Regulation of Transcription in the rpoBC Operon in Escherichia coli K12

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ABSTRACT

The transcriptional organisation of the rpoBC operon in E.coli K12 has been elucidated, principally by molecular cloning and functional analysis of restriction fragments of E.coli DNA. The rpoBC genes (encoding the β and β' subunits of RNA polymerase) lie immediately downstream from a cluster of 4 ribosomal protein genes, rplKAJL, and were found to be co-transcribed with 2 of these ribosomal protein genes, the operon structure being: promoter rplJL rpoBC.

Evidence from other laboratories places the rplKA genes in a second transcription unit upstream, and establishes the presence of a partially-effective transcription termination site (rpot1) between rplJL and rpoBC.

A set of fusions has been constructed in which the promoters from these 2 transcription units and the rpot1 site are linked to the easily-assayed lacZ gene. Studies with these fusions indicate that:

(i) transcription initiated at the rplKA promoter can contribute to rpoBC expression;

(ii) the efficiency of termination at rpot1 is reduced by the addition of rifampicin, an inhibitor of the initiation of transcription.

The implications of these findings for the regulation of rpoBC transcription are discussed with reference to the literature, and a model is proposed for the regulation of rplJL rpoBC gene expression in E.coli.

Declaration

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

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Abbreviations

bp	base-pair
BSA	Bovine Serum Albumin
CTAB	Cetyl Trimethyl Ammonium Bromide
DTT	Dithiothreitol
EtBr	Ethidium Bromide
gp	gene product
hr	hour
kb	kilobase
kdal	kilodalton
min	minute
MW	Molecular Weight
Rif	Rifampicin
RPase	RNA Polymerase (EC 2.7.7.6)
s	second
Stl	Streptolydigin
ts	temperature-sensitive
trpL	transcribed region of the trp operon preceding the first structural gene, trpE

CONTENTS

CHAPTER 1

1.1 The Role of Nucleic Acid Sequences in Initiation and Termination of Transcription

The genome of Escherichia coli is organised into discrete units of transcription whose boundaries are defined by specific nucleotide sequences at which RNA polymerase is instructed to initiate or terminate transcription. The ability of RNA polymerase to recognise and respond to such signals can be enhanced or diminished by the action of a variety of regulatory molecules, many of which also recognise sequence information in the template.

In this brief introduction I shall not discuss the influence of regulatory molecules on the initiation of transcription (indeed, such a discussion would be of questionable relevance to the thesis). Rather, since the expression of rpoBC in E.coli may be modulated by transcription termination events, the introduction will be biased towards the latter.

A large number of promoter sequences are now known, and although these sites exhibit a wide range of efficiencies and accessory factor dependencies, a degree of structural uniformity is evident. Pribnow (1975) and Schaller (1975) first drew attention to a relatively conserved seven base-pair sequence (the consensus sequence is TATPuATPu with A and G favoured at positions 4 and 7 respectively) centred about -12 (12 bp upstream of the mRNA start-point). Subsequent work has made it clear that all promoters tend towards the consensus sequence in this region. Although only the T at position 6 is present (so far) in every case, in any given promoter at least 5 of the positions agree with the consensus sequence. Positions 1, 2 and 6 are the most strongly conserved. The G at

position 7 and in addition the 6 nucleotides immediately upstream of the heptamer are only weakly conserved (Rosenberg and Court, 1979).

A further consensus sequence is detectable in most promoters at a region about 35 bp upstream of the mRNA start-point (Takanami et al, 1976). A highly conserved trinucleotide TTG occurs adjacent to and upstream of a less stringently conserved ACA sequence (and again, the consensus spreads beyond this, at least downstream). This region is thought to function in the initial recognition of the promoter by RNA polymerase prior to formation of the tightly-bound initiation complex. Mild DNAase I digestion of promoter DNA protected by RNA polymerase ("footprinting") suggests that RNA polymerase covers the promoter region for some 60-70 bp upstream of the mRNA initiation site (Schmitz and Galas, 1979). However, although the sequences upstream of the -35 region reveal a tendency to AT-richness they otherwise show no homology.

Most promoter point mutations affect promoter function adversely, and the majority are clustered in the conserved region around -12 (Rosenberg and Court, 1979). Furthermore, almost all the known -12 region mutations occur within the three most highly conserved residues of the Pribnow heptamer. Promoter mutations also cluster in the -35 conserved region. Those mutations which enhance the activity of promoters are also concentrated in the -12 and -35 regions and the majority of them are found to increase the homology to the consensus sequence (Rosenberg and Court, 1979).

Studies on the effects of bound RNA polymerase upon chemical modification of promoter DNA have revealed that nucleotide positions thus detected as contact sites fall principally within the regions implicated in promoter function by sequence homology and mutational

data. There are also, however, (for example, in the lacUV5 promoter: Johnsrud, 1978) several nucleotide positions lying outside the conserved sequence regions which show enhanced reactivity towards methylation with DMS as a result of polymerase binding, and so presumably RNA polymerase contact sites are not restricted simply to the regions of conserved sequence.

In general, however, the good agreement between these 3 very different approaches encourages the belief that the promoter sequence features described above are of some functional significance.

Comparisons of the sequences of termination sites reveal that: (i) a region of hyphenated dyad symmetry (i.e. an inverted repeat) precedes the termination site in almost every known case; (ii) the 3' terminus of the RNA transcript frequently contains a series of uridine residues; (iii) the DNA preceding the termination site is often, but not invariably, GC-rich. There is substantial variation among the terminators in the length of the sequence repeated and in the distance between the two repeats, but termination generally occurs 20 (±4) nucleotides beyond the centre of dyad symmetry. It is rare for all transcripts to end at a unique residue; usually two or more adjacent base-pairs can encode the terminal ribonucleotide (Rosenberg and Court, 1979).

In vitro, these sites exhibit a spectrum of dependence on rho factor for termination. Rho is a protein (monomer MW 50 kdal) isolated from E.coli (Roberts, 1969) and encoded by the rho gene (Ratner, 1976). Rho factor exhibits two activities: the catalytic release of RNA from transcription complexes at specific sites on DNA and an RNA-dependent hydrolysis of nucleoside triphosphates. The

former is dependent on the latter, and both activities require the persistence of the transcript (Darlix, 1973; Galluppi et al, 1976).

λt_{R1}, the $tRNA_{tyr}$ terminator and a terminator in the λcro gene all exhibit absolute dependence on rho factor for termination in vitro (Rosenberg et al, 1978a; Küpper et al, 1978; Calva and Burgess, 1980), but note that in the absence of rho RNA polymerase is known to pause at t_{R1} before continuing to elongate the transcript (Adhya and Gottesman, 1978). Rho does not affect the termination efficiency at the trp attenuator in vitro but may stimulate release of the leader transcript (Fuller and Platt, 1978). The λ6S transcript (probably an aborted late gene mRNA) is terminated and released in the absence of rho factor (Rosenberg et al, 1975) although addition of rho affects the yield and 3' terminus of the transcript.

There may be some significance in the fact that none of the rho-dependent terminators (λt_{R1}, $tRNA_{tyr}$ and λcro) has a series of U residues at the 3' end of the transcript (the $tRNA_{tyr}$ terminator, furthermore, has no dyad symmetry either). However, in T7 tec1 the run of U residues is short and interrupted (Dunn and Studier, 1980), and yet this terminator is completely rho-independent. In short, there seems to be no simple relationship between terminator structure and rho-dependence.

The molecular mechanism of rho action is unclear, but genetic evidence on the interaction between various rho and rpo alleles, and the site-specificity of the phenotypic effects of different rho alleles suggests that rho almost certainly interacts with RNA polymerase as well as the transcript (Das et al, 1978; Guarente and Beckwith, 1978).

Which features of termination sequences are important in the termination event? The inverted repeat sequences could, *a priori*, be involved in the formation of intra-molecular stem-loop structures in the DNA or RNA, or in DNA:RNA pairing. The first of these possibilities seems to be eliminated by the observation that if GTP is replaced by ITP in transcription of the *trp* attenuator (*trpL*$^+$), polymerase fails to terminate (IC base-pairs are weaker than GC base-pairs), clearly implicating base-pairing involving the RNA in the termination event (Lee and Yanofsky, 1977). Mutations in λt_{R1} which increase (*cin*) or decrease (*cnc*) the efficiency of termination also increase or decrease the stability of the stem structure (Rosenberg *et al*, 1978ab), and point mutations in *trpL* which relieve termination are concentrated in the GC-rich region and mostly convert GC to AT base-pairs (Stauffer *et al*, 1978). These changes could destabilise RNA:DNA or RNA:RNA base-pairing. However, among these mutations are a GC to CG change in *trpL*, and 2 *cnc* mutations at t_{R1} which convert AT to GC base-pairs. These should not affect RNA:DNA pairing. Hence the phenotypes of these termination mutants are likely to result from changes in stem-loop formation in the transcript.

Farnham and Platt (1980) have extended these observations in a study of the effects of analogue incorporation and of a polymerase mutation on readthrough at *trpL*$^+$, and at a deletion derivative *trp*∇*LC*1419. This deletion removes the distal 4 TA base-pairs in the run of 8 beyond the stem-loop in the *trp* attenuator, and abolishes termination *in vitro*. Polymerase can terminate, however, when the analogue iodo-CTP (which forms stronger base-pairs with G) replaces CTP. A mutant polymerase (*rpo*203: Guarente and Beckwith, 1978)

which restores termination in rho-defective strains, also terminates at *trpVLC*1419 (at a point just past the deletion junction). Analogues such as Br-UTP and allylamine-UTP (which form stronger base-pairs with A) elevate readthrough at *trpL*$^+$, as does low temperature for transcription by wild-type polymerase (although not by *rpo*203 polymerase). These observations suggest a mechanism for termination in the absence of rho involving both RNA:RNA and RNA:DNA interactions. Farnham and Platt propose that polymerase terminates or continues to transcribe on the basis of two parameters: the stability of the terminator stem-loop in the RNA and the strength of polymerase-stabilised base-pairing between the distal nucleotides of the transcript and the DNA. Martin and Tinoco (1980) suggest that a duplex between the template and an oligo-U tract at the 3' end of a transcript would be markedly unstable, (and a 3' run of A residues is not a likely alternative) on the basis that the RNA:DNA hybrid complex $rC(pU)_5pG:dC(pA)_5pG$ is ≥200 times less stable at room temperature than the corresponding complex containing $rC(pA)_5pG:dC(pT)_5pG$. In the absence of additional factors then, polymerase should require both a stem-loop and a 3' stretch of uridine residues in the transcript for termination. Bearing in mind the absence of a series of TA base-pairs from the λt_{R1}', *λcro* and $tRNA_{tyr}$ terminators, Farnham and Platt suggest that in these cases the action of rho might be to confer on a paused ternary complex the instability which would otherwise derive from weak 3' terminal rU:dA pairing. The *rpo*203 polymerase presumably stabilises rU:dA regions less effectively than the wild-type, so that its termination complexes would be more prone to terminate.

Is there information essential for termination distal to the

DNA sequence encoding the 3'-terminal ribonucleotides of the transcript? The published sequences show little obvious homology in this region (Rosenberg and Court, 1979). Furthermore, of two insertions of new DNA 7 bp and 17 bp beyond t_{R1}, and a deletion in trpL (∇147) whose promoter-proximal end was 11 nucleotides beyond the termination site, none had any effect on termination (Rosenberg et al, 1978ab; Bertrand et al, 1977).

Wu et al (1980) reported that several deletions which abolished termination at the end of the trp operon in vivo had their proximal end-points 12-73 nucleotides beyond the apparent point of termination of trp mRNA. However, more recent results indicate that in vitro there are two functional termination sites at the end of the trp operon: (i) a weak, rho-independent terminator with stem-loop and oligo-U features; (ii) a strong rho-dependent terminator in the region removed by the deletions, which shows a tendency toward AT-richness but no remarkable sequence features. The first (upstream) site corresponds to the 3' terminus of trp mRNA isolated in vivo, but this 3' end is apparently the result of termination principally at the second site and subsequent trimming back of the transcript by processing enzymes (L. Guarente, pers. comm.). Hence there is no evidence to suggest that sequences distal to the region encoding the 3' end of the transcript are required for termination.

To conclude this short section, it should be emphasised that it is not possible at present to recognise promoters or terminators definitively (still less to assess their efficiencies) merely by inspection of a nucleotide sequence.

1.2 Attenuation of Transcription

The presence of efficient transcription termination sites at the ends of operons is essential to prevent uncontrolled effects on the expression of genetic information lying downstream. It has recently become apparent, however, that termination sites also occur within bacterial operons, and that the expression of distal genes in the same transcription unit can be modulated ("attenuated") by varying the efficiency of termination at such sites. One example of attenuation of transcription will be considered in detail, since the mechanism may be of some relevance to regulation in the rpoBC operon.

Expression of the 5 structural genes trpEDCBA of the trp operon in E.coli is controlled by the tryptophan apo-repressor, a 47 kdal homodimer encoded by the unlinked trpR gene (Gunsalus et al, 1979). RNA polymerase cannot initiate transcription at the trp promoter when the repressor complex (aporepressor:tryptophan) is bound at the overlapping operator site. Various observations, however, have long suggested that trp regulation involves additional mechanisms.

For example, several trpS mutants (trpS is the structural gene for trp-tRNA$_{trp}$ synthetase) display abnormal regulation of the trp operon (Ito et al, 1969). Furthermore residual regulation by tryptophan (or a product derived from tryptophan) is shown by all trpR mutants, including several trpR amber strains which synthesise little or no active repressor (McGeoch et al, 1973; Morse and Morse, 1976). Since trp expression in trpR strains is reduced in response to 7-azatryptophan (which is inactive as a co-repressor but recognised as a substrate by trp-tRNA$_{trp}$ synthetase), but is not affected by 6-methyltryptophan (an active corepressor), the

possibility that leakiness of the trpR mutations is responsible for this residual regulation is effectively excluded. Additionally, a trpS mutant which cannot charge $tRNA_{trp}$ with 7-azatryptophan no longer shows any reduction in trp expression in response to 7-azatryptophan (Morse and Morse, 1976). Evidently part of the regulation of the trp operon is related in some way to the activity of trp-$tRNA_{trp}$ synthetase.

Certain internal deletions of the trp operon were found to produce a co-ordinate increase (up to 9-fold) in the expression of the trp structural genes under conditions of derepression (Jackson and Yanofsky, 1973). These deletions have their upstream end-points in the leader region (a 162 bp segment preceding the first structural gene, trpE) and their distal termini in one of the 5 trp structural genes. They do not prejudice normal trp operator-promoter function (Jackson and Yanofsky, 1973; Bertrand et al, 1976). This suggested that there might be a regulatory site between the trp promoter-operator and trpE, which normally functions to reduce trp operon expression independently of the repressor-operator interaction. Furthermore, the regulatory consequences of these deletions are cis- not trans-dominant, indicating that their action is to remove a cis-acting site in the trp leader region rather than to abolish the expression of a diffusible gene product (Morse and Morse, 1976).

Using quantitative hybridisation analysis of RNA from cells grown in the presence of excess tryptophan it was shown that trpR prototrophs synthesise leader RNA in 8 to 10-fold excess over structural gene mRNA (Bertrand et al, 1976), presumably as a result of transcription termination in the leader region. Consistent

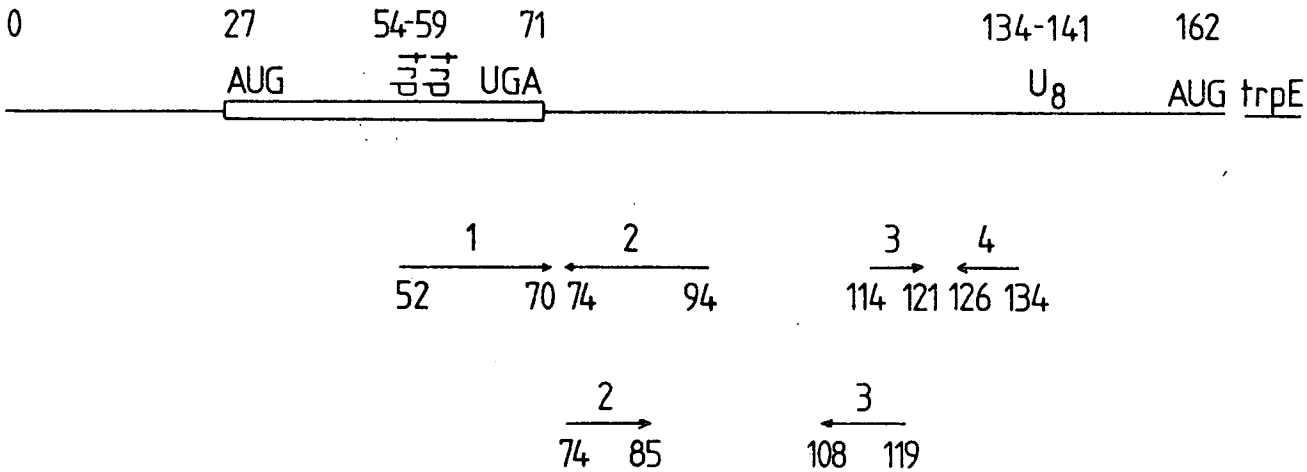
with this conclusion is the detection *in vivo* of a discrete *trp* mRNA species corresponding to the initial *ca.* 140 nucleotides of the leader region (Bertrand *et al*, 1976). Furthermore, the presence *in vivo* of mRNA containing both leader and structural gene sequences (Bronson *et al*, 1973) demonstrates that termination is less than 100% efficient and that transcription of *trpEDCBA* results from polymerases transcribing through the leader region. These results suggest that attenuation could be achieved by regulating the proportion of RNA polymerases which continue into the *trp* structural genes.

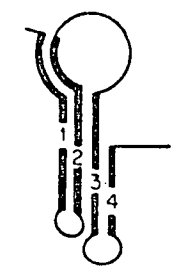
The nucleotide sequence of the 162 bp *trp* leader region (Squires *et al*, 1976; Lee *et al*, 1978) has several features bearing on the mechanism of termination. An AUG initiation codon occurs at positions +27 to +29 adjacent to a Shine-Dalgarno ribosome binding sequence in the leader RNA, and ribosomes bind tightly at this region as well as to the *trpE* AUG at +163 to +165 (Platt *et al*, 1976). Furthermore there is a UGA stop codon at +69 to +71, in phase with the leader AUG codon, suggesting that the leader could code for a 14 amino-acid peptide, which would have tandem tryptophan residues (an unusual feature) at positions 10 and 11. The sequence also reveals a GC-rich region of dyad symmetry followed by a run of 8 TA base-pairs at +134 to +141, reminiscent of the structure of other known terminators in *E.coli* (Fig. 1.1); the *trp* leader transcript could therefore form a stem-loop by intra-molecular base-pairing.

This terminator-like sequence does indeed function as such, both *in vivo* and *in vitro*. Thus transcription of the *trp* leader region *in vitro* produces transcripts terminated at nucleotides

Fig. 1.1 The left-hand side shows the RNA transcribed from the *trp* leader region in *E.coli*, with the leader peptide coding region represented by an unfilled box. The figures refer to the nucleotides from the startpoint of transcription to the *trpE* AUG initiation codon, and the arrows beneath represent inverted repeat sequences, which may allow the transcript to adopt extensive secondary structure. Attenuated transcripts end at nucleotides 140-141 in the sequence of U residues.

The right-hand side shows the proposed secondary structures in terminated *trp* leader RNA. The calculated free energies of formation of the stem and loop structures (in kcal mol^{-1}) are also shown (from Oxender *et al*, 1979).





ΔG s
1·2 : -11.2
2·3 : -11.7
3·4 : -20

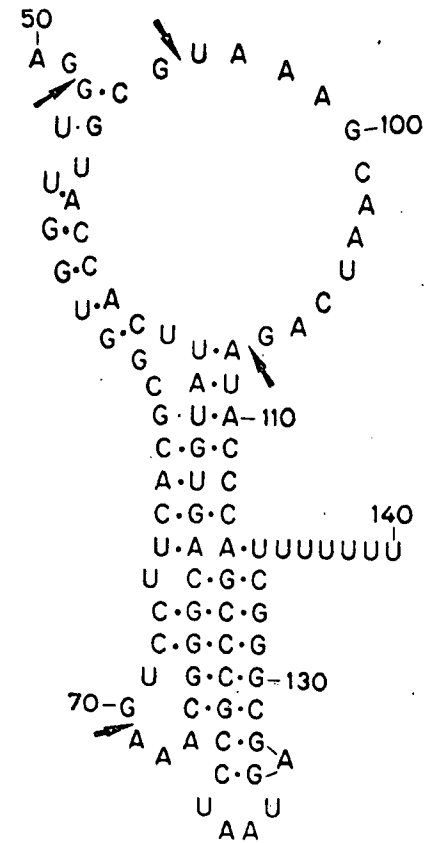


Fig.1.1

+140 and +141, that is with 7 or 8 3'-terminal U residues (Lee and Yanofsky, 1977). In vivo, transcription terminates at any of five adjacent positions in the sequence of 8 TA base-pairs, producing transcripts ending in CU_n-OH where n is 4-8 (Bertrand et al, 1977; Fig. 1.1). The importance for termination of the sequence in this part of the leader is shown by the observation that a deletion whose promoter-proximal end-point is in the centre of the potential stem-loop completely abolishes termination, whereas one ending 11 nucleotides beyond the normal RNA stop-site has no detectable effect (Bertrand et al, 1977).

Partial hydrolysis of leader RNA with RNAase T1 (which cleaves at G residues and shows a strong preference for single-stranded substrates) revealed that other regions of the transcript in addition to the terminator stem-loop are resistant to nuclease attack, and hence probably involved in base-pairing. Comparison of these data with the leader RNA sequence led to the proposal that 4 distinct segments of the transcript might be involved in base-pairing, and that 3 stem-loop structures (1.2, 2.3, 3.4) were feasible (Lee and Yanofsky, 1977; Oxender et al, 1979; Fig. 1.1). Furthermore, formation of stem-loops 2.3 and 3.4 (the terminator) are mutually exclusive, suggesting that transcription termination might be controlled by shifting the equilibrium between these alternative configurations.

Starvation for tryptophan (or arginine, see below), but not for other amino-acids, results in a relief of termination at the attenuator (Bertrand and Yanofsky, 1976; Zurawski et al, 1978). Mutations in trpS leading to defective charging of $tRNA_{trp}$ produce the same result, and indeed there is an inverse correlation between

amino-acylation of $tRNA_{trp}$ and relief of attenuation (Morse and Morse, 1976). Mutations in *miaA* (formerly *trpX*) and *trpT* ($tRNA_{trp}$) can also decrease the frequency of termination at the attenuator (Yanofsky and Soll, 1977). In these two cases, however, the evidence favours the idea that defective tRNA-ribosome interactions rather than inefficient charging of $tRNA_{trp}$ are responsible for relief of attenuation (see below). The elevated *trp* operon expression seen when *trpS*, *trpT*, or *miaA* mutations are introduced into a *trpR* strain does not arise if the strain is also deleted for the attenuator. Furthermore, the increases in *trp* gene expression attributable to *trpS*, *trpT* and *miaA* alleles do not exceed the 10-fold maximum expected to result from loss of attenuator function (Bertrand *et al*, 1976).

The results discussed above imply strongly that translational effects, presumably involving the leader-encoded peptide, are important in *trp* attenuation. Efforts to demonstrate the synthesis of intact leader peptide *in vivo* have been unsuccessful, but ribosomes are known to bind at the leader peptide initiation codon *in vitro* (Platt *et al*, 1976), and indirect evidence for leader translation *in vivo* is compelling. Deletions which fuse the initial segment of the *trp* leader to *trpE* (Miozzari and Yanofsky, 1978) or to the *lacI* gene (Schmeissner *et al*, 1977) lead to the synthesis *in vivo* of novel fusion polypeptides whose N-termini are identical in sequence to the first residues of the putative *trp* leader peptide. In an analogous system (the *his* attenuator) there is further genetic evidence for the importance of leader peptide synthesis. A single base change in the *his* leader region generates an ochre (UAA) codon early in the peptide-coding unit, and results

in a His^- phenotype. Ochre suppressors restore a His^+ phenotype, presumably by allowing translation of the leader peptide to continue (Johnstone et al, 1980).

Oxender et al (1979) have proposed a model in which interactions dependent on the movement of ribosomes translating the peptide-coding region, the concentration or function of charged $tRNA_{trp}$ in the cell, and the secondary structure of leader RNA can account for the regulation of transcription termination at the trp attenuator. Their model assumes that the stem-loop 3.4 functions to signal RNA polymerase to terminate transcription and postulates 3 regulatory conditions of attenuation:

1. Under conditions of tryptophan excess, a ribosome translates the leader RNA as it is being synthesised and masks regions 1 and 2 (Fig.1.1). If the ribosome is not released before region 4 is synthesised, then stem-loop 3.4 forms and polymerase terminates transcription.
2. Under conditions of limiting tryptophan, when $tRNA_{trp}$ is largely uncharged, the ribosome stalls at the trp codons in region 1. Regions 2 and 3 can therefore base pair as they are synthesised, pre-empting formation of stem-loop 3.4 and allowing polymerase to continue into the trp structural genes.
3. In the absence of translation (transcription in vitro, or starvation for some amino-acid near the N-terminus of the leader peptide) region 1 base pairs with region 2, and as regions 3 and 4 are synthesised stem-loop 3.4 forms and signals polymerase to terminate transcription.

This model is consistent with the effects of deletion and point mutations in the trp leader region (Bertrand et al, 1977; Stauffer

et al, 1978; Zurawski et al, 1978). Mutations that relieve transcription termination at the attenuator map in regions 3 and 4, immediately proximal to the site of termination (Stauffer et al, 1978). All these mutations destabilise stem-loop 3.4 (in accord with the idea that integrity of stem-loop 3.4 is required for termination). Furthermore mutations which destabilise stem-loop 3.4 alone show greater relief of termination than those which destabilise both stem-loops 2.3 and 3.4, consistent with the proposed action of 2.3 in pre-empting formation of 3.4. Two mutations which increase transcription termination at the trp attenuator have also been characterised (Zurawski et al, 1978), and their effects on termination suggest that there is normally some competition between stem-loops 2.3 and 3.4 in $trpL^+$ cells even when growing in excess tryptophan. This could arise if the ribosome translating the leader is occasionally released prior to the synthesis of region 4, thereby allowing stem-loop 2.3 to form (note that although 3.4 is more stable than 2.3, 3 is synthesised before 4: Fig.1.1). The trpL 75 G → A mutation destabilises stem-loop 2.3, and increases transcription termination relative to $trpL^+$ (under conditions of tryptophan starvation or tryptophan excess), presumably by favouring formation of stem-loop 3.4 (Zurawski et al, 1978). The trpL 29 G → A mutation changes the leader peptide translation start codon AUG to AUA. When the translation start site for the coat protein gene of Qβ is changed from AUGA to AUAA, the efficiency of ribosome binding is reduced by 93% (Taniguchi and Weissman, 1978). trpL 29 presumably reduces translation of the leader transcript and should allow stem-loop 1.2 to form under all circumstances. The increased transcription

termination seen in trpL29 cells would then result from the reduced competition between 2.3 and 3.4 by prior formation of 1.2. A similar mechanism of action presumably applies to the chain termination mutation in the his attenuator mentioned above.

The model proposed by Oxender et al (1979) suggests that the response of the trp attenuator to starvation for particular amino-acids should depend on the positions of the codons for those amino-acids relative to regions 1 and 2. Relief of transcription termination should only occur when the ribosome stalls at a codon where it will prevent formation of 1.2 but allow formation of 2.3. This provides an explanation for the observation that starvation for arginine is almost as effective as starvation for tryptophan in relieving attenuation in vivo (Zurawski et al, 1978), since there is a single codon for arginine immediately distal to the tandem trp codons (Lee et al, 1978). It is not clear whether this effect of arginine plays any particular physiological role.

The mechanisms by which trpT and miaA mutations relieve attenuation (Yanofsky and Soll, 1977) are also susceptible to interpretation on the basis of this model. The mutant trpT allele produces a $tRNA_{trp}$ with a single base change in the CCA stem which disrupts its secondary structure, and cells carrying this allele show decreased attenuation when unstarved, but restored attenuation when starved for isoleucine. The proposed explanation for this is that the mutant tRNA is defective in a late step in translation (it exhibits normal activity in the $tRNA_{trp}$ charging reaction) and hence stalls ribosomes at the trp codons, mimicking tryptophan starvation (Eisenberg and Yarus, 1980). The mechanism by which isoleucine starvation restores attenuation is probably by stalling ribosomes

at the ile codon (the fourth amino-acid of the peptide) thereby preventing leader translation c.f. *trpL* 29. The *miaA* gene presumably codes for a tRNA modifying enzyme, since the *miaA* mutant is deficient in N^6-(Δ^2-isopentenyl)-2-methylthioadenosine (Yanofsky and Soll, 1977; Vold *et al*, 1979). This hypermodified base has been implicated as a requirement for efficient tRNA-ribosome interaction (Gefter and Russell, 1969) and its absence may lead to inefficient translation of the tandem *trp* codons in the leader sequence (even when the $tRNA_{trp}$ is fully charged), thereby stalling ribosomes in region 1.

Implicit in the model described above is the suggestion that there is a separation in the *trp* leader RNA of structural features that function to *regulate* the transcription termination signal (stem-loops 1.2 and 2.3) from the features that *constitute* the transcription termination signal (stem-loop 3.4 and the oligo-U sequence). Zurawski and Yanofsky (1980), starting from the *trpL* 29 and *trpL* 75 mutants, obtained a series of second site *trpL* mutations which relieved the increased transcription termination caused by *trpL* 29 and *trpL* 75. 18 out of 19 such mutations were single base changes in the 3.4 region which destabilised this stem-loop (the other was a T → G change in the run of 8 TA pairs at the termination site). Furthermore, irrespective of the presence or absence of *trpL* 29 or *trpL* 75, any of these mutations which destabilised stem-loop 3.4 resulted in 2 to 4-fold increased *trp* operon expression relative to *trpL*$^+$. Hence, in agreement with the suggestion above, mutations which destabilise the termination loop 3.4 are *cis*-dominant to those which influence the secondary structure adopted by stem-loops 1.2 and 2.3.

The operation of this elegant attenuation system, then, extends the range and subtlety of the regulation of trp enzyme synthesis. It ensures that although tryptophan starvation elicits increased trp operon expression, general amino-acid starvation does not lead to the futile production of trp mRNA. Finally, several other amino-acid biosynthesis pathways in gram-negative bacteria (e.g. his, thr, ile, phe) utilise very similar mechanisms to regulate expression of the relevant enzymes.

1.3 Control of RNA Polymerase Synthesis in E.coli

The DNA-dependent RNA polymerase of E.coli (EC 2.7.7.6) consists of 4 distinct polypeptide sub-units; α, β, β' and σ (Burgess, 1969), encoded by the genes rpoA-D respectively (Hayward and Scaife, 1976). The protomeric form ("holoenzyme") has the structure $\alpha_2\beta\beta'\sigma$ (Travers and Burgess, 1969; Berg and Chamberlin, 1970). The approximate molecular weights of these polypeptides are:
α, 36.6 kdal; β, 145 kdal; β', 150 kdal; σ, 82 kdal
(Ovchinnikov et al, 1977; Lowe et al, 1979).

Location and Organisation of rpo genes

The gene for the α subunit of RNA polymerase, rpoA, was discovered within a cluster of genes coding for components of the translational machinery (the str-spc region) near 72' on the E.coli linkage map. A series of transducing phages carrying progressively larger segments of E.coli DNA from this region was isolated from a strain into which a lambda prophage had been inserted near aroE at 72' (Jaskunas et al, 1975ab).

These phages were shown to code for ribosomal proteins both in a

DNA-dependent *in vitro* protein synthesising system, and following infection of UV-irradiated bacteria. λ*fus*2, carrying the most extensive bacterial substitution (48 kb of *E.coli* DNA) encoded 27 ribosomal proteins, elongation factors EF-Tu and EF-G, and, in common with two phages carrying smaller stretches of *E.coli* DNA, a protein with the size, antigenic properties and tryptic fingerprint of RPase subunit α (Jaskunas *et al*, 1975b). Hybridisation of mRNA labelled *in vivo* to the separated strands of these phages demonstrated that all these *E.coli* genes were transcribed in the same direction: anticlockwise on the genetic map (Jaskunas *et al*, 1975c). An analysis of the effects of deletions and polar insertion mutations in this gene cluster has established that the genes are disposed in 4 transcription units (Fig. 1.2). Furthermore, *rpoA* is co-transcribed with the genes for 4 ribosomal proteins (S13, S11, S4, L17), the gene order being promoter, *rpsM*, *rpsK*, *rpsD*, *rpoA*, *rplQ* (Jaskunas *et al*, 1975c, 1977ab). The 25 bp *rpsD-rpoA* intercistronic region includes a Shine-Dalgarno sequence (ribosome-binding site) but no suggestion of any promoter or terminator-like entity, supporting the contention that these genes are transcribed from a single upstream promoter (Post and Nomura, 1979).

The *rpoA* 109 mutation blocks late gene transcription in bacteriophage P2 (Sunshine and Sauer, 1975), and is complemented by λ*rpoA* phages (Jaskunas *et al*, 1976). In *rpoA* 109 strains there is a his for leu substitution in the α polypeptide, and furthermore all the α subunits carry this alteration (Fujiki *et al*, 1976). Hence there is only a single active copy of *rpoA* in *E.coli*. More recently two temperature-sensitive mutations in *rpoA* were isolated, after localised mutagenesis of the *aroE-strA* region, by screening for

Fig. 1.2 Map of the λfus3 genome, including the genes for 27 ribosomal proteins, EF-Tu, EF-G and RNA polymerase subunit α. The organisation of these genes into 4 transcription units is shown.

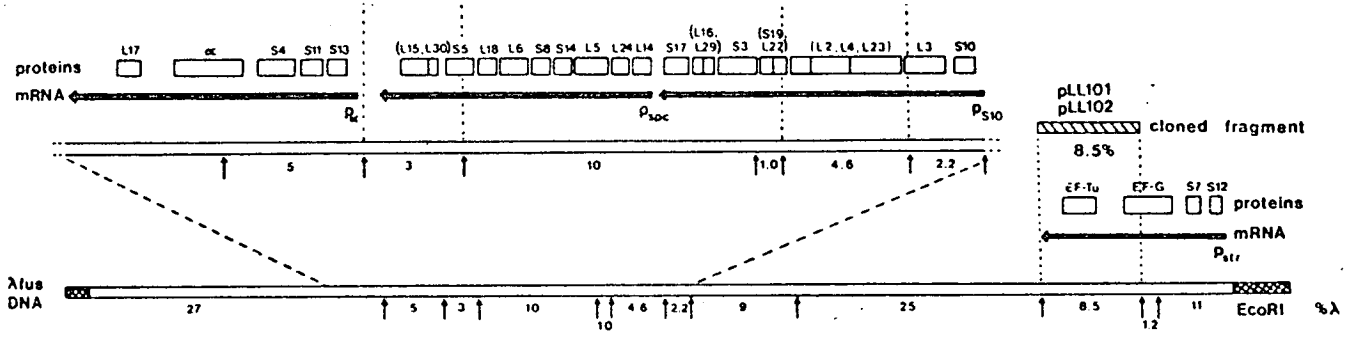

proteins
mRNA
L17
α
S4
S11
S13
(L15,L30)
S5
L18
L6
S8
S14
L5
L24
L14
S17
(L16, L29)
S3
(S19, L22)
(L2,L4,L23)
L3
S10
P_R
P_{soc}
P_{S10}
5
3
10
1.0
4.6
2.2
pLL101
pLL102
8.5%
cloned fragment
EF-Tu
EF-G
S7 S12
proteins
mRNA
P_{str}
λfus
DNA
27
5
3
10
10
4.6
2.2
9
25
8.5
1.2
11
EcoRI
%λ

Fig.1.2

strains defective in RNA synthesis at 43°C. RNA polymerase purified from two such mutants (*rpoA* 101 and *rpoA* 112) exhibited altered thermostability and fidelity of transcription *in vitro*, in each case associated with the α subunit (Ishihama *et al*, 1980).

The structural gene for the β subunit, *rpoB*, was first defined by mutations to rifampicin-resistance, which are found at a single locus (Babinet, 1971), near 89.5' on the latest linkage map (Bachmann and Low, 1980) and have been shown by enzyme reconstitution experiments to cause an alteration in the β subunit (Heil and Zillig, 1970). Furthermore, one rifampicin-resistance mutant, *rpoB7*, alters the electrophoretic mobility of a CNBr fragment of the β polypeptide (Boyd *et al*, 1974). The β subunit is also the target for streptovaricin (Heil and Zillig, 1970) and streptolydigin (Iwakura *et al*, 1973).

Matzura *et al* (1971) provided the first evidence that *rpoB* and *rpoC* (the structural gene for β') might lie in close proximity on the chromosome. Reinitiation of transcription after removal of rifampicin from a culture was succeeded by the appearance of complete, newly-synthesised β subunit after 2 min, but full-size β' did not appear for a further 90s. This lag in the appearance of β' after synchronous reinitiation of transcription is consistent with successive translation of β and β' from a single polycistronic mRNA.

Genetic evidence also suggested that *rpoB* and *rpoC* must lie in the same transcription unit. Amber mutations in *rpoB* were isolated by a method exploiting the dominance of rifampicin-sensitivity over rifampicin-resistance: rifampicin-resistant derivatives of an *rpoB*$^+$ (Rif-S)/F' *rpoB* (Rif-R) merodiploid include nonsense, missense and deletion mutants of *rpoB*$^+$. Such mutants are dependent on the

presence of the F' for survival (Austin and Scaife, 1970; Austin et al, 1971; Errington et al, 1974). One of these amber nonsense mutations in rpoB affected the synthesis of both β and β' subunits in merodiploids (Hayward et al, 1974) and was unable to complement temperature-sensitive mutations in either rpoB or rpoC (Claeys et al, 1976). Furthermore a deletion (∇18) fusing rpoB to the nearly argCBH operon puts rpoC under the control of the arg repressor (Errington et al, 1974), demonstrating that rpoBC are normally co-transcribed, and in the same direction as argCBH (clockwise on the genetic map).

Further investigation of the rpoBC operon was greatly facilitated by the isolation of specialised transducing phages carrying segments of the E.coli chromosome including rpoBC (Kirschbaum and Konrad, 1973; Konrad et al, 1973). One of these phages, λrif18 (carrying the dominant rifampicin-resistance allele, rpoB3) was shown to complement both non-polar and polar amber mutations in rpoB (Kirschbaum and Scaife, 1974); hence it clearly expresses both rpoB and rpoC.

In λrif18 most of the λ sequences to the left of the attachment site are replaced by a 30 kb segment of E.coli DNA (Lindahl et al, 1977). Infection of UV-irradiated bacteria with this phage elicits the synthesis of not only the β and β' subunits of RNA polymerase, but of a number of 50S ribosomal proteins viz. L1, L7/12, L10, L11, encoded by the genes rplA, rplL, rplJ, rplK (L7 is an N-acetylated derivative of L12); EF-Tu (encoded by tufB) and an unidentified 20 kdal protein "20 k" (Lindahl et al, 1975 and 1977; Jaskunas et al, 1975d). The bacterial substitution in λrif18 also includes a ribosomal RNA transcription unit (rrnB) and the genes for 5 tRNAs

(tgtB, thrU, tyrU, glyT, thrT). Thus λrif18 carries a cluster of genes for components of the cellular transcriptional and translational machinery (Lund et al, 1976; Yamamoto et al, 1976; Yamamoto and Nomura, 1979).

Heteroduplexes formed between λrif18 and the related but independently-isolated phages λrif12 and Ø80rif (Kirschbaum and Konrad, 1973; Konrad et al, 1973) revealed complete homology in the region coding for EF-Tu, the ribosomal proteins and ββ' (Lindahl et al, 1977). This strongly suggests that no significant sequence rearrangements have occurred during transducing phage genesis, so that the bacterial DNA in λrif18 should be a faithful facsimile of the corresponding region of the E.coli chromosome.

Physical mapping of λrif18, in concert with the use of purified restriction fragments as templates in vitro in a DNA-dependent protein synthesising system, revealed the order and locations of the bacterial genes. Significantly, the 4 ribosomal protein genes map together upstream of, and adjacent to the rpoBC genes (Lindahl et al, 1977). Furthermore, hybridisation of in vivo labelled RNA to the separated strands of λrif18 has established that all the bacterial genes on λrif18 are transcribed leftward (Jaskunas et al, 1976). In view of the observed cotranscription of rpoA with ribosomal protein genes, it seems reasonable to speculate that rpoBC might be in the same transcription unit as one or more of the upstream 50S ribosomal protein genes.

The first information on the location of the structural gene for the σ subunit of RNA polymerase, rpoD, came from a study of the differential synthesis rate of σ in strains harbouring various F' factors, which were thereby merodiploid for different regions of

the chromosome. It was found that all F' strains diploid for the metC-argG region exhibited a 2-3 fold increase in the differential synthesis rate of σ (Nakamura et al, 1977). Furthermore, the σ subunits of E.coli K12 and S.typhimurium are resolvable by SDS-polyacrylamide gel electrophoresis, and strains of S.typhimurium harbouring the appropriate F' plasmids produced E.coli σ in addition to S.typhimurium σ. There is also a mobility difference between the σ subunits of E.coli K12 and E.coli C, and this characteristic was more than 90% co-transducible with dnaG, suggesting that the σ gene should map at ∿66' on the linkage map (Harris et al, 1977).

Several mutations characterised by the production of altered or defective σ subunit were also found to map close to dnaG. Gross et al (1978) demonstrated that σ subunit from a mutant designated rpoD1 is thermolabile in vitro, although rpoD1 strains show no unusual thermosensitivity. The alt-1 mutation, selected for its ability to allow cAMP-independent expression of the arabinose operon (Silverstone et al, 1972), was later found to be an alteration of the σ subunit leading to thermosensitivity in vivo and altered promoter preference in transcription assays in vitro (Travers et al, 1978). Two additional ts mutants, rpoD 285 and rpoD 303, produced σ subunit thermolabile in vitro, and having (in the former case) altered electrophoretic mobility (Harris et al, 1978; Nakamura, 1978). All these mutations mapped close to dnaG at 66'.

Transducing phages coding for the σ subunit have recently been isolated by virtue of their ability to transduce $dnaG^+$ or $rpoD^+$ and so enable lysogenic derivatives of $dnaG^{ts}$ or $rpoD^{ts}$ strains to grow at 42°C. Physical mapping and deletion analysis of the cloned DNA

fragment (a 9 kb HindIII fragment) revealed that rpoD and dnaG are in close proximity, with the promoter for rpoD probably lying between the two genes (Gross et al, 1979; Scaife et al, 1979). Nakamura (1980) has independently isolated a recombinant multicopy plasmid carrying the same 9 kb HindIII fragment, using complementation of rpoD285 as the selection.

Expression of rpo genes

Measurements of the differential synthesis rates of the α, β and β' subunits have established that they are produced in quantities closely matched to their molar ratios in the core enzyme ($\alpha_2\beta\beta'$) over a wide range of growth rates, although most estimates suggest a slight relative overproduction of α subunit (Matzura et al, 1973; Iwakura et al, 1974; Hayward and Fyfe, 1978a). Exponentially growing cells do indeed contain a detectable amount of metabolically-stable, free α subunit (Ishihama et al, 1976). Values obtained for the differential synthesis rate of the σ subunit suggest a figure around 0.3 moles σ per mole β (Iwakura et al, 1974; Engbaek et al, 1976; Hayward and Fyfe, 1978a). In haploid strains the polymerase subunits are as stable metabolically as the bulk of cellular protein during balanced exponential growth (Iwakura et al, 1974; Hayward and Fyfe, 1978a).

In contrast to the close growth rate dependency of the differential synthesis rate of components of the translational apparatus in E.coli (Schleif, 1967), the rate of polymerase core subunit synthesis is less rigidly coupled to growth rate (Matzura et al, 1973; Iwakura et al, 1974; Blumenthal et al, 1976). This in itself suggests that RNA polymerase is not produced

constitutively.* Other observations also point emphatically to the existence of a mechanism able to regulate subunit synthesis.

For example, strains of E.coli carrying the rpoB70 rifampicin-resistance allele on an F' and rpoB$^+$ on the chromosome express both copies of the rpoBC operon, and contain both sensitive and resistant polymerase molecules (Austin et al, 1971). The addition of rifampicin swiftly inactivates the rif-sensitive polymerases. This causes a rapid decrease in the global rate of RNA and protein synthesis, but also elicits a co-ordinate, transient increase in the rate of ββ' synthesis (Hayward et al, 1973; Tittawella and Hayward, 1974). Furthermore this response is observed irrespective of which allele of rpoB is dominant, and occurs also in rpoB$^+$ haploid strains of E.coli treated with sub-saturating concentrations of rifampicin (Nakamura and Yura, 1976). Evidently the ββ' polypeptides are normally synthesised at rates below the maximum of which the cells are capable: there must be a control system superimposed on the biosynthetic limits set by the rpoBC operon copy number and the general supply of transcription-translation machinery.

Hayward et al (1974) observed that in a strain carrying an unsuppressed rpoBam allele on the chromosome and rpoB$^+$ on an F' (at relative copy numbers of about 2:1 during exponential growth)

Footnote:* In the case of σ, the differential synthesis rate scarcely changes over a range of growth rates, so that the core:σ ratio is greater at rapid growth rates (Iwakura et al, 1974; Iwakura and Ishihama, 1975; Engbaek et al, 1976).

the β synthesis rate is not one third of that given by a normal merodiploid, as might be imagined. In fact it is twice this rate, and hence indistinguishable from the normal hapoid β synthesis rate. These results tentatively suggested that a specific regulatory mechanism in some way relates the rate of polymerase subunit synthesis inversely to the amount of active polymerase available, and can thus compensate for a shortfall in effective rpoB copy number.

Glass et al (1975) provided much clearer evidence of such a compensatory mechanism by demonstrating that in strains carrying amber nonsense mutations in rpoB together with an inefficient amber suppressor (giving ∿20% suppression efficiency) β subunit was nevertheless produced at a rate very close to that of the isogenic rpoB$^+$ strain. Moreover, β' (and the N-terminal amber fragment of β: Glass, 1977) was overproduced, the former particularly so when the rpoB amber mutation was only weakly polar. Glass et al also observed that if the suppression efficiency were further reduced (to about 1%) the ability of the system to compensate was overwhelmed, and β synthesis now fell below that seen in the rpoB$^+$ control strain.

Joint induction of β and β' (by rifampicin, or in response to inefficient suppression of an amber mutation) might be explained if their synthesis were autogenously regulated, i.e. directly by product(s) of the rpoBC operon. Various instances of this form of control are known: the following examples show that it can operate at the level of transcription or translation. T4 gp32 binds tightly and co-operatively to all available single-stranded DNA during infection. The amount of gp32 synthesised is coupled to

the amount of ssDNA produced, and is therefore affected by mutations in T4 DNA metabolism. Autoregulation of gp32 synthesis is mediated at the level of translation: gp32 binds first to all available ssDNA but any excess then represses further gp32 synthesis by binding to gene 32 mRNA and inhibiting its translation (Gold et al, 1976; Russell et al, 1976; Lemaire et al, 1978).

λcI repressor mediates negative control of λ early gene expression at O_L and O_R, and also regulates its own synthesis by binding at O_R. O_R contains 3 17 bp repressor binding sites which partially overlap 2 associated promoters P_R and P_{RM}. These promoters direct the divergent synthesis of cro and cI mRNA, respectively. The 3 repressor binding sites differ slightly in their nucleotide sequence, and repressor binds to these sites with an affinity order $O_R1 \equiv O_R2 > O_R3$ (the repressor affinity of O_R3 is approximately 25-fold lower than that of O_R1 and O_R2). Repressor bound to the strong binding sites O_R1 and O_R2 prevents transcription of cro from P_R (by steric exclusion of RNA polymerase) but stimulates transcription of cI from P_{RM}. At relatively high concentrations of repressor, the weakest operator site O_R3 is also occupied and repressor synthesis from P_{RM} is shut off (again by direct exclusion of polymerase). Thus the order of repressor binding to the sites in O_R ensures that at low repressor concentrations transcription of cro is turned off and transcription of cI is turned on, while at high repressor concentrations transcription of both genes is repressed (Ptashne et al, 1976; Sauer et al, 1979).

One of the characteristics expected of an autogenous regulation circuit is the ability to compensate for variations in gene dosage. Indeed a synchronously dividing culture shows no detectable

perturbation of ββ' synthesis upon replication of the rpoBC operon (Matzura et al, 1973). On the other hand a merodiploid harbouring KLF10, and hence having roughly 1.5 times the normal rpoBC operon complement, does exhibit a corresponding gene dosage effect on ββ' synthesis; the resulting additional ββ' subunits are rapidly degraded (Hayward et al, 1974).

This apparent paradox can be rationalised by postulating that rpo control is mediated by a molecule which includes at least the 3 subunits of core polymerase. The rpoA and rpoBC genes (at 72' and 89.5' respectively) both lie near, but on opposite sides of the origin of replication (oriC) at 83.5' (Sugimoto et al, 1979; Meijer et al, 1979). Since the chromosome is replicated bidirectionally (Masters and Broda, 1971) rpoA and rpoBC should be replicated more or less in unison. Accordingly in the haploid cell significant overproduction of the core subunits at the time of rpo gene replication should be prevented by a rise in the concentration of the autogenous 'repressor'.

In the KLF10 merodiploid only the rpoBC genes are in the diploid state, so that this strain is likely to maintain an equilibrium concentration of polymerase molecules similar to that of the haploid. If E.coli regulates rpoBC expression simply through this parameter one would predict that in the F' strain ββ' will be synthesised as a linear function of rpoBC gene copy member, as appears to be the case in practice. Hayward et al (1974) suggested that the observed metabolic instability of a fraction of the newly-synthesised ββ' in the merodiploid might result from the failure of excess β and β' to be incorporated into complete core enzyme.

There is an accumulation of evidence suggesting that expression of the rpoBC operon is regulated at the level of transcription. Dennis (1977a) examined the rate of rpoBC transcription (and concomitant synthesis of the ββ' subunits) in a strain carrying rpoC56, a temperature-sensitive allele affecting the initiation of RNA synthesis (Gross et al, 1976). After a shift to 42°C labelling of RNA stops almost immediately in this strain, but at 39°C RNA synthesis continues at a reduced rate: initiation is restricted by about 2-fold. Nevertheless, rpoBC transcription (assayed by hybridisation of RNA, pulse-labelled in vivo, to suitable plasmid probes) and ββ' synthesis were stimulated by the temperature shift, so that after 30 min at 39°C both transcription and translation of the polymerase subunits had increased 5 to 6-fold relative to total incorporation. This evidently represents an absolute increase in the rates of synthesis.

When bacterial cultures are treated with rifampicin (which inhibits RNA chain initiation without interfering with elongation and completion of nascent RNA transcripts: Sippel and Hartman, 1968) a transient increase in the rates of synthesis of the β and β' subunits (and, indeed, of α and σ) is observed (Hayward et al, 1973; Nakamura and Yura, 1976; Hayward and Fyfe, 1978b). Analogous experiments with streptolydigin (an inhibitor of RNA chain elongation: Schlief, 1969) showed no such stimulation of the synthesis of rpo gene products, although total RNA synthesis in the culture was inhibited to the same extent as above (Tittawella and Hayward, 1974; Nakamura and Yura, 1976). (Note that this failure of streptolydigin to affect specifically the rate of ββ' subunit synthesis in the same way as rifampicin was one of the early

pieces of evidence for autogeny, since it suggested that polymerase might have distinct roles in transcription and self-regulation.) Furthermore, Blumenthal and Dennis (1978) demonstrated that the induction of rpo gene product expression by rifampicin was a transcriptional effect and that within limits, the stimulation of rpoBC transcription was greater when the general restriction on initiation of transcription was more severe. A reduction in transcription initiation can also be imposed by shifting an rpoD285 strain (which produces thermolabile σ subunit) to 42°. In this case transcription of rpoBC and production of ββ' are again selectively increased (Blumenthal and Dennis, 1980).

The evidence presented above is consistent with the operation of a mechanism in E.coli which regulates transcription of rpoBC in inverse proportion to the level of initiation-competent RNA polymerase in the cell. Studies on mutants with defects in polymerase assembly may also be interpreted in this light. The temperature-sensitive alleles rpoC4 and rpoB2 rpoB7 are defective in core polymerase assembly at the restrictive temperature, although the pre-assembled cores are stable and remain active in transcription (Ishihama et al, 1976; Taketo and Ishihama, 1976; Taketo et al, 1976). (The rpoC4 strain overproduces ββ' 2 to 3-fold even at 30°, although at this temperature general transcription is apparently completely normal: further evidence for dual roles of polymerase in transcription and self-regulation.) After a shift to 42°C the previously exponential accumulation of cellular mass and RNA soon becomes arithmetic. In both these temperature-sensitive assembly mutants there is a gradual increase in rpoBC transcription (and a similar change in ββ' synthesis rate) at 42°C, consistent with a

slow dilution during growth of polymerase assembled before the temperature shift (Ishihama *et al*, 1976; Taketo *et al*, 1976; Little and Dennis, 1979).

Is the synthesis of β and β' controlled only at the level of *rpoBC* transcription? There are a number of recent observations, in fact, which clearly suggest that ββ' production is also subject to post-transcriptional regulation. For example, amplification of the *rpoBC* operon (in a strain carrying this transcription unit on a multicopy plasmid) leads to amplified transcription of *rpoBC* (as is presumably the case in the gene dosage effect of KLF10 on ββ' synthesis: Hayward *et al*, 1974), and, furthermore, the strain contains elevated levels of *rpoBC* mRNA. Despite this, the synthesis rates of β and β' were elevated only 2-fold (Dennis and Fiil, 1979). Evidently under these conditions of marked overproduction of *rpoBC* mRNA the synthesis of ββ' is restricted by some post-transcriptional mechanism. The operation of such a mechanism would also explain why induction of ββ' synthesis after addition of rifampicin is a transient phenomenon (Hayward *et al*, 1973) whereas *rpoBC* mRNA synthesis continues at an elevated level for a prolonged period (Blumenthal and Dennis, 1978). The mechanism which limits translation of *rpoBC* mRNA under these conditions is unknown. It is of some interest, however, that in a DNA-dependent protein-synthesising system RNA polymerase holoenzyme represses the synthesis of β and β' without causing any specific alteration of mRNA synthesis coded by λ*rif*18 (Ishihama and Fukuda, 1980). The possible physiological roles played by the post-transcriptional and translational regulation systems will be discussed in Chapter 6.

The mechanisms controlling the synthesis of the α and σ subunits

of RNA polymerase have been subjected to less intensive study and are not at present understood. During exponential growth rpoA is transcribed at the same frequency as the ribosomal protein genes in the adjacent spc operon (Blumenthal and Dennis, 1980). Recall that rpoA is flanked by ribosomal protein genes, with which it shares a promoter, and that the intercistronic region upstream of rpoA is very short and contains no plausible termination site. Yet α synthesis exhibits a growth-rate dependency and response to amino-acid starvation in relA and relA$^+$ strains like that of ββ' rather than that of the translational machinery (Iwakura et al, 1973; Blumenthal et al, 1976). Rifampicin does elicit a modest stimulation of α synthesis (Nakamura and Yura, 1976; Hayward and Fyfe, 1978b) and although it is not known whether any modulation of transcription is involved in this response, a similar restriction of transcription initiation caused by shifting an rpoD285 strain to 42°C is known to result in increased production of α mRNA (Blumenthal and Dennis, 1980). It seems likely that α synthesis, like that of β and β', can be modulated at both transcriptional and translational levels.

As discussed above, merodiploids harbouring an F' carrying rpoD show an increased rate of σ synthesis roughly commensurate with the elevated rpoD gene copy number. When the number of rpoD copies is amplified further, however, by the presence of a multicopy plasmid carrying rpoD (roughly 50 copies per cell), the differential synthesis rate of σ is increased only 4-5 fold (Nakamura, 1980), suggesting a considerable degree of regulatory compensation for the increased gene dosage. Curiously, merodiploids harbouring KLF10 synthesise σ at twice the haploid rate, although KLF10 does not

carry rpoD (Hayward and Fyfe, 1978a). Perhaps this disturbance of rpoD regulation is related to the presence of an additional copy of the rpoBC operon in KLF10. Hayward and Fyfe (1978a) noted that under these conditions sigma is metabolically unstable, and suggested that the excess, unstable β or β' subunits might sequester and cause degradation of a proportion of the sigma pool.

Rifampicin elicits a vigorous stimulation of σ synthesis, but as is the case for the α subunit it is not clear whether this is a transcriptional or post-transcriptional effect, which renders difficult any interpretation of the result.

Progress towards understanding the regulation of α and σ subunit synthesis awaits data on the synthesis of their respective mRNAs.

The experiments described in Chapters 3, 4 and 5 of this thesis are concerned with the regulation of the transcription of the rpoBC operon. They were designed with the following objectives in mind:

1. To reveal whether there are any other genes in the rpoBC transcription unit;
2. To identify the site (or sites) involved in regulating the transcription of rpoBC and of any other co-transcribed genes.

CHAPTER 2

1. Growth Media

L-broth contained, per litre: Difco Bacto Tryptone, 10g; Bacto Yeast Extract, 5 g; NaCl, 5 g; adjusted to pH 7.2. L-agar contained, in addition: Difco agar, 15 g. (Agarose replaced agar for plate lysate DNA preparations - see 2H.)

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

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

Lactose MacConkey agar contained, per litre: Oxoid L37 Peptone, 20 g; Lactose, 10 g; Bile Salts No. 3 (Oxoid L56), 1.5 g; NaCl, 5 g; Neutral red, 0.05 g; Crystal violet, 0.001 g; Oxoid L13 agar No. 3, 15 g.

M9 minimal medium contained, per litre: KH_2PO_4, 1.2 g; Na_2HPO_4, 2.8 g; NaCl, 0.2 g; NH_4Cl, 0.4 g; 1.0 M $MgSO_4$, 5 ml. Final concentrations of supplements were: sugars or glycerol, 0.1% (w/v); amino acids, 20 µg/ml; vitamin B_1, 2 µg/ml. M9 minimal agar contained, in addition: Difco agar, 15 g.

2. Lambda Techniques

A. Titrations. Phage suspensions were serially diluted in phage buffer. 0.1 ml of a suitable dilution was mixed with 0.2 ml of a suitable indicator strain (grown to stationary phase and resuspended in 10 mM $MgSO_4$) and 3 ml BBL Top agar (46°C) and poured onto a BBL plate. Plaques were scored after 12-18 hr incubation at 37°C.

B. Plate lysates. Single plaques were picked into 1 ml phage buffer plus 1 drop chloroform. Approximately 10^6 pfu were mixed with 0.2 ml plating cells and 3 ml BBL Top agar (46°C) and poured onto a fresh, wet L plate. Plates were incubated at 37°C for 5-8 hr to achieve confluent lysis; 5 ml L broth were then added and the plates were refrigerated overnight. The broth was removed, shaken gently with chloroform, clarified (5000 rpm for 10 min; 20°C) and titrated: titres were in the range 5.10^8-1.10^{11} pfu/ml.

C. Liquid lysates. A fresh overnight culture was diluted 1:50 in L-broth supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) glucose and grown shaking at 37°C to OD_{650} 0.45 (2.10^8 cells/ml). Phage (moi 1-2) were then added and the OD_{650} was followed at intervals; when it reached a minimum, 2 ml l^{-1} chloroform were added. Phage were purified from lysates as described in 2D or 2E.

D. Precipitation of phage with PEG 6000. After addition of chloroform to the lysate NaCl was added (40 g l^{-1}) and the flask was shaken at 37°C for a further 10 min. Pancreatic RNAase A (Sigma) and DNAase I (Sigma) were added (1 µg/ml of each) and the lysate was left at 20°C 1-3 hr before clarification (10,000 g for 10 min at 4°C). 0.25 vol 50% (w/v) PEG 6000 were added to the clear supernatant to give a final concentration of 10% (w/v), and the suspension was left at 4°C 1-12 hr. The precipitate was pelleted (10,000 g for 10 min, 4°C), resuspended by gentle rotary shaking in 0.02-0.05 vol phage buffer, and further purified by CsCl step gradient and equilibrium gradient centrifugation as described in 2E.

E. Phage preparation by ultracentrifugation. Phage were harvested from clarified lysates by ultracentrifugation (3 hr at 40,000 g, 4°C),

and the pelleted phage were resuspended in 0.05 vols phage buffer by gentle rotary shaking overnight at 6°C. Debris was sedimented (10 min at 10,000 g, 4°C) and the supernatant was treated at 20°C for 1-3 hr with RNAase A and DNAase I (final 10 µg/ml of each). The phage suspension was then layered onto a preformed CsCl step gradient with steps of densities 1.4, 1.5 and 1.6, and centrifuged in a Spinco SW 50.1 rotor (2 hr at 100,000 g, 4°C) or Spinco SW 25.1 rotor (3 hr at 40,000 g, 4°C). The bluish phage band was collected through the side of the tube using a syringe and hypodermic needle, and mixed with 41.5% (w/v) preclarified CsCl solution. Phage were then centrifuged in a Spinco 50 Ti rotor (48 hr at 80,000 g, 4°C) and the bluish phage band recovered as above.

F. Preparation of λrif18. A single colony of strain H105 (λcI857 Sam7, λrif18) was suspended in 25 ml Oxoid nutrient broth containing Rifampicin (Sigma or Lepetit, 100 µg/ml) and grown shaking overnight at 32°C. This overnight was diluted 50-fold in fresh Oxoid and grown shaking at 32°C to OD_{650} 0.45, and prophage were induced by gentle heating to 42°C. The flask was then shaken for a further 2 hr at 39°C and the cells were pelleted (10 min at 10,000 g, 4°C) and resuspended in 0.02 vols phage buffer. Chloroform was added (1 ml/50 ml suspension) and the flask was shaken at 37°C for 10 min before addition of DNAase I (final 10 µg/ml). After a further 10 min shaking at 37°C cell debris was pelleted (10 min at 10,000 g, 4°C) and the concentrated phage suspension was purified by CsCl step gradient and equilibrium centrifugation as described in E. above, except that λrif18 and helper were separated by collecting 0.5 ml fractions of the equilibrium gradient after puncturing the bottom of the tube.

G. Phage DNA preparation. CsCl was removed by dialysis against TE buffer (10 mM Tris-Cl (pH 7.8), 1 mM EDTA) for 1 hr at 4°C, and the phage solution was extracted four times by gentle rolling with an equal volume of freshly-distilled phenol pre-equilibrated against 0.5 M Tris-Cl (pH 7.8) at 20°C. Residual phenol was removed by dialysis against TES buffer (10 mM Tris-Cl (pH 7.8), 1 mM EDTA, 300 mM NaCl) and subsequently several changes of TE buffer at 4°C over a 24 hr period. Finally the absorbance of the DNA solution was measured at 260 nm, 280 nm and 320 nm.

H. Preparation of phage DNA from plate lysates (Cameron et al, 1977). Approximately 10^6 pfu derived from a single plaque were mixed with 0.2 ml plating cells and 3 ml BBL-agarose (0.65%) at 46°C, and poured onto an L-agarose plate. After incubation at 37°C until lysis was confluent (5-8 hr) 5 ml 10 mM Tris-Cl (pH 7.5), 10 mM EDTA were added and the plate was left overnight at 4°C. To 4 ml of the supernatant were added 0.4 ml 0.5 M EDTA, 0.2 ml 2 M Tris base, and 0.2 ml 10% (w/v) SDS, followed by 10 µl Diethylpyrocarbonate (Sigma). The solution was heated at 65°C for 30 min in open tubes, followed by chilling on ice. 1 ml 5 M potassium acetate (unbuffered) was then added and the solution was left on ice for 1 hr before centrifugation (27,000 g for 10 min, 4°C). The clear supernatant was decanted, mixed with 11 ml ethanol in a siliconised Corex tube, and left overnight at -20°C, or for 10 min in an ethanol/dry ice bath. Nucleic acids were pelleted (12,000 g for 30 min, -10°C), washed with cold 80% ethanol, dried in a vacuum desiccator and finally dissolved in 200 µl TE buffer.

I. Phage crosses. Cells were grown shaking at 37°C in L-broth for 2 hr after a 1:20 dilution from a fresh overnight culture, and then pelleted (5,000 g for 10 min, 20°C) and resuspended twice in 1 mM $MgSO_4$. Approximately 2.10^8 cells and 1.10^9 pfu of each phage were mixed in a final vol $\lesssim$1 ml, and left for 20 min at 20°C for adsorption before pelleting the cells (5,000 g for 10 min, 20°C). The infected cells were resuspended in 1 ml of prewarmed L-broth and diluted 100-fold into warm broth in a flask, which was then shaken at 37°C for 1.5 hr. A few drops of chloroform were added and the flask was shaken for a further 5 min before titration of total phage, and plating on selective bacteria.

J. Construction and testing of lysogens. Cells were grown shaking at 37°C in L-broth to $\sim 5.10^8$ cells/ml, pelleted (5000 g for 10 min, 20°C), and resuspended in 10 mM $MgSO_4$. The bacteria were then infected with transducing phage (and, if required, with a hetero-immune, integration-proficient, helper phage), each at moi 1-2. After 20 min at 20°C for adsorption the mixture was diluted into an equal vol of prewarmed broth and incubated at 32°C or 37°C for 1 hr. Lysogens were selected by the addition, before plating, of approximately 10^9 pfu/plate of a homoimmune $\underline{c}I^-$ phage, or in some cases a mixture of two such phages with the same immunity but different host ranges. Where helper-mediated integration was involved selection was for the immunity of the integration-deficient phage. Putative lysogens were tested for immunity to infection by homoimmune phage, and sensitivity to λvir, by spotting phage dilutions ($\sim 10^9$ pfu/ml) onto streaks.

Table 2.1 Bacterial Strains

Strain	Genotype	Reference or Donor
C600	*lac leu thi thr tonA supE*	Appleyard, 1954
Ymel	*supF*	N. Murray
Ymel ø80^{r}	*supF tonA*	"
H105 lysogen	*recA1* HfrKL16 (λcI857 Sam7)(λ*rif*18)	Kirschbaum and Konrad, 1973
S159	*gal strA uvrA*	Jaskunas *et al*, 1975b
S159 (λ)	*gal strA uvrA* (λ*ind*$^{-}$)	" " "
S159(λimm21)	*gal strA uvrA* (λ*imm*21)	Linn and Scaife, 1978
AA125	∇(*gal-uvrB*) ∇(*lacZ*)M15 ∇(*trpED*)102 *lacI3 his tonA tsx*	N. Murray
ED8538	*lacZ*$_{am}$ *leu trpA*33	Murray *et al*, 1977
JG47	*recA*56 Hfr *ilv spc str thr*	R. Hayward
AW11	*recA*56 *metB* KLF10 *metB rpoB*70	"
LE102	*recA1 argH bfe his lac metB rif str thi* KLF10 *argH*$^{+}$ *metB*$^{+}$ *rpoB*$^{+}$	Linn *et al*, 1979
ED8741	*recA*56 *hsdR hsdM*$^{+}$ *metB trp supE*	N. Murray
HB101	*recA1 gal pro str*	D. Finnegan
CR63	Hfr *supD*	Appleyard, 1954
NEM49	*lac leu thi thr tonA supE* (P2)	Zissler *et al*, 1971

AJ7	recA1 argG lac mal metB rpoB70 str	Boyd et al, 1974
ED8641	recA56 hsdR hsdM$^+$ ∇(trpE-B)9 trpR	N. Murray
MC1000	∇(araABOIC-leu)7697 ∇(lacIZY)X74 araD139 galU galK strA	Casadaban and Cohen, 1980
RSH10	his mal metB rcs40 str thi	R. Hayward
RSH21	his mal metB rcs$^+$ str thi	"
NEM246	lig^{ts} supF	N. Murray
NEM197	groN	Georgopoulos & Herskowitz, 1971
NEM36	polA thi	N. Murray
QR47	supE	Weil and Signer, 1968
AJN1	recA56 metB rcs40 str thi	Newman et al, 1979
AJN5	recA1 argG lac metB rpoB70 str	Newman and Hayward, 1980
AJN10	recA56 metB rcs$^+$ str thi	" "
AJN20	recA56 mal$^+$ metB rcs$^+$ str thi (ANλ172)	" "
AJN21	recA56 mal$^+$ metB rcs$^+$ str thi (ANλ232)	This work
AJN30	his mal$^+$ metB rcs$^+$ str thi	" "

Table 2.2 Phage Strains

Phage Number	Genotype	Reference or Donor
NMλ5	λ^+	N. Murray
NMλ14	b2 immλ cI	"
NMλ54	imm21	"
NMλ243	vir	"
NMλ507	b2 imm21 cI	"
NMλ508	h80 imm21 cI	"
NMλ549	h80 immλ cI	"
NMλ570BV2	$sb1^{\circ}$ $sb(2-3)^{\nabla}$ imm21 nin5	Klein and Murray, 1979
NMλ590	b538 imm434 $shn6^{\circ}$	Murray et al, 1977
NMλ676R	h80 att80 cI857 nin5 Sam7	N. Murray
NMλ761	$srI(1-2)^{\nabla}$ supF $(att\text{-}red)^{\nabla}$ imm21 nin5	Murray et al, 1977
NMλ904	chiA131 red3 gam210	N. Murray
NMλ908	$srI(1-2)^{\nabla}$ $polA^{L}$ $(att\text{-}red)^{\nabla}$ imm21 nin5	"

ANλ20	srI(1-2)$^{\nabla}$ rplL rpoBC^{L} (att-red)$^{\nabla}$ imm21 nin5
ANλ23	srI(1-2)$^{\nabla}$ rplL rpoBC^{R} (att-red)$^{\nabla}$ imm21 nin5
ANλ34	srI(1-2)$^{\nabla}$ tufB rplKA^{R} (att-red)$^{\nabla}$ imm21 nin5
ANλ63	srI(1-2)$^{\nabla}$ rplL rpoBC^{L} (att-red)$^{\nabla}$ imm21 nin5
ANλ81	b538 imm434 cI::(rpot1, P4) shn6°
ANλ172	srI(1-2)$^{\nabla}$ rplL rpoBC^{L} (att-red)$^{\nabla}$ cI857 nin5
ANλ232	srI(1-2)$^{\nabla}$ rplL rpoBC^{R} (att-red)$^{\nabla}$ cI857 nin5
ANλ261	chiA131 srI(1-2)$^{\nabla}$ rplJL rpoBC^{L} (att-gam)$^{\nabla}$ cI857 nin5
ANλ321	sb1° sb(2-3)$^{\nabla}$ rplJL rpoB^{R} imm21 nin5
ANλ363	sb1° sb(2-3)$^{\nabla}$ rplJL rpoB^{L} imm21 nin5
ANλ514	srI(1-2)$^{\nabla}$ rpoBC^{L} (att-gam)$^{\nabla}$ cI857 nin5

Notes: (1) All AN strains were constructed in the course of this work.

(2) L and R denote the transcriptional orientation of cloned DNA fragments.

3. DNA Techniques

A. Ethanol precipitation. DNA was precipitated in siliconised Corex tubes or plastic microcentrifuge tubes at -20°C (2-12 hr) or in ethanol/dry ice (10 min) after the addition of 0.1 vol 3 M potassium acetate and 2.5 vol ethanol, and pelleted (12,000 g for 30 min, -10°C, or 6 min in a Quickfit microcentrifuge, 4°C). Pellets were normally washed with 80% ethanol, spun again, and dried in a vacuum desiccator before resuspension in TE buffer.

B. Restriction endonuclease digestions. Reactions were carried out either in heat-sealed glass capillaries or in plastic snap-cap tubes. Appropriate quantities of DNA, dH_2O, 10x reaction buffer and restriction endonuclease were mixed thoroughly and incubated at 37°C (30°C for BamHI), typically for 1 hr. Reactions were stopped by the addition of excess EDTA and/or heating to 70°C for 10 min. Table 2.4 describes the reaction conditions for each endonuclease.

C. DNA fragment purification.

(i) Sucrose gradient sedimentation: Restriction endonuclease digests (50-100 µg DNA/200 µl) were layered onto sterile preformed linear 5-20% (w/v) sucrose gradients in TE buffer (17 ml polypropylene tubes, Sorvall AH 627 rotor) and centrifuged for 16 hr at 35,000 g, 4°C. 1 ml fractions were collected after puncturing the bottom of the tube, and the fragment of interest was located by running 25 µl of each fraction on an agarose gel. Sucrose was then removed by exhaustive dialysis against TE buffer at 4°C (several changes over 36 hr) and the DNA was concentrated by ethanol precipitation.

Table 2.3 Restriction Endonucleases

Endonuclease	Bacterium of origin	Specificity	Source
AluI	Arthrobacter luteus	AGCT	A. Newman
AvaI	Anabaena variabilis	CPyCGPuG	J.C. Boothroyd*
BamHI	Bacillus amyloliquefaciens H	GGATCC	Mrs K. Mileham*
BglII	Bacillus globigii	AGATCT	Boehringer
EcoRI	Escherichia coli RY13	GAATTC	Mrs K. Mileham*
HaeIII	Haemophilus aegyptius	GGCC	A. Smith (Cambridge)
HhaI	Haemophilus haemolyticus	GCGC	K. O'Hare (Strasbour
HindIII	Haemophilus influenzae R_d	AAGCTT	Mrs S. Bruce*
KpnI	Klebsiella pneumoniae OK8	GGTACC	BRL
PstI	Providencia stuartii 164	CTGCAG	A.M.C. Brown*
SalI	Streptomyces albus G	GTCGAC	Boehringer

* Department of Molecular Biology, Edinburgh

Table 2.4 Reaction conditions for restriction endonucleases

	Tris-Cl		2-mercaptoethanol	DTT	$MgCl_2$	NaCl	BSA*	Triton X-100	KCl
R.endonuclease	mM	pH	mM	mM	mM	mM	µg/ml	% (v/v)	mM
AluI	10	7.6	7		7				
AvaI	10	7.5	10		10				
BamHI	10	7.5	10		10	50			
BglII	10	7.4		1	10				6
EcoRI	10	7.5	10		10	100		0.005	
HaeIII	6.6	7.4		1	6.6				
HhaI	6	7.9	6		6	100			
HindIII	10	7.5	10		10	50		0.005	
KpnI	6.6	7.4		1	6.6				
PstI	100	7.5			10				
SalI	6	7.4	6		6	150	100		

* BSA was freed of any contaminating nuclease activities by several cycles of freezing and thawing.

(ii) Electroelution (McDonell et al, 1977): Restriction endonuclease digests were run out on agarose horizontal gels (Section 4) and two marker tracks were stained in EtBr and photographed under UV. The area from the unstained tracks containing the fragment of interest was then cut out, sliced (2 mm slices) and put into a 1" dialysis sac with ∿2 vol 1x EE buffer (5 mM Tris base, 2.5 mM Acetic acid). The DNA was eluted by immersing the sac in 400 ml 1x EE buffer in a tank with 150 V applied across its electrodes, usually for 3 hr. The buffer was then removed from the sac, filtered through siliconised glass wool, extracted twice with phenol and twice with diethyl ether, ethanol-precipitated, washed with 80% ethanol and dried in vacuo before resuspension in TE buffer.

(iii) Passive elution: The region of the gel containing the fragment of interest was smashed by forcing it twice through a fine hypodermic needle, and shaken (12-24 hr at 20°C) in 10 vols 150 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA. Agarose fragments were removed by filtering through siliconised glass wool, and the solution was passed through a 1 ml DEAE-cellulose (Whatman, DE52) column in a siliconised pasteur pipette at 5 ml hr^{-1}. DNA bound to the column was eluted with 2 ml 1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and concentrated by ethanol precipitation.

Methods (i) and (iii) gave good yields (20-50%) and purified fragments were recut easily with other restriction endonucleases; method (ii), although more rapid, gave lower yields (10-20%) and purified fragments were sometimes difficult to recut.

D. Recombination of DNA fragments in vitro. Restriction endonuclease digests were diluted to 10-30 µg/ml with 10 mM Tris-Cl (pH 7.5), 100 mM NaCl. 0.1 vol of 10x T4 ligase cocktail (660 mM Tris-Cl (pH7.2),

10 mM EDTA, 100 mM $MgCl_2$, 100 mM DTT, 1 mM ATP) were added together with T4 DNA ligase (generously provided by Mrs K. Mileham or R. Jefferson). Ligation was at 10°C for 3-6 hr, after which samples were withdrawn for transfection/transformation.

E. Transfection/transformation of recombinant DNA. A fresh overnight culture was diluted 1:50 in L-broth and grown, shaking, to OD_{650} 0.55 at 37°C. The cells were chilled on ice for 20 min, pelleted (5000 g for 10 min, 20°C), and resuspended in 0.5 vol ice-cold 0.1 M $MgCl_2$. The cells were immediately pelleted, resuspended in 0.05 vol ice-cold 0.1 M $CaCl_2$ and left on ice 0.5-6 hr before use. The DNA was diluted to 0.1 ml in SSC:$CaCl_2$ (3 vol 1x SSC:4 vol 0.1 M $CaCl_2$), and 0.2 ml competent cells were added. After 30 min on ice the cells were heat-shocked (2 min at 42°C): for transfections the cells were now returned to ice for 30 min before plating out in BBL Top agar supplemented with 10 mM $MgSO_4$; for transformations 1 ml L-broth was added and the mixture was incubated at 37°C for 1 hr before plating on selective media.

4. Gel Electrophoresis

A. Horizontal agarose gels (McDonell et al, 1977). Gels (0.8%-1.7% w/v) were prepared by dissolving agarose in 200 ml TAE buffer (40 mM Tris-acetate (pH 8.3), 33 mM sodium acetate, 1 mM EDTA) with vigorous stirring, on an electric hot plate/magnetic stirrer. After refluxing for 1 min the agarose solution was cooled to 50°C and poured into the perspex mould (30 x 14 x 0.5 cm) with a 13-tooth slot former in place near one end. After the agarose had set the slot former was removed and paper wicks (Whatman 3 MM) saturated with TAE buffer were hung from each end to make electrical contact with the buffer

chambers (each holding 400 ml TAE buffer). DNA samples for analysis were mixed with 5 µl TAE loading buffer (0.5 x TAE, 0.125 M EDTA, 0.1% (w/v) bromophenol blue, 50% (w/v) glycerol) and loaded by micropipette. Gels were run at 80 V, (30 V across the gel itself) typically for 20 hr; after ∿1 hr the gel surface was wetted thoroughly and a Saran wrap covering was applied. DNA was visualised after staining with Ethidium bromide (Sigma, final 1µg/ml) for 20 min, destaining for 40 min in H_2O and transillumination with long wave UV (Ultraviolet Products Inc, Chromatovue C-62, 365 nm peak transmission). Photography was with Ilford FP4 film, using 15 s exposure through a red filter.

B. 3-10% gradient polyacrylamide gels (Maniatis et al, 1975).

Stock solutions for 3-10% gels were as follows:

29% (w/v) acrylamide, 1% (w/v) bisacrylamide (Serva)

10x TBE buffer (0.9 M Tris base, 0.89 M boric acid, 25 mM EDTA, pH 8.3)

10% (w/v) ammonium persulphate.

Two 30 ml solutions, respectively 3% and 10% in acrylamide, were made up as follows: 3 ml or 10 ml acrylamide:bisacrylamide, 3 ml 10x TBE buffer, 0.28 ml 10% $(NH_4)_2S_2O_8$, dH_2O to 30 ml; both solutions were de-gassed (2 min swirling on a water pump) and 7 µl TEMED (N,N,N',N'-tetramethylethylenediamine, Serva) were added to each. A 2-chamber linear gradient-marker was used to pour a 3-10% gradient into a glass sandwich (40 x 14 x 0.15 cm) with perspex spacers sealed with water agar. The gradient was overlaid with 2-butanol and allowed to polymerise (2 hr). The 2-butanol was then decanted and the gel surface was rinsed with 1x TBE buffer. An upper gel mixture was prepared as follows: 1.67 ml acrylamide-bisacrylamide, 2 ml 10x TBE

buffer, 0.28 ml $(NH_4)_2S_2O_8$, 16 ml dH_2O. The mix was degassed and a small quantity used to rinse the gel surface. 7 µl TEMED were then added and the upper gel was poured by pipette. A 13-well slot-former was inserted and 1 hr allowed for polymerisation, after which slot-former and bottom spacer were removed and the gel was set up in a perspex gel apparatus (Raven), with 400 ml 1x TBE buffer in each reservoir. DNA samples for analysis were ethanol-precipitated after digestion, dried in vacuo, and resuspended in 20 µl TE buffer. 5 µl of TBE loading buffer (0.5x TBE, 0.125 M EDTA, 0.1% Bromophenol blue, 50% (v/v) glycerol) were added, and the samples were dried down to 10-15 µl before loading with a Hamilton microsyringe. Gels were run at 250 V for 1 hr, and then at 160 V for 14 hr. Staining was for 5 min in ethidium bromide (3 µg/ml), followed by 2 min destaining in H_2O. Gels were photographed against a black background by overhead shortwave UV illumination (Mineralight C5, 254 nm peak transmission): 5-8 min exposure through a red filter, Ilford FP4 film.

Fragments in the size range 40-3000 bp were normally visible on these gels; between 40 and 1000 bp there was a linear relationship between mobility and log MW.

C. SDS-polyacrylamide gels (Laemmli, 1970)

Stock solutions used were as follows:

A:BA	29.2% (w/v) acrylamide; 0.8% bisacrylamide
4x lower Tris	18.17% (w/v) Tris base, 0.4% (w/v) SDS, to pH 8.8, with conc. HCl
10% SDS	10% (w/v) Sodium dodecylsulphate
4x upper Tris	6.06% (w/v) Tris base, 0.4% (w/v) SDS, to pH 6.8 with conc. HCl

10% AMPS	10% (w/v) Ammonium persulphate
4x running buffer	1.2% (w/v) Tris base, 5.76% (w/v) glycine (pH 8.6)

Preparation of gradient gels was essentially as above (4B), except that gradients were 7-15% or 10-20%, with a 3% stacking gel, and the dimensions of the glass sandwich were 23 x 14 x 0.08 cm. For the lower gel, 15 ml of each solution contained the following:

	7%	15%	10%	20%
dH_2O	7.7 ml	3.7 ml	6.2 ml	1.3 ml
4x lower Tris	3.75	3.75	3.75	3.75
A:BA	3.5	7.5	5.0	9.8
10% AMPS	27 µl	17 µl	23 µl	17 µl
TEMED	8 µl	8 µl	9 µl	15 µl

The 3% stacking gel contained the following in a final vol. of 10 ml: 6.5 ml dH_2O, 2.5 ml 4x upper Tris, 1.0 ml A:BA, 40 µl 10% (w/v) AMPS, 15 µl TEMED.

5% SDS-polyacrylamide gels contained the following: 22.4 ml dH_2O, 10.0 ml 4x lower Tris, 6.8 ml A:BA, 60 µl 10% AMPS, 20 µl TEMED. The stacking gel was as described above.

Protein samples for analysis (sample preparation is described in METHODS 6) were loaded using a Hamilton microsyringe, usually in a volume of 20 µl, and a constant voltage of 80 V was applied until 'stacking' was complete. Gels were subsequently run at 105 V until the bromophenol blue reached the bottom (~16 hr). The stacking gel was removed and discarded, and the lower gel was fixed and stained in 500 ml Westerberg stain (30% (v/v) methanol, 12% (w/v) TCA, 3.6% (w/v) sulphosalicylic acid, 0.2% (w/v) Coomassie brilliant blue) for 30 min at 37°C. Destaining was in 5% (v/v) methanol, 7.5% (v/v)

acetic acid at 37°C. The gel was then rinsed briefly in dH_2O, and dried down onto Whatman 3 MM paper (2 hr on a Bio-rad gel drier) for autoradiography (24-72 hr exposure, without intensification and at room temperature, Kodak XH-1 or BB-5 film).

After autoradiography, the radioactivity in specific protein bands on the gel was sometimes determined by cutting out the region of interest and incubating the gel slice at 37°C, shaking, in 3 ml solubilising scintillant (Petri, 1972: 1.44 g PPO, 40.4 mg dimethyl POPOP, 3.6 ml 1% (w/v) SDS, 40 ml Soluene, 360 ml Toluene) for 12-18 hr. Vials were counted on a Packard Tri-carb scintillation counter.

5. Plasmid Techniques

A. Small scale preparation of plasmid DNA for screening purposes.

A 10 ml overnight culture in L-broth + ampicillin (40 µg/ml) was pelleted (10,000 g for 10 min, 4°C) and the cells were resuspended in 0.5 ml 25% (w/v) sucrose in 50 mM Tris-Cl (pH 8.0). 50 µl of a freshly-made solution of lysozyme (50 mg/ml in 0.25 M Tris-Cl (pH 8.0), 11,000 units/mg, from Worthington) were added, and the suspension was swirled for 1 min at 37°C. After 5 min on ice 0.65 ml 0.25 M EDTA (pH 8.0) was added, and the cells were left on ice for a further 5 min, before addition of 0.5 ml 1% (w/v) BRIJ 58, 0.4% (w/v) sodium deoxycholate in TE buffer. The cells were left on ice to lyse, and a cleared lysate was then obtained by centrifugation (30,000 g for 30 min at 4°C). 1 ml of cleared lysate was extracted with phenol 3 times, and with chloroform:isopropanol (24:1) twice, and nucleic acids were precipitated (after the addition of 0.1 vol 3 M sodium acetate (pH 5.0) and 0.56 vol. isopropanol) by freezing at

-70°C, and thawing at -20°C. The precipitate was pelleted (max. speed in microfuge, 6 min at 4°C), washed with cold isopropanol and dried in a vacuum desiccator. The pellet was then resuspended in 200 µl TE buffer, dialysed against TE buffer at 4°C for 2-12 hr, and returned to a plastic snapcap tube. RNA was degraded by the addition of 5 µl RNAase A (1 mg/ml, pre-treated for 5 min at 80°C) and 30 min incubation at 37°C. 10-30 µl were used for restriction endonuclease digests.

B. Large-scale preparation of plasmid DNA (Clewell and Helinski, 1970)

An overnight culture in L-broth + 1% glucose + ampicillin (40 µg/ml) was diluted 1:50 in the same medium and grown shaking at 37°C to an OD_{590} of 1.0. Chloramphenicol (free base) was added to give 150 µg/ml and the culture was shaken at 37°C for a further 12 hr. The cells were then pelleted (10,000 g for 10 min, 4°C) and washed with 0.5 vol. TE buffer (pH 7.4). Cells from a 500 ml culture were resuspended in 5 ml 25% (w/v) sucrose, 50 mM Tris-Cl (pH 8.1), 40 mM EDTA. 1.5 ml lysozyme (10 mg/ml in the same solution) were added. The suspension was mixed, left on ice for 5 min, and 1.5 ml 0.5 M EDTA (pH 8.1) were added. After a further 5 min on ice 13.5 ml "Triton lysis mix" (0.1% (w/v) Triton X-100, 62.5 mM EDTA (pH 8.1), 50 mM Tris-Cl (pH 8.1)) were added and the cells were left on ice for lysis to occur. The lysate was cleared by centrifugation (40,000 g for 30 min at 4°C), and 0.95 g CsCl plus 0.1 ml 5 mg/ml ethidium bromide solution were added per ml of supernatant. The solution was then centrifuged (50 Ti rotor, 90,000 g for 48 hr at 15°C) and the tube was viewed under long-wave UV. The lower fluorescent band (containing supercoiled plasmid DNA) was withdrawn through the side of the tube using a syringe and

hypodermic needle, extracted 4 times with isopropanol (pre-equilibrated with 0.95 g ml^{-1} CsCl solution) to remove ethidium bromide, and dialysed exhaustively against TE buffer. Plasmid DNA preparations were sometimes further purified by phenol extraction, and were normally concentrated by ethanol precipitation.

C. Enrichment for tetracycline-sensitive transformants (Bolivar et al, 197

Transformants harbouring derivatives of pBR322 lacking a functional tetracycline-resistance determinant* were enriched for as follows: 1 ml transformed cells in L-broth (see 3E) were diluted 1:100 in L-broth + ampicillin (40 µg/ml) and grown overnight at 37°C to eliminate non-transformed cells. 2 ml of this culture were used to inoculate 200 ml L-broth, and the flask was shaken at 37°C for 60 min. Tetracycline was added (4 µg/ml) and after a further 60 min at 37°C D-cycloserine was added (100 µg/ml) and shaking was continued for 2.5 hr at 37°C. The culture was then centrifuged (10,000 g for 10 min, 4°C) and the pellet was resuspended in 100 ml L-broth + ampicillin (40 µg/ml). The flask was shaken overnight at 37°C and dilutions were replica-plated and screened for the desired recombinants.

* Due to insertion of foreign DNA at the HindIII site in the promoter for the tetracycline-resistance gene. Note, however, that such insertions frequently carry or generate functional promoters.

6. Phage infection of UV-irradiated cells and ^{35}S-methionine labelling of proteins (Jaskunas et al, 1975c).

Bacteria (S159 and lysogenic derivatives) were grown overnight in M9 minimal medium with maltose as sole carbon source, diluted to Klett 12, and grown shaking at 37°C to Klett 60 (3.10^8 cells/ml). 15 ml of the culture were centrifuged (4000 rpm for 10 min, 20°C), the supernatant was carefully removed by pasteur pipette and the cells were resuspended in 4.5 ml 1x M9 salts (to which 20 mM $MgSO_4$ and 0.01 µg/ml L-methionine had been added immediately before use). 4 ml of the suspension were irradiated in a sterile glass petri dish on ice (7.5 min at 600 ergs/mm^2/sec) in the dark, and 50 µl of cells (5.10^7 bacteria) were dispensed into plastic snap-cap tubes. Phage were then added (5 µl of 10^{11} pfu/ml, moi 10) and the tubes were incubated at 37°C for 20 min. 200 µl of pre-warmed 1x M9, 1 mM $MgSO_4$, 0.01 µg/ml L-methionine, 100 µCi/ml L-[^{35}S]-methionine (∿1500 Ci/mmol, SJ204 from Amersham) were then added to each tube. After 3 min at 37°C 30 µl of 2 mg/ml L-methionine were added, and the tubes were incubated at 37°C for a further 3 min before chilling on ice and addition of 5 µl 0.2 M sodium azide. The cells were pelleted (max. speed in microfuge, 6 min at 4°C) and the supernatant was carefully removed by aspiration. Pellets were resuspended in 1 ml Matzura buffer(62.5 mM Tris-Cl (pH 7.4), 0.1 mM DTT, 0.2 mg/ml L-methionine) and centrifuged as before, and finally resuspended in 100 µl SDS sample buffer (0.5x Upper Tris, 80 mM 2-mercaptoethanol, 3% (w/v) SDS, 8.5% (v/v) glycerol, 0.01% (w/v) bromophenol blue). Samples were incubated at 100°C (2 min) and loaded on an SDS-polyacrylamide gel (see 4C).

7. Plaque Detection by DNA:DNA Hybridisation

A. ^{32}P-labelling of DNA by nick translation (Rigby et al, 1977)

50 µl aliquots of DNAase I (Sigma, 1 mg/ml in 10 mM HCl) were stored frozen at -20°C, and thawed before use into 0.45 ml renaturation buffer (10 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 1 mg/ml BSA). After 2 hr renaturation on ice the DNAase I solution was diluted 10^3-fold in dH_2O for use (final concentration 10^{-4} mg/ml). Nick translation reactions (total volume 50 µl) were made up as follows: the labelled nucleoside triphosphate (α-^{32}P-dCTP, 400 Ci/mmole or α-^{32}P-dGTP, 400 Ci/mmole, PB165 and PB166 from Amersham) was dried down in a snap-cap tube; 5 µl 10x NT buffer (500 mM Tris-Cl (pH 7.8), 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol), 1 µg DNA, unlabelled dNTPs (stock solutions 0.1 mM) to give final concentrations of 20 µM each, 1 µl DNAase I (10^{-4} mg/ml) and dH_2O to 50 µl were then added. The reaction was started by adding 1 unit DpolI (generously provided by Mrs Barbara Will) and incubating at 15°C. After 1-3 hr the reaction was stopped by adding excess EDTA. Unincorporated nucleotides were removed by passage over a Sephadex G-75 (fine) column (10 x 0.7 cm). Elution was with 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and fractions were monitored for Cerenkov radiation. The presence of ^{32}P-DNA in the first peak was then confirmed by determining the fraction of ^{32}P which was TCA-insoluble, using 10 µl samples of the peak fractions.

B. Filter preparation, hybridisation and autoradiography

(Benton and Davis, 1977)

Plates carrying plaques for screening were chilled at 4°C for 30 min to harden the agar, and a nitrocellulose filter (8 cm diameter, from Millipore) was placed carefully onto each plate. After 2 min in

contact with the plate each filter was taken through the following treatments, 'plaque' side uppermost:

Whatman 3 MM paper/saturated with 0.5 M NaOH	(5 min)	immersion
0.1 M NaOH, 1.5 M NaCl	(20 s)	
0.5 M Tris-Cl (pH 7.5), 1.5 M NaCl	(2 x 20s)	
2x SSCP	(20 s)	

The filters were then blotted dry and baked for 2 hr at 80°C in vacuo. The ^{32}P-labelled probe DNA ($\geq 5.10^5$ Cerenkov cpm) was denatured (10 min at 90°C) and added to 10-15 ml hybridisation solution (25% (v/v) formamide, 30 mM Tris-Cl (pH 7.6), 750 mM NaCl, 1 mM EDTA, 0.2% (w/v) SDS) at 37°C. Filters were hybridised in this solution for 18 hr at 37°C either in a sealed plastic bag or under paraffin in a petri dish. Filters were then washed as follows: 45 min in 4x SSC (20°C); 45 min in 4x SSC (65°C); 30 min in 2x SSC (20°C); and dried at 37°C. Autoradiography was at -70°C using flash-sensitised X-ray film and an intensifying screen (Ilford Fast Tungstate), usually for 12-48 hr.

8. Preparation of AluI (Roberts et al, 1976)

Arthrobacter luteus, ATCC 21606, was grown on nutrient broth (Difco) and cultures were harvested in stationary phase. 5 g cells were disrupted by sonication in 2 vols 10 mM Tris-Cl (pH 7.5), 10 mM 2-mercaptoethanol, at 4°C, and the lysate was centrifuged (100,000 g for 1 hr at 4°C) to pellet debris. The supernatant was made 1.0 M in NaCl, and DNA was removed by fractionation on a column (50 cm x 2 cm) of Bio-gel A 0.5 m (Bio-rad), which was eluted with 1.0 M NaCl, 10 mM Tris-Cl (pH 7.5), 10 mM 2-mercaptoethanol. Fractions were assayed for nuclease activity by incubation with λ DNA

followed by agarose gel electrophoresis. Those containing endonucleolytic activity were combined and dialysed against 10 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol ("phosphocellulose buffer") and applied to a column (21 cm x 1.4 cm) of phosphocellulose (Whatman, P11) previously equilibrated with the same buffer. Elution was with 150 ml of a linear gradient from 0 to 1.0 M KCl in phosphocellulose buffer: AluI eluted between 0.60 and 0.75 M KCl. Of the peak fractions, some were dialysed against phosphocellulose buffer containing 50% glycerol and stored at -20°C, and others were dialysed against phosphocellulose buffer. The latter were applied to a column (25 cm x 0.9 cm) of DEAE-cellulose (Whatman, DE-52) previously equilibrated with phosphocellulose buffer. Elution was with 100 ml of a linear gradient from 0 to 0.3 M KCl in phosphocellulose buffer: AluI eluted between 0.21 and 0.26 M KCl. The peak fractions were dialysed against phosphocellulose buffer containing 50% glycerol, and stored at -20°C.

About 3000 units of AluI were obtained, apparently free of other nuclease activities, and stable at -20°C ≥ 6 months. There was no observable difference between the phosphocellulose and DEAE-cellulose column fractions in terms of purity.

9. Use of Synthetic Molecular Recombination Linkers (Maniatis et al, 1978)

2 µg HindIII molecular recombination linker (dCCAAGCTTGG, from Collaborative Research Inc.) were phosphorylated using T4 polynucleotide kinase (24 units) in a total volume of 5 µl PNK buffer (66 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM Spermidine, 15 mM DTT) for 1 hr at 37°C, and mixed with an AluI digest of the donor

DNA (ethanol-precipitated, washed with cold 80% ethanol, and resuspended in 10 µl TE buffer). 0.2 vols 5x PNK buffer and 1 µl T4 DNA ligase were added and the solution was incubated at 15°C for 3 hr (total DNA concentration ≥ 300 µg/ml). The efficiency of the phosphorylation/blunt-end ligation steps was determined by running one-tenth of the product on a 3-10% polyacrylamide gradient gel to visualise linker oligomers by ethidium bromide fluorescence. The remainder, after 10 min incubation at 70°C, was digested at 37°C for 1 hr with 40 units HindIII. Linker fragments were then removed on a Sephadex G-75 (Pharmacia) column (8 cm x 0.5 cm). Elution was with TE buffer, and fractions (10 x 150 µl) were assayed for DNA fragments >50 bp long by ethidium bromide visualisation of each, run on a 3-10% polyacrylamide gel. Fractions containing DNA >50 bp were ethanol-precipitated and resuspended in 15 µl TE buffer. AluI fragments with synthetic HindIII cohesive termini thus attached to their ends were finally recovered by ligation with HindIII-digested λ590 DNA and transfection: recombinants were recognised by virtue of their clear plaque morphology.

10. Assay of β-galactosidase (Miller, 1972)

Cultures were grown at 37°C on an orbital shaker at 200 rpm in M9 minimal medium containing ampicillin (40 µg/ml) and, where desired, arabinose at 0.1% (w/v) as non-metabolisable inducer of lacZ in strains harbouring pMC81 (Casadaban and Cohen, 1980) or its derivatives. β-galactosidase levels were determined from samples taken at OD_{600} 0.26-0.40. Each assay containing the following: 0.8 ml PM2 reducing buffer (120 mM sodium phosphate, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, 10 mM 2-mercaptoethanol); 0.2 ml 0.1% (w/v) CTAB; 0.01 ml 1% (w/v) sodium

deoxycholate; 1.0 ml of a suitable dilution of cells; and 0.6 ml 13.5 mM o-nitrophenyl-β-D-galactopyranoside (Sigma). Assays were incubated at 28°C and were stopped after sufficient colour had developed (usually 20-100 min) by the addition of 1.3 ml 1 M Na_2CO_3. The A_{550} and A_{420} were recorded using a Zeiss spectrophotometer.

CHAPTER 3

3.1 Construction and Physical Analysis of Recombinant Phage

The approach for this initial investigation of the transcriptional organisation of the *rplKAJL rpoBC* gene cluster entailed sub-cloning restriction fragments of λ*rif*18 in a phage vector: this is formally analogous to the mapping of promoters by deletion.

The λ vector used was NMλ761, a *Hin*dIII replacement vector derived from λ742 (Murray *et al*, 1977) by replacement of the *trpE* segment by a *supF* fragment (Fig. 3.1). Phage arising from the ligation of mixed *Hin*dIII digests of λ*rif*18 and λ761 were recovered by transfection of strain AA125, and recombinants were recognised by transferring plaques onto ED8538 ($lacZ_{am}$) on lactose MacConkey agar: recombinants gave colourless plaques whereas phage carrying the *supF* fragment gave red plaques. 55% of the plaques recovered were recombinants.

Recombinant phage were purified via single plaques and the size and orientation of their inserted *Hin*dIII fragment were determined by agarose gel electrophoresis of *Hin*dIII and *Eco*RI digests of plate lysate DNA preparations (Cameron and Davis, 1977). In this way it proved possible to identify phage carrying most of the large *Hin*dIII fragments from λ*rif*18 (Fig. 3.1).

Fig. 3.2 shows *Hin*dIII and *Eco*RI digests of purified DNA of 3 recombinants designated λ17, λ23 and λ34, and of the parent phages λ761 and λ*rif*18. λ17 and λ23 give identical *Hin*dIII cleavage patterns consistent with replacement of the 8.8 kb *supF* fragment of λ761 with the 11 kb *rplL rpoBC* fragment of λ*rif*18 (Lindahl *et al*, 1977). The *Eco*RI patterns of λ17 and λ23 both show the left arm of the vector (22 kb) and the 3.2 kb and 3.6 kb fragments from the vector's right arm in addition to the 4 internal *Eco*RI fragments

Fig. 3.1 The upper part shows the physical map of λrif18 DNA for EcoRI and HindIII, with λ DNA represented by a single line, and the E.coli DNA substitution by an unfilled box. Known E.coli genes carried on λrif18 are also indicated: LJAK are the genes for 50S ribosomal proteins L7/12, L10, L1 and L11, respectively. The E.coli DNA is transcribed leftwards. EcoRI and HindIII fragment sizes are in kb. The data are derived from Lindahl et al (1977).

The lower part shows sites for EcoRI and HindIII in the replacement vector NMλ761 (Murray et al, 1977). The 8.8 kb HindIII fragment is of E.coli DNA, and includes a supF gene.

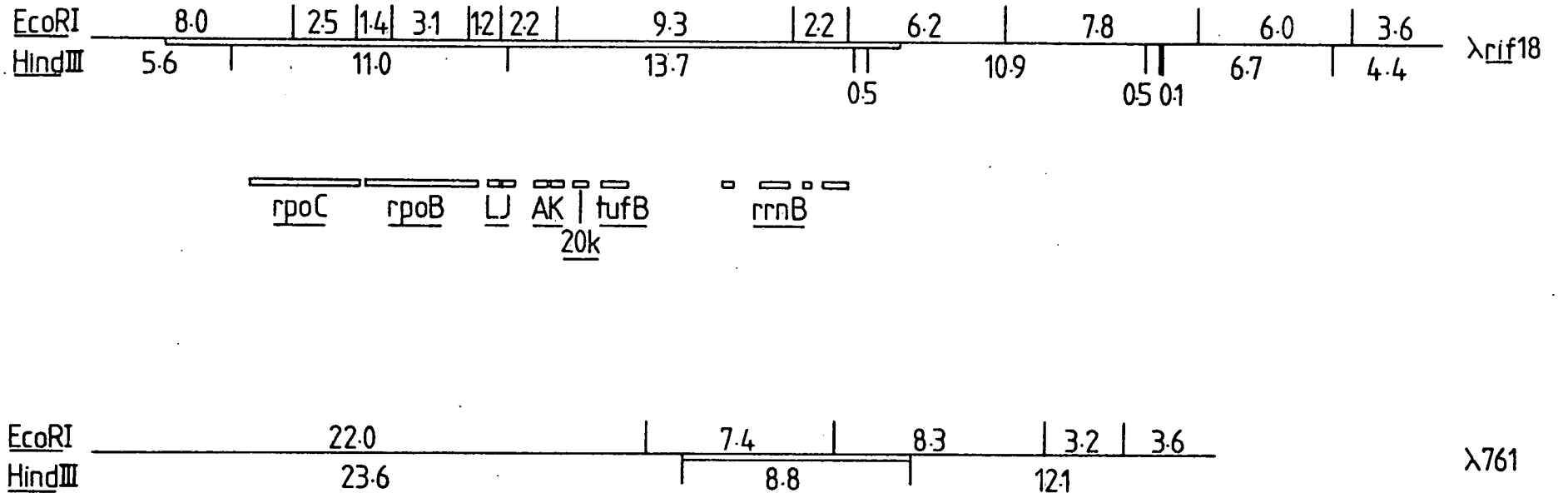

EcoRI
HindIII
8·0
2·5
1·4
3·1
1·2
2·2
9·3
2·2
6·2
7·8
6·0
3·6
λrif18
5·6
11·0
13·7
0·5
10·9
0·5
0·1
6·7
4·4
rpoC
rpoB
LJ
AK
tufB
20k
rrnB
EcoRI
HindIII
22·0
23·6
7·4
8·8
8·3
3·2
12·1
3·6
λ761

Fig. 3.1

Fig. 3.2 1% agarose gel electrophoresis of λ761, λ*rif*18 and *in vitro* recombinants after digestion with *Hin*dIII or *Eco*RI.

Track number	DNA	Restriction endonuclease
1	λ*rif*18	*Hin*dIII
2	λ232	"
3	λ172	"
4	λ23	"
5	λ17	"
6	λ*rif*18	*Eco*RI
7	λ17	"
8	λ23	"
9	λ172	"
10	λ232	"
11	λ761	"
12	λ761	"
13	λ34	"
14	λ*rif*18	"
15	λ761	*Hin*dIII
16	λ34	"
17	λ*rif*18	"

Notes:

1 The marker tracks are λ*rif*18/*Hin*dIII (H) and λ*rif*18/*Eco*RI (E), with fragment sizes given in kb.

2 In tracks 6-11 the digests were not 70°C-treated after digestion, so that the 3.6 kb (and 8.0 kb in the λ*rif*18 track) bands are present in less than stoichiometric yield owing to cohesive end annealing.

3 In this and other agarose gels the fragment sizes given are considered accurate to within ±0.2 kb for fragments in the size range 2.0-8 kb.

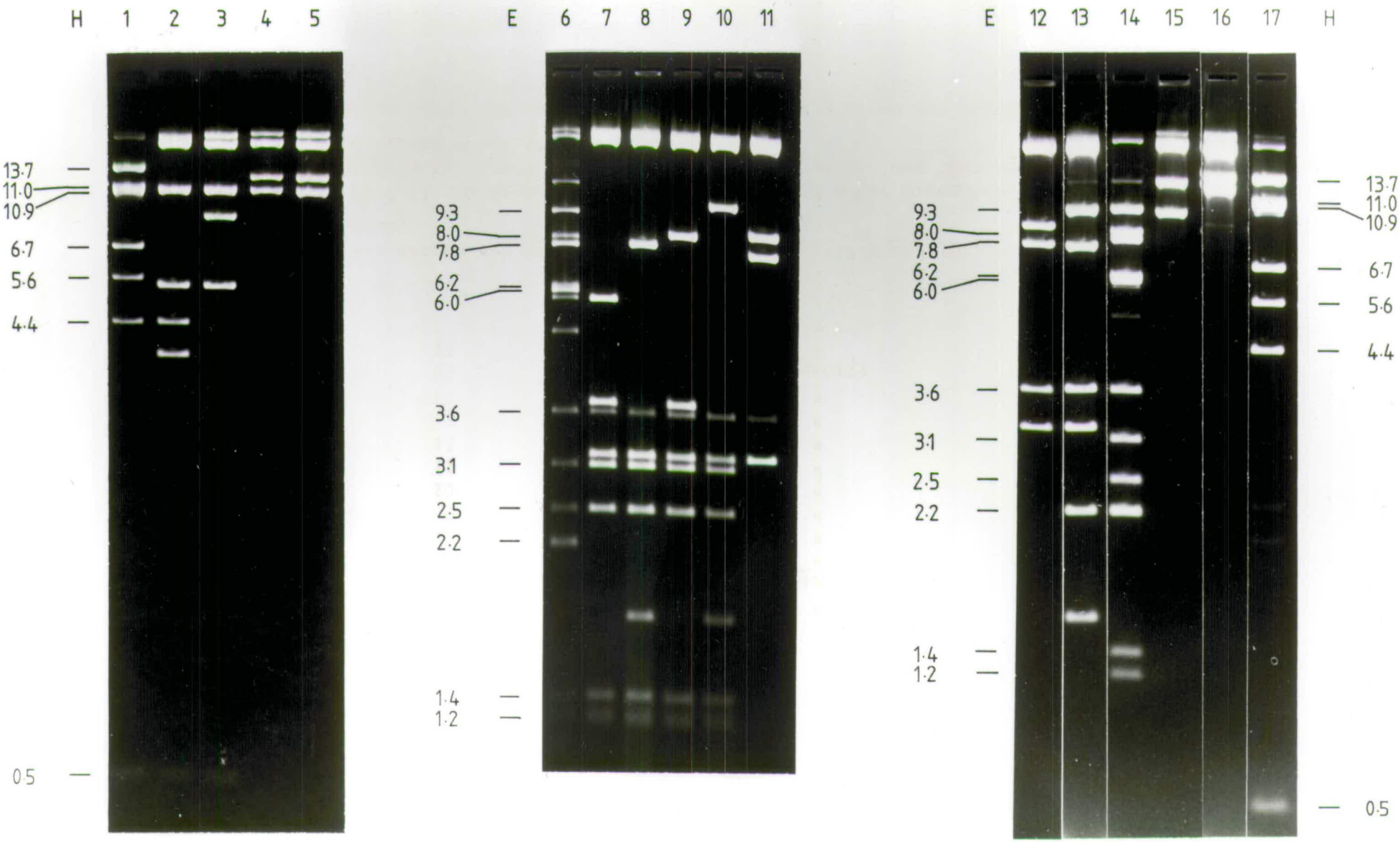
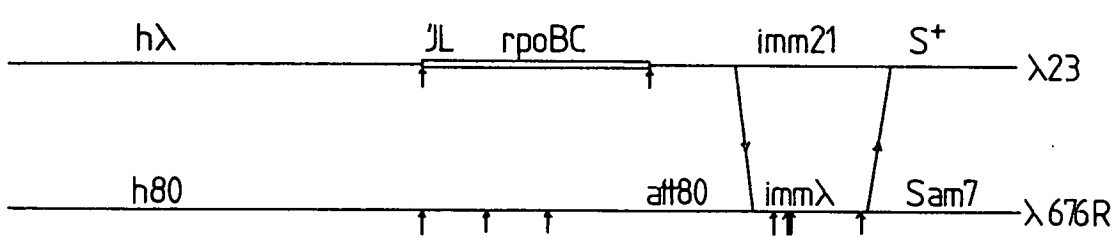

H
1
2
3
4
5
13·7
11·0
10·9
6·7
5·6
4·4
0·5
E
6
7
8
9
10
11
9·3
8·0
7·8
6·2
6·0
3·6
3·1
2·5
2·2
1·4
1·2
E
12
13
14
15
16
17
H
9·3
8·0
7·8
6·2
6·0
3·6
3·1
2·5
2·2
1·4
1·2
13·7
11·0
10·9
6·7
5·6
4·4
0·5

Fig. 3.2

derived from the 11 kb HindIII fragment (Lindahl et al, 1977). The two patterns differ, however, in that λ17 gives fragments of 5.7 kb and 4.0 kb, whereas λ23 yields 7.7 kb and 2.0 kb fragments, indicating that in λ17 the 11 kb HindIII fragment is in the L orientation (correctly oriented for transcription from λP_L) whereas in λ23 the fragment is in the R orientation (Fig. 3.3).

λ34 gives a HindIII cleavage pattern consistent with replacement of the supF fragment of λ761 by the 13.7 kb HindIII fragment of λrif18. The EcoRI digest confirms this: the 2 internal EcoRI fragments of 9.3 kb and 2.2 kb are present, and two additional bands (representing the hybrid λ-bacterial insert fragments) of 7.4 kb and 1.7 kb also appear, indicating that the 13.7 kb fragment is in the R orientation. The identity of the inserted fragment was determined for 44 recombinants, but no phage carrying the 13.7 kb fragment in the L orientation was found.

Initially, infections of UV-irradiated λimm21 lysogens with these imm21 recombinants suggested that repression of phage promoters was less well maintained than in analogous λ-lysogenic infections. Accordingly, to facilitate their functional characterisation, λ17 and λ23 were crossed with NMλ676R to yield immλ derivatives of λ172 and λ232 (see below).



λ23×λ676R cross, yielding λ232. HindIII sites are marked.

Fig. 3.3 Restriction maps of λ761 derivatives λ17, λ23 and λ34 and of imm λ versions of λ17 and λ23 (λ172 and λ232). The sizes of EcoRI and HindIII fragments are given in kb. L, J, A and K represent the 50S ribosomal subunit protein genes rplKAJL. J' and 'J signify that the gene has been severed by HindIII and is carried incomplete. λ34 also carries most of the rrnB rRNA transcription unit, but this is not shown.

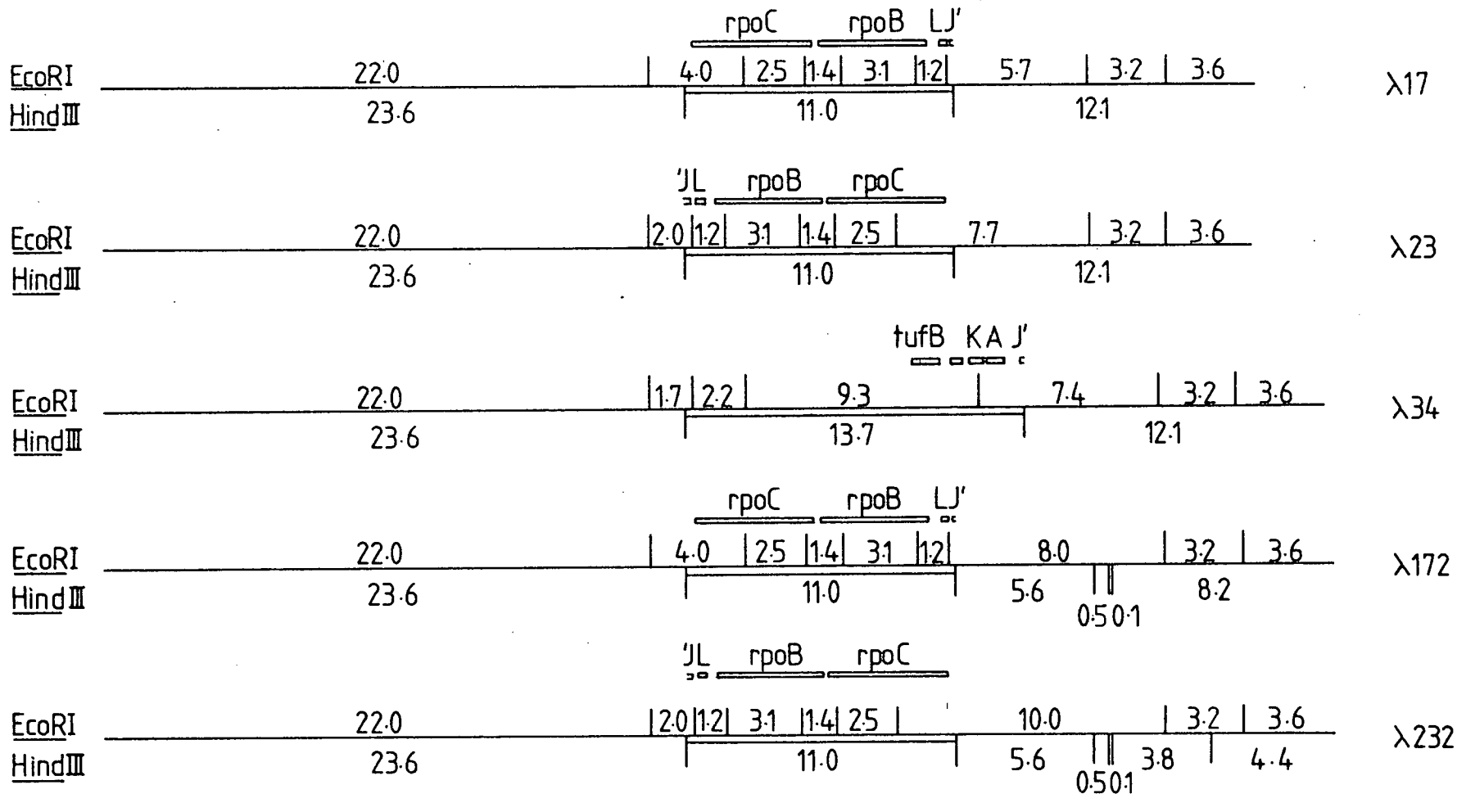

rpoC
rpoB
LJ'
EcoRI
HindIII
22·0
23·6
4·0
2·5
1·4
3·1
1·2
11·0
5·7
12·1
3·2
3·6
λ17
'JL
rpoB
rpoC
2·0
1·2
3·1
1·4
2·5
7·7
λ23
tufB
KA
J'
1·7
2·2
9·3
13·7
7·4
λ34
8·0
5·6
0·5
0·1
8·2
λ172
10·0
3·8
4·4
λ232

Fig. 3.3

The desired hλcI857 recombinants were recognised by their clear plaque morphology at 37°C on a supF tonA host, and checked for their ability to transduce Ymel to Rif-R. HindIII and EcoRI digests of λ172 and λ232 are also shown in Fig. 3.2. HindIII digestion of both phages gives the left arm fragment (23.6 kb) and 11 kb rplL rpoBC insert as in λ17 and λ23. λ232 gives additionally bands of 5.6 kb, 4.4 kb, 3.8 kb and 0.5 kb, consistent with a cross-over in the gam-cIII region onto the right arm of the immλ parent λ676R. λ172 differs only in that it yields an 8.2 kb fragment instead of 4.4 kb and 3.8 kb: presumably this is due to a second unselected crossover in λ172 to include the shn6° replacement present in the right arm of the imm21 parent. Unselected multiple exchanges are common in the QS region of λ (N. Murray, pers. comm.). Indeed, genetic analysis (data not shown) of the recombinants λ172 and λ232 indicated that both carry the S^+ allele of the imm21 parent.

The EcoRI digestion patterns of λ172 and λ232 differ from those given by their imm21 parents only in the size of the fragment flanking the right end of the 11 kb HindIII fragment: in each case the fragment given by the immλ derivative is 2.3 kb longer as a result of the replacement of imm21 with immλ.

The data shown in Fig. 3.2 are interpreted and summarised in Fig. 3.3 in the form of physical and genetic maps of λ17, λ23, λ34, λ172 and λ232.

3.2 Expression of rpoB by λrplL rpoBC in vivo

The physical mapping data of Lindahl et al (1977) suggested that the bacterial DNA fragment cloned in λ17 and λ23 should include complete copies of the structural genes for rplL, rpoB and, probably, rpoC.

A test for the expression of the cloned Rif-R copy of rpoB in the absence of transcription from phage promoters is afforded by the construction of lysogens of these phages in a recA rpoB$^+$ (Rif-S) background. Recombinants derived from λ761 are att P.∇' int$^-$ xis$^-$ and 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. The end result is usually tandem insertion of helper and transducing phage at the λ attachment site (Gottesman and Yarmolinsky, 1968).

Dilysogens of AJN10 (recA56 rpoB$^+$) were isolated after simultaneous infection with λ$^+$ and λ17 or λ23. 21-immune colonies could only be obtained after co-infection with λ$^+$ helper, and were invariably immune to both λ and 21. All such lysogens tested (32/32) were Rif-S, in contrast to analogous λrif18, λimm21 dilysogens, which were Rif-R (8/8).

The straightforward interpretation of this result is that the normal promoter for rpoB lies outside the 11 kb HindIII fragment. Another plausible explanation, however, might be that the phage-borne, Rif-R copy of rpoB is still being expressed from its usual promoter, but has been separated from a second mutation in λrif18 which conferred dominance to the Rif-R allele in cis. If this were the case lysogens of λ17, λ23 and λrif18 might contain comparable amounts of Rif-S and Rif-R RPase, but only the latter would display Rifampicin-resistance.

Evidence against this possibility was obtained by examining the properties of an additional set of lysogens, derived from AJN1 (isogenic with AJN10 except that it carries a mutation, rcs-40, mapping in or near rpoB). The rcs-40 allele alters the structure

of RPase without significantly affecting its sensitivity to Rif and allows phenotypic expression of Rif-resistance mutations such as rpoB70 in merodiploids, which would otherwise remain Rif-S. Hence AJN1 harbouring KLF10 rpoB70 is Rif-R, whereas the AJN10 merodiploid is Rif-S (Hayward, 1976). However, the AJN1 lysogens of λrplL rpoBC were Rif-S like their AJN10 counterparts and in contrast to the AJN1 (λrif18) control. This provides further evidence against the presence of an effective bacterial promoter for transcription of the rif allele of rpoB cloned in λrplL rpoBC.

The properties of λrplL rpoBC-lysogenic derivatives of a recA$^+$ rpoB$^+$ strain (Ymel) were also examined. In the absence of an integration-proficient helper these int$^-$ xis$^-$ phages lysogenise by generalised recombination with the chromosomal rplL rpoBC region. Fig. 3.4 shows 3 possible modes of integration: (1) involves two crossovers and generates a Rif-R haploid (which could become lysogenic by a secondary event); (2) and (3) involve single reciprocal crossovers upstream and downstream, respectively, of the rifampicin-resistance mutation. Both generate lysogens, but their Rif phenotype depends on whether the cloned bacterial fragment carries a strong promoter for rpoBC. If so, either (2) or (3) will yield lysogens in which both copies of rpoB are expressed: the phenotype would be the same in either case, depending only on which allele of rpoB is dominant. In the absence of a cloned promoter (2) would generate Rif-R lysogens, whilst (3) would yield Rif-S. Finally, if the cloned fragment carries neither an effective promoter nor a functionally intact rpoC gene, lysogens produced by either (2) or (3) would be inviable through inability to synthesise β' subunit.

Fig. 3.4 3 possible modes of rec-mediated integration of λrplL rpoBC. The rplKAJL genes are shown as a continuous block, and transcription is rightwards. The majority of lysogens produced in this fashion were Rif-S, presumably having arisen by mode 3, and reflecting the position of the rif mutation in the N-terminal half of rpoB. All such lysogens, however, readily gave rise to rif-R clones when challenged with rifampicin.

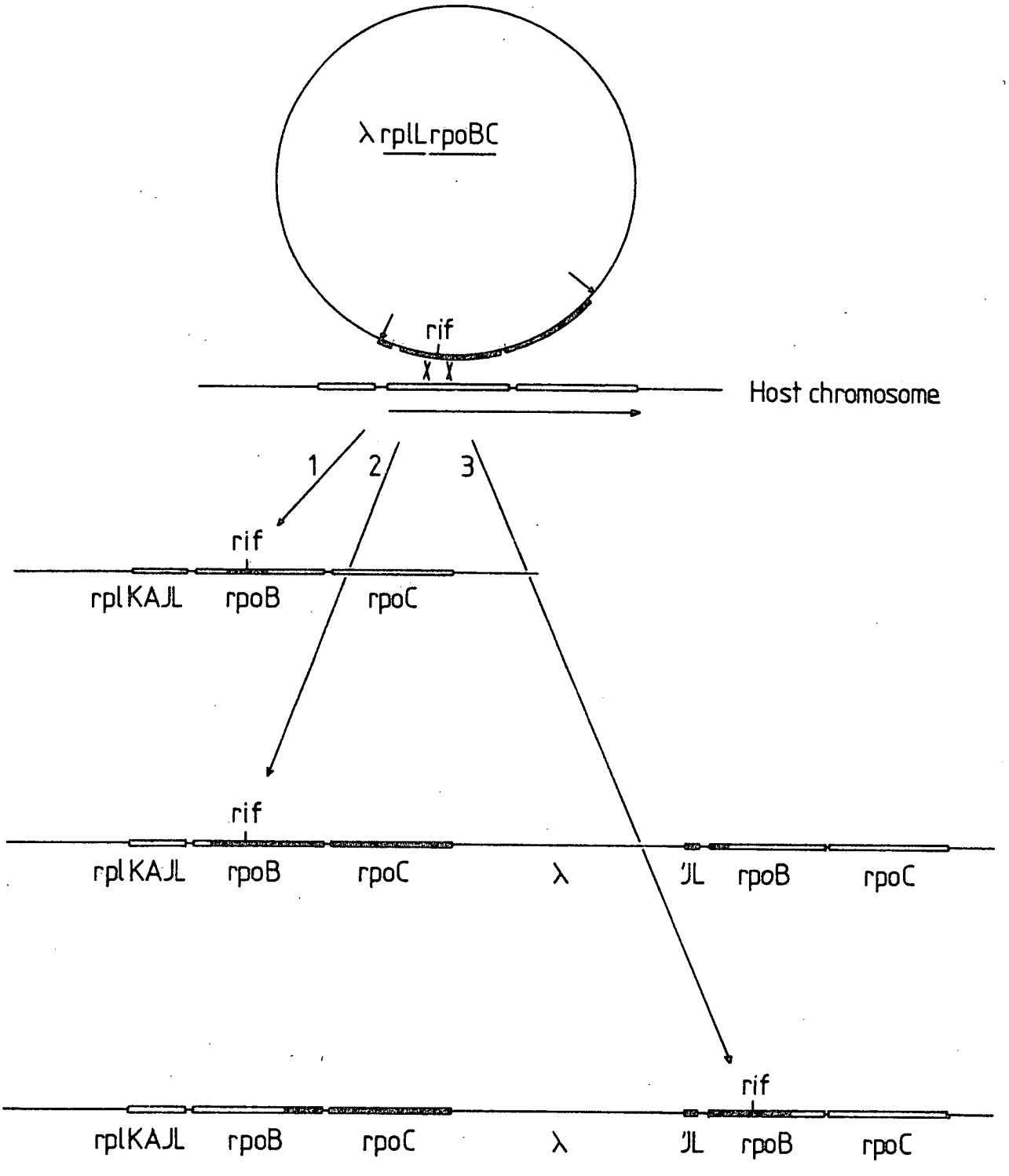

λ rplL rpoBC
rif
Host chromosome
1
2
3
rif
rplKAJL
rpoB
rpoC
rif
rplKAJL
rpoB
rpoC
λ
'JL
rpoB
rpoC
rif
rplKAJL
rpoB
rpoC
λ
'JL
rpoB
rpoC

Fig. 3.4

In practice both Rif-R and Rif-S imm21 lysogens were readily isolated from Ymel infected with λ17 and λ23 (18/19 such lysogens were Rif-S), but the Rif-S clones always gave rise to Rif-R colonies when challenged with Rifampicin. The implication of this is that the Rif-R clone arose by mode (2) and the Rif-S clones by mode (3), and that the second rpoB allele in either case is transcriptionally inactive. Rec-mediated excision and re-integration of the prophage, however, leads to the frequent appearance of Rif-R colonies in which the resistant rpoB allele is now expressed and the $rpoB^+$ copy has been separated from its promoter.

These results confirm that the 11 kb HindIII fragment does not include an effective rpoBC promoter, and they also indicate that the fragment carries a functionally intact copy of rpoC and of any other essential genes which might lie distal to rpoC in the same operon.

3.3 Expression of Cloned Genes in UV-irradiated Bacteria

A convenient test for the presence of bacterial structural genes on fragments cloned in transducing phages is provided by infection and L-[^{35}S]-methionine pulse-labelling of bacteria pre-irradiated with UV to abolish transcription of the host genome. Furthermore, in UV-irradiated lysogenic bacteria transcription from major phage promoters is eliminated: under these conditions cloned genes can be transcribed only if a suitable promoter(s) is present on the bacterial DNA fragment (Jaskunas et al, 1975b).

Fig. 3.5 shows autoradiographs of ^{35}S-methionine-labelled proteins synthesised in E.coli S159 and its λind$^-$ and λimm21 lysogens following infection with various transducing phages. Infection of the S159 (λind$^-$) lysogen with λrif18 elicits the synthesis of a

Fig. 3.5 Autoradiographs of ^{35}S-methionine-labelled proteins synthesised in UV-irradiated *E.coli* S159 and its λ*ind*$^-$ and λ*imm*21 lysogens following phage infection. The proteins were fractionated on a 7-15% SDS-polyacrylamide gel (tracks 1-8) or a 10-20% SDS-polyacrylamide gel (tracks 9-15).

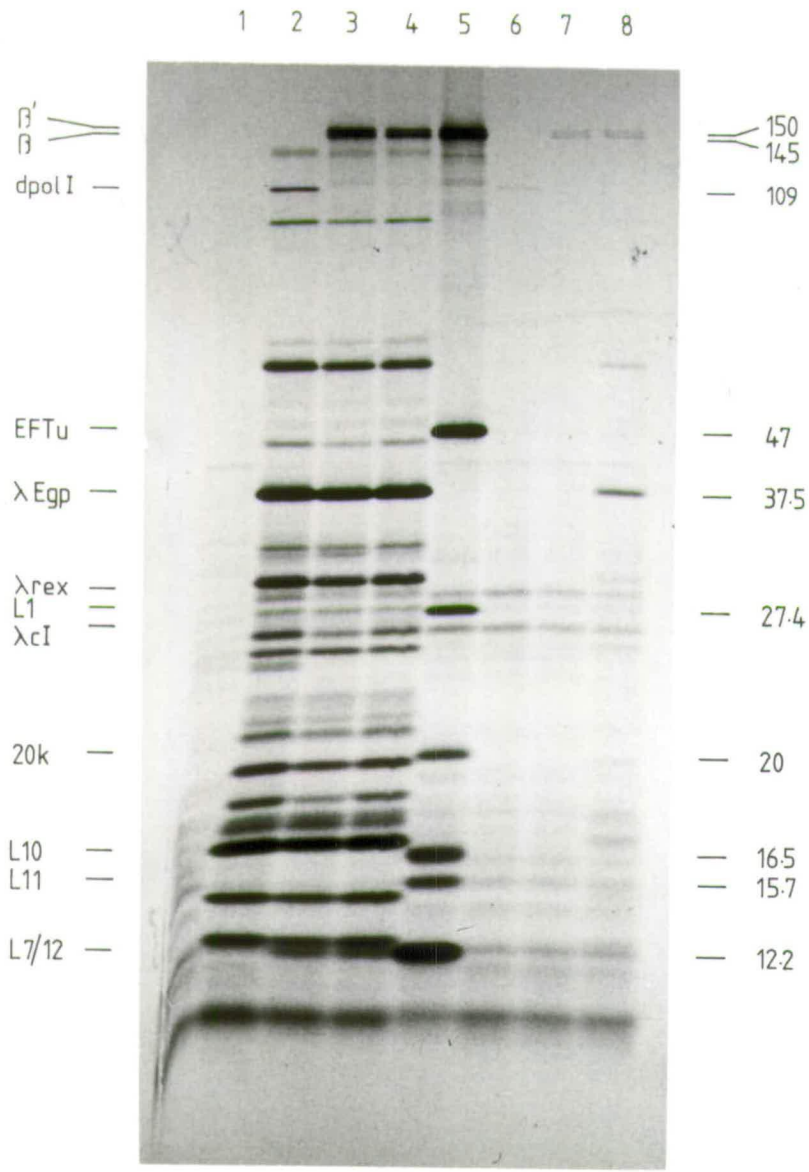
Tracks number	Phage	Host
1	-	S159
2	λ*polA*	"
3	λ172	"
4	λ232	"
5	λ*rif*18	S159 (λ*ind*$^-$)
6	λ*polA*	"
7	λ172	"
8	λ232	"
9	λ34	S159 (λ*imm*21)
10	λ34	"
11	λ232	S159
12	λ34	"
13	λ34	"
14	λ*rif*18	"
15	-	"

Notes: [1] Proteins of interest are marked; the figures are polypeptide MWs in kdal. The ribosomal proteins and EFTu were identified in collaboration with T. Linn through comparison with purified marker proteins and/or by study of several deletion phages.

[2] λ*polA* carries the *polA* gene in the L orientation; it is analogous in structure to λ172, being the result of a cross between λ908 and λ676R (Kelley *et al*, 1977). The inserted *Hin*dIII fragment appears to encode d*polI* and little else.

[3] The cpm present in bands of interest from the λ*rif*18 track (5) were as follows (with the cpm present in the uninfected track (1) in brackets): β', 8267 (31); β, 10217 (33); EFTu, 20814 (167); L1, 6004 (388); L10, 10535 (1369); L11, 7950 (1704); L7/12 28463 (2926).

[4] The molar ratio of β to L7/12 synthesis was about 2-fold higher for λ172 in S159 than for λ*rif*18 in either S159 or S159 (λ*ind*$^-$): T. Linn, pers.comm. This may reflect the presence of a transcriptional terminator, responsive to λNgp, between *rplL* and *rpoB*.


1 2 3 4 5 6 7 8
β'
β
dpol I
EFTu
λ Egp
λrex
L1
λcI
20k
L10
L11
L7/12
150
145
109
47
37·5
27·4
20
16·5
15·7
12·2

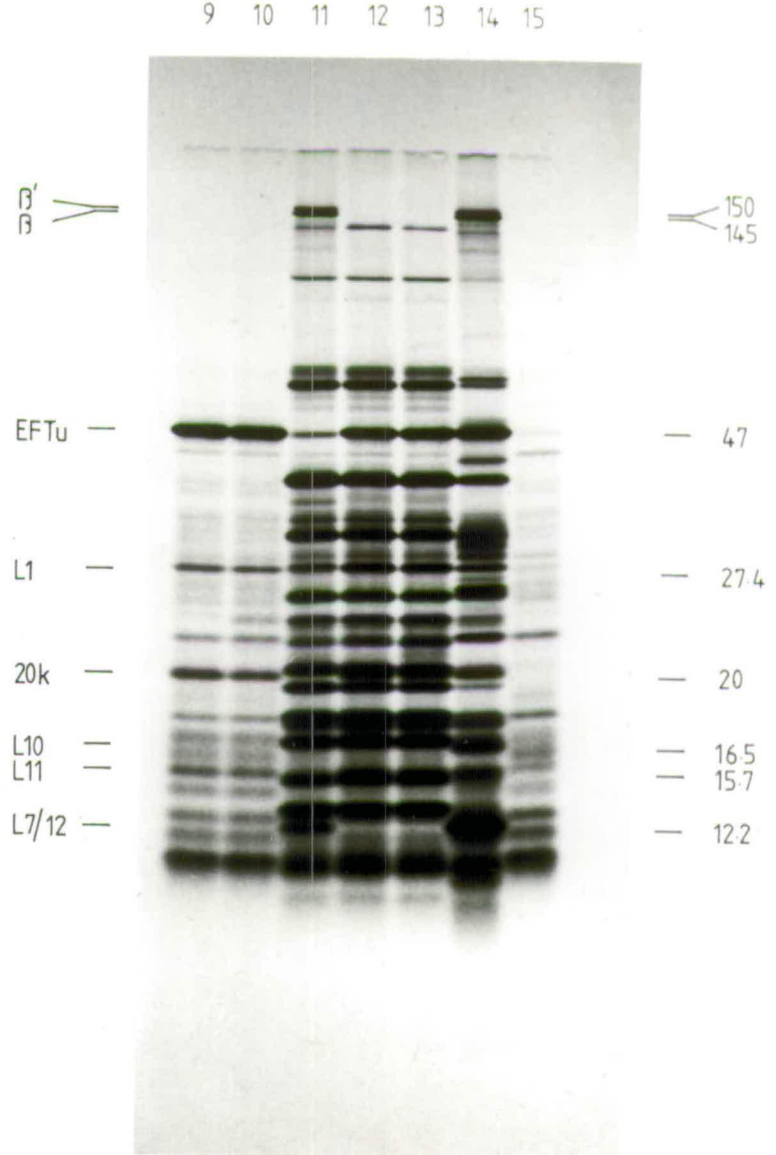

9 10 11 12 13 14 15
β'
β
EFTu
L1
20k
L10
L11
L7/12
150
145
47
27·4
20
16·5
15·7
12·2

Fig. 3.5

range of proteins (β, β', EFTu, L1, cIgp, rexgp, 20k, L10, L11 and L7/12) in agreement with the data of Lindahl et al (1977); 20k is an unidentified protein of 20 kdal first noted by Kirschbaum et al (1976). The approximate molar ratios of synthesis of the bacterial proteins were determined by excising and counting the bands (for typical results see the Fig. 3.5 legend) and allowing for the molecular weights and methionine contents of the various polypeptides (Kaltschmidt et al, 1970; Arai et al, 1973; Wittmann, 1974; Burgess, 1976). Results for the experiment shown in Fig. 3.5 are as follows (relative to β as 1.0): β' 0.83; β 1.0; EFTu 9.2; L1 3.0; L10 7.6; L11 3.9; L7/12 32 (see Discussion below).

In the non-lysogenic host S159 infection with λ172 and λ232 clearly leads to the synthesis of β, β' and L7/12 in addition to the background of λ proteins, which are also given by λpolA (analogous to λ172, but the inserted HindIII fragment codes for the 109 kdal DNA polymerase I polypeptide, and little else). The UV-irradiation does not completely abolish the host-coded synthesis of small proteins, including L7/12, but with this proviso no other polypeptides ascribable to the cloned rplL rpoBC fragment are detectable.

Significantly, infection of the lysogenic host S159 (λind$^-$) with λ172 or λ232 leads to the synthesis of β and β' at only 2-3% of the rate given by λrif18 (whereas the rates of cIgp and rexgp synthesis are similar). Hence the cloned DNA fragment does not carry a strong bacterial promoter for transcription of rpoBC, confirming the results of studies on these phage in vivo.

The low-level synthesis of β and β' seen in the λ172 and λ232 infections of the lysogen probably reflects transcription from a weak

promoter(s) on the cloned DNA fragment, rather than from lambda promoters. In this context it is relevant that: (i) both L and R orientations give equivalent low levels of ββ' synthesis; (ii) repression of λEgp synthesis is effectively total in λ172 infections (see Footnote*); (iii) the λ integrase promoter is removed by the att-red deletion. Low-level expression of β and β' is discussed further in Chapter 4.

Because of the residual levels of expression of small host proteins it is not possible to say whether synthesis of L7/12 also responds to the action of this low-level promoter(s); certainly, however, rplL is not significantly expressed in the absence of transcription from λP_L or P'_R.

λ34 (carrying the 13.7 kb HindIII fragment of λrif18 in the R orientation) elicits the vigorous synthesis of EFTu, L1, L11 and 20k in both S159 and its λimm21 lysogen: this fragment evidently carries bacterial promoter(s) capable of transcribing these genes. Neither λ34 nor the rplL rpoBC phages appear capable of directing the synthesis of L10, the product of rplJ, irrespective of whether λ promoters are functioning or not. The simplest explanation of this is that the rplJ gene contains the HindIII site separating the 11.0 kb and 13.7 kb fragments of λrif18.

* Footnote: After infection with λ232 (the R orientation phage) synthesis of λEgp and of several other λ proteins was slightly derepressed. One explanation for this might be that rightward transcription initiated within the inserted bacterial fragment is able to read through into the left arm of the phage. According to Barry et al (1979), however, the 11 kb HindIII fragment includes the strong terminator rpot2 beyond rpoC, and does not detectably activate a downstream lacZ gene. Conceivably a sequence at the rpo-lambda junction is capable of acting as a weak promoter.

3.4 Discussion

The data for the synthesis rates of the 50S ribosomal proteins and EFTu in UV-irradiated cells infected with λ*rif*18 indicated that relative to β (1.0) the molar yields were about 3.0-7.6 for L1, L10 and L11, 32 for L7/12 and 9.2 for EFTu. Bearing in mind that ribosomes are synthesised at 5-7 times the molar rate of RNA polymerase (Kjeldgaard and Gausing, 1974), that they contain single copies of all component proteins except L7/12 (which occur as a tetramer: Hardy, 1975; Subramanian, 1975) and that ribosomal proteins are efficiently assembled into ribosomes (Kjeldgaard and Gausing, 1974), these relative rates of synthesis correspond reasonably with those observed *in vivo*. The EFTu synthesis rate is 8-14 times that of ribosomes *in vivo* (Furano, 1975), but there are two genes for EFTu (*tufA* and *tufB*: Jaskunas *et al*, 1975d), and *tufB*, the copy carried by λ*rif*18, is 2-3 times less productive than *tufA* (Pedersen *et al*, 1976). Hence the relative rate of EFTu synthesis elicited by λ*rif*18 is also reasonably compatible with measurements made *in vivo*. These results imply that the *E.coli* DNA carried by λ*rif*18 probably retains the normal transcriptional and translational regulators for these genes.

The main conclusion from the experiments described in this chapter is that since there is no strong promoter for *rpoBC* in a DNA fragment including *rplL* and a portion of *rplJ*, *rpoBC* must be co-transcribed with *rplL* and presumably *rplJ*. This has been confirmed in other laboratories. By sub-cloning partial *EcoRI* digestion products of λ*rif*18 in a λ vector Yamamoto and Nomura (1978) were able to show that the *rpoB* promoter lay in the 2.2 kb *EcoRI* fragment (Fig. 3.1) upstream of *rplJ*, demonstrating that

the rpoBC transcription unit was: promoter rplJL rpoBC. Furthermore, phage carrying the 2.2 kb EcoRI fragment (which includes only the 3' end of rplK) could not express rplA except by read-through from λ promoters. Although this does not locate the normal promoter for rplA, it suggests that rplA and rplK are co-transcribed from a promoter lying somewhere upstream of rplK. In experiments extending our joint work (Newman, Linn and Hayward, 1979) Linn and Scaife (1978) reached the same conclusions. They located a promoter between the HindIII site in rplJ and the EcoRI site in rplK which was sufficient, when fused to the 11 kb HindIII fragment in a transducing phage, for autonomous expression of rplJL rpoBC (but not rplA) in UV-irradiated bacteria and for expression of the dominant rifampicin-resistance allele rpoB3 in the prophage state.

The contents and arrangements of transcription units upstream of the rplJL rpoBC operon on λrif18 have been deduced by functional analysis of several deletion and fusion derivatives (Yamamoto and Nomura, 1979; Linn et al, 1979a,b). In the argB-rpoBCV9 fusion the synthesis of EFTu responds to control by argR, whereas that of the downstream genes for "20k" and the 50S ribosomal proteins is unaffected. This implies a separate transcription unit for tufB, followed by an efficient transcription termination site (Linn et al, 1979a). Further studies of the effects of various deletions on the expression of genes in this region (Yamamoto and Nomura, 1979) establish the following arrangement of strong promoters (P) and terminators (t):

PrrnB PthrU tyrU P?glyT thrT PtufBt P20kt? PrplKA PrplJL rpoBC

Dennis (1977b) has examined the rates of synthesis and steady-state levels of the mRNAs for rpoBC, the adjacent rplKAJL genes,

and part of the ribosomal protein gene cluster at 72' (encoding 15 ribosomal proteins and polymerase subunit α) by hybridisation of mRNA labelled *in vivo* to probes representing these regions of the genome. His results are subject to systematic errors, since the endpoints of the probes used (partial *Eco*RI digestion products of λ*rif*18, cloned in a plasmid vector) are not ideally placed for accurate discrimination between *rplKA*, *rplJL* and *rpoBC* mRNA. Nevertheless, it is clear that the average transcription frequencies of the ribosomal protein genes at 72' and 89' are similar and 4-5 times the transcription frequency of *rpoBC*. This proportionality does not vary with changes in growth rate. On the other hand the steady-state levels of *rpoBC* mRNA were found to remain essentially constant over a 3-fold range of growth rates, whereas the levels of ribosomal protein mRNA doubled over this range.

These data imply that a marked stability difference exists between *rpoBC* and ribosomal protein gene mRNAs (*rpoBC* mRNA being relatively less stable at rapid growth rates than ribosomal protein gene mRNA). They also suggest the presence of a site in the *rplJL rpoBC* transcription unit, presumably between *rplL* and *rpoB*, which acts as a partially effective terminator of transcription, and under normal circumstances allows only 20-25% of RNA polymerases which initiate upstream to proceed into *rpoBC*. (The latter estimate of readthrough efficiency assumes that *rplKA* and *rplJL* are transcribed equally frequently, a point not established by the data.)

The obligatory co-transcription of *rpoBC* with *rplJL*, and of *rpoA* with a separate set of ribosomal protein genes, suggests that *E.coli* normally exploits this arrangement to ensure a co-ordinated

synthesis of mRNA for ribosomal proteins and RNA polymerase. The apparent invariance of transcriptional readthrough from rplJL into rpoBC over a considerable range of growth-rates supports this view. However, the distinct growth-rate dependencies of the differential synthesis rates of RNA polymerase and ribosomes, and the comparable growth rate-dependent stability difference between their respective mRNAs, suggest that additional post-transcriptional effects must also be operative: in particular, for modulating overall gene expression at different growth rates. A further level of regulation is suggested by evidence that in response to some physiological constraints (other than simple variation in growth rate) transcription of rpoBC can be adjusted relative to that of rplJL (Section 4.4).

All the studies on the arrangement of transcription units discussed in this chapter were performed using mutant DNA. The transducing phage λrif18 was isolated by virtue of its dominant expression of rifampicin-resistance (Kirschbaum and Konrad, 1973). The basis of the dominance of the rpoB3 allele carried by λrif18 is obscure; a possibility which has not yet been satisfactorily excluded is that dominance might result from a cis-acting regulatory mutation. Accordingly, the transcriptional organisation of the rpoBC operon in λrif18 might be anomalous. The approach adopted to investigate this question was to isolate and analyse the rpoBC structural sequences and regulatory sites directly from the bacterial chromosome, without imposing any genetic selection for their function.

CHAPTER 4

4.1 Isolation and Characterisation of rpoBC Structural Genes from the E.coli chromosome

Direct isolation of rpoBC sequences from the E.coli chromosome was expedited by insertion of the 11 kb HindIII fragment of λrif18 into the single HindIII site of pBR322 (Bolivar et al, 1977) to produce plasmid pNA219. This recombinant was readily identified by agarose gel electrophoresis of HindIII and EcoRI digests of plasmid DNAs from Amp-R Tet-S transformants. A library of HindIII fragments of E.coli K12 CR63 DNA cloned in NMλ761 was kindly provided by Dr N. Murray, and was screened for plaques hybridising to [^{32}P]-labelled pNA219 DNA. Several such plaques were detected, and analysis of the phage DNA showed that they all carried an 11 kb HindIII fragment. One such phage, λ63, was purified for further study: HindIII and EcoRI digestions of λ63 yielded fragments indistinguishable from those given by λ17 (Fig. 4.1). Presumably the 11 kb insert in λ63 carries rpoBC sequences, and is in the L orientation.

L-[^{35}S]-methionine pulse-labelling of S159 after infection with λ63 initially revealed the synthesis of only L7/12 and β' in addition to λ proteins: contrast the behaviour of λ17, which yielded L7/12, β and β' under similar conditions (Fig. 4.2). This apparent anomaly was resolved by running the labelled proteins on a 5% SDS-polyacrylamide gel (Fig. 4.2) which clearly separated the λ17-encoded β and β' subunits. The single band of the mobility of β' given by λ63 in Fig. 4.2 was likewise barely resolved into two bands, apparently representing normal-sized β' and a β subunit of reduced mobility. This reduction in mobility would be consistent with a MW some 3-4 kdal in excess of the 145 kdal characteristic of β subunit from other K12, B, and MRE600 strains (Lowe et al, 1979).

Fig. 4.1 1% agarose gel electrophoresis

Track number	DNA	Restriction endonuclease
1	λ17	HindIII
2	λ63	"
3	λ17	EcoRI
4	λ63	"
5	λrif18	AvaI
6	λ261	"
7	λ172	"
8	λrif18	HindIII
9	λ261	"
10	λ172	"
11	λrif18	EcoRI
12	λ261	"
13	λ172	"
14	λ261	PstI
15	λ172	"

Notes:

[1] The marker tracks are λrif18/HindIII (H) and λrif18/EcoRI (E), with fragment sizes in kb.

[2] The faint bands of 2.0 and 2.3 kb in track 8 are due to λcI857 Sam7 contamination of the λrif18 DNA.

[3] The AvaI digests (tracks 6 and 7) did not quite reach completion.

[4] The fragments marked in tracks 14 and 15 are those which differ between λ172 and λ261; the marker track used for this gel is not shown.

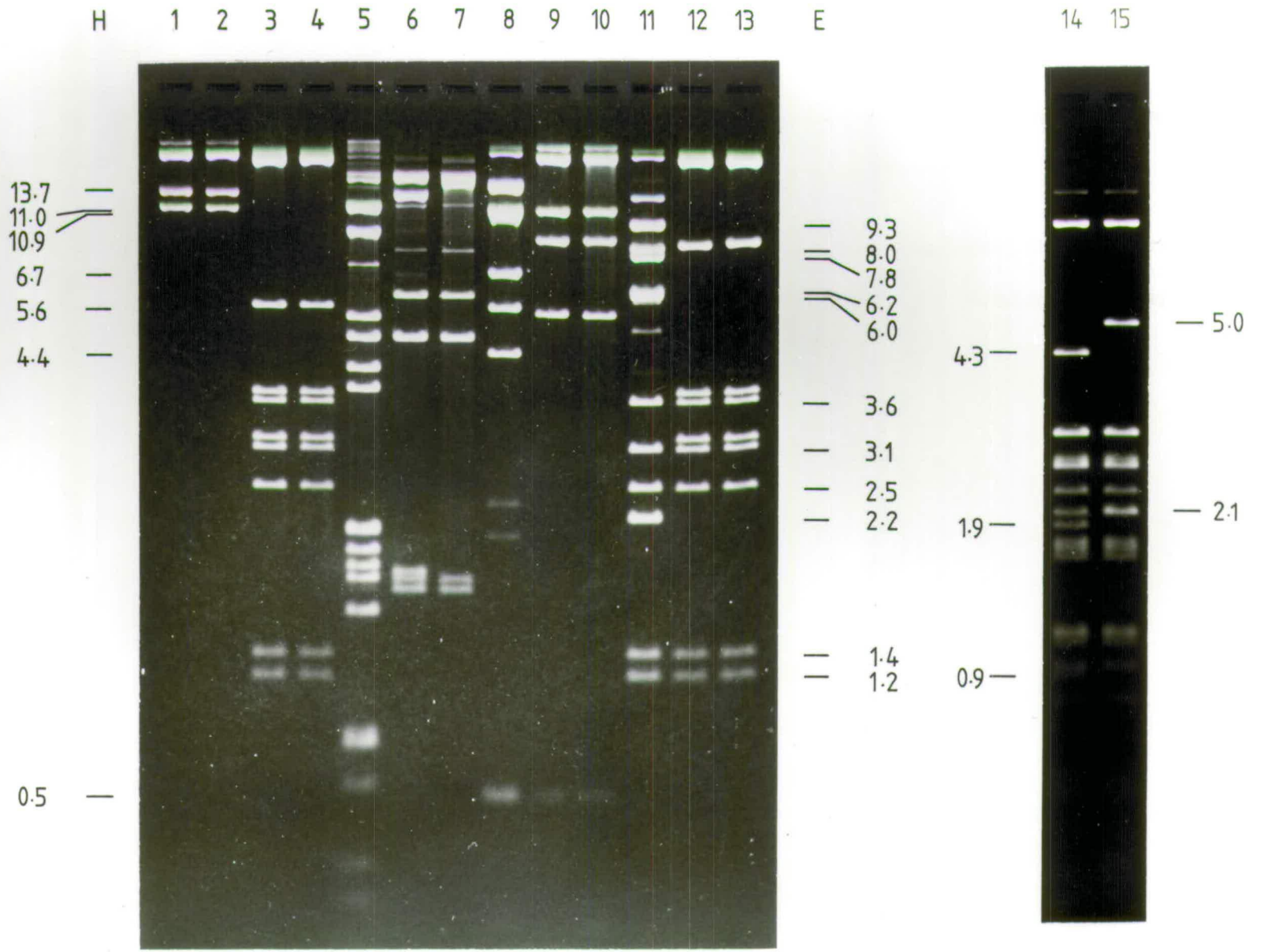

H
1
2
3
4
5
6
7
8
9
10
11
12
13
E
14
15
13·7
11·0
10·9
6·7
5·6
4·4
0·5
9·3
8·0
7·8
6·2
6·0
3·6
3·1
2·5
2·2
1·4
1·2
4·3
1·9
0·9
5·0
2·1

Fig. 4.1

This situation obtains also in CR63 itself: the same anomalous mobility of β was observed in an SDS-lysate of CR63 labelled with L-[^{35}S]-methionine *in vivo* (see Fig. 4.2). The nature of the mutation responsible for this size alteration is unknown; it may be relevant that CR63 was derived from CR67 by UV curing of a λ prophage.

Except for this difference in the size of the β subunit, however, the pattern of bacterial gene expression displayed by λ17 and λ63 appears to be the same. Infection of the S159 (λ*imm*21) lysogen elicits only a low-level synthesis of β and β' in each case, and L7/12 synthesis is not detectably increased above background levels. This points to the conclusion that in the *E.coli* K12 chromosome, as in the bacterial DNA carried by λ*rif*18, the transcription of *rplL* and *rpoBC* is effectively dependent upon sequences lying outside the cloned *Hin*dIII fragment.

This was confirmed by examining the expression of *rpoB* by λ63 in a suitable lysogen. The K12 strain AJN5 is *recA1 metB rpoB*70; *rpoB*70 is a recessive rifampicin-resistance allele. AJN5 itself is Rif-R, but introduction of an episome, KLF10 (from strain LE102) expressing *rpoB*$^{+}$ resulted in the loss of rifampicin-resistance, as expected if both alleles are being expressed simultaneously (see Austin *et al*, 1971).

Tandem dilysogens of AJN5 were isolated after simultaneous infection with λ63 and λ^{+}: they retained rifampicin-resistance, however, indicating a lack of expression of the rifampicin-sensitive allele of *rpoB* carried on λ63, and confirming the conclusions drawn from the UV-irradiated cell infections.

Fig. 4.2 Autoradiographs of ^{35}S-methionine-labelled proteins synthesised in UV-irradiated E.coli S159 (tracks 14-15 were unirradiated) and its λind$^-$ and λimm21 lysogens following phage infection. Proteins were fractionated on a 10-20% SDS-polyacrylamide gel (tracks 1-12) or 5% SDS-polyacrylamide gel (tracks 13-16).

Track number	Phage	Host
1	-	S159 (λind$^-$)
2	λrif18	"
3	λpolA	"
4	λ172	"
5	λ261	"
6	λ63	S159 (λimm21)
7	λ17	"
8	λpolA	S159
9	λ172	"
10	λ261	"
11	λ63	"
12	λ17	"
13	λ17	S159
14	-	unirradiated S159
15	-	unirradiated CR63
16	λ63	S159

Notes

[1] Proteins of interest are marked, with their MWs in kdal.

[2] The basis for the CR63 β mobility change is not known. Note, however, that a TAA → sense codon mutation at the end of rpoB would give an altered β polypeptide with 19 additional amino-acids before the next TAA codon. Since the rpoB-rpoC inter-gene sequence is 88 nucleotides long (Ovchinnikov et al, 1980) this would not entail reading into rpoC.

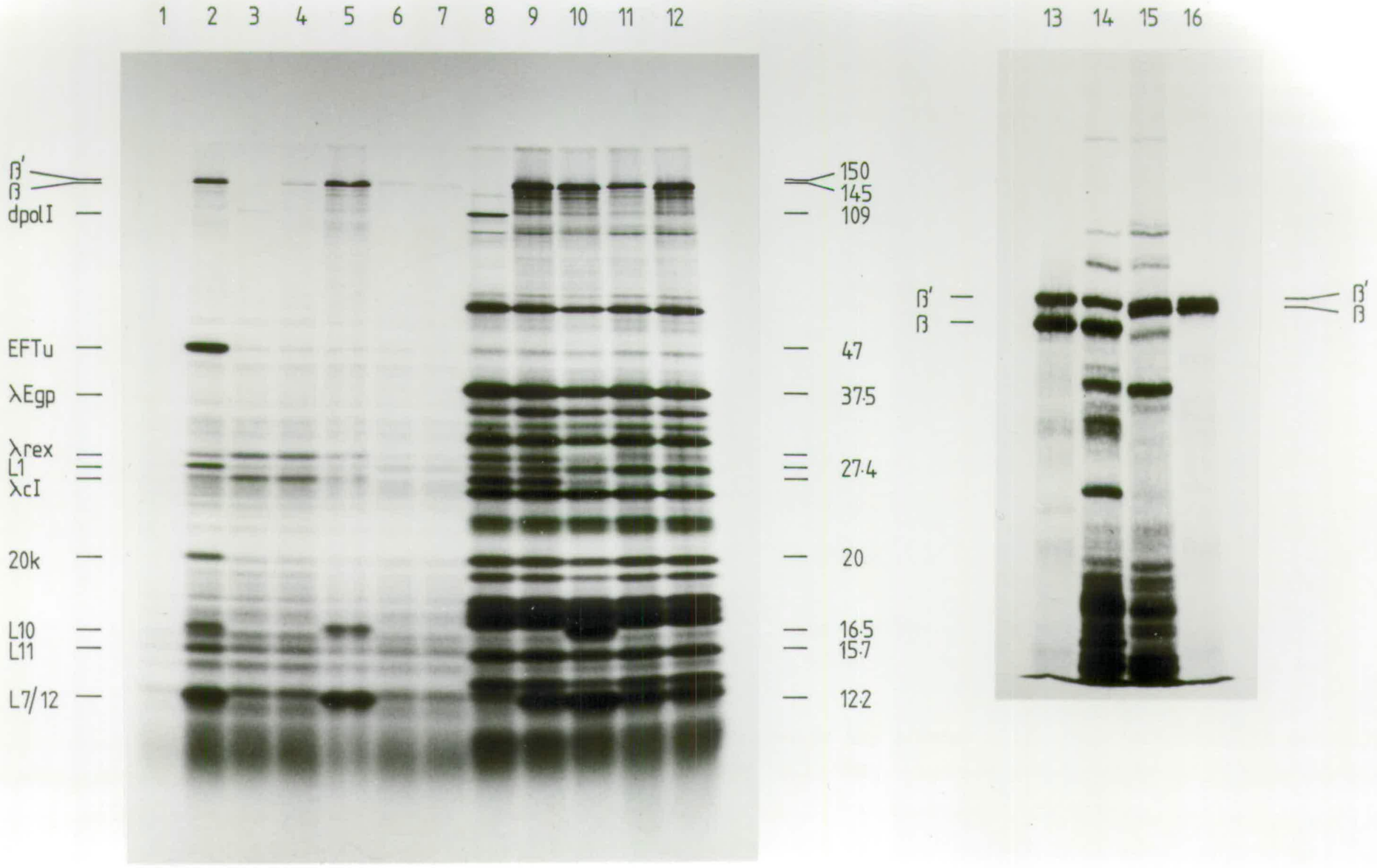

1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16
β'
β
dpolI
EFTu
λEgp
λrex
L1
λcI
20k
L10
L11
L7/12
150
145
109
47
37·5
27·4
20
16·5
15·7
12·2
β'
β
β'
β

Fig. 4.2

4.2 Expression of rplJL rpoBC from a Single Promoter in E.coli K12

The results of Yamamoto and Nomura (1978) and Linn and Scaife (1978) disclosed that in λrif18 a site located between the HindIII target in rplJ and the EcoRI target in rplK was both necessary and sufficient for expression of the rplJL rpoBC genes. In this section I describe experiments designed to discover whether the corresponding region of the E.coli K12 chromosome reveals a similar transcriptional organisation (see Chapter 3, Discussion).

Starting from λ172, phage incorporating additional DNA from the E.coli chromosome upstream of the HindIII site in rplJ were isolated as follows, employing the approach suggested by Shimada et al (1975). The starting E.coli strain was RSH21 (a rec$^+$ his Arg$^+$ Pur$^+$ Rif-S transductant of X174: Hayward, 1976); since RSH21 is λ-R (mal) a Mal$^+$ derivative, AJN30, was obtained by transduction with $P1_{vir}$ and checked for λ-sensitivity. AJN30 was then lysogenised with λ172 and λ232, to yield lysogens with the prophage integrated into the chromosomal rpoBC region.

These two strains were then made his$^+$ recA56 by interrupted mating with JG47 (an Hfr which transfersthese markers early) to yield strains AJN20 (recA56 and lysogenic for λ172) and AJN21 (recA56 and lysogenic for λ232). Phage produced by temperature induction of AJN20 and AJN21 were plated on C600 lysogenic for P2 to select phage which had become phenotypically Spi$^-$ via deletion of the λgam gene (Zissler et al, 1971). In NMλ761 and its derivatives the gam gene is adjacent to the inserted HindIII fragment (Fig. 4.3), so selection of phage deleted for gam is likely to enrich for phage carrying additional bacterial DNA instead.

The rationale behind the conversion of the lysogens to recA

Fig. 4.3 Rec-mediated integration of λ172 at the chromosomal *rpoBC* locus, and genesis of λ261 by subsequent illegitimate excision of the prophage. λ261 was isolated by selection for Spi$^-$ phages able to plate on C600 (P2), followed by screening for plaques which hybridised to ^{32}P-pNA38 (see text section 4.2 for details).

Sequences derived from λ172 are shown as filled boxes, whilst those from the RSH21 genome are shown as open boxes, but note that there is some uncertainty as to the precise junction between RSH21—derived and *λ172*-derived DNA: all sequences downstream of the Rif mutation presumably originate from λ172, whilst all sequences upstream of the *HindIII* site in *rplJ* must come from RSH21. The origin of DNA lying between these two points remains undetermined.

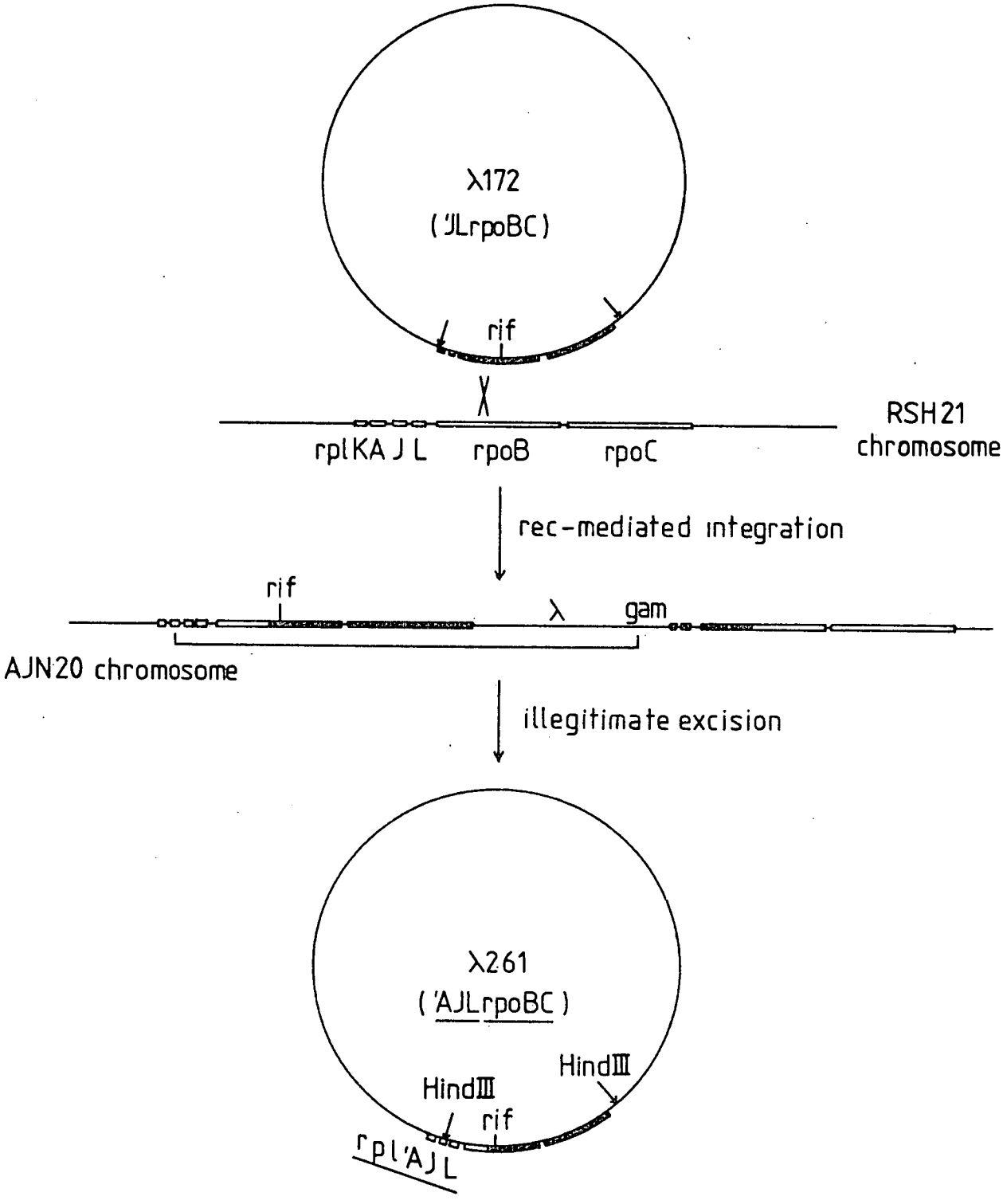

λ172
('JLrpoBC)
rif
RSH21
chromosome
rplKA J L
rpoB
rpoC
rec-mediated integration
rif
λ
gam
AJN20 chromosome
illegitimate excision
λ261
('AJLrpoBC)
HindIII
HindIII
rif
rpl'AJL

Fig. 4.3

before temperature induction was to increase the relative frequency of the aberrant excisions required to generate phage of the desired kind. In retrospect it is likely that the benefit of this procedure was reduced by poor maturation of red⁻ gam⁻ phage in the recA background. Nevertheless, Spi⁻ derivatives of λ172 and λ232 were readily obtained and screened for plaques hybridising to [^{32}P]-labelled pNA38 DNA. pNA38 is a derivative of pBR322 carrying a 1.9 kb EcoRI-HindIII fragment adjacent to and upstream of the 11 kb HindIII fragment in λrif18; it was constructed and kindly donated by Dr A. Nicolaidis.

The Spi⁻ phage from induction of the λ172 lysogen included a number of plaques which hybridised to [^{32}P]-pNA38, with various degrees of strength. In contrast, and demonstrating the specific nature of this method, the corresponding λ232 Spi⁻ phage gave no plaques capable of hybridising to [^{32}P]-pNA38: presumably Spi⁻ derivatives of λ232 would include phage carrying extensions of bacterial DNA beyond the downstream end of the 11 kb HindIII fragment (Fig. 4.3).

One of the λ172 Spi⁻ phage which hybridised strongly to [^{32}P]-pNA38, designated λ261, was purified for further study. λ261, in common with the majority of λ172 derivatives able to plate on C600 (P2), produced very small plaques, making large-scale phage preparation difficult. For this reason a chi site (Henderson and Weil, 1975; Lam et al, 1974) was crossed into the left arm of λ261 from NMλ904 (λchiA131 red3 gam210), resulting in much improved plaque size, but no detectable alteration in restriction pattern.

Digestion of DNA from λ261 and its parent λ172 with HindIII or EcoRI revealed no major change in size of the restriction fragments

Fig. 4.4 AvaI restriction maps of λ172 and λ261, illustrating the presence of a new AvaI site near the right-hand end of the E.coli DNA in λ261. Fragment sizes are in kb. The EcoRI and HindIII cleavage patterns given by λ172 and λ261 are identical (Figs. 4.1 and 3.3).

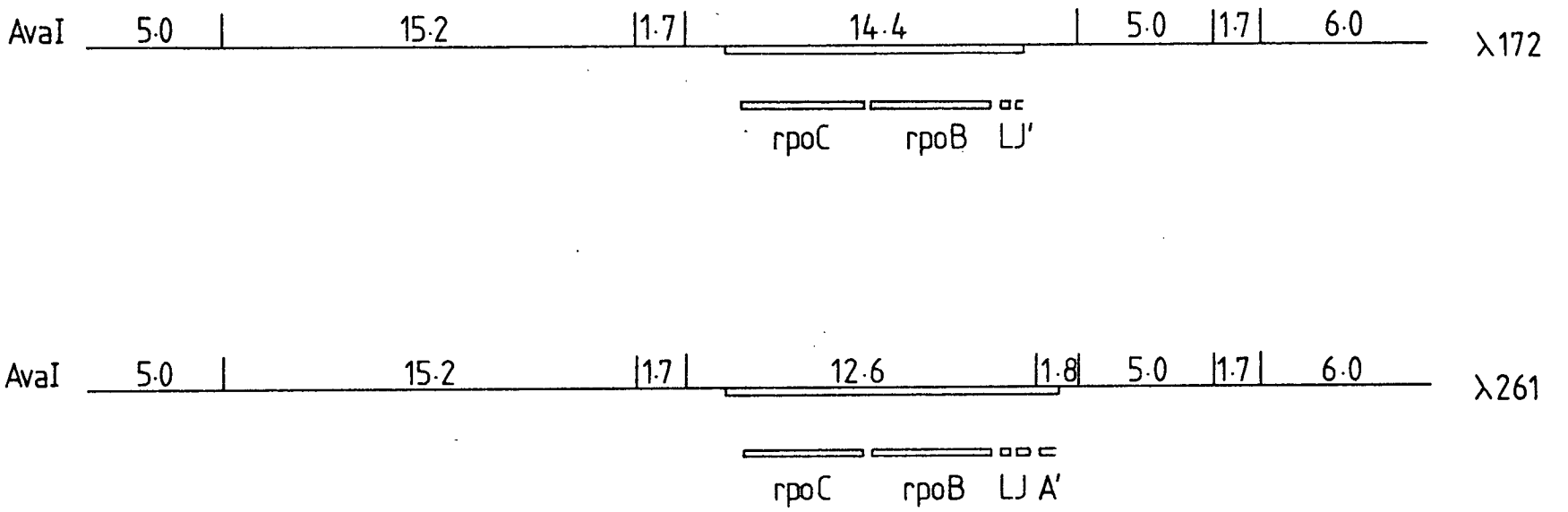

AvaI
5·0
15·2
1·7
14·4
5·0
1·7
6·0
λ172
rpoC
rpoB
LJ'
AvaI
5·0
15·2
1·7
12·6
1·8
5·0
1·7
6·0
λ261
rpoC
rpoB
LJ A'

Fig. 4.4

obtained (Fig. 4.1). Digestion with AvaI, however, gave fragments of 12.6 kb and 1.8 kb from λ261, replacing a 14.4 kb fragment in the λ172 digest (Figs. 4.1 and 4.4). Furthermore, digestion with PstI (Fig. 4.1) revealed that fragments of 5.0 kb and 2.1 kb arising from λ172 were replaced in the λ261 digest by new fragments of 4.3 kb, 1.9 kb and 0.9 kb. From these data it can be concluded that: (i) the novel sequences present in λ261 contain no sites for EcoRI or HindIII; (ii) the length of the novel E.coli DNA present in λ261 closely matches the length of λ DNA lost; (iii) the novel E.coli DNA includes an AvaI site. The data are also consistent with the presence of two PstI sites in the novel E.coli DNA, and one PstI site in the λ DNA lost from λ172.

In this context it is relevant that there are an AvaI site and two PstI sites some 170 bp, 340 bp and 1200 bp, respectively, upstream of the HindIII site in the corresponding region of λrif18 DNA (Post et al, 1979).

In order to define more accurately how much extra E.coli DNA has been added to the 11 kb fragment in λ261 (and further to test its structural identity with the sequences present in λrif18 DNA) the 8 kb EcoRI fragments of λ172 and λ261 DNA (which contain the region of interest - Fig. 3.3) and the 1.9 kb EcoRI-HindIII fragment of pNA38 were purified by electroelution from agarose gels. The three fragments were then recut with HaeIII, HhaI and PstI and analysed on 3-10% polyacrylamide gradient gels alongside markers of defined size (Fig. 4.5). With each enzyme the λ261 8 kb fragment gave rise to new sub-fragments not given by the λ172 8 kb fragment, and co-migrating with sub-fragments of the 1.9 kb fragment from pNA38. Thus with HaeIII the λ261 8 kb fragment yielded 237 bp, 207 bp,

Fig. 4.5 3-10% polyacrylamide gradient gel electrophoresis

Track number	DNA	Restriction endonuclease
1	pBR322	HpaII
2	f 8.0 kb/λ172	HaeIII
3	f 8.0 kb/λ261	"
4	f 1.9 kb/pNA38	"
5	pBR322	"
6	pBR322	"
7	f 1.9 kb/pNA38	PstI
8	f 8.0 kb/λ172	"
9	f 8.0 kb/λ261	"
10	f 1.9 kb/pNA38	HhaI
11	f 8.0 kb/λ172	"
12	f 8.0 kb/λ261	"

Notes

[1] The marker tracks are pBR322/HpaII (pa) and pBR322/HaeIII (ae) with fragment sizes in bp (Sutcliffe, 1978).

[2] The f 8.0 kb tracks display digests of the purified 8.0 kb EcoRI fragments of λ172 or λ261 (Fig. 3.3); these fragments include the λ-rpl junction.

[3] The f 1.9 kb tracks display digests of the purified 1.9 kb EcoRI/HindIII fragment of pNA38; this fragment extends from the HindIII site in rplJ to the EcoRI site in rplK (Fig. 4.13).

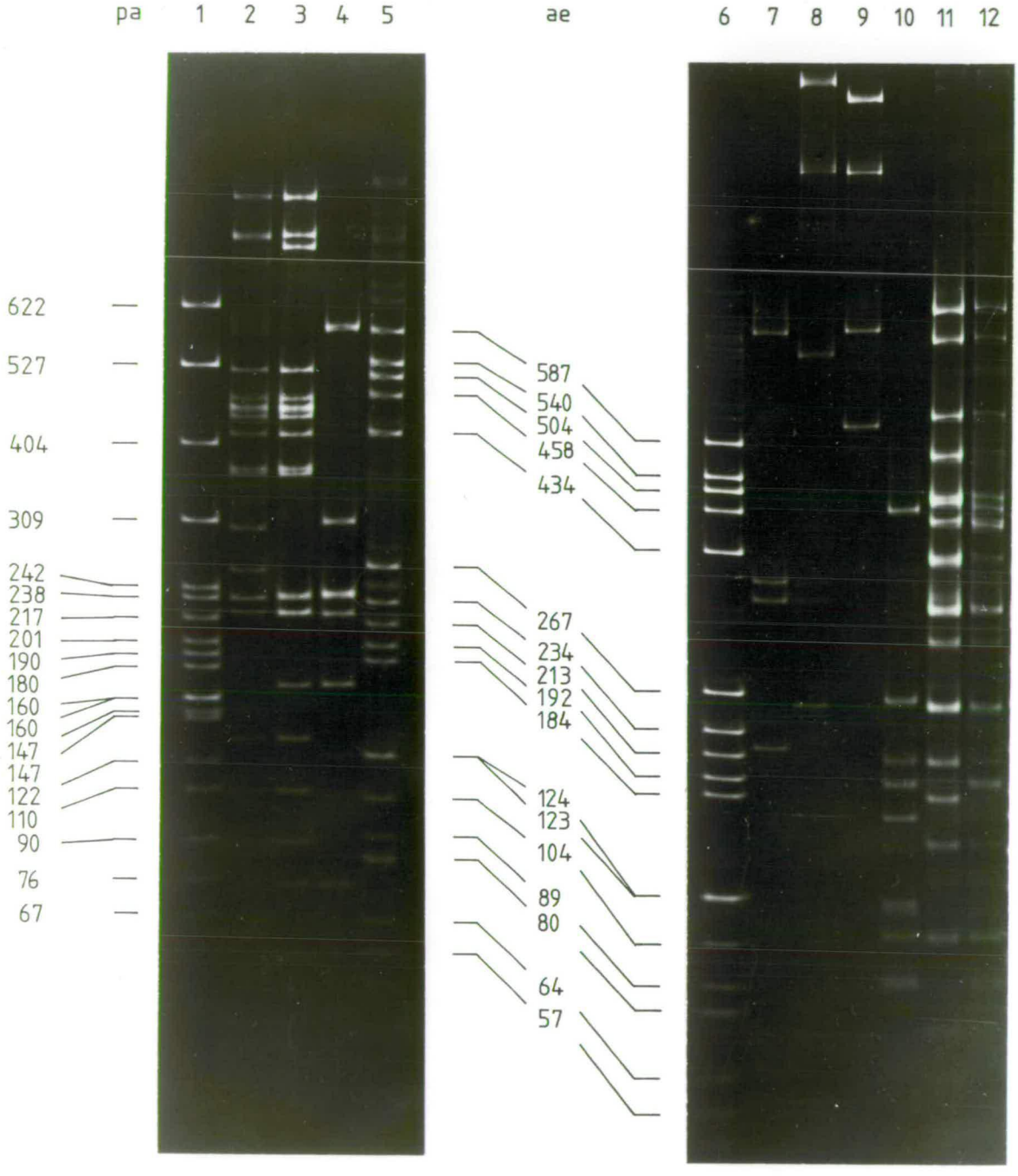

pa
1
2
3
4
5
ae
6
7
8
9
10
11
12
622
527
404
309
242
238
217
201
190
180
160
160
147
147
122
110
90
76
67
587
540
504
458
434
267
234
213
192
184
124
123
104
89
80
64
57

Fig. 4.5

Table 4.1

Rif-phenotype of *rpoBC*-merodiploids

Host	AJN10 (*rcs*$^+$)	AJN1 (*rcs*-40)
Phage or F' present		
KLF10 *rpoB*70	S	R
λ*rif*18, λ*imm*21	R	R
λ172, λ*imm*21	S	S
λ261, λ*imm*21	S	R

166 bp and 72 bp; with HhaI, 490 bp, 207 bp, 185 bp, 106 bp and 91 bp; and with PstI, 922 bp (see Fig. 4.6 for interpretation and summary of these data).

The data shown in Fig. 4.5 and summarised in Fig. 4.6 suggest that the additional sequences cloned in λ261 share close structural similarity with the corresponding region of λrif18. Furthermore, reference to the nucleotide sequence of this region of λrif18 (Post et al, 1979) indicates that in λ261 the E.coli DNA should extend beyond rplJ and the rplJ-rplA interval into rplA, but stop short of the 5' terminus of this structural gene.

Two lines of evidence indicate that the additional E.coli DNA cloned in λ261 includes a site allowing vigorous autonomous transcription of rpoBC. Firstly, in the S159 (λind$^-$) lysogen, where λP_L is repressed, λ261 directed the synthesis of L10, L7/12, β and β', whereas under the same conditions λ172 elicited only a very weak synthesis of β and β' (Fig. 4.2). λ261 did not direct the synthesis of L1 in either S159 or S159 (λind$^-$); this is consistent with the fragment-recutting data presented above, which indicated that the 5' end of rplA is absent.

Secondly, a λ261, λimm21 dilysogen of strain AJN1 (recA56, rcs-40) is Rif-R, and grows normally at a rifampicin concentration of 200 μg/ml (Table 4.1). The λ172, λimm21 dilysogens of both AJN1 and AJN10 were Rif-S, and the λrif18 lysogens were Rif-R, as expected. Curing of λ261 and of λrif18 from these lysogens, by selection of survivors at 42^{o}C, restored rifampicin-sensitivity. The survivors retained imm21, but were λ$^-$ and λ-sensitive.

Surprisingly, (λ261, λimm21)-lysogenic derivatives of AJN10 (rcs$^+$) were found to be Rif-S, in contrast to the analogous AJN1

Fig. 4.6 Restriction sites in the rplKAJL region used in determining the extent of the additional rpl sequences cloned in λ261. Transcription is from right to left. The lower line shows the sites for HaeIII and HhaI in the 1.9 kb HindIII-EcoRI DNA fragment, with the fragment sizes in bp. The end-point of the E.coli sequences in λ261 lies in the region common to the 230 bp HaeIII and 254 bp HhaI fragments, within the rplA gene. Restriction sites in the rplKAJL region were taken from Post et al (1979).

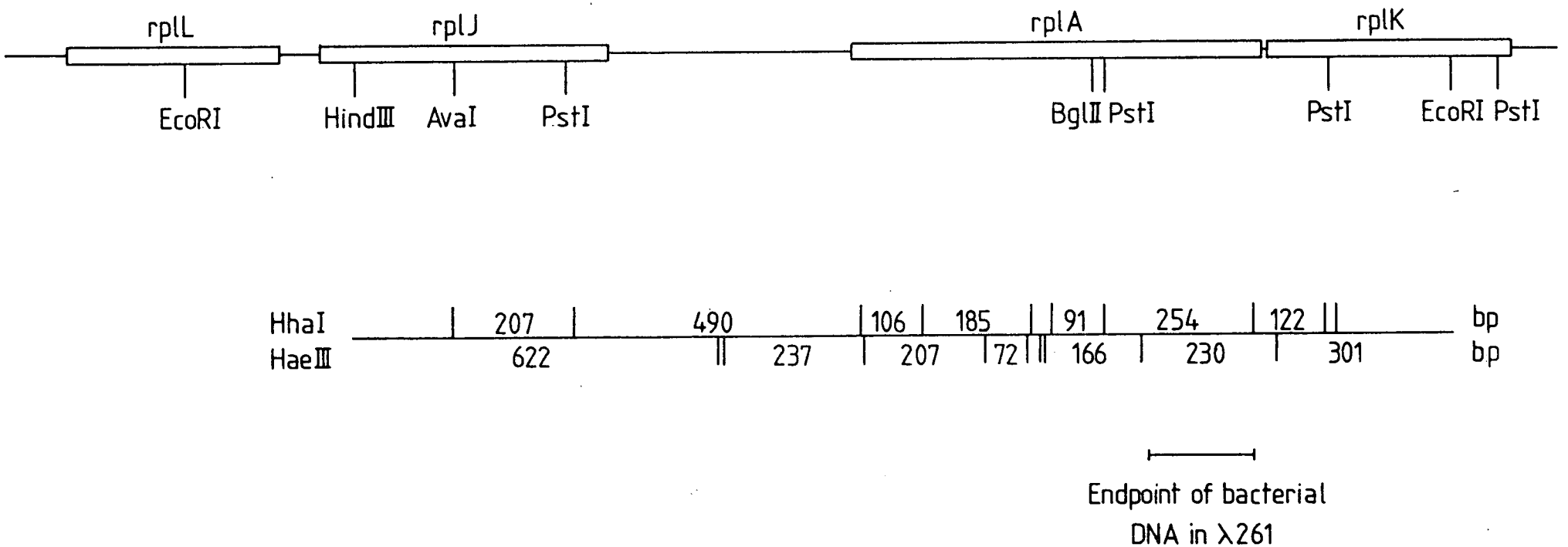

rplL
rplJ
rplA
rplK
EcoRI
HindIII
AvaI
PstI
BglII PstI
PstI
EcoRI PstI
HhaI
207
490
106
185
91
254
122
bp
HaeIII
622
237
207
72
166
230
301
bp
Endpoint of bacterial
DNA in λ261

Fig. 4.6

lysogens, and despite the fact that the rifampicin-resistance mutation present in λ261 was derived from λ*rif*18, which as a prophage confers rifampicin-resistance on both AJN1 and AJN10. The implication of this finding, considered further in the Discussion below, is that the dominant expression of rif-resistance by λ*rif*18 was the result of an additional alteration(s), now separated from the rif-resistance mutation in λ261.

Nevertheless, the data presented above suggest that all four genes *rplJ rplL rpoB rpoC* are expressed from a single promoter, presumably lying in the *rplJ-rplA* intercistronic region. However, data presented by Fiil *et al* (1979) raised the possibility that *rplJ* and *rplL rpoBC* might form two distinct transcription units. This idea was based upon a study of the proteins synthesised in strains harbouring multicopy plasmids carrying various regions of the *rplKAJL rpoBC* gene cluster. In particular, plasmids carrying a *Bgl*II fragment extending from the *Bgl*II site in *rplA* (see Fig. 4.6) downstream into *rpoC*, and thus carrying complete copies of *rplJ rplL rpoB* (and the entire *rplJ-rplA* intercistronic region), expressed L7/12 and β, but not L10. Their interpretation of this result was that *rplJ* is expressed from a separate promoter lying upstream of the *Bgl*II site, within the structural gene (*rplA* or *rplK*) for L1 or L11: the corollary of this would be that both hypothetical promoters ("P_J" and "P_{LBC}") would be separated from their respective structural genes by a long leader sequence.

Recently λ vectors capable of accepting *Bam*HI-restriction fragments have become available (Klein and Murray, 1979). These phages can also accept *Bgl*II-generated fragments (*Bam*HI cuts at the sequence G▽GATCC, and *Bgl*II at A▽GATCT, both producing GATC cohesive

Fig. 4.7 1% agarose gel electrophoresis

Track number	DNA	Restriction endonuclease
1	λ363	EcoRI
2	λ321	"
3	λ570BV2	"
4	λ261	"

Notes: 1 The marker track is λ261/EcoRI (E)

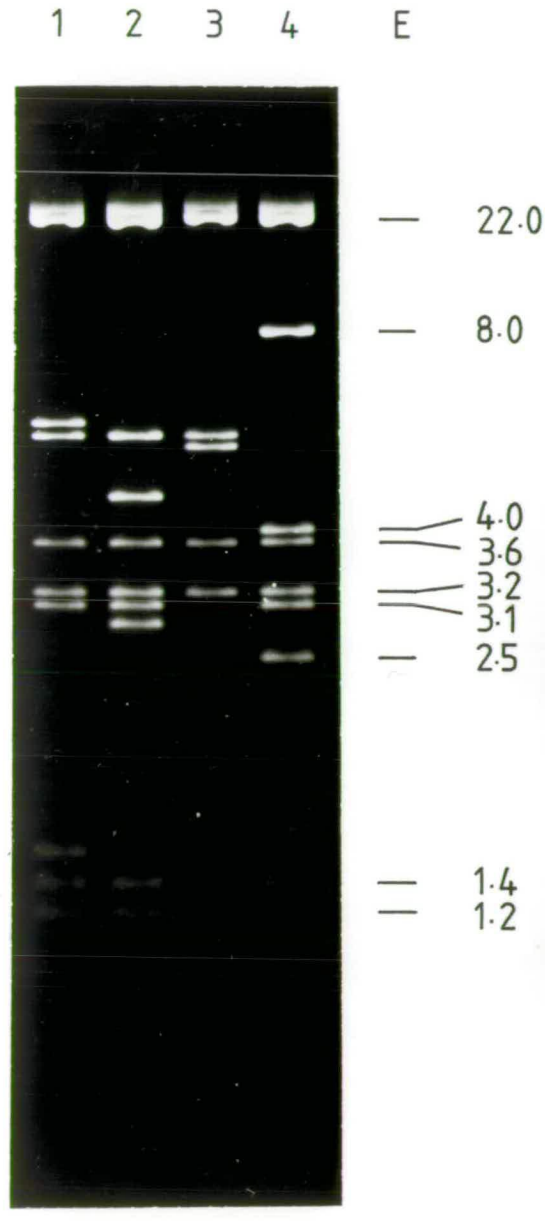


Fig. 4.7

Fig. 4.8 EcoRI restriction maps of the BamHI vector λ570 BV2 (Klein and Murray, 1979), and of two recombinants λ363 and λ321 carrying a 7.6 kb BglII fragment from λrif18 (shown as an unfilled box). This fragment includes rpl'AJL rpoBC'; it is in the L orientation in λ363 and in the R orientation in λ321. The vector's single BamHI site is destroyed by insertion of the BglII fragment.

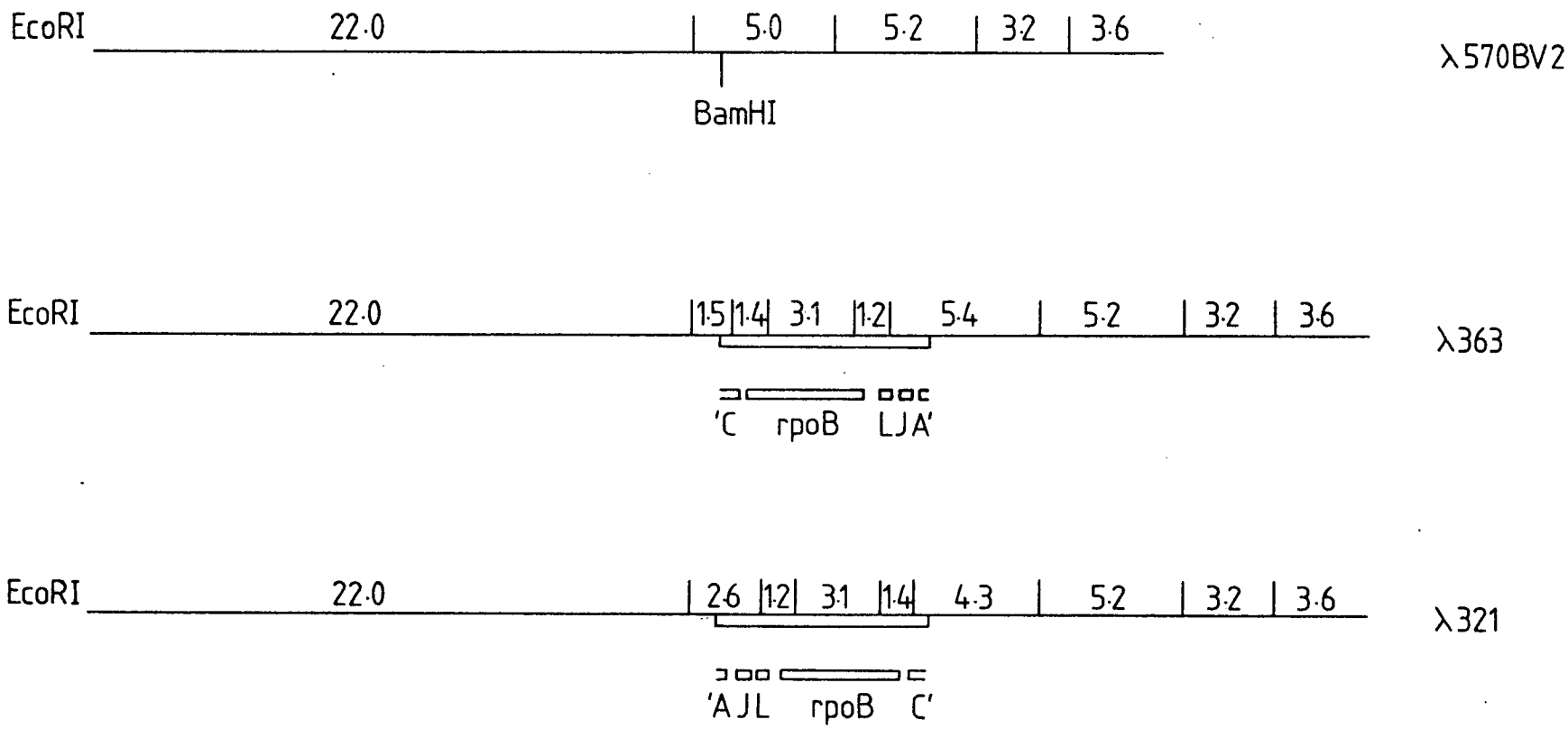

EcoRI
22·0
5·0
5·2
3·2
3·6
BamHI
λ570BV2
EcoRI
22·0
1·5
1·4
3·1
1·2
5·4
5·2
3·2
3·6
'C rpoB LJA'
λ363
EcoRI
22·0
2·6
1·2
3·1
1·4
4·3
5·2
3·2
3·6
'AJL rpoB C'
λ321

Fig. 4.8

ends). Hence the suggestion of Fiil et al could be tested by cloning the relevant BglII fragment in a λ vector (λ570 BV2, kindly donated by Ms B. Klein) and investigating the expression of rplJ rplL and rpoB by the recombinant phages.

Phage carrying the 7.6 kb rplJ-rpoB BglII fragment were isolated from a ligation of BglII-cut λrif18 and BamHI-cut λ570 BV2 DNAs, their initial identification being achieved by plaque-hybridisation with [^{32}P]-pNA26. pNA26 is a derivative of pSF2124 (So et al, 1976) carrying the 1.2 kb rplL-rpoB EcoRI fragment of λrif18. It was kindly donated by Dr A. Nicolaidis.

Composite BamHI-BglII sites are not a substrate for either endonuclease. The identity and orientation of the inserted DNA fragments were determined, therefore, by cleavage with EcoRI (Fig. 4.7). λ363 and λ321 are two phage which hybridised with [^{32}P]-pNA26. Upon EcoRI digestion they both give fragments of 1.2 kb, 1.4 kb and 3.1 kb, which are also common to λ261 and are derived from the rplL-rpoB region. Moreover, both λ363 and λ321 lack the 5.0 kb EcoRI fragment (present in the λ570 BV2 digest) which contains the vector's single BamHI site. The recombinants differ, however, in that λ363 gives fragments of 5.4 kb and 1.5 kb, whereas λ321 gives 4.3 kb and 2.6 kb. These data establish that λ363 carries the 7.6 kb BglII fragment in the L orientation, whereas λ321 has the R orientation (see Fig. 4.8 for interpretation and summary of the data from Fig. 4.7).

The result of a UV-irradiated cell experiment investigating gene expression by these recombinant phages is shown in Fig. 4.9. λ321 and λ363 elicited the synthesis of L10, L7/12 and β in the non-lysogen, confirming that both recombinants carry intact copies of the rplJ,

Fig. 4.9 Autoradiograph of ^{35}S-methionine-labelled proteins synthesised in UV-irradiated E.coli S159 and its λind$^-$ and λimm21 lysogens following phage infection. Proteins were fractionated on a 10-20% SDS-polyacrylamide gel.

Track number	Phage	Host
1	-	S159 (λimm21)
2	λ570BV2	"
3	λ321	"
4	λ363	"
5	λ261	S159 (λind$^-$)
6	λ570BV2	S159
7	λ321	'
8	λ363	'

Notes:

[1] The cpm present in the β, L7/12 and L10 bands in tracks 7 and 8 were as follows:

	track 7 (λ321)	track 8 (λ363)
β	3312	28444
L7/12	51825	84451
L10	27837	39967

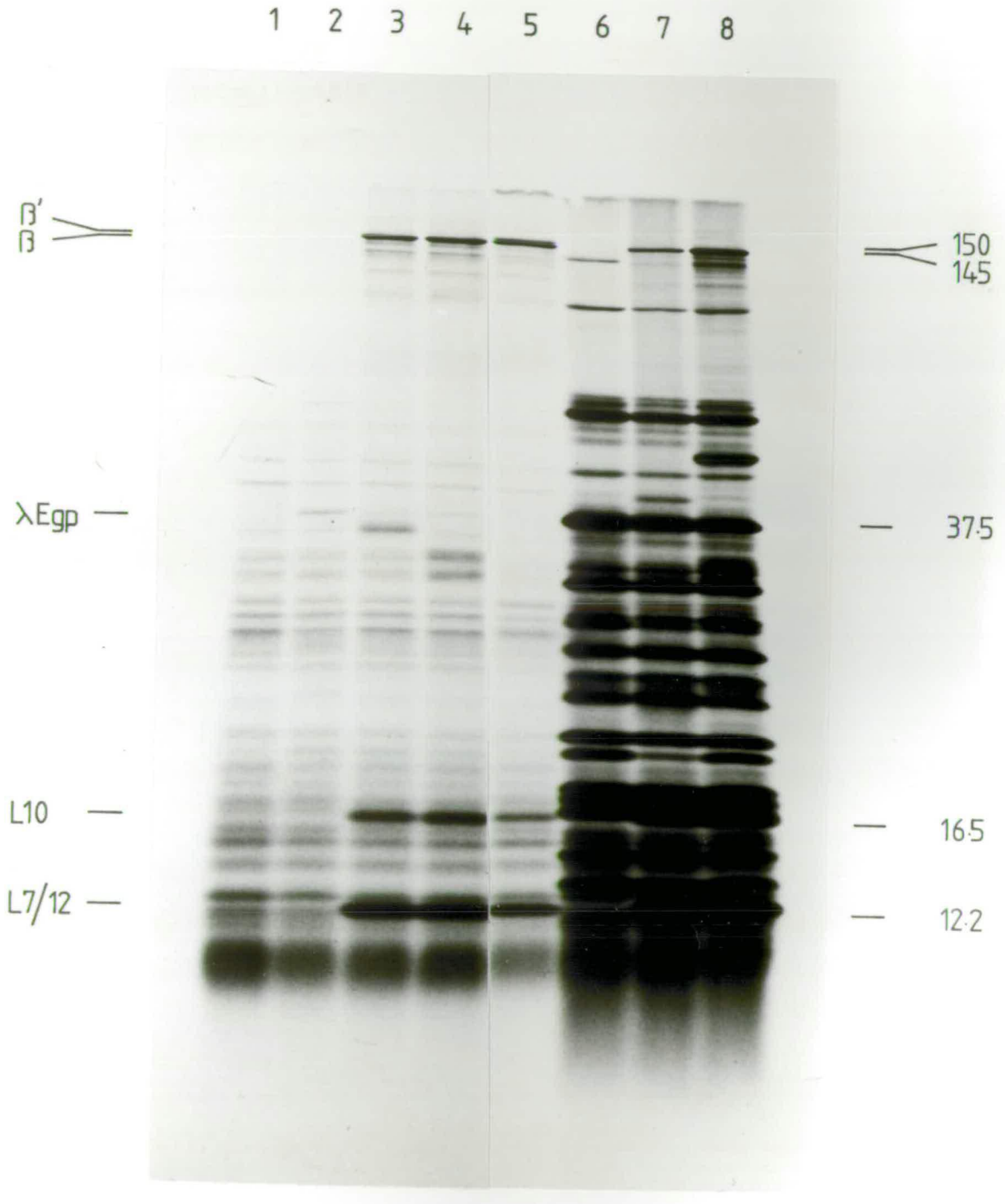


Fig. 4.9

rplL and rpoB genes. It is apparent that after infection with the R orientation phage (λ321) less β was made than with the L orientation phage (λ363) although synthesis of L10 and L7/12 was comparable (Fig. 4.9 legend). A likely explanation for this is that in λ321 convergent transcription from λP_L preferentially reduces the expression of rpoB. Consistent with this hypothesis, in the λimm21 lysogen (λ promoters repressed) both λ321 and λ363 directed the synthesis of L10, L7/12 and β, the proportions of these proteins being indistinguishable in the two cases.

Evidently, the 7.6 kb BglII fragment does include a promoter able to express rplJ. This conclusion is in disagreement with the findings of Fiil et al (1979), which implied that "P_J" lies upstream of the BglII site in rplA (see Discussion at end of this Chapter). The present results are consistent with expression of all 4 genes rplJ rplL rpoB rpoC from a single promoter.

4.3 Low Level Transcription of rpoBC in the Absence of the Major Promoter

Despite the absence of the major promoter of the operon, a weak but reproducible expression of rpoBC was detectable (independently of λ promoter activity) after infection of UV-irradiated lysogenic bacteria with phage carrying the 11 kb HindIII fragment containing rplL rpoBC (Fig. 3.5).

Further information on the site or sites responsible for this expression of rpoBC was provided by the fortuitous isolation of λ514, a Spi^- deletion derivative of λ172 (Chapter 4.2). Fig. 4.10 shows HindIII and EcoRI digests of DNA derived from λ514 and its progenitor λ172. The 11.0 kb and 5.6 kb HindIII fragments of λ172 are replaced by a single fragment of 13.0 kb in λ514, whilst the EcoRI digests

Fig. 4.10 1.2% agarose gel electrophoresis.

Track number	DNA	Restriction endonuclease
1	λ514	EcoRI
2	λ172	"
3	λrif18	"
4	λ514	HindIII
5	λ172	"
6	λrif18	"
7	λ514	SalI
8	λ172	"
9	λrif18	"

Notes:

[1] The marker tracks are λrif18/EcoRI (E) and λrif18/HindIII (H)

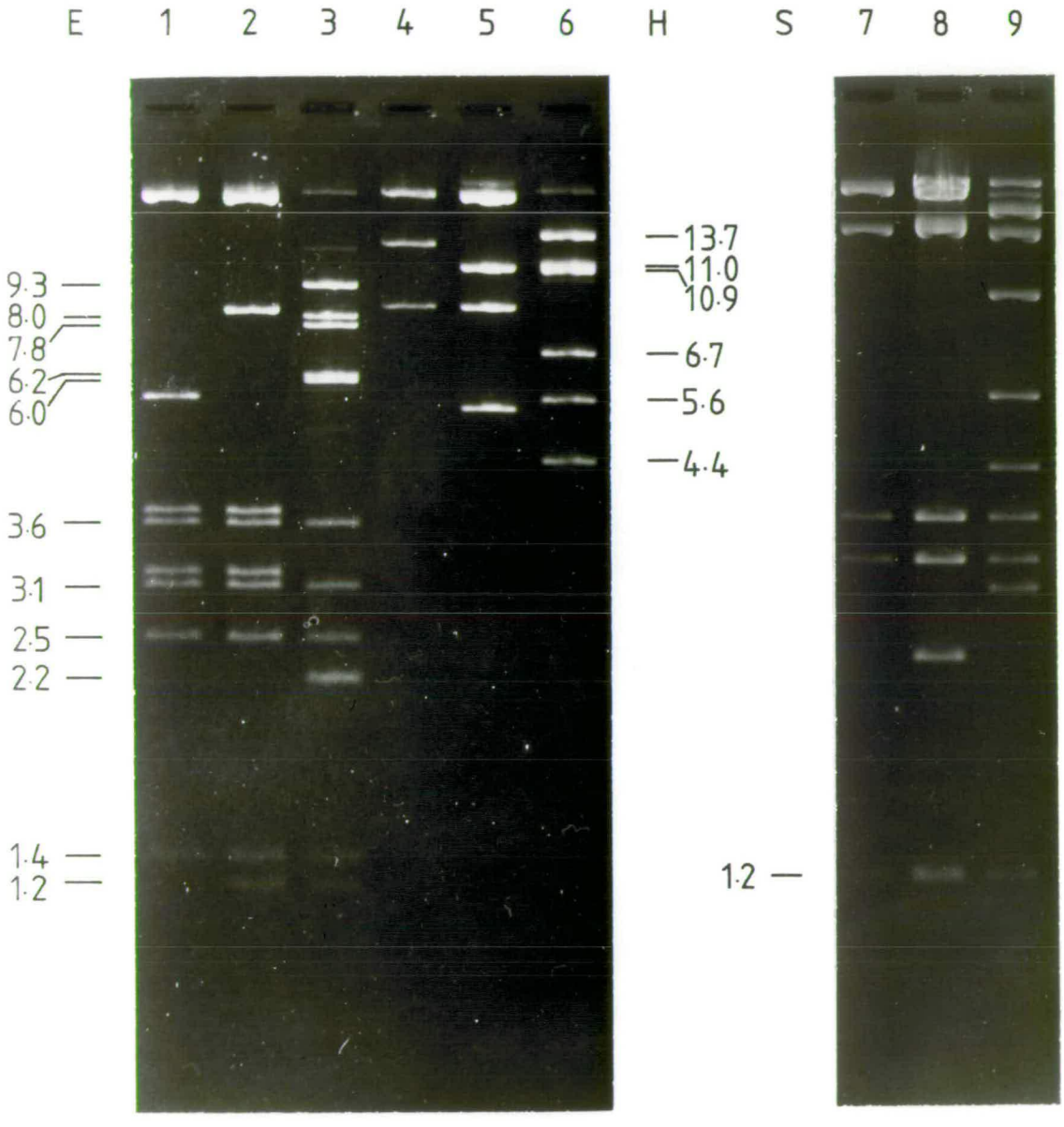
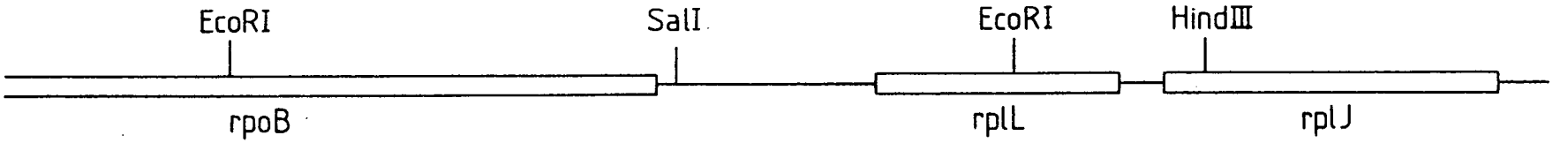
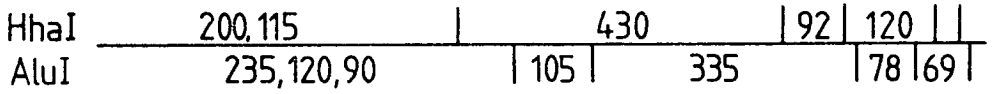

E
1
2
3
4
5
6
H
S
7
8
9
9·3
8·0
7·8
6·2
6·0
3·6
3·1
2·5
2·2
1·4
1·2
13·7
11·0
10·9
6·7
5·6
4·4
1·2

Fig. 4.10

Fig. 4.11 Restriction sites in the rplJL-rpoB' region. Transcription is from right to left. The line below shows the HhaI and AluI sites in the 1.2 kb rplL-rpoB EcoRI fragment, with fragment sizes in bp: fragments smaller than 50 bp are not given sizes. The rpl-rpo sequences in λ514 end within the 78 bp AluI fragment in rplL. Data from Post et al (1979) and J-C. Ma (pers. comm.)





rpl sequences deleted
by illegitimate excision
to generate λ514

Fig. 4.11

disclose the replacement of the 1.2 kb and 8.0 kb EcoRI fragments with one of 5.6 kb. These data indicate that λ514 arose from λ172 by deletion of 3.6 kb of DNA from the region surrounding the upstream end of the rplL rpoBC fragment, including the HindIII site in rplJ, the EcoRI site in rplL, and part or all of the λgam gene. Also shown in Fig. 4.10 are SalI digests of λrif18, λ172 and λ514, showing that all 3 phages gave rise to the 1.2 kb fragment which includes the 5' end of rpoB (Post et al, 1979; Fiil et al, 1979). Hence the deletion in λ514 extends at least as far as the EcoRI site in rplL, but stops short of the SalI site in the rplL-rpoB intercistronic space. I am grateful to J.C. Ma for further information on this: recutting of appropriate purified fragments with AluI and HhaI (data not shown) indicated that the deletion ends within a 78 bp AluI fragment near the 3' end of rplL. Thus λ514 carries a small (3') portion of rplL, the entire rplL-rpoB intercistronic space, and rpoBC (see Fig. 4.11).

Are the sequences retained in λ514 still capable of initiating low-level transcription of rpoBC? The data presented in Fig. 4.12 indicate that λ514 directed the synthesis of β and β', but not L7/12, after infection of S159, consistent with the mapping work described above. After infection of the λind$^-$ lysogen, moreover, λ514 still directed a weak synthesis of β and β'. Excision and counting of the relevant bands established that the weak expression of β and β' by λ172 was reduced further by the deletion which gave rise to λ514.

These data suggest the possibility that at least two minor promoters for rpoBC are present on the bacterial DNA fragment. The more effective of these, possibly lying between rplJ and rplL, is

Fig. 4.12 Autoradiograph of ^{35}S-methionine-labelled proteins synthesised in UV-irradiated S159 and S159 (λ*ind*$^-$) following phage infection. Proteins were fractionated on a 7-15% SDS-polyacrylamide gel.

Track number	Phage	Host
1	λ261	S159 (λ*ind*$^-$)
2	λ172	"
3	λ514	"
4	-	"
5	λ261	S159
6	λ172	"
7	λ514	"

Notes:

[1]The cpm present in the β and β' bands in tracks 1, 2 and 3 are as follows:

	Track 1 (λ261)	Track 2 (λ172)	Track 3 (λ514)
β	10940	300	80
β'	10082	306	105

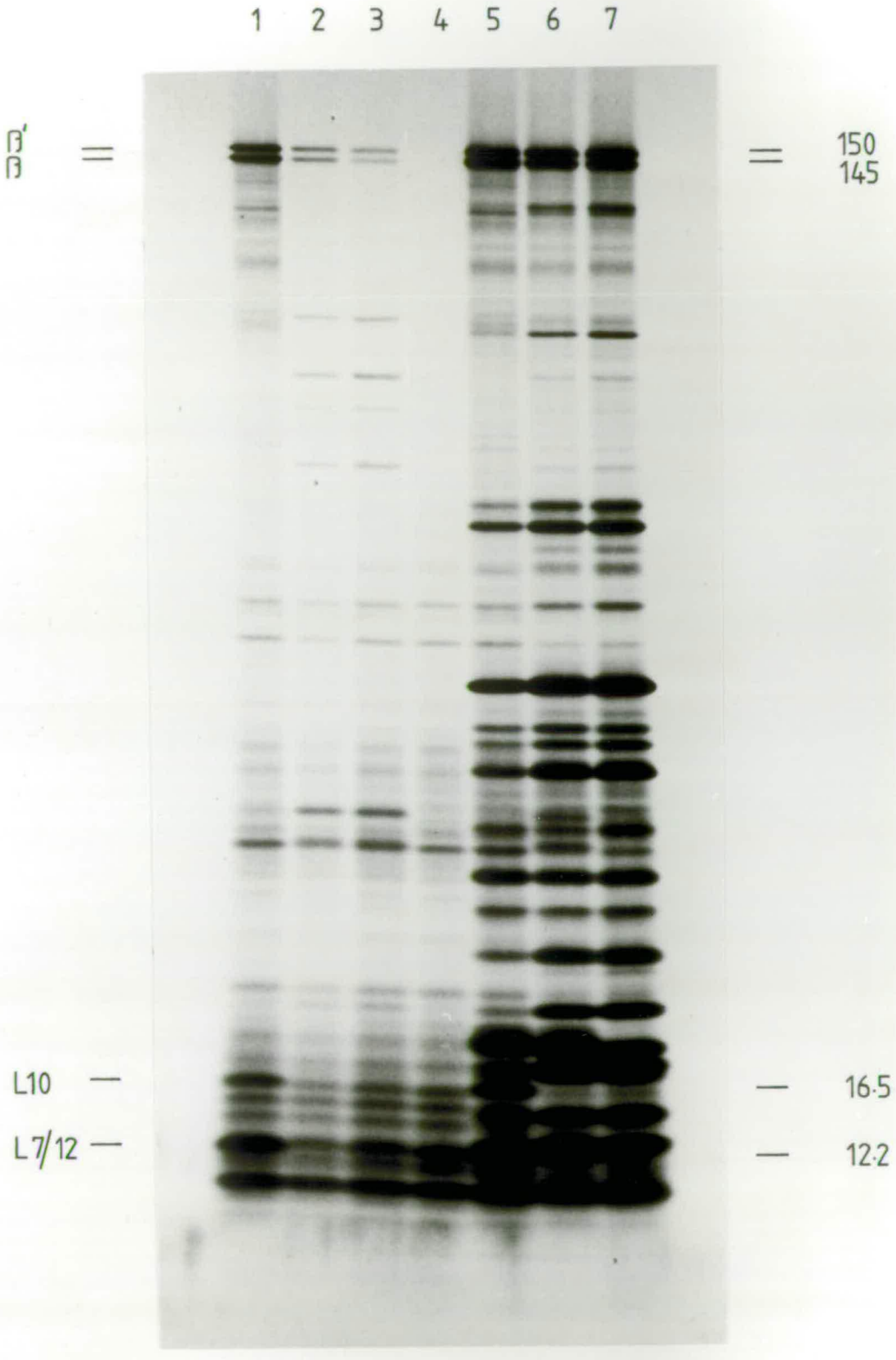


Fig. 4.12

deleted in λ514, whereas the other, almost certainly lying in the rplL-rpoB intercistronic space, is retained.

4.4 Discussion

The results of the experiments described in this Chapter confirm the conclusion, previously deduced from studies using DNA from λrif18, that rpoBC are co-transcribed with rplJL in E.coli.

λ261 carries a functional copy of the rpoBC operon. It directs synthesis of L10, L7/12, β and β' in UV-irradiated cells, and as a prophage confers rifampicin-resistance on the rcs-40 host AJN1. In an rcs^+ background, however, the λ261 prophage is unable to confer rifampicin-resistance. Since no selection was imposed for expression of rifampicin-resistance during the isolation of λ261, it seems unlikely that the phage carries a new rifampicin-resistance allele (rifampicin-resistance mutations arise spontaneously at a frequency of only 1 in 10^8: Babinet, 1971). On the contrary, λ261 is very likely to have inherited the rifampicin-resistance mutation from λrif18, which carries the dominant rifampicin-resistance allele rpoB3. This suggests that the rpoB3 allele has a complex genetic basis: the rifampicin-resistance component of rpoB3 has been recovered in λ261, but has apparently been separated from some other feature(s) which conferred dominance in λrif18. The results do not reveal whether the additional dominance feature lies within rpoB (on the N-terminal side of the rifampicin-resistance mutation) or external to it. Linn and Scaife (1978) observed that when a phage structurally rather similar to λ261 was constructed in vitro, using bacterial DNA derived entirely from λrif18, the dominant rifampicin resistance characteristic of rpoB3 was retained. Hence if the dominance of rpoB3 is the result of a second mutation

external to rpoB, this must lie between rpoB and the EcoRI site in rplK, which defines the upstream end of the E.coli DNA present in the rpoB3-transducing phage constructed by Linn and Scaife.

Although the properties of λ261 indicate that the dominance of rpoB3 is the result of more than one mutation, the mechanism of the dominance remains unclear. The weight of evidence, however, is against the idea that dominant rifampicin-resistance is the result of a cis-effective regulatory change increasing the proportion of resistant enzyme in the cell: rpoB3/rpoB$^+$ heterodiploid bacteria, in common with other heterodiploids showing the more usual dominance of the sensitive allele, appear to contain roughly equal quantities of Rif-S and Rif-R enzyme (Kirschbaum and Konrad, 1973; Hayward et al, 1973; Newman and Hayward, 1979). The additional alteration in rpoB3 seems more likely to act on β to alter the competition at promoters between Rif-S and Rif-R polymerases, upon which the normal dominance relationship is based (Bordier, 1974; Hayward, 1976).

There seems to be no simple explanation for the result reported by Fiil et al (1979), namely, that multicopy plasmids carrying the BglII fragment of λrif18, which includes 'rplA rplJL rpoBC', express rplL and rpoB but not rplJ; whereas a similar pBR322 derivative carrying an additional 410 nucleotides of DNA upstream of the BglII site in rplA (Fig. 4.13), up to the PstI site in rplK, expresses both rplJ and rplL strongly in the same host. The present work shows that the BglII fragment, whenpresent in a transducing phage, expresses all 3 genes normally in UV-irradiated lysogenic cells, in accord with the evidence (see below) that the rplJL rpoBC major promoter resides in the rplA-rplJ intercistronic region. Possibly the synthesis of plasmid-encoded L10 is prevented post-transcriptionally

in the case of the plasmids carrying the BglII fragment, although it is not clear why these should differ from the PstI plasmid in this respect.

Infection of UV-irradiated lysogenic bacteria with phage carrying the 11 kb HindIII fragment of λrif18 (λ172 and λ232) or the homologous fragment from the genome of CR63 (λ63) elicited a weak but reproducible synthesis of ββ' (at 2-3% of the rate given by λ261 using the major rpo promoter). This low-level expression was further curtailed in λ514, in which a deletion has removed most of rplL and all the bacterial sequences upstream (including the rplL-rplJ intercistronic region). This suggests that low-level transcription of rpoBC might be initiated at 2 distinct sites, only one of which is deleted from λ514: these would most likely be located in the rplJ-rplL and rplL-rpoB intercistronic regions.

The physiological significance, if any, of this low-level expression of ββ' is not known. It is clear that λ172, λ232 and λ63 are all incapable, as prophages, of effective expression of their rpoB genes in circumstances where the prophage rpoB allele would, if expressed, be dominant. Note, however, that in quantitative terms it compares favourably with the expression in UV-irradiated lysogens of dpolI from the λpolA phage (Fig. 3.5), which is thought, from genetic evidence, to carry the normal polA promoter (Kelley et al, 1977). Expression from this promoter is responsible for the presence of some 400 molecules of dpolI per cell (Kornberg, 1974).

Independent evidence for minor promoter activity in the rplJL rpoBC operon has been obtained by Barry et al (1979) from measurements of β-galactosidase expression in fusions linking

Fig. 4.13 DNA sequence of the ribosomal protein gene cluster from λrif18 (Post et al, 1979). Restriction enzyme sites relevant to the experimental work described in this thesis are shown.

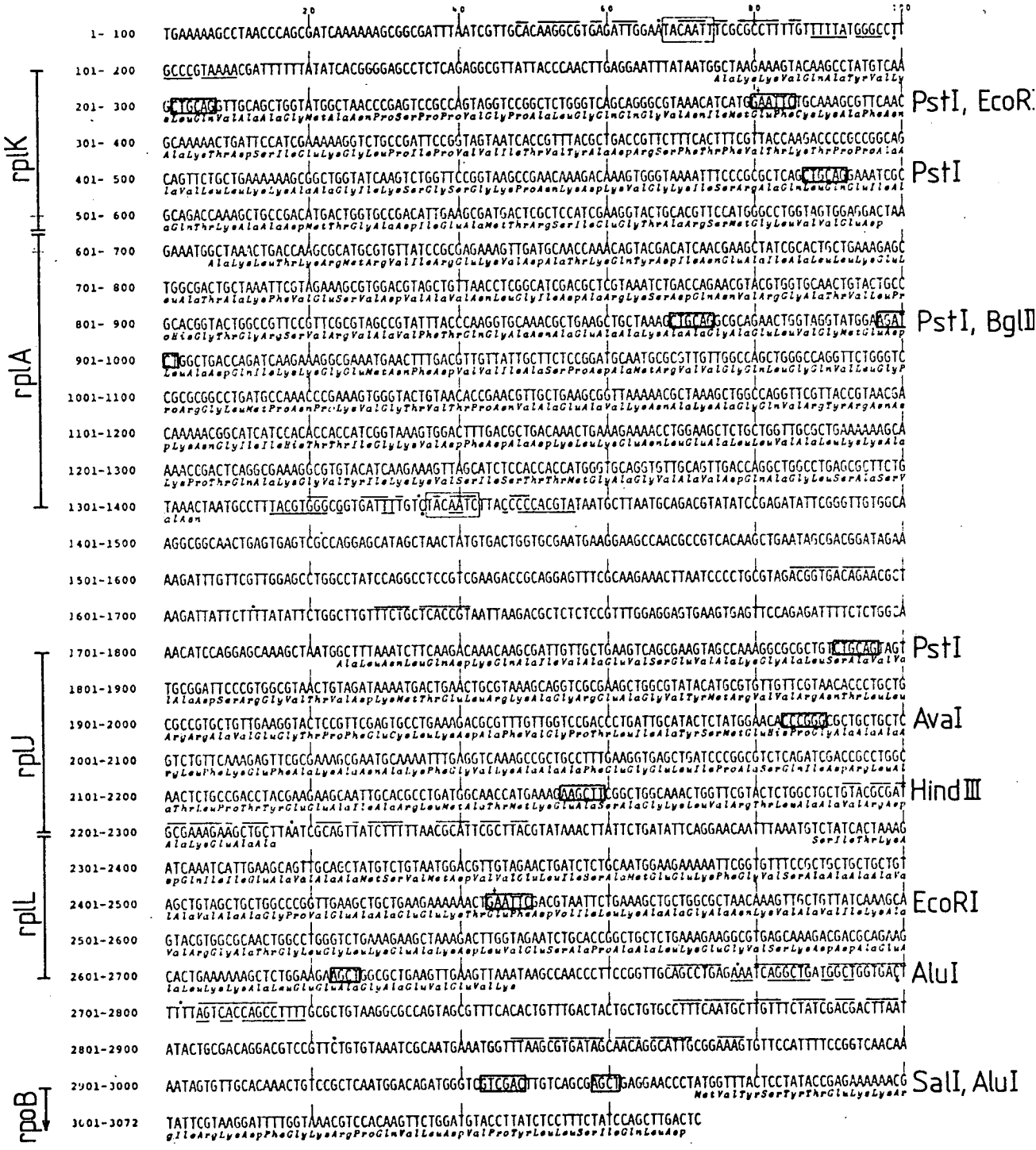

rplK
rplA
rplJ
rplL
rpoB
PstI, EcoRI
PstI
PstI, BglII
PstI
AvaI
HindIII
EcoRI
AluI
SalI, AluI

Fig. 4.13

different segments of the rpo region to lacZ. Their results indicated that initiation of transcription occurred in the rplJ-rplL intercistronic space, and possibly also in the rplL-rpoB and rpoB-rpoC intercistronic regions. Hence it is possible that there is a major or minor promoter upstream of each structural gene in this operon.

This set of fusions also enabled Barry et al to locate a strong (>99% efficient) terminator lying within the 11 kb rplL rpoBC HindIII fragment a short distance beyond rpoC.

The evidence presented so far on the identification and location of promoters and terminators in the rplKAJL rpoBC region is summarised in Fig. 4.14 and can now be assessed with reference to the nucleotide sequence of this ribosomal protein gene cluster (Post et al, 1979; Figs. 4.13 and 4.15).

rplKA are co-transcribed from a promoter located between rplK and the 20k gene (Yamamoto and Nomura, 1979). Experiments using small DNA fragments as templates indicate that a transcript is initiated in vitro near nucleotide 79 in the sequence, a few bp downstream of a sequence TACAATT, which corresponds to the Pribnow consensus sequence at 5 positions (Taylor and Burgess, 1979). Moreover about 35 bp upstream from nucleotide 79 is a sequence TTGCACA, similar to the -35 consensus sequence. This region probably represents the rplKA promoter P1 (Figs. 4.14 and 4.15). It also contains a hyphenated inverted repeat (nucleotides 48-86) followed at nucleotides 87-91 by TTTTT, which together resemble several known terminators. Perhaps this represents a termination signal for the 20k message. If these signals function in vivo the rplKA and 20k transcripts would overlap in a manner reminiscent of the transcripts at the geneH-geneA boundary in ØX174 (Sanger et al, 1977).

Fig. 4.14 Promoters and terminators in the rplKAJL rpoBC gene cluster. rpot1 functions at about 75-80% efficiency during exponential growth (Dennis, 1977b), and rpot2 is highly efficient (>99%: Barry et al, 1979). The locations of P3 and P4 are not known with any accuracy, although P4 almost certainly lies somewhere in the rplL-rpoB intercistronic space (see Section 4.3).

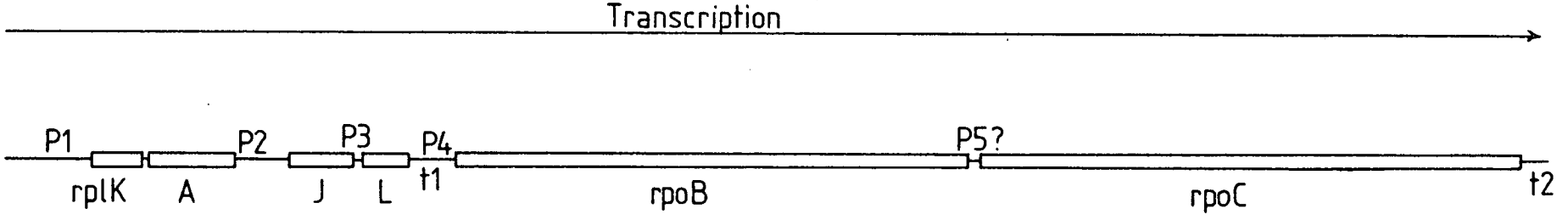


Fig. 4.14

Consistent with the co-transcription of rplK and rplA there are only 3 bp between these 2 genes, and the Shine-Dalgarno sequence for rplA lies within the rplK structural gene. In contrast to this, though, the other intercistronic distances are 413, 67 and 321 bp between rplA and rplJ, rplJ and rplL and between rplL and rpoB, respectively.

RNA polymerase binds strongly in vitro to a HaeIII fragment (1287-1523) from the rplA-rplJ intercistronic region (Taylor and Burgess, 1979) and initiates a transcript at 1348±1 (Post et al, 1979; Taylor and Burgess, 1979). Moreover strains harbouring a multicopy plasmid carrying an EcoRI-SmaI fragment (281-1986) from this region contain an RNA species whose 5' end corresponds to the region around nucleotide 1350. Upstream of this position are the sequences TACAATC (1336-1342) and TTTACG (1313-1318) similar to the Pribnow and -35 consensus sequences. This site in all likelihood represents the rplJL rpoBC major promoter P2.

As observed in the Introduction it is not feasible, in the absence of corroborative evidence, to identify promoters merely by inspecting a nucleotide sequence. Thus although promoter-like elements are present in both rplJ-L and rplL-rpoB intercistronic regions (Fig. 4.15) their significance is unclear. None of the small fragments from this region are retained on nitrocellulose filters in assays for binding or initiation by RNA polymerase in vitro (Taylor and Burgess, 1979). However, this is not surprising in view of the difference of nearly 2 orders of magnitude between the effectiveness of the putative minor rpo promoters and the major P2 promoter, as deduced from the UV-irradiated cell work and gene fusion studies (Fig. 4.12; Barry et al, 1979).

Fig. 4.15. DNA sequence of the ribosomal protein gene cluster from λrif18 DNA (Post et al, 1979). Features bearing on the regulation of transcription are shown, as follows:

a) P1 promoter -35 sequence

b) P1 promoter Pribnow sequence

c) rplKA transcript start-point

d) P2 promoter -35 sequence

e) P2 promoter Pribnow sequence

f) rplJL(rpoBC) transcript start-point

gh) Possible -35 and Pribnow sequences for P3

i) rpot1 terminator stem-loop sequence

jk) Closely-spaced ribosome-binding site and translation start-stop signals near the RNAase III cleavage site in the transcript.

lm) Possible -35 and Pribnow sequences for P4.

Notes: [1]Several regions of dyad symmetry are shown (in addition to the rpot1 potential stem-loop-coding sequence) by over-lining or underlining, with a dot at the centre of symmetry.

[2]The rplJ-rplL and rplL-rpoB regions contain several promoter-like sequences in addition to those shown.

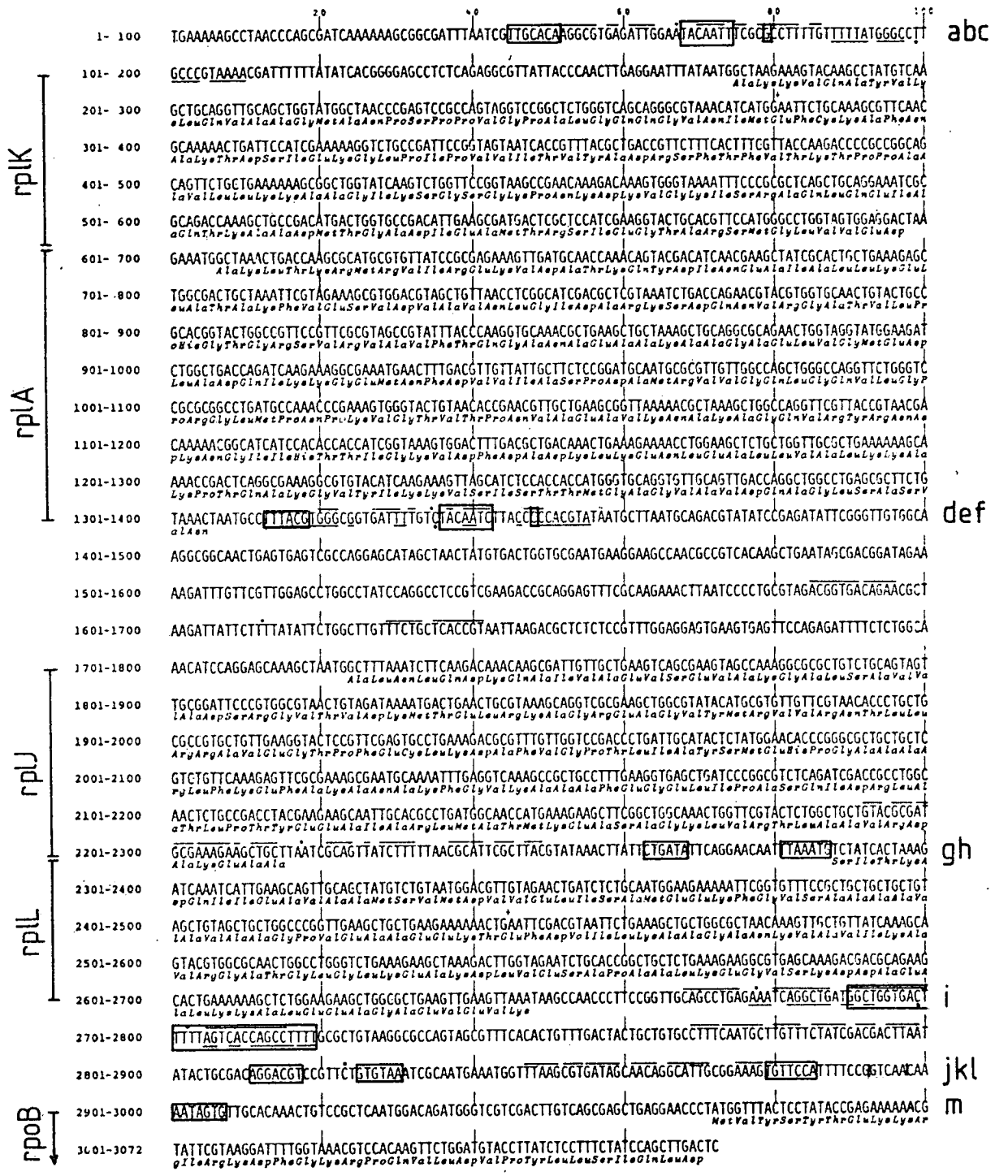


Fig 4.15

The results of hybridisation analysis of mRNA labelled *in vivo* (Dennis, 1977b) suggested that there should be a partially effective transcription termination site in the *rplJL rpoBC* operon, responsible for the 4 to 5-fold lower frequency of transcription of *rpoBC* relative to *rplKAJL* observed *in vivo*. The 321 nucleotide *rplL-rpoB* intercistronic space, in which such a termination site might be expected to lie, contains at least 3 separate regions of hyphenated dyad symmetry, so that RNA transcribed from this region might possess extensive secondary structure. In particular the second inverted repeat sequence, centred at nucleotide 2702, might form a stem-loop in the RNA with a perfect 11 bp stem, which would be followed by a run of 4 U residues (Fig. 4.15). Sequences such as this have been found in several terminators and attenuators, just upstream of the transcription stop site. The structure and function of this region of the *rplJL rpoBC* operon, called *rpot1*, is discussed in more detail in Chapters 5 and 6.

The constant proportionality between the transcription frequencies of *rpoBC* and *rplKAJL*, observed at various growth rates, does not apply under conditions which differentially affect the activity of, or requirement for, ribosomes and RNA polymerase. For example, a non-specific reduction in the rate of transcription initiation elicits an increase in the transcription frequency of *rpoBC* relative to that of *rplKAJL* (Dennis, 1977a; Kirschbaum, 1978; Blumenthal and Dennis, 1978; Little and Dennis, 1979; Blumenthal and Dennis, 1980). Amino-acid starvation modulates *rplKAJL* transcription in *relA*$^+$ and *relA* strains, but does not affect the *rpoBC* transcription frequency (Maher and Dennis, 1977).

If these induced regulatory changes were absolutely specific for

the polymerase genes (in response to reduced transcription initiation) and for the ribosomal proteins (in response to amino-acid starvation) modulation of transcription termination at rpot1 might alone account for the observed changes. However, the response of rpoBC transcription to constraints on initiation is apparently accompanied by a less marked, but reproducible, increase in rplKAJL transcription, implying that some modulation of promoter activity may also be involved. Errors in the hybridisation data, both experimental and systematic (since the probes used did not discriminate clearly between rpoBC, rplKA and rplJL mRNA), further complicate this issue.

One approach to solving these uncertainties would be to construct fusions linking rpot1, and the rpo promoters, to an easily-assayed downstream gene. The activity of these isolated transcription initiation and termination sites could then be examined under various experimental conditions, perhaps allowing identification of the site(s) involved in regulating transcription of rpoBC.

CHAPTER 5

5.1 Construction of Fusion Strains Carrying the rpo (t1, P4) Sites Inserted Between araI and lacZ

This work has exploited an ara-trp-lac fusion (Casadaban, 1975) carried on pMC81, a ColE1-derived plasmid vector which also carries a gene for ampicillin-resistance (Casadaban and Cohen, 1980). The plasmid contains single sites for HindIII and KpnI, by means of which DNA fragments can be inserted between the araBAD promoter (araI) and the lacZ structural gene (Fig. 5.5).

The disposition of KpnI and HindIII sites in the rplL-rpoB area is such that the P4 and t1 regulatory sites cannot be inserted directly between araI and lacZ using these two restriction enzymes. The strategy adopted was instead to fragment the relevant DNA with AluI, which yields many fragments, including one of 335 bp containing almost all the region of interest between rplL and rpoB (Post et al, 1979). This AluI fragment was then inserted at the HindIII site in pMC81 by means of synthetic molecular recombination linkers (Bahl et al, 1977), via an intermediate phage vector.

The plasmid pNA26 (see Chapter 4.2) includes a 1.2 kb EcoRI fragment which straddles the region of interest. Digestion of the purified 1.2 kb fragment with AluI produced sub-fragments of sizes (bp) 335, 235, 120, 105, 90, 78 and 69 (Fig. 5.1); smaller fragments would have escaped detection on this gel. Note that the 335 bp fragment is the largest from this region, and that it contains a site for SalI (Post et al, 1979; Fig. 5.2).

Attempts at construction of the araI-rpo (t1, P4)-lacZ fusions by direct cloning in pMC81 were unsuccessful, and prompted the use of a HindIII "immunity-insertion" phage, NMλ590 (Murray et al, 1977), as an intermediate vector. λ590 has the following relevant

Fig. 5.1 3-10% polyacrylamide gradient gel electrophoresis

Track number	DNA	Restriction endonuclease(s)
1	f 1.2 kb	AluI
2	pBR322	HaeIII
3	pBR322	HaeIII
4	HindIII linker oligomers	-
5	pNA38	AluI, HindIII
6	pNA38	AluI
7	λ590 recombinant	HindIII
8	"	"
9	"	"
10	"	"
11	pBR322	AluI
12	f 1.2 kb	AluI
13	λ590 recombinant	HindIII
14	"	"

Notes

[1] The marker tracks are f 1.2 kb/AluI (A'), pBR322/HaeIII (ae) and pBR322/AluI (A). The fragments in the latter digest differ from those suggested by the published sequence of pBR322 (Sutcliffe, 1978): a ∿720 bp fragment appears instead of predicted fragments of 659 bp and 63 bp (which are adjacent in the pBR322 physical map). This change is consistent with loss of the AluI recognition site between these two fragments by mutation.

[2] f 1.2 kb is the 1.2 kb rplL-rpoB EcoRI fragment (Fig. 5.2).

[3] AluI digestion of f 1.2 kb produced the 90 bp fragment with variable yield.

[4] In track 4 oligomers (up to 13 mers) are visible as discrete bands, and above that merge into a continuum.

[5] In track 5 the AluI fragments of pNA38 are each reduced in mobility by the attachment of HindIII termini. The fragments shown range in size from 49 to 910 bp.

[6] Tracks 7-10 and 13-14 display HindIII digests of plate lysate DNA from λ590 recombinants, each with a single small HindIII fragment inserted in cI.

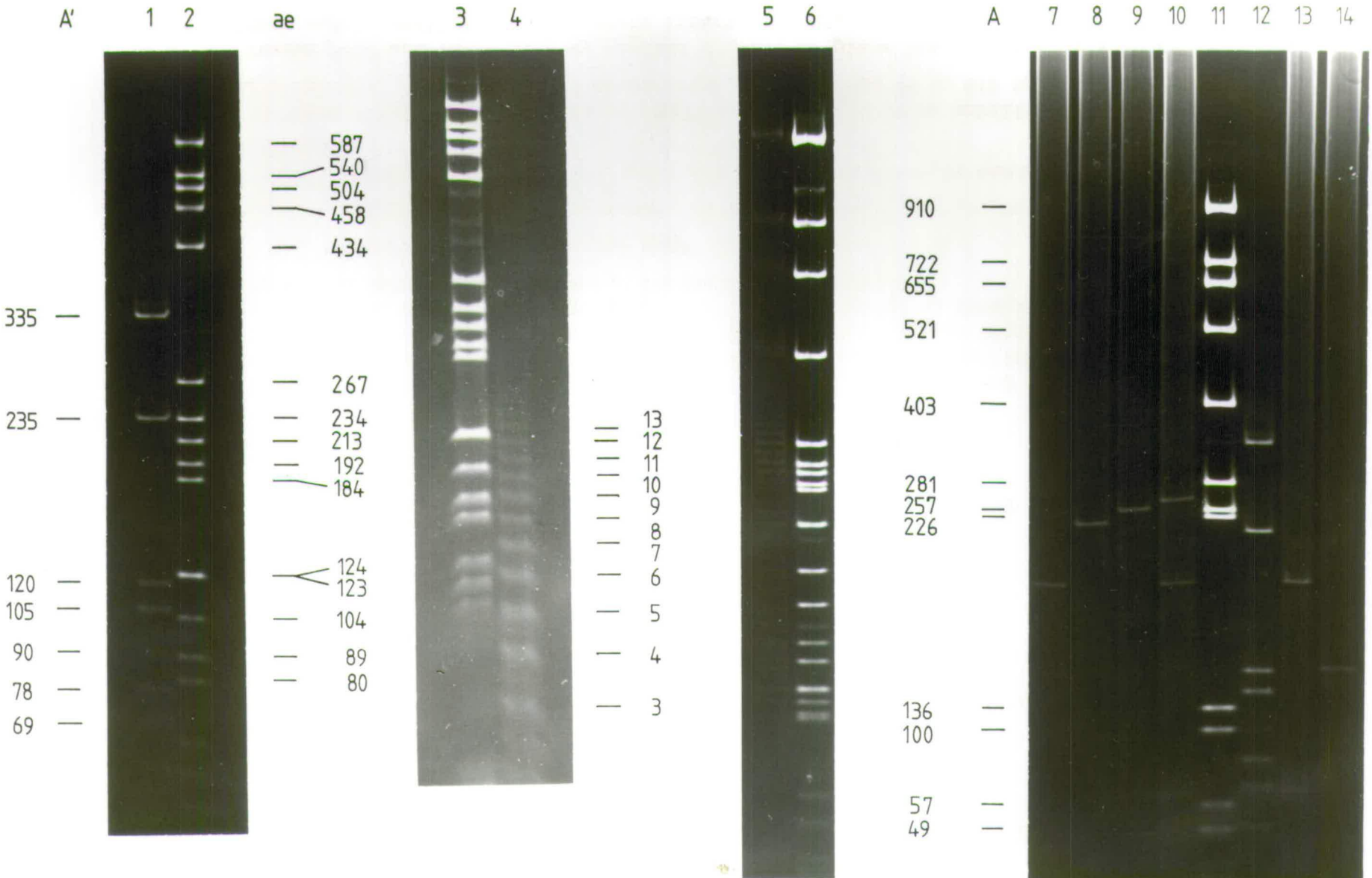

A'
1
2
ae
3
4
5
6
A
7
8
9
10
11
12
13
14
335
235
120
105
90
78
69
587
540
504
458
434
267
234
213
192
184
124
123
104
89
80
13
12
11
10
9
8
7
6
5
4
3
910
722
655
521
403
281
257
226
136
100
57
49

Fig.5.1

Fig. 5.2 A. Restriction enzyme sites in the rplKAJL gene cluster, relevant to the construction and physical analysis of the araI-rpo-lacZ fusions described in this chapter (data from Post et al, 1979).

B. Sites for AluI and SalI in the 1.2 kb rplL-rpoB EcoRI fragment. The Fragments originating from the downstream (rpoB) end of the fragment were not ordered. rpot1 is present on the 335 bp AluI fragment which includes most of the rplL-rpoB intercistronic DNA.

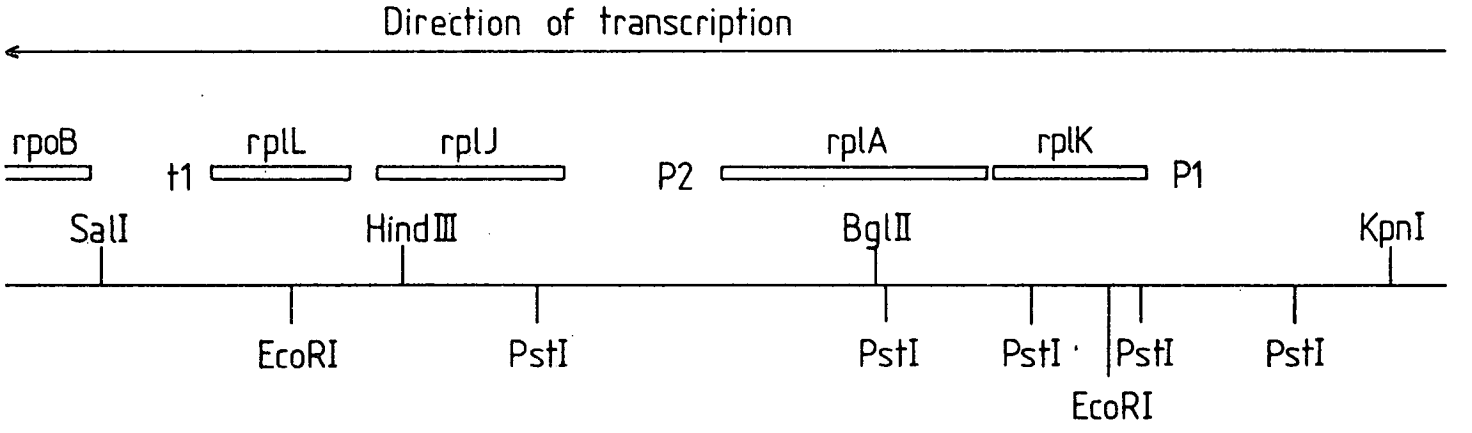


Fig.5.2A

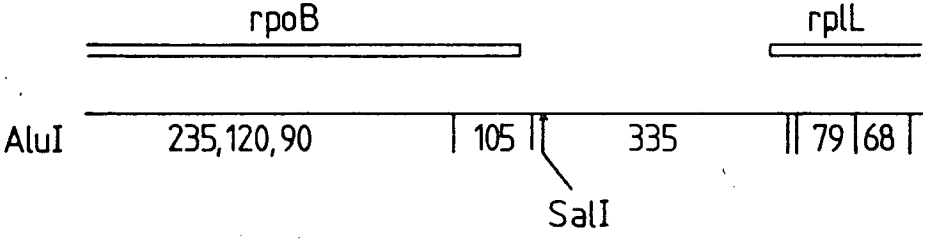


Fig.5.2B

advantages over pMC81 itself: (i) high transfection efficiency; (ii) easy identification of recombinants by their clear plaque morphology; (iii) easy screening for recombinants of interest by plaque hybridisation (more specific than colony hybridisation). Thus the approach finally employed was to digest pNA26 with AluI (>50 fragments), add HindIII molecular recombination linkers, cut with HindIII to generate HindIII cohesive termini, and ligate with HindIII-digested λ590 DNA. Phage carrying HindIII fragments inserted in cI were identified as clears after transfection, and further screened by plaque-hybridisation with $[^{32}P]$-1.2 kb fragment as probe. Finally, plate-lysate DNA was prepared from individual phages and analysed by HindIII digestion and polyacrylamide gel electrophoresis. In this way were identified phage carrying most of the individual AluI fragments from the region of interest, each slightly reduced in mobility by the added HindIII termini. Of particular interest was a recombinant, λ81, carrying a 345 bp HindIII fragment (Fig. 5.1 shows gels illustrating the stages in this process).

That this 345 bp HindIII fragment includes the 335 bp rplL-rpoB AluI fragment, carrying t1 and P4, was confirmed (Fig. 5.3) by SalI digestion of λ81 and the vector λ590: the 335 bp AluI fragment includes the sole SalI site present in pNA26 (Post et al, 1979; data not shown). λ590 has two SalI sites and yields fragments of ∿25 kb, ∿16 kb and 0.5 kb. In the λ81 digest, however, the 16 kb fragment is replaced by fragments of 3.4 kb and ∿13 kb, confirming the identity of the inserted HindIII fragment.

Transfer of the 345 bp HindIII fragment into the pMC81 HindIII site was facilitated by the development of a screening procedure based

on insertional inactivation of the trpB gene in which the HindIII site lies. trpB is present intact and in the correct orientation for expression from araI in the ara-trp-lac fusion (Casadaban, 1975). Strain ED8641 is recA56 hsdR hsdM$^+$ ∇(trpB-E)9 trpR; it is auxotrophic for tryptophan and cannot grow on indole, an intermediate in the tryptophan biosynthesis pathway. ED8641 harbouring pMC81, however, can grow on indole if arabinose is added to activate transcription of trpB from araI, allowing the formation of tryptophan synthetase, product of the trpA and trpB genes.

Mixed HindIII-digested λ81 and pMC81 DNA was ligated and used to transform ED8641 to ampicillin-resistance. Individual transformants were scored for trp phenotype by transfer to indole media ± tryptophan, and small-scale plasmid DNA preparations were made from the few trp auxotrophs obtained. Fig. 5.3 shows EcoRI and EcoRI + SalI digests of pMC81 and of plasmids prepared from two such trp auxotrophs. There is no site for SalI in pMC81 and hence a SalI + EcoRI double digestion produces the same EcoRI fragments of 7.9 kb and 17.7 kb given by EcoRI alone (Casadaban and Cohen, 1980). The EcoRI digestion patterns of plasmids pHR4 and pHR5 (isolated from trp auxotrophic clones) are indistinguishable from that of pMC81. The EcoRI + SalI double digests, however, disclose the presence of a SalI site in pHR4 and pHR5: the 17.7 kb EcoRI fragment is replaced by fragments of ∿13.8 kb and 4.2 kb (pHR4) or ∿13.5 kb and 4.5 kb (pHR5). Since the SalI site lies very near the downstream end of the 335 bp AluI fragment (Post et al, 1979) these data establish both the identity and orientation of the inserted fragment. In pHR4 the transplanted fragment is in the normal orientation relative to the araI promoter, whilst pHR5 has the reverse arrangement.

Fig. 5.3 1.4% agarose gel electrophoresis

Track number	DNA	Restriction endonuclease(s)
1	λrif18	EcoRI
2	λ590	SalI
3	λ81	"
4	pMC81	EcoRI
5	"	EcoRI + SalI
6	pHR4	EcoRI
7	"	EcoRI + SalI
8	pHR5	EcoRI
9	"	EcoRI + SalI

Notes

[1] The marker track is λrif18/EcoRI (E)

[2] The plasmid DNA displayed on this gel was from small-scale preparations; hence the background of E.coli fragments visible.

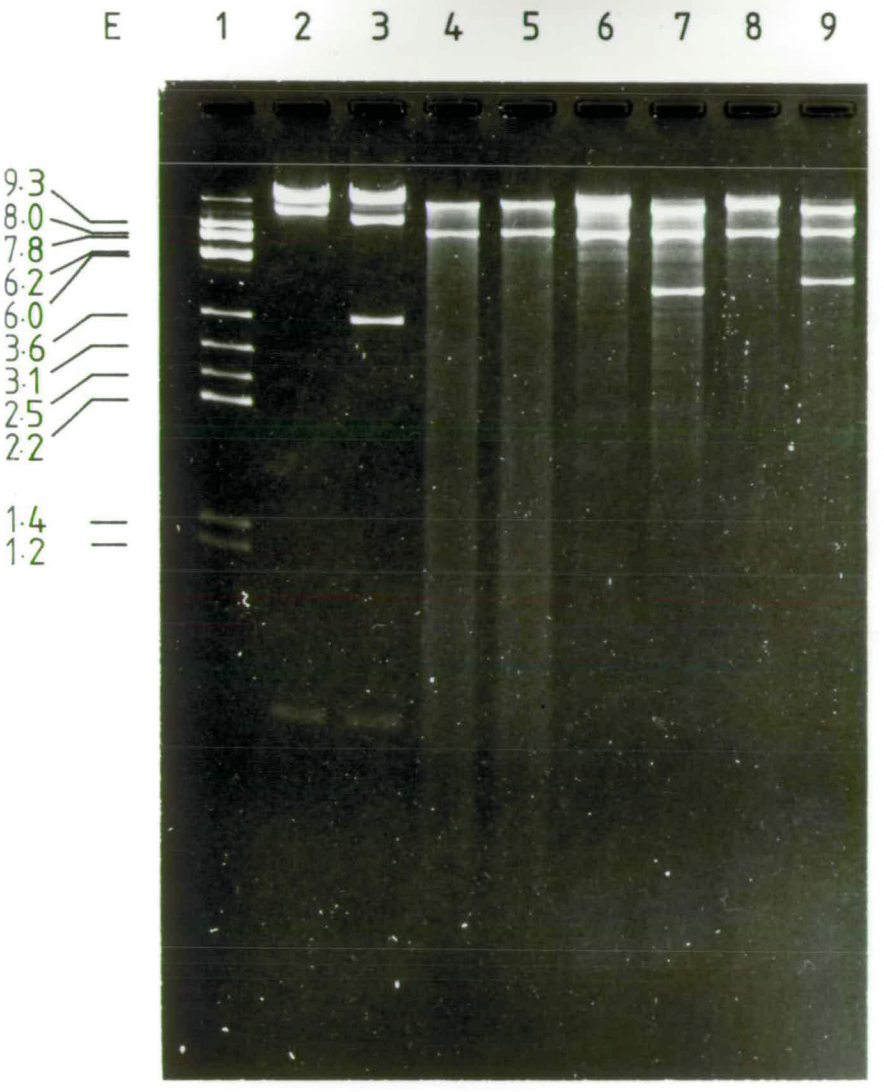


Fig. 5.3

Fig. 5.4 Flow diagram summarising the steps involved in the construction of the pHR4 and pHR5 fusions, in which rpot1 is inserted between araI and lacZ. Further details may be found in sections 2.9 and 5.1; see also Fig. 5.1. The Sephadex G-75 chromatography step is included in order to separate the desired AluI fragments with attached HindIII termini from the large molar excess of linker fragments. Plaque hybridisation used (^{32}P)-1.2 kb rplL-rpoB EcoRI fragment as probe.

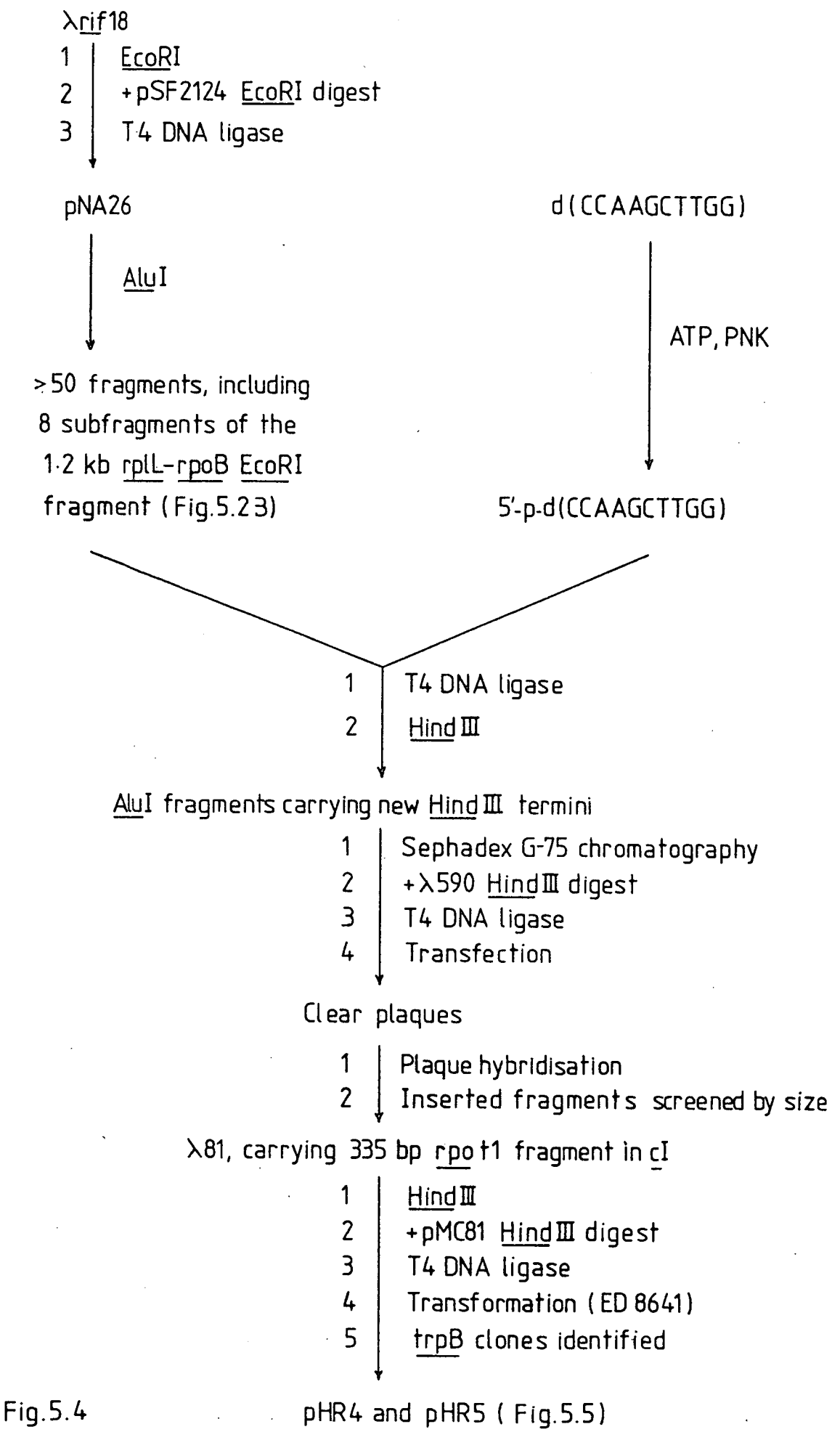

λrif18
1 EcoRI
2 +pSF2124 EcoRI digest
3 T4 DNA ligase
pNA26
AluI
>50 fragments, including
8 subfragments of the
1.2 kb rplL-rpoB EcoRI
fragment (Fig.5.23)
d(CCAAGCTTGG)
ATP, PNK
5'-p-d(CCAAGCTTGG)
1 T4 DNA ligase
2 HindIII
AluI fragments carrying new HindIII termini
1 Sephadex G-75 chromatography
2 +λ590 HindIII digest
3 T4 DNA ligase
4 Transfection
Clear plaques
1 Plaque hybridisation
2 Inserted fragments screened by size
λ81, carrying 335 bp rpo t1 fragment in cI
1 HindIII
2 +pMC81 HindIII digest
3 T4 DNA ligase
4 Transformation (ED 8641)
5 trpB clones identified
pHR4 and pHR5 (Fig.5.5)

Fig.5.4

Finally, pHR4 and pHR5 were transferred (by transformation) into strain MC1000 for the experiments described in 5.3. The construction of pHR4 and pHR5 is summarised in Fig. 5.4, and genetic and physical maps are shown in Fig. 5.5.

5.2 Construction of Fusions Linking rpo P2 to lacZ

A derivative of pMC81 in which rpo P2 is fused to lacZ was constructed by replacing the 1.6 kb BamHI-HindIII fragment of the vector with a 1.2 kb BglII-HindIII fragment of λrif18 which includes a 3' portion of rplA, the P2 promoter, and a 5' portion of rplJ (Post et al, 1979; Casadaban and Cohen, 1980; Figs. 5.2 and 5.5). The products of a ligation of a BglII + HindIII double digest of λrif18 DNA with a HindIII (limit) + BamHI (partial) digest of pMC81 (the vector has 3 BamHI sites) were used to transform MC1000 to ampicillin-resistance. The desired recombinant pHR6 was identified by virtue of its vigorous expression of lacZ in the absence of arabinose (giving a red colony on lactose MacConkey agar; contrast the barely pink colonies harbouring the vector pMC81, which expresses lacZ only very weakly under these conditions).

Another fusion designated pHR1 was produced by replacing the 0.8 kb KpnI-HindIII fragment of pMC81 with a 2.6 kb KpnI-HindIII fragment of λrif18 which includes both the P1 and P2 promoters, the rplK and rplA structural genes, and the same 5' terminal portion of rplJ as in pHR6 (Post et al, 1979; Fiil et al, 1979; Casadaban and Cohen, 1980; Figs. 5.2 and 5.5). The products of a ligation of KpnI-HindIII double digests of λrif18 and pMC81 were used to transform MC1000 to ampicillin-resistance, and recombinants of the desired kind were identified as described above for pHR6.

Fig. 5.5 The upper part shows the genetic organisation and physical map of pMC81, linearised at one of its 3 BamHI sites (Casadaban and Cohen, 1980). Transcription from the araOI region proceeds leftwards into araC, and rightwards through araB', Mu', trp'CBA' into lacZ. The restriction sites marked are given co-ordinates (in kb) starting from the BamHI site lying between rep colE1 and araC.

The lower part shows, at an expanded scale, the first 10 kb of the 3 fusions pHR1, pHR6 and pHR4. Fused rpo sequences are represented as unfilled boxes below each line, with vector genes of interest above the line. Relevant restriction sites within the fusion are also shown; but in pHR1 there are 4 PstI sites between the KpnI and BglII sites which have been omitted for the sake of clarity. pHR5 is similar to pHR4, with the inserted rpot1 fragment reversed in orientation. The total lengths of these fusion plasmids are: pHR1, 27.4 kb; pHR6, 25.2 kb; pHR4, 25.9 kb. Exact distances between sites in the rpo portions of these fusions may be found in Fig. 4.15.

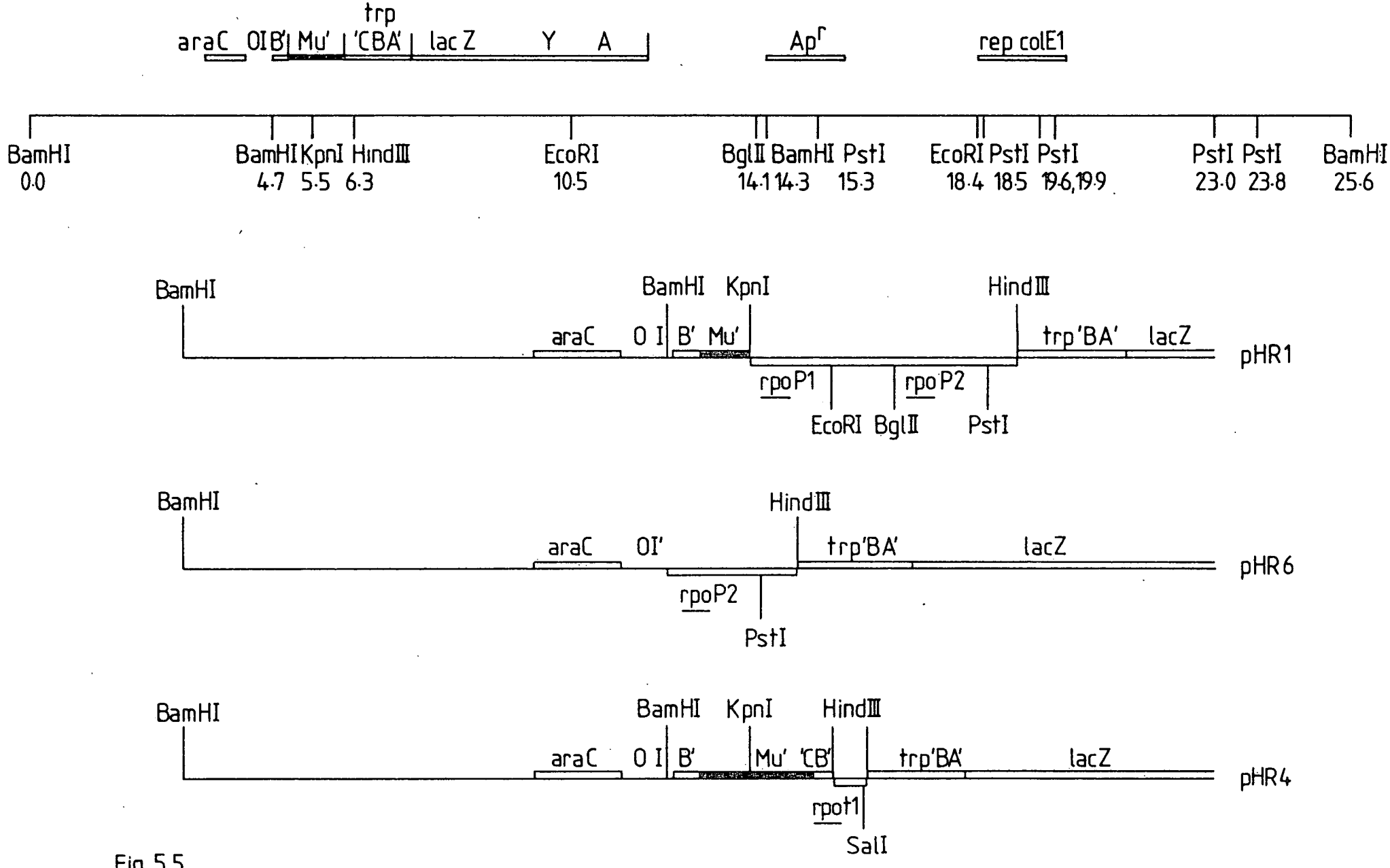

trp
araC OI B' Mu' 'CBA' lacZ Y A
Ap^r
rep colE1
BamHI 0·0
BamHI 4·7
KpnI 5·5
HindIII 6·3
EcoRI 10·5
BglII 14·1
BamHI 14·3
PstI 15·3
EcoRI 18·4
PstI 18·5
PstI 19·6,19·9
PstI 23·0
PstI 23·8
BamHI 25·6
BamHI
BamHI KpnI
HindIII
araC O I B' Mu'
trp'BA' lacZ
pHR1
rpoP1
rpoP2
EcoRI BglII PstI
BamHI
HindIII
araC OI'
trp'BA' lacZ
pHR6
rpoP2
PstI
BamHI
BamHI KpnI HindIII
araC O I B' Mu' 'CB'
trp'BA' lacZ
pHR4
rpot1
SalI

Fig.5.5

Fig. 5.6 1.2% agarose gel electrophoresis (tracks 1-18).
3-10% polyacrylamide gradient gel electrophoresis (tracks 19-22)

Track number	DNA	Restriction endonuclease	Track number	DNA	Restriction endonuclease
1	λrif18	KpnI + HindIII	12	pMC81	BamHI
2	"	HindIII	13	unknown	"
3	"	EcoRI	14	pHR6	"
4	pHR1	KpnI + HindIII	15	pHR1	PstI
5	"	"	16	pMC81	"
6	"	"	17	unknown	"
7	pHR1	EcoRI	18	pHR6	"
8	pMC81	"	19	pBR322	AluI
9	unknown	"	20	pNA38	"
10	pHR6	"	21	pHR1	PstI
11	pHR1	BamHI	22	pMC81	"

Notes

1 The marker tracks are λrif18/EcoRI, pMC81 cut with EcoRI, BamHI and PstI (Fig. 5.5), and pBR322/AluI (A).

2 The DNA in tracks 4-6 is from 3 independently-isolated transformants from a ligation of mixed HindIII + KpnI-digested λrif18 and pMC81 DNAs; all 3 expressed lacZ in the absence of arabinose.

3 The DNA in tracks 9-10, 13-14 and 17-18 is from 2 transformants from a ligation of mixed HindIII + BglII-digested λrif18 DNA and HindIII + BamHI (partial)-digested pMC81 DNA; both expressed lacZ in the absence of arabinose.

4 All 4 plasmids in tracks 7-18 give a single large fragment after digestion with HindIII (data not shown).

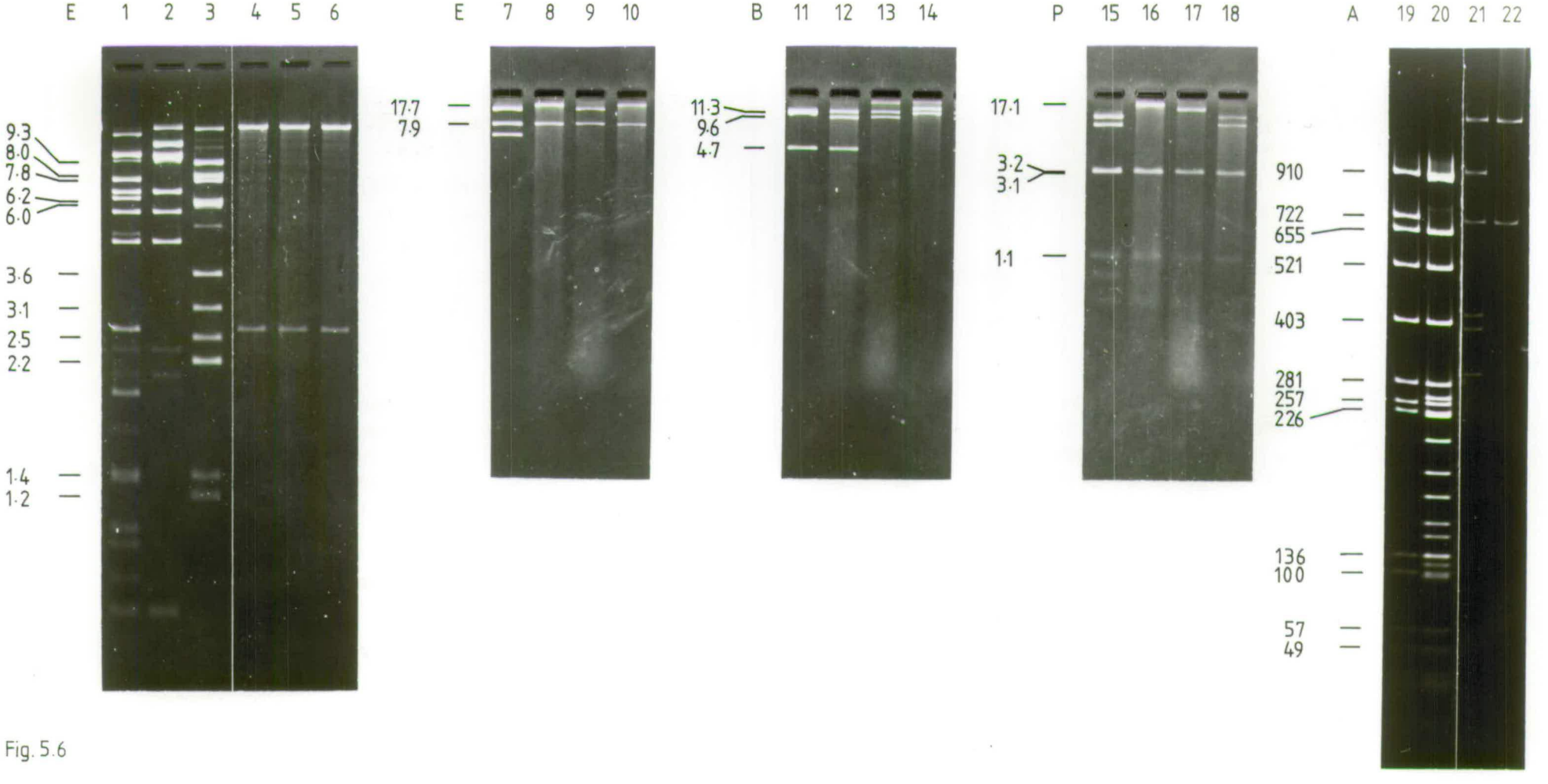

E 1 2 3 4 5 6
9·3
8·0
7·8
6·2
6·0
3·6
3·1
2·5
2·2
1·4
1·2
E 7 8 9 10
17·7
7·9
B 11 12 13 14
11·3
9·6
4·7
P 15 16 17 18
17·1
3·2
3·1
1·1
A 19 20 21 22
910
722
655
521
403
281
257
226
136
100
57
49

Fig. 5.6

The predicted structures of pHR1 and pHR6 were confirmed by the following observations (Fig. 5.6; Casadaban and Cohen, 1980): (i) KpnI + HindIII double digestion of pHR1 yields fragments of 24.8 kb and 2.6 kb, the latter comigrating with a fragment in the analogous digest of λrif18; (ii) EcoRI digestion of pHR1 yields fragments of 5.8 kb and 13.5 kb replacing a 17.7 kb fragment in the pMC81 digest, consistent with the presence of a new EcoRI site in the inserted fragment; (iii) BamHI digestion of pHR6 yields a new fragment of 13.9 kb replacing the fragments of 9.6 kb and 4.7 kb given by pMC81 (consistent with loss of the BamHI site in ara by ligation with the BglII terminus of the inserted fragment); (iv) PstI digestion of pHR6 yields new fragments of 9.3 kb and 7.4 kb instead of a 17.1 kb fragment in the pMC81 digest, consistent with the presence of a new PstI site at the expected position in the inserted fragment; (v) PstI digestion of pHR1 yields new fragments of 9.3 kb, 7.6 kb, 0.9 kb, 0.4 kb, 0.38 kb and 0.28 kb, consistent with the presence of 5 PstI sites in the inserted fragment as predicted by the data of Post et al (1979) and Fiil et al (1979).

5.3 Expression of lacZ by the novel fusions

The steady-state levels of β-galactosidase activity in MC1000 strains harbouring pMC81 and the rpo fusion derivatives were determined during exponential growth (Table 5.1). L-arabinose, when present as a non-metabolised inducer, was at a final concentration of 0.1%. Each result is the mean of 3 or more independent determinations on separate cultures.

The results in Table 5.1 indicate that each fusion displays

Table 5.1

Plasmid	Known promoters and terminators upstream of *lacZ*	β-galactosidase activity[a] -Arabinose	β-galactosidase activity[a] +Arabinose[b]
pMC81[c]	*araI*	105(± 5)	11800 ± 500
pHR1	*araI* *rpo* P1 P2	1260(± 90)	NT
pHR6	*rpo* P2	890(± 40)	NT
pHR4	*araI* *rpo*(t1, P4)	45(± 4)	530 ± 50
pHR5	*araI* *rpo*(t1, P4)[d]	51(± 6)	1210 ± 60

Notes

[a] The figures are units of β-galactosidase (Miller, 1972) with the range of results in brackets.

[b] The addition of arabinose to the growth medium reduced the growth rate of these strains. This may be due to the induction of a high rate of synthesis of gene products fused to the *araI* promoter and/or to the accumulation by active transport of high concentrations of non-metabolised L-arabinose (Casadaban and Cohen, 1980).

[c] In MC1000 harbouring pMC81 the reduction in growth rate caused by L-arabinose was particularly marked.

[d] In pHR5 the fragment carrying *rpo*(t1, P4) is inserted in the reverse orientation.

characteristic levels of lacZ expression in the presence and absence of arabinose. In interpreting these data it should be remembered that the amount of transcription proceeding into the lac genes in each fusion will reflect not only the action of promoters and terminators, but also the influence of polarity which could arise in some fusions as a consequence of translational frame-shifts. Since the HindIII site in trpB occurs within the first 200 bp of the gene (Hopkins et al, 1976), DNA inserted at this site could be extremely polar. Hence caution is needed when making comparisons between the levels of lacZ expression from different fusions. Furthermore, it must be assumed that the various fusions are present in the same number of copies per cell. Accurate assessment of plasmid copy number is difficult, but small-scale preparations of plasmid DNA, when displayed on agarose gels, revealed no major differences in plasmid yield (data not shown).

The pHR1 and pHR6 fusions share complete homology downstream from the BglII site in rplA through rpo P2, rplJ' 'trpB into lacZ (Fig. 5.5). In the absence of araI-mediated transcription, any difference in lacZ expression between these two fusions must be attributed to the influence in pHR1 of the additional rpo sequences upstream of the BglII site. The reproducibly higher level of lacZ expression given by pHR1 (Table 5.1) indicates that transcription initiated at the P1 promoter upstream of rplK contributes significantly to lacZ expression. The implication of this is that there is no strong terminator between rplKA and rpo P2, so that at least some P1-initiated RNA polymerases continue into rplJL rpoBC in vivo.

Insertion of the 335 bp AluI fragment from the rplL-rpoB

intercistronic space, via HindIII linkers, into the trpB HindIII site in pMC81 results in a reduction of L-arabinose-induced lacZ expression 22-fold (for the normal orientation of insertion) or 10-fold (for the reverse orientation). The region between rplL and rpoB is responsible both in its normal genetic environment (Dennis, 1977b) and in several fusions analysed by Barry et al (1979) for a 5 to 6-fold reduction in the transcription of distal genes. Presumably the stronger termination observed in the pHR4 fusion is related to additional polar effects (see also Chapter 5 Discussion).

The data for pHR4 and pHR5 further indicate that significant expression of lacZ only occurs in the presence of arabinose. Hence the majority of polymerases transcribing lacZ must also have traversed the rpo (t1, P4) insert. Any contribution of P4 to lacZ expression in pHR4 is obscured by the basal level of β-galactosidase synthesis in the absence of arabinose and the complicating influence of polarity, and cannot be quantified.

Despite much attention to the effects of constraints, which differentially affect the activity of (or requirement for) RNA polymerase and ribosomes, on transcription of the rplKAJL rpoBC gene cluster the sites involved in the resulting regulatory changes have still not been clearly identified (Dennis, 1977a; Maher and Dennis, 1977; Blumenthal and Dennis, 1978). The fusion of individual rpo regulatory sites to lacZ may allow the effects of these regulatory mechanisms to be assayed by alterations in the level of lac expression under different experimental conditions.

Low levels of rifampicin cause a partial inhibition of the initiation of transcription, but specifically stimulate transcription of rpoBC in vivo (Blumenthal and Dennis, 1978). Does lacZ expression show an analogous response when linked up to any of the rpo regulatory sites?

Fig. 5.7 illustrates the result of an experiment designed to test the influence of t1 on *lacZ* expression, following induction by arabinose of the upstream *araI* promoter in MC1000 harbouring pHR4. In the control strain (MC1000 harbouring pMC81) rifampicin inhibits induction of *lacZ*; in contrast, identical treatment of pHR4 leads to a slight net potentiation of *lacZ* expression, although initiation of transcription at *araI* is presumably inhibited to the same extent as for pMC81.

Analogous experiments with streptolydigin (an inhibitor of RNA chain elongation) revealed that in this case induction of *lacZ* was inhibited to a similar extent with either pMC81 or pHR4 (Fig. 5.7). Note that rifampicin, but not streptolydigin, induces increased ββ' synthesis in *E.coli* (Tittawella and Hayward, 1974). Hence for pHR4 the addition of rifampicin leads to a reduction in transcriptional termination, presumably at *rpo* t1. Furthermore this effect is observable even when t1 is transplanted out of its normal genetic environment.

An alternative explanation for the effect of rifampicin on *lacZ* expression in MC1000 harbouring pHR4 would involve increased transcription from the P4 promoter thought to be present on the 335 bp *AluI* fragment. The addition of low levels of rifampicin *without* arabinose, however, did not elicit any induction of *lacZ* expression in MC1000 harbouring either pMC81 or pHR4 (Fig. 5.7).

Similarly, addition of low levels of rifampicin to MC1000 harbouring pHR6, in which *lacZ* is transcribed from P2, revealed no discernible stimulation of β-galactosidase synthesis (data not shown). In this case, however, a modest stimulation of P2-initiated transcription might have been undetectable in view of the background

Fig. 5.7 Profiles of β-galactosidase induction in MC1000 harbouring pMC81 and pHR4. Arabinose (final 0.1%) was added at 0 min, with or without Rif or Stl (see below for symbols), and β-galactosidase activity was assayed at intervals. At the antibiotic concentrations used growth continued unchecked for ≥1 hr, as judged by OD_{600} readings.

Symbols:

+ + Rifampicin (20 μg ml^{-1})

× + Arabinose (0.1%)

o + Arabinose (0.1%) + Rif (20 μg ml^{-1}) or Stl (80 μg ml^{-1})

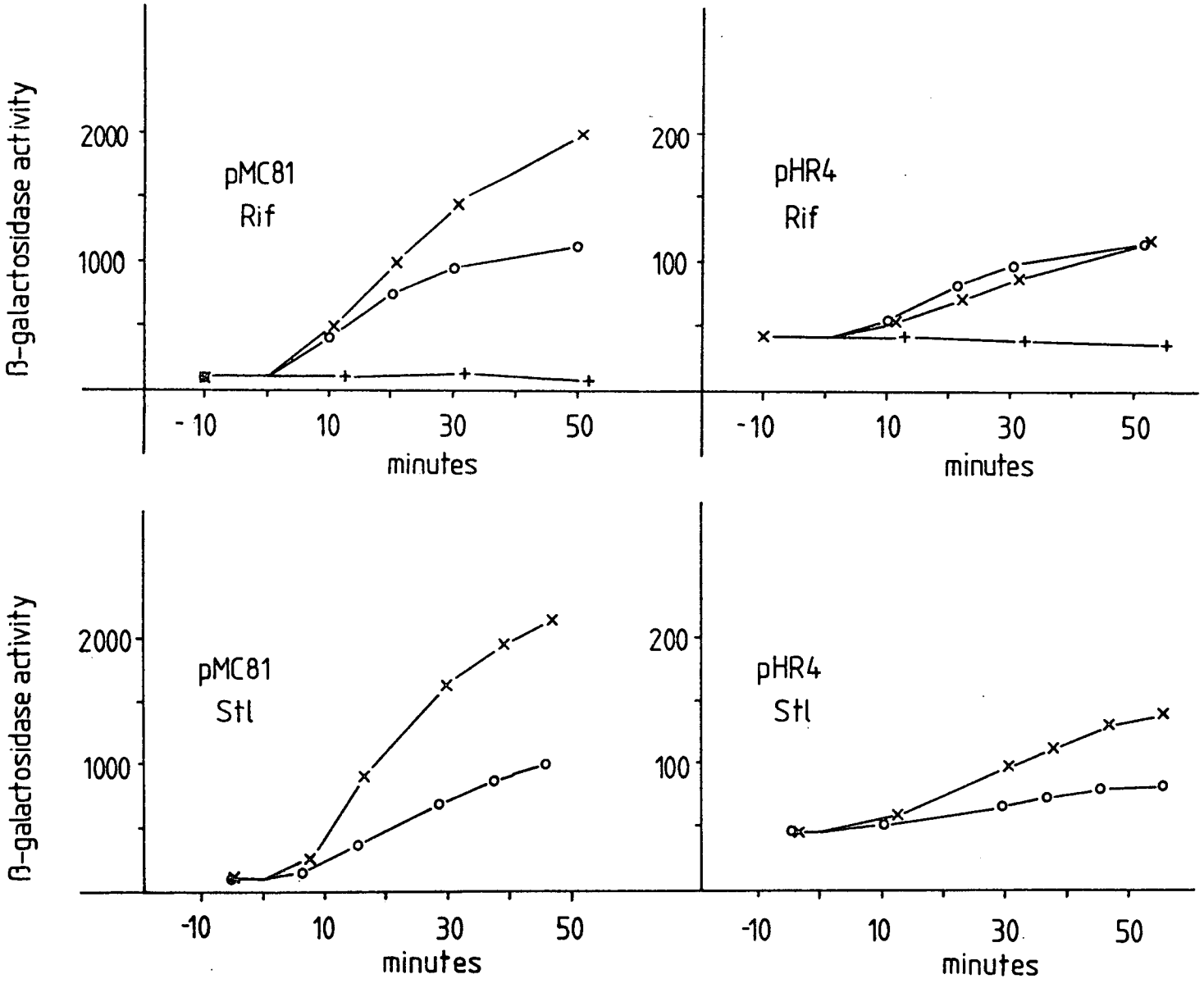

β-galactosidase activity
2000
1000
pMC81
Rif
-10
10
30
50
minutes
200
100
pHR4
Rif
-10
10
30
50
minutes
β-galactosidase activity
2000
1000
pMC81
Stl
-10
10
30
50
minutes
200
100
pHR4
Stl
-10
10
30
50
minutes

Fig.5.7

of pre-existing β-galactosidase activity. Lack of time prevented a more satisfactory investigation of the influence of rifampicin on transcription initiated at P2.

5.4 Discussion

A comparison of the β-galactosidase levels in strains harbouring pHR1 and pHR6 (Table 5.1) indicates that sequences upstream of the BglII site in rplA contribute significantly to lacZ expression in pHR6.* By inference, at least some transcripts initiated in this region (presumably at rpoP1) will extend in vivo beyond rpoP2 and into the rplJL rpoBC genes.

Consistent with this idea, the nucleotide sequence of the rplA-rplJ region does not include any obvious terminator-like sequences. Moreover, P2 is situated rather close to rplA, leaving little room for a termination site for P1-initiated transcripts (Fig. 4.15). Recall that although the P2 promoter appears to be both necessary and sufficient in vivo for rplJL rpoBC operon expression, this has been demonstrated only under conditions in which the upstream promoter rpoP1 was absent (Yamamoto and Nomura, 1978; Linn and Scaife, 1978; Newman and Hayward, 1980).

Barry et al (1979) constructed several derivatives of pMC81 in which various parts of the 11 kb rplL rpoBC HindIII fragment were

*Footnote. Some lacZ expression occurs in the strain harbouring pMC81 even in the absence of arabinose, presumably as a result of incomplete repression of araI (Table 5.1). The araI promoter is present in pHR1, but absent in pHR6 (BamHI cuts about 45 bp upstream of the araBAD mRNA start-site: Greenfield et al, 1978; Fig. 5.5). In pHR1, however, the inserted KpnI-HindIII fragment extends ≥400 bp upstream of rpoP1 and almost certainly includes the termination signal for the 20k transcript (Post et al, 1979; Yamamoto and Nomura, 1979). Transcription from araI should not therefore contribute to lacZ expression in either pHR1 or pHR6.

interposed between araI and lacZ. By comparing lacZ expression from these fusions they were able to deduce the presence of a partially effective transcription termination site on the 1.2 kb EcoRI DNA fragment which spans the rplL-rpoB intercistronic gap. This element reduced the level of distal gene expression 5 to 6-fold, suggesting that it could be the site responsible in vivo for the observed 4 to 5-fold difference between rplKAJL and rpoBC transcription frequencies (Dennis, 1977b).

When the 335 bp AluI fragment from the rplL-rpoB intercistronic space (Fig. 4.13) is interposed between araI and lacZ, β-galactosidase expression is reduced ∿22-fold (Table 5.1). Presumably this marked reduction results from a combination of specific transcription termination in the inserted fragment and polar effects beyond the site of insertion in trpB. Polarity can certainly be an important influence on lacZ expression in these fusions: insertion of a 567 bp 16S rDNA fragment (containing nonsense codons in every reading frame) at the trpB HindIII site in pMC81 results in a 10-fold decrease in arabinose-induced lacZ expression (Barry et al, 1979).

Constraints on the general level of transcription initiation in the cell can be induced by the addition of rifampicin, or by shifting mutants with temperature-sensitive defects in transcription initiation (certain rpoC or rpoD mutations) to a semi-restrictive temperature. The marked stimulation of rpoBC transcription which follows such treatments is thought to reflect the action of a regulatory system which normally maintains transcription of rpoBC at a sub-maximal rate. The restraining influence of this system appears to be relaxed under conditions which reduce the amount of initiation-competent RNA polymerase in the cell. A priori this

might be achieved by an increase in the activity of one or more of the rpo promoters, or by reducing the frequency of termination at rpot1.

The results of an experiment displayed in Fig. 5.7 are consistent with the latter mechanism. In the strain harbouring pHR4 lacZ expression is maintained (or slightly increased) at a rifampicin concentration which significantly reduces the level of transcription initiation at araI (viz. the effect of rifampicin on lacZ expression in the control strain harbouring pMC81). This effect is dependent on arabinose-induced transcription and therefore is not the result of increased transcription from a promoter within the 335 bp AluI fragment in pHR4. Rather, it must result from a reduction in the efficiency of a termination site on this fragment in the presence of rifampicin. Furthermore, a general inhibition of transcription at the level of elongation (caused by addition of Streptolydigin) reduces lacZ expression to equal extents in strains harbouring either pMC81 or pHR4 (Fig. 5.7). Here again the expression of lacZ by pHR4 is responding to a constraint in essentially the same way as the expression of ββ' by the chromosome (Nakamura and Yura, 1976).

The specific response of lacZ expression in the strain harbouring pHR4 following addition of rifampicin encourages the belief that the 335 bp AluI fragment carries the rpot1 site, and that regulation of rpoBC transcription can be achieved, at least in part, by modulation of transcription termination.

The question of a possible enhancing effect of rifampicin (and/or streptolydigin) on the rate of transcription initiation at the rpo promoters, which is not resolved in this work, might best be approached by pulse-labelling RNA in the strains harbouring the

fusion plasmids, and quantitative hybridisation of labelled lacZ mRNA to a suitable lac DNA probe. The fusion plasmids could also be used to determine whether relief of termination at rpot1 is implicated in the increased transcription of rpoBC observed in ts initiation-defective strains at semi-restrictive temperatures.

The ara-rpo-lac fusions described in this chapter may be useful in the isolation of regulatory mutations in the rpo control sites, since they should allow the application of powerful selective techniques borrowed from classical lac genetics, in combination with localised mutagenesis in vitro. A pre-requisite for such experiments would be the transfer of the fusions to a phage vector, since the presence of multiple copies of the fusion would probably defeat the selective techniques. The identity of hypothetical control proteins functioning at rpo regulatory sites might then be established by demanding secondary mutations capable of suppressing the primary alteration in trans.

Finally, investigation of the structure and function of rpot1 in vitro should be facilitated by a ready source of the 345 bp HindIII fragment, which includes rpot1, from the λ590 derivative, λ81.

CHAPTER 6 - Final Discussion

6.1 Post-transcriptional regulation of the synthesis of ribosomal proteins and RNA polymerase subunits

Much recent experimental evidence favours the idea that the synthesis of ribosomal proteins and polymerase subunits is regulated post-transcriptionally. For example, in a strain lysogenic for λspc2, which carries the spc and rpoA operons (Fig. 1.2), the synthesis rate of spc and rpoA operon mRNAs is doubled, but there is no gene dosage effect on the synthesis of the corresponding ribosomal proteins. Furthermore, the spc and rpoA operon mRNAs have shorter than normal half-lives in this lysogenic strain, to such an extent that their steady-state levels are only slightly higher than in the control strain (Fallon et al, 1979a). Similarly in a strain harbouring a multicopy plasmid carrying the spc promoter and the 7 promoter-proximal spc operon genes the mRNA for these genes is made at 10 to 20-fold the normal rate, but only the 2 promoter-proximal proteins, L14 and L24, are significantly over-produced. Again, the overproduced mRNA is abnormally unstable (Fallon et al, 1979b). Furthermore, the introduction of a recA mutation into the plasmid-carrying strain produces a cold-sensitive phenotype, associated with an inability to degrade over-produced L14 and L24 at 30°C. It therefore seems that accumulation of excess free ribosomal proteins is detrimental to growth. The very low levels of free ribosomal proteins in the normal cell (Gausing, 1974; Lindahl, 1975) are apparently maintained firstly by post-transcriptional feedback regulation of ribosomal protein synthesis, and, if this level of control proves inadequate, by degradation of free ribosomal proteins. One can speculate that

unassembled ribosomal proteins might bind to mRNA or DNA and consequently interfere with gene expression.

The rplJL rpoBC operon gene products are also subject to post-transcriptional regulation. When this operon is present on a multicopy plasmid, the synthesis rate of mRNA for rplJL rpoBC is increased by 10-fold roughly in proportion to gene dosage; the steady state level of rplJL rpoBC operon mRNA is also increased, albeit to a lesser extent. Notwithstanding this, the synthesis rate of L10 and L7/12 is increased by no more than 30%, and that of β and β' by no more than 2-fold, in the plasmid-carrying strain (Dennis and Fiil, 1979).

Keys to understanding the mechanism of these regulatory processes have come from studies in vitro, and from the isolation of mutants with altered post-transcriptional regulation. Fiil et al (1980) observed that the presence of a multicopy plasmid carrying rpoP2 and rplJ, but no other complete genes, is severely detrimental to growth of the host bacterium, and causes a marked oversynthesis of both rplJ mRNA and L10. Six independent point mutations which overcame this detriment were found to lie in the 375 nucleotide-long leader sequence between rpoP2 and the rplJ translation start-site, and 4 of these lay in a region of hyphenated dyad symmetry in the centre of the leader sequence. The physiological effect of these alterations was to reduce L10 synthesis about 20-fold, without altering the rate of transcription of rplJ. The implication of this result is that translation of rplJ message is normally regulated by the interaction of some regulatory element with a region in the rplJ leader sequence. The mutants presumably alter the structure adopted by the leader RNA and thereby mimic the

translational inhibition effect normally achieved by binding of the regulatory element.

L7/12 and L10 interact *in vitro* to form a stable 4:1 complex which binds 23S rRNA (although neither protein on its own interacts with 23S rRNA: Dijk *et al*, 1977). The behaviour of the mutants described above is best interpreted on the basis of a model in which the synthesis of L10 is normally regulated by binding of L10:$(L7/12)_4$ complex to the *rplJ* leader region. Studies *in vitro* are consistent with this idea: addition of L10:$(L7/12)_4$ complex specifically inhibits synthesis of L10 and L7/12 in DNA-dependent protein synthesis directed by λ*rif*18 DNA, without affecting the synthesis of β, β', EFTu or L1 (Ishihama and Fukuda, 1980). Furthermore, synthesis of L10 and L7/12 is inhibited differentially, and the inhibition is a reversible one, acting at the level of translation rather than by mRNA cleavage or degradation (Brot *et al*, 1980). In view of the fact that L7/12 are made at 4 times the rate of L10 from the same polycistronic mRNA, it seems reasonable that if the translation of both is regulated by L10:$(L7/12)_4$ complex, there should be separate regulatory sites on the mRNA for L10 and L7/12 regulation. Consistent with this, the *rplJ* and *rplL* genes are separated by a 67 nucleotide space. The RNA transcribed from this region might include the *rplL* regulation site.

The synthesis of *both* L1 and L11 is specifically inhibited *in vitro* by the addition of L1 (but not by L11), whereas the synthesis of other proteins encoded by λ*rif*18 is unaffected (Yates *et al*, 1980; Brot *et al*, 1980). Inhibition of L1 synthesis is maximal at an L1 concentration of 0.5 μM, which would correspond *in vivo* to only about 300 molecules of free L1 per cell (c.f. $5\text{-}10.10^4$ ribosomes), in accord with the observed small size of the free ribosomal protein

pool in E.coli. The observation that L1, but not L11, controls the synthesis of both proteins is consistent with a mechanism in which ribosome initiation at the start of rplK is regulated by the concentration of free L1, and that ribosomes which translate rplK then continue to translate rplA without further regulatory restraint. If ribosomes do not independently initiate at the beginning of rplA, equimolar synthesis of L1 and L11 will be assured. Fittingly, there is an intergenic space of only 3 nucleotides between rplK and rplA (Post et al, 1979).

Similar behaviour is also seen with S4, which selectively inhibits the synthesis of 3 proteins of the rpoA operon, S13, S11 and S4 itself, when this is directed by λspc1 DNA in vitro. Analogous effects are demonstrable in vivo: Lindahl and Zengel (1979) fused an internal fragment of the S10 operon including the genes for L2, L4 and L23 to the lac promoter on a hybrid plasmid. Activation of lac-promoted transcription of the fused ribosomal protein genes leads to their oversynthesis, rapidly followed by an almost complete halt in the synthesis of S3, S19, L3, L16, L22 and L29, whilst synthesis of other ribosomal proteins is unaltered. All the affected genes lie in the same chromosomal operon as L2, L4 and L23. In contrast to this behaviour, a similar induction of the oversynthesis of the spc operon-encoded proteins L15 and L30 did not result in repression of the synthesis of any ribosomal protein. In view of the evidence that only L1 regulates translation of the two rplKA operon proteins, it may be general for only certain gene products of each particular operon to be involved in its translational regulation.

Nomura et al (1980) suggest a basis for feedback repression of

ribosomal protein synthesis at a molecular level. The ribosomal proteins known to be involved in inhibition of translation, such as S4 and S7, all bind strongly to rRNAs in ribosome assembly reactions *in vitro*. The nucleotide sequences of their binding sites on rRNA are known, and when compared with the sequences of those regions of the mRNA to which they bind in their autoregulatory capacity, it is evident that both regions could adopt secondary structures of striking similarity.

Translation of mRNAs for ribosomal proteins might therefore be regulated by competition, between specific binding sites on rRNA and on the mRNA, for regulatory ribosomal proteins (of which there may be one or more per transcription unit). The evidence suggests that the mRNA-binding is reversible; presumably it interferes with the attachment or movement of ribosomes. The reduced stability of ribosomal protein mRNAs under conditions of autoregulation by repressor ribosomal proteins would, on this basis, be a secondary effect of reduced ribosome coverage leading to enhanced susceptibility to nuclease degradation.

Post-transcriptional regulation of the synthesis of ββ' is less well characterised, but the evidence for its role in the regulation of RNA polymerase synthesis is nonetheless compelling. Ishihama and Fukuda (1980) find that the synthesis of ββ' is specifically inhibited by RNA polymerase holoenzyme (but not by the individual subunits) in a DNA-dependent protein synthesis system primed with λ*rif*18 DNA, without specific alteration in the pattern of RNA synthesis or in the synthesis of other proteins coded by the phage. Similar effects operate *in vivo*. For example, Dennis (1977b) showed that the synthesis rates of *rpoBC* and *rplKAJL* mRNAs remained

proportional to one another over a range of growth rates, whereas the relative differential synthesis rate of ββ' was lower during rapid growth. By analogy with the mechanism for ribosomal protein feedback regulation discussed above, the reduced stability of rpoBC mRNA at fast growth rates could likewise be a secondary consequence of the inhibition of its translation. Post-transcriptional limitation of ββ' synthesis is also seen in the response to rifampicin treatment (leading to its transience on the translational level: Hayward et al, 1973) and in a strain carrying multiple copies of the rplJL rpoBC operon (see above). Note an important difference between ribosomal protein and ββ' post-transcriptional regulation: the former is mediated by unassembled ribosomal proteins, whereas the latter apparently depends on fully-assembled RNA polymerase.

6.2 Termination of transcription at rpot1

Barry et al (1980) have examined the transcripts produced in vivo from the rplJL rpoBC operon by hybridising total cellular RNA to purified restriction fragments covering the rplL-rpoB area, and then analysing the nuclease S1-resistant RNA:DNA hybrids on polyacrylamide gels. When the 1.2 kb rplL-rpoB EcoRI fragment is used as the probe in this way the major hybridised RNA proves to be about 0.3 kb long, and there are also several other RNA species (in lower yield) intermediate in length between the major RNA and the restriction fragment (the absence of a hybrid corresponding to full-size probe is discussed below). If the analysis is repeated using the 11 kb HindIII fragment as probe (this extends 289 bp further upstream than does the 1.2 kb EcoRI fragment: Fig. 4.13), the major hybrid RNA is one of 0.59 kb, with several additional bands,

Fig. 6.1 RNA sequences within the rplL-rpoB intercistronic region. The numbers correspond to the positions in the corresponding DNA sequence (Post et al, 1979; Figs. 4.13 and 4.15).

(A) Possible secondary structure near the 3' end of the attenuated mRNA.

(B) RNA sequence and possible secondary structure in the RNAase-III-sensitive region. The ribosome-binding sequence and adjacent translation start-stop codons in the loop are also marked.

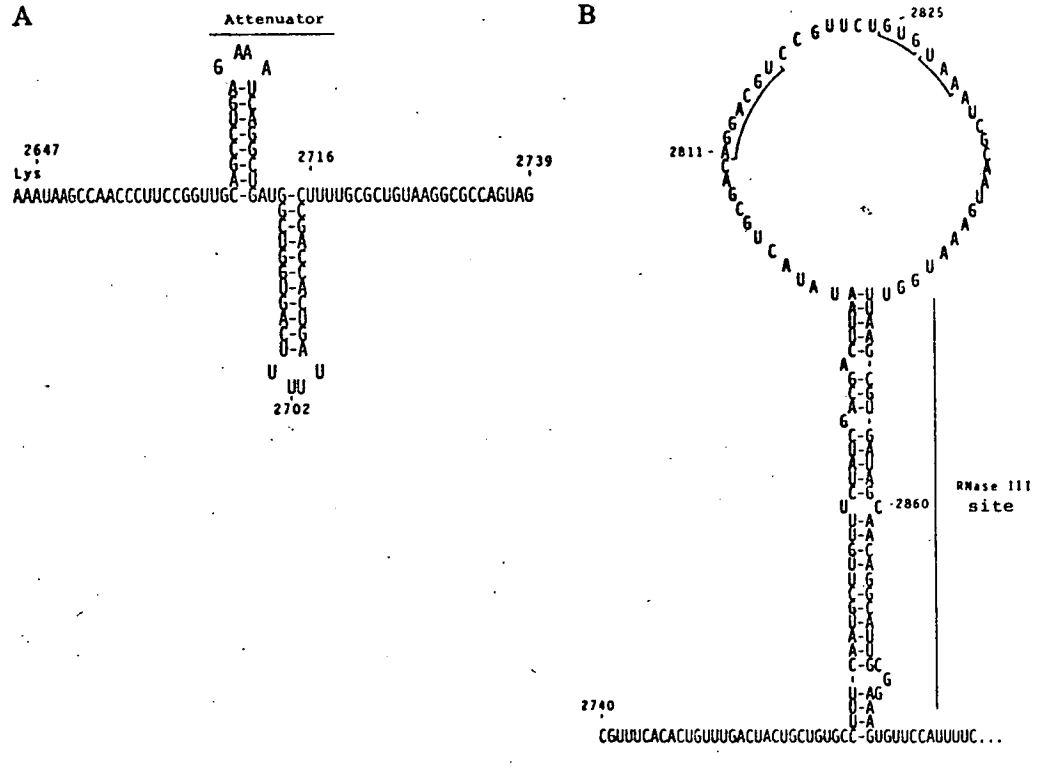

A
Attenuator
2647
Lys
2716
2739
AAAUAAGCCAACCCUUCCGGUUGC-GAUG-CUUUUGCGCUGUAAGGCGCCAGUAG
2702
B
2825
2811
2860
RNase III
site
2740
CGUUUCACACUGUUUGACUACUGCUGUGCC-GUGUUCCAUUUUC...

Fig.6.1

including 2 of 0.70 and 0.71 kb replacing bands of 0.41 and 0.42 kb seen with the EcoRI probe. This information places these RNAs at the upstream ends of the probe restriction fragments, and locates their 3' ends in the intercistronic region. The 3' terminal sequence of the major hybrid RNA was also determined, revealing that it ends at nucleotide 2716 (Fig. 4.15), 69 nucleotides beyond the end of rplL.

The absence of an RNA hybrid corresponding to the entire length of the EcoRI probe fragment, and the presence of smaller discrete bands, suggested some form of processing of the readthrough RNA. The sizes of the intermediate length RNA species would be consistent with a cleavage at about nucleotide 2860 (Fig. 4.15). Indeed, S1 nuclease treatment of hybrids formed between the 1.2 kb EcoRI fragment and RNA isolated from an rnc (RNAase III-deficient) strain yielded RNA species of ∿0.3 kb and 1.2 kb, and no other discrete bands.

Barry et al (1980) have thus demonstrated the presence in vivo of a prominent RNA species whose 3' end is located 69 bp downstream from rplL, beyond two adjacent regions of dyad symmetry, which could form stem-loop structures in the transcript (Fig. 6.1). The second region of symmetry is followed by a run of 4 TA base-pairs, a feature resembling that seen in several known terminators. Hence the RNA species isolated in vivo seems very likely to be a direct product of transcription termination at or near nucleotide 2716.

Comparison of the RNA species found in rnc^+ and rnc strains indicated that transcripts which continue beyond rpot1 are normally subject to cleavage by RNAase III near nucleotide 2860, in a region of possible secondary structure (Fig. 6.1) which is reminiscent of

Fig. 6.2 Summary of transcriptional and post-transcriptional controls expected to influence expression of the rplKAJL rpoBC genes. All the interaction sites shown are on the transcripts, (which are represented by the horizontal lines) with the exception of the rpot1 site at which polymerase is postulated to regulate termination of transcription; this site may be either on the transcript or on the DNA in the rplL-rpoB interval. Binding of the repressor proteins to site(s) on their mRNAs is thought to reduce translation by inhibiting the attachment or movement of ribosomes. The ? above the Rpol regulation site between rpoB and rpoC reflects uncertainty as to whether there are separate sites for independent post-transcriptional regulation of β and β' synthesis.

Symbols


L1 interaction site on rplKA mRNA for post-transcriptional regulation of L1 and L11 synthesis.


L10:$(L7/12)_4$ interaction sites on rplJL mRNA for post-transcriptional regulation of L10 and L7/12 synthesis.


RNA polymerase interaction sites for post-transcriptional regulation of ββ' synthesis.


RNA polymerase interaction site for regulation of transcription termination at rpot1.


RNAase III-sensitive site.

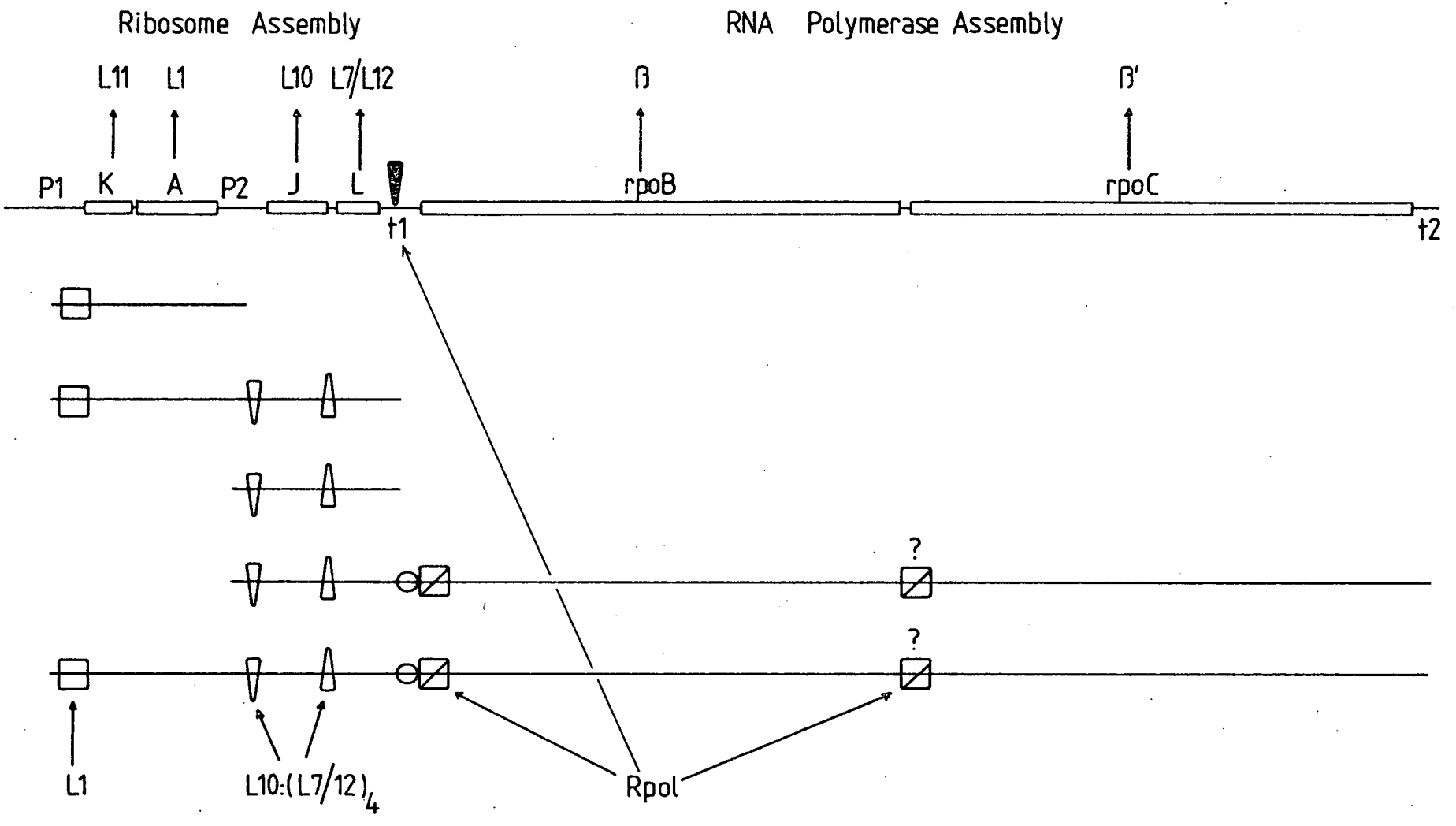

Ribosome Assembly
RNA Polymerase Assembly
L11
L1
L10
L7/L12
β
β'
P1
K
A
P2
J
L
rpoB
rpoC
t1
t2
?
?
L1
L10:(L7/12)4
Rpol

Fig.6.2

RNAase III-sensitive sites in 16S precursor rRNA and in T7 mRNA (Young and Steitz, 1978; McConnell, 1979). Furthermore, this region of the transcript contains a ribosome-binding sequence and closely-spaced transcription start and stop signals; these features are also present in RNAase III sites in T7 (McConnell, 1979). The RNAase III cleavage of the rplJL rpoBC transcript is the first known example of such processing in a bacterial mRNA. Its function is unclear, and is apparently not mandatory, but it might facilitate independent post-transcriptional regulation of rplJL and rpoBC mRNAs.

6.3 Model for rplKAJL rpoBC regulation

The evidence presented up to this point is summarised in Fig. 6.2, which shows the transcriptional and post-transcriptional controls expected to operate in the regulation of the rplKAJL rpoBC gene cluster. The most important uncertainty in relation to the model shown in Fig. 6.2 concerns the mechanism by which termination of transcription at rpot1 is controlled.

The specific induction of rpoBC mRNA synthesis associated with inhibition of RNA chain initiation by rifampicin and in some ts polymerase mutants (even at permissive temperatures) is consistent with RNA polymerase having the dual functions of RNA synthesis, and of autogenous regulation of the rpoBC structural genes at the level of transcription. One can speculate that the amount of active polymerase in the cell is continuously monitored via transient interaction of holoenzyme, in its regulatory capacity, with rpot1. This interaction could be at the DNA or RNA level; if the former, polymerase would bind in a mode distinct from that adopted at promoters. Whichever is the case, the effect on termination at rpot1 should depend in some way upon whether the regulating

polymerases are in a state competent to initiate a transcript.

In the trp attenuator, the efficiency of termination is controlled via ribosome-mediated changes in the secondary structure of the leader transcript (Oxender et al, 1979), which affect the formation of a stem-loop in the RNA immediately preceding the termination site. By analogy, the stability or formation of the putative terminator stem-loop in rpot1 might be influenced by transient interaction between regulatory holoenzyme molecules and the rpot1 transcript. RNA polymerase is known to interact with many different polyanions, including RNA itself, and will even use RNA as a template for transcription in vitro (reviewed in Chamberlin, 1974). It is also plausible that termination may involve interaction of RNA polymerase with RNA stem-loops in its own products. Finally note that in any case the post-transcriptional regulation of ββ' synthesis seems very likely to involve binding of holoenzyme to its own mRNA.

Models for rpot1 regulation which require interaction of holoenzyme with the DNA must account for the fact that small restriction fragments containing rpot1 are not retained by holoenzyme in nitrocellulose filter-binding assays (Taylor and Burgess, 1979). Any rpot1:polymerase interaction must therefore be either weak or reversible. Nevertheless, transient binding of holoenzyme to rpot1 (either sense or antisense strand) might conceivably affect termination, perhaps by 'blocking' or destabilising a transcribing polymerase paused at the terminator.

Some authors have suggested that there is an additional step in the putative autoregulatory system for rpoBC transcription. Tittawella (1974) observed that in an rpoB3 (Rif-R)/rpoB8 (Rif-S)

merodiploid (which is phenotypically rif-resistant) only a short exposure to rif is necessary to induce maximal transient induction of ββ' synthesis. Furthermore, induction was reduced considerably if a constraint on the fidelity of protein synthesis was applied during (but not after) exposure to rifampicin. Nakamura and Yura (1975) isolated a conditionally lethal amber mutation (am100) which, when temporarily unsuppressed, led to a gradual decline in the rate of ββ' synthesis. The authors claimed that am100 lies outside the RNA polymerase structural genes (mapping near polA at 86'). These observations would be consistent with the presence of an additional component in the rpo regulation system. The synthesis of this hypothetical intermediate control protein would be repressed by RNA polymerase, and it would regulate positively at rpot1. Several objections can be raised against these experiments: a) in Tittawella's experiments removal of rifampicin may have been incomplete, and in any case he did not examine rpoBC transcription; b) the am100 strain probably contained multiple mutations, and Nakamura and Yura did not in fact verify that they had mapped the mutation responsible for the effect on rpoBC expression.

What roles do transcriptional and post-transcriptional regulation of this operon play in vivo? In normal growth conditions post-transcriptional regulation of polymerase synthesis may be the more important control point: the efficiency of the rpot1 terminator does not change over a 3-fold range of growth rates (Dennis, 1977b). Alterations in the frequency of termination at rpot1 have been described most often as a result of unusual constraints; for instance after addition of rifampicin (Blumenthal and Dennis, 1978) or when initiation-defective ts rpo mutants are shifted to semi-restrictive

temperatures (Dennis, 1977a): circumstances under which there rapidly develops a shortfall in transcription initiation capacity. One observation in particular, however, provides *prima facie* evidence that regulation at *rpot1* also operates in more "natural" circumstances. When a *relA*$^+$ strain is subjected to partial amino-acid starvation the intracellular concentration of ppGpp rises, and the transcription of rRNA and ribosomal proteins, including *rplKAJL*, is reduced. *relA* strains respond to this constraint by increasing *rplKAJL* transcription (and do not accumulate ppGpp). In neither the *relA* nor the *relA*$^+$ strain, however, is the rate of *rpoBC* transcription detectably altered under these circumstances (Maher and Dennis, 1977), presumably because variable termination at *rpot1* compensates (*in either direction*) for changes in the transcription initiation rate at the *rpo* promoters.

In summary, transcription of the *rplKAJL rpoBC* genes can be regulated both at the level of initiation (for example, by the stringent control system) and, under some circumstances, by variation in termination at *rpot1*. Superimposed on this transcriptional regulation is a set of post-transcriptional feedback loops which ensures that synthesis of the *rplKAJL* and *rpoBC* gene products is closely tailored to demand.

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