 retains the rpoB3 mutation at base-pair 4561. Further, as no selection for rifampicin resistance was imposed during the original isolation of λAJN261 (see Fig. 3.2) it seems unlikely that a different rif^r mutation was acquired by this phage. 2) There is a second, cis-acting regulatory mutation in λrif^d18, which is absent from λAJN261

and increases the relative expression of the *rif*^{r} allele (by acting either at the transcriptional or the post-transcriptional level) in heterodiploids. If this is so, then it must lie upstream of position 2444 in *rplL* or downstream of position 6401 in *rpoB*. This rules out some of the most likely candidates for the site of a regulatory mutation which could affect relative *rpoBC* expression: i.e. the partial terminator t_{L7}, the RNaseIII processing site, the weak promoter P_{β}, the translational initiation site of *rpoB*, and the proposed translational regulatory sequence for *rpoB* (Dennis, 1984). These are all situated within the region which I have shown to display no sequence differences between wild-type, λ*rif*^{d}18, and λAJN261 DNA. 3) There is a second structural mutation in *rpoB*, *downstream* of position 6401, which is necessary for the dominance of the *rif*^{d}18 allele and which was lost from λAJN261 during its original construction. For this explanation to be true a number of crossovers between λAJN172 and the chromosome would have been required to retain *rpoB3*, but remove another mutation downstream (cf. Fig. 3.2). Although this possibility is unlikely, it has not yet been excluded. 4) DNA present in λ*rif*^{d}18, and necessary for dominance, is missing entirely from λAJN261. Perhaps the most obvious candidate of this type would be P_{L11}, the promoter upstream of *rplKA* which is normally responsible for all expression of the *rpoBC* operon (Brückner and Matzura, 1981; C. Squires, pers. comm.). This is missing from λAJN261, and might be stronger than, or in some other relevant way differ from, P_{L10} (the promoter between *rplA* and *rplJ*, which is normally occluded by P_{L11} (Brückner and Matzura, 1981; Morgan and Hayward, 1985; C. Squires , pers. comm.; Chapter 5 of this thesis), but which is essential for *rpoBC* expression by the λAJN261 prophage). Another potentially relevant feature of λ*rif*^{d}18 is that it carries

extensive regions of bacterial DNA (especially upstream of the rpoBC operon) which are absent from λAJN261, and which might conceivably include some gene essential for the dominance phenotype.

Hypotheses 2) and 4) will be discussed in more detail in the next chapter, which describes attempts to compare translation and subsequent assembly of the rpoB3 - encoded β subunit into complete R-pol in λAJN261 and λrif^d18 lysogens. Although the properties of λAJN261 indicate that the dominance of rif^d18 must require a second mutation additional to rpoB3, or else some DNA present in λrif^d18 but "deleted" from λAJN261, the mechanism of dominance of rif^d18 remains unclear. This question will also be examined and discussed more fully in Chapter 4.

The results presented in this chapter, and the clones constructed to obtain them, will hopefully be of use in the mutational analysis of the rplL - rpoB intercistronic region, which contains so many interesting known or putative regulatory features, both at the transcriptional and post-transcriptional levels, affecting the control of rpoBC expression.

CHAPTER 4

The nature of dominance of the *rif*^{d}18 mutation

4.1 Introduction

In *E. coli* mutations conferring resistance to the antibiotic rifampicin, *rif*^{r}, (all of which occur in the *rpoB* gene) are usually recessive to the wild-type sensitive allele, *rif*^{s} (*rpoB*$^+$). However, a special class if rifampicin resistance mutations, *rif*^{d}, are dominant over *rif*^{s} in heterodiploids. The most studied example of such mutations is *rif*^{d}18 (Kirschbaum and Konrad, 1973). Taken as a whole the sequencing studies performed on *rif*^{d}18 DNA agree with the proposal, originally made by Kirschbaum and Konrad, that a single mutation within *rpoB*, *rpoB*3, is sufficient for dominance of the *rif*^{d}18 allele (Ovchinnikov *et al.*, 1983; Morgan *et al.*, 1984; see also Chapter 3). However, as previously outlined in Chapter 3,there is strong genetic evidence to suggest that the dominance and *rif*^{r} components of *rif*^{d}18 can be separated, in that λAJN261, a derivative of λ*rif*^{d}18, expresses *rif*^{r} but not *rif*^{d} (Newman and Hayward, 1980). Moreover I have shown by direct sequence studies that λAJN261 retains the *rpoB*3 mutation (Chapter 3). Earlier, P1 transduction experiments performed by Kirschbaum (1973a) indicated that the drug-resistance and dominance genotypes are closely linked. However, insufficient data were obtained to prove that the *rif*^{d}18 phenotype was the result of a single mutation. If more than one mutation is responsible for the *rif*^{d}18 phenotype, it is possible that the second mutation is regulatory and *cis*-active in nature, such that a high proportion of RNA polymerase molecules in *rif*^{s}/*rif*^{d}18 heterodiploids contain the resistant

β subunit (encoded by rpoB3) rather than the sensitive (rpoB$^+$) polypeptide. This proposal has been discussed in more detail in 3.1.

To investigate this possibility further I lysogenised λrif^{d}18, and the non-dominant derivative λAJN261, separately into each of two E. coli strains containing mutant chromosomal rpoB alleles. These two alleles each encode a β polypeptide with aberrant mobility on SDS polyacrylamide gels. This allowed me to distinguish chromosomal expression of rpoB from that of the prophage, and therefore to quantitate the relative levels of expression of the sensitive and resistant β-subunits in the λrif^{d}18 and λAJN261 heterodiploid lysogens.

I have also attempted to compare the dominance properties of λAJN261 and λrif^{d}18 in genetic backgrounds differing from those previously reported (Newman and Hayward, 1980) so as further to explore the differing dominance properties of λrif^{d}18 and λAJN261.

4.2 Strain constructions

The two host strains chosen for the expression analysis of the λrif^{d}18 and λAJN261 prophages were both derivatives of E. coli K12, termed CR63 and W3110-del1, each expressing a β polypeptide which, although fully functional, can be resolved from wild-type β using SDS polyacrylamide gel electrophoresis.

The β polypeptide of CR63 has decreased mobility on SDS polyacrylamide gels (Newman and Hayward, 1980; see Fig. 4.1). The nature of the mutation responsible for this size alteration of β is unknown, although it may be relevant that CR63 was derived from CR67 by UV curing of a λ prophage (Appleyard, 1954). The basis of the mobility change will be discussed in more detail in 4.3.

In contrast to CR63 the β polypeptide of W3110-del1 has increased

mobility on SDS polyacrylamide gels (Nene and Glass, 1984; see Fig. 4.4). The β polypeptide of W3110-del1 is thought to be the product of an N-terminal deletion of rpoB (R.E. Glass, pers. comm.). This strain was kindly donated by Dr R.E. Glass.

As can be seen in Table 2.1 both CR63 and W3110-del1 are Rec^+. Hence to prevent Rec-mediated recombination between the chromosome and either of the phages λAJN261 and λrif^d18, I decided to convert both strains to Rec^-. Additionally a λ^S derivative of CR63 was required.

The course chosen was to transfer recA56 from JC5088 (see Table 2.1), an Hfr strain which transfers thy^+ and recA56 early, by interrupted mating (see 2.5(ii)). To allow selection of ex-conjugants, CR63 and W3110-del1 were first made Thy^- by selection of trimethoprim-resistant derivatives, followed by screening for thy. The CR63 thy strain (BM1) was then made Str-R, by transduction with $P1_{vir}$ grown on AB1886, in order to provide counter-selection against the JC5088 (Str-S) donor. Next, since CR63 and its thy Str-R derivative are λ^R, the latter was made λ^S by transduction with $P1_{vir}$ grown on the malE::Tn10 λ^S strain CGSC6137 (see Table 2.1). Tn10 (tetracycline-resistant) transductants were selected, purified, and then screened for co-transduction of λ^S (which is determined by a gene closely linked to λ^R).

The two strains BM6 (CR63 thy Str-R λ^S malE::Tn10) and BM7 (W3110-del1 thy Str-R λ^S) were then made Thy^+ recA56 by interrupted mating with JC5088, selection of Str-R Thy^+ ex-conjugants, and screening for UV sensitivity (diagnostic for Rec^-), to yield strains BM5 and BM9 respectively.

Both λrif^d18 and λAJN261 are integration deficient; hence in the absence of generalised recombination stable lysogens of these phages can be isolated only if a helper phage is present to provide integration

functions. Lysogens of BM5 and BM9 were therefore isolated after simultaneous infection with λNM54 (λimm21), kindly provided by Dr N.E. Murray, and the immλ-cI^{ts}857 phages λrif^{d}18 or λAJN261. Lysogens were selected for immλ, then checked for temperature sensitivity (see 2.4(viii)). Finally their rifampicin-resistance properties were examined. The rifampicin resistance test involved spreading dilutions of an overnight culture of the lysogen onto oxoid and oxoid-rifampicin (100μg/ml) agar plates. After allowing the plates to dry they were incubated at 30°C for 24-48 hours. The plates were then analysed for survival of the lysogens. When the λrif^{d}18 and λAJN261 lysogens of BM5 and BM9 were tested in this manner a surprising result was obtained; all 4 lysogens survived at a frequency of approximately 1 in 10^{3}-10^{5} on the oxoid-Rif plates compared with the drug-free plates. This contrasted with a Rif-R control, which displayed complete survival, and a Rif-S control, which did not show any growth, on the oxoid-Rif plates. The rifampicin resistance phenotype observed for the λrif^{d}18 and λAJN261 lysogens of BM5 and BM9, which I have termed "quasi-resistance" (QR), will be discussed in 4.7(i).

4.3 β and β' protein synthesis in the λrif^{d}18 and λAJN261 lysogens of the CR63 derivative BM5

As described above, the λrif^{d}18 and λAJN261 lysogens of the CR63 recA56 strain exhibit, unexpectedly, an identical phenotype with respect to rifampicin resistance. I suspected that this might be due to the presence of the β^{63} polypeptide, which might for example confer different behaviour of the drug-sensitive RNA polymerase molecules in DNA blockade (see 3.1), as compared with wild-type β. Alternatively the Rif-sensitive alleles rpoB^{+} and rpoB-CR63 might differ in their degree of

expression relative to rpoB3 (rif^{d}18) in heterodiploids, if such differences in rpoB allele expression play a role in rif^{d}18's (or can modify) dominance in wild-type strains. Therefore, the lysogens BM5 (λrif^{d}18)(λimm21) and BM5 (λAJN261)(λimm21) were grown in minimal medium at 30°C to mid-log phase. Samples were then removed, pulse-labelled with L-[^{35}S]-methionine, and chased with unlabelled methionine (see 2.10). The β and β' polypeptides were then isolated by SDS polyacrylamide gel electrophoresis of crude cell extracts. An autoradiograph from such an experiment is shown in Fig. 4.1. Tracks 1 and 6 are from the control strains CR63 and C600, respectively. Tracks 2-5 represent the BM5 (λAJN261), and 3 separately isolated BM5 (λrif^{d}18), lysogens. As can be seen all four lysogens express a wild-type β polypeptide. Quantitative analysis of the relative expression of the prophage-encoded β and the chromosomal product β^{63} proved difficult, due to the presence of a CR63 encoded polypeptide which has a similar mobility to the wild-type β polypeptide (see track 1). However, it seems clear that the λrif^{d}18-product and β^{63} are produced at very similar rates in the BM5 (λrif^{d}18) strain. If anything it is the λAJN261-encoded β subunit which may be expressed at a higher rate relative to β^{63} (track 2). Although not striking, this apparent difference between λrif^{d}18 and λAJN261 in the degree of expression of their rpoB3 allele has proved reproducible. It might be due, for example, to expression from two different promoters (P_{L11} for λrif^{d}18, P_{L10} for λAJN261).

I interpret these data to suggest that if quasi-resistance can be equated with dominance, rif^{d}18 can be dominant without there being any overexpression of the rpoB3 gene. Moreover, the CR63 background suppresses the difference in dominance between λrif^{d}18 and λAJN261, observed previously in AJN10 (Newman and Hayward, 1980). On the other

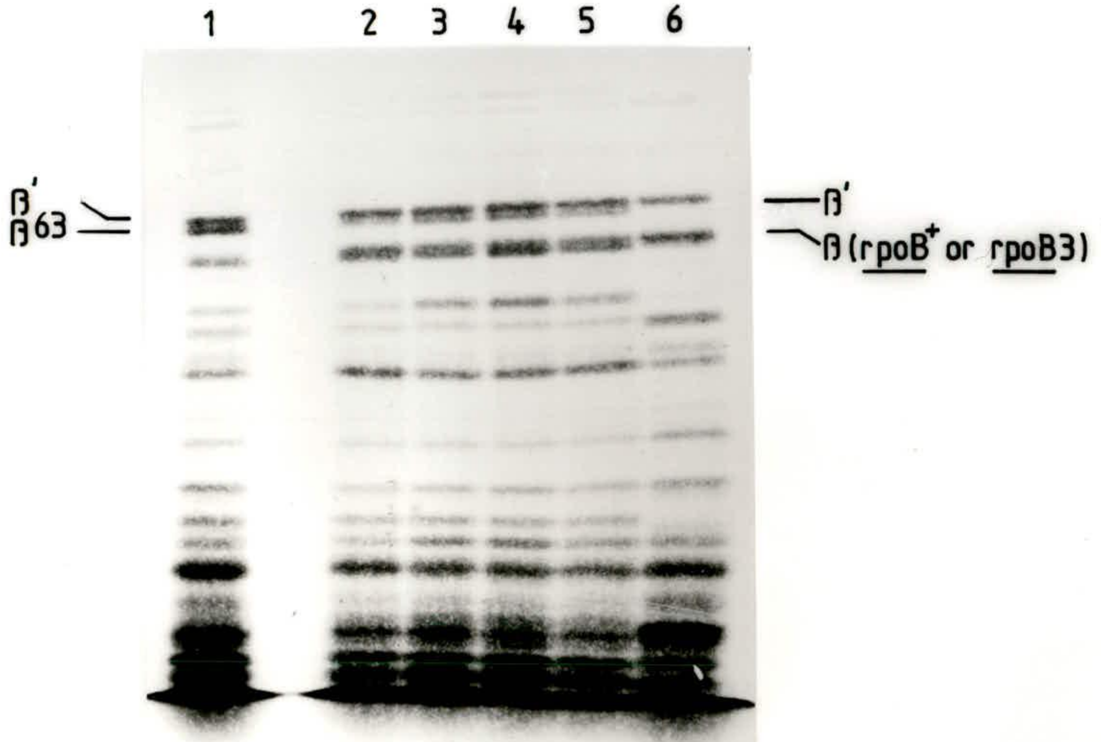


Fig. 4.1

<u>Fig. 4.1</u> An autoradiograph of L-[^{35}S]-methionine labelled proteins synthesised in <u>E. coli</u> CR63 (track 1), C600 (track 6), a λAJN261 lysogen of CR63 (track 2) and 3 independent λ<u>rif</u>^{d}18 lysogens of CR63 (tracks 3-5). Proteins were fractionated on a 5% SDS polyacrylamide gel.

hand if quasi-resistance does not represent rpoB3-dominance, then the CR63 background suppresses the normal dominance of λrif^{d}18, making it indistinguishable in this respect from λAJN261.

It is relevant at this point to consider the nature of the β^{63} mutation. I already showed by DNA sequence analysis of CR63 (Chapter 3) that no sequence differences exist between rpoB3, rpoB255 and rpoB-63 DNA in the region encoding the first 188 N-terminal amino acids of β. The DNA sequence of rpoBC shows that mutation of the translational stop codon of rpoB, TAA → sense codon, would give an altered β polypeptide with 19 additional amino acids, stopping at another TAA codon, without reading into the rpoC gene (Ovchinnikov et al., 1981b). To test this hypothesis for the nature of the rpoB-63 by DNA sequencing, I digested λAJN63 with the restriction endonucleases EcoRI and PstI (see Fig. 4.2) and ligated the resultant fragments into the M13 vector mp10 (Messing and Vieira, 1982) digested with the same enzymes. The correct clone was identified by T-screening (discussed in 3.2) and then sequenced. It can be clearly seen in Fig. 4.3 that CR63 has the normal TAA stop codon at the end of rpoB. In addition comparisons of the DNA sequence of CR63 obtained in this region with the published DNA sequence of λrif^{d}47 (Ovchinnikov et al., 1981b) revealed no differences between them. Hence the aberrant mobility of the CR63 β polypeptide on SDS polyacrylamide gels is not due to a mutation in the first 564bp or last 171bp (and stop codon) of rpoB. A single amino acid substitution could affect the mobility of the denatured polypeptide on the gel (Noel et al., 1979) although a small insertion in rpoB has not been ruled out.

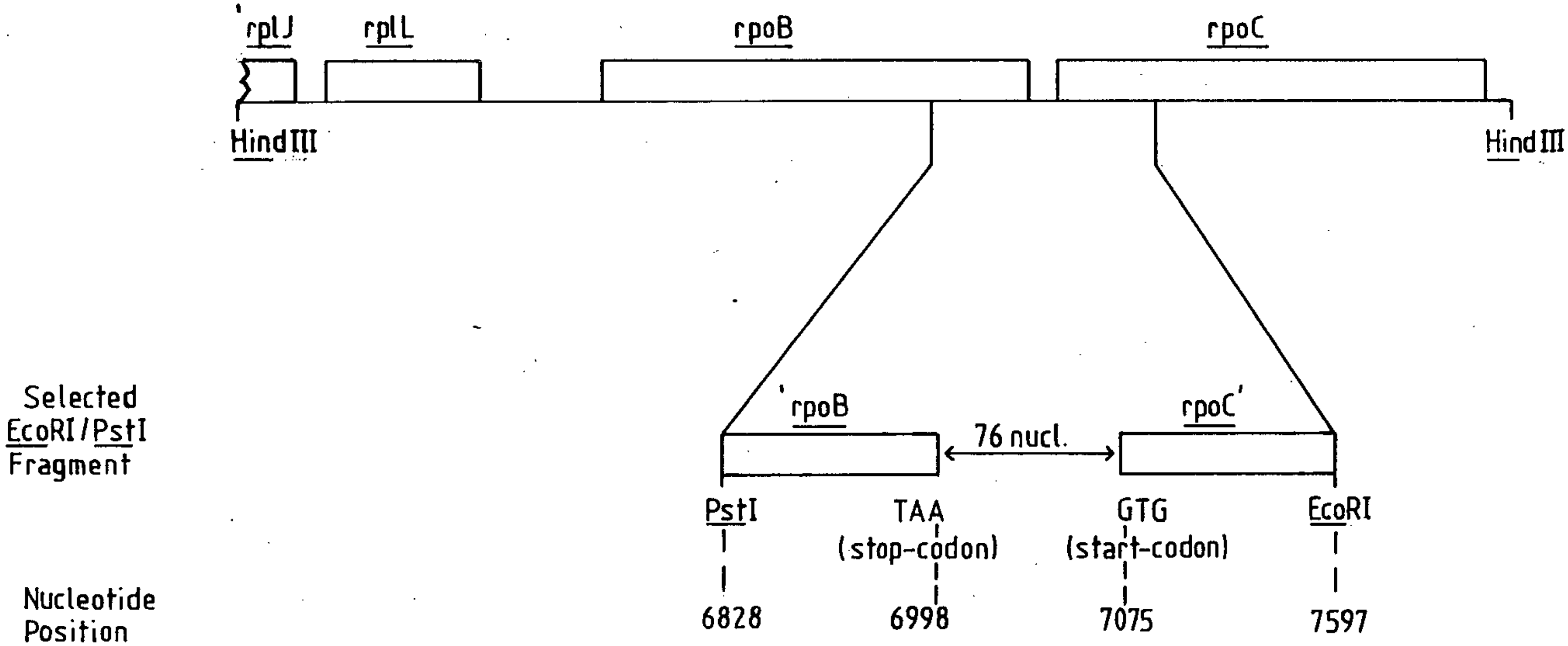


Fig. 4.2

Fig. 4.2 A map of the translational stop site of *rpoB* and the translational start site of *rpoC* in the 10.14 kb *Hind*III fragment (of the *rplKAJLrpoBC* operon) carried by λAJN63.

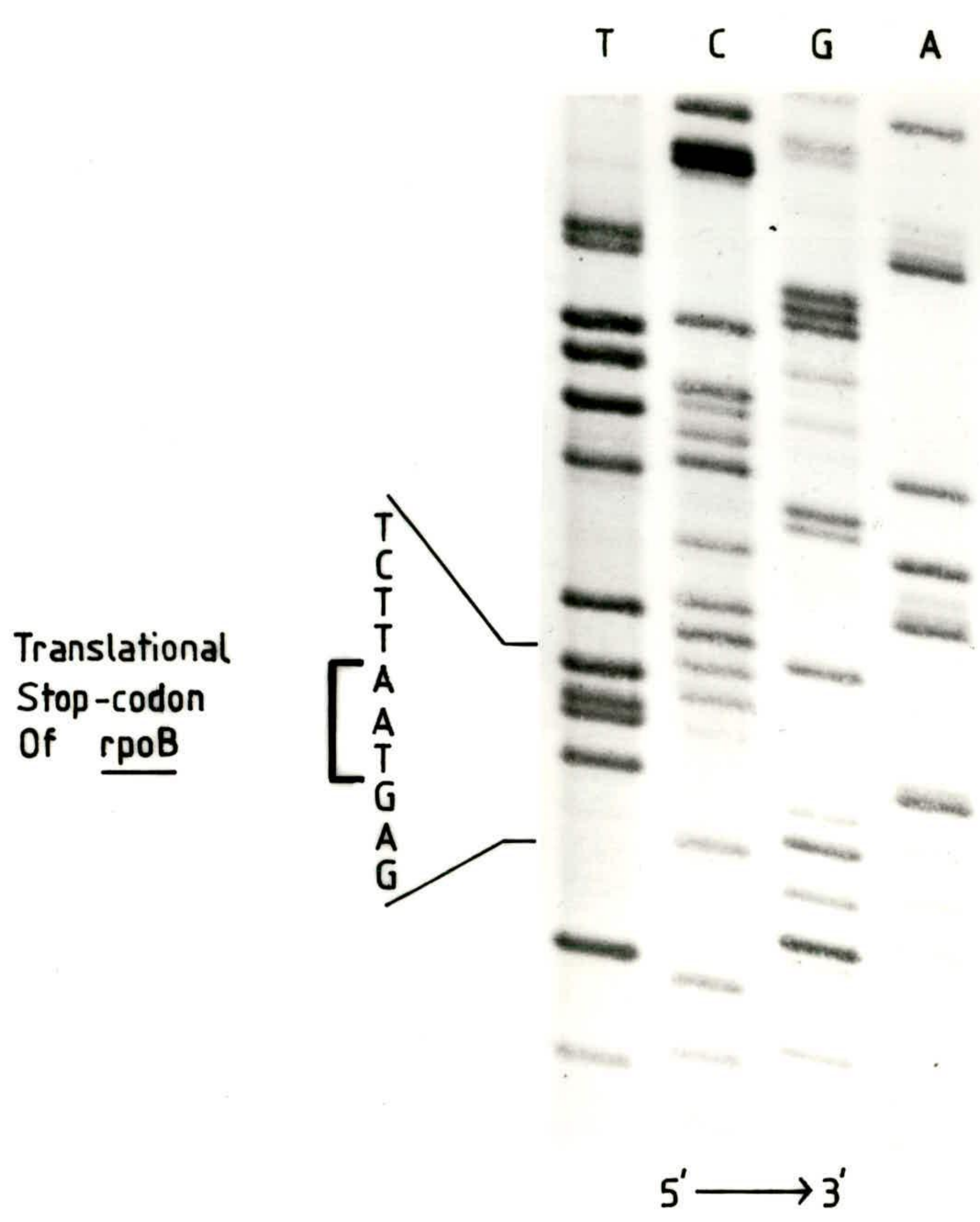


Fig.4.3

Fig. 4.3 A sequencing analysis of the translational stop codon of *rpoB* in the appropriate *Eco*RI/*Pst*I restriction fragment (see Fig. 4.2) isolated from CR63.

Notes:

1 The direction of the sequencing analysis with respect to the *rpoB* coding strand is 5' → 3'.

4.4 β and β' protein synthesis in the λ*rif*^{d}18 and λAJN261 lysogens of W3110-del1

The λ*rif*^{d}18 and λAJN261 lysogens of the *recA*56 derivative of W3110-del1 (BM9), constructed as previously described, both showed a very similar rifampicin resistance phenotype to that of the CR63 lysogens, i.e. "quasi-resistance". To determine whether either W3110-del1 lysogen showed any differential rate of synthesis of the phage-encoded β, relative to the truncated β encoded by the chromosomal locus, lysogens were grown in minimal medium at 30°C to mid-log phase, and then pulse-labelled with L-[^{35}S]-methionine as before. The proteins were analysed by SDS polyacrylamide gel electrophoresis. An autoradiograph of one such gel is presented in Fig. 4.4. Tracks 1 and 2 show the controls BM9 and C600, respectively. Tracks 3 and 6 indicate that both phages express their *rpoB*3 gene. Further it can be seen that the λ*rif*^{d}18-encoded β subunit and β_{del} are synthesised at approximately equal rates (track 3), while in contrast the λAJN261-encoded β is synthesised at a higher rate than the β_{del} encoded by the host's own *rpoB* gene (track 6). These results are in good agreement with those of the CR63 analysis.

Although the BM5 and BM9 lysogens of λ*rif*^{d}18 and λAJN261 were screened for their rifampicin phenotype, the colonies propagated for the protein analyses had never been exposed to the drug. Hence one model to explain the *rif*^{d}18 genotype is that after addition of rifampicin to a *rif*^{d}18/*rif*^{s} heterodiploid, expression of the λ*rif*^{d}18-encoded *rpoB* is preferentially stimulated. Rifampicin is known to have a stimulatory effect on the transcription of *rpoBC*, and consequently on β and β' protein synthesis (although this effect is transient in normal Rif-S/Rif-R heterodiploids: see Chapter 1). Therefore if *rif*^{d}18 carries a regulatory mutation which allows greater or more prolonged stimulation of *rpoB*3-

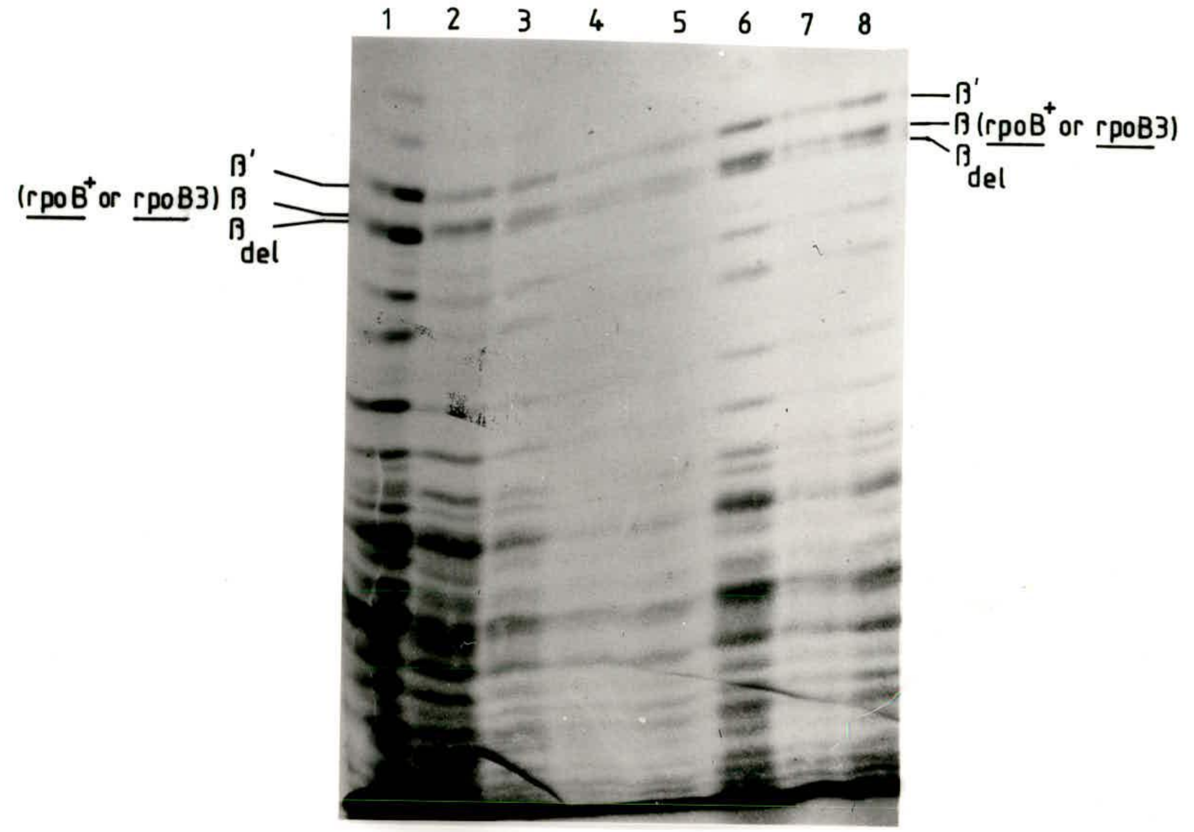


Fig. 4.4

Fig. 4.4 An autoradiograph of L-[^{35}S]-methionine labelled proteins synthesised in *E. coli* W3110-dell (track 1), C600 (track 2), a λ*rif*^{d}18 lysogen of W3110-dell (track 3: no rifampicin addition; track 4: 12 min. after addition of 50μg/ml rifampicin; track 5: 25 min. after drug addition) and a λAJN261 lysogen of W3110-dell (tracks 6-8, in the same order as described for the λ*rif*^{d}18 lysogen). Proteins were fractionated on a 5% SDS polyacrylamide gel.

expression by rifampicin, then a higher proportion of λ<u>rif</u>^{d}18-encoded β^R relative to the chromosomal (drug-sensitive) β could arise. To test this hypothesis λ<u>rif</u>^{d}18 and λAJN261 lysogens of BM9 were grown as before to mid-log phase. At this point rifampicin to a final concentration of 50 μg/ml was added and the cells left at 30°C for a further 12 minutes (rifampicin stimulation of $\beta\beta'$ reaches a peak 10-15 minutes after drug addition; Hayward and Fyfe, 1978a) or 25 minutes (a time point at which $\beta\beta'$ synthesis is expected to decline: ibid.). The cells were then pulse-labelled with L-[^{35}S]-methionine as before, and the proteins analysed by SDS polyacrylamide gel electrophoresis. The results are shown in Fig. 4.4. As expected the λ<u>rif</u>^{d}18 lysogen (tracks 4 and 5) and the λAJN261 lysogen (tracks 7 and 8) show a stimulation of β, β_{del} and β' syntheses relative to total protein at 12 minutes, and some recovery of total protein synthesis at 25 minutes. However no difference in the rate of synthesis of β_{del} relative to that of β encoded by either λ<u>rif</u>^{d}18 or λAJN261 could be detected, at either time point.

From these results, as from those obtained with CR63, I conclude that the dominance of <u>rif</u>^{d}18 can be expressed without there being any significant relative over-expression of its <u>rpoB</u>3 gene in heterodiploids, before <u>or</u> after drug addition, whether arising at the transcriptional or post-transcriptional level. Moreover W3110-dell suppresses the difference in dominance between λ<u>rif</u>^{d}18 and λAJN261, observed in AJN10 (Newman and Hayward, 1980). Alternatively if the "quasi-resistance" observed in W3110-dell lysogenised with either phage is NOT equatable with dominance, then this <u>E. coli</u> strain abolishes the dominance of <u>rif</u>^{d}18. The "quasi-resistant" phenotype and the unexpected similarity of the λ<u>rif</u>^{d}18 and λAJN261 lysogens of BM5 and BM9 will be discussed further in 4.7(i).

4.5 Stability of the λ*rif*^{d}18- and λAJN261-encoded β polypeptides

Another model to explain the dominance of *rif*^{d}18 is that the *rif*^{d}18 encoded β polypeptide is more stable than the *rif*^{s} β in heterodiploids, perhaps as a result of a structural mutation which either stabilises the individual subunit or allows preferential incorporation of the *rif*^{d}18 β into complete RNA polymerase enzyme. (It is already known that *rpoBC* diploids synthesise excess β and β', and that the excess subunits are unstable; e.g. Hayward and Fyfe, 1978b.) Hence a higher than normal fraction of cellular RNA polymerase would contain drug-resistant β.

To examine this possibility λ*rif*^{d}18 and λAJN261 lysogens of BM9 were grown to mid-log phase, as before. Following a short pulse with L-[^{35}S]-methionine the cells were chased with excess unlabelled methionine for 30, 60 or 120 minutes. Protein samples were then analysed by SDS polyacrylamide gel electrophoresis. The autoradiograph is presented in Fig. 4.5. The polypeptides show an unexpected stability in the lysogens (tracks 5-10) after the 30, 60 and 120 minute chase and therefore it is difficult to assess any change in half lives of β$_{del}$ and the β encoded by λ*rif*^{d}18 or λAJN261.

4.6 The *rif*^{d}18 genotype: more than one mutation?

Evidence presented earlier in this chapter has provided no further insight into the difference between the rifampicin resistance phenotypes of λ*rif*^{d}18 and λAJN261, especially as I have shown that λAJN261 retains *rpoB3*, reported to be the single mutation responsible for the dominance of *rif*^{d}18 (Ovchinnikov *et al.*, 1983; Chapter 3). Hence the only direct evidence supporting a secondary mutation hypothesis

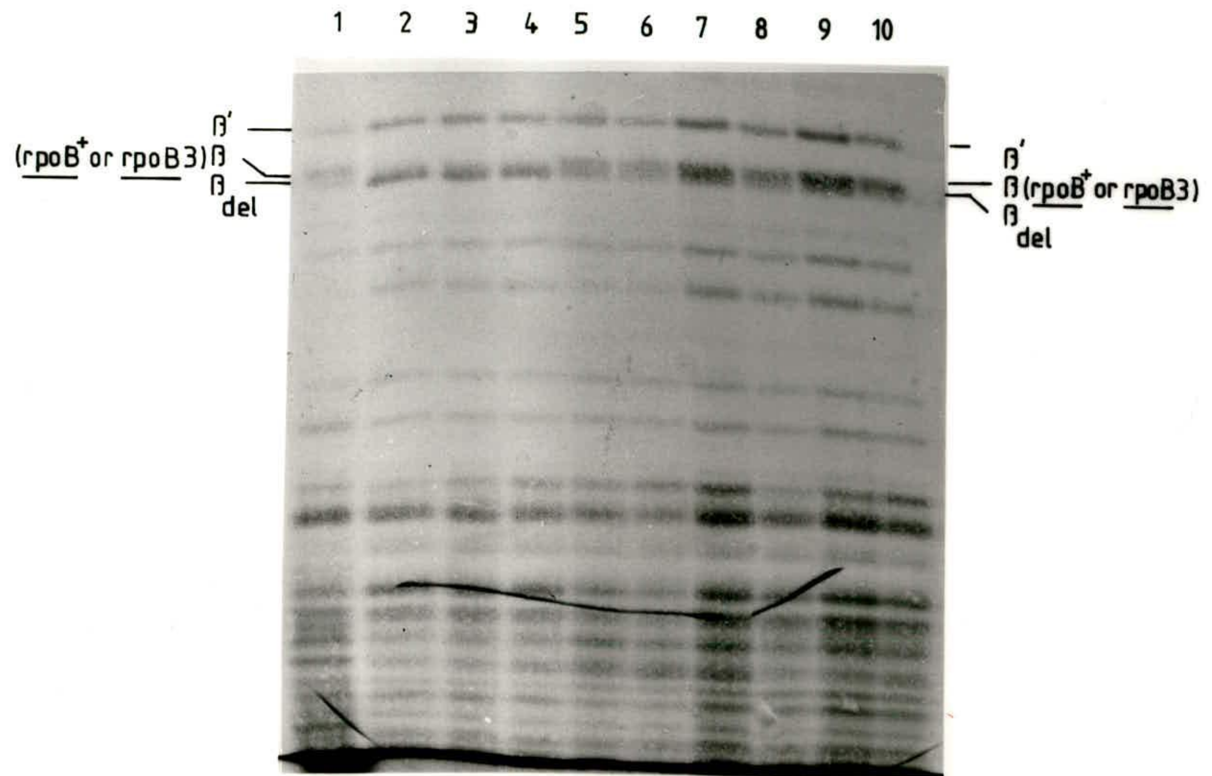


Fig. 4.5

Fig. 4.5 An autoradiograph of L-[^{35}S]-methionine labelled proteins synthesised in *E. coli* C600 (track 1), W3110-dell (after a 3 min. pulse labelling with L-[^{35}S]-methionine followed by a 30, 60 or 120 min. chase with excess unlabelled methionine: tracks 2, 3 and 4 respectively), a λ*rif*^{d}18 lysogen of W3110-dell (after pulse and chases as described above: tracks 5, 6 and 7 respectively) and a λAJN261 lysogen of W3110-dell (after pulse and chases as described above: tracks 8, 9 and 10 respectively). Proteins were fractionated on a 5% SDS polyacrylamide gel.

arises from the report of Newman and Hayward (1980) that a λAJN261 lysogen of the rpoB^{+} (rif^{s}) strain AJN10 is Rif-S, whereas the λrif^{d}18 lysogen of the same strain is Rif-R (see Table 3.1).

To re-examine this difference I relysogenised AJN10 and AJN1 (the recessive-sensitive derivative of AJN10) with λAJN261, using the phage λNM54 (imm21) as a helper. After confirmation of the presence of λ-immunity and temperature sensitivity,(characteristic of λAJN261, which is immλ cI^{ts}857), the λAJN261 lysogens of AJN10 and AJN1 were checked for their rifampicin resistance phenotype. As expected the AJN10 lysogen was Rif-S; however, instead of showing the expected clearcut Rif-R phenotype, the AJN1 lysogen was "quasi-resistant" (see Table 4.1). This phenotype was observed with two independent preparations of λAJN261, one of which was the repackaged λAJN261 DNA used in the sequencing analysis described in 3.3. Quasi-resistance will be discussed in 4.7(i), but the results obtained lead me to suggest that this phenotype actually represents dominance of the drug-resistant allele, at least in rif^{d}18 heterodiploids.

I also attempted to relysogenise AJN10 and AJN1 with λrif^{d}18. Despite much effort this proved unsuccessful, probably for three main reasons. Firstly both these strains have a strong tendency to be mucoid (especially at 30°C), which makes them less susceptible to λ infection. (Because of this problem considerable effort was required to obtain the λAJN261 lysogens.) Secondly my source of λrif^{d}18 for these experiments was heat induction of the strain H105 (λrif^{d}18)(λcI^{ts}857 Sam7) (Kirschbaum and Konrad, 1973). Thus the phage lysate contained two phages of the same immunity, and therefore λrif^{d}18 lysogens could not be reliably selected as immλ. Attempts to separate these 2 phages depended on density fractionation on a CsCl equilibrium gradient, which at best was

Table 4.1

PHAGE / STRAIN	λ<u>rif</u>^{d}18	λAJN261	λAJN321	λAJN363
CR63	QR	QR	-	-
W3110-del1	QR	QR	-	-
W3110-del2*	-	QR	-	-
W3110	-	S	QR	QR
H105	QR/R†	S	QR	QR
AJN10	-(R)	S (S)	-	-
AJN1(rcs)	-(R)	QR(R)	-	-

The rifampicin phenotype of strains lysogenic for the various λrpoBC derivatives mentioned. QR - quasi-resistant, S - sensitive, (R)(S) - fully resistant and fully sensitive (as defined by previous studies). A dash is inserted where lysogens have not been tested, in some cases (see text) due to difficulty in obtaining the relevant lysogen.

* - see text for details

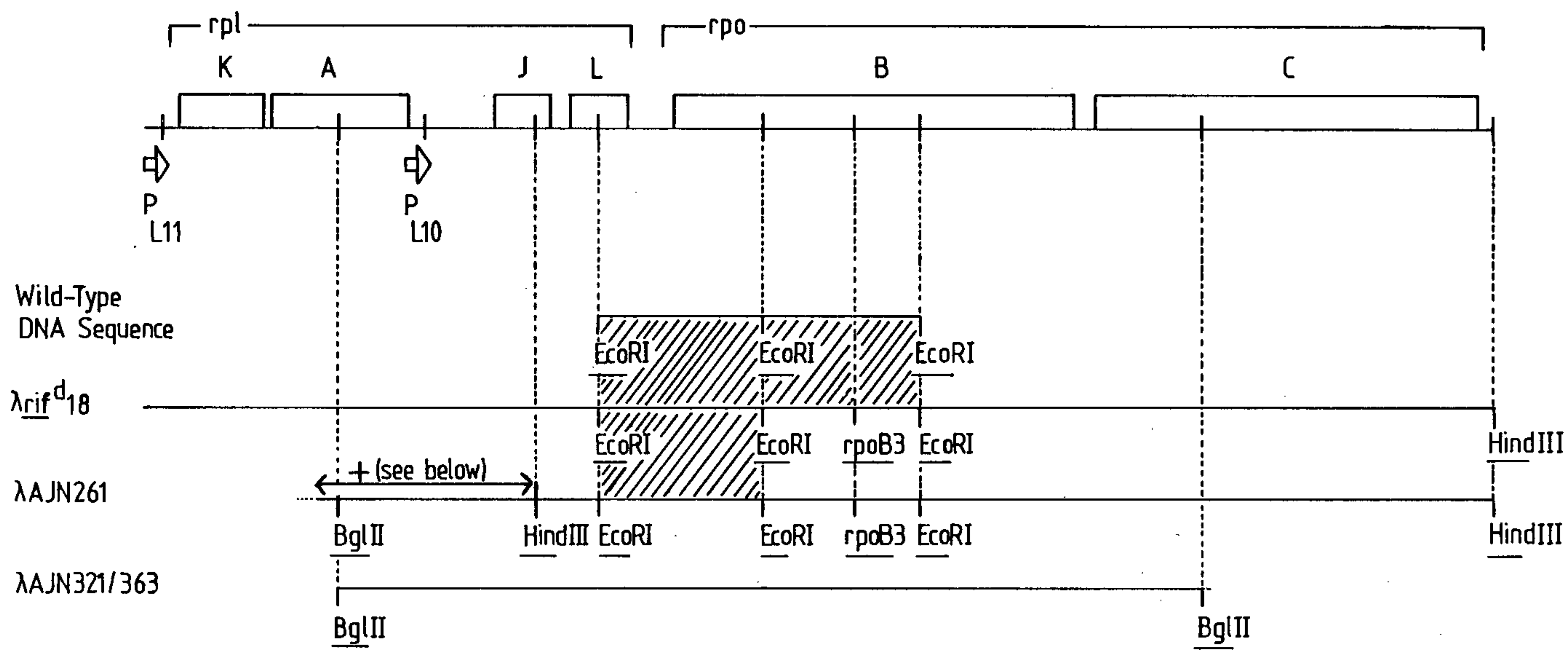
† - R in the strain provided by Kirschbaum: QR when freshly constructed.

incomplete. Thirdly, λ$\underline{rif}^d$18 is not a plaque former, so its titre could only be guessed at. This is a serious disadvantage, because correct multiplicity of infection is important for successful lysogenisation.

Does λAJN261 yield Rif-S lysogens in any other strains of E. coli? I decided to lysogenise H105, in whose Rec^- background λ$\underline{rif}^d$18 was originally characterised (Kirschbaum and Konrad, 1973) with λAJN261 and λ$\underline{rif}^d$18. The H105 for these experiments was obtained by heat-curing the H105 (λ$\underline{rif}^d$18) (λ$\underline{c}I^{ts}$857 Sam7) lysogen, looking for survivors on oxoid agar plates at 42°C. Heat-resistant colonies were then checked for the correct genetic characteristics, including λ-sensitivity and Rif-S. When λAJN261 and λ$\underline{rif}^d$18 were lysogenised into H105 the λAJN261 lysogen was Rif-S, whereas the λ$\underline{rif}^d$18 lysogen was quasi-resistant (see Table 4.1). Hence the genetic difference observed between these two phages in AJN10 lysogens (Newman and Hayward, 1980), is confirmed in H105.

What could be the basis of this difference? One possibility is that rpoBC-transcription initiated from P_{L10} (as in λAJN261) is weaker than that from the "real" operon promoter, P_{L11}, which normally occludes P_{L10} (Brückner and Matzura, 1981; C. Squires, pers. comm. λAJN261 lacks, but λ$\underline{rif}^d$18 retains, P_{L11}). The data in Figs 4.1 and 4.2 suggest that if anything P_{L10} is stronger than P_{L11}. However, assuming for the moment that P_{L11} really is required for dominance (for whatever reason), we would expect that expression of rpoBC from any derivative of λ$\underline{rif}^d$18 which has lost the P_{L11} promoter would mimic the λAJN261 rifampicin phenotype, when lysogenised in the appropriate strain.

The structures of two phages, λAJN321 and λAJN363, carrying the 7.6kb BglII fragment from λ$\underline{rif}^d$18 are shown in Fig. 4.6. These two phages,



— Identical DNA sequence excluding the rpoB3 mutation.

+ — All sequences upstream of the HindIII (rplJ) target in λAJN261 must be wild-type (see Fig.3.2). Note also that the precise 5′ endpoint, within rplA, is unknown.

Fig.4.6

Fig. 4.6 A map of the *rpoBC* operon including the sequence analysis of λ*rif*^{d}18, λAJN261 and wild-type DNA (Ovchinnikov *et al.*, 1983; Morgan *et al.*, 1984; Chapter 3). Note that the complete DNA sequence of the *rpoBC* operon in λ*rif*^{d}18 is known except for the distal portion of *rpoB* (Post *et al.*, 1979; Delcuve *et al.*, 1980; Squires *et al.*, 1981; Ovchinnikov *et al.*, 1983; Morgan *et al.*, 1984). The published wild-type DNA sequence, the extent of which is shown, has been found to be identical to λ*rif*^{d}18 (apart from the *rpoB*3 mutation: Ovchinnikov *et al.*, 1983; Morgan *et al.*, 1984).

The structure of the λ derivatives λAJN321 and λAJN363, and the positions of the strong promoters P_{L11} and P_{L10} are also shown.

constructed by cloning this BglII fragment in either orientation into the single BamHI site of vector λ570-BV2 (Klein and Murray, 1979), were kindly donated by Dr A.J. Newman. The BglII restriction site in rplA is very close to the mapped end point of the operon carried by λAJN261 (Newman and Hayward, 1980). More importantly rpoB expression from these phages in the lysogenic state is dependent on the P_{L10} promoter. Unfortunately λAJN321 and λAJN363 do not contain a complete rpoC gene; but I assume rather confidently that this has no bearing on the rif^{d} phenotype. Both these phages are imm21 and integration-deficient; hence lysogens of H105 were isolated after simultaneous infection with λNM1 (immλ $cI^{ts}857$) as a helper. Lysogens were checked for imm21 and temperature sensitivity, and then examined as to their rifampicin phenotype. Both H105(λAJN321) and H105(λAJN363) lysogens were quasi-resistant to rifampicin (see Table 4.1). This contrasts with the rifampicin phenotype of H105(λAJN261), suggesting that the loss of dominance observed in the case of λAJN261 is not due to expression of rpoB from the P_{L10} rather than the P_{L11} promoter. This result strongly suggests that λAJN261 has indeed lost a "second" mutation present in the rplKAJLrpoBC operon of $\lambda rif^{d}18$, which confers dominance upon rpoB3. By inference this mutation must lie within the BglII restriction fragment.

I have confirmed this difference between λAJN261 and both λAJN321 and λAJN363 in another strain of *E. coli* K12, W3110. BM26, a recA56 derivative of W3110 was first constructed as described in 4.2. BM26 was then lysogenised with λAJN261, λAJN321 and λAJN363 as previously described. The resulting lysogens were checked for their resistance to rifampicin; the λAJN261 lysogen was Rif-S, while the λAJN321 and λAJN363 were quasi-resistant (see Table 4.1).

4.7 Discussion

(i) The rifampicin-resistance phenotype of rif^d18.

The results above indicate that when λrif^d18 or certain derivatives are lysogenised into a strain, in which a fully Rif-R phenotype had been expected on the basis of earlier published work, the actual phenotype is what I have termed quasi-resistance. What does this phenotype mean in genetic terms? One hypothesis to explain it is that the dominance of rif^d18 is incomplete, but sufficient to allow the lysogen to survive and multiply long enough, in the presence of rifampicin, for additional mutations to be selected. Such mutations might be rif^r, rif^{s-rcs} or even null mutations in the chromosomal rpoB; or a further rif^r mutation of rpoB in the prophage, increasing the drug-resistance of its product (or its ability to overcome "DNA blockade": cf. Chapter 3). Indeed one can imagine several other kinds of secondary mutation which would confer full Rif-R. If this explanation is true then the fully Rif-R phenotype - (as judged by my protocol for testing rifampicin resistance, described in 4.2) - which is displayed by the H105(λrif^d18)(λ$cI^{ts}857Sam7$) double lysogen provided by Dr J.B. Kirschbaum, could mean that the prophage or the H105 rpoBC operon has acquired one of these additional mutations. Depending upon the precise nature of such further mutation(s), induction of λrif^d18 from a fully Rif-R clone, and relysogenisation into a heat-cured (non-lysogenic) derivative of the same clone, might restore full Rif-R. In fact, when λrif^d18 was induced from the double lysogen, separated from the λ$cI^{ts}857Sam7$ helper, and relysogenised back into H105 in the presence of λNM54 (imm21), a quasi-resistant phenotype was obtained (see Table 4.1). This excludes, for example, stable rif^r or rif^{s-rcs} mutations in the chromosomal rpoB, or stable mutations in the prophage. Instead it points to some

chromosomal mutation which is permissive for dominance, but renders the strain inviable in the absence of λ<u>rif</u>^{d}18 (for example, a null mutation of the chromosomal <u>rpoB</u>, or alteration of some other gene affecting the cells response to the drug; cf. rifampicin-dependent growth: Dabbs, 1982).

If the model proposed above is true then one would expect the "fully Rif-R" colonies to remain Rif-R even after prolonged growth in the absence of rifampicin. To test this, 10 fully Rif-R colonies were picked from the QR growth of the λAJN261/BM9 lysogen on oxoid rifampicin plates, and grown on L-broth agar plates or in L-broth medium. This had no effect on the rifampicin resistance phenotype, ie. all 10 isolates remained fully Rif-R (cf. the original H105(λ<u>rif</u>^{d}18)(λ<u>c</u>I^{ts}857<u>S</u>am7)lysogen). Hence the mutation, although reversed when H105 and λ<u>rif</u>^{d}18 are separated by heat-induction and the lysogen reconstructed, is maintained in a fully Rif-R lysogen despite removal of the drug. One way in which this mutation could be mapped is by P1 transduction from a fully Rif-R lysogenic strain, in which a large number of Tn<u>10</u>(Tet-R) insertions had been made, into a strain showing a QR phenotype (see Table 4.1): preferably the same host strain. Tet-R colonies could then be screened for cotransduction of full Rif-R, and the relevant Tn<u>10</u>-insertion sites would be mapped by conventional methods.

All the strains in which the quasi-resistant phenotype has been observed are Rec^{-}; hence explanations which involve homologous recombination of the phage into the chromosomal <u>rpoB</u> locus can be discounted. The lysogens were purified by 2 to 3 single colony isolations before they were exposed to rifampicin: hence the quasi-resistant phenotype cannot be ascribed to colony impurity.

There are probably two reasons why this phenomenon has not been observed previously. The first is that rifampicin has been used by a number

of groups to select λ*rif*^{d}18 lysogens directly; hence it would be impossible to detect quasi-resistance. Secondly, in the few cases where an *imm*λ selection procedure has been used (*without* rifampicin) to isolate lysogens, the colonies have not been screened for Rif-R in any quantitative way.

As an interesting footnote, comparisons between the rifampicin resistance phenotypes of the various λAJN261 lysogens I constructed (see Table 4.1) suggest that the strains CR63, W3110-del1 and W3110-del2 have a *rif*$^{s-rcs}$ genotype (compare the H105, W3110, AJN10 and AJN1 lysogens with the CR63, W3110-del1 and W3110-del2 lysogens). W3110-del2 is a derivative of *E. coli* K12 which expresses a fully functional β polypeptide which has an increased mobility on SDS polyacrylamide gels (Nene and Glass, 1984). This strain, kindly provided by Dr R.E. Glass, has a viable deletion of approximately 165bp within the distal portion of *rpoB*. I constructed a *recA*56 derivative and λAJN261 lysogen of this strain as described above.

(ii) The *rif*^{d}18 genotype: a single or double mutation required for the quasi-resistant phenotype?

Most of the previously reported evidence suggests that *rif*^{d}18 is one of a special class of single *rif*^{r} mutations, which occur at a low frequency (see Chapter 3). However, the difference between the rifampicin phenotypes of λ*rif*^{d}18, λAJN321, and λAJN363 lysogens and those of λAJN261 lysogens require to be explained; especially as my sequence analysis (Chapter 3) has proven that λAJN261 retains *rpoB*3 (the mutation reported, by Ovchinnikov *et al.* (1983), to be solely responsible for the *rif*^{d}18 genotype). One proposed explanation holds that λAJN261 has lost a second, *cis*-acting regulatory mutation which in λ*rif*^{d}18 increases the expression of the resistant (*rpoB*3) gene relative to that of the sensitive (*rpoB*$^{+}$) gene in heterodiploids. The protein synthesis analyses of the λ*rif*^{d}18 and λAJN261 lysogens of W3110-del1 and CR63 clearly disprove this hypothesis in its simplest form, because these lysogens synthesise the λ*rif*^{d}18-encoded β polypeptide at a rate approximately equal to that of the host chromosome's own *rpoB*-product. I further demonstrated that addition of rifampicin had no detectable effect on the relative expression of the β polypeptides in the W3110-del1/λ*rif*^{d}18 lysogen. Interestingly the prophage-encoded β subunit in the W3110-del1 and CR63 λAJN261 lysogens was synthesised at a somewhat higher rate than the "chromosomal" β, although again rifampicin had no detectable differential effect. This difference in degree of expression between β_{261} and $\beta_{rif^{d}18}$ in the lysogens presumably reflects the fact that *rpoB* is being expressed from two different promoters (P_{L11} for λ*rif*^{d}18, P_{L10} for λAJN261).

It must be noted that one cannot necessarily extend the results of the expression analyses presented in this chapter, to draw conclusions about the difference in rifampicin resistance phenotypes between λ*rif*^{d}18 and λAJN261 when lysogenised in other strains. This *caveat* arises because the CR63 and W3110-dell hosts behave as S-recessive, so that the two phages produce an identical rifampicin phenotype. This point will be reconsidered below.

A model difficult to exclude states that after rifampicin addition to a *rif*^{d}18/*rif*^{s} heterodiploid, the initially equal distribution of sensitive and resistant RNA polymerase molecules between the "actively transcribing" and the (substantial) "currently idle" pools (Matzura *et al.*,

1973; Iwakura et al., 1974; Matzura, 1980) is altered such that the $rif^{d}18$ RNA polymerase becomes preferentially active. However, against this "redistribution" hypothesis is the evidence that when RNA synthesis by an H105 ($\lambda rif^{d}18$)($\lambda cI^{ts}857Sam7$) lysogen was studied by pulse-labelling with [5 - ^{3}H] - uridine in vivo, labelling reached a minimum of 56% (relative to a drug-free control) 8 minutes after exposure of the lysogen to rifampicin (Newman et al., 1979). This is essentially the same result as that obtained for $rpoB^{+}$/rpoB-Rif-R (phenotype Rif-S) and rpoB-Rif-Srcs40/Rif-R (phenotype Rif-R) heterodiploid cells, known to be synthesising sensitive and resistant RNA polymerase in nearly equal amounts (Hayward et al., 1973). Similar experiments by Tittawella (1976a) had produced closely similar results for a $rif^{s}{}_{KM7}/rif^{d}18$ (phenotype Rif-R) heterodiploid strain ($rif^{s}{}_{KM7}$ being a "reversibly sensitive" rpoB allele). More importantly Tittawella's results suggested that there was no alteration in the ratio of the resistant to sensitive enzyme even after prolonged growth in the presence of rifampicin (ibid.). Finally, note that the above results of Newman et al. (1979) are clearly compatible with the hypothesis that there is not an unusually high proportion of resistant RNA polymerase in the cells of a heterodiploid $rpoB^{+}/rif^{d}18$ strain; i.e. that my observations with the rpoB-CR63/- and rpoB-del1/$rif^{d}18$ heterodiploids can in fact be extrapolated to the $rpoB^{+}/rif^{d}18$ strains, despite the recessiveness of the CR63 and W3110-del1 alleles. However, some doubt must remain. The results of Hayward et al. (1973) were clearcut on account of the unusually high drug-resistance of the rif^{r}(rpoB70) β polypeptide product involved in their experiments. The $rif^{d}18$ product is very much less resistant than that of rpoB70 and, further, binds rifampicin more strongly than the sensitive enzyme in vitro (Smith, 1982); as a result the experiments of

Kirschbaum and Konrad (1973), Tittawella (1976a) and Newman et al. (1979) cannot be so unambiguously interpreted.

What then is the nature of the rif^d18 genotype? An answer to this question may come from a complete analysis of the difference in resistance properties between λrif^d18 and λAJN261 heterodiploid lysogens. Preliminary evidence presented in this chapter has shown that two phages derived from λrif^d18, λAJN321 and λAJN363, which are structurally very similar to λAJN261 upstream of rpoB (but carry only part of rpoC) display the rif^d18 quasi-resistance phenotype in both H105 and W3110 (see Table 4.1). In contrast λAJN261 lysogens of the same strains are Rif-S. Since all three prophages must express rpoB from P_{L10}, and lack all the DNA upstream of rplA, it seems clear that λAJN261 has lost, through the recombination with wild-type DNA which occurred during its construction, a second mutation which is present in the BglII('rplA - rpoC')fragment of λrif^d18, and which is necessary for quasi-resistance: Fig. 4.6 summarises the relevant information. The most plausible hypotheses for the site and nature of the second mutation are: 1) a structural mutation within rpoB3, directly or indirectly affecting the ability of RNA polymerase containing its rifampicin-resistant β product to compete for "blockaded" promoters. Such a mutation would have to map downstream of rpoB3 (see Fig. 4.6) and consequently would have required multiple crossovers, when λAJN261 was first generated, to remove it; 2) a mutation in the P_{L10} promoter (or, for example, an up-mutation in the unmapped P_{L7} promoter: Ma et al., 1981) increasing the expression of the rpoB3 (resistant) allele relative to the sensitive allele in heterodiploids; 3) some mutation (presumably upstream of the EcoRI target in rplL) which increases the stability and/or efficiency of translation of the mRNA encoding the rpoB3-product.

All of the evidence summarised in the immediately preceding paragraphs suggests that hypotheses 2) and 3) are incorrect; but they cannot be entirely excluded, for the reasons given. One way in which the putative second mutation could be mapped would be to replace restriction fragments of λAJN261 with the corresponding fragment from λ*rif*^{d}18, and then screen *in vitro* recombinants for *rif*^{d}.

Although it is apparent that the *rif*^{d}18 genotype is complex, the same may not apply to the other *rif*^{d} mutations which have been isolated. An interesting experiment would be to test the rifampicin phenotypes of other specialised transducing phages carrying *rif*^{d} mutations, such as λ*rif*^{d}47. Are they also quasi-resistant, or is this phenotype peculiar to λ*rif*^{d}18? A more detailed analysis of the quasi-resistant phenotype is required, and could yield further insights not only into the nature of rifampicin resistance in *E. coli*, but into the detailed mechanism of RNA polymerase/promoter DNA interactions which may be involved in determining the outcome of DNA blockade by drug-inactivated polymerase molecules.

CHAPTER 5

SI-nuclease analysis of rpoBC operon expression

5.1 Introduction

A partial uncoupling of rpoBC from rplKAJL transcription is known to occur under certain conditions: e.g. after challenge with the antibiotic rifampicin (Blumenthal and Dennis, 1978) or during induction of the stringent response by partial amino acid starvation (Maher and Dennis, 1977). Similar effects on rpoBC expression are observed when certain rpo^{ts} mutants or most ts-suppressed rpo-amber mutants are transferred to a partially restrictive temperature (Blumenthal and Dennis, 1980b; Little and Dennis, 1980).

Treatment with the antibiotic rifampicin strongly stimulates rpoBC transcription, but that of rplKAJL only weakly (Blumenthal and Dennis, 1978). The rates of β and β' protein syntheses at first mimic the transcriptional changes. However, the protein stimulation is transient, reaching a peak at 30 minutes. In contrast, examination of the results (ibid.) suggests that rpoBC transcription may remain at the higher level up to 60 minutes, although the authors did not comment upon this observation, and it is based on limited data. The partial transcriptional uncoupling clearly arises, at least partly, from increased readthrough of the partial terminator, t_{L7}, which lies in the intercistronic space between rplL and rpoB (Howe et al.,1982; Newman et al., 1982; Fukuda and Nagasawa-Fujimori, 1983). Our unpublished observations on lacZ fusion plasmids have also suggested that rifampicin might activate the strong promoter, P_{L10}, which lies in the intercistronic space between rplA and rplJ, and is normally occluded

by P_{L11} (Brückner and Matzura, 1981; C. Squires, pers. comm.). There is no direct evidence as to whether t_{L7}, or P_{L10}, or both are involved in the uncoupling of rpoBC from rplKAJL transcription observed when the stringent response is induced (Blumenthal et al., 1976; Reeh et al., 1976; Blumenthal and Dennis, 1980a). Increased readthrough of t_{L7} is almost certainly implicated in the stimulation of rpoBC transcription which is observed when certain rpo^{ts} or most ts-suppressed rpo-amber mutants are exposed to partially restrictive temperatures (Blumenthal and Dennis, 1980b; Little and Dennis, 1980). Interestingly, in these two studies of rplJL transcription was also stimulated, though to a lesser extent than that of rpoBC. Although no measurements of rplKA transcription were made, it is tempting to speculate that P_{L10} stimulation may have a role to play in the increased expression of rpoBC demonstrated under these conditions.

In the work described in this chapter I have investigated the roles of P_{L10} and t_{L7} in the uncoupling of rpoBC - from rplKAJL-mRNA synthesis, by applying SI-nuclease mapping to examine transcription in vivo through the DNA regions carrying these signals, after partial amino acyl-tRNA limitation, or treatment with rifampicin.

5.2 Construction of M13 probes

The M13 DNA probes for the SI-analysis were constructed by ligation of the appropriate restriction endonuclease fragment, which contains the relevant region of the rplKAJLrpoBC operon, into the M13 cloning vectors mp10 and mp11 (Messing and Vieira, 1982). A map of all the probes used is shown in Fig. 5.1. I required the fragment in both orientations in M13 to obtain, from single-stranded M13 DNA preparations, a positive (+ve), which hybridises to the sense mRNA, and a negative (-ve)

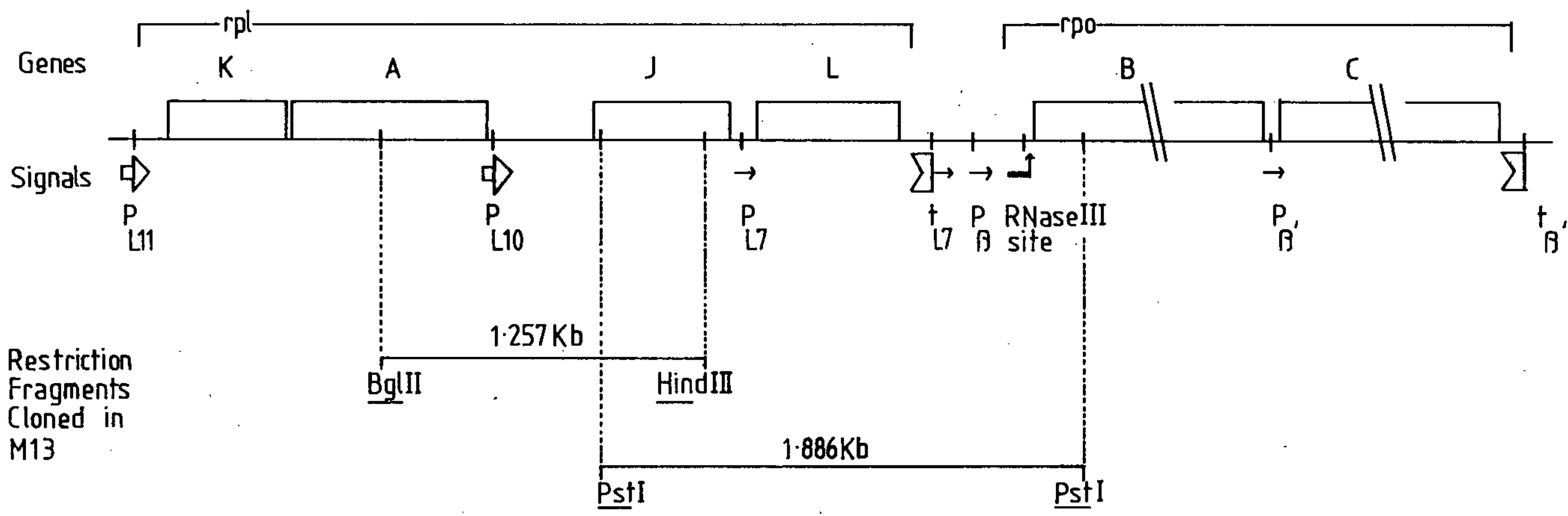


Fig. 5.1

Fig. 5.1 A map of the rplKAJLrpoBC operon including a number of potential regulatory signals: [strong promoter symbol], a strong promoter; ⟶ a weak promoter; [partial terminator symbol] a partial terminator and [RNaseIII site symbol], an RNaseIII processing site (Newman *et al.*, 1979; Post *et al.*, 1979; An and Friesen, 1980; Barry *et al.*, 1980; Ma *et al.*, 1981). Note that the precise locations of the weak promoters P_{L7}, P_{β} and $P_{\beta'}$ are unknown. Also shown are the restriction fragments, and the regions they contain, cloned into M13 for the SI analyses (described later).

probe, which would hybridise to any antisense mRNA. Hence, where two different restriction sites were used to provide the DNA fragment, it was ligated into mp10 and mp11. However, where two identical restriction sites were used the fragment was ligated only into mp10, and representatives of both orientations were identified by DNA sequencing. All the probes used in this study were characterised by restriction analysis (Fig. 5.2) and DNA sequencing. The restriction and sequencing analyses confirmed that all the probes contained inserts of the correct size and orientation (see Fig. 5.1). If there are any additional inserts in the probe then these must be of insignificant size and lie upstream of the main insert.

5.3 Transcription in the P_{L10} region of unconstrained *E. coli*

RNA isolated from ED3867, a derivative of *E. coli* K12, was hybridised to excess +ve and -ve single-stranded M13 probes, carrying the p_{L10} region on a *Bgl*II/*Hin*dIII restriction fragment (Fig. 5.1), and then digested with SI-nuclease. SI-resistant hybrids were separated on neutral 1.5% or 2% (w/v) agarose gels, transferred to nitrocellulose and hybridised to [^{32}P]-pNA38 DNA (described in the legend of Fig. 5.3). Hybrids were highlighted by autoradiography. An example of one such experiment is shown in Fig. 5.3A. As can be seen in track 2, a major hybrid (a) whose size is approximately 1.26kb is produced with the +ve probe. This is equivalent in size to the insert in the M13 probes (track 1), and therefore represents the full-length "readthrough" transcript. A number of smaller hybrids were also obtained, of estimated sizes, (b) 1.09kb, (c) 0.81kb, (d) 0.72kb, (e) 0.67kb, (f) 0.59kb, (g) 0.54kb and (h) 0.45kb. Hybrids (d) and (e),and (f) and (g) were poorly resolved, and hybrid (b) was very faint. Apart from hybrid (b)

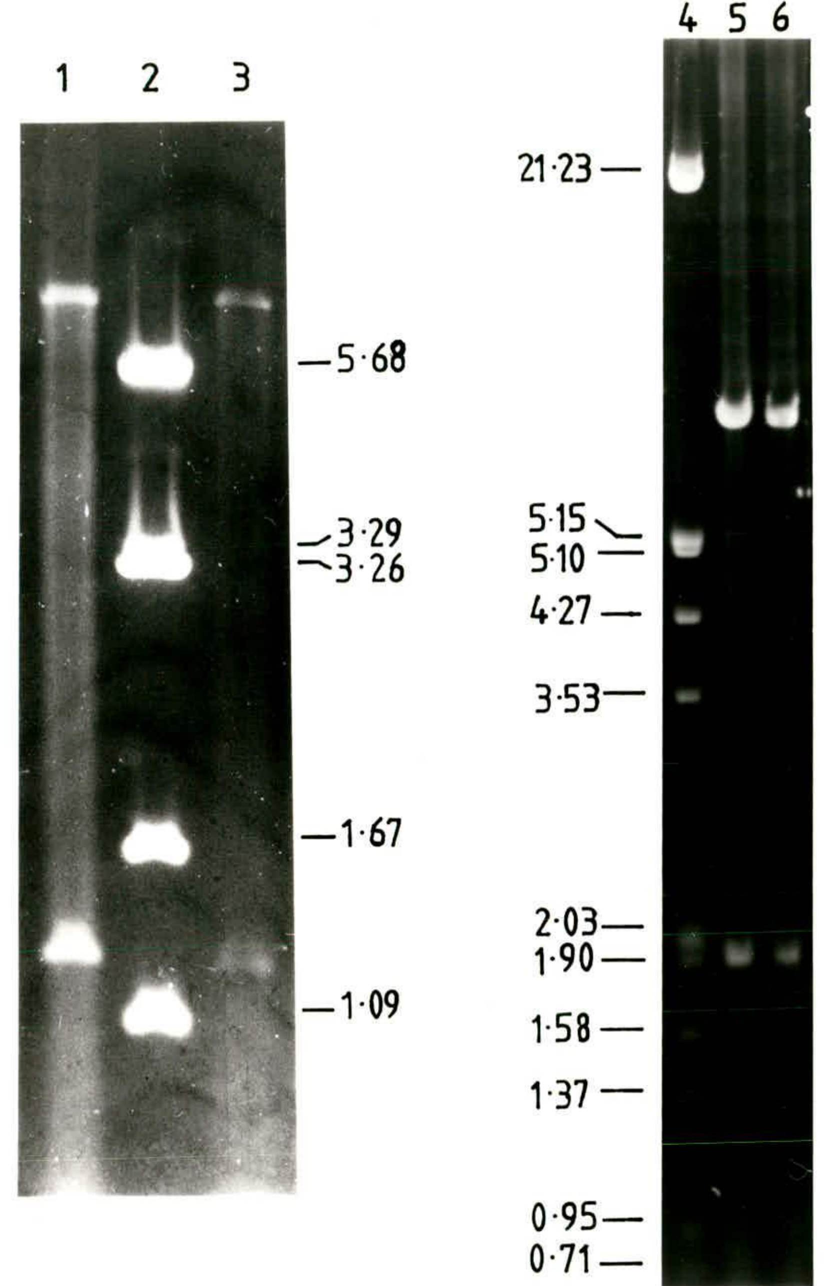


Fig. 5. 2

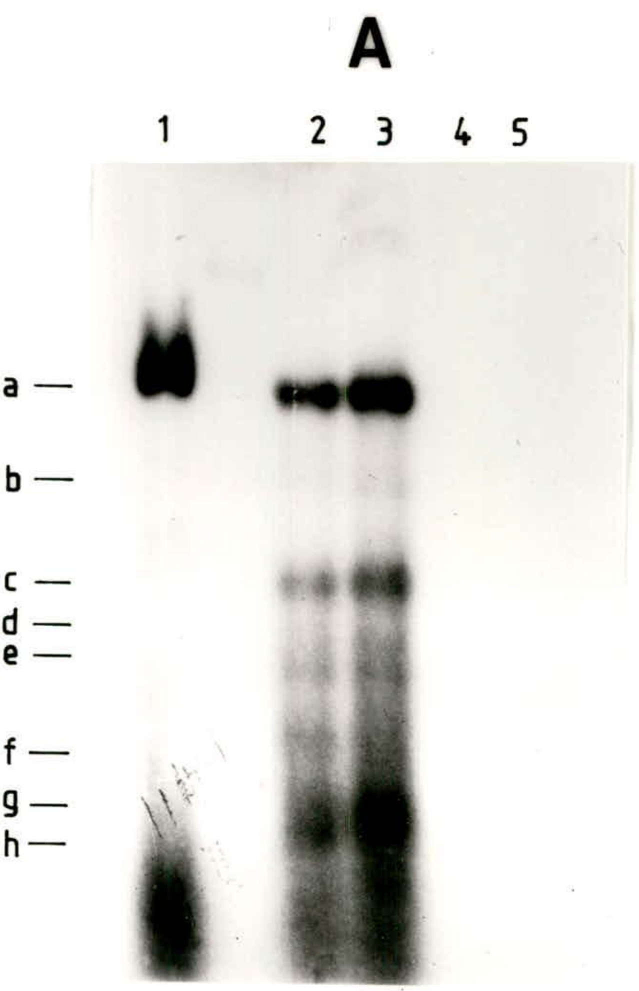
Fig. 5.2 Restriction analysis of the M13 hybridisation probes.

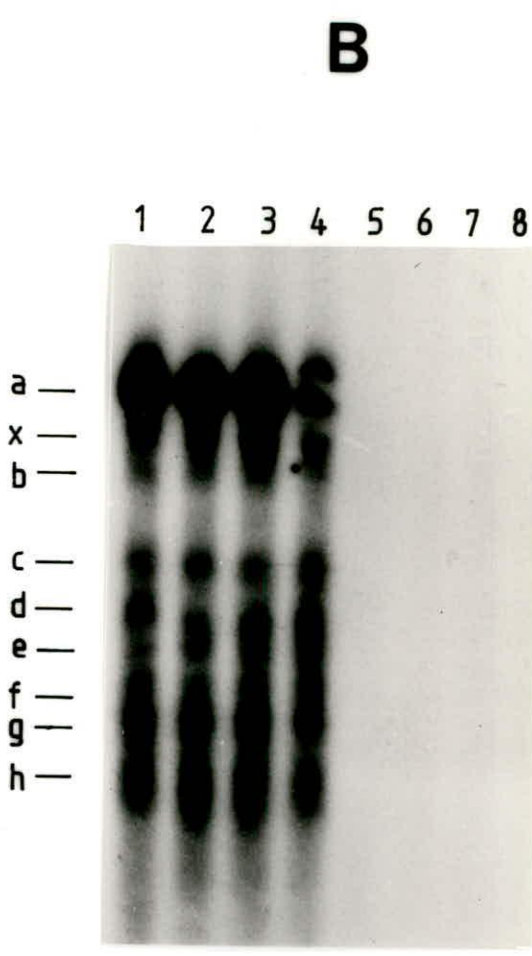
1% agarose gel electrophoresis.

Track number	DNA	Restriction endonuclease
1	BglII/HindIII insert in mp10	EcoRI + HindIII
2	pHR14	PstI
3	BglII/HindIII insert in mp11	EcoRI + HindIII
4	λcI^{ts}Sam7	EcoRI + HindIII
5	PstI insert in mp10	PstI
6	PstI insert in mp10	PstI

Notes:

1. The marker tracks are pHR14/PstI and λcI^{ts}Sam7/EcoRI/HindIII. The sizes of the marker DNA fragments are shown in kb.
2. The -ve and +ve probes for the P_{L10} region (tracks 1 and 3 respectively) were digested with EcoRI (15bp outside the BglII/HindIII insert) because the BglII sites were destroyed in cloning.
3. The -ve and +ve probes for the t_{L7} region are in tracks 5 and 6, respectively.





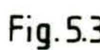


Fig.5.3

Fig. 5.3 Autoradiographs of SI-resistant hybrids formed between the 'rplA P_{L10} rplJ' single-stranded M13 probes and RNA isolated from A) ED3867, and B) EMR3 ($valS^+$) and AB4141 (valS(Ts)).

A	Track	Probe/Source of RNA
	1	+ve/(-ve DNA probe)
	2	+ve/10 min., no Rif
	3	+ve/10 min., + Rif
	4	-ve/10 min., no Rif
	5	-ve/10 min., + Rif

B	Track	Probe/Source of RNA
	1	+ve/10 min. $valS^+$
	2	+ve/10 min. valS(Ts)
	3	+ve/25 min. $valS^+$
	4	+ve/25 min. valS(Ts)
	5	-ve/10 min. $valS^+$
	6	-ve/10 min. valS(Ts)
	7	-ve/25 min. $valS^+$
	8	-ve/25 min. valS(Ts)

Notes:

1 "+ve" signifies the DNA probe complementary to rpl mRNA; "-ve" the opposite strand.

2 For sizes of hybrids a-h and x see text.

3 RNA was isolated, in A: from ED3867 10 minutes after rifampicin (Rif) addition, and from a drug-free control and in B: from $valS^+$ and valS(Ts) strains, 10 or 25 minutes after a temperature shift from 30°C to 38°C. It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. After fractionation of SI-resistant hybrids by (1.5%) agarose gel electrophoresis, they were transferred to nitrocellulose, then labelled using [^{32}P]-pNA38 DNA as probe. pNA38 is pBR322 containing the 'rplKA-rplJ' (EcoRI/HindIII) fragment. It was constructed and kindly provided by Dr A.J. Newman (Newman et al., 1982).

these results are in good agreement with those of Brückner and Matzura (1981) for the P_{L10} region. In their study they used two probes which extended from the EcoRI site in rplK to either the HindIII site in rplJ, or the EcoRI site in rplL. They hybridised excess RNA (which in theory could yield misleading multiple-hybrid products following SI-digestion) to a denatured double-stranded probe. Hence my results, using excess single-stranded probe, provide a useful confirmation of their data. My technique can, in addition, distinguish between normal operon transcription and "anti-mRNA" transcripts, complementary to the opposite DNA strand. On the other hand, because I did not use a pair of probes with one common and one different end point, Brückner and Matzura's data are essential for my interpretation.

Further confirmation of these results for "unconstrained" bacteria came from the hybridisation of RNA isolated from E.coli K12 EMR3 (AB4141 $ValS^+$), 10 or 25 minutes after a temperature shift from 30°C to 38°C, to the +ve and -ve "P_{L10}" probes (Fig. 5.3B - tracks 1 and 3). Although these are not strictly speaking unconstrained cells (due to the temperature shift) the hybrids obtained were qualitatively very similar to those for ED3867, and are better resolved. In addition to hybrid (b) a hybrid (x) of approximate size 1.15kb was observed (contrast ED3867). Both of the bands, (b) and (x), are unexplained and were not observed by Brückner and Matzura.

The main conclusion from my data and those of Brückner and Matzura is that the great majority of transcription through this region initiates at P_{L11} (yielding the full-length hybrid). Thus, P_{L10} is severely occluded. The presence of most minor bands is indicative of slow endonucleolytic processing of the transcripts from this region. Bands (e) and (f), and (d) and (g) are thought to represent RNA processing sites at

or around nucleotides 1485 and 1615 respectively (the scale of Post et al. (1979) is used), in the intercistronic region between rplA and rplJ (Fig. 5.4). This hypothesis is further confirmed by the observation that bands (d) and (g), and (e) and (f), are present in rough molar equivalences. In contrast weak transcriptional initiation and termination provide the more likely explanations for bands (c) and (h) respectively (see Fig. 5.4). This is suggested for two reasons: 1) P_{L10} would allow initiation at nucleotide 1347-1349; and 2) there appears to be more of hybrid (h) than of hybrid (c), especially in molar terms. However, the possibility that these bands reflect RNA processing at 1340, followed by rapid digestion of hybrid (c), cannot be excluded. Thus it is possible that P_{L10} is so completely occluded by P_{L11} that it does not contribute any detectable transcription of downstream genes. Note also that there was no detectable transcription in the opposite, "anti-sense" direction under these conditions (Fig. 5.3A, tracks 4 and 5; Fig. 5.3B, tracks 5 to 8).

5.4 Transcription in the P_{L10} region following rifampicin treatment

The strain ED3867 was grown, as before, to mid-log phase. The culture was then incubated for a further 10 or 25 minutes in the presence of rifampicin (10μg/ml), after which the RNA was isolated and treated as before. Hybrids obtained with the +ve P_{L10} probe are shown in Fig. 5.3A, track 3. Densitometric analysis of the hybrids formed with RNA isolated after 10 minutes treatment showed no change in the relative amounts of individual hybrids, compared with the rifampicin-free control (track 2). Therefore there is no evidence of increased initiation at P_{L10} (band (c) is not more prominent), nor of decreased termination near P_{L10} (band (h) is not reduced). The other main feature of this experiment is

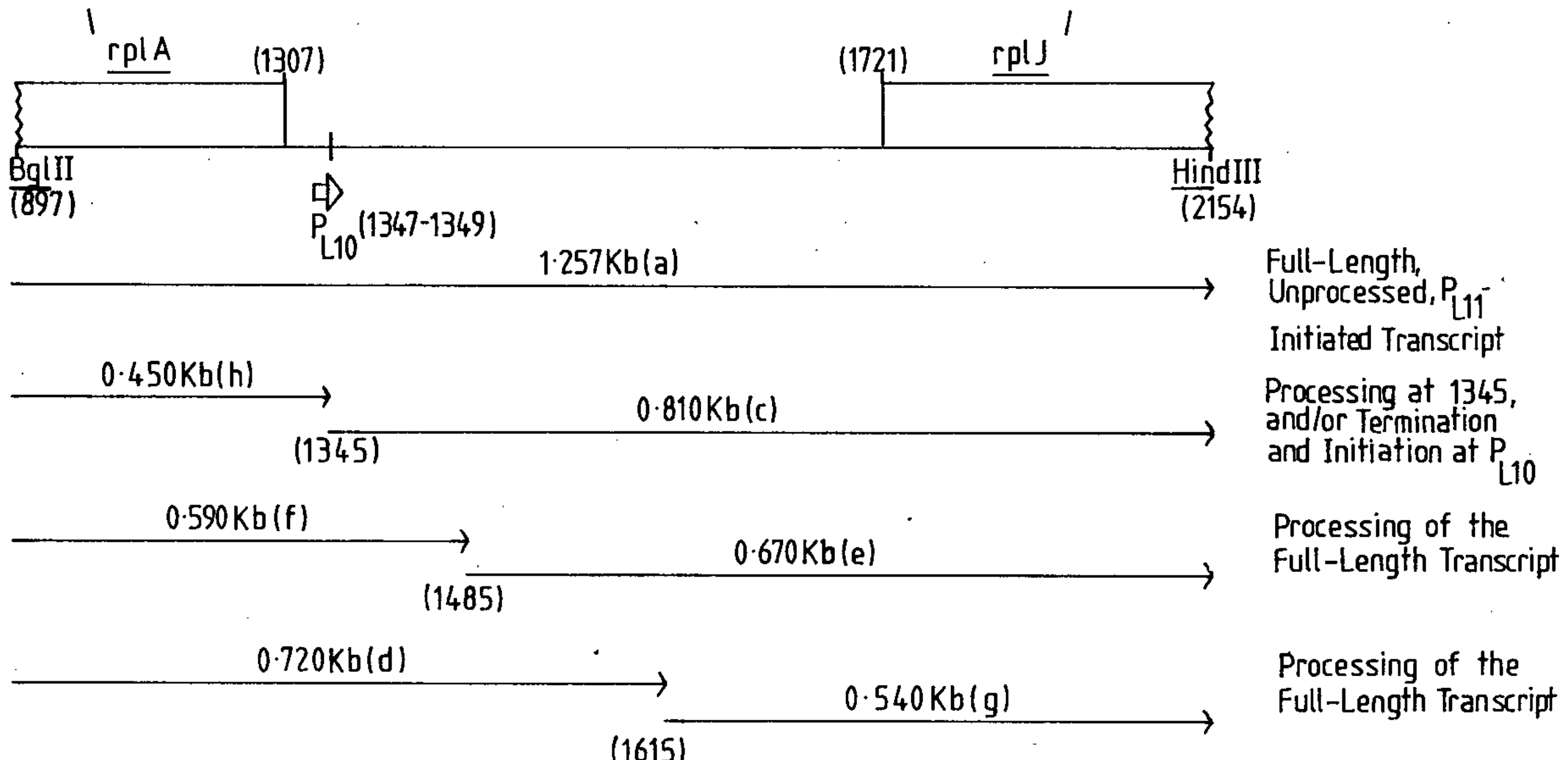


Fig. 5.4

Fig. 5.4 Transcription and RNA-processing in the *rplA-rplJ* intercistronic region. Numbers in brackets reflect the nucleotide scale of Post *et al.* (1979). Letters in brackets identify the hybrids observed in Fig. 5.3A and B. The interpretations shown on the right are those of Brückner and Matzura (1981), supported by my own data.

that the -ve M13 probe did not reveal any "convergent" transcription (tracks 4 and 5), even after prolonged exposure of the autoradiograph (data not shown).

To confirm that ββ' protein synthesis <u>is</u> stimulated under these conditions, ED3867 was grown as before (except in minimal medium) and after the relevant treatments cells were pulse-labelled with L-[^{35}S]-methionine. The labelled protein extract was fractionated on a 5-15% SDS polyacrylamide gradient gel. An autoradiograph is shown in Fig. 5.5. Densitometric analysis of tracks 1, 2 and 4 versus 5 indicated that a possible stimulation of ββ' synthesis had occurred, relative to a number of other cellular proteins, 10 minutes after treatment with rifampicin (cf. Hayward and Fyfe, 1978a) although results were inconclusive here. The mRNA analyses demonstrated, in contrast, that no stimulation of P_{L10} (nor changes in termination or processing of RNA in its vicinity) occurred at this time point. Hence it is unlikely that alterations in the quantity or quality of mRNA, mediated by sites in the '<u>rplA</u>-P_{L10}-<u>rplJ</u>' region, explain the ββ'-stimulation observed. There is some preliminary evidence to suggest that, after 25 minutes treatment with rifampicin, initiation at P_{L11} may have decreased and/or the general rate of mRNA processing may have increased (Fig. 5.6, tracks 1 and 2). It is relevant to note that the stimulation of β and β' syntheses had passed its peak by this time (Fig. 5.5; densitometric analysis of tracks 3 and 6). Interestingly there is an indication of convergent transcription, giving rise to hybridisation with the -ve M13 probe (Fig. 5.6, track 4), after 25 minutes of drug treatment. None whatever is detectable without rifampicin treatment, nor after 10 minutes drug treatment (Fig. 5.3A, track 5). The hybrids formed are heterogeneous, in the size range of 300-600bp.

Fig.5.5

Fig. 5.5 An autoradiograph of L-[^{35}S]-methionine labelled proteins isolated from the *E. coli* strain ED3867. Tracks 1-3 represent the 0, 10 and 25 min. drug-free controls respectively. Tracks 4-6 represent proteins labelled after the same intervals following rifampicin (10μg/ml) addition. Proteins were fractionated on a 5-15% SDS polyacrylamide gradient gel.

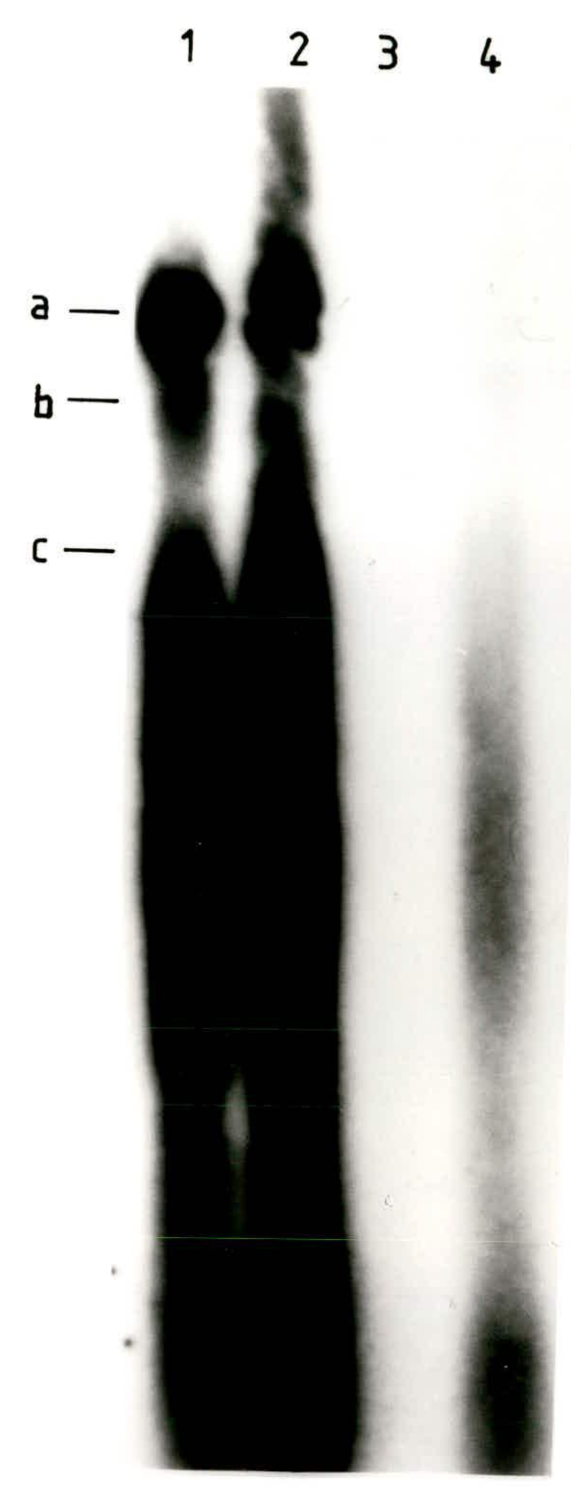


Fig. 5.6

Fig. 5.6 An autoradiograph of SI-resistant hybrids formed between the '*rplA* P_{L10} *rplJ*' single-stranded M13 probes and RNA isolated from ED3867.

Track	Probe/Source of RNA
1	+ve/25 min., no Rif
2	+ve/25 min., + Rif
3	-ve/25 min., no Rif
4	-ve/25 min., + Rif

Notes:

1 "+ve" signifies the DNA probe complementary to *rpl* mRNA; "-ve" the opposite strand.

2 For sizes of hybrids a-c see text.

3 RNA was isolated from ED3867 25 minutes after rifampicin (Rif) addition, and from a drug-free control. It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. The SI-resistant hybrids were fractionated on a 2% agarose gel, transferred to nitrocellulose, and labelled using [^{32}P]-pNA38 DNA (see Fig. 5.3).

5.5 Transcription in the P_{L10} region following amino acyl-tRNA limitation

Induction of the stringent response was achieved (see below for verification) by shifting the temperature of the valS (Ts) strain AB4141 from 30°C to either 38°C, or 42°C. RNA isolated 10 or 25 minutes after the temperature shift to 38°C from the valS (Ts) strain and the isogenic $valS^+$ derivative (EMR3) displayed no detectable change in the pattern of transcription through the P_{L10} region (Fig. 5.3B). Although hybrid (a) shows a reduction in track 4 this is almost certainly an artefact of the Southern transfer step, and indeed has not been observed in repeat experiments. As can also be seen, the -ve probe has not detected any significant "convergent" transcription (and certainly no alterations of its level) under these conditions (tracks 5 to 8).

Similarly, after a temperature shift to 42°C there is again no change in the transcription pattern for either strain, at the same time points (Fig. 5.7, tracks 1 to 4). However, in contrast to the 38°C temperature shift results, the negative probe in this experiment has detected heterogeneous hybrids in the 200bp range, using RNA isolated 10 minutes after shifting the valS (Ts) strain to 42°C (track 6). These have increased in size to the 300-600bp range, 25 minutes after the shift (track 8). Again nothing significant could be detected using RNA from the $valS^+$ strain (tracks 5 and 7), even after prolonged exposure of the autoradiograph. [^{3}H]-uridine pulse-labelling studies showed that at 42°C (although not at 38°C) a strong reduction in stable RNA synthesis occurred in the valS (Ts), but not the $valS^+$ strain, as expected if limitation of valyl-$tRNA^{val}$ synthesis in the former strain was producing a strong stringent response. These temperatures have previously been shown to elicit a strong stringent response (as judged by RNA and protein accumulation, and ppGpp synthesis) in another *E. coli* strain JF858 carrying the valS (Ts) mutation (Reeh *et al.*, 1976).

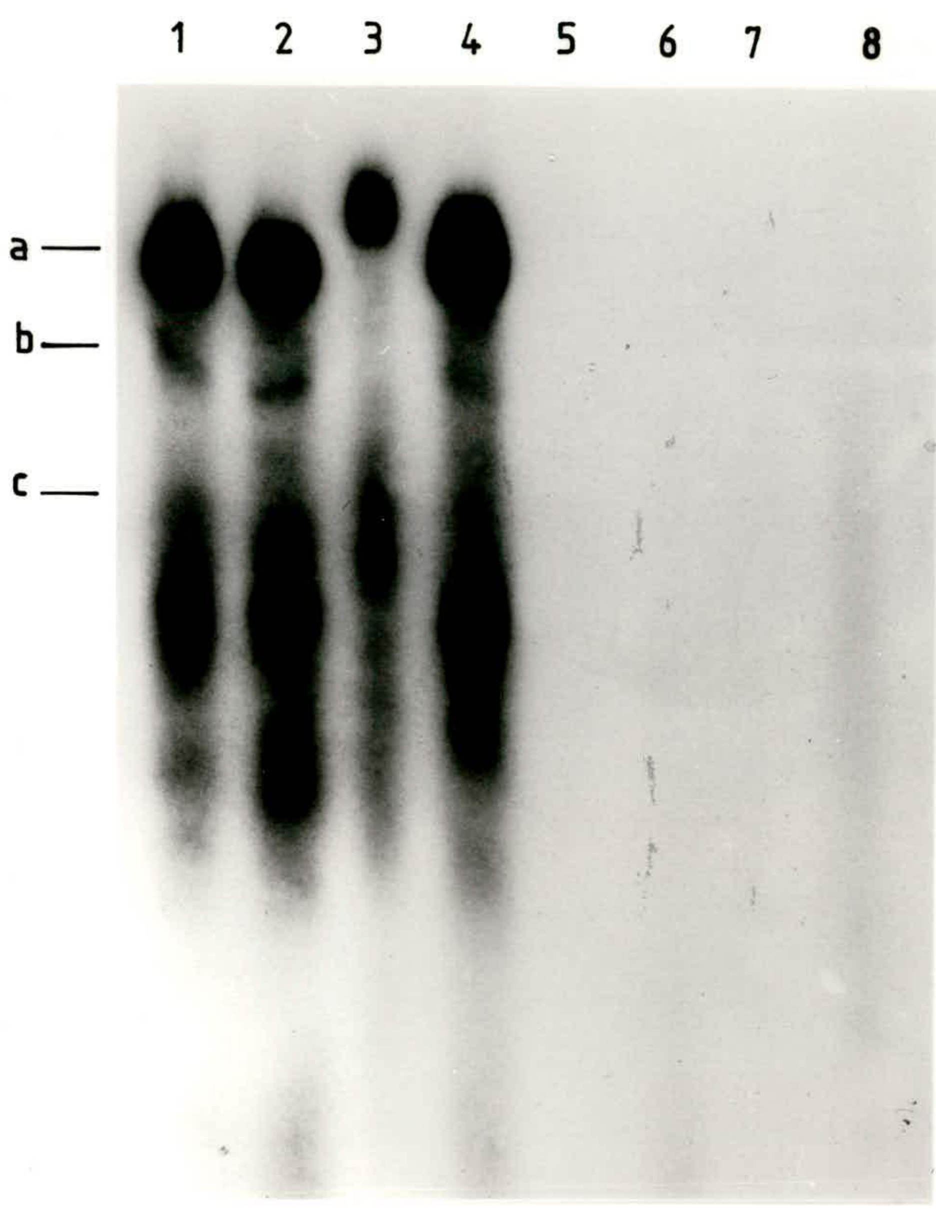


Fig. 5.7

Fig. 5.7 An autoradiograph of SI-resistant hybrids formed between the '*rplA* P_{L10} *rplJ*' single-stranded M13 probes and RNA isolated from EMR3 (*valS*$^{+}$) and AB4141 (*valS*(Ts)).

Track	Probe/Source of RNA
1	+ve/10 min. *valS*$^{+}$
2	+ve/10 min. *valS*(Ts)
3	+ve/25 min. *valS*$^{+}$
4	+ve/25 min. *valS*(Ts)
5	-ve/10 min. *valS*$^{+}$
6	-ve/10 min. *valS*(Ts)
7	-ve/25 min. *valS*$^{+}$
8	-ve/25 min. *valS*(Ts)

Notes:

1 "+ve" signifies the DNA probe complementary to *rpl* mRNA; "-ve" the opposite strand.

2 For sizes of hybrids a-c see text.

3 RNA was isolated from EMR3 (*valS*$^{+}$) and AB4141 (*valS*(Ts)) 10 or 25 minutes after a temperature shift from 30°C to 42°C. It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. The SI-resistant hybrids were fractionated on a 2% agarose gel, transferred to nitrocellulose, and labelled using [^{32}P]-pNA38 DNA (see Fig. 5.3).

5.6 Transcription in the rplL-rpoB intercistronic region in unconstrained *E. coli*

To study transcription through this region derivatives of M13 mp10 were constructed, containing the appropriate 1.9kb *Pst*I fragment in both orientations (Fig. 5.1). This region of the operon contains a variety of interesting features including the partial terminator, t_{L7}; the RNaseIII processing site; P_{β}; and probably P_{L7} (Barry *et al.*, 1979; Newman *et al.*, 1979; Barry *et al.*, 1980; An and Friesen, 1980). The major RNA species expected for this region, in a wild-type strain of *E. coli*, are shown in Fig. 5.8. All these predictions are based on earlier studies of this region by Barry *et al.* (1980), using pairs of probes with one common and one different end-point, and by Fukuda and Nagasawa-Fujimori (1983), using a probe labelled at the 3'-end. The transcripts shown are the full-length, unterminated, unprocessed transcript; the t_{L7}-terminated transcript (expected to be the major species, as the terminator is 80-85% efficient); and RNaseIII-processed derivatives of the readthrough transcript (cut at two sites). Apart from the hybrid generated by the full-length transcript the remainder of the hybrids obtained with the *Pst*I probe have very similar mobility on agarose gels. However, a much simpler pattern of only two hybrids, corresponding to the terminated transcript and the full-length transcript, is predicted for an RNaseIII$^-$ strain. Hence RNA from a normal RNaseIII$^+$ strain (A19) and its virtually isogenic RNaseIII$^-$(*rnc*) derivative (AB301-105: see Table 2.1) was isolated, and hybridised to the +ve and -ve *Pst*I probes to confirm the expected transcriptional pattern in unconstrained *E. coli*; and also to identify a suitable strain in which to perform SI-analysis of transcription through t_{L7}, under conditions known to uncouple *rpl* from *rpoBC* transcription. The results

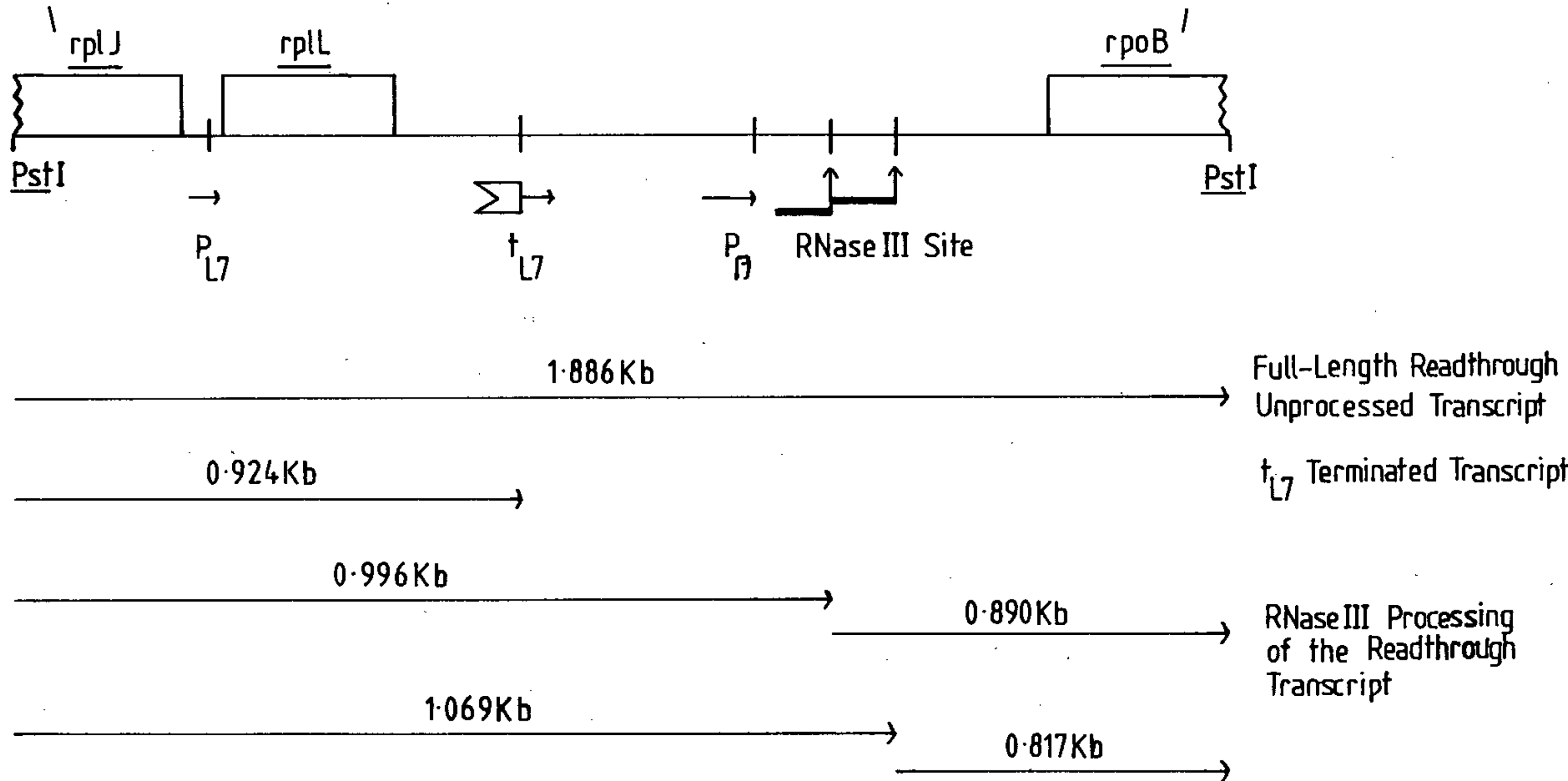


Fig.5.8

Fig. 5.8 The mRNA species complementary to the '*rplJL* t_{L7} *rpoB*' *Pst*I fragment, as predicted by the work of Barry *et al.* (1980) and Fukuda and Nagasawa-Fujimori (1983). ⟶ indicates a weak promoter; ⟩⊢→ a partial transcriptional terminator and ▬↑ an RNaseIII processing site (Newman *et al.*, 1979; An and Friesen, 1980; Barry *et al.*, 1980; Ma *et al.*, 1981). Note that the precise locations of P_{L7} and P_{β} are unknown.

are shown in Fig. 5.9. The $RNaseIII^+$ pattern obtained with the +ve probe (track 5) is complicated, with a number of poorly resolved hybrids present (as expected) in the 0.8 - 1.0kb size range. Evidently RNA-processing in this region is much more rapid than between rplA and rplJ. Hybrid (a) has a size of 1.88kb, and is therefore the full-length unprocessed transcript. A much simpler pattern, in contrast, was obtained from the $RNaseIII^-$ strain, track 4. Apart from hybrid (a) only two other hybrids, (b) and (c), of respective sizes 1.48kb and 0.91kb, were observed. Hybrid (c) has the correct mobility for the expected t_{L7}-terminated transcript (see Fig. 5.8). However hybrid (b), although admittedly a minor product, was unexpected. The most likely explanation is that this hybrid represents initiation at the weak promoter P_{L7}, for which tentative evidence exists from cloning studies in λ vectors (Newman et al., 1979; Ma et al., 1981) and lacZ fusion experiments (Barry et al., 1979). These studies could not map P_{L7} accurately; it was clear only that it must lie between rplL and the HindIII target in rplJ. If in fact it lies between rplJ and rplL, initiation at this site would yield a hybrid of approximate size 1.45kb. P_{L7}-initiated transcript would not have been detected in the earlier studies, because of the probes used. Indeed it is not observed among the $RNaseIII^+$ strain products here (track 5), presumably because of processing of an already minor transcript. Other explanations for hybrid (b) are possible, and a more detailed investigation using different probes would be required to map precise start and/or endpoints in the region. No anti-sense transcription was detected with the -ve probe, tracks 2 and 3, for either the $RNaseIII^+$ or $RNaseIII^-$ strain under these conditions.

The results presented in this section provide a useful confirmation

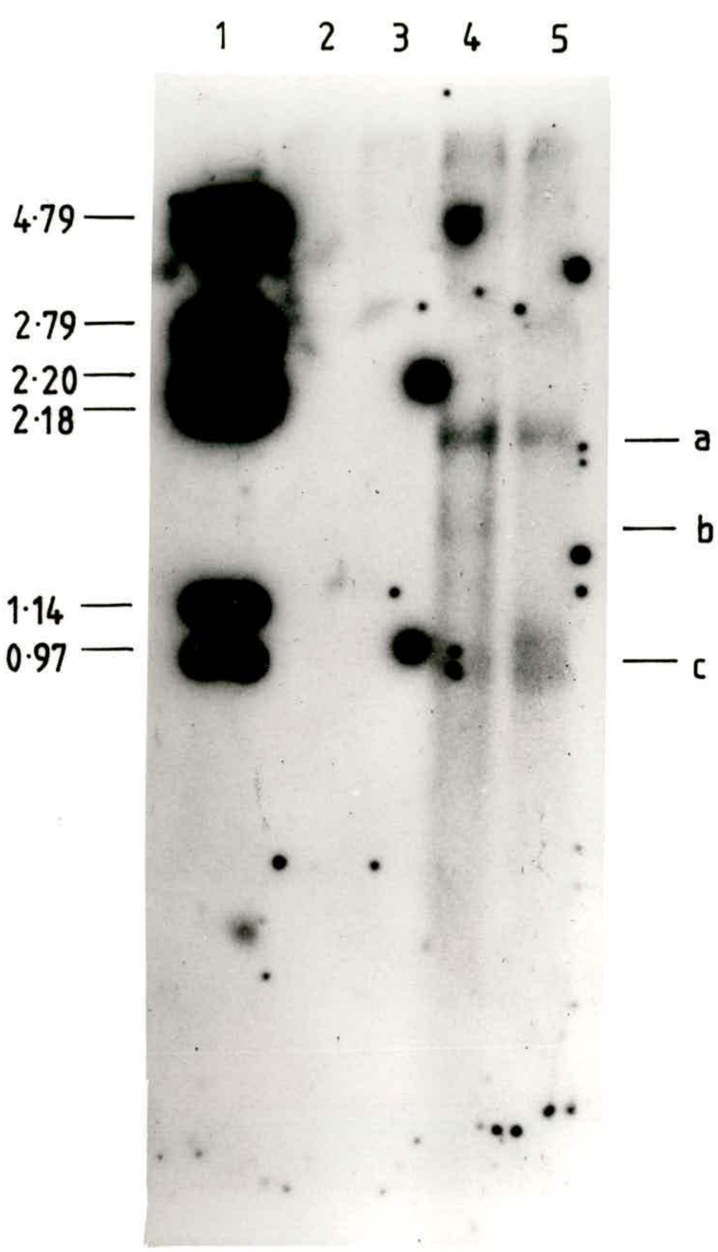


Fig. 5.9

Fig. 5.9 An autoradiograph of SI-resistant hybrids formed between the '*rplJL* t_{L7} *rpoB*' single-stranded M13 probes and RNA isolated from A19 (RNaseIII^{+}) and AB301-105 (RNaseIII^{-}).

Track	Probe/Source of RNA
1	(pHR3 digested with *Pst*I)
2	-ve/RNaseIII^{-}
3	-ve/RNaseIII^{+}
4	+ve/RNaseIII^{-}
5	+ve/RNaseIII^{+}

Notes:

1 "+ve" signifies the DNA probe complementary to *rpl*-*rpo* mRNA; "-ve" the opposite strand.

2 The marker track is pHR3/*Pst*I (track 1). The sizes of the marker DNA fragments are shown in kb.

3 For the sizes of hybrids a-c see text.

4 RNA was isolated from A19 (RNaseIII^{+}) and AB301-105 (RNaseIII^{-}). It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. The SI-resistant hybrids were fractionated on a 2% agarose gel, transferred to nitrocellulose, and labelled using [^{32}P]-pHR3 DNA (see Fig. 5.3). pHR3 (a.k.a. pNA219) is pBR322 containing the '*rplJLrpoBC* (10.14kb *Hin*dIII) fragment. It was constructed and kindly provided by Dr A.J. Newman (Newman and Hayward, 1980).

of the earlier analyses of this region, and additionally give preliminary evidence for the location of the weak promoter P_{L7}. In the next section I investigate the roles of the sites highlighted above in the regulation of β and β' syntheses.

5.7 Transcription in the rplL-rpoB intercistronic region following rifampicin treatment

If, as predicted, rifampicin increases readthrough of t_{L7}, I expect to be able to detect an increase in the amount of the full-length transcript relative to the terminated transcript, in the $RNaseIII^-$ strain. If, however, the rifampicin-stimulated increase in rpoBC transcription results from stimulation of a minor promoter in this region, e.g. P_{L7} or P_β, then I should also be able to detect this. It should be noted that Howe et al. (1982) have provided clear evidence that P_β is not stimulated by rifampicin using a lacZ fusion.

I isolated RNA from AB301-105 grown in the presence or absence of rifampicin for 10 or 25 minutes, and then performed an SI-analysis with the PstI +ve and -ve probes (Fig. 5.10). In tracks 2-5 two hybrids are seen to have formed with the +ve probe, of sizes 1.95kb and 0.96kb. These clearly represent the full-length and terminated transcripts, respectively. Heterogeneous smaller hybrids also seen in these tracks and the "smearing down" from the main bands may be indicative of normal mRNA turnover. Densitometric analysis of tracks 2 and 4, derived from the drug-free control, indicates a ratio of full-length: terminated transcript of 0.24 and 0.19 respectively, indicating termination efficiencies at t_{L7} of 80% and 84% respectively. These values are in close agreement with previous estimates of the efficiency of termination at t_{L7}, in unconstrained cells (for reviews see Yura and Ishihama,

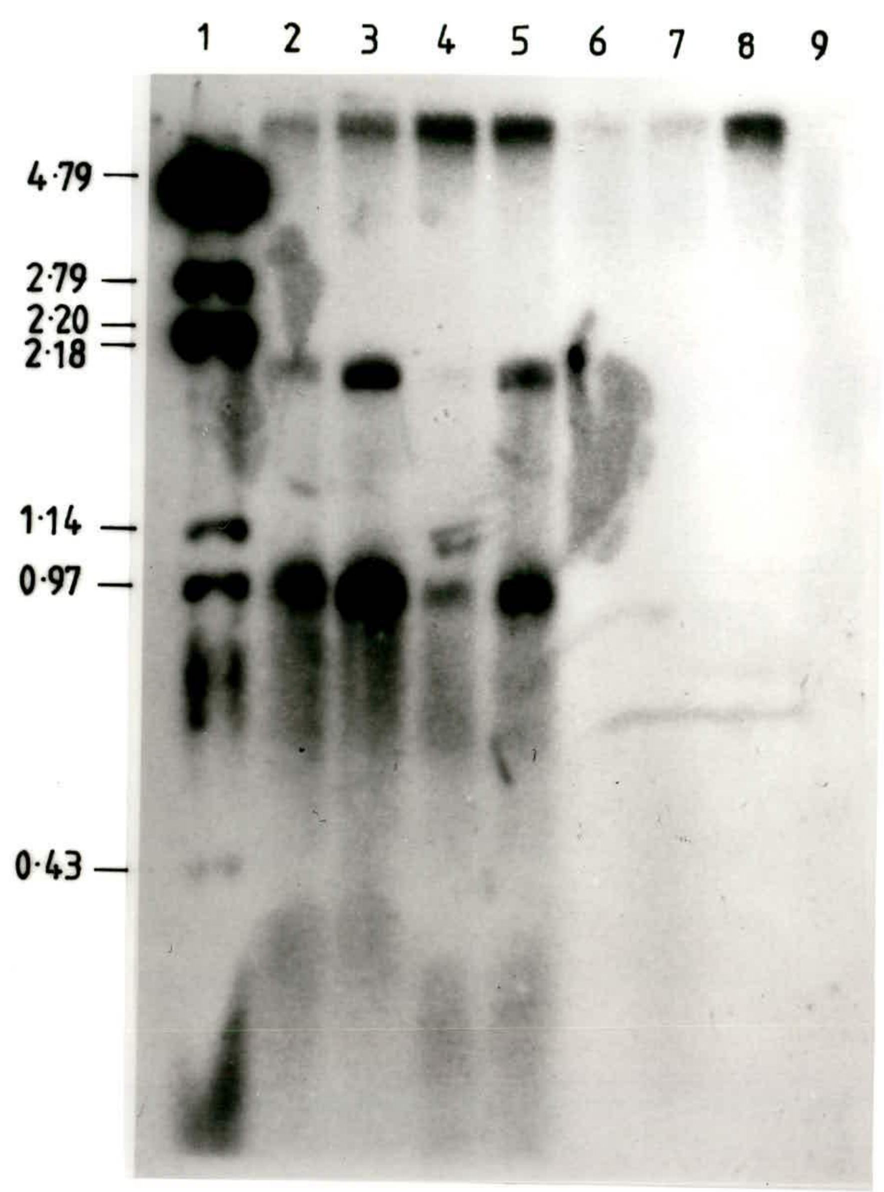


Fig. 5.10

Fig. 5.10 An autoradiograph of SI-resistant hybrids formed between the '*rplJL* t_{L7} *rpoB*' single-stranded M13 probes and RNA isolated from AB301-105 (RNaseIII$^-$).

Track	Probe/Source of RNA
1	(pHR3 digested with *PstI*)
2	+ve/10 min., no Rif
3	+ve/10 min., + Rif
4	+ve/25 min., no Rif
5	+ve/25 min., + Rif
6	-ve/10 min., no Rif
7	-ve/10 min., + Rif
8	-ve/25 min., no Rif
9	-ve/25 min., + Rif

Notes:

1. "+ve" signifies the DNA probe complementary to *rpl-rpo* mRNA; "-ve" the opposite strand.
2. The marker track is pHR3/*PstI* (track 1). The sizes of the marker DNA fragments are shown in kb.
3. RNA was isolated from AB301-105 (RNaseIII$^-$) 10 or 25 minutes after rifampicin (Rif) addition, and from a drug-free control. It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. The SI-resistant hybrids were fractionated on a 2% agarose gel, transferred to nitrocellulose, and labelled using [^{32}P]-pHR3 DNA (see Fig. 5.9).

1979; Matzura, 1980; Lindahl and Zengel, 1982). A similar analysis of the RNA isolated 10 or 25 minutes after rifampicin had been added (tracks 3 and 5) demonstrated, in contrast, that the full-length: terminated transcript ratio had increased to 0.44 and 0.58 respectively. This shows that readthrough of t_{L7} in this strain has been increased by approximately 2-fold. These results are consistent with a previous study which demonstrated that rifampicin stimulates rpoBC transcription 1.5- to 2-fold, and hinted that this stimulation is less transient than that of β and β' protein syntheses (Blumenthal and Dennis, 1978). In addition to showing increased readthrough of t_{L7} my experiments indicate 1) that production of the minor 1.5kb (putative P_{L7}-initiated) transcript, observed in Fig. 5.9, is not stimulated by rifampicin and 2) that there is no detectable synthesis of anti-mRNA complementary to the PstI (t_{L7}) probe under these conditions (tracks 6-9), even after 25 minutes of drug treatment. (Contrast the anti-mRNA complementary to the P_{L10} region, detectable after 25 minutes drug treatment of ED3867.)

The effect of rifampicin on β and β' protein syntheses was checked as before, by pulse-labelling with L-[^{35}S]-methionine and fractionating total labelled protein on a 5-15% SDS polyacrylamide gradient gel (Fig. 5.11). As expected densitometric analysis confirmed a strong stimulation of ββ' synthesis (2- to 3-fold) 10 minutes after rifampicin treatment (tracks 1 and 2). However this preliminary analysis shows, in contrast to previous studies (e.g. Hayward and Fyfe, 1978a), that the stimulation of ββ' synthesis by rifampicin has not passed its peak at 25 minutes (tracks 3 and 4). If, as one might suggest, this is merely a strain-dependent difference, then one would expect similar results for the isogenic RNaseIII^{+} strain, A19.

Fig. 5.11

Fig. 5.11 An autoradiograph of L-[^{35}S]-methionine labelled proteins isolated from the *E. coli* strain AB301-105 (RNaseIII^{-}). Tracks 1 and 3 represent the 10 and 25 minute drug-free controls. Tracks 2 and 4 represent proteins labelled 10 or 25 minutes, respectively, after addition of rifampicin (10μg/ml). Proteins were fractionated on a 5-15% SDS polyacrylamide gradient gel.

When I performed an analysis of proteins synthesised in A19 following rifampicin treatment, just as described for AB301-105, β and β' protein syntheses were stimulated approximately 3-fold (Fig. 5.12, tracks 1 and 2) 10 minutes after rifampicin addition. However, in contrast to AB301-105, this stimulation was past its peak by 25 minutes dropping to 1.5- to 2-fold (tracks 3 and 4). Although results refer to only two time points, and clearly require more careful quantitation (e.g. by double-labelling techniques: cf. Hayward and Fyfe, 1978a), it is tempting to speculate that the prolongation of rifampicin-induced stimulation of ββ' synthesis in AB301-105 may be connected with the lack of mRNA processing by RNaseIII in this strain.

I have attempted to confirm that the stimulation of rpoBC transcription observed in AB301-105 (Fig. 5.10) is mimicked in A19, by SI-nuclease analysis. The results are shown in Fig. 5.13. The three major hybrids detectable, in tracks 2-5, have estimated sizes of 1.75kb, 0.98kb and 0.90kb respectively. However, only the "1.75kb" hybrid can be assigned unambiguously (evidently the 1.89kb, full-length unprocessed transcript), since only two bands migrating in the 0.8-1.0kb size range can be distinguished clearly, whereas at least five hybrids have been predicted (see Fig. 5.8). On the other hand, if RNaseIII always cuts quickly at both sites; or if one site is very strongly preferred; only 3 hybrids are expected (if the minor P_{L7}- and P_{β}-products are ignored). Hence it is impossible by this technique to quantitate the terminated transcript, or the processed fraction of the readthrough transcript, and so assess the effect of rifampicin on termination at t_{L7} in this strain. Elizabeth Marson and I have initiated measurements of the rates of synthesis of rpoC- and rpl'AJ'-mRNA by $[^3H]$-uridine pulse-labelling and filter hybridisation analyses. The

Fig. 5.11

Fig. 5.12

Fig. 5.12 An autoradiograph of L-[^{35}S]-methionine labelled proteins isolated from the *E. coli* strain A19 (RNaseIII^{+}). Tracks 1 and 3 represent the 10 and 25 minute drug-free controls. Tracks 2 and 4 represent proteins labelled 10 or 25 minutes, respectively, after addition of rifampicin (10μg/ml). Proteins were fractionated on a 5-15% SDS polyacrylamide gradient gel.

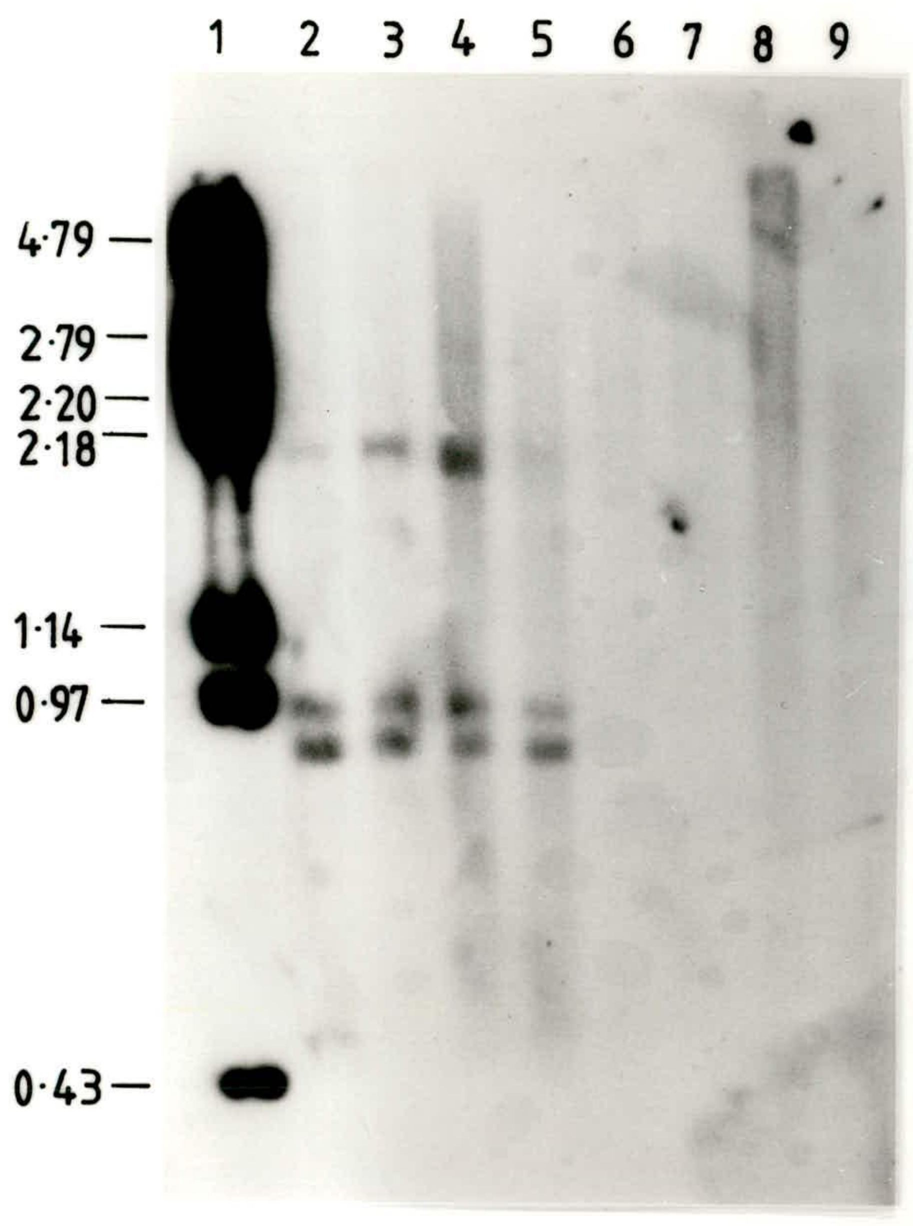


Fig. 5.13

Fig. 5.13 An autoradiograph of SI-resistant hybrids formed between the 'rplJL t_{L7} rpoB' single-stranded M13 probes and RNA isolated from A19 ($RNaseIII^{+}$).

Track	Probe/Source of RNA
1	(pHR3 digested with PstI)
2	+ve/10 min., no Rif
3	+ve/10 min., + Rif
4	+ve/25 min., no Rif
5	+ve/25 min, + Rif
6	-ve/10 min., no Rif
7	-ve/10 min., + Rif
8	-ve/25 min., no Rif
9	-ve/25 min., + Rif

Notes:

1 "+ve" signifies the DNA probe complementary to rpl-rpo mRNA; "-ve" the opposite strand.

2 The marker track is pHR3/PstI (track 1). The sizes of the marker DNA fragments are shown in kb.

3 RNA was isolated from A19 ($RNaseIII^{+}$) 10 or 25 minutes after rifampicin (Rif) addition, and from a drug-free control. It was then hybridised to the +ve and -ve probes, and treated with SI-nuclease. The SI-resistant hybrids were fractionated on a 2% agarose gel, transferred to nitrocellulose, and labelled using [^{32}P]-pHR3 DNA (see Fig. 5.9).

results so far indicate that rifampicin treatment (10μg/ml) stimulates rpoC relative to rpl transcription in A19, as expected, and that moreover this stimulation is maintained for at least 45 minutes after drug addition. This is in agreement with the limited data reported by Blumenthal and Dennis (1978) for similar experiments with another RNaseIII^{+} strain (NC3: a derivative of E. coli B/r); and the indirect evidence obtained by gene fusion studies (pHR7: Howe et al., 1982; and pHR11: Newman et al., 1982).

More work is required to establish whether RNaseIII-processing really plays a necessary role in post-transcriptional regulation of ββ' synthesis, as suggested by the above results. The necessary experiments include careful quantitation of the levels of stimulation of ββ' protein synthesis by rifampicin in A19 and AB301-105, using double-labelling of proteins, e.g. with [^{14}C]- and [^{3}H]-leucine; and extensive mRNA analyses by filter hybridisation with appropriate probes.

Attempts to demonstrate by SI-analyses that rifampicin or amino acyl-tRNA limitation stimulate readthrough of t_{L7}, in the strains ED3867 and AB4141 (valS(Ts)) respectively, have to date proved unsuccessful because of the RNaseIII-processing problem. This might be overcome in two ways: 1) A different, short M13 probe for this region could be used. However, this might prove difficult to construct because of the paucity of suitably located restriction sites in the vicinity, and would require a complete change of hybrid-detection technique. 2) An RNaseIII^{-} derivative could be constructed. I already know that this approach should work, as I have shown in this chapter that rifampicin stimulates readthrough of t_{L7} in AB301-105. Although I have found that RNaseIII-processing may be invovled in post-transcriptional regulation of rpoBC, this is unlikely to have any effect on the transcriptional regulation of t_{L7}.

5.8 Discussion

My results concerning transcription of the P_{L10} region of unconstrained E.coli K12 strains ED3867, EMR3 (valS^{+}) and AB4141 (valS(Ts)) confirm and extend the earlier studies of this region (Brückner and Matzura, 1981). The combined data suggest that most, if not all, transcription of rpoBC is initiated at P_{L11}, the promoter upstream of rplKA, with severe or complete occlusion of P_{L10}. Further, it suggests that there may be slow endonucleolytic processing of the mRNA in the intercistronic space between rplA and rplJ, near nucleotide positions 1500, 1605 and perhaps 1340. In the latter case there might alternatively (or additionally) be very weak transcriptional termination of the P_{L11}-initiated mRNA, and/or initiation at P_{L10}. Interestingly I have found that there are reproducible strain-dependent differences in the processing rates at all three sites (including the possible initiation/termination near P_{L10}). EMR3 and AB4141 seem in this respect to resemble the strain W3350 (Brückner and Matzura, 1981), while ED3867 gives results reminiscent of the hybridisation data obtained from the strain AJ5002 (Dennis et al., 1985). It is tempting to speculate that the processing observed in these studies is the first step in the degradation of the rplKA mRNA. At each of the putative processing sites it is possible to identify potential secondary structures in the mRNA (Brückner and Matzura, 1981). Post and his colleagues, in an earlier study, reported that processing and/or termination might occur in the region of nucleotide 1600 (Post et al., 1979).

It is known from deletion and fusion analyses that the P_{L10} promoter is capable of vigorous expression of the downstream genes in the absence of a functional P_{L11} (e.g. Linn and Scaife, 1978; Yamamoto and Nomura, 1978; Howe et al., 1982; Newman et al., 1982). However, when

P_{L11} is functional P_{L10} appears to be entirely occluded. There are a number of mechanisms which can be proposed to explain this phenomenon, all of which will be discussed in greater detail in Chapter 6. However, one can speculate that if pausing occurs in the near vicinity of P_{L10} (even if it leads to little or no termination) this could increase the mean dwell-time of functioning core RNA polymerase (arriving from P_{L11}) over the P_{L10} recognition sequences, and therefore block de novo access to P_{L10}.

My transcriptional studies of the 'rplJL-rpoB' region in the strains A19 and AB301-105 have given results agreeing closely with the published SI-analysis of this region (Barry et al., 1980). A19 in addition to the full-length transcript shows a number of hybrids in the 0.8-1.0kb size range, as expected if RNaseIII-processing of the message occurs. AB301-105, the RNaseIII-deficient strain, gives a much simpler pattern of hybrids. My results confirm that partial termination of transcription occurs at "t_{L7}" near nucleotide 2700 (scale of Post et al., 1979). I have presented direct evidence that termination at this site in vivo is 80-85% efficient, in agreement with earlier estimations. I have also provided preliminary evidence for weak initiation at or near nucleotide 2220. This may well represent the P_{L7} promoter which has been postulated (on the basis of cloning/ deletion studies) to lie in (or upstream of) the intercistronic region between rplJ and rplL (2219-2284). A more detailed study would have to be performed to confirm this definitively. It is not surprising that this particular transcript was not observed in the previous SI-analyses by Barry et al. (1980), because it is minor and therefore outwith the range of sensitivity of their technique.

The main purpose of the experiments outlined in this chapter

was to investigate whether the partial uncoupling of rpoBC from rplKAJL transcription, observed when E. coli is treated with rifampicin or upon induction of the stringent response by partial valyl-tRNA limitation, was in part due to a stimulation of the occluded promoter P_{L10}; and to show directly the involvement of altered readthrough of the partial terminator t_{L7} under these conditions. The experiments presented here strongly suggest that P_{L10} is not activated by either constraint, at least in the strains used. Rifampicin does cause a 2-fold decrease in termination of mRNA at t_{L7}, thus closely reflecting (and explaining) the well-established stimulation of ββ' protein synthesis. There is no reason to suppose that this effect reflects any specific regulatory feature of the rpoBC operon, since rifampicin causes readthrough of all tested transcriptional terminators both rho-independent and rho-dependent (Howe et al., 1982; Newman et al., 1982; Cromie and Hayward, 1984). This of course does not exclude variations in readthrough of t_{L7} as a specific regulatory response to other constraints.

The increased yield of hybrid (b) from RNA of rifampicin-treated cells, annealed with the probe for the P_{L10} region (Fig. 5.3A, track 3), remains to be explained. One intriguing, although preliminary observation is that at the later times, for both the studied constraints, detectable synthesis of anti-mRNA (complementary to the opposite DNA strand) occurred in the P_{L10} region (but not in the 'rplJrplLt_{L7}rpoB' region). Preliminary attempts to map the origin of this transcription more precisely, using further probes, have proved unsuccessful. It seems unlikely that it plays any significant role in the stimulation of rpoBC transcription or of ββ' protein synthesis, as it is undetectable at times where such stimulation is already strong. Convergent transcription might, in principle, play a role in the transience of the rifampicin

stimulation of ββ' synthesis which, as hinted by the results of Blumenthal and Dennis (1978) and confirmed in this chapter, involves post-transcriptional inhibition of rpoBC-mRNA translation. However, the -ve probe spanning the rpl'JLrpoB' region did not detect any anti-mRNA synthesis after 25 minutes of rifampicin treatment, either in A19 or AB301-105. As this probe "covers" the translational start region of rpoB, and all of the upstream intercistronic RNA (some of which has been implicated in translational regulation of rpoB: Dennis, 1984), it is unlikely that convergent transcription *is* involved in the inhibition of ββ' translation under these conditions. If it *has* any physiological significance, it might (speculatively) be implicated in post-transcriptional inhibition of rplKA and/or rplJL expression during severe limitation of amino acyl-tRNA supply. Under these conditions it may be essential to maintain significant *transcription* through the rplKAJL genes, to allow continued expression of rpoBC.

Evidence presented in this chapter also suggests that RNaseIII-processing of the mRNA in the rplLrpoB intercistronic space has a role in the post-transcriptional regulation of β and β' synthesis. Although it has been known for several years that RNAseIII cleaves the mRNA in this region *in vivo*, the physiological function of this processing (if any) has been unknown until now. Similarly, it is well established that RNaseIII is involved in the processing of the bacteriophage T7 early and late polycistronic transcripts *in vivo*; but the available evidence suggests that this processing is not *essential* for translation of the T7 mRNA, nor for any other function in successful host infection: T7 grows on RNaseIII-deficient strains (Dunn and Studier, 1973; Yamada and Nakada, 1976). Perhaps the best characterised examples of the role of RNaseIII-processing are its involvement in the regulation of int

gene expression in bacteriophage λ (Echols and Guarneros, 1983) and of gene 1.2 in T7 (Saito and Richardson, 1981). It has been suggested that the processing of rpoBC mRNA, by separating the rpo- from rpl- message, ensures that the former is unaffected by the post-transcriptional regulation of the ribosomal protein genes (Nomura et al., 1984). However, an RNaseIII-deficient strain shows no detectable change in rpoBC mRNA synthesis or ββ' protein production compared to its isogenic $RNaseIII^+$ derivative, under normal growth conditions (Dennis, 1984; and this chapter). In the same report Dennis showed, by studying a number of deletions of the intercistronic space in plasmid fusions, that sequences in the vicinity of the RNaseIII target are important in efficient translation of rpoBC mRNA. Dennis was also co-author of an earlier paper which clearly established the existence of post-transcriptional regulation of ββ' synthesis in vivo: strong over-production of rpoBC mRNA from a multicopy plasmid led only to a comparatively modest over-synthesis of β and β' (Dennis and Fiil, 1979). My results now suggest that RNaseIII-processing may be essential to allow post-transcriptional negative regulation of ββ' synthesis, to compensate for over-production of rpoBC mRNA (such as that reported by Dennis and Fiil). This finding is clearly worthy of further investigation, to verify it and then (if proven) to explore its underlying mechanism. A model for transcriptional and post-transcriptional regulation of rpoBC is presented in Chapter 1 and Chapter 6.

The SI-nuclease mapping experiments described in this chapter are subject to at least two criticisms. 1) I have not examined pulse-labelled RNA. 2) The method of labelling hybrids by transfer and probing is subject to the vagaries of differential transfer efficiency. With respect to the first point, however, the functional half-lives of

βmRNA and L7/L12mRNA have been estimated to be short, and similar; 60 and 80 seconds respectively (Pedersen et al., 1978). Thus the intensities of the hybrid bands should be essentially a measure of rates of transcription. The very fact that reasonably compact bands are detected argues that pulse-labelling of the RNA is unlikely to lead to very different results. Most importantly my control data closely resemble those of Brückner and Matzura (1981) for the P_{L10} region, those of Barry and colleagues (1980) for the t_{L7} region, and independent estimates (by other methods) of the efficiency of the transcriptional terminator t_{L7} (for reviews see Yura and Ishihama, 1979; Matzura, 1980; Lindahl and Zengel, 1982; also the papers by Howe et al., 1982; Newman et al., 1982; and Ralling and Linn, 1984). This lends credence to my data regarding the effects of growth constraints.

CHAPTER 6

Final Discussion

6.1 Transcriptional regulation of rpoBC

There are a number of transcriptional signals present in the rplKAJLrpoBC operon which could be utilised for the regulation of rpoBC (Fig. 6.1). These include (besides the chief promoter P_{L11}) the occluded promoter, P_{L10}; a number of minor promoters, P_{L7}, P_{β} and $P_{\beta'}$; and a partial terminator of transcription, t_{L7}.

I have shown in Chapter 5 that P_{L10} is not stimulated detectably either by treatment with rifampicin or by partial valyl-tRNA limitation. Does this mean that the P_{L10} promoter has no role to play in rpoBC expression? Stimulation of rpoBC mRNA synthesis is also observed when certain rpo^{ts} or ts-suppressed rpo-amber mutants are exposed to partially restrictive temperatures (Blumenthal and Dennis, 1980b; Little and Dennis, 1980). Some of these mutants also exhibit increased rplJL transcription, although to a lesser extent than rpoBC. There are insufficient data in these reports to draw a clear conclusion about P_{L10} (in particular no simultaneous assays for rplKA mRNA were performed), but it is possible that activation of P_{L10} plays a role at least in these cases.

In order to propose a mechanism for such activation, it would be necessary to understand how P_{L10} is normally occluded by P_{L11}. There are three main possibilities, of which two (numbers 1 and 3 below) have previously been suggested by Adhya and Gottesman (1982).

1) Simple steric exclusion: RNA polymerase initiates so frequently at P_{L11} as to pack transcription complexes over the complete operon,

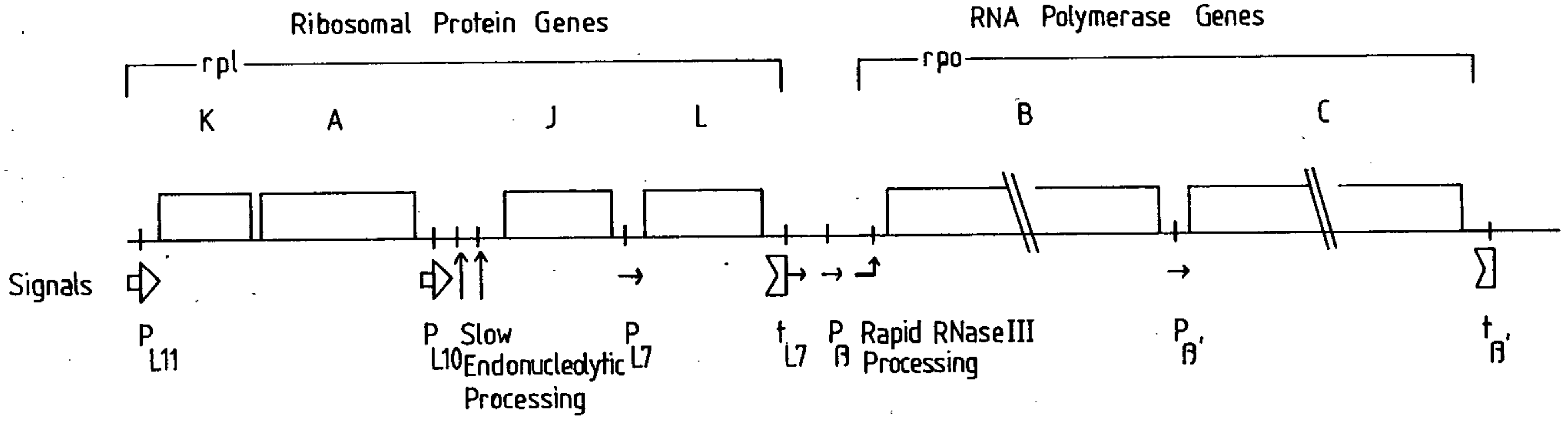


Fig. 6.1

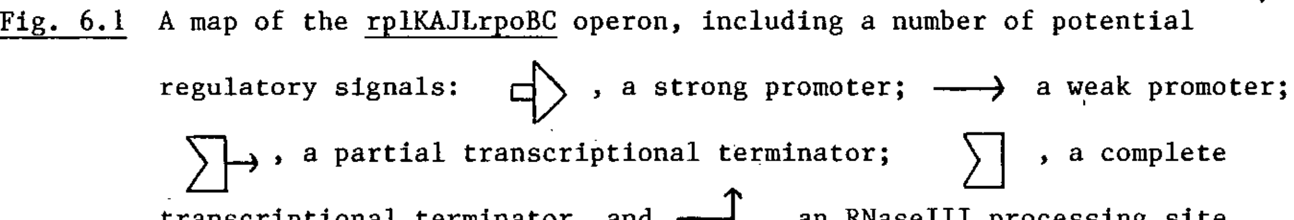


Fig. 6.1 A map of the rplKAJLrpoBC operon, including a number of potential regulatory signals: , a strong promoter; a weak promoter; , a partial transcriptional terminator; , a complete transcriptional terminator, and , an RNaseIII processing site.

thereby preventing access to the P_{L10} recognition sequences. 2) A kinetic exclusion mechanism, based on the hypothesis that in order to initiate, free RNA polymerase must bind unspecifically to the DNA template near the P_{L10} promoter, and then "search" by moving back and forth along the DNA until it "recognises" the promoter sequences ("search" reference, e.g. von Hippel et al., 1984). My proposal here is that the searching RNA polymerase is "knocked off" the template before it can find or bind to P_{L10}. 3) An extended dwell-time mechanism, whereby a "pause" site (whether or not associated with any transcriptional termination) lies in close proximity to P_{L10}, causing transcribing RNA polymerase to halt at P_{L10} sufficiently long enough to block access to it by free RNA polymerase. It should be noted here that there is potential for RNA stem-loop formation (needed for pausing) in the nucleotide sequence near P_{L10}. Model 1 is improbable because only rRNA promoters initiate often enough, about one transcript per second under optimal growth conditions (von Hippel et al., 1984), to give close packing of RNA polymerase on the template. Regarding model 3, my work (Chapter 5) shows that if there is a transcriptional terminator near P_{L10} it must be very weak (see Fig. 5.3A and B); moreover the addition of rifampicin has no effect on the degree of termination (see Fig. 5.3A, compare tracks 2 and 3). However, the techniques I have used could not identify pause sites unless they produced very prolonged pauses (or significant termination). Accordingly models 2 and 3 both remain open as explanations of P_{L10} occlusion.

The most obvious means by which occlusion could be lifted is by prevention of transcriptional initiation at P_{L11}. Therefore progress towards the understanding of the role of promoter occlusion in the expression of this operon requires detailed studies of rplKA-relative

to rplJL-mRNA synthesis. Both the σ and α operons also contain promoters which are normally occluded (Cerretti et al., 1983; Lupski and Godson, 1984). It is therefore tempting to think that promoter occlusion may have a role in the regulation of RNA polymerase synthesis.

The rpoBC operon contains a number of weak promoters (Fig. 6.1). These have been generally identified by deletion and fusion studies (Barry et al., 1979; Newman et al., 1979; An and Friesen, 1980; Ma et al., 1981); hence their contribution to normal operon expression is unclear. In Chapter 5 I provided preliminary evidence that P_{L7} is weakly active in the natural operon (which raises an interesting side issue: why should this inherently weak promoter not be occluded, in contrast to the vigorous P_{L10}?). However, I clearly showed that neither this promoter nor P_{β} are significantly stimulated by rifampicin, a constraint which stimulates transcription of rpoBC (Blumenthal and Dennis, 1978). The fusion studies of Howe et al. (1982) had already suggested that the drug gives no detectable activation of P_{β}. Therefore it is unlikely that any of the weak promoters identified in this operon make a significant contribution to its expression, or have any major role in the regulation of rpoBC, in the conditions of growth so far studied. Interestingly one of the minor promoters discovered in the σ operon, Phs, has been shown to be stimulated by induction of the heat-shock response (described in Chapter 1) and is therefore directly responsible for the increased σ synthesis observed under these conditions (Lupski et al., 1984; Taylor et al., 1984).

The major transcriptional regulation of rpoBC expression is likely to be mediated by the partial transcriptional terminator, t_{L7}. Termination at this site is known to occur at nucleotide position 2716, approximately 70bp beyond the translational stop codon of rplL, as

judged by SI-nuclease mapping of the 3' ends of *in vivo* transcripts (Barry *et al.*, 1980). Immediately preceding the termination site there are two potential regions of hyphenated dyad symmetry (Fig. 6.2A). Such regions are typical of rho-independent terminators, which classically consist of a G-C rich stem-loop structure followed by a sequence of 10 or 11 nucleotides, including at least 7 T's (Brendel and Trifonov, 1984) or 6 T's (R.S. Hayward, pers. comm.) respectively, in which termination occurs (Platt and Bear, 1983). Results presented in Chapter 5 demonstrate directly, for the first time, that rifampicin treatment causes a 2-fold increase in readthrough of t_{L7} on the chromosome. I hope in the near future to study transcription through this site under a variety of other conditions known to stimulate *rpoBC* transcription.

What is the mechanism by which readthrough of t_{L7} is regulated? The evidence to date suggests that constraints which inhibit the initiation of transcription, e.g. rifampicin (Blumenthal and Dennis, 1978) or elevated growth temperatures applied to certain *rpo*^{ts} mutants (Blumenthal and Dennis, 1980b; Little *et al.*, 1981), specifically induces *rpoBC* mRNA synthesis. This is consistent with the hypothesis that there is an autogenous regulatory mechanism for *rpoBC* transcription, which is sensitive to the level of initiation-competent RNA polymerase molecules present in the cell. This could be mediated by a direct interaction of free active RNA polymerase with t_{L7} at the DNA, RNA, or transcription-complex level, or perhaps through the regulation of expression of an intermediary protein by free RNA polymerase. Rifampicin stimulates the readthrough of all tested terminators, both rho-dependent and -independent (Howe *et al.*, 1982; Newman *et al.*, 1982; Cromie and Hayward, 1984). Therefore it is possible that in this case the effect on t_{L7} is non-specific, and has no bearing on the normal regulation of

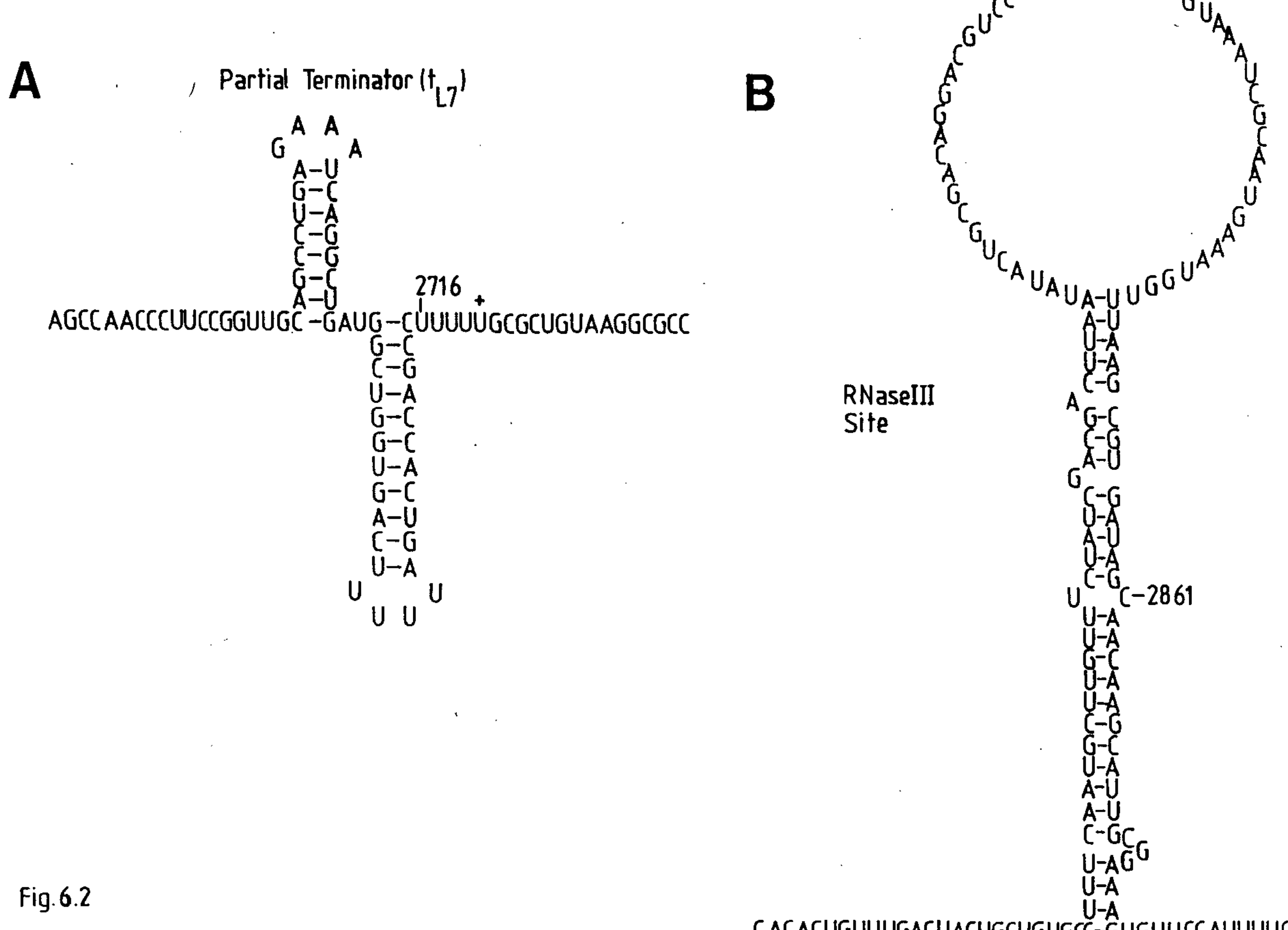


Fig. 6.2

Fig. 6.2 A) The nucleotide sequence of the partial terminator t_{L7} (Post *et al.*, 1979) and the potential secondary structure which is present within the sequence (Barry *et al.*, 1980).

\+ Note that the site of termination at position 2716 (Post *et al.*, 1979 scale) has 5 U's rather than 4 U's (Morgan *et al.*, 1984).

B) The nucleotide sequence and potential secondary structure of the RNaseIII processing site (Post *et al.*, 1979; Barry *et al.*, 1980; Morgan *et al.*, 1984). The approximate cut site is at position 2861. An extra 5' end has been observed in RNA *in vivo* by Fukuda and Nagasawa-Fujimori (1983) at position 2787, which they suggest may be a promoter. Alternatively it may reflect a second cut by RNaseIII.

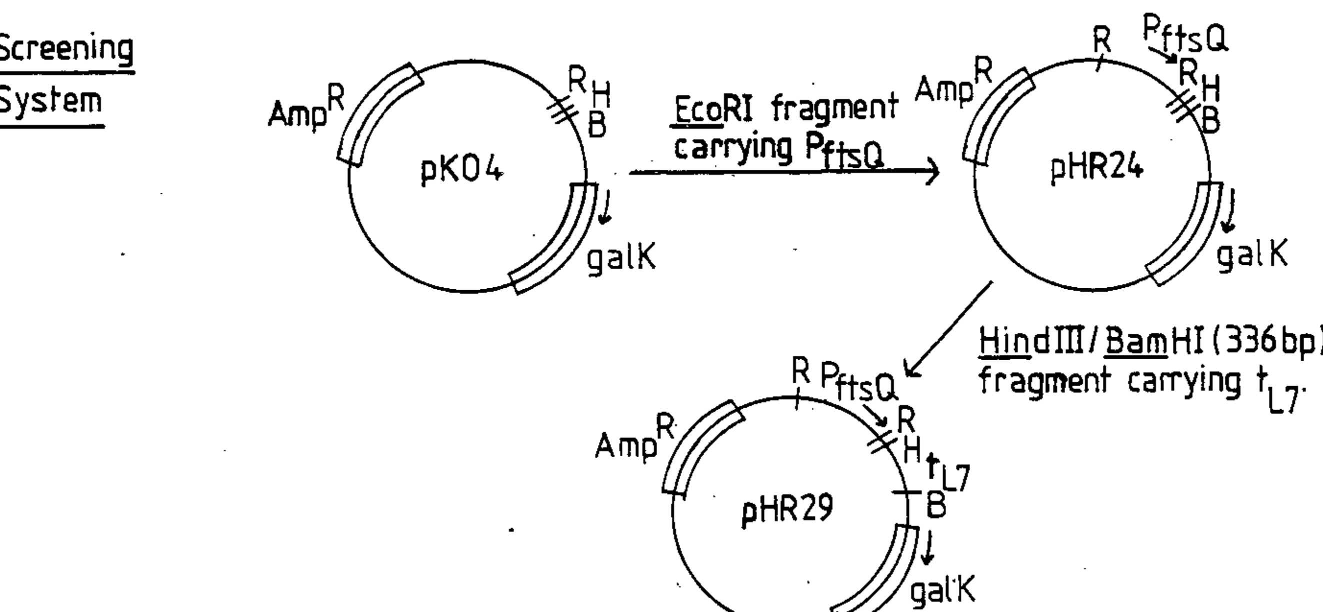
rpoBC expression. It may simply reflect the binding of rifampicin to transcribing RNA polymerase (Yarbrough et al., 1976), causing a change in the enzyme such that it no longer recognises terminators efficiently. In this respect it is relevant to note that some rpoB mutations which confer rif^r also affect the ability of RNA polymerase to recognise transcriptional terminators in the absence of rifampicin (Yanofsky and Horn, 1981).

Tittawella, however, proposed that synthesis of an unstable protein termed Π, whose expression is normally repressed by complete holoenzyme, was induced when rifampicin was added to the cell; and that Π was responsible for the increased readthrough of t_{L7} (Tittawella, 1976b). His proposal was based on the observation that the application of a constraint on protein synthesis during (but not after) the first four minutes of rifampicin treatment reduced the degree of induction of ββ' protein synthesis. Moreover Nakamura and Yura isolated a conditionally lethal mutation, am100, which when temporarily unsuppressed led to a gradual decline in the rate of ββ' synthesis (Nakamura and Yura, 1975). However, in this study the authors did not verify that am100 was the mutation responsible for the phenotype, in their heavily mutated strain. It should be remembered that a number of ancillary factors distinct from the RNA polymerase subunits have been discovered in E. coli, which affect the transcriptional termination process, e.g. the NusA and Rho proteins (Platt and Bear, 1983), and perhaps tau (Briat and Chamberlin, 1984). The λN antitermination protein (and its instability) are also noteworthy here (Greenblatt, 1984).

Although it can be argued that rpo^{ts} or ts-suppressed rpo-amber mutations at partially restrictive temperatures could also affect termination in a non-specific way, this is not so easy to envisage for

rpoD285 (Blumenthal and Dennis, 1980b), a temperature sensitive mutation of σ (which is believed to play no role in termination: cf. O'Hare and Hayward, 1981). This causes increased synthesis of ββ' and rpoBC mRNA at the restrictive temperature (Blumenthal and Dennis, 1980b). Nor is it easy to dismiss as unspecific the effect of induction of the stringent response, a constraint which reduces rplKAJL transcription in the Rel^+ strain (and increases it in a Rel^- isogenic strain) but leaves rpoBC relatively unaffected (Maher and Dennis, 1977). This constraint is of particular interest since it is likely to be suffered by E. coli in its normal existence.

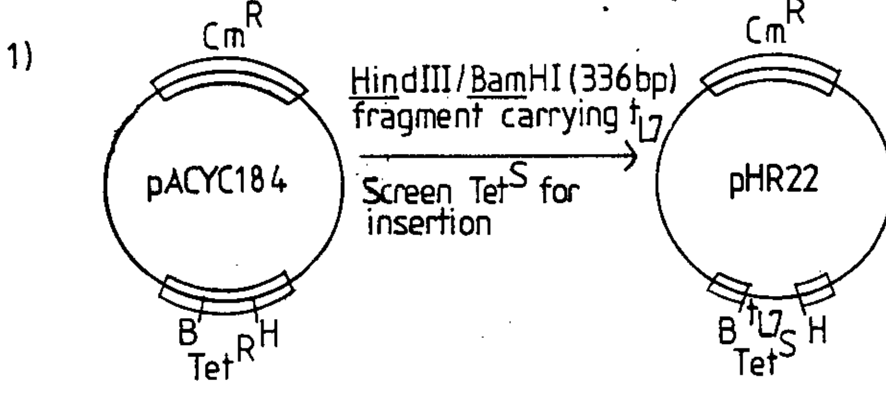
How then is the expression of rpoBC regulated through t_{L7}? In an attempt to answer this question I have constructed a screening system for t_{L7} mutants using galK plasmids (McKenney et al., 1981; Fig. 6.3). One of the advantages of this system is that the terminator fragment can be mutagenised in isolation from the rest of the screening plasmid (for example as shown in Fig. 6.3), either in another plasmid or in M13. The mutagenised t_{L7} fragment can then be reintroduced into the screening plasmid, and possible mutants identified by comparison of galactokinase expression by these fusions and the "wild-type" plasmid. In this particular study I have constructed an expression vector which allows detection of possible terminator mutations (showing increased readthrough) by simply observing colony colour-phenotype on the appropriate indicator plates. This technique relies on the fact that a $galK^-$ strain carrying a plasmid with very low galK expression gives white colonies on MacConkey-galactose plates, while high expression yields red colonies. Therefore I chose a fairly weak promoter, P_{ftsQ} (Robinson et al., in press) which when fused upstream of t_{L7} gives rise to colonies which are white/pink. Hence one class of red colony



pHR29 gives pink/white colonies when transformed into E.coli and streaked on gal-mac-amp indicator plates (see text). The order in pHR29 is P_{ftsQ} t_{L7} galK, where t_{L7} is in the wild-type orientation. Translation is closed in all 3 reading frames immediately upstream and downstream of the terminator, thus mimicking its wild-type environment. Mutations decreasing terminator efficiency should give red colonies; those increasing it, white colonies.

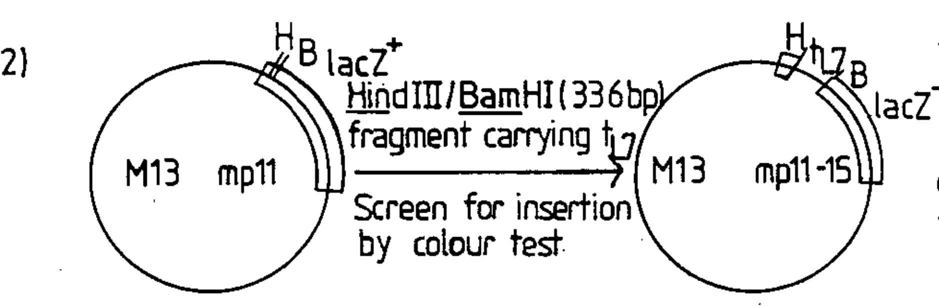
Mutagenesis (Two approaches)

1)



Mutagenise for example by growing pHR22 in a mutD strain, then isolate the plasmi DNA and use it as a source of the t_{L7} fragment for insertion into the screening plasmid. The different anti-biotic resistances aid isolation of the desired recombinants.

2)



The single stranded M13 form o mp11-15 can be used for muta-genesis eg by bisulphite, or directed by synthetic oligonucle tides. t_{L7} can then be cloned bac into the screening system.

Blue plaque colour on appropriate IPTG/X-gal indicator plates (see Chapter 2).

White plaque colour on the same indicator plates.

Fig. 6.3

Fig. 6.3 The strategy followed to construct the screening system for detection of t_{L7} mutants, and to mutagenise the terminator. R, H and B represent *Eco*RI, *Hin*dIII and *Bam*HI restriction sites, respectively.

obtained when the mutagenised terminator fragment is reintroduced should represent reduced termination at t_{L7}. Any "hopefuls" can then be further characterised by DNA sequencing. The t_{L7} terminator could be mutagenised in a variety of ways, including passage through a mutD strain, or deliberate alteration of specific base pairs using oligonucleotides and M13 clones. Hence this is a potentially powerful approach to study sequences important for termination at t_{L7} (provided that copy number mutations affecting the plasmids are not very frequent). Additionally the system would allow the mutant terminators to be tested for altered regulation in genetic backgrounds and/or conditions known to uncouple rpoBC transcription from that of rplKAJL.

Recently a number of regions have been identified in the E. coli chromosome which have structural similarities to the BoxABoxBBoxC nut sites involved in λN protein-directed antitermination (Friedman and Olson, 1983; Lupski et al., 1983; Li et al., 1984). One of these sites, in the rrnG operon, was identified in a small DNA fragment which was shown to have antitermination activity (Li et al., 1984). Interestingly a 67bp TaqI restriction fragment isolated from the rplJ gene also displays antitermination activity in this test system (C. Squires, pers. comm.). Initial sequence analysis of this fragment has identified a BoxABoxB-like sequence. However, unlike the case in rrnG, this sequence lies in a region which is normally translated. Moreover translation of the rrnG "antitermination" fragment has been shown to block its antitermination function (Li et al., 1984). Perhaps if translation of rplJ were blocked, for example by convergent transcription, this might allow activation of its antitermination site. Extensive experimentation would obviously be required to test the relevance of this sequence for the regulation of rpoBC. If it is important, then it is

possible to envisage an _E. coli_ _N_-like protein (Π?) interacting at the site, perhaps in conjunction with other cellular factors such as NusA, to alter the "state" of the transcribing RNA polymerase into a form or a complex which does not recognise t_{L7}. It should be noted that conflicting evidence exists as to the effect of NusA on t_{L7} termination (Peacock _et al._, 1982; R.S. Hayward, pers. comm.). The synthesis of σ has been shown to respond positively to _N_ (Nakamura and Yura, 1976b), and _nut_-like sequences have been identified in _rpsU_ upstream of a strong transcriptional terminator (Lupski _et al._, 1983). Hence it is conceivable that σ synthesis, which is also stimulated by rifampicin (Nakamura and Yura, 1976a; Hayward and Fyfe, 1978a), is under Π regulation. Although α production is also stimulated by rifampicin (_ibid._) there is as yet no evidence of any transcriptional activation of _rpoA_, nor of any likely terminator upstream of this gene.

The above model for autogenous transcriptional regulation of _rpoBC_ invokes an indirect effect, whereby the expression of an antiterminator protein is regulated by the level of free RNA polymerase in the cell. However, other models exist: for example it is possible that free RNA polymerase interacts directly with one or both stem-loop structures at t_{L7} (Fig. 6.2A), in a way reminiscent of the S10 attenuator (Lindahl _et al._, 1983). A constraint which affects the activity or supply of free RNA polymerase might also affect the ability of the enzyme to bind to the mRNA and/or DNA, in such a way as to influence formation of the stem-loops and so affect readthrough of t_{L7}. Evidence against interaction at the DNA level, however, arises from the observation that RNA polymerase does not bind to a t_{L7} restriction fragment _in vitro_ (Taylor and Burgess, 1979).

6.2 Post-transcriptional regulation of *rpoBC*

It is clear from a series of overexpression studies that post-transcriptional repression of ββ' synthesis occurs (e.g. Kirschbaum and Scaife, 1974; Dennis and Fiil, 1979; Meek and Hayward, in press). In contrast to the transcriptional regulation of *rpoBC*, relatively little is known concerning the sites or specific mechanisms involved. Dennis has found that sequences, distinct from the translational initiation signals of *rpoB*, and extending from the region of the RNaseIII site upstream of *rpoB* (Figs 6.1 and 6.2B), are important for the efficient translation of *rpoB* mRNA (Dennis, 1984). A later study has shown, however, that removal of most of the DNA upstream of *rpoB*, including the RNaseIII site, has no effect on the post-transcriptional repression of ββ' synthesis (Meek and Hayward, in press). *In vitro* experiments have indicated that holoenzyme ($\alpha_2\beta\beta'\sigma$) or a subcomplex ($\alpha_2\beta$: a normal intermediate in the assembly of the enzyme) can repress the translation of *rpoBC* mRNA (Fukuda *et al.*, 1978; Kajitani *et al.*, 1980; Lang-Yang and Zubay, 1981; Peacock *et al.*, 1982). In the light of this evidence Meek has proposed a model in which $\alpha_2\beta$ or holoenzyme binds to the *rpoBC* mRNA in such a way as to prevent initiation of translation of *rpoB*; either by directly blocking the translation initiation site, or by stabilising a secondary structure of the RNA in which the *rpoB* translational start signals are sequestered, blocking access by the ribosome (Meek and Hayward, in press; see Fig. 1.8). This model is very similar to that established for the post-transcriptional regulation of ribosomal protein synthesis (Nomura *et al.*, 1984). The latter model differs mainly in that individual ribosomal proteins are the translational regulators, whereas complete RNA polymerase (or a major subassembly) is the proposed regulator of *rpoBC*; and in that

the relevant ribosomal proteins appear to act by binding to mRNA sites closely resembling the ribosomal RNA targets with which they interact in ribosome assembly. Hence perhaps the activation of ββ' translation observed with certain poorly suppressed rpoB amber mutations (rpoB12 and rpoB1603: Dennis et al., 1985) represent a lifting of repression; either by an active mechanism sensitive to the levels of functional polymerase, or by interference of the β amber polypeptide with the repressor molecule. Obviously the post-transcriptional activation observed by Dennis and his colleagues requires further investigation.

There are two published reports that show that RNaseIII-processing has no effect on rpoBC translation under normal growth conditions (Barry et al., 1980; Dennis, 1984). However, extrapolation of these findings to suggest that the processing has no role in the regulation of rpoBC translation, under any conditions, may be misleading. Results which I have described in Chapter 5 suggest that RNaseIII-processing is important for post-transcriptional repression of ββ' synthesis, following stimulation of rpoBC mRNA synthesis by rifampicin. It will therefore be interesting to study the effects of RNaseIII-processing deficiency under other conditions in which post-transcriptional regulation of rpoBC has been observed.

Little clear evidence exists for post-transcriptional regulation of α and σ synthesis. It is known that α synthesis is stimulated by partial amino acid starvation, and the indications are that this may occur at the post-transcriptional level (Blumenthal et al., 1976; Reeh et al., 1976; Blumenthal and Dennis, 1980a). However, in contrast to the rpoBC operon, the short stretch of intercistronic DNA between rpsD and rpoA (25bp) is devoid of any recognisable regulatory features (Post and Nomura, 1979). Hence the mechanism by which post-transcriptional

regulation of rpoA could take place remains obscure. Interestingly, recent experiments have shown that a 2- to 3-fold overproduction of ββ', due to induction of a lysogenic λ derivative carrying rpoBC, leads to a 20-40% reduction in α-production directed by the chromosome and a 40-80% reduction in the case of α-synthesis encoded by a multi-copy plasmid, where rpoA is expressed from the inducible strong λ promoter P_L (Meek et al., manuscript in preparation). Although these results require to be complemented by transcriptional data, it is tempting to speculate that post-transcriptional regulation of α is taking place under these conditions.

6.3 Regulation of rpoBC expression under normal growth conditions

The syntheses of β and β' can be regulated at the transcriptional and post-transcriptional levels. What part do these alternative mechanisms normally play in regulating RNA polymerase synthesis?

The rates of β and β' synthesis have been shown to be invariant relative to total protein synthesis over a range of different growth rates (Ralling et al., 1985). In addition growth rate changes over a wide range have no effect on the transcription of rpoBC relative to rplKAJL (Dennis, 1977b; Ralling et al., 1985), implying that there is no change in the amount of readthrough of t_{L7}. Hence the extra active RNA polymerase required for the increase in total RNA synthesis, as the growth rate increases, is presumably supported by the parallel increase in the expression of the RNA polymerase subunits. If any shortfall in active RNA polymerase occurs, due to a sudden increase in global RNA synthesis, there is evidence to suggest that this is accommodated by the substantial idle pools of core enzyme within the cell rather than an alteration in the regulation of RNA polymerase

expression (Matzura *et al.*, 1973; Shepherd *et al.*, 1980). Therefore the transcriptional and post-transcriptional regulation of ββ' synthesis is unlikely to play any major role under these conditions although perhaps the post-transcriptional mechanism fine tunes ββ' synthesis during steady state growth. A different and harder problem to understand is the mechanism by which a sudden increase in total RNA synthesis activates hitherto dormant molecules.

What roles, then, do the transcriptional and post-transcriptional regulatory mechanisms play in the response of *rpoBC*-expression to various growth constraints? A simplistic model is presented in Fig. 6.4. The hypothesis is that if there is either a drop in the rate of ββ' synthesis, or a constraint on the ability of RNA polymerase to initiate transcription, the system detects a drop in the level of active RNA polymerase and responds (perhaps via increased output of an ancillary protein: Π) by inducing increased transcription through t_{L7} into *rpoBC*. If however ββ' synthesis becomes too high, a post-transcriptional feedback mechanism is brought into play which involves translational repression of *rpoBC*, mediated by the binding of holoenzyme or the $\alpha_2\beta$ subassembly to sequences involved in translational initiation of *rpoB*. Recent evidence suggests that the regulatory molecule of *rpoB* translation may be different from the *rpoC* regulator (Meek and Hayward, in press). Although less is known about α and σ synthesis it is likely that the production of both these subunits is co-ordinated with that of β and β'. For example, when ββ' is overproduced by induction of a λ*rpoBC* prophage, a repression of α synthesis and of chromosomally-encoded β synthesis occurs (Meek and Hayward, in press; Meek *et al.*, manuscript in preparation). Similarly, following rifampicin treatment all the subunits are synthesised at a higher rate (Nakamura and Yura,

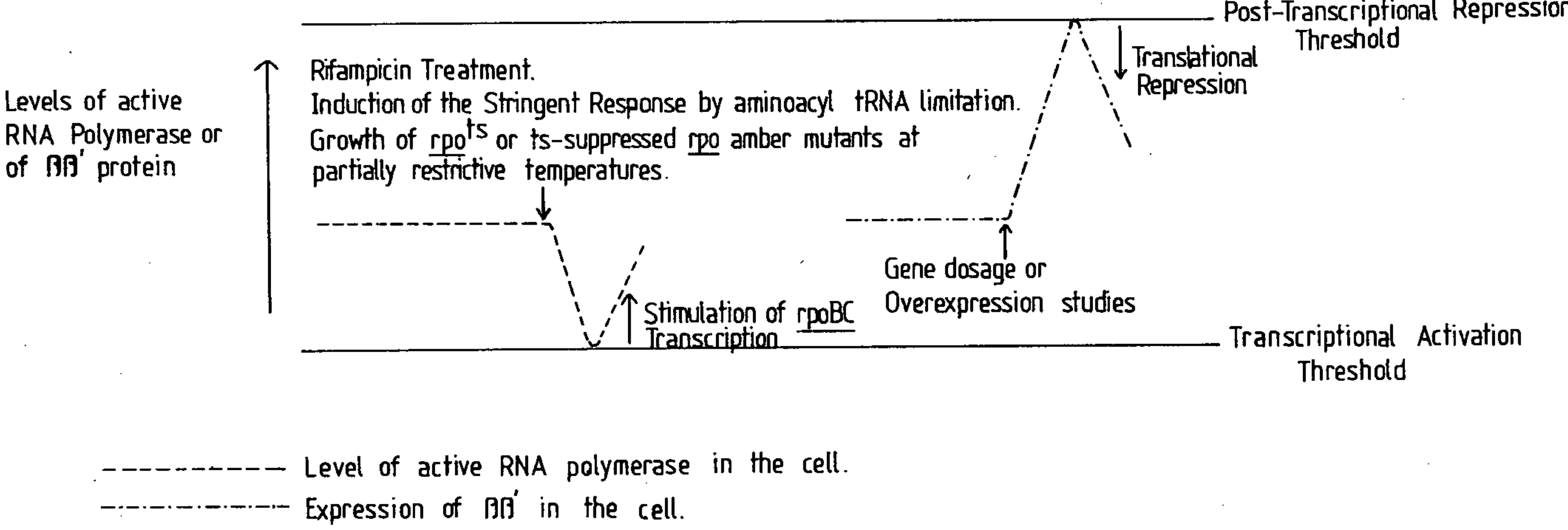


Fig. 6.4

Fig. 6.4 Suggested roles of the two levels of regulation of *rpoBC* expression in maintaining β and β' concentrations within defined limits.

1976a; Hayward and Fyfe, 1978a), perhaps through the action of Π. Hence it is clear that the cell can co-ordinate repression or activation of the production of RNA polymerase subunits.

My study of the $\underline{rif}^{d}$18 genotype (Chapters 3 and 4) has not revealed any associated regulatory mutation, although it is clear from my data that (at least one) secondary mutation, distinct from rpoB3, is involved. The nature of the extra genetic change is unclear and it is possible, if the W3110-del1 and CR63 analyses were misleading (due to the similar rifampicin resistance phenotypes of the $\lambda\underline{rif}^{d}$18 and λAJN261 lysogenic derivatives of these two strains: see Table 4.1 and discussed in 4.7(ii)), that it is a regulatory mutation. I know from the sequencing analysis that it does not lie within the translational start signals for rpoB, or in the t_{L7} region; however, mutations for example in the P_{L10} region, or in the nut-like sequence withing rplJ, have not been excluded. Therefore further investigation of the $\underline{rif}^{d}$18 genotype may yet provide insight into the regulation of rpoBC; and it would have additional relevance because $\lambda\underline{rif}^{d}$18 has been the primary source of DNA for the regulatory analyses of β and β'.

Apart from further study of $\lambda\underline{rif}^{d}$18, other series of experiments are suggested by the results I have presented. These include:

1) Detailed SI-analysis of the t_{L7} region in $RNaseIII^{-}$ strains (or using different M13 probes) to demonstrate directly the increased readthrough of t_{L7}, under various further conditions known to partially uncouple rpoBC from rplKAJL transcription. The sensitivity of my technique allows mRNA transcribed from the chromosomal locus to be tested, obviating the need to amplify mRNA levels by using plasmid strains.

2) A study of the role of RNaseIII-processing in post-transcriptional regulation of <u>rpoBC</u>. This work needs protein double-labelling experiments to allow real quantitation of the effects of a processing constraint on ββ' synthesis.

3) Mutagenesis of t_{L7}, and use of the plasmid screening system, to identify mutations which may not only affect the efficiency of termination at t_{L7}, but more interestingly its regulation.

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APPENDIX

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The wild-type nucleotide sequence of the rpoBC-attenuator region of Escherichia coli DNA, and its implications for the nature of the $rif^{d}18$ mutation.

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The wild-type nucleotide sequence of the *rpoBC*-attenuator region of *Escherichia coli* DNA, and its implications for the nature of the *rif*^{d}18 mutation

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ABSTRACT

To investigate the possibility that the unusual dominant rifampicin-resistance characteristic of the rif^{d}18 allele of E.coli rpoB is due to a secondary, regulatory mutation, we have determined the nucleotide sequence of a 1.1 Kbp wild-type DNA fragment, including the transcriptional attenuator and translational start-site of rpoB. We have also re-investigated the previously published sequences of this region in λrif^{d}18 and λrif^{d}47 DNA. Our results indicate that all three sequences are identical, and reveal some errors in the published data. We discuss the basis of dominance of rif^{d}18.

INTRODUCTION

The entire DNA nucleotide sequence of the rplKAJLrpoBC operon, encoding ribosome- and RNA polymerase subunits in Escherichia coli, has been reported by Post et al (1), with extension and correction by Delcuve et al (2); together with Gurevitch et al (3), Ovchinnikov and Colleagues (4,5), and Squires et al (6). The Soviet groups (3-5) studied DNA derived from λrif^{d}47 (7); the rest, λrif^{d}18 (8). These rpoBC-transducing phages carry (different) mutations of the rpoBC operon which have the unusual property of conferring not only resistance to rifampicin, but dominance over the wild-type (rpoB^{+}, RifS) allele in terms of drug response. The sequence of wild-type DNA has been reported (9) only for the EcoRI-generated internal fragment of rpoB, basepairs 3528 through 6401(Fig.1). (We use the Post et al scale (1) as corrected in (2), and adjusted for two other discrepancies discussed below). The only reported differences between wild-type, rif^{d}18 and rif^{d}47 DNA within this fragment are shown in Table 1 (base pairs 4516 and 4561), and are believed to generate the amino acid changes in the β-subunit of RNA polymerase which lead to rifampicin-resistance. The published work also suggests that, upstream of the above EcoRI fragment, there is a surprising deletion of AGC in rif^{d}18 as compared with rif^{d}47, and that the "tail" sequences of the transcriptional attenuator which lies between rplL and rpoB (10) may differ as between these two alleles (Table 1).

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We have previously reported evidence (11) which suggested that the dominance property of λrif^{d}18 - rpoBC DNA might be genetically separable from the rifampicin-resistance mutation at base pair 4561. Specifically, a λ-derivative carrying the HindIII-'rplJL rpoBC fragment of λrif^{d}18 (constructed in vitro) was recombined in vivo into rpoB^{+} (RifS) DNA originally derived from E.coli AJ1 (12). The lysogen was then induced, and aberrant excision products were selected; among these was λAJN261, which had inherited a functional promoter for rplJL rpoBC from the chromosome. This phage expresses the rifampicin-resistance, but not the dominance property of λrif^{d}18 (11). This finding, together with other unpublished, preliminary data led us to consider the possibility that dominance arises from a closely linked, cis-acting regulatory "up" mutation, located upstream of the rifampicin resistance mutation. This could cause dominance by increasing the proportion of drug-resistant molecules among the RNA polymerase population. One possible mutation of this type would lead to an improved ribosome-binding sequence for initiation of rpoB translation (or reduced feedback regulation of this translation); another possible class would reduce the efficiency of transcriptional termination at the attenuator between rplL and rpoB. The reported difference in attenuator sequence between λrif^{d}18 and λrif^{d}47 DNA (1, 3) lent some credibility to the latter model (see Table 1). We have tested these ideas by sequencing of the appropriate DNA.

EXPERIMENTS AND DISCUSSION

We have compared the DNA sequences of the rpoBC-attenuator region from four existing clones in λ: λrif^{d}47 (7); λrif^{d}18 (8); λAJN63, carrying a HindIII-'rplJL rpoBC fragment from the wild-type (RifS) chromosome of E.coli K12 strain CR63 (11); and λAJN261 (11), obtained as described above via recombination between DNA derived from λrif^{d}18 and from the chromosome of E.coli AJ1 (rpoB^{+}, Rifs). Appropriate EcoRI - SalI fragments were sub-cloned into M13 mp10 and -mp11 (13), and sequenced by a recent modification of Sanger's dideoxynucleotide approach (14). In this way sequences were determined for at least one strand throughout the EcoRI ('rplL-attenuator-rpoB') fragment (basepairs 2444 through 3533) of λrif^{d}18, λAJN63, and λAJN261; and for both strands between the following limits: wild-type (CR63) DNA, basepairs 2645 to 2731 and 3175 to 3318; λrif^{d}18 DNA, 2534 to 2836 and 3200 to 3348; and λAJN261 DNA, 2659 to 2785 and 3188 to 3291. Note that the attenuator region (circa 2660 to 2725) and the AGC at 3286 to 3288 have been determined on both strands. Note also that two independent pre-

Table 1. Differences between wild-type and other DNA sequences in the 'rplL-rpoB' region of E.coli DNA, base pairs 2444 to 6401.[a]

Basepair(s)	Locus	Sequence observed				Ref.
		Wild-type[b]	λAJN261[c]	λrif[d]18	λrif[d]47	
2543	rplL	-	-	T	-	1
		-	-	-	C	3
		C	C	C*	C	This work
2714-2720	Transcriptional attenuator (3'-tail)	-	-	C_2T_4	-	1, 2
		-	-	-	C_1T_7	3
		C_2T_5*	C_2T_5*	C_2T_5*	C_2T_5	This work
3286-3288	rpoB	-	-	-	AGC[d]	4
		-	-	deleted	-	2
		AGC*	AGC*	AGC*	-	This work
4515-4517	rpoB	GAC	GAC	GAC	GTC[e]	1, 9
4560-4562	rpoB	TCC	TTC[f]	TTC[f]	TCC	1, 9 This work

a On the scale of Post et al (1) as corrected by (2) and by the present work.
b From E.coli CR63, via λAJN63 (11), in the present work.
c The DNA sequenced was obtained by recombination between a derivative of λrif[d]18, and E.coli AJ1 ($rpoB^+$): see text.
d Confirmed by amino acid sequencing (4).
e GTC encodes val, replacing asp; this is the rpoB255 (RifR) mutation of λrif[d]47.
f TTC encodes phe, replacing ser; the rpoB3 (RifR) mutation of λrif[d]18.
* Determined by us on both strands of the DNA.

parations of λrif[d]18 DNA were sub-cloned and sequenced in these critical areas; and that the batch of λAJN261 DNA used was re-packaged, and shown to produce the same Rif phenotypes as bona fide λAJN261 when lysogenised in AJN1 and AJN10 (11). Finally, λrif[d]47 DNA (from a plasmid sub-clone kindly provided by Professor R.B. Khesin) was sequenced (on one strand only) between basepairs 2444 and 2729.

Our main conclusion is that the wild-type, λrif[d]18, and λAJN261 sequences are identical throughout the above EcoRI fragment (basepairs 2444-3533; Fig.1). λrif[d]47 is also identical to wild-type in this region, assuming that where

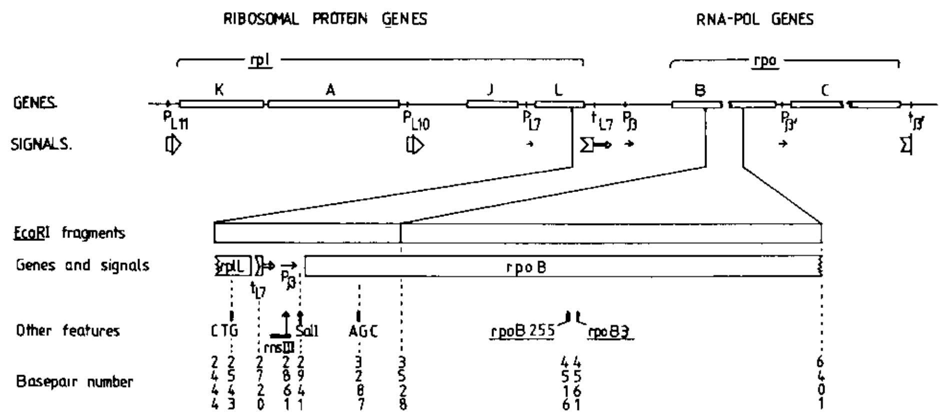

Fig.1 Map of the rplKAJL rpoBC operon of E.coli K12 (map position 90 min), based on refs. 1,2,4,9,10 and 18. The expanded segment has now been sequenced for wild-type DNA (9, and this work). ⇨,strong promoter; ⟶, weak promoter: Σ⇨ ,attenuator; Σ| , strong terminator; ⊤ , RnaseIII target. See Table I and text for the significance of the other features marked, and the basepair scale adopted.

the early sequence of Gurevitch et al. (3) disagrees with our data (2444 to 2729), or those (4) of Ovchinnikov et al. (2889 to 3533), it is the Gurevitch et al. results which are in error. Even in those regions which we have sequenced on only one strand, and where "compressions" or "pile-ups" engender possible ambiguities in reading the sequence, the band patterns of all four clones are so identical that we are convinced there could be no hidden differences. Taken together with the work of Ovchinnikov's group (9) the known wild-type sequence now extends from basepair 2444 (near the down-stream end of rplL) to 6401 (near the downstream end of rpoB). The only difference between rif^d18 and wild-type in this region is the rpoB3 (RifR) mutation at 4561 (9), supporting the argument of Ovchinnikov et al (ibid.) that this single mutation causes both resistance and dominance. Similarly, the only difference between rif^d47 and wild-type is the rpoB255 (RifR) mutation at 4516 (9). These findings are consistent with the original suggestion (8,and Kirschbaum, pers. comm.) that rif^d mutations are merely a special class of rif^r mutations, perhaps producing an RNA polymerase which is unusually competent at displacing rifampicin-inactivated sensitive enzyme from blockaded promoters (15-17). However, direct evidence for this hypothesis is not yet available.

We have recently found that λAJN261 fails to express the dominant rifampicin-resistance displayed by its parent, λrif^d18, not only when

lysogenised in E.coli AJN10 (11) but also in H105, the Rec$^-$ genetic background in which λrif^{d}18 was originally characterised (8). We have also proven, by direct sequencing of the appropriate PvuII-generated DNA fragment cloned into the SmaI target of M13mp10, that λAJN261 is identical in sequence to wild-type DNA (4, 9) between basepairs 4497 and 4824, except that (as expected) it retains the rpoB3 mutation of λrif^{d}18 (Table I). The difference in dominance properties between these two phages therefore remains to be explained.

Our results have also revealed four apparent errors in published sequences (Table 1). (i) C, not T occurs at position 2543 in all four clones. Thus the leucine residue determined here is encoded by CUG, as in 43 of the other 46 leucines in rplKAJL (1), rather than the rare UUG. (ii) There are five rather than four successive T residues in the "3'- tail" of the attenuator (basepairs 2716-2720), in both λrif^{d}18 and wild-type DNA. (iii) The attenuator in λrif^{d}47 is indistinguishable from that of λrif^{d}18 and wild-type. (iv) λrif^{d}18 does not have a deletion of AGC at nucleotides 3286-8. The contrary conclusion of Delcuve et al. (2) may have arisen from a "compression" tendency which makes sequencing in this region tricky. The reported failure of endonuclease FnuDII to cut λrif^{d}18 DNA at this point (2), as it should if AGC were present, seems to have been a misleading negative result. We have found that the isoschizomer ThaI does indeed cut λrif^{d}18 DNA here.

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SI ANALYSIS OF P_{L10} ACTIVITY IN THE E.COLI rpoBC OPERON AFTER AMINOACYLtRNA LIMITATION OR RIFAMPICIN TREATMENT[1]

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ABSTRACT The rpoBC (RNA polymerase) genes of E.coli are usually cotranscribed with four ribosomal protein genes from a single promoter, P_{L11}. Another strong promoter, P_{L10}, lies between rplA and -J, but is normally occluded. Rifampicin treatment and aminoacyl-tRNA limitation partially uncouple rpoBC from rpl transcription. We have used S1 mapping to ask whether or not P_{L10} plays a role in this uncoupling.

INTRODUCTION

The RNA polymerase of E.coli consists of 4 non-identical subunits $\alpha, \beta, \beta', $ and σ (1) encoded by genes rpoA, B, C and D respectively. rpoBC share an interesting operon with four genes encoding 50S ribosomal proteins: rplK, A, J and L. The basic structure and main transcriptional signals of the operon are shown in Fig.1 (see reviews 2 - 4). Strong promoters lie upstream of rplK (P_{L11}), and between rplA and -J (P_{L10}). Under normal conditions, however, transcription of the operon initiates mainly if not solely at P_{L11}; P_{L10} is occluded (5; andC. Squires, pers. comm.). When P_{L11} is deleted, P_{L10} shows its innate strength (2-4).

Under various constraints, such as a mild challenge with the RNA polymerase inhibitor rifampicin, or partial limitation of aminoacyl-tRNA supply, a partial uncoupling of rpoBC- from rplKAJL- transcription occurs (6,7). The drug challenge stimulates transcription of rpoBC strongly,

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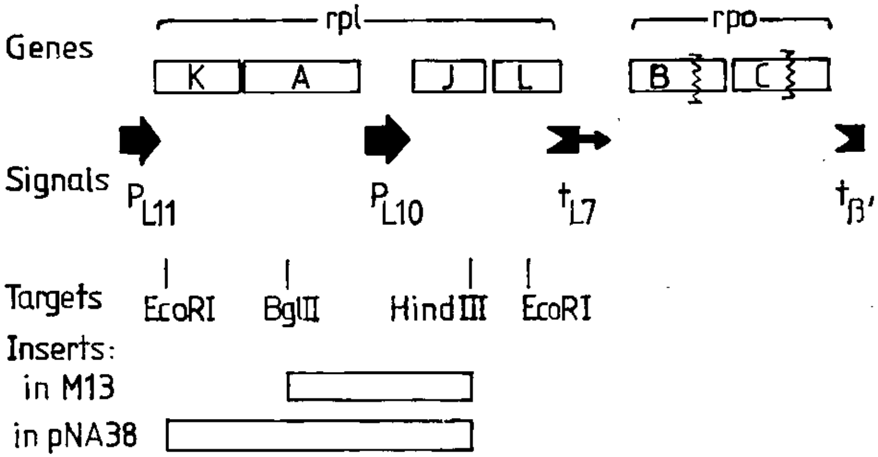

FIGURE 1. Map of the E.coli rplKAJL rpoBC operon, showing the genes, strong promoters(➡), partial terminator (➡), strong terminator( ), relevant restriction targets, and extents of operon DNA in the M13 ss DNA and plasmid ds DNA probes used (not to scale). For sources of data see refs. 2-4, and 16).

but rplKAJL only weakly (6). Valyl-tRNA limitation, achieved by shifting a culture of a rel^{+} $valS^{ts}$ strain to partially restrictive temperature, reduces transcription of rplKAJL (stringent response) with scarcely any effect on that of rpoBC (7). The mechanism of uncoupling by rifampicin clearly includes increased readthrough of the partial transcriptional terminator (normally 80% effective) lying between rplL and rpoB (Fig.1): (8, 9, 10). However, our unpublished observations have suggested that rifampicin might also activate P_{L10}, whether passively (by reducing initiation at P_{L11}) or by some more active mechanism. There is no direct evidence as to whether the partial terminator and/or $P_{L10}$are involved in the uncoupling by valyl-tRNA limitation. However, stimulation of rpoBC-transcription is also observed when certain rpo-ts mutants, or ts-suppressed rpo-amber mutants are exposed to partially restrictive temperatures (2-4, 11).Insome of these cases rplJL transcription was also increased, though to a lesser extent than for rpoBC. Accordingly, it is again possible

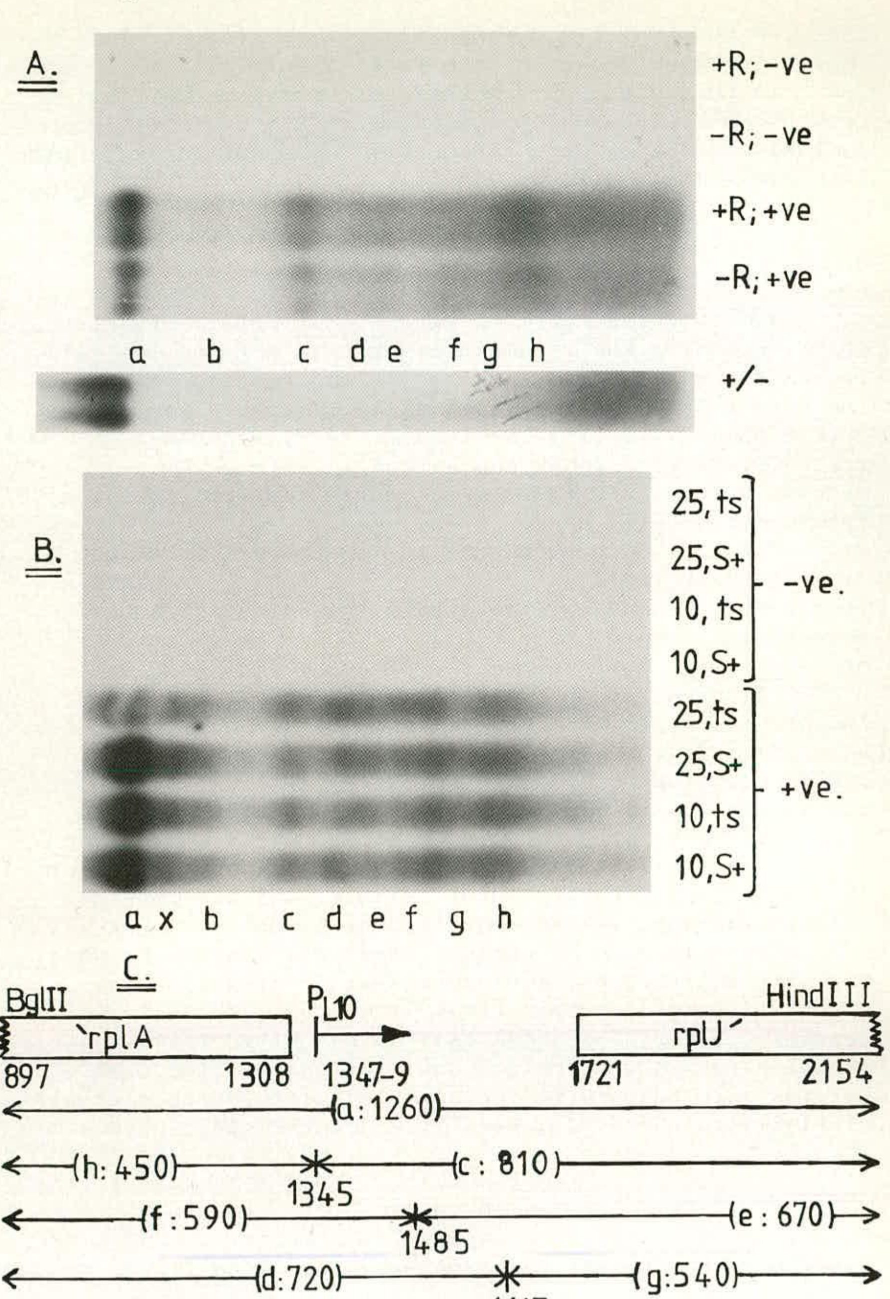

FIGURE 2.

that the constraint may activate P_{L10} as well as increasing readthrough of the partial terminator.

In this paper we demonstrate, however, that two growth constraints which stimulate rpoBC-transcription: partial inhibition with rifampicin, and partial starvation for valyl-tRNA: give no significant stimulation of P_{L10}.

MATERIALS AND METHODS

All bacterial strains were E.coli K-12 derivatives. ED3867 (used in the rifampicin work) is thi trp lys galK lac-delX74 malA rpsL(StrR) tsx (J. Maule, pers. comm.). The strain facilitating valyl-tRNA limitation was AB4141: metC56 thi-1 valS7(Ts) lacY1 galK2 xyl-5 ara-14 rpsL69(StrR) tfr5 tsx57 supE44 rel^{+}. Its $valS^{+}$ control derivative, EMR3, was constructed from AB4141 by P1 transduction (E.A. Marson, pers.comm.).

The M13 DNA probes used in S1 analysis were made by cloning the BglII/HindIII ('rplA-P_{L10}-rplJ') fragment from pNA38 (8: and Fig.1) between the BamHI and HindIII targets of M13mp10 and -11 (12). They were characterised by restriction analysis, and by DNA sequencing (13).

Cultures were grown to an A^{650} of 0.5 in L-broth. In the drug experiment the cultures (37°C) were grown for a further 10 or 25 min in the presence or absence of rifampicin (10μg/ml). In the valyl-tRNA limitation work the isogenic $valS^{+}$ and valS(ts) strains, grown at 30°C, were shifted to 38°C (which should be sufficient to produce partial limitation: cf. 7) or 42°C and grown for 10 or 25 min further. Total cellular RNA was then isolated essentially as described (14).

Hybridisation of excess (25μg) M13 single-stranded DNA with RNA (1.5mg), and subsequent S1 digestion was essentially as described (14). Hybrids were analysed on neutral 1.5% or 2% agarose gels as described (15). After staining (ethidium bromide) and UV-visualisation the hybrids were transferred to nitrocellulose, probed using ^{32}P-labelled pNA38 DNA (Fig.1), and autoradiographed.

RESULTS

Hybrids Formed by RNA from Unconstrained E.coli.

As shown in Fig.2A, RNA from untreated ED3867

FIGURE 2. S1-resistant hybrids formed between the '*rplA*-P_{L10}-*rplJ*' ssDNA probes and RNA extracted (A) from ED3867 or (B) from AB4141 (*valS*(ts)) and EMR3 (*valS*$^+$). In *A*, RNA was extracted after 10 min growth in presence (+R) or absence (-R) of rifampicin; "+ve" signifies the DNA probe complementary to *rpl*-mRNA; "-ve" the opposite strand; a to h are hybrid bands discussed in text. The lowest track in A represents the +ve and -ve probes mixed, hybridised, S1-treated, and run in a parallel gel track. In *B*, RNA was extracted *10* or *25* min after shifting a *valS*-ts (ts) or *valS*$^+$ (S+) culture from 30° to 38°C. Note that in both A and B, no significant hybrids of lower mobility were detected, with the exception of faint bands near the wells (presumably due to chromosomal DNA in the RNA preps). *C*. Interpretation of the hybrids seen in *A*, sized by comparison with markers (not shown) and assigned with the aid of ref 5. Numbers in brackets are sizes in bp; other numbers are nucleotide coordinates on the scale of Post *et al* (16). The positions of probe and gene termini and of P_{L10} start nucleotides are shown near the top of the diagram; and the putative RNA processing sites, below the lower lines. For b and x, see text.

produces a major hybrid band (a) whose estimated size is 1.26kb, equivalent to that of the probe used (1.257 kb), and indeed parallel to the S1-resistant DNA formed by hybridisation of the positive and negative strand probes. Other positive-probe hybrids observed, with their mean estimated lengths in kb, are b (1.09; very faint); c (0.81); d (0.72) and e (0.67), poorly resolved; f (0.59) and g (0.54), poorly resolved; and h (0.45). There is no evidence of transcripts complementary to the other DNA strand, even after prolonged exposure of the autoradiographs. Although not strictly from unconstrained cells, the hybrids formed by RNA extracted from EMR3 (or AB4141) 10 or 25 min after a shift from 30° to 38°C are qualitatively very similar, and better resolved (Fig. 2B), providing us with alternative size estimates. Our probes extend from the *BglII* site in *rplA* to the *HindIII* site in *rplJ*. In their similar studies Bruckner and Matzura (5) used two probes, extending from the *EcoRI* site in *rplK* to the same *HindIII* site, or to the *EcoRI* target downstream (in *rplL*) (*cf*. Fig.1). All of our hybrids (except b and x) can be interpreted (Fig. 2C) in the same way as by Bruckner and Matzura. This provides a useful confirmation

of their conclusions, because we have used ss instead of ds probe DNA, and have used excess probe instead of their excess RNA (which could in principle have yielded misleading multiple-hybrid/S1 products). The overall conclusion from both studies is that the great majority of the mRNA is initiated upstream of rplKA (presumably at P_{L11}) and continues beyond rplJ, without any rapid processing. However, there is evidence of slow endonucleolytic processing in the rplA to rplJ intercistronic region (see Fig. 2C): firstly at nucleotide 1605 ($\pm$ 10) (scale of Post et al, 16); secondly at 1500 ($\pm$ 15); and thirdly, perhaps, at 1340 ($\pm$25). In the first two cases the rough molar equivalence of bands d and g, and of f and e, respectively (and of the corresponding hybrids in 5) favour the hypothesis that they result from RNA processing - although the alternative of weak transcriptional termination and re-initiation is not formally excluded. With respect to the 1340 region, however, this alternative (or additional) possibility is favoured both because P_{L10} is known to allow initiation at nucleotides 1347-1349, and because there appear to be more moles of hybrid h than of c. If so, then although all of c might arise from processing, part of h should reflect transcriptional termination near P_{L10}. We cannot say whether or not there is any initiation at P_{L10}; it might be entirely occluded (cf. 5), or might contribute roughly one fourth (ED3867) to one tenth (EMR3/AB4141) of downstream transcription.

Hybrid b is very faint. Its size is compatible with initiation (or processing) near nucleotide 1045 (inside rplA); or termination (or processing) near nucleotide 2010 (inside rplJ); or some intermediate explanation. We cannot yet distinguish between these possibilities; all are unexpected. Hybrid x is equally faint, and unexplained.

Effects of Rifampicin.

10 min after treatment of ED3867 with rifampicin there is strong stimulation of β and β' synthesis (data not shown: comparable to ref. 17); but no striking change in the pattern of RNA hybrids formed with our probe (Fig. 2A; and densitometry). There is an increase in hybrid b, but it remains a minor band. There is no evidence of decreased termination near P_{L10} (band h is not reduced), nor of increased initiation there (band c is not more prominent). The results after 25 min of drug treatment (not shown) were

similar, except for some preliminary indication that initiation at P_{L11} may have decreased, and/or the general rate of processing increased. At this stage the stimulation of $\beta\beta'$ synthesis had passed its peak.

Effects of Temperature Shift in valS(ts) and valS+ Strains

Neither at 10 nor 25 min after shifting valS(ts) or valS+ cultures from 30° to 38°C does the extracted RNA show any detectable change in hybridisation patterns with our probe (Fig.2B. The seeming reduction in hybrid A in one track is almost certainly an artefact. It was not observed in repeat experiments). The same absence of change applies (with the positive M13 probe) after a shift to 42°C (not shown). ^{3}H-uridine pulse-labelling studies, moreover, confirmed a strong reduction in stable RNA synthesis at 42°C in the valS(ts) strain, only, as expected if valyl-tRNA limitation were producing a strong stringent response.

However, we have preliminary evidence (not shown) that there is weak convergent transcription, giving rise to hybridisation with the negative strand probe, when the 42°C constraint is applied to AB4141; and also after 25 min rifampicin treatment in ED3867. The observed hybrids are heterogeneous, averaging 200bp after 10 min, and with further material in the 300-600 bp range after 25 min. We have no evidence yet as to the point of initiation of convergent transcription. Although preliminary, these data are striking because the "minus" probe shows no hint of RNA hybridisation after milder challenges (Fig.2) - or in the valS+ strain after 42°C shift - despite prolonged exposure of the autoradiographs.

DISCUSSION

Our results confirm those of Bruckner and Matzura (5) in suggesting (i) that most rplJL rpoBC mRNA is initiated at P_{L11} (upstream of rplKA), so that P_{L10} must be largely (if not entirely) occluded; (ii) that there is slow intercistronic endonucleolytic processing of the mRNA between rplA and rplJ, near positions 1605, 1500 and perhaps 1340; and (iii) that there may well be partial termination near the latter position, i.e. in the vicinity of P_{L10}. Regarding (i) and (iii), it is interesting to

speculate that if there *is* termination near P_{L10}, the associated pausing (18) could increase the mean dwell-time of polymerase (originating from P_{L11}) over P_{L10}, and thus help to explain its occlusion. Regarding (ii), one might speculate that the slow endonucleolytic processing could be the first step in degradation of *rplKA* mRNA. Bruckner and Matzura have already noted that there are potential RNA secondary structures near each site, which might act as processing targets; and Post *et al* (16) earlier presented independent evidence for processing (or termination) in the vicinity of nucleotide 1600. Our results also suggest reproducible strain-dependent differences in rates of processing, at all 3 sites and/or in initiation and termination near P_{L10}. In this respect EMR3 and AB4141 may resemble the strain W3350 (5), whereas ED3867 gives results more compatible with the hybridisation data of Dennis *et al* (19) for strain AJ5002.

Our main purpose in these experiments was to investigate whether the relative stimulation of *rpoBC* versus *rplKA(JL)* mRNA synthesis, by mild challenge with rifampicin or by valyl-tRNA limitation (*cf*. Introduction), might be due in part to reduced occlusion of P_{L10}. We have found no evidence of this. We have also seen no indication that the proposed termination of transcription near P_{L10} is opposed by rifampicin, despite the drug's observed readthrough effect on many other terminators (8,20). The increase in hybrid b following drug treatment requires further investigation. Our most intriguing, although preliminary observation is that the more extreme constraints may lead to significant transcription of the opposite DNA strand, a phenomenon not detectable under the other conditions studied. Although we do not yet know the point of origin of this backwards transcription, it seems unlikely to be relevant for the stimulation of $\beta\beta'$ synthesis, because *inter alia* it is not detectable after 10 min in rifampicin, when $\beta\beta'$ stimulation is already strong. However, if the convergent transcription has any physiological significance it could play a role in the unexplained transience of stimulation, which may (6) reflect a post-transcriptional inhibition. Note also that convergent transcription could, in principle, help to limit continued initiation of translation of *rplKA*- and/or *rplJL*-mRNA during extreme valyl-tRNA limitation, when it may be essential to maintain significant basal transcription through *rpl(KA)JL* to ensure *rpoBC* expression.

Our data are subject to two general caveats because

of the protocols used. We have not yet examined pulse-labelled RNA preparations; such work is now in progress. Moreover, our method of labelling hybrids by transfer and probing is subject to the vagaries of differential transfer efficiency. However, our control data closely resemble those of ref.5, to which the second criticism does not apply. We shall be pleasantly surprised if pulse-labelling reveals a strong stimulation of P_{L10} initiation entirely hidden in our present data.

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