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IN VIVO REGULATION OF TRANSCRIPTION OF THE *Escherichia coli* RNA POLYMERASE GENES *rpoB* and *rpoC*

by

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Department of Microbiology & Immunology
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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada

November, 1985

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ABSTRACT

The transcriptional control of two genes *rpoB* and *rpoC* encoding β and β', the major protein subunits of *E. coli* RNA polymerase, was examined *in vivo*. Restriction fragments from the *rplKAJLrpoBC* cluster of genes were fused to a *lacZ* gene carried on a lambda phage vector. The transcriptional activity of each cloned fragment was examined by measuring the β-galactosidase level in a lysogen carrying a transcriptional fusion phage. Two strong promoters, *rplKp* and *rplJp*, were identified along with a weak promoter, *rplLp*. A transcriptional attenuator, *atn*, located between *rplL* and *rpoB*, terminated 60 percent of transcription reading through it regardless of which promoter initiated transcription.

The expression of ribosomal proteins and of RNA polymerase proteins have both been reported to increase with respect to total protein, but to diverge from each other as the growth rate of a culture of *E. coli* was increased. However, using transcriptional fusions, this study found that the frequency of transcription initiation at *rplKp*, *rplJp* and *rplLp* relative to total protein synthesis remained constant at growth rates greater than 0.8 doublings per hour. The ratio of the frequency of transcription initiation at *rplKp* or *rplJp* compared to transcription which read through *atn* remained constant at all growth rates. Therefore, transcriptional controls do not account for the increasing level of expression of these proteins with respect to growth rate, nor for the divergence in expression of ribosomal proteins with respect to RNA polymerase proteins as the growth rate increases.

Hybridization studies to estimate the level of *rplJL* transcripts with respect to *rpoBC* transcripts in strains carrying *rho*, *nus* or *sfrB* mutations implicated the Rho, NusA and SfrB proteins in termination at *atn*. SI mapping experiments confirmed the location of *atn*, 72 bp 3' to *rplL*, but suggested that the RNaseIII processing sites were 80 bp 5' to the positions previously reported. SI mapping studies on *rho*, *nus*, and *sfrB* mutants did not reveal an altered pattern of termination in mutants carrying lesions in any of these genes.

From these studies it is concluded that regardless of which of the two strong promoters, *rplKp* or *rplJp*, is used to initiate transcription which reads through *atn* to transcribe *rpoBC*, transcription is reduced at *atn* by 60 percent. Minor promoters do not contribute substantially to *rpoBC* transcription. Because the frequency of transcription of *rplKAJL* and *rpoBC* remains constant at all growth rates, the changing patterns of expression seen for the proteins encoded by these genes is probably controlled post-transcriptionally. On the basis of the effects of *rho* and *nus* lesions on the *rplJL*/*rpoBC* transcript ratio, the terminator *atn* is designated Rho-dependent.

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LIST OF ABBREVIATIONS

Antibiotics

Amp	ampicillin
Cam	chloramphenicol
Kan	kanamycin sulphate
Rif	rifampicin
Spc	spectinomycin dihydrochloride
Str	streptomycin sulphate
Tet	tetracycline hydrochloride

Chemicals

BME	β-mercaptoethanol
BPB	bromophenol blue
DEP	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetic acid
EtBr	ethidium bromide
IPTG	isopropyl-β-D-thiogalactoside
NFT	nitrofurantoin
ONPG	o-nitrophenol-β-D-galactoside
PEG	polyethylene glycol (MW6000)
PIPES	1,4-piperazinediethanesulfonic acid
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane (Base)
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Other

β-gal	β-galactosidase
L agar	Luria agar
L broth	Luria broth
MCA	MacConkey agar
moi	multiplicity of infection
O.D.	optical density
pfu	plaque forming units
TE	Tris-EDTA (Tris-HCl 10 mM pH 7.5; EDTA 1 mM)
SSC	saline sodium citrate (NaCl 0.15 M; Na citrate 15 mM)

INTRODUCTION

1.1 BACKGROUND

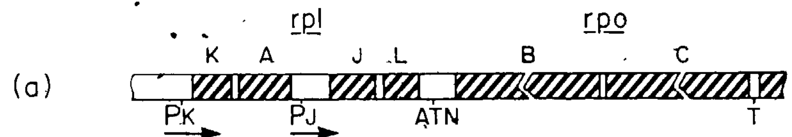
1.1.1 RNA polymerase and the rplKAJLrpoBC genes

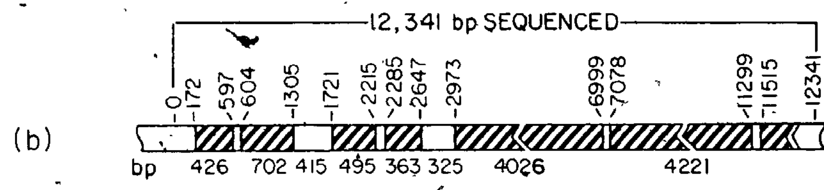
This work was undertaken in order to gain a better understanding of how Escherichia coli controls the expression of its only transcriptase, RNA polymerase (EC 2.7.7.6, nucleoside triphosphate:RNA nucleotidyltransferase). This large (400,000D) multimeric enzyme is made up of three proteins designated α, β and β' (alpha, beta and beta prime) which, assembled as $\alpha_2\beta\beta'$, form what is called core enzyme. Another protein designated σ (sigma) or its recently discovered sister σ-32 (sigma-32) associates reversibly with core enzyme to confer upon it transcriptional specificity. After transcription begins, the sigma protein dissociates from the transcriptional complex (Travers and Burgess 1969; Hansen and McClure 1980). In it's role as a catalytic subunit, the sigma protein is found at lower levels than core polymerase proteins. The α protein is found in relative molar excess and the assembly of RNA polymerase core enzyme appears to be limited by the synthesis of β and β' (Iwakura et al. 1974; Engbaek et al. 1976; Hayward and Fyfe 1978). Therefore the expression of RNA polymerase can be measured by examining the expression of β and β'. These two very large proteins (151,000D and 155,000D) are among the largest proteins to resolve on SDS acrylamide gels of crude extracts of E. coli making them easily accessible for study.

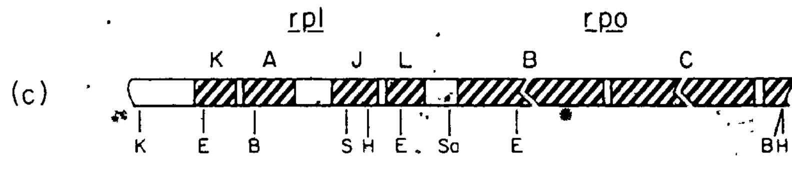
Both rpoB and rpoC, located at about 90 minutes on the 100 minute map of the E. coli chromosome (Bachmann 1983) were found to be under the control of a common promoter placing them in a single operon (Linn and Scaife 1978; Yamamoto and Nomura 1978). Their shared promoter, rplJp, was found to also be responsible for the transcription of two ribosomal protein genes, rplJ and rplL (ibid). The genes, rplJL, were located between rplJp and rpoBC giving an operon structure of rplJLrpoBC. More recently evidence has been published that suggests rplKp, a promoter further upstream, before the adjacent ribosomal protein genes rplK and rplA, is responsible for

FIGURE 1: Genetic and physical organization of rplKAJLrpoBC.

This group of genes is located at 90 min on the 100 min map of the *E. coli* chromosome (Bachmann 1983). The hatched regions indicate coding sequences, the open regions indicate non-coding sequences. The figure is drawn to scale (bar) except *rpoB* and *rpoC* which have been compressed. **(a)** Four ribosomal protein genes *rplKAJL* and two RNA polymerase genes *rpoBC* are illustrated along with their transcriptional regulatory features. Transcription is from left to right. **(b)** The entire cluster has been sequenced (Post et al. 1979; Ovchinnikov et al. 1981; Squires et al. 1981; Ovchinnikov et al. 1982; Morgan et al. 1984) and is shown here numbered according to the coordinates of Post et al. **(c)** The restriction sites referred to in the text are marked. Fragments can be sized precisely from the sequence data.







1000 bp

B - BglII
E - EcoRI
H - HindIII
K - KpnI
Sa - SalI
S - SmaI

initiating the transcription of all six genes, rplKAJL and rpoBC (Bruckner and Matzura 1981). Therefore the study of rpoBC expression includes the study of ribosomal protein gene expression or at least the expression of rplKAJL encoding L11, L1, L10, and L7/12 respectively.

1.1.2 Measurement of RNA polymerase and ribosomal proteins

At the outset of this work, both RNA polymerase and ribosomal proteins in E. coli had been the objects of extensive research (for reviews see: Losick and Chamberlin 1976; Chambliss et al. 1980). The nature of the relationship between the rate of synthesis of ribosomal proteins (r-proteins) and total protein was well established and although the relationship between the rate of synthesis of RNA polymerase and total protein was well studied, the nature of this relationship was not agreed upon (Shepherd et al. 1980).

Each of the 52 r-proteins that make up the E. coli ribosome (aggregate mol. wt. approx. 800,000D) is found in a single copy per ribosome with the exception of L7/12 (Hardy 1975; Subramanian 1975). Some copies of this protein are modified (acetylated) and resolve as separate spots on 2-D gels leading to original designations of L7 and L12. Up to four copies of the L7/12 protein are found in each ribosome.

Because free pools of ribosomal proteins are negligibly small (2 - 3 percent) and because ribosomal proteins do not turn over at a rate different from bulk protein (Dennis 1974a), the level of a given r-protein can be estimated from measuring the fraction of total protein present in ribosomes. This fraction has a value of about 10 percent at low growth rates and increases to almost 30 percent at high growth rates (Dennis and Bremer 1974), suggesting an almost three-fold increase in the amount of all r-proteins with respect to total protein as the growth rate increases from about one to three doublings per hour.

Estimates had been made of the level of RNA polymerase proteins. At low growth rates (about one doubling per hour), RNA polymerase was found to comprise about one percent of total protein (Iwakura et al. 1974; Engbaek et al. 1976). Given that ribosomes have about twice the molecular weight of protein as RNA

polymerase (800,000D vs 400,000D), the stoichiometry of most r-proteins with respect to RNA polymerase proteins β and β' is about 5:1 at low growth rates. Because L7/12 is found at four copies per ribosome, the ratio of L7/12 to polymerase proteins β and β' is 20:1:1. Therefore the stoichiometry of the proteins encoded by rplJLrpoBC is about 5:20:1:1 at low growth rates.

The relationship between the rate of synthesis of r-protein and the rate of synthesis of total protein, defined as $\alpha_{(r)}$ (Schleif 1967) has been determined to increase in direct proportion to the increase in the growth rate (Schleif 1967; Gausing 1974; Dennis and Bremer 1974). This is not surprising because ribosomes are known to incorporate amino acids into protein at a constant rate and the rate of protein synthesis is known to increase in proportion to the growth rate. Therefore the number of ribosomes synthesizing protein at higher growth rates must increase relative to total protein.

The relationship of RNA polymerase to total protein, defined as $\alpha_{(p)}$, the equivalent measurement for RNA polymerase as $\alpha_{(r)}$ is for ribosomes, has not been so well established. Numerous estimates of $\alpha_{(p)}$ had been reported (summarized in Shepherd et al. 1980) but there has been little agreement on the precise relationship of $\alpha_{(p)}$ to growth rate. The general consensus of these measurements has been that, although $\alpha_{(p)}$ may increase with growth rate, it does so much less rapidly than $\alpha_{(r)}$. What is clear then, is that the synthesis of r-proteins and RNA polymerase proteins appears to be differentially regulated in response to changes in the growth rate. Using the values of $\alpha_{(r)}$ and $\alpha_{(p)}$ reported by Shepherd et al. (1980), in molar terms, the ratio of ribosomes to RNA polymerase has been seen to increase from a value of about 5:1 at low growth rates to about 7:1 at high growth rates.

At the time that rplJLrpoBC was defined as as operon, bacterial gene expression was understood primarily in terms of the operon model. Operons were defined as groups of genes under the coordinate transcriptional control of a promoter. Bacterial proteins of a related function were expressed coordinately by the transcription of a polycistronic message and translated independently from a ribosome binding site prior to each cistron. Although post-transcriptional controls

were an admitted formal possibility, the very short half lives of bacterial transcripts did not seem to warrant such a complication. The grouping of genes into an operon implied that the gene products were co-regulated and predicted that the stoichiometry of those gene products would be constant. The discovery of an operon that included both ribosomal protein genes and RNA polymerase genes implied that the expression of ribosomes and RNA polymerase was controlled by a common transcriptional promoter. It was thought that perhaps the regulation of growth might occur at the level of regulation of the transcription of the rplJLrpoBC operon. The arrangement of r-protein genes and RNA polymerase genes in a single operon was however paradoxical. Recalling the protein measurements cited, describing the divergent levels of ribosomal and RNA polymerase proteins, the gene products of this operon appeared to defy the prevailing model of how bacterial gene expression was regulated. The work described in this thesis and the work of many others was motivated by the intriguing arrangement of the rplJLrpoBC genes in an operon.

1.1.3 Transcription of rplKAJLrpoBC

In their initial report of a common promoter for rplJLrpoBC, Linn and Scaife (1978) made two additional observations that are of relevance to this work. Their experiments were performed in a highly perturbed *in vivo* system that used lambda phage vectors carrying chromosomal DNA as templates for transcription in lethally UV-irradiated cells. The phage-borne DNA in such a system provides a template for the cell's transcriptional apparatus and these transcripts are the only messages available for translation. They presented evidence that, even in the absence of the restriction fragment carrying the strong promoter rplJp, a low level of synthesis of L7/12, β, and β' occurred from a template carrying rplL and rpoBC, suggesting to them a weak promoter downstream of rplJ. They also speculated that, on the basis of the protein stoichiometry they observed (that is, a much lower molar level of β and β' than L10 and L7/12), a transcriptional attenuator might function between the ribosomal protein genes and the RNA polymerase genes. The term attenuator used here refers to a transcriptional terminator which allows some transcription to proceed through it. Using an *in vivo* approach that utilized cloned restriction

fragments from rplJLrpoBC ligated between arap and lacZ, Barry et al. (1979) confirmed the existence of both of these transcriptional features. They estimated that 83 percent of transcription initiated at arap was terminated by an attenuator located between rplL and rpoB. Furthermore, they identified a terminator, rpoCt, located on a restriction fragment that carried the 3' end of rpoC. Contemporaneous in vitro studies confused the picture somewhat. Observing the efficient synthesis of L7/12, β and β' in an in vitro system primed by a DNA template that carried rplLrpoBC without rplJp, Goldberg et al. (1979) suggested that rplL and rpoBC might form their own operon. In a similar vein, Fiil et al. (1979), working with recombinant plasmids in vivo, also suggested that rplLrpoBC might form a transcriptional unit. However in vivo, they observed little synthesis of L7/12 or β using the same template as Goldberg's group. What was not disputed was the picture of a complicated set of transcriptional controls that was emerging from both in vivo and in vitro studies on the rplJLrpoBC operon.

Although some of these reports were difficult to reconcile with one another, some outstanding questions with regard to rplJL and rpoBC transcription seemed to be resolved. The existence of a common promoter for rplJLrpoBC and a transcriptional attenuator between rplJL and rpoBC agreed well with a previous hybridization study (Dennis 1977) which had reported that the ratio of transcripts from rplKAJL and rpoBC remained constant but with a value of about five. This study had implied that these genes were co-regulated and that an attenuator functioned between them to reduce the transcription of the promoter distal genes rpoBC.

The preliminary reports on the control of rplJLrpoBC transcription hinted that the ultimate protein stoichiometry, might be explained by some complex transcriptional controls. The rplJ and rplL genes were both transcribed and expressed at a higher level than the rpoB or rpoC genes. A promoter located downstream of rplJ might account for the higher level of L7/12 synthesis or alternatively might allow the independent transcription of rpoBC under some conditions. For instance there had been a report that the transcription of rpoBC could, under a condition of amino acid starvation which elicits

the stringent response, increase in frequency with respect to that of rplJL (Maher and Dennis 1977). This might be explained if a promoter specific for rpoBC, normally quiescent, becomes active under some conditions, such as stringency. Or perhaps, the divergent expression of ribosomal and polymerase proteins under conditions of increasing growth rate might be explained, if termination before rpoBC is modulated by attenuation.

1.1.4 Control of rplKAJLrpoBC expression

Emerging at about the same time as the present work began, was evidence that translational controls, or at least post-transcriptional mechanisms, could function in E. coli to affect gene expression. The first report confirming this idea examined the effect that gene dosage had on the transcription and expression of eleven ribosomal protein genes (Fallon et al. 1979). Although the transcription of the plasmid-amplified ribosomal protein genes increased in approximate proportion to the gene dosage, no concomitant increase in r-protein synthesis occurred. Furthermore, the overproduced transcripts seemed to be specifically degraded. Studies on other ribosomal protein genes quickly followed and established that most ribosomal protein genes were susceptible to post-transcriptional control including rplKA (Dean and Nomura 1980) and rplJL (Yates et al. 1981). Similarly there was mounting evidence that rpoBC expression was subject to post-transcriptional control that reduced expression of β and β' by an autogenous mechanism. In an early gene copy experiment, Kirschbaum (1973) observed that β and β' levels merely doubled when a lambda transducing phage carrying rpoBC was induced and the copy number of rpoBC increased several hundred-fold. Dennis and Fiil (1979) induced a six-fold overtranscription of rplJL and rpoBC and observed only a two-fold increase in β and β' synthesis. In more direct experiments, others working in vitro demonstrated that the addition of RNA polymerase holoenzyme or an assembly intermediate could repress the translation of rpoBC transcripts (Kajitani et al. 1980; Ishihama and Fukuda 1980).

Therefore as the present work began, it was becoming clear that post-transcriptional controls could play a major role in the control of bacterial gene expression. It was also becoming clear

that one could no longer expect to account for the overall control of expression, ultimately measured as the level of a given protein, solely in terms of transcription, despite the success of such an approach when dealing with some operons such as lactose and tryptophan. However, this did not diminish the relevance or importance of a study that would establish the nature of the transcriptional controls functioning within the rplKAJLrpoBC cluster of genes, in order to define the contribution transcription makes to the regulation of RNA polymerase expression.

1.1.5 Attenuation

The belief that transcription played a major role in the regulation of rpoBC expression stemmed not only from faith in the prevailing operon model, as typified by the paradigmatic lac operon. Already cited is a report demonstrating that transcription of rplJL could be uncoupled from that of rpoBC (Maher and Dennis 1977) and several reports suggested that this phenomenon could occur under other conditions. Blumenthal and Dennis (1980) reported a better than two-fold increase in rpoBC transcription upon temperature shift of a strain, carrying a temperature sensitive lesion in rpoD, without a corresponding increase in rplJL transcription. They proposed that either attenuation was relaxed or that perhaps a normally quiescent promoter, rpoBp, was responsible, becoming active under these conditions. In another strain, which carried an amber mutant in rpoB and a temperature sensitive amber suppressor, an increase in temperature resulted in a four-fold increase in rpoBC transcription and β synthesis, with only a two-fold increase in rplJL transcription and no increase in L10 or L7/12 synthesis (Little and Dennis 1980). Similar observations had been made using low levels of rifampicin to perturb the normal regulation of β and β' synthesis although in this case transcripts were not examined directly (Tittawella and Hayward 1974). It seemed quite clear that under some conditions transcription of rplJL and rpoBC could become uncoupled. This unusual situation was worth examining as a novel regulatory mechanism in procaryotic gene expression.

The presence of a structure that can terminate transcription in the middle of an operon is a highly unusual feature and atn within rplJLrpoBC deserves special attention, particularly in regard to its potential to regulate rpoBC transcription independently of

initiation at the promoter. Before proceeding, it is perhaps a good idea to emphasize here that attenuation at atn in rplJLrpoBC is conceptually very distinct from the attenuation which occurs in the leader sequence of many biosynthetic operons, the most familiar example of which may be the trp operon (Yanofsky 1981). This latter type of attenuation modulates termination in a leader sequence found between the promoter and the structural genes of the operon. The original definition of an operon accommodates such transcriptional attenuation. That is, despite the complication of a transcriptional attenuator in the leader, all of the genes in the operon are still transcribed in concert, as a polycistronic message, the transcription of which is directed by shared control mechanisms. In contrast to this situation, atn permits the independent control of two genes, rpoBC, within an operon, revealing a dimension in the control of bacterial gene expression not previously appreciated.

There are two types of transcriptional terminators known to function in E. coli; variously called simple, factor independent, or Rho-independent; and complex, factor dependent, or Rho-dependent (Adhya and Gottesman 1978; Holmes et al. 1983). Because it now appears that all factor dependent terminators share a requirement for the rho gene protein, the two types will be referred to here as Rho-independent and Rho-dependent.

The first type, Rho-independent terminators, are structurally well defined and are able to direct termination of transcription in vitro, without any proteins other than RNA polymerase being present (von Hippel et al. 1984). They share a consensus sequence, striking enough to allow their prediction from DNA sequence data alone. They are characterized as having a 7-10 bp inverted repeat centered 20 bp upstream of a stretch of thymidine residues in the non-coding strand. The inverted repeat allows a stem-loop structure to form in the transcript with a calculated free energy value of -20 to -30 kcal/mole. The transcript is terminated within or just distal to the stretch of uridines (von Hippel et al. 1984). Rho-independent terminators are generally very efficient and are the type commonly found at the end of operons or as attenuators in the leader regions of biosynthetic operons.

Rho-dependent terminators have an absolute requirement for Rho in vitro (Adhya and Gottesman 1978) and require a relatively long, untranslated transcript sequence (>70 bp) in order to terminate transcription. Other than these two features, the sites of Rho action are poorly characterized, being without a consensus sequence or any obvious shared secondary structure.

Although Rho-dependent terminators are not well defined, the function of the Rho protein itself is established (von Hippel et al. 1984). Rho is able to bind to an untranslated transcript and if the appropriate sequence is present on the transcript, Rho can mediate the dissociation of RNA polymerase from the transcript. Sites required for Rho action all cause polymerase to pause in vitro, but not all pause sites in vitro are sites of Rho action in vivo (Kingston and Chamberlin 1981). Transcriptional polarity, the premature termination of transcription due to interrupted translation, is also mediated by the Rho protein. An untranslated stretch of transcript can be bound by Rho and if an appropriate site is available, transcription will be terminated.

The rho gene was originally identified as coding for a protein that facilitates the accurate termination of transcription at specific sites on lambda templates (Roberts 1969) and it is at two of these sites, λt_{L1} and λt_{R1}, that the nature of Rho-dependent termination has been best characterized (Roberts 1976). At the delayed early gene terminators of phage lambda, transcription termination is not only Rho-dependent but an anti-termination mechanism also functions. The N protein of lambda can prevent termination at Rho terminators, if the transcription complex passes over the nut sequence and is modified by the N protein. The N protein has a requirement for several host proteins in order to function properly. These host genes are called nus genes and were originally identified as mutations that prevented N protein function (Friedman 1970). One of these, nusA, codes for NusA, a protein which binds to RNA polymerase when the transcribing polymerase traverses a sequence called a boxA site (Friedman and Olson 1983). After the sigma protein dissociates and NusA has bound to RNA polymerase, NusA binds the N protein and somehow N modifies the transcription complex, when RNA polymerase transcribes the lambda nut site to render it insensitive to subsequent termination signals

(Greenblatt 1984). This rather complicated series of events allows transcription to proceed through the Rho-dependent terminators found on the lambda phage genome.

For some time there has been suggestive evidence that a similar mechanism might function in E. coli. Morgan (1980) inserted Tn10 into an rrn operon and found that, unlike the insertion of Tn10 into a structural gene, the insertion in rrn was incompletely polar. Aksoy et al. (1984b) have shown that a site in the rrnG operon leader sequence carries a Rho-dependent termination sequence that is ignored when transcribed from a fragment carrying rrnGp but not when transcribed from arap. This seems to suggest an anti-terminaton mechanism similar to that employed by lambda phage. Morgan (personal communication) has evidence from in vitro studies that not only is there a Rho-dependent site in rrn operons, but that NusA mediates anti-termination at these sites. This suggests that anti-termination in E. coli may be exactly analagous to the mechanism in lambda. This contention gathers indirect support from the observation that an overtranscription from λp_L not only titrates out nus gene products (Nakamura et al. 1983), but that the overproduction of a sequence utilizing NusA protein decreases the transcription of stable RNA operons (Sharrock et al. 1985).

Barry et al. (1980) used an S1 mapping technique to locate the site at which transcription terminates in the rplL-rpoB intercistronic space, 69 bp downstream of rplL. By examining the sequence of Post et al. (1979), they were able to determine the sequence at the 3' end of the atn terminated transcript. Termination at atn occurs in a run of five uridines after a sequence that closely resembles a typical Rho-independent terminator. The attenuator in rplJLrpoBC looks like a simple terminator. However, other reports could be interpreted as suggesting that transcription termination in rplJLrpoBC is Rho-dependent in vivo (Holowachuck et al. 1980) and NusA sensitive in vitro (Zarucki-Shultz et al. 1979). Therefore the nature of atn has been ambiguous, perhaps having characteristics of both kinds of transcriptional terminator.

Proteins other than NusA and Rho have been implicated in the termination process. Five nus genes had originally

been identified by Friedman and workers (Friedman 1970; Friedman et al. 1983). Two of these nusA and nusB, encode proteins that have no known function outside of transcription termination. The other three, nusC, D, and E, map in rpoB, rho, and rpsJ, respectively. The role of NusA in anti-termination is well studied, but that of other nus products is not at all clear. Another host protein, SfrB, the product of the sfrB gene, has been reported to function as an anti-termination protein in the tra operon of the F plasmid (Beutin and Achtman 1979; Beutin et al. 1981), although this protein too is poorly studied to date. RNaseIII, the product of the rnc gene, has been reported to affect the level of transcription in two lambda operons (Lozeron et al. 1983) and is well known to affect the expression of several genes by modifying transcripts. An RNaseIII site is found in the rplL-rpoB intercistronic space downstream of atn (Barry et al. 1980). The roles of these proteins in the control of rplJLrpoBC transcription had not been determined.

1.2 THE PROBLEM

At the inception of the work described in this thesis, several transcriptional regulatory features functioning in the rplJLrpoBC operon had been described and their locations and approximate strengths assessed. Two groups reported a strong promoter, rplJp, to be responsible for initiating the transcription of rplJLrpoBC (Linn and Scaife 1978; Yamamoto and Nomura 1978). Working in vivo, with recombinant plasmids, others had corroborated a minor promoter, rplLp, located downstream of rplJ (Barry et al. 1979) and probably located between rplJ and rplL; had confirmed an attenuator to function between rplL and rpoB to reduce transcription by 83 percent; and had located the end of the operon defined by a terminator that stopped all transcription after rpoC (Barry et al. 1979; An and Friesen 1980). Still other evidence argued that a promoter specific for rpoBC must also exist (Maher and Dennis 1977; Little and Dennis 1979; An and Friesen 1980; Blumenthal and Dennis 1980). The sequence of rplKAJL (Post et al. 1979) supported the location of these features but could not give any indication of their physiological significance. In vitro studies had not contributed to a coherent picture of rplJLrpoBC transcriptional

regulation.

The problem was then to examine the transcriptional regulatory features of rplJLrpoBC in order to confirm their positions and assess the relative contribution that each of the regulatory structures made to the overall control of rplJLrpoBC expression in vivo. Further, it was important to examine the transcriptional control of rplJLrpoBC under conditions reported to elicit changes in the pattern of transcription of different parts of the operon in order to determine how such changes were controlled. It was not clear whether such control was a result of modulated attenuation or of transcriptional initiation from an internal promoter. This study was designed to address these questions, to unravel the complex pattern of transcriptional regulation in rplJLrpoBC and to determine the contribution transcriptional controls made to the control of expression of RNA polymerase.

Shortly after this work began, a report was published suggesting that rplKp, (Linn et al. 1979; Yamamoto and Nomura 1979; An and Friesen 1980) was the promoter normally responsible for directing the transcription of rplKAJLrpoBC in vivo (Bruckner and Matzura 1981), which of course implied that all six genes were co-regulated. This evidence broadened the scope of this work to include rplKA, since rplKp might then control the transcription of rpoBC and hence the synthesis of β and β'.

The transcription of rpoBC might have been initiated at any one of three promoters, but it was also quite clearly regulated at atn, perhaps the major transcriptional element functioning to control the transcription of rpoBC. Because of the unusual nature of this structure, terminating transcription in the middle of an operon, and because it did not conform to either a description of a Rho-dependent or a Rho-independent terminator, but had attributes of both, it was of interest to identify conditions and proteins that affected its in vivo function.

1.3 THE APPROACH

1.3.1 Gene Fusions

The primary approach used in this work to examine the transcriptional control of rpoBC expression was one that employed

gene fusions. DNA fragments from rplKAJLrpoBC were fused in vitro to a sequence carried on a lambda vector that included lacZ, the gene that encodes β-galactosidase. This was designed to place the transcription of lacZ under the control of the cloned sequence. By examining the expression of β-gal from such fusions, transcriptional regulatory features of rplKAJLrpoBC could be both located and their relative strengths assessed. When E. coli is grown under steady state conditions, all cell constituents increase at the same rate so that each comprises a constant fraction of cell mass. There is no turnover of protein under steady state growth conditions (Goldberg and Dice 1974). Therefore the level of β-galactosidase expressed by a strain carrying a gene fusion and growing under steady state conditions can be measured relative to any other cell constituent to assess the frequency at which the cloned fragment directs the transcription of lacZ. In this way, the simplicity and convenience of assaying β-galactosidase, the lacZ product, allows the assessment of rplKAJLrpoBC transcriptional regulation under a wide variety of conditions or in a wide variety of genetic backgrounds.

To appreciate why such an apparently indirect approach was both necessary and desirable, one must keep in mind the nature of the genes under consideration. All six genes encode proteins that are essential and as such are not amenable to the sort of genetic analysis applied with such success to non-essential genes. One does not have the luxury of being able to select regulatory mutants that overproduce or underproduce proteins from these genes in order to identify transcriptional control mechanisms. In any case, the measurement of a protein represents a measure of the sum of all regulatory mechanisms affecting the expression of that protein and does not distinguish transcriptional controls from post-transcriptional controls.

Not only is an undersupply of the proteins from rplKAJLrpoBC deleterious to the cell, as one might expect, an oversupply of at least some of them, or even fragments of some, can be lethal or deleterious to the cell. This is one reason why multi-copy plasmids carrying fragments of these genes do not allow a complete or accurate analysis of their regulation. For instance, it had been reported that when cloned on a multi-copy plasmid, a fragment of

rplKAJLrpoBC that produced only the amino terminus of L10 could be deleterious or lethal (Friesen et al. 1983). If a cloned fragment is poorly tolerated by the host cell, there will be a negative selection imposed on a plasmid carrying such a fragment and the copy number of the plasmid will be reduced. Measurements derived from such a construction are difficult to compare with measurements made with other constructions which allow the normal vector copy number. A second potential problem, much more insidious in nature, was perceived with the use of multi-copy plasmids. If the transcription of a cloned sequence is regulated by a factor, perhaps a protein, that is normally found at a low level in the cell, the provision of a high copy number of that sequence might bind and titrate out the protein, thereby affecting the normal regulation of that or a similar sequence. The pertinence of this concern to the regulation of transcription through atn has recently been provided (Sharrock et al. 1985). This work demonstrated the effect an over-expression of the lambda nut sequence carried on a high copy number plasmid had on stable RNA operon transcription. This reduction of transcription is quite possibly due to the titration of NusA.

For the reasons outlined above, the work described in this thesis employed gene fusions painstakingly constructed on lambda phage vectors rather than the relatively simple assembly of these fusions on plasmids. By using lambda phage as the vector, a single copy of the assembled fusion could be stably introduced into the host chromosome as a lysogen. It was hoped that this would perturb the normal regulation of rplKAJLrpoBC as little as possible and allow the cloning of any fragments of these genes. That the logic employed to justify this approach is valid, was demonstrated by the successful cloning on a lambda vector of a fragment carrying both rplKp and rplJp, which had been reported by others to be lethal when carried on a multi-copy plasmid (Friesen et al. 1983).

For this study, true transcriptional fusions have been constructed. That is, the transcription of lacZ is dependent on transcription initiated upstream of the lacZ gene, but translation is controlled by the endogenous lacZ ribosome binding site. This is in contrast to some previous fusions made with rplJLrpoBC fragments that

placed the expression of lacZ under the transcriptional and translational control of a cloned sequence (Holowachuck et al. 1980; Hui et al. 1982).

To make such transcriptional fusions, DNA fragments from rplKAJLrpoBC were cloned in a lambda vector phage λJDW36 (Windass and Brammar 1979) which carries lacZ downstream from trpBA. Fragments of DNA cloned into the HindIII site in trpB can direct transcription of the downstream lacZ gene. Most lacZ fusion vectors used for the analysis of regulatory sequences are based on one of several trp-lac fusions (Mitchell et al. 1975). The fusion carried by λJDW36 carries the W205 deletion, fusing trp and lac such that the transcriptional regulatory elements normally found at the end of trp and beginning of lac are missing. The functional trpt' terminator is gone (Wu et al. 1981), as is the lac promoter (Xian-Ming et al. 1984), but the lacZ ribosome binding site remains intact. The W209 deletion by comparison, used by others, extends into the 5' lacZ sequence removing the first few codons of β-galactosidase and expresses a TrpA-LacZ fusion protein. Because trpA is translationally coupled to trpB, the expression of β-galactosidase is dependent on both the transcription and translation of the sequence cloned upstream of trpB.

One of the pitfalls of any gene fusion system in procaryotes is transcriptional polarity caused by the binding of Rho protein to an untranslated transcript sequence and resulting in premature transcriptional termination. The presence of an untranslated sequence is not necessarily a cause for polarity (see background) but the trpB sequences downstream of the HindIII cloning site in trpB have been reported to be notoriously polar (Aksoy et al. 1984a). One way of avoiding polarity is to ensure that there are no untranslated sequences between the cloned fragment and the lacZ gene. Because the sequences of all of the DNA fragments used in the construction of the fusions described in this work are known, the fusions were designed such that the reading frame into trpB was preserved, resulting in the translation of both trpB and trpA sequences.

A collection of fusion phages was made using in vitro recombinant DNA techniques so that each of the promoters from rplKAJLrpoBC could be examined both separately, and in combination

with each other, and in combination with atn. These phages were introduced as lysogens into host strains completely deleted for lac genes. Such lysogenized hosts were grown under steady-state conditions and β-galactosidase levels were measured to assess the transcriptional activity of the fragment cloned on the lambda vector. In this way the location and relative activities of the rplKAJLrpoBC transcriptional regulatory sites were established.

By subjecting lysogens to a variety of growth conditions, the effect of those conditions on the transcriptional regulation of rplKAJLrpoBC could be assessed. Because growth rate had been reported to elicit a differential rate of expression of ribosomal proteins and RNA polymerase proteins, the appropriate lysogens were grown at a number of different growth rates and β-galactosidase measured at each.

1.3.2 Direct assessment of transcripts

To assess the role that proteins known to be involved in termination might have on transcription through atn, attempts were made to lysogenize a variety of E. coli termination protein mutants with transcriptional fusion phages. These attempts failed, probably because such mutations prevent normal lambda regulation and lysogeny. In the case of rho, a successful transfer of rho112 was made to lysogens of recombinant phages carrying rplJp and rplJp+atn by P1 transduction, but this success was not repeated with other lesions. Therefore, strains carrying mutations in rho, nusA, nusB, nusE, sfrB, rnc and rpo203 were assessed by directly measuring the ratio of rplJL and rpoB transcripts using hybridization probes, and comparing the level of each. The transcripts of interesting mutants were then examined directly by S1 mapping techniques.

1.4 SUMMARY OF RESULTS

Two strong promoters, rplKp and rplJp, were located on restriction fragments carrying DNA sequences 5' to rplK and rplJ respectively. A weak promoter, rplLp, was identified on a fragment carrying the rplJ-rplL intercistronic space and found to initiate one-tenth the level of transcription as rplJp. A transcriptional attenuator was located on a fragment carrying the rplL-rpoB

intercistronic space. Whether initiated at rplKp or rplJp, transcription through atn was reduced by about 60 percent. Transcription initiated at rplLp did not read through atn at levels distinguishable from background. No promoter specific for rpoBC was observed.

Regardless of the rate at which lysogens carrying rplKp, rplJp, or rplLp, or any of these promoters plus atn were grown, no increase in β-galactosidase levels was observed. It was concluded that the divergent expression of L10 and L7/12 from that of β and β' with increasing growth rate is not due to the uncoupling of transcription of rplJL from that of rpoBC. Because no increase in the frequency of rplKAJL transcription was observed it is concluded that the increasing level of synthesis of these proteins as the growth rate increases is controlled post-transciptionally by an autogenous mechanism(s) that prevents the translation of excess transcripts.

When the rho112 lesion was introduced into a lysogen carrying rplJp+atn β-galactosidase levels doubled, suggesting that the Rho protein is normally involved in termination at atn. When the ratio of rplJL and rpoB transcripts was determined in two rho mutants, it fell to about one-half the value of the ratio seen in the isogenic parent strain. This also implicates the Rho protein in attenuation. In similar experiments with nus and sfrB mutants, NusA and SfrB proteins were both implicated in modulating the frequency of termination; both mutations decreased read-through as judged by higher rplJL/rpoB transcript ratios. This is consistent with the previously defined role of these proteins in anti-termination.

S1 mapping of transcripts which terminated within the rplL-rpoB intercistronic region, confirmed the location of atn about 72 bp 3' to the end of rplL (Fig 15). Although it was difficult to assess the relative amounts of the various S1 fragments, most transcription quite clearly stopped at the attenuator sequence. Two of the longer fragments seen were shown to be RNaseIII processing products, by comparing the pattern of transcripts seen in a mutant carrying the rnc105 mutation with the wild type pattern. This located the processing sites about 80 bp 5' to the position mapped by Barry et al. (1980). This places the processing site on the 5' side of the proposed ribosome binding site and supports the contention that processing of the rpoB

transcript plays no part in the control of its translation (Dennis 1984). No changes in transcription patterns were obvious when mutant strains with rho, nus or sfrB lesions were examined by S1 mapping. This supports the contention that termination in the rplL-rpoB intercistronic space is occurring primarily at a single discrete site. The S1 technique did not resolve transcript bands with sufficient contrast to allow a comparison of the relative intensities of the various fragment bands by laser densitometry. Such a comparison would be required to confirm more directly the involvement of Rho, NusA and SfrB proteins in the attenuation process.

MATERIALS AND METHODS

2.1 REAGENTS

All chemicals used in this work were reagent grade unless otherwise specified. Most reagents were purchased from Fischer Scientific (Fairhaven, New Jersey), J. T. Baker (Phillipsburg, New Jersey), Sigma Chemical Co. (St. Louis, Missouri), BioRad (Richmond, California), Bethesda Research Laboratories (BRL) (Gaithersburg, Maryland), Boehringer-Mannheim (West Germany) or Schwartz-Mann (Spring Valley, New York). Media ingredients were from Difco (Detroit, Michigan). Antibiotics were from Sigma. Biologicals, primarily nucleic acid modifying enzymes, were purchased from BRL or Boehringer-Mannheim or occasionally from New England Biolabs (Beverley, Massachusetts) or Pharmacia (Montreal, Quebec). Isotopes were from New England Nuclear (Canada) (Lachine, Quebec) or ICN Radiochemicals (Irvine, California). Products from other sources are noted in the text.

2.2 ESCHERICHIA COLI STRAINS

All *E. coli* used in this work were derived from *E. coli* K-12. They are described in Table 1.

2.3 PLASMIDS

The recombinant plasmids constructed for this work were made using the cloning vector pBR325 (Fig A1: Bolivar 1978). All other plasmids used in this work are listed in Table 2.

2.4 BACTERIOPHAGE

All of the recombinant phage made for the work are immunity 21, *N* independent, competent lysogens. All of the M13 constructions were made using mp8 (Messing and Vieira 1982). Transductions were performed with P1(vir) and P1(clr100). All bacteriophage are listed in Table 3.

STRAIN MANIPULATIONS

2.5 CULTURE AND GROWTH OF E. COLI

2.5.1 Maintenance of strains

Standard bacteriological techniques as described in Miller (1972) were used for the culture, maintenance and manipulation of *E. coli* throughout this work. Strains were recovered from storage and maintained for frequent use on L agar (Luria agar: W/V Bacto tryptone 1%; Bacto yeast extract 0.5%; NaCl 1%; glucose 0.1%; pH to 7.2 with NaOH) plates. For long term storage (> 3 mo), strains were kept as stabs in nutrient agar (W/V Difco nutrient broth 1.0%; NaCl 0.5%; Difco agar 0.6%) at room temperature and at 4°C, and as glycerolized frozen cultures in nutrient broth (W/V Difco nutrient broth 2.5%) at -30°C and -70°C.

2.5.2 Growth of cultures

Most cultures were grown in L broth (Luria agar: w/o agar and glucose) either as overnight cultures of 10 ml volumes in MacCartney bottles rotated at 37°C or, for exponential cultures, shaken at 200 rpm in baffled side-arm flasks.

2.5.3 Measurement of growth rate – turbidimetry

Growth of shaken cultures was monitored turbidimetrically with a Klett-Summerson colourimeter (Klett-Summerson Co., New York, New York) holding a red filter transmitting light of 640-700 nm. Klett O.D. values were approximately 100 x $O.D._{600}$ values (i.e, a Klett O.D. of 10 = $O.D._{600}$ of 0.1) measured with a Gilson 250 spectrophotometer (Gilson Instrument Laboratories Inc., Oberlin, Ohio). A calibration curve was established in this laboratory for C600 grown in L broth. A Klett O.D. value of 40 is equivalent to a viable cell count of 2 x 10^8 cells/ml; a Klett O.D. value of 70 is equivalent to a viable cell count of 5 x 10^8 cells/ml. The relationship between Klett O.D. and cells/ml is linear until a Klett O.D. value of 75, and can be extrapolated through the origin.

2.6 IDENTIFICATION OF E. COLI STRAINS

Single colonies grown on L plates were re-streaked on minimal medium supplemented with thiamine (2 μg/ml), glucose or other carbon source (W/V 0.2%), and appropriate amino acids (20 μg/ml) to confirm auxotrophic markers, or on L plates containing an antibiotic (20 μg/ml) to confirm drug resistance markers. Normally only amino acids, carbon sources and antibiotic resistances were confirmed except as where noted. Antibiotics were filter sterilized (0.22 μm nitrocellulose filters) as were some sugars, and added to media cooled after autoclaving. Other sugars, amino acids and thiamine were autoclaved separately, then added to media cooled after autoclaving. MacConkey lactose agar (MCA) was purchased pre-mixed (Difco). Plates were incubated at 37°C unless the strain had a temperature sensitive lesion.

2.7 CONFIRMATION OF SPECIFIC GENOTYPES

2.7.1 lac

Much of the work described in this thesis depends upon the measurement of β-galactosidase synthesized from a lacZ gene carried on a lambda phage in strains normally unable to synthesize β-gal. The absence of a functional lac operon was confirmed on minimal plates supplemented with lactose or on MCA plates. The specific absence of lacZ was confirmed on L agar plates containing 5-bromo-4-chloro-3-indole-β-D-galactosidase (X-gal:30 μg/ml).

2.7.2 recA

All of the strains used as hosts for lambda lysogens were made recA to reduce the induction frequency of lambda. The recA phenotype was confirmed by the inability of a strain to grow on L plates supplemented with nitrofurantoin (NFT 3.0 μg/ml). NFT in acetone was added to L agar just before pouring.

2.7.3 rho

Rho mutants are hypersensitive to rifampicin (Rif; Guterman and Howitt 1979) and will not grow on L plates supplemented with 10 μg/ml Rif. Rif plates were prepared by dissolving Rif in dimethyl formamide (60 mg/ml) and adding this stock solution to molten L agar just before pouring.

2.7.4 nus

A lawn of the strain to be checked was made on a BBL agar plate (BBL Trypticase[R], Becton, Dickinson and Co., Cockeysville, Maryland; BBL agar W/V: BBL Trypticase[R] 1.0%; NaCl 0.5%; $MgSO_4$ 10 mM; agar 1.0%). Serially diluted lambda phage, nin5 and cI7 (Table 3), were spotted on the lawn, dried, and incubated overnight. Only nin lambda will form plaques on nus strains (Friedman 1970).

2.8 CONSTRUCTION OF STRAINS OF E. COLI

2.8.1 Growth of P1 lysates on donor strain

A P1 lysate was first grown on the donor strain. P1(vir) was grown by infection and lysis on R plates (W/V Bacto tryptone 1.0%; Bacto yeast extract 0.1%; NaCl 0.8%), P1(clr100) by heat induction from the lysogenized donor, and the lysates titred on C600. Most of the constructions described here are from donors carrying a transposon (Tn10 or Tn5) near the mutation of interest.

2.8.2 Transduction of genetic markers

A recipient strain was grown overnight in L broth plus 5 mM $CaCl_2$, spun down and resuspended in 10 ml of 10 mM $MgSO_4$, 5 mM $CaCl_2$. The resuspended cells were rotated for 15 min at 37°C then 0.3 ml removed and infected with 10^9 P1 (grown on the donor strain) by slow rotation for 20 min at 37°C. Infected cells were overlaid on L plates, supplemented with either tet (10 µg/ml, for Tn10) or kan (20 µg/ml, for Tn5) by resuspending 0.1 ml cells in 2.5 ml L top-agar (0.8% agar) which did not contain any antibiotic. For constructions using P1(clr100), the overlaid plates were incubated at 42°C to prevent lysogeny. Colonies arising after 24-36 hr incubation were streaked on selective plates (20 µg/ml antibiotic) to confirm the presence of the proximal transposon, then the phenotype of the isolate was checked.

2.8.3 Removal of transposons

To remove a transposon from a strain, that strain [illegible] either subjected to 2 cycles of a penicillin selection protocol (Miller 1972) using ampicillin as the lytic agent or, in the case of Tn10 strains, streaked on rich medium containing chlortetracyline (50 µg/ml)

and fusaric acid (12 μg/ml) to directly select for tetracycline sensitive clones (Maloy and Nunn 1981).

2.9 LYSOGENIZATION OF Δlac-recA STRAINS

The strain to be lysogenized, usually GR50-7 or MG4 (Table 1), was grown overnight in 10 ml L broth supplemented with maltose (W/V: 0.2%) and 10 mM $MgSO_4$, spun down and resuspended in 10 ml phage buffer (W/V: KH_2PO_4 0.3%; Na_2HPO_4 0.7%; NaCl 0.5%; Gelatin 10 μg/ml; 0.1 mM $CaCl_2$; 1 mM $MgSO_4$) supplemented with additional $MgSO_4$ (10 mM). To 0.3 ml cells (10^9) were added 5×10^7 lambda phage (moi=0.05) and this mixture gently rotated at 32°C for 20 min. The infected culture was serially diluted in the same solution and plated at a final dilution of 10^{-6} on MCA or L broth X-gal plates, both of which contained 10 mM $MgSO_4$. Red colonies on MCA or blue colonies on X-gal plates were streaked to obtain clones.

2.10 DETERMINATION OF MULTIPLICITY OF LYSOGENIZATION

2.10.1 β-galactosidase assay

The majority of lysogenized clones prepared from this low-multiplicity protocol are single lysogens. By picking 5 to 10 clones and performing β-galactosidase assays (see following), multiple lysogens were distinguished from single lysogens as expressing two or three times the level of β-gal as the majority of the group screened. If the β-gal values were the same for all clones, they were presumed to be single lysogens.

2.10.2 Ter testing

Providing the Ter protein in trans (by superinfection) and determining the number of phage particles produced of the lysogen type, mono- and multiply-lysogenized strains could be distinguished (Gottesman and Yarmolinsky, 1968). The strain to be Ter tested was grown to a Klett O.D. value of 40 in $MgSO_4$ (10 mM) supplemented L broth and 2.0 ml was superinfected with λ762 at an moi of 2.0 and grown for a further 2 hr. Five drops of $CHCl_3$ were added and the culture rotated for 10 min at 37°C. Dilutions of this were spotted on a fresh lawn of *E. coli* 159*ind*⁻ grown on L agar with

Table 1: Strains of *E. coli* used in this work

STRAIN	GENOTYPE	SOURCE or REFERENCE
159 ind⁻	uvr gal str^R(λcI857ind⁻)	Linn and Scaife 1978
AD1600	SA1030 rho15ts	Das et al. 1976
AD1919	SA1030 rho112	S. Garges
AD1919-1	AD1919 ilv-6960::Tn10	this work by P1 transduction from G6960
BU7026	recA56 Δ(lac-pro)	G. Chaconas
BU7026-3	BU7026 (λGR3)	this study, lysogen
BU7026-3-1919	BU7026-3 rho112	this study by P1 transduction from AD1919-1
C205	N99 nusA1	D. Ward
C347	SA500ilv Δ(chlD-blu)8 nusB5	D. Ward
C600	thi-1 thr-1 leuB6 lacY1 tonA21 supE44	Appleyard 1954
CSH50	ara Δ(lac-pro) strA thi	Miller 1972
G6960	ilv-6960::Tn10	Guterman et al. 1982
GR50-7	CSH50 ΔTn10::srl-1300 recA56	this work by P1 transduction from JC10240
GR50-8	GR50-7 (λGR1)	this work, lysogen
GR50-9	GR50-7 (λGR2)	this work, lysogen
GR50-10	GR50-7 (λGR3)	this work, lysogen
GR50-11	GR50-7 (λGR4)	this work, lysogen
GR50-12	GR50-7 (λGR5)	this work, lysogen
GR50-16	GR50-7 (λGR6)	this work, lysogen
GR50-19	GR50-7 (λGR7)	this work, lysogen
GR50-21	GR50-7 (λGR8)	this work, lysogen

Table 1: continued

STRAIN	GENOTYPE	SOURCE or REFERENCE
GR50-23	GR50-7 (λGR11)	this work, lysogen
GR50-24	GR50-7 (λGR12)	this work, lysogen
GR50-26	GR50-7 (λGR15)	this work, lysogen
GR50-28	GR50-7 (λGR10)	this work, lysogen
GR50-30	GR50-7 (λTL103)	this work, lysogen
GR50-8-1919	GR50-8 rho112	this work by P1 transduction from AD1919-1
HB101	hsdS20 recA13 ara-14 lacY1 galK2 mtl-1 xyl-5 rpsl20 supE44 proA2	Maniatis et al. 1982 (H.Boyer)
JC10240	HfrKL16 recA56 rpsE2300 relA1 ilv-318 spoT1 srl-1300::Tn10 thi-1 (P1clr100 Cam)	G. Chaconas
JG148	thi Δ(ara-leu)498 Δ(lac)X74	J. Greenblatt
JG148-3	JG148 ΔTn10::srl-1300 recA56	this study by P1 transduction from JC10240
JG148-4	JG148-3 (λGR1)	this study, lysogen
JG148-5	JG148-3 (λGR2)	this study, lysogen
JG148-6	JG148-3 (λGR3)	this study, lysogen
JG148-7	JG148-3 (λGR4)	this study, lysogen
JM103	thi Δ(lac-pro) strA endA sbcB15 hsdR4 supE (F' $proA^+$ $proB^+$ $lacI^q$ traD Δ(lacZ)M15	Messing et al. 1981
K556	N99 nusE71	D. Ward
MG1655	prototrophic (F^-,λ^-)	Guyer 1981

Table 1: continued

STRAIN	GENOTYPE	SOURCE or REFERENCE
MG4	MG1655 Δ(arg-lac)U169 recA56 ΔTn10::srl-1300 ΔTn10::zah-135	this study by P1 transduction from JC10240 and SH205
MG5	MG4 (λGR3)	this study, lysogen
MG7	MG4 (λGR11)	this study, lysogen
MG9	MG4 (λTL102)	this study, lysogen
MG10	MG4 (λTL108)	this study, lysogen
MG14	MG4 (λGR9)	this study, lysogen
N99	rpsL galK	Friedman 1970
N2076	thi-1 argH1 nadB4 lacY1 gal-6 malA1 xyl-7 ara-13 mtl-2 str-9 tonA2 supE44	Apirion and Watson 1975
N2077	N2076 rnc105	Apirion and Watson, 1975
NS428	recA (λAam11 Sam7 b2 red3 cI857)	Enquist 1979
NS433	recA (λEam4 Sam7 b2 red3 cI857)	Enquist 1979
RR1	HB101 $recA^{+}$	Maniatis et al. 1982 (H. Boyer)
SA500	rpsL his	Das et al. 1976
SA1030	SA500 galp3	Das et al. 1976
SA2244	trp lys lac gal str^{R} thi	K. Sanderson
SA2243	SA2244 sfrB14	K. Sanderson
SH205	HfrC phoA8 glpD3 glpR2 relA1 zah-735::Tn10 tonA22 Δ(argF-lac)U169	Schweizer and Boos 1983
X10015	rpsL Δ(argF-lac)U169 Δ(tonB-φ80att)X8605 rpo203 rho201	Guarente and Beckwith 1978

10 mM $MgSO_4$. Non-lysogens yield (approx) 10^2 pfu/ml; monolysogens, 10^4 pfu/ml and multiple lysogens, 5 x 10^6 pfu/ml.

CLONING

2.11 RECOMBINANT TECHNIQUES - GENERAL

Most of the techniques used here for cloning DNA fragments and confirming the structure of recombinant molecules are similar to or adapted from Maniatis et al. 1982 or Davis et al. 1980. Phage manipulations are adopted from Schleif and Wensink 1981. To facilitate the development of cloning strategies and to aid in the restriction analysis of recombinant molecules, a set of computer programs written by R. Staden (Staden 1977 and Staden 1978) was adapted for use on the University of Western Ontario Digital Equipment Corporation DECsystem10 computer.

2.12 PRODUCTION OF DNA

2.12.1 Plasmid DNA - large scale

Large amounts of plasmid DNA (>100 μg, to 2.0 mg) were prepared by first growing the host strain, transformed by the plasmid of interest (host strains were HB101 or RR1, Table 1) at 37°C in 400 ml volumes of L broth to a Klett O.D. value of 85. Chloramphenicol (170 μg/ml), where appropriate, or spectinomycin (300 μg/ml) was added and the cultures continued incubating overnight. Plasmid DNA was harvested, usually from a total volume of 800 ml, essentially as described by Clewell and Helinski (1972), using a Brij-sodium deoxycholate solution (W/V 1% : 0.4%) to lyse cells pre-treated with lysozyme and suspended in isotonic sucrose (W/V 25%). CsCl equilibrium gradients containing 0.2 mg/ml EtBr were centrifuged 48 hr at 45K rpm (Beckman Ti75 rotor, approx 150,000 x g). Plasmid bands were removed by side puncture, butanol extracted, dialysed, phenol extracted and finally dialysed overnight against DNA buffer (2.12.6).

2.12.2 Plasmid DNA - small scale

Small amounts of plasmid DNA (50 - 100 μg) were prepared from 25 ml L broth cultures, first grown to a Klett O.D. value of 50 before the addition of chloramphenicol (160 μg/ml), where

appropriate, or spectinomycin (300 μg/ml). Cultures were incubated overnight, washed with TE buffer and resuspended in 0.5 ml isotonic sucrose buffer (W/V: sucrose 25%; Tris 50 mM; pH 8.0; diethylpyrocarbonate 0.1%) and lysozyme (1 mg) added. Gentle lysis was accomplished by the addition of Brij-sodium deoxycholate (as above) followed by centrifugation at 22,000 x g (Sorvall[R] - GSA rotor, 13.5K rpm) for 20 min. The supernatent was phenol extracted, ether extracted, ethanol precipitated (2 x) and the pellet resuspended in 2.5 ml of an RNase solution (RNaseA 25 μg/ml; RNaseT1 400 U/ml). This was incubated 30 min at 37°C and the plasmid DNA recovered by spermine precipitation. The final pellet was resuspended in 100 μl TE buffer. This protocol yielded 50 - 100 μg plasmid DNA, sufficiently pure to be useful for all cloning manipulations and free of RNA as assessed by agarose gel electrophoresis.

2.12.3 Lambda DNA

Preparative amounts of lambda DNA (mg) were prepared by inoculating a large volume (400 ml) L broth supplemented with Mg^{++} ($MgSO_4$ 10 mM) with an inoculum prepared by infecting 2.0 ml of substrate cells (C600) with a low multiplicity of lambda (moi = 0.01) and shaking the culture until it was visibly lysed (Maurer et al. 1980). Phage were recovered from the culture supernatant by polyethylene glycol/NaCl precipitation (PEG 6000), after treatment by RNaseA (1 mg/ml) and DNAseI (1 mg/ml) as described in Schleif and Wensink 1981. PEG pellets were centrifuged on step gradients (Beckman, SW25 rotor, 25K rpm) and the phage recovered in a 1.0 ml vol of CsCl by dripping the gradient. The CsCl was removed by dialysis and phage DNA recovered by phenol extraction (2.13.1).

2.12.4 M13 dsDNA

M13 clones were used for the production of ssDNA probes but for cloning, the dsDNA replicative form was prepared. mp8 (Messing and Vieira 1982) dsDNA was grown by infecting a 400 ml culture of JM103 (Table 1) grown to a Klett O.D. value of 70 with mp8 (infectious particles) at a multiplicity of 10. This infected culture was shaken for 2 hr then treated as a large scale plasmid preparation after overnight growth in the presence of antibiotic (2.17.1).

2.13 PREPARATION OF DNA

2.13.1 Phenol extraction to remove protein

Proteins were removed from DNA preparations by extraction with re-distilled phenol (flushed with N_2, saturated with buffer (Tris 0.5 M; EDTA 1 mM)) and stored at -30°C. An equal volume of phenol was mixed by inversion with a DNA solution by slow rotation at room temperature. The phases were separated by low speed centrifugation (approx. 3000 x g) and the aqueous phase put in a clean tube. Plasmid DNA generally required 3 or 4 such extractions, bacteriophage required more (up to 10 x for M13). Following phenol extraction, a single extraction with phenol:chloroform (1:1) was made, followed by extraction with chloroform alone.

2.13.2 Ether extraction to remove phenol

For very small volumes of DNA (<1.0 ml), an equal volume of ether was used to extract the phenol/chloroform from the DNA. The ether was removed by pipette, the solution heated to 70°C for 10 min, then held in a vacuum desiccator for 5 - 10 min. Larger volumes of DNA were dialyzed for 24 hr.

2.13.3 Dialysis to remove phenol or salts

Traces of phenol or large amounts of CsCl left after gradient centrifugation were removed by dialysis. Lengths of dialysis tubing of an appropriate diameter (>1.0 ml vol) were sealed with clips and stirred in the cold (4°C) in a volume of 500 ml DNA buffer. About one hr was required to remove CsCl, whereas 24 hr and 4 buffer changes were required to remove phenol.

2.13.4 Butanol extraction to remove EtBr

DNA buffer saturated sec-butanol was used to remove EtBr. Equal volumes of saturated butanol and DNA solution were mixed for 20 min by inversion following which the butanol phase was removed. Extraction was continued until no pink colour appeared in the butanol. Butanol extraction was usually followed by phenol extraction.

2.13.5 Ethanol precipitation

To concentrate DNA or to remove salts from solutions of DNA, two volumes of re-distilled 95% ethanol were added to DNA having an NaCl content of 0.1 M. DNA solutions having little

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salt were brought to 0.3 M sodium acetate before the addition of ethanol. Ethanol:DNA solutions were held overnight at -20°C or for 15 min in a dry ice ethanol bath (-78 °C). The precipitated DNA was pelleted at 4°C by a 15 min spin in a microfuge (Eppendorf 5412, Brinkman Instruments (Canada) Ltd., Rexdale, Ontario) or, if a larger volume, at 20,000 x g for 20 min at 0°C in corex tubes (Sorvall[R] Centrifuge, SS-34 rotor). Pellets were drained, rinsed with 80% ethanol, respun for 5 min, drained, dried under vacuum desiccation for 2 min or until dry, and finally resuspended in DNA buffer or TE buffer (Tris 10 mM; pH 7.5; EDTA 1 mM).

2.13.6 Spermine Precipitation

A fresh spermine solution (0.1 M in H_2O) was added to a DNA solution on ice, to a final concentration of 10 mM (Hoopes and McClure 1981). This was held on ice 15 min then, spun at 4°C in a microfuge for 10 min. Pellets were drained, then washed with spermine wash (sodium acetate 0.3 M; magnesium acetate 10 mM; in 75% ethanol) by gentle vortexing, and held on ice for 1 hr. A 5 min spin followed, then pellets were drained, spun quickly (15 sec) and dried.

2.14 ESTIMATION OF DNA CONCENTRATION AND PURITY

2.14.1 Spectrophotometry

DNA solutions were diluted in H_2O and their absorbance at 260 nm and 280 nm determined in quartz cuvettes. Based on an $O.D._{260}$ of 1.0 for a 50 µg/ml solution of nucleic acids, the concentration of an unknown solution was established. An $O.D._{260}$:$O.D._{280}$ ratio of about 2 was used as an indication that the DNA was sufficiently free of protein.

2.14.2 Gel Electrophoresis

DNA solutions were assessed for damage, presence of chromosomal DNA and RNA, and for concentration by agarose gel electrophoresis (see following).

2.15 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction digests were done in a final volume of 40 µl on 1 - 2 µg DNA. A standard buffer (Tris 10 mM; pH 7.5; $MgCl_2$ 10 mM;

BME 10 mM; W/V Bovine serum-albumin 50 μg/ml) was supplemented with one of 100 mM (high), 50 mM (medium) or 10 mM (low) NaCl, according to the manufacturer's recommendation. Normally 2 Units of enzyme were added per μg DNA and the digest held at 37°C for 2 hr. Reactions were stopped by heating to 70°C for 10 min, then held on ice.

2.16 SEPARATION OF RESTRICTION FRAGMENTS BY SIZE

2.16.1 Agarose gel electrophoresis

Agarose gels (W/V 0.5% to 0.9%) were electrophoresed in a Tris-acetate buffer (Tris 40 mM; sodium acetate 20 mM; EDTA 1 mM; pH to 8.2 with acetic acid) at 40 volts overnight. EtBr (1 μg/ml) was added to the molten, cooled agarose before pouring. The apparatus used was of the submarine type holding a gel slab 15.0 cm wide, 28.0 cm long and 0.6 cm thick. Sample wells usually held a 50 μl volume.

2.16.2 Polyacrylamide gel electrophoresis

Polyacrylamide gels (W/V 6% to 8%: acrylamide: bis-acrylamide 29:1) were electrophoresed in a Tris-borate buffer (Tris 89 mM; Boric acid 89 mM; EDTA 25 mM; pH to 8.3 with acetic acid) at 40 volts overnight or for 3 hr at 200 volts. Gels were stained by soaking in an EtBr solution (0.5 μg/ml) for 15 min after electrophoresis. Polyacrylamide gels were run in a vertical apparatus holding gels 3 mm thick, 15 cm wide and 18 or 25 cm long. Sample wells held a 25 μl sample volume.

2.17 GEL PHOTOGRAPHY

EtBr stained DNA in agarose and polyacrylamide gels was visualized by placing the gel on a U.V. transilluminator which transmitted ultra-violet light of 366 nm (Ultra-violet Products Inc., San Gabriel, California). Illuminated in this fashion, gels were photographed with a large format (4 in x 5 in) camera using Tri-X film (Kodak ASA 400). The camera was mounted about 50 cm from the surface of the gel and exposures were for 20 sec.

2.18 PURIFICATION OF RESTRICTION FRAGMENTS FROM GELS

2.18.1 Excision of DNA fragments from gels

All restriction fragments for this work were recovered from agarose or polyacrylamide gels by excising the desired fragment band from a gel with a sterile scalpel. Bands were visualized by staining the gel lightly with EtBr (15 min, 0.5 μg/ml) after electrophoresis, and placing the gel, on a glass plate (2.5 mm) to attenuate U.V. light, on a U.V. transilluminator emitting light at 366 nm. Three types of gel were used.

2.18.2 Electro-elution from agarose

Early in this work, DNA fragments of all sizes were recovered by placing the gel slice carrying the fragment into dialysis tubing, adding 1.0 ml Tris-acetate electrophoresis buffer, and electrophoresing the tubing, at right angles to the current, at 80 volts for 3 hr at 4°C (Smith 1980). The gel slice was then removed, the end of the tubing re-sealed and the current reversed for 2 min. The buffer/DNA solution was filtered through a polycarbonate membrane before butanol extraction and ethanol precipitation. The final pellet was resuspended in TE or ligation buffer (2.21).

2.18.3 Extraction from low-melting-temperature agarose

Gel slices cut from low-melting-temperature agarose gels (usually 0.6%) were first weighed then heated to 68°C for 10 min, before being extracted at 30°C with an equal weight of pre-warmed phenol. Two further phenol extractions at room temperature were performed, followed by one each of phenol:chloroform (1:1), chloroform, and ether. DNA was recovered by spermine precipitation.

2.18.4 Elution from polyacrylamide

Small restriction fragments (<500 bp) were recovered from slices of polyacrylamide gels by a modified crush-and-elute protocol (Maxam and Gilbert 1980). A 1.0 ml disposable pipette tip was first plugged with silanized glass wool, then the tip was heat sealed. The gel slice was placed in this tip, ground to a paste with a glass rod, then resuspended in elution buffer (ammonium acetate 0.5 M; magnesium acetate 10 mM; EDTA 1 mM) and allowed to stand overnight at 37°C. The end of the pipette tip

was cut off and the elution buffer forced out of the tip by using a squeeze bulb. The disposable tip was rinsed with an additional 0.5 M elution buffer, the DNA recovered by ethanol precipitation, and the pellet rinsed several times with 80% ethanol.

2.19 DE-PHOSPHORYLATION OF RESTRICTED VECTOR DNA

In order to reduce the self-ligation of plasmid or M13 vectors cut with a single restriction enzyme, the vector was treated with calf intestinal alkaline phosphatase (CIP) before the ligation step (Chaconas and van de Sande 1980; Maniatis et al. 1982). After restriction and heating to $68^{o}C$ to inactivate the restriction enzyme, the sample was diluted to 100 µl in CIP buffer (Tris 50 mM pH 9.5; $MgCl_2$ 1 mM; $ZnCl_2$ 0.1 mM) and 1 Unit CIP added. The reaction was held at 37 ^{o}C for 30 min, a second 1 Unit aliquot of CIP added, and the sample held for a second 30 min incubation. The reaction was stopped by the addition of 5 mM EDTA and then heating to $70^{o}C$ for 10 min. Two spermine precipitations followed.

2.20 LIGATION OF DNA *IN VITRO*

2.20.1 For transfection and transformation

Restriction fragments and vector sequences to be ligated, purified as described, were mixed in equimolar (approx) or fragment excess quantities and co-precipitated in ethanol or spermine. The dried pellet was resuspended in 100 µl freshly prepared ligation buffer (Tris 10 mM pH 7.2; EDTA 5 mM; $MgCl_2$ 10 mM; ATP 10 mM; BME 10 mM) and 1 Unit T4 DNA ligase added. Cohesive end ligations were incubated at $12^{o}C$, blunt end ligations, at $16^{o}C$.

2.20.2 For *in vitro* packaging

Reaction conditions were identical except the ligation volume was kept to a minimum (10 to 20 µl). This was done to enhance the formation of concatemeric DNA, the substrate for lambda packaging proteins.

2.21 ADDITION OF SYNTHETIC LINKERS

2.21.1 Kinasing linkers

About 20 pmoles of synthetic linkers were kinased using 20 Units of polynucleotide kinase in 25 µl of kinase buffer (Tris 70 mM pH 7.6; $MgCl_2$ 10 mM; 5 mM DTT; ATP 0.25 mM). The reaction was carried out for 45 min at 37°C. Kinased linkers were stored frozen at -30°C.

2.21.2 Filling in 3' ends of DNA

The recessed 3'-OH ends left by HindIII digestion were filled in using the Klenow fragment of DNA polymerase I. About 2.0 µg of cut plasmid DNA, precipitated from the restriction reaction, was resuspended in a final volume of 25 µl nick translation buffer (Tris 50 mM pH 7.2; $MgSO_4$ 10 mM; BME 10 mM; dNTPs 0.1 mM) and 1 Unit Klenow fragment added. The reaction was incubated on the bench for 15 min, then extracted once with phenol and spermine precipitated.

2.21.3 Ligation of linkers to end-filled vector

To 1.0 µg of end-filled vector (0.2 pmole) was added 10 pmole of kinased linkers and a standard ligation protocol followed. This ligation mix was ethanol precipitated, overdigested with XbaI, electrophoresed and the linear band recovered and re-ligated before being used to transform HB101 (see following section).

2.22 IDENTIFICATION OF RECOMBINANT PLASMIDS

2.22.1 Transformation with plasmid DNA

Recombinant plasmids contained in ligation reactions were used to transform *E. coli* strains HB101 or RR1 (Table 1), made competent for transformation by a simple $CaCl_2$ heat shock procedure derived from that of Mandel and Higa (1972). Briefly, cells were grown in L broth to a Klett O.D. of 70, iced for 20 min, washed first with 0.5 vol of 0.1 M $MgCl_2$, then with 0.05 vol of 0.1 M $CaCl_2$ and held on ice for a further 30 min. To 200 µl of cells about 1.0 µg ligated DNA was added either in ligation buffer or diluted to 100 µl with TE buffer. This mixture was held a further 30 min, heated to 42°C for 2 min by swirling gently in a water bath, held for a further 30 min and finally incubated for 60 min at 37°C with an added 1.0 ml vol of L broth.

2.22.2 Screening transformed clones

Transformed cells prepared as described above were serially diluted in L broth and 100 μl volumes mixed with 2.5 ml of L top agar (L broth + 0.8% agar) without antibiotic and overlaid on L agar plates containing ampicillin at 20 μg/ml. Although it was always difficult to estimate the amount of DNA in ligation reactions and the efficiency at which ligation occurred, control transformations with intact plasmid DNA yielded approximately 10^6 transformants per ug DNA.

Colonies arising on the selective media containing ampicillin were patched onto rich plates containing tetracycline or chloramphenicol (both at 20 μg/ml) to identify clones carrying plasmids of the appropriate antibiotic resistance/sensitivity profile (see Fig A1). Such clones were re-streaked on L plates to obtain single colonies and the phenotype of these confirmed on antibiotic plates.

2.22.3 Screening plasmids by restriction

Clones of the correct phenotype were grown in culture in order to grow sufficient plasmid DNA to be analysed by restriction digests. Early in this work, a protocol similar to that described in 2.6.2 was used allowing 6 clones to be screened per day. More recently, using the methodology of Holmes and Quigley (1981), up to 24 clones could be screened each day. This protocol relies on a 60 sec boiling step to lyse 1.4 ml of an overnight culture of each clone, resuspended in isotonic sucrose (W/V 25 %) and lysozyme (1.0 mg/ml). Enough plasmid DNA is recovered after isopropanol precipitation to permit several restriction digests. To visualize restriction fragments less than 1.0 kb in length, the restricted sample is treated with 2.5 g RNaseA for 10 min at room temperature just prior to loading. This removes the large amount of RNA that would otherwise obscure small fragments.

2.23 IDENTIFICATION OF RECOMBINANT LAMBDA PHAGE

2.23.1 Transfection with recombinant lambda DNA

Recombinant lambda phage in ligation reactions were used to transfect TGL72, an E. coli strain which transfects efficiently, made competent for DNA uptake in exactly the same

manner as strains for transformation (2.[illegible].1). After heat shocking the cell/DNA solution, the mixture was held on ice for 30 min, diluted in phage buffer (2.8) with 10 mM $MgSO_4$ and overlaid on BBL agar plates with BBL top agar (2.8), both containing 10 mM $MgSO_4$. To diluted aliquots, it was necessary to add 0.3 ml substrate cells, either TGL72 or C600 grown in L broth plus 10 mM $MgSO_4$, then resuspended in phage buffer plus 10 mM $MgSO_4$. Transfection efficiency with intact lambda DNA was approximately 10^5 plaques / μg DNA.

2.23.2 In vitro packaging of lambda DNA

If no plaques arose after the ligation and transfection protocols, the constructions were repeated by ligating the fragments in a small volume (2.20.2) and packaging the recombinants in vitro (Hohn and Murray, 1977), using a simplified version of that protocol (Davis et al. 1980). Rather than preparing the cell extracts containing the lambda proteins necessary for packaging DNA separately, the two strains, NS428 and NS433 (Table 1), are grown to a Klett O.D. value of 35 at 32°C, induced at 42°C for 15 min, grown a further 3 hr and then mixed and resuspended in packaging buffer (Tris 40 mM; V/V BME 0.1%; DMSO 7%). To 20 μl of these induced packaging strains was added 1 μg of lambda DNA in a 10 μl vol and 1 μl ATP (0.1 M) and the mixture incubated 1 hr at 37°C. The mixture was serially diluted and overlaid with 0.3 ml C600 on BBL agar as described previously (2.23.1). The protocol yielded approximately 10^6 plaques / μg DNA, an order of magnitude greater than the transfection protocol.

2.23.3 Screening phage by restriction

Plaques obtained by either transfection or in vitro packaging were picked with a pasteur pipette and stored in 1.0 ml of SM buffer (Tris 20 mM pH 7.5; NaCl 0.1 M; $MgSO_4$ 1 mM; W/V gelatin 0.01%). 100 μl of each plaque lysate were added to 0.3 ml C600 that had been grown overnight in L broth supplemented with maltose (0.2%) and $MgSO_4$ (10 mM) and resuspended in phage buffer (2.8) and phage allowed to absorb to these cells for 20 min at 30°C. The infected culture was overlaid with BBL top agarose on BBL agarose plates (both with 10 mM $MgSO_4$), incubated at 37°C and monitored for confluent lysis (6 - 8 hr). Confluently lysed plates were cooled for 15 min at -10°C, overlaid with 5.0 ml phage elution buffer (Tris 10 mM pH 7.6;

EDTA 10 mM) and allowed to stand overnight at 4°C. The elution buffer containing phage was removed by pipette and stored at 4°C with a drop of $CHCl_3$. Lambda DNA was extracted from 0.5 ml aliquots of this phage solution according to a protocol described in Davis et al. (1980) which permitted enough lambda DNA to be purified to visualize restriction fragments on agarose gels. Briefly, to 0.5 ml phage solution was added 1 µl DEP and 11.5 µl SDS (W/V 10%), the tubes mixed by inversion and to heated to 70°C for 10 min. After incubation 65 µl of 5.0 M potassium acetate was added, the tubes inverted once and then held on ice for 30 min before a 15 min microfuge spin at 4°C. The supernatant was removed and nucleic acids precipitated by the addition of 1.0 ml ethanol at room temperature and a 5 min microfuge spin. The pellet was dried and resuspended in 250 µl of a TE/RNaseA (10 µg/ml) solution and incubated at 37°C for 30 min. DNA was recovered by a spermine precipitation. The final pellet was resuspended in 40 µl of an appropriate restriction buffer. After restriction, the sample was loaded directly into agarose gels without the heating step normally used to stop restriction reactions. Heating at this point to 70°C prevented the resolution of discrete bands.

2.24 IDENTIFICATION OF RECOMBINANT M13 PHAGE

2.24.1 Transfection with recombinant M13

Ligation reaction products were used to transform E. coli strain JM103 (Table 1) made competent by a $CaCl_2$ heat shock protocol (2.22.1) except that after heat shock, the transformed cells were plated immediately. Transformed cells were diluted, mixed with 0.3 ml of exponentially growing JM103, and overlaid on L agar in 2.5 ml L top agar containing X-gal (70 µl of W/V X-gal 2%) and IPTG (10 µl of IPTG 0.1 M). Plates were incubated at 37°C overnight.

2.24.2 Screening of recombinant M13 by size

Recombinant plaques appeared white after overnight incubation, whereas a recircularized vector plaque, without an insert, appeared blue. White plaques were picked by Pasteur pipette and added to 2.0 ml of JM103 grown to a Klett of 30. These infected cultures were incubated at 37°C for 6.5 hr, spun in a microfuge for 5 min, then the supernatants containing M13 phage were removed and stored at

4°C. To 40 µl of these phage lysates were added 10 µl DIGE buffer (Messing et al. 1981: NaCl 1.0 M; EDTA 0.2 M; W/V SDS 10%; BPB 1%) and the mixture electrophoresed on 0.7% agarose gels. Recombinant M13 clones are larger than the vector and, therefore, can be distinguished as slower running bands.

2.24.3 Determination of insert orientation

Singlely restricted vector DNA yields recombinants carrying the restriction fragment in either orientation. Before proceeding to a restriction analysis of M13 recombinants, it was useful to confirm that the clones to be screened carried fragments in both orientations. Only one of the two possible orientations is useful for preparing ssDNA probe. Phage with the same fragment cloned, but in opposite orientations, will hybridize via their complementary insert and can be distinguished on agarose gels as running slower than unhybridized ssDNA. 20 µl of 2 phage lysates were mixed, 10 µl DIGE buffer added, and the mixture incubated at 65°C for 1 hr and electrophoresed immediately.

2.24.4 Screening M13 phage by restriction.

Recombinant M13 lysates (2.24.3) were used to infect a 25 ml culture of JM103 grown to a Klett O.D. value of 70 at a moi of 2 and the infected culture grown 2 hr. This culture was then processed as a small scale plasmid prep (2.17.2) and the resulting dsDNA analyzed by agarose gel electrophoresis of restricion enzyme digests.

2.25 CONFIRMING THE STRUCTURE OF RECOMBINANT MOLECULES.

The screening of recombinant plasmids, lambda and M13 molecules depended on visualizing a restriction fragment of the correct size generated from a small scale crude DNA preparation. To confirm that the fragment cloned was the correct fragment, a larger amount of DNA was prepared, purified, and a more extensive restriction analysis done. This involved visualizing all of the predicted restriction fragments from the vector and insert with several restriction enzymes to confirm their sizes. The size and orientation of the insert was, where possible, confirmed by digesting the construction with an enzyme that cut the

Table 2: Plasmids used in this work

PLASMID	DESCRIPTION	SOURCE or REFERENCE
pBR325	Cam^R Amp^R Tet^R	Bolivar 1978
pGR1	Cam^R Amp^R [rplJ'L rpoBC']	this study
pGR3	Cam^R Amp^R [rplJ'L' rpoC']	this study
pGR4	Cam^R Amp^R [rplJ'L' rpoB'C']	this study
pGR5	Cam^R Amp^R [trpB'A lacZ'](HindIII)	this study
pGR6	Cam^R Amp^R [trpB'A lacZ'](XbaI)	this study
pGR7	Amp^R [rpoB', rplL', rplK'A prplJ trpB A lacZ]	this study
pGR8	Amp^R [rplL']	this study
pGR9	Amp^R [rpoB']	this study
pRJ1	Amp^R Tet^R [rplK'AJL rpoB']	R. Jones
pTL20	Cam^R Amp^R [rplK'AJL rpoBC]	T. Linn and this study
pTL512	Tet^R [rplJ'L']	T. Linn
pBal-12	Amp^R Tet^R [rplK'A prplJ rplL' rpoB]	T. Linn and this study

Table 3: Bacteriophage used in this work

LAMBDA	DESCRIPTION	SOURCE or REFERENCE
762	cI imm nin5 (att-red) [supF]	Murray et al. 1977
c17	c17 cI	Friedman 1970
cI21	cI21 imm21	lab stock
cI857	cI857Ts Sam7	lab stock
drif^{d}18	cI857 Sam7 [rrnB tufB rplKAJL rpoBC]	Kirschbaum and Konrad 1973
GR1	imm21 nin5 [rplK'AJ' trpB'A lacZY']	this study
GR2	imm21 nin5 [rplL'B' trpB'A lacZY']	this study
GR3	imm21 nin5 [rplKAJL,rpoB' trpB'A lacZY]	this study
GR4	imm21 nin5 [rplJ'L' trpB'A lacZY']	this study
GR5	imm21 nin5 [trpB'A lacZY']	this study
GR6	imm21 [plac5 lacZYA']	this study
GR7	imm21 nin5 [rplKAJ' trpB'A lacZY']	this study
GR8	imm21 nin5 [rplK' trpB'A lacZY']	this study
GR9	imm21 nin5 [rrnB' trpB'A lacZY']	this study
GR10	imm21 nin5 [rplK'A,rplJp trpB'A lacZY]	this study
GR11	imm21 nin5 [rplKAJL,rpoB' trpB'A lacZY]	this study
GR12	imm21 nin5 [rplJ'L,rpoB' trpB'A lacZY]	this study
GR15	imm21 nin5 [rpoC'orfX',trpB'A lacZY]	this study
JDW36	imm21Ts nin5 [trpED'B'A lacZY']	Windass and Brammar 1979
KV1	imm21 nin5 [lacZ rpoB']	this study

Table 3: continued

LAMBDA	DESCRIPTION	SOURCE or REFERENCE
metA20	cI857 Sam7 [purD rrnE metA]	Yamamoto and Nomura 1976
NM540	imm21 nin5 att	Wilson and Murray 1979
NM616	imm21 nin5 att [lacZ]	Wilson and Murray 1979
plac5	cI857Ts Sam7 [lacZYA']	G. Mackie
p(rpl-rpo)76	imm21 [rplK'AJL rpoBC]	Linn and Scaife 1978
TL25	imm21 nin5 [trpED'A' lacZY']	Linn and Ralling 1985
TL102	imm21 nin5 [rplK'AJ' trpA' lacZY']	T. Linn
TL103	imm21 nin5 [rplK'A, rplJp trpB'A lacZY']	T. Linn
TL108	imm21 nin5 [rplK' trpA' lacZY']	Linn and Ralling 1985
UT1	imm21 cts nin5 [rplJ'L rpoB', trpB A lacZY']	this study

Other

φX174	am16	J. Colasanti
M13 mp8	[lacZ]	Messing and Vieira 1982
MP8-JL	mp8 [rplJ'L']	this study
MP8-587	mp8 [rpoB']	this study
MP8-B	mp8 [rpoB']	T.Linn, this study
P1vir	vir	lab stock
P1clr	clr100Ts Cam^R(Tn9)	A. Białkowska

fragment asymetrically internally, visualizing the resulting fragments, and confirming that they also met with predictions of the fragment sizes from knowledge of the DNA sequence.

ASSAY OF TRANSCRIPTIONAL FUSIONS

2.26 GROWTH OF LYSOGENIZED STRAINS

2.26.1 Media

Strains of E. coli lysogenized with recombinant lambda phage were grown in a base medium, AB (Clarke and Maaloe 1967), supplemented with 2 μg/ml thiamine and as follows: AB1 - 0.4% glycerol; AB2 - 0.4% glucose; AB3 - 0.4% glucose and 40 μg/ml of 20 amino acids; AB4 - 0.4% glucose and 0.4% casamino acids; AB5 - 0.4% glucose and 0.4% casamino acids and 0.2% yeast extract; AB6 - 0.4% glucose and 1.0% casamino acids and 0.5% yeast extract. AB1 and AB2 were also supplemented with individual amino acids as needed to satisfy the auxotrophic requirement(s) of some host strains. In the case of the very slow growth rate experiments, glycerol supplementation of AB1 was replaced by acetate at 0.2%.

2.26.2 Preparation of cultures

A single colony of the strain to be assayed was inoculated into a 10 ml aliquot of the same medium to be used and grown overnight at the temperature to be used for growth of the culture (usually 37°C). The next day, a 20 ml vol of this medium was pre-warmed and shaken at 200 rpm (15 - 30 min) before being inoculated with 20 - 200 μl of the overnight culture. These 20 ml cultures were shaken at 200 rpm in baffled side-arm flasks and their growth monitored by Klett readings taken at 15 - 30 min intervals. Growth rate was calculated from a plot of Klett O.D. values against time on $\log_{10}$ paper (Appendix Fig A2).

2.26.3 Steady state growth

Most of the data cited in this work is from cultures of lysogenized E. coli, grown in media AB1 - AB6 under steady state conditions, manifest as a straight line plot of log Klett O.D. vs. time (Appendix Fig A2). Cultures were sampled at Klett O.D. values of 7 - 8 and 15 - 16, having allowed at least 2 doubling times under

the assay conditions before sampling. After sampling, cultures were monitored to ensure exponential growth continued for at least another doubling time.

2.26.4 Calculation of growth rate

Growth rate is normally expressed as doublings/hr, that is the number of times in one hour that the mass of a culture doubles in value. The reciprocal of this number, the doubling time, is the time taken for a culture's mass to double in value. In this work, growth rate values are always expressed as doublings/hr. A culture with a growth rate of 2.0 is undergoing 2 doublings/hr or is doubling its mass every 30 min (0.5 hr). The growth rate is calculated by determining the slope of a line drawn through the $\log_{10}$ Klett O.D. values plotted against time (Appendix Fig A2).

2.27 DETERMINATION OF β-GALACTOSIDASE LEVELS

2.27.1 β-galactosidase assay

β-galactosidase assays were performed according to Miller (1972), with minor modifications. From cultures grown to Klett O.D. values of 7 - 8 and 15 - 16 were removed 2 ml vol of cells which were iced immediately and held for 20 min with gentle shaking. Duplicate 20 - 400 µl aliquots (depending on β-gal levels) were added to Z buffer (Na_2HPO_4 0.6 M; NaH_2PO_4 0.4 M; KCl 10 mM; $MgSO_4$ 1 mM; BME 20 mM) to give a final volume of 800 µl. A drop of $CHCl_3$ was added, then 2 drops 0.1% SDS, the mixture vortexed vigorously for 10 sec and incubated at 28°C for 5 min. 200µl of fresh ONPG (4 mg/ml in Z buffer) was added and a stop watch started. The tube was vortexed quickly to mix the contents and replaced in the 28°C waterbath. At 15 - 30 min, the first tube of a duplicate pair was removed and the reaction stopped by the addition of 0.5 ml 1.0 M Na_2CO_3. The second tube was incubated for twice the length of time and stopped. After stopping, reactions were held on ice until the $O.D._{600}$ value could be measured.

2.27.2 Total protein assay

A sufficient volume of cells was removed and iced for β-gal assays to allow a 1.0 ml volume to be frozen at -30°C for subsequent total protein measurement. Total protein was measured

according to Bradford (1976) using BioRad dye reagent concentrate (V/V ethanol 4.7%; phosphoric acid 8.5%; Coomassie brilliant blue G250 0.01%). Frozen cells were thawed and divided into three 300 μl aliquots for assay in triplicate. To each 300 μl sample was added 300 μl TCA (W/V 10%) and the mixture held on ice for 15 min prior to a 10 min microfuge spin at 4°C. The resulting pellet was resuspended in 1.0 ml 0.05 M NaOH and 250 μl BioRad dye reagent concentrate added. After 30 min at room temperature, the tubes were held on ice until their $O.D._{595}$ could be measured. Protein content was determined by constructing a standard curve from a solution of bovine gamma globulin (1 mg/ml).

2.27.3 Calculation of β-galactosidase units

The β-galactosidase assay protocol used in this work was developed by Miller (1972) and is based on the hydrolysis of the chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG). The activity of β-gal is assessed by determining the increase in the $O.D._{420}$ (absorbance) of the sample. Therefore β-gal Units are $O.D._{420}$/min per ml per $O.D._{600}$ sample. This effectively normalizes Units to the mass or dry weight of the culture which is the parameter measured by O.D. (Koch 1981).

The equation used in Miller reads:

$$\beta\text{-gal units} = 1000 \times \frac{\text{sample } O.D._{420} - (1.75 \times \text{sample } O.D._{550})}{t(\text{min}) \times \text{vol(ml)} \times O.D._{600}(\text{cells})}$$

where sample = cells plus ONPG

t = time of incubation at 28°C

vol = volume of cells assayed

$O.D._{600}$(cells)= optical density at 600 nm of the culture

To relate β-gal synthesis to the rate of synthesis of total protein rather than mass, total protein assays were made on samples taken coincidentally with those for β-gal assay.

Miller's formula was changed to read:

$$\beta\text{ - gal units} = 1000 \times \frac{\text{sample O.D.}_{420} - (1.75 \times \text{sample O.D.}_{550})}{t(\text{min}) \times \text{vol(ml)} \times \text{protein}(\mu g)}$$

This normalizes β-gal values to weight of protein.

No statistical analysis was performed on the β-gal values measured by either approach. Rather the four values derived from duplicate samples, taken at two sampling points, were averaged and any value differing from the mean by more than 10 percent was discarded, a situation which seldom arose. If two experiments were done on the same lysogen under the same conditions and the growth rates were identical, the values of the experiments were again simply averaged. A rule of thumb for comparing values from different lysogens is to consider each value as plus or minus 10 percent.

DIRECT EXAMINATION OF TRANSCRIPTS

2.28 HYBRIDIZATION OF ^{3}H-RNA TO ssDNA

2.28.1 Preparation of pulse labeled RNA

Prior to labeling E. coli, strains were grown as for β-gal assay in AB5. At a Klett of 30, a 1.0 ml aliquot of the culture was added to 300 prewarmed [5,6-^{3}H] uridine (^{3}H-uridine) dissolved in 100 μl water (6 mM ^{3}H-uridine, final concentration). After a 50 sec pulse, the culture was transferred to 0.3 ml frozen sodium azide (0.04 mM in AB), mixed and held on ice. The labeled cells were harvested by microfuge centrifugation for 5 min at 4°C, then the RNA extracted in a manner fashioned after Summers (1970). Simply, the cell pellet was resuspended in 1.0 ml protoplasting buffer (Tris 10 mM pH 8.0; EDTA 8.0 mM; sucrose 0.45 M; W/V lysozyme 80 μg/ml) held 10 min on ice and microfuged for 5 min at 4°C. Protoplasts were resuspended in 0.2 ml lysing buffer (Tris 10 mM; NaCl 10 mM; sodium citrate 1.0 mM; W/V SDS 1.5%) to which 3 μl DEP were added. After mixing, the protoplasts were held at 37°C for 5 min, then 0.1 ml. of cold saturated NaCl added and mixed by single inversion and held on ice for 10 min. Proteins were pelleted by a 15 min microfuge spin at 4°C and nucleic

acids recovered from the supernatent by overnight ethanol precipitation. The final pellet was resuspended in 100 µl 2 x SSC and stored at -30°C.

2.28.2 **Preparation of ssDNA probes**

Single strand complementary DNA was prepared by growing M13 phage carrying inserts cloned as described previously (2.24). The protocol used to grow ss infectious M13 (2.24.3) was scaled up, using the single plaque recovered from transfection and grown in 2.0 ml culture as the source of the first inoculum. Scaling up was accomplished in 2 steps: the first to 25 ml, the second to 400 ml. The large scale preparations were clarified of debris and infectious M13 recovered from the supernatent by PEG precipitation (2.17.3). PEG pellets were resuspended in 12 ml TE buffer and mixed with 6.0 ml W/V CsCl 65% to give a solution with a density of 1.31 g/ml. This was centrifuged at 50K rpm (Beckman TI75 rotor) for 48 hr to equilibrium. The phage band was removed by side-puncture in a 2 - 3 ml volume yielding a total of approximately 10^{14} phage. After removing CsCl by dialysis against TE, the phage DNA was purified away from phage protein by extensive extraction with phenol. M13 ssDNA preparations had a final concentration of .15 - .3 µg/ml.

2.28.3 **Hybridization of ^{3}H-RNA to ssDNA in liquid**

Hybridization reactions were carried out in triplicate and as described by Maniatis et al. (1982), in a volume of 100 µl held at 45°C for 18 hr. Each reaction contained 2.0 µg RNA and 0.2 µg DNA, providing at least a 10-fold excess of DNA (Appendix, Fig A3) in hybridization solution (V/V Formamide 50%; NaCl 0.6 M; Tris 80 mM pH 8.4; EDTA 4.0 mM; W/V Yeast RNA 10 µg/ml). Formamide was freshly de-ionized by the addition of 2 - 3 gm of ion exchange resin (BioRad AG501-X8, 20 - 50 mesh) to 5 ml formamide and standing for 30 min. After mixing, the tubes were heated to 70°C for 10 min and immediately transferred to 45°C.

2.28.4 **Determination of mRNA hybridized to DNA**

RNA that was not hybridized to DNA was degraded by the addition of 0.9 ml of an RNase mixture (RNaseA 25 µg/ml; T1 20 units/ml) in TE buffer. The samples were held at room temperature for 50 min and then incubated at 37°C for 10 min before being diluted

into 15 ml wash solution (KCl 0.5 M; Tris 10 mM pH 7.2; Nygaard and Hall 1963) in stainless steel filter holders, held on a Hoefer 10 place filter apparatus (Hoefer Scientific Instruments, San Francisco, California). The solution was filtered slowly (15 mm Hg) through 25 mm nitrocellulose filters (Schleicher Schuell, Keene, New Hampshire) with a pore size of 0.45 μm. Each filter was washed 5 times with further 15 ml volumes of wash solution. The filters were then dried, suspended in 5.0 ml scintillation fluid (toluene: omnifluor^R; New England Nuclear Omnifluor^R 4 g/l), dark adapted overnight and the cpm determined by scintillation counting (Beckman LS7500, Beckman Instruments). Background counts were determined for each RNA preparation by hybridizing to mp8 DNA without a cloned insert.

2.29 S1 MAPPING OF TRANSCRIPTS

2.29.1 Preparation of ^{35}S-DNA probe

Plasmid pGR8 (Fig. 6) was grown and purified as described previously (2.17.1). To remove all traces of chromosomal DNA, 300 μg of pGR8 in 1.5 ml of TE were electrophoresed on a 0.5% low melting temperature agarose gel, the plasmid band excised and plasmid DNA was recovered by phenol extraction and ethanol precipitation. Gel purified DNA (2.0 μg) was restricted with EcoRI and labeled by an end-fill reaction as in 2.21.2, except ^{35}S-dTTP (10 μCi; 10 pmole dTTP) was added, the reaction allowed to stand 15 min at room temperature, then unlabeled dTTP added to 1.0 nM for an additional 5 min. The reaction mixture was extracted with a 1:1 solution of phenol:chloroform, then passed over a G50-Sepharose (Pharmacia) column with a void volume of 1.0 ml. Four fractions of 250 μl were collected with the end-labeled plasmid found in fraction 3. Labeled DNA was precipitated with ethanol, dried and resuspended in high salt restriction buffer (2.14) and restricted with SalI.

2.29.2 Hybridization of RNA to ^{35}S-DNA probe

The hybridization protocol used was derived from that originally described by Berk and Sharp (1977) with modifications as described by Barry et al. (1980). Cold RNA was prepared as in 2.25.1, omitting the labeling step and scaling up the protocol to 5.0 ml instead of 1.0 ml. Each hybridization reaction contained 25 μg RNA, 10 μg

yeast RNA and approximately 50 ng of end-labeled pGR8 DNA. This mixture was co-precipitated, dried and resuspended in 35 µl hybridization solution (28 µl freshly de-ionized Formamide; 7 µl PIPES salts: PIPES 40 mM pH 6.4; EDTA 1.0 mM; NaCl 0.4 M) and then heated to 80°C for 10 min before being transferred immediately to 53°C and incubated for 3 hr.

2.29.3 S1 digestion of unhybridized nucleic acids

After 3 hr under hybridizing conditions, the tubes were opened while still in the water bath and 1000 units S1 nuclease added in 350 µl S1 buffer (NaCl 0.28 M; sodium acetate 50 mM; $ZnSO_4$ 4.5 mM). The tubes were closed, vortexed quickly and placed immediately in a 37°C water bath for 30 min. The reaction was stopped with 50 µl of 4.0 M ammonium acetate and put on ice. Hybrids were recovered by the addition of 20 µg yeast RNA, 50 µl 1.0 M NaCl and ethanol precipitation. The final pellet was resuspended in 20 µl TE for native gel electrophoresis or in 90% formamide for denaturing gel electrophoresis.

2.29.4 Resolution of S1 resistant fragments on native gels

RNA/DNA hybrids resistant to S1 nuclease digestion (S1 hybrids) were electrophoresed on polyacrylamide gels, run as described previously (2.15.2) using 6% acrylamide and electrophoresing for 3 hr at 200 volts. To accurately size the fragments which survived S1 nuclease digestion and to better resolve the intensity of the different size bands, denaturing gels were run to visualize the ^{35}S-ssDNA portion of the hybrid. These gels were 8% acrylamide containing 7.0 M urea and 1.5 mm thick. S1 hybrids were prepared as described except after the final ethanol precipitation, the pellets were resuspended in a denaturing solution (V/V Formamide 90%; W/V Bromophenol blue 0.02%; Xylene cyanol 0.02%), heated to 90°C for 1 min and loaded quickly on the denaturing gel. The gel had been pre-run for 1 hr to raise its temperature to 55°C (surface thermometer) and this temperature was maintained by a voltage of 1000. Samples were electrophoresed for 1.5 to 2.0 hr.

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2.30 AUTORADIOGRAPHY OF GELS

Both native and denaturing gels were dried onto filter paper and autoradiographed without intensifying screens for 24 - 48 hr at -70°C. Kodak X-omat XAR-5 film was used for all autoradiography. Autoradiograms were scanned with a laser densitometer (2202 Ultrascan, LKB Bromma) to estimate the relative intensity of the fragment bands within a track on a gel.

FIGURE 2: Transducing lambda phages that provided chromosomal DNA for sub-cloning. All of the recombinant DNA work described in this thesis utilized E. coli chromosomal fragments carried on lambda specialized transducing phages whose physical maps had been established. Lambda sequences are shown as narrow lines; E. coli chromosomal sequences are shown as wide cross hatched lines or where more detail is shown, coding sequences are hatched and non-coding sequences are open. A map of the wild type lambda genome is shown above the figure for reference. **(a)** $\lambda drif^{d}18$ (Kirschbaum and Konrad 1973). This phage carries more than 20 kb of chromosomal DNA including the entire rplKAJLrpoBC cluster, shown here beneath the figure enlarged for clarity. **(b)** λp(rpl-rpo)76 (Linn and Scaife 1978). A 10 kb fragment of $\lambda drif^{d}18$ is carried in the λp(rpl-rpo)76 genome. The smaller number of restriction fragments made this phage more useful for sub-cloning rplJL-rpoBC DNA. **(c)** λmetA20 (Yamamoto and Nomura 1976). This transducing phage was used as a source of a ribosomal RNA operon promoter, carried as shown, on an 800 bp EcoRI-HindIII fragment (Brosius et al. 1978).

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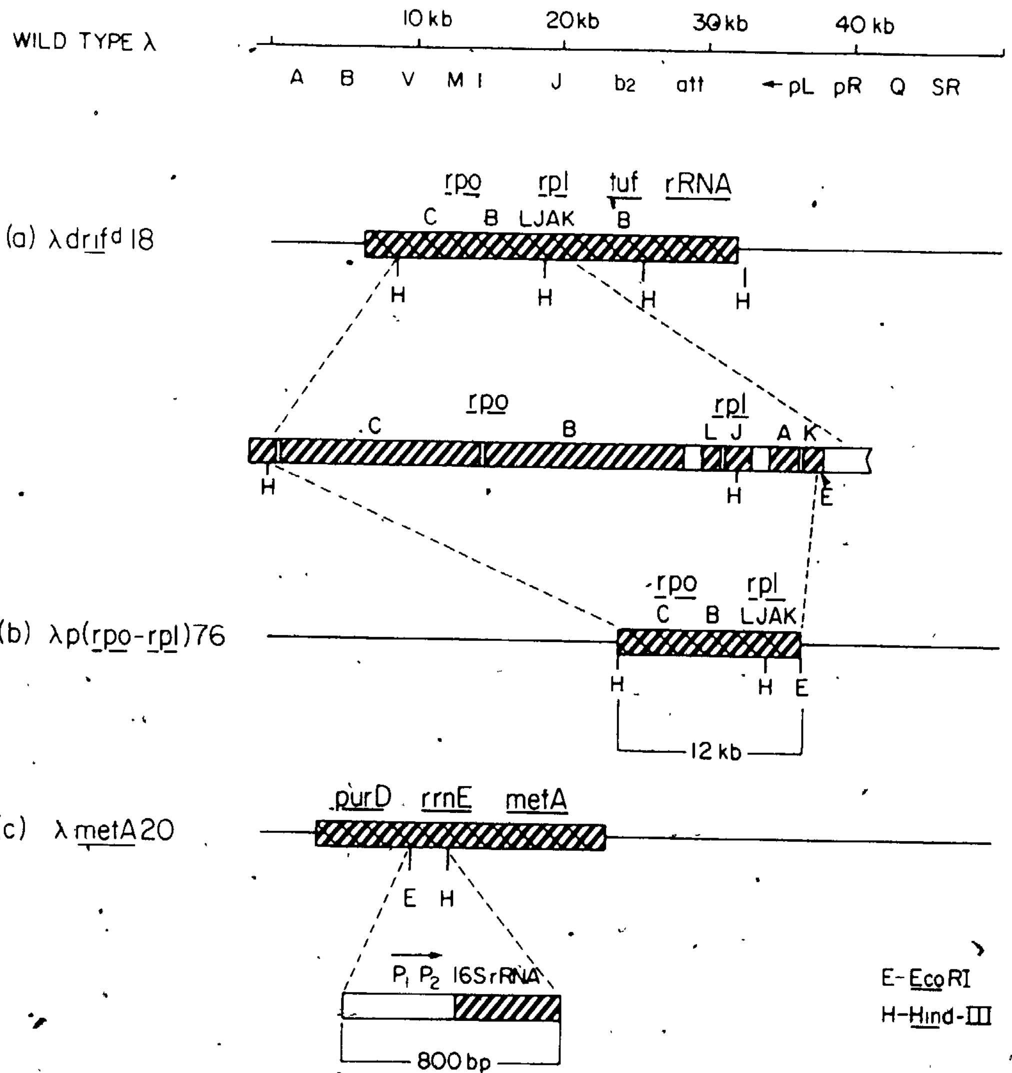
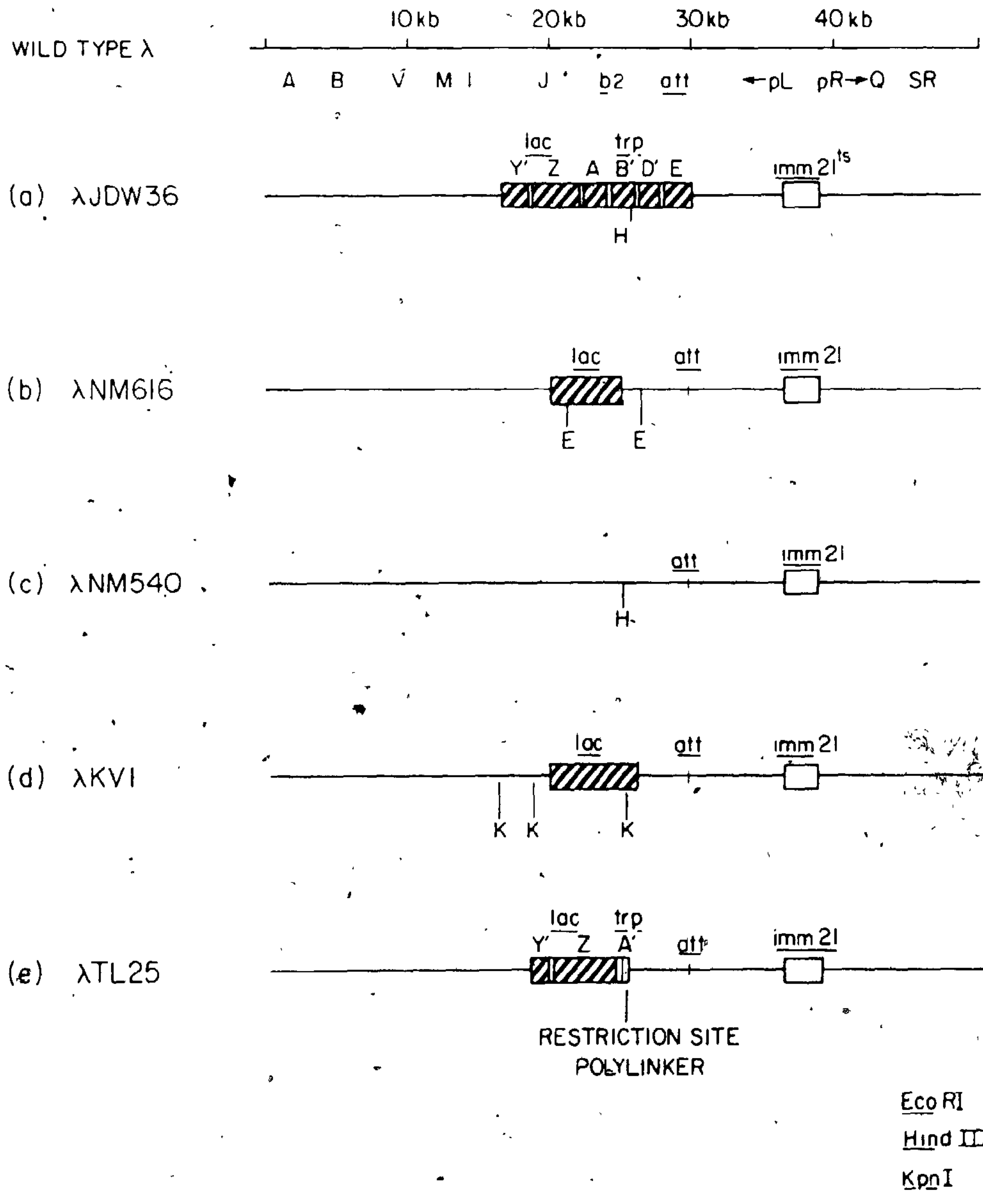
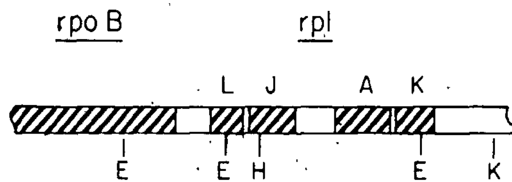


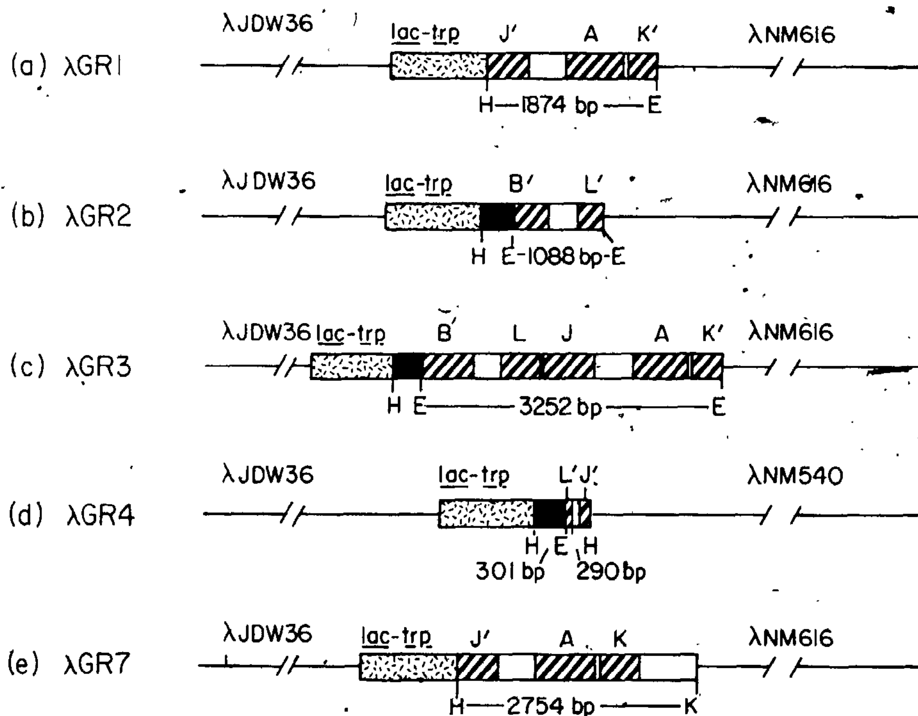
FIGURE 3: Phage lambda vectors used for the *in vitro* construction of transcriptional fusions. Lambda sequences are shown as narrow lines except the immunity regions which are shown as open boxes. *E. coli* chromosomal sequences are shown as wide lines with coding sequences hatched and non-coding sequences open. A map of the wild type lambda genome is shown above the figure for reference **(a)** λJDW36 (Windass and Brammer 1979). This transducing phage carries the W205*trp*-*lac* deletion (Barnes et al. 1974; Mitchell et al. 1975) which has removed most of the sequences between *lac* and *trp* including *trpt* but leaving the *lacZ* ribosome binding site intact (see 1.3 for details). The *Hind*III site in *trpB* was used as the cloning site for most of the constructions described here. Because there are no other *Hind*III sites between the site in *trpB* and the left end of the JDW36 lambda genome, this left arm can be purified easily from an agarose gel separating *Hind*III digested λJDW36 DNA. **(b)** λ NM616 (Wilson and Murray 1979). This lambda phage provided a source of lambda right arm DNA for many of the constructions described. The right arm was obtained by digesting λNM616 with *Eco*RI and purifying the DNA fragment as described in the text. The right arm of λNM616 carries the lambda *att* site and a recombinant phage carrying this site can lysogenize *E. coli*. **(c)** λNM540 (Borck et al. 1976). This phage is as λNM616 except the right arm was generated by cleavage with *Hind*III. **(d)** λ KV1 (this work). This phage was derived from λNM616 by cloning an *Eco*RI fragment in λNM616 carrying a *Kpn*I site. Cleavage of λKV1 with *Kpn*I generates a right arm terminated by a *Kpn*I site. **(e)** λ TL25 (Linn and Ralling 1985). This phage was derived from λJDW36 and lacks most of the *trpA* and all of the *trpB* sequences. A polylinker allows fragments generated with many different enzymes to be cloned into this vector.



WILD TYPE λ
10kb
20kb
30kb
40kb
A B V M I J b2 att ←pL pR→Q SR
(a) λJDW36
lac
trp
Y' Z A B' D' E
imm21ts
H
(b) λNM616
lac
att
imm21
E E
(c) λNM540
att
imm21
H
(d) λKV1
lac
att
imm21
K K K
(e) λTL25
lac
trp
Y' Z A'
att
imm21
RESTRICTION SITE
POLYLINKER
Eco RI
Hind III
KpnI

FIGURE 4: Transcriptional fusion lambda phages GR1, GR2, GR3, GR4, GR7. Restriction fragments from rplKAJLrpoBC were fused to trpB carried on the left arm of λJDW36 and a viable lysogenic phage assembled by providing a right arm from another lambda vector (Fig 3). Lambda and *E. coli* sequences are shown as in previous figures but with stippled lines indicating lac-trp sequences and solid lines indicating the 301 bp adapter fragment (Fig 7). The scale bar applies to *E. coli* sequences only. Pictured above the figure is a physical map of the relevant rpl-rpo fragment. **(a)** λGR1. An 1874 bp fragment was recovered from an EcoRI/HindIII digest of λp(rpl-rpo)76 and ligated between the HindIII left arm of λJDW36 and the EcoRI right arm of λNM616, leaving rplJ' fused in phase to trpB' (Crawford et al. 1980). **(b)** λGR2. A complete HindIII and partial EcoRI digest of pGR4 (Fig 7) yielded a 1385 bp fragment that was ligated between the HindIII left arm of λJDW36 and the EcoRI right arm of λNM616. The reading frame from rplL is restored into trpB by the 301 bp EcoRI-HindIII adapter (Fig 7). **(c)** λGR3. This phage was made in 2 steps. First, a 1679 bp HindIII fragment was purified from pGR4 and ligated between the HindIII arms of λJDW36 to create λUt1 (Fig 6). A HindIII partial digest of this phage yielded the left arm of λJDW36 fused to the 1679 bp fragment. This was purified and ligated in the presence of the 1874 bp EcoRI-HindIII fragment of λGR1 and the EcoRI right arm of λNM616 to yield λGR3. **(d)** λGR4. A HindIII digest of pGR3 (Fig 7) yielded a 591 bp fragment which was ligated between the HindIII left arm of λJDW36 and the EcoRI right arm of λNM616, again using the 301 EcoRI-HindIII adapter to restore the reading frame. **(e)** λGR7. A 2.8 kb KpnI-HindIII fragment derived from a restriction digest of λdrif^{d}18 was cloned between the HindIII left arm of λJDW36 and the KpnI right arm of λKV1.

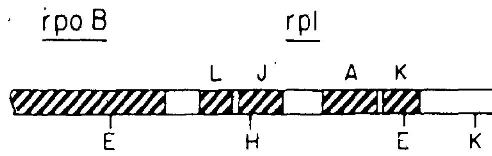


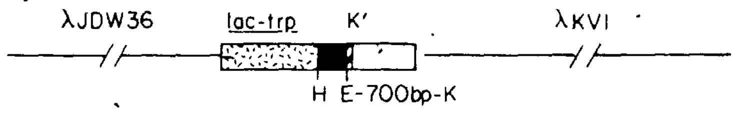


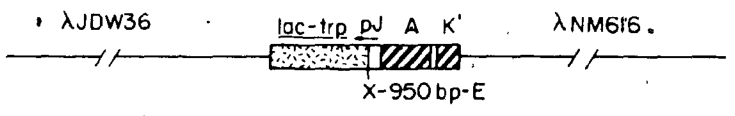
1000 bp

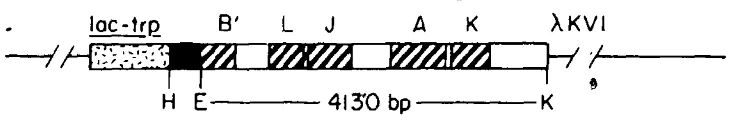
E - EcoRI
H - Hind III
K - KpnI

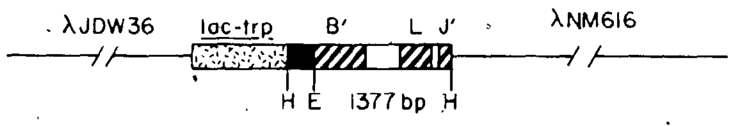
FIGURE 5: Transcriptional fusion lambda phages GR8, GR10, GR11, GR12, GR15. As in previous figures E. coli chromosomal sequences are shown as wide lines and lambda sequences as narrow lines. The scale bar applies to E. coli sequences only. Pictured above the figure is a physical map of the relevant rpl-rpo fragment. **(a)** λGR8. A 700 bp KpnI-EcoRI fragment was ligated between the HindIII left arm of λJDW36 and the KpnI right arm of λKV1. **(b)** λGR10. An EcoRI digest of pGR7 (Fig 8) yields a 6.1 kb fragment which was ligated between the EcoRI left arm of λJDW36 and the EcoRI right arm of λNM616. This fragment carries the same promoter as λGR1, but has the rplJ structural sequences removed by a Bal31 in vitro deletion technique (Fig 8). **(c)** λGR11. A double restriction digest of λdrif^{d}18 with SalI and KpnI yielded a 3.3 kb fragment extending from a KpnI site about 600 bp before rplK to a SalI site located just before rpoB at position 2944. This fragment was ligated between a SalI left arm from λGR2 and the KpnI right arm of λKV1. **(d)** λGR12. A partial HindIII digest of λUt1 (Fig 6) yielded the left arm fragment of λGR12. It carried a fragment extending from the HindIII site in rplJ through the EcoRI site in rpoB fused to the left arm of λJDW36 by the 301 bp adapter. The left arm was provided by a HindIII digest of λNM540. **(e)** λGR15. The 301 bp adapter was recovered from an EcoRI-HindIII digest of pGR3 and ligated between the HindIII left arm of λJDW36 and the EcoRI right arm of λNM616.

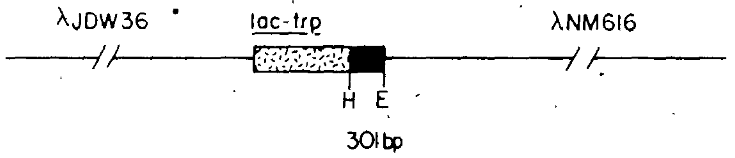


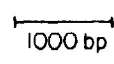






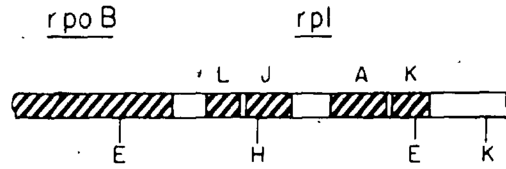




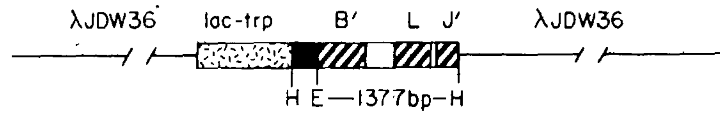


E - EcoRI
H - HindIII
K - KpnI
X - XbaI

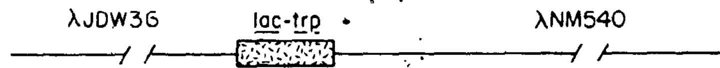
FIGURE 6: Transcriptional fusion construction intermediate lambda phage Utl, and control lambda phages, GR5, GR6, GR9. As in previous figures, E. coli chromosomal sequences are shown as wide lines and lambda sequences as narrow lines. The scale bar applies to E. coli sequences only. Pictured above the figure is a physical map of the relevant rpl-rpo fragment. **(a)** λUtl. The 1679 bp HindIII fragment of pGR4 was ligated into the HindIII site of λJDW36. This permitted the construction of λGR3 (Fig 4c) to be made in two steps. **(b)** λGR5. This phage, made by fusing the left arm of λJDW36 to the right arm of λNM540, is without a cloned sequence. A lysogen of λGR5 provides a background value for β-gal assays. **(c)** λGR6. This phage was made by a genetic cross not by in vitro recombinant techniques. λplac5 and λcimm21 (Table 3) were used to co-infect CSH50 (Table 1) and pink lysogens, able to grow at 37°C, selected. λplac5 carries the lac operon without a repressor gene. This phage served as a means of assessing the β-gal assay protocol. **(d)** λGR9. A 800 bp HindIII-EcoRI fragment of λmetA20 (Fig 2) was cloned between the HindIII left arm of λJDW36 and the EcoRI right arm of λNM616. This served as a positive control for the growth rate portion of this work.



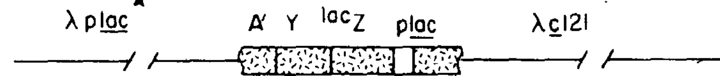
(a) λUT1



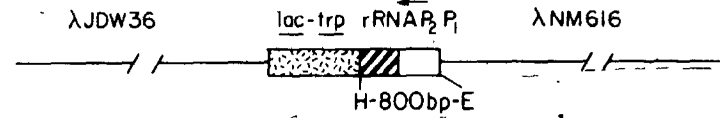
(b) λGR5



(c) λGR6



(d) λGR9



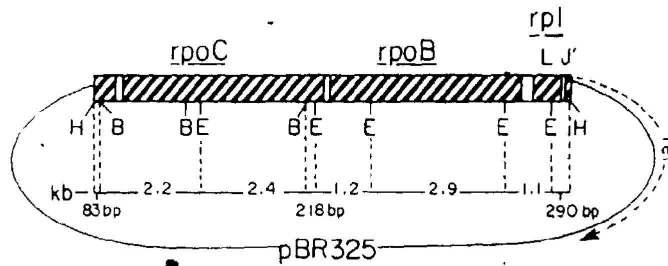
1000 bp

E - EcoRI
H - HindIII
K - KpnI

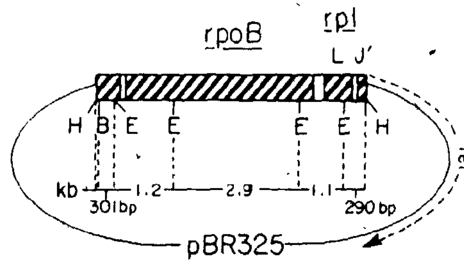
FIGURE 7: Recombinant plasmids pTL20, pGR1, pGR3, pGR4.

Plasmids constructed for this study were based on the cloning vector pBR325 (Bolivar 1978; Fig A1). Vector sequences are shown as narrow lines. E. coli chromosomal sequences are shown as wide lines with hatching indicating structural sequences and open regions indicating non-coding sequences. The solid wide line in (c) and (d) indicates the 301 bp adapter sequence. **(a)** pTL20. The 10 kb HindIII fragment of λp(rpl-rpo)76 was sub-cloned in the HindIII site of pBR325 (T.Linn). From the collection of transformants, this plasmid was identified, with the 10 kb fragment cloned in the orientation shown. **(b)** pGR1. pTL20 was digested to completion with BglII and re-ligated. A plasmid, pGR1, which had a single BglII site remaining and which had lost both BglII fragments, was identified. **(c)** pGR3. pGR1 was digested to completion with EcoRI and re-ligated. A plasmid, pGR3, which had lost all three EcoRI fragments of pGR1, was identified. The 301 bp HindIII-EcoRI fragment was used as a linker in several of the recombinant phage constructions in which it serves to re-align the reading frame into trpB. **(d)** pGR4. A partial EcoRI digest of pGR1 followed by re-ligation yielded a plasmid pGR4 which carried a 1088 bp EcoRI fragment spanning the intercistronic space between rplL and rpoB.

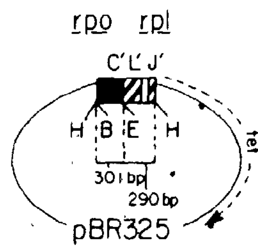
(a) pT.L20



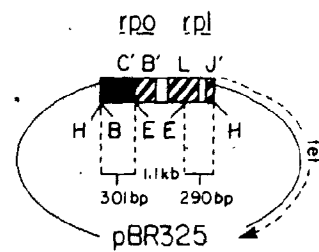
(b) pGR1



(c) pGR3



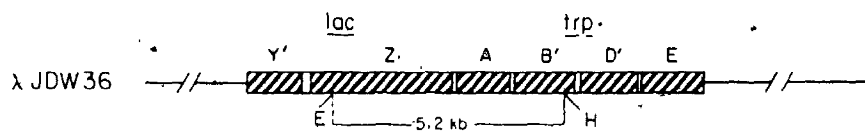
(d) pGR4



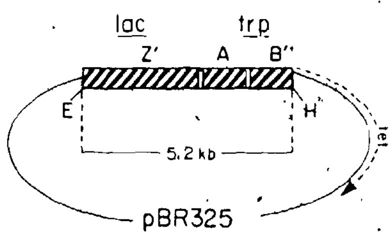
B - Bgl II
E - Eco RI
H - Hind III

FIGURE 8: **Recombinant plasmids pGR5.2, pGR6, pGR7, pBal-12.**

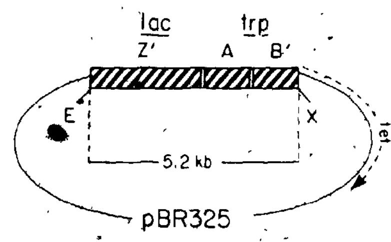
These plasmids were made in order to clone a fragment carrying rplJp, a promoter located between rplA and rplJ, but without the structural genes for rplJ. A collection of Bal31 deletion plasmids was generated by opening pRJ1 at the SmaI site in rplJ, digesting with Bal31 and adding XbaI linkers before re-ligating (T.Linn). This collection of deletions was screened and pBal-12 identified as having lost all of rplJ and some of the untranslated leader, but with 130 bp remaining downstream of the promoter. **(a)** pGR5. To facilitate the construction of λGR10, the EcoRI-HindIII fragment of λJDW36 was sub-cloned in pBR325 to yield pGR5.2. **(b)** pGR6. The HindIII site was converted to an XbaI site by end-filling after HindIII restriction and adding XbaI linkers. **(c)** pBal-12. This plasmid lacks the structural gene sequence for rplJ and some of the rplJ leader sequence. **(d)** pGR7. For this plasmid, pGR6 was opened at its XbaI site and the XbaI-EcoRI fragments from pBal-12 recovered. The ligation of these sequences produced pGR7 carrying rplJp in the orientation shown. A 6.15 bp EcoRI fragment from this plasmid was re-cloned between vector arms to yield λGR10.



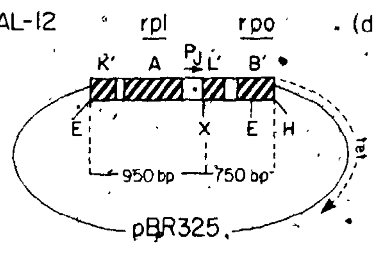
(a) pGR5.2



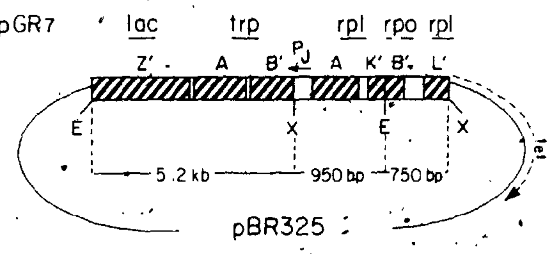
(b) pGR6



(c) p BAL-12



(d) pGR7



E - EcoRI
H - HindIII
X - XbaI

FIGURE 9: Recombinant plasmids pRJ1, pGR8, pGR9.

As in previous figures vector sequences are shown in narrow lines and E. coli chromosomal sequences are shown in wide lines. **(a)** pRJ1 (R. Jones, this lab). This plasmid carries the same rplKAJLrpoBC sequence as λGR3 except with a 400 bp deletion at the 5' end of rplK. It is a more useful donor of fragments for sub-cloning than the phage because the smaller vector carries fewer restriction sites and when restricted, generates fewer fragments. **(b)** pGR8. This plasmid carries a 503 bp SalI-EcoRI fragment used as a probe in the S1 mapping section of this work. **(c)** pGR9. This plasmid carries a 587 bp fragment which was further sub-cloned in M13 (Fig 10) for use as a hybridization probe.

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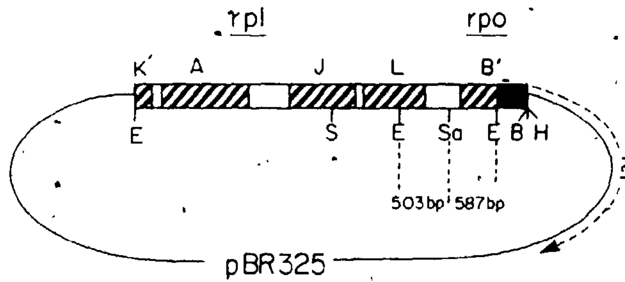
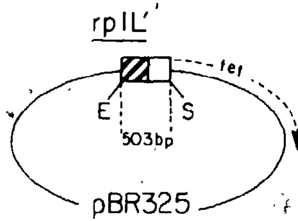
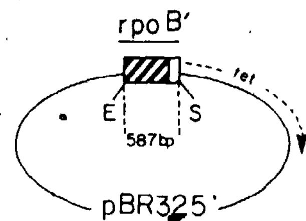


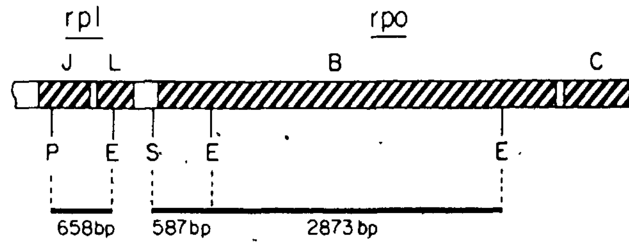




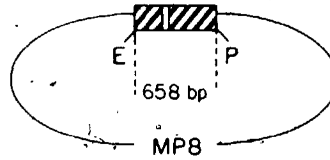
B - *Bgl* II
E - *Eco*RI
H - *Hin*d III
Sa - *Sal* I
S - *Sma*I

FIGURE 10: Recombinant M13 phages MP8-JL, MP8-587, MP8-B.

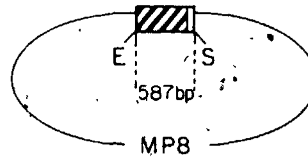
Three hybridization probes were cloned in the M13 vector mp8 (Messing and Vieira 1982). As in previous figures, vector sequences are shown in narrow lines and E. coli chromosomal sequences are shown in wide lines. Pictured above the figure is the physical map of the relevant rpl-rpo sequence with the probe sequences illustrated. **(a)** MP8-658 carries a 658 bp fragment carrying only rplJL sequences, sub-cloned from pTL512 (Ralling et al. 1985). **(b)** MP8-587 carries the last 24 bp of the rplL-rpoB intercistronic sequence and the first 563 bp of the rpoB structural sequence sub-cloned from pGR9 (Fig 9). **(c)** MP8-B carries 2873 bp of the rpoB sequence sub-cloned from pTL413 (Ralling et al. 1985).



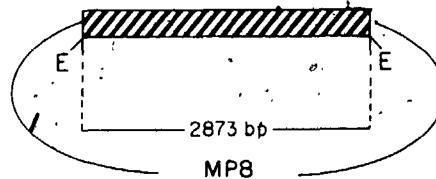
(a) MP8-JL



(b) MP8-587



(c) MP8-B



E - Eco RI
P - PstI
Sa - SalI

RESULTS

3.1 RELATIVE TRANSCRIPTIONAL ACTIVITIES OF REGULATORY SITES WITHIN rplKAJLrpoBC

The transcriptional fusion phages whose construction is described in Chapter 2 (Fig 4, 5 and 6) were made in order to assess the relative contribution each of the previously suggested transcriptional regulatory sites makes to the overall transcriptional control of the rplKAJLrpoBC genes. As discussed in Chapter 1, in vitro, in vivo, and sequence data had identified several promoters and an attenuator, but a coherent picture of the transcriptional control of rplKAJLrpoBC had not emerged, nor had a single comprehensive study been done. To accomplish this, each of the previously suggested transcriptional control sites was cloned in a lambda vector such that transcription initiated within the cloned fragment continued into a lacZ gene carried on the vector. Each transcriptional fusion phage was introduced into the chromosome of a lac deletion host strain, GR50-7, as a lysogen. By growing such lysogenized strains under identical conditions, and measuring the level of β-galactosidase expressed by each, the transcriptional activity of the phage-cloned fragment could be assessed and the relative activity of each fragment compared. The results of this study are shown in Table 4.

For clarity, the results presented below are identified by the regulatory feature carried on the phage-cloned fragment. The strain designation is specified in brackets following.

rplJp (GR50-8) Previous work had demonstrated that a strong promoter was located on a DNA fragment extending from the EcoRI site in rplK to the HindIII site in rplJ (Fig 1: Linn and Scaife 1978; Yamamoto and Nomura 1978; An and Friesen 1980). To measure the strength of the promoter found on this fragment, it was first cloned by fusing the HindIII site in rplJ directly to the HindIII site in trpB creating λGR1 (Fig 4a). A lysogen of λGR1, GR50-8, expressed 2983 U of β-gal (Table 4a), indicating that rplJp is indeed a strong promoter since GR50-16, carrying λGR6 transcribing lacZ from the derepressed

lacp, expresses 2023 U (Table 4d). The precise location of rplJp on this 1874 bp fragment is known from sequence data (Post et al. 1979), footprinting studies (Taylor and Burgess 1979) and an insertion study which localized the position of rplJp to within 80 bp of the end of rplA (Hui et al. 1982).

rplLp (GR50-11) Several studies had suggested the existence of a promoter that might independently initiate transcription before rplL (Linn and Scaife 1978; Barry et al. 1979; Fiil et al. 1979; Goldberg et al. 1979; Brot et al. 1980). To assess the strength of this promoter, the intercistronic region between rplJ and rplL, the most probable location of this promoter, extending from the HindIII site in rplJ to the EcoRI site in rplL, was cloned creating λGR4 (Fig 4d). A lysogen, GR50-11, carrying λGR4 expressed 228 U of β-gal (Table 4a). This amount suggests rplLp is only eight percent the strength of rplJp (2983 U).

rpoBp (GR50-9) A variety of experiments had suggested that rpoBC might be independently transcribed from a promoter specific for these two genes (Barry et al. 1979; An and Friesen 1980; Holowachuck et al. 1980; Ma et al. 1981). One group (An and Friesen) had isolated enhanced promoter mutations and located the site of these mutations 165 bp upstream of rpoB by sequence analysis. This placed rpoBp in the rplL-rpoB intercistronic region. A DNA fragment extending from the EcoRI site in rplL to the EcoRI site in rpoB was cloned creating λGR2 (Fig 4b). A lysogen, GR50-9, carrying λGR2 expressed no β-gal above background values (Table 4a). Therefore the putative promoter, rpoBp, does not appear to direct any transcription of rpoBC, under these conditions.

rplJp+rplLp+atn (GR50-10) A transcriptional terminator, which allowed some transcription to proceed past it and hence was named an attenuator, had been located in the intercistronic region between rplL and rpoB (Barry et al. 1980). This site, atn, had previously been shown to reduce transcription initiated at arap by 83 percent (Barry et al. 1979) or from rplJp, cloned downstream of arap, by 88 percent (Howe et al. 1982). To determine the efficiency with which atn terminated transcription initiated at the endogenous promoter, rplJp, a DNA sequence extending from the EcoRI site in rplK to the EcoRI site in

rpoB was cloned creating λGR3 (Fig 4c). A lysogen of this phage, GR50-10, expressed 962 U of β-gal (Table 4a), demonstrating that about 68% of transcripts initiated at rplJp are terminated at atn.

rplLp+atn (GR50-24) The previous transcriptional fusion carried both rplJp and rplLp upstream of atn. The simplest model of attenuation would suggest that all transcripts be terminated with equal efficiency at atn. However, it has been shown for some lambda operons that only appropriately modified transcription complexes can read through some terminators (Friedman and Gottesman 1983). That this may be pertinent stems from the observation that, with different promoters, the degree of read-through permitted by atn varies from 6 percent with galp to 2 percent with arap and is about 12 percent with rplJp cloned downstream of arap (Howe et al. 1982). Therefore, although rplLp appears much weaker than rplJp, transcription initiated at rplLp might be qualitatively different from that initiated at rplJp and read through atn at a greater efficiency. To assess this efficiency, a DNA sequence extending from the HindIII site in rplJ to the EcoRI site in rpoB was cloned creating λGR12 (Fig 5d). A lysogen of λGR12, GR50-24, expressed background levels of β-gal (Table 4a). This suggests that transcription initiated at rplLp is terminated with at least the efficiency of that initiated at rplJp and that transcription of rpoBC under these conditions is primarily directed by initiation at rplJp.

rplKp (GR50-21) A promoter, rplKp, had been previously identified for rplKA (Linn et al. 1979; Yamamoto and Nomura 1979; An and Friesen 1980). More recently, there have been a reports proposing that rplKp initiates transcription that can read through rplJp and is, therefore, responsible for the transcription of the six genes rplKAJLrpoBC (Bruckner and Matzura 1981; Friesen et al. 1983). To compare the strength of rplKp to the other promoters already examined, a DNA sequence extending from the KpnI site upstream of rplK to an EcoRI site within rplK was cloned creating λGR8 (Fig 5a). A lysogen of this phage, GR50-21, expressed 3986 U of β-gal (Table 4a) suggesting that rplKp is about 34 percent stronger than rplJp (2983 U). Previous estimates of the strength of rplKp relative to rplJp, made using fragments cloned in multi-copy plasmids had suggested rplKp was

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between 10 percent (Howe et al. 1982) and 50 percent (An and Friesen 1980) stronger than rplJp.

rplKp+rplJp (GR50-19) Although rplJp is a strong promoter and was reported to be capable of directing the synthesis of wild type levels of β and β' in a UV-irradiated cell system (Linn and Scaife 1978) or when a polar insertion was placed upstream of rplJp in vivo (Hui et al. 1982), biochemical evidence demonstrated that transcripts initiated at rplKp carried all four genes rplKAJL (Bruckner and Matzura 1981). Other genetic and transcriptional fusion evidence suggested that when rplKp was upstream of rplJp, some read-through of rplJp occurred (An and Friesen 1983; Howe et al. 1982). Because rplKp was found to be substantially stronger than rplJp in the present work, the question of rplKp read-through of rplJp could be addressed, albeit indirectly, with a transcriptional fusion carrying both rplKp and rplJp. A DNA sequence extending from the KpnI site upstream of rplK to the HindIII site in rplJ was cloned creating λGR7 (Fig 4e). A lysogen of this phage, GR50-19, expressed 3280 U of β-gal (Table 4a), 10 percent more than rplJp alone (2983), but an 18 percent reduction over rplKp alone (3986 U). This suggested that at least some transcription initiated at rplKp terminates before rplJp.

rplKp+rplJp+atn (GR50-23) As discussed previously, termination of transcripts initiated at different promoters may be subject to varying termination efficiencies at atn. Therefore, although together rplKp and rplJp do not transcribe lacZ much more frequently than rplJp alone, if some or all of this transcription is in fact initiated at rplKp, it might read through atn with a different efficiency than transcription initiated at rplJp. To address this suggestion, a DNA fragment extending from the KpnI site upstream of rplK to the EcoRI site in rpoB was cloned creating λGR11 (Fig 5c). A lysogen of this phage, GR50-23, expressed 1082 U of β-gal, a value not substantially different from that of GR50-10 carrying only rplJp upstream of atn (962 U). This suggests that even if transcription of rpoBC relies on initiation at rplKp, the termination efficiency at atn is no different than if rplJp is the promoter.

Table 4: **β-Galactosidase levels expressed by strain GR50-7 lysogens of transcriptional fusion phages.** The host strain GR50-7 (Δlac, recA; Table 1) was lysogenized by each of the transcriptional fusion phages made for this study. Each lysogen is assigned a strain designation (column 1) and the lambda phage carried by each strain is indicated (column 2). For complete descriptions of the strains refer to Table 1; for the lambda phage Table 2; or for illustrations of the phage Figures 4, 5, and 6. Each lysogen was grown under steady state conditions and sampled at two points for β-galactosidase. β-gal levels were calculated according to Miller (1972) normalized to $O.D._{600} = 1$ (see 2.27:3). **(a)** Lysogens carrying transcriptional fusion phages with DNA restiction fragments from rplKAJLrpoBC. **(b)** Lysogens of two phages similar to λGR1 (Fig 4a) but with in vitro dilutions of the rplJ structural sequences. The lambda phage λGR10 is illustrated (Fig 5b) and its construction detailed (Fig 8). The lambda phage λTL103 (T.Linn) is not illustrated because it is identical to λGR10 except it has a slightly shorter deletion. **(c)** Control strains express very low levels of β-gal. Some of the transcriptional fusions use a 301 bp linker to join the cloned DNA sequences to trpB (see Fig 4 legend) while others have cloned sequences joined directly to trpB. The β-gal level expressed by GR50-12 carrying a phage λGR5 (Fig 6b) without any cloned sequences fused to trpB is used to correct the β-gal levels measured in strains with fusions of DNA directly to trpB. The β-gal level expressed by GR50-26 carrying lambda λGR15 which has the 301 bp linker fused to trpB is used to correct β-gal levels from fusions that employ this linker. **(d)** A lysogen which carries λGR6 (Fig 6c) expresses β-gal from a constitutively transcribed lacZ gene under the control of the endogenous lacp.

	STRAIN	LAMBDA	β-GAL	β-GAL (corrected)	GROWTH RATE dblgs/hr
(a)	GR50-8	GR1	3004	2983	0.57
	GR50-9	GR2	51	-	0.75
	GR50-10	GR3	1037	962	0.90
	GR50-11	GR4	303	228	0.80
	GR50-19	GR7	3301	3280	0.45
	GR50-21	GR8	4061	3986	0.94
	GR50-23	GR11	1103	1082	0.95
	GR50-24	GR12	70	-	0.88
(b)	GR50-28	GR10	21	-	0.77
	GR50-30	TL103	203	182	0.70
(c)	GR50-7	--	0	-	0.85
	GR50-12	GR5	21	-	0.90
	GR50-26	GR15	75	-	0.74
(d)	GR50-16	GR6	2023	-	0.87

rplJp, (rplJ) (GR50-28, GR50-30) Lysogens of the transcriptional fusion, λGR1, carrying rplJp and most of the rplJ structural sequences, grew slowly compared to other lysogens (see growth rate data - Table 4a). A collection of Bal31 in vitro generated deletions was available on a multi-copy plasmid (T. Linn) and one of these, pBal-12 (Fig 8) with a deletion that removes all but 130 bp of DNA downstream of rplJp was used as a source of rplJp. This fragment was fused to trpB in the same manner as other rplJ fragments. Presumably the trpB sequences, left untranslated in λGR10 now exerted a strong polar effect on downstream transcription. A lysogen, GR50-28, carrying GR10 expressed no β-gal discernible from background levels (Table 4b). To confirm this, a second deletion was cloned in an identical fashion to create λTL103 (T. Linn - not shown). This phage carries a deletion which does not extend as far into the untranslated leader sequence of rplJ. A lysogen of this phage expressed 182 U of β-gal, still far below that expected (2983).

3.2 TRANSCRIPTIONAL ACTIVITIES OF rplKAJLrpoBC REGULATORY SITES IN RESPONSE TO GROWTH RATE

3.2.1 Host strain GR50-7 lysogens

As discussed in Chapter 1, the expression and transcription of rplJL have been shown to diverge from that of rpoBC under a variety of conditions. The most thoroughly investigated parameter which effects a divergence in the expression of L10 and L7/12 from that of β and β' is growth rate. Recall that the values $\alpha_{(r)}$ and $\alpha_{(p)}$, the relative synthesis rates of ribosomal and polymerase proteins, increase but at different rates as the growth rate increases.

To determine how the transcription of rplKAJLrpoBC is affected by growth rate, strains of GR50-7 lysogenized with transcriptional fusions carrying one of rplJp, rplLp, rpoBp or rplJp+atn were grown in different media to achieve a range of growth rate, and β-gal levels were assessed in each. The results of such measurements are shown in Fig 11. Although no transcription was apparent from rpoBp in the previously described experiments, it was included in the growth rate experiments to examine the possibility that such a promoter, normally inactive, might become active at high or low growth

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rates. However, the lysogen, GR50-9, carrying λ GR2 expressed only background levels of β-gal at all growth rates (data not shown). The measurements obtained with GR50-9 were taken as evidence that background levels of β-gal did not change measureably at any growth rate and these GR50-9 values substituted for control values. Because these background values remained so consistent and comprised such a small fraction of the experimental values, all of the growth rate data are uncorrected for background.

GR50-8, lysogenized with λGR1, carrying rplJp, expressed levels of β-gal that declined markedly from a value of 4371 U of β-gal at a growth rate of 0.2 doublings per hour to 2976 U at 0.9 doublings per hour (Fig 11a). Above a growth rate of 0.9 doublings per hour, the decline in β-gal was much less marked, reaching a value of 2894 U at a growth rate of 1.5 doublings per hour, the fastest growth rate this lysogen achieved (Fig 11a). GR50-10 behaved in a similar fashion, except β-gal values declined from 2158 U to 963 U over a growth rate range of 0.2 to 2.6 doublings per hour (Fig 11b). The curves drawn through each set of values (Fig 11a and b) are approximately parallel. The amount of β-gal expressed from GR50-8 and GR50-10 is constant above a growth rate of 0.9 doublings an hour. A lysogen of GR50-11, with λ GR4 carrying rplLp, expresses a constant low amount of β-gal, about 300 U at all growth rates (Fig 11c).

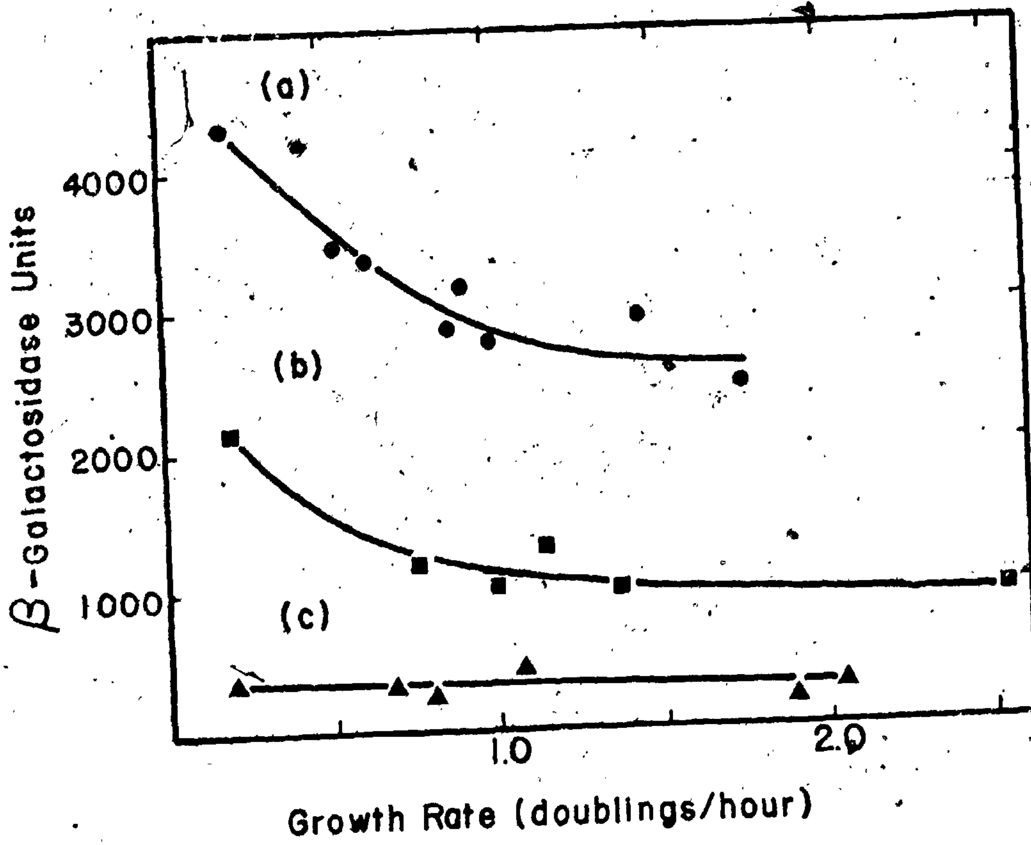
These data support several conclusions. Firstly, neither of the two previously proposed minor promoters, rplLp or rpoBp, contributes substantially to the transcription of rplL or rpoBC under the conditions of growth employed in this study. Secondly, termination at atn remains a constant fraction of transcription initiated at rplJp resulting in a constant ratio of β-gal levels expressed by GR50-8 and GR50-10. This observation suggests, that to resolve the diverging values of $\alpha_{(r)}$ and $\alpha_{(p)}$, post-transcriptional controls must be invoked. Finally, because β-gal levels expressed by lysogens carrying λGR1 and λGR3 quite clearly decrease in response to growth rate increases, it is concluded that the transcription of these genes decreases in response to increasing growth rate until a value of about 0.9 doublings per hour is reached and thereafter remains approximately constant. This last

FIGURE 11: β-galactosidase levels in GR50-7 lysogens grown at different rates. GR50-7 lysogens were grown in a series of increasingly rich media to achieve successively faster growth rates. Although only six different media were used, there are eight points in panel (a). This results from growth rate differences upon repetition in the same medium. In other words, a given strain does not grow at precisely the same rate even in the same medium. β-gal values were calculated according to Miller (1972) normalized to $O.D._{600} = 1$. Except for the lowest growth rate for each lysogen, achieved by supplementing AB with acetate and culturing at 30°C, cultures were grown at 37°C as described (2.26).

(a) ● - GR50-8 (λGR1)

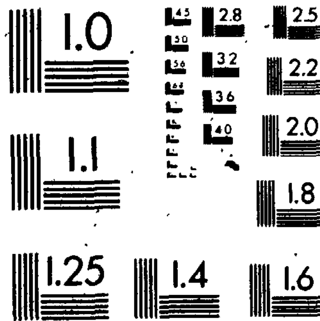
(b) ■ - GR50-10 (λGR3)

(c) ▲ - GR50-11 (λGR4)



(a)
(b)
(c)
4000
3000
2000
1000
β-Galactosidase Units
1.0
2.0
Growth Rate (doublings/hour)

2 OF/DE 2



conclusion is paradoxical in light of the clearly demonstrated increase in the value of $\alpha_{(r)}$ in response to growth rate.

Because of the unexpected results seen when lysogens of these transcriptional fusions were grown at increasing rates of growth and because the lysogen GR50-8 grew slower than other lysogens on almost all of the media used, it was decided that a repetition of these growth rate experiments be made in a different strain of E. coli.

3.2.2 Host strain JG148-3 lysogens

The data collected with lysogens of the host strain JG148-3 are presented in Table 5 to demonstrate three points.

The β-gal values obtained with this strain are consistent with those obtained using host strain GR50-7 which lends credence to the methodology employed to select lysogens and assay β-gal. The decrease in β-gal levels expressed by GR50-8 and GR50-10 are reflected in the decreasing levels of JG148-4 and JG148-7, respectively. Finally, the presence of λGR1 in JG148-4 results in a reduced growth rate in the richer of the two media used compared to the growth rate of the other lysogen JG148-7 whose growth rate was determined (Table 5).

The growth rate experiments with GR50-7 and JG148-7 suggested that transcription of rplJLrpoBC decreased or remained constant as the growth rate increased, a result surprising in light of the reported level of synthesis of these proteins with respect to growth rate. However, this conclusion remained tentative in light of two difficulties encountered with this work. As demonstrated by the data, lysogens of λGR1 grew very poorly in some media. Not only did these lysogens grow poorly but in the host strain GR50-7, the increasing complexity of the media did not always correlate with an increase in the growth rate of λGR1 lysogens (not shown). Additionally, there was some concern as to whether balanced growth was being achieved in all lysogens of GR50-7 and JG148-3. To feel confident a culture is undergoing balanced growth when sampled, it must have been growing exponentially for at least two doubling times and continue to grow exponentially for one doubling time after sampling. Some of the GR50-7 and JG148-3 lysogens showed exponential kinetics for only a

STRAIN	LAMBDA	β-GAL	GROWTH RATE*	β-GAL	GROWTH RATE+
JG148-4	GR1	3723	0.53	2655	1.2
JG148-5	GR4	434	0.58	N.D.	N.D.
JG148-6	GR2	140	0.50	N.D.	N.D.
JG148-7	GR3	1589	0.53	1128	1.76

* Grown in AB2 at 37°C

\+ Grown in AB4 at 37°C

TABLE 5: β-galactosidase levels in JG148-3 lysogens. A second lac deletion strain, JG148-3, was lysogenized with the phages listed above to confirm the results obtained with GR50-7. Because GR50-7 and its lysogenized derivatives grew poorly in some media, the growth rate experiments were repeated in this strain. Only enough β-gal data were obtained with the JG148 lysogens to confirm that β-gal levels decreased as the growth rate was increased.

few doubling times in some media, making the assumption that balanced growth was occurring at the time of sampling difficult.

3.2.3 Host strain MG4 lysogens

Because of the growth problems encountered, several prototrophic strains of E. coli were evaluated for their growth behaviour in AB media. One of these, MG1655 (Table 1), displayed excellent growth kinetics with increasing growth rates corresponding to an increasing complexity of the AB media. A strain named MG4 was constructed from the parent and showed increasing growth rates with increasingly complex media and long exponential growth intervals that permitted at least two doublings at an exponential rate of growth both before and after sampling (not shown). Because lysogens of λGR1 and λGR8 grew slowly, presumably because a fusion protein translated from the hybrid rplJ-trpB or rplK-trpB was expressed, the left arms of these two phages were exchanged for the left arm of λTL25 (Fig 3). The vector, λTL25, has had the trpB and most of the trpA sequences removed (Linn and Ralling 1985) and carries stop codons in all three reading frames in the short sequence of trpA that remains. Therefore, fusion proteins are not synthesized from transcripts reading through trpA into lacZ. A new phage, λTL102 (not shown), carries the same rplKAJL sequences as λGR1 and the new phage, λTL108 (not shown), carries the same rplK sequences as λGR8. MG4 lysogens of either of these transcriptional fusion phages grew acceptably although still not as quickly as other lysogens (see Fig 12a and b).

MG4 lysogens of five transcriptional fusion phages were grown at five different growth rates and β-gal levels were determined at each (Fig 12). The pattern of β-gal expression from a strain, MG9, lysogenized by λTL102 carrying rplJp and from MG5 with λGR3 carrying rplJp+atn, closely resembled that seen with GR50-7 lysogens of the equivalent fusion phages (compare Fig 12a with Fig 11a and b). Experiments with MG10, lysogenized by λTL108 carrying rplKp, and MG7, carrying rplKp+rplJp+atn, gave a similar result and the same conclusions as made for rplJp and atn can be drawn. Transcription from rplKp did not increase with growth rate, nor did the ratio of transcription of rplKAJL vary with respect to that of rpoBC in response to changes in the growth rate. Strain MG14 is lysogenized by λGR9

FIGURE 12: β-galactosidase levels in MG4 lysogens grown at different rates. A prototropic strain, MG1655 (Table 1), that showed much better growth kinetics than GR50-7, was used to construct MG4, its Δlac, recA daughter. This strain was lysogenized with transcriptional fusion phages carrying the major transcriptional regulatory sites from rplKAJLrpoBC, then grown at different rates and assayed for β-gal. The trend of decreasing β-gal levels with increasing growth rate is again evident. β-gal is calculated according to Miller (1972) and normalized to $O.D._{600} = 1$. Shown in panel (b) are values for MG14 which is lysogenized by λGR9 carrying rrnEp cloned in the trpB site of λJDW36 (Fig 5).

(a) ● - MG9 (λTL102); ■ - MG5 (λGR3)

(b) ● - MG10 (λTL108); ■ - MG7 (λGR11); ▲ - MG14 (λGR9)

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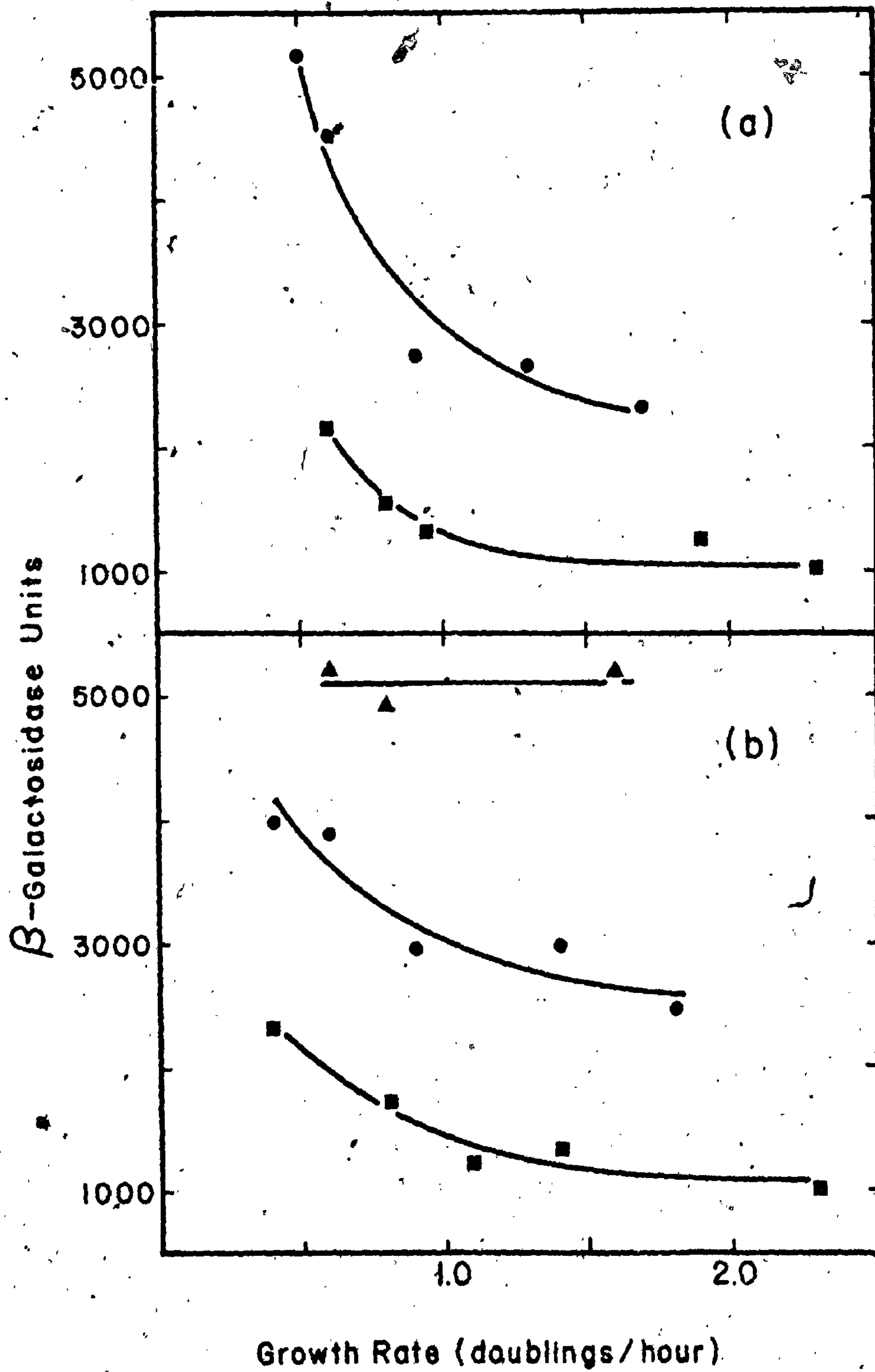

(Fig 6d), which has the rrnE promoter derived from λmetA20 (Fig 2c) fused to trpB. A lysogen of λGR9 expressed high levels of β-gal that increase slightly in response to growth at an increased rate (Fig 12b). This transcriptional fusion was designed to provide a positive control for the growth rate experiments described here. However, it is unlikely that this fusion carries an intact rrnE promoter (see Discussion 4.2.4).

3.2.4 Host strain MG4 β-gal normalized to protein

Rather than normalizing β-gal values to the mass of a sample, the parameter effectively measured by $O.D._{600}$ values (Koch 1981), it may be more appropriate to normalize β-gal values to the total amount of protein in a sample (see Discussion 4.2.3). Therefore, β-gal levels in four MG4 lysogens and two GR50-7 lysogens were measured and total protein concentrations determined for each sample, allowing β-gal levels to be normalized to total protein (Fig 13). The calculation of β-gal values is made in a similar fashion to that of Miller (1972), except mg of protein is substituted for the $O.D._{600}$ reading (see 2.27.3).

The results shown in Fig 13 are in most respects identical to those presented in previous figures (Fig 11, 12). The decrease in β-gal levels expressed by lysogens MG9, MG5, MG10 and MG7 is less marked at low growth rates, and above a growth rate of 0.8 doublings per hour, β-gal values remain constant. When lysogens carrying λGR4 (GR50-11) and λGR2 (GR50-9), expressing β-gal from rpoBp and rplLp, were assessed and normalized to total protein, the same result is obtained as with previous experiments which normalized their β-gal levels to $O.D._{600}$ (Fig 11). GR50-9, carrying λGR2 with the putative rpoBp, expressed background levels of β-gal indicating it is not active at these growth rates. GR50-11, carrying λGR4 with rplLp, expressed a constant low level of β-gal, indicating it is not responsible for a significant level of rpoBC transcription at these growth rates.

3.3 EFFECT OF A rho MUTATION ON TRANSCRIPTION THROUGH atn

The transcriptional terminator atn resembles a simple terminator but may be susceptible to the action of accessory proteins which help effect termination at Rho-dependent terminators (Chapter 1).

FIGURE 13: β-galactosidase levels normalized to total protein in MG4 lysogens grown at different rates. Miller (1972) calculates β-gal levels normalized to $O.D._{600} = 1$, which in effect normalizes β-gal levels to an estimate of the mass of the sample. A more appropriate parameter is total protein (see Discussion). Therefore, the MG4 lysogens shown here were re-grown and sampled for β-gal and total protein. The lower panel (c) shows the results from similar experiments done with two GR50-7 lysogens which express very low levels of β-gal.

(a) ● - MG9 (λTL102); ■ - MG5 (λGR3)

(b) ● - MG10 (λTL108); ■ - MG7 (λGR11)

(c) ● - GR50-11 (λGR4); ■ - GR50-9 (λGR2)

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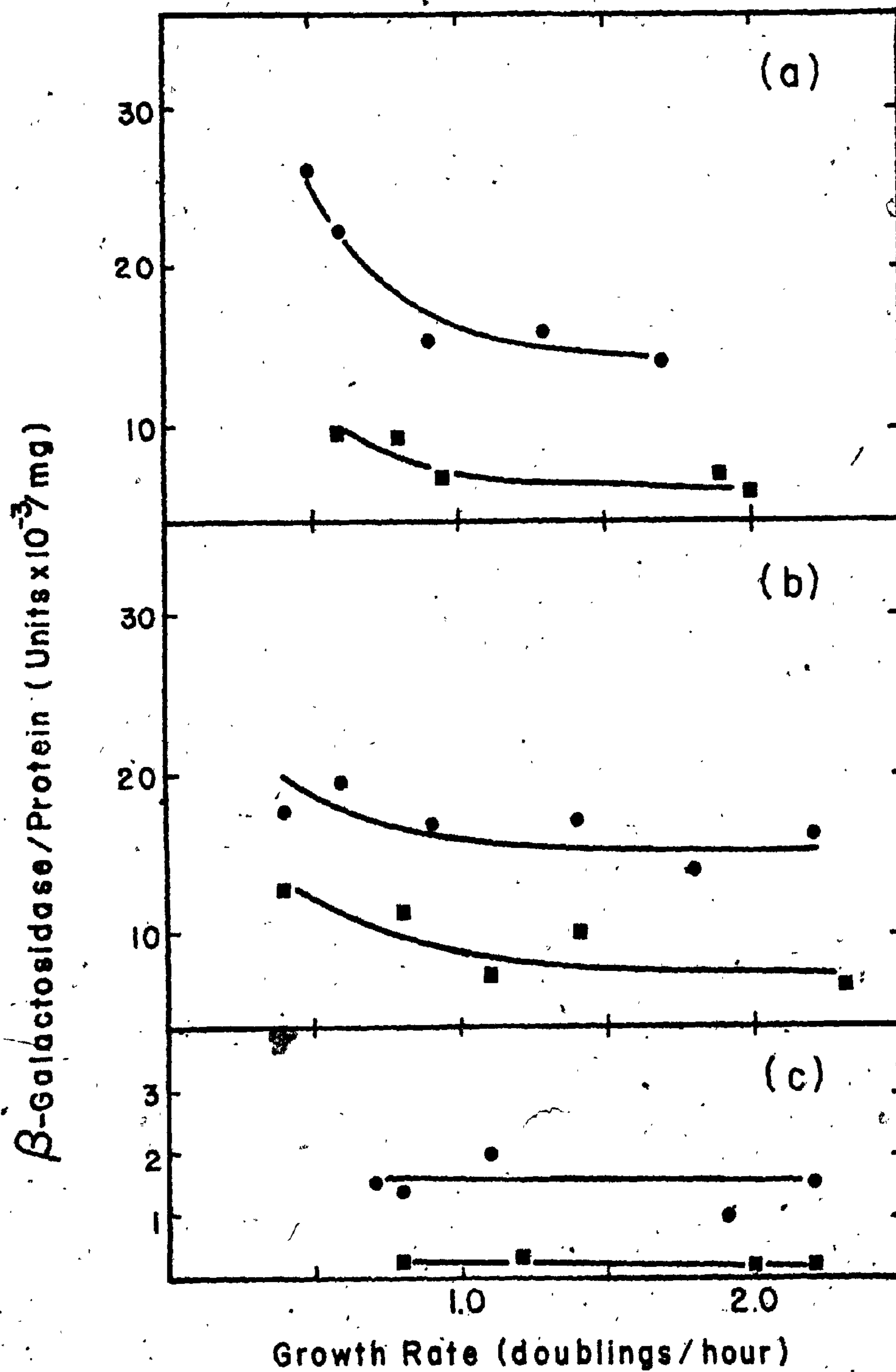


STRAIN	LAMBDA	Rho	β-GAL	GROWTH RATE
GR50-8	GR1	+	2868	0.9
GR50-8-1919	GR1	-	2680	0.2
BU7026-3	GR3	+	1139	1.8
BU7026-3-1919	GR3	-	2532	1.1

Table 6: β-galactosidase levels in a rho strain.
A GR50-7 lysogen, GR50-8, which carries λGR1 was made rho⁻ by introducing the rho112 lesion from AD1919-1 by P1 transduction. A similar construction was attempted with the isogenic strain, GR50-10, carrying λGR3, without success. Therefore BU7026-3, a lysogen which also carries λGR3, was used for comparison. The lysogens and their rho112 derivatives were grown in AB4 and β-gal assays were performed.

One way to identify proteins involved in termination at atn is to examine the effect that a mutation in the gene(s) encoding such a protein(s) has on β-gal expression directed by a transcriptional fusion carrying atn. The original intention of this work was to introduce fusion phages into such E. coli mutants as lysogens. This proved impossible. However, some success was achieved crossing a rho mutation, rho112, into two lysogens by P1 transduction. A strain, GR50-8 carrying λGR1 (Fig 4a), was made rho112 by P1 transduction. This had no effect on the β-gal level (Table 6), suggesting that rplJp was unaffected by Rho and confirming that the fusion carried by λGR1 was not polar. A second strain, BU7026-3 carrying λGR3 (Fig 4c), was made rho112 and the β-gal level doubled (Table 6). This suggested that transcription through atn initiated at rplJp was susceptible to Rho-mediated termination and that in a genetic background of reduced Rho function, termination at atn was reduced by a factor of 2.

3.4 rplJL AND rpoB TRANSCRIPT LEVELS MEASURED BY HYBRIDIZATION TO ssDNA PROBES

The attenuator in rplJLrpoBC terminates transcription in the intercistronic space between rplL and rpoB (Barry et al. 1980). If the transcript levels of rplJL and rpoB are estimated by hybridization, transcription of rplJL exceeds that of rpoB by a ratio of about 5:1 (Dennis 1977; Ralling et al. 1985). This ratio remains constant regardless of the growth rate (ibid). Therefore, one way of assessing the efficiency of termination at atn is to determine the ratio of rplJL and rpoB transcripts by measuring the amounts of labeled rplJL transcripts and rpoB transcripts that hybridize to rplJL or rpoB specific DNA probes.

Mutations in genes which increase the termination efficiency at atn should increase the rplJL/rpoB transcript ratio and mutations in genes which decrease the termination efficiency should decrease the rplJL/rpoB transcript ratio. To determine the transcript ratios in mutant strains of E. coli, ssDNA probes complementary to rplJL and rpoB transcripts were cloned in an M13 vector (Fig 10). Equal amounts of pulse-labeled RNA, harvested from mutant strains and their isogenic parents, were hybridized to each probe. By determining the cpm

STRAIN	RELEVANT GENOTYPE	JL/B
SA1030	w.t.	4.8
AD1600	rho15	1.6
AD1919	rho112	2.5
X10015	rho201, rpoB203	10.5
N99	w.t.	4.9
C205	nusA1	7.8
C347	nusB5	4.5
K556	nusE71	4.9
N2076	w.t.	4.9
N2077	rnc105	6.7
SA2244	w.t.	4.3
SA2243	sfrB	7.2
TGL177	pRJ1	3.6+

+ The smaller rpoB probe, MP8-587, was used in this experiment.

TABLE 7: rplJL/rpoB transcript ratios in a variety of E. coli strains. E. coli mutant strains and their isogenic parents were screened to determine the effect of the lesion carried on JL/B transcript ratios as assessed by a hybridization assay. A reduced ratio suggested that transcription of rpoB had increased due to decreased termination at atn, whereas an increased ratio suggested that termination at atn was increased.

hybridized to each probe, correcting for the size of the probe and expressing the result as a ratio, the effect of a number of mutations on the rplJL/rpoB transcript ratio, and hence termination efficiency at atn, was determined (Table 7).

The parental ratio ranges from 4.3 to 4.9, agreeing with the previous estimates of this ratio (Dennis 1977; Ralling et al. 1985). Two rho strains, AD1600 and AD1919, with lesions in rho (Table 1), show greatly reduced rplJL/rpoB transcript ratios, resulting in the conclusion that termination at atn is less frequent in the presence of rho lesions, implicating the Rho protein in normal termination at atn. A strain, X10015, carrying a lesion in rho and a compensating lesion in rpoB, shows an increased transcript ratio consistent with the previously defined phenotype of this mutation (Guarente and Beckwith 1978). Of the three nus mutations screened, only C205 carrying a nusA lesion showed a ratio which differs significantly from the isogenic parent N99. This increased ratio suggests termination is more frequent in the absence of normal nusA function and implicates nusA in normal anti-termination at atn.

Apparently nusB and nusE do not affect termination at atn. The strain N2077 carries a mutation in rnc which encodes RNaseIII, a protein responsible for processing RNA and reported not to affect the expression of rpoB (Dennis 1984). This strain shows a clear increase in the rplJL/rpoB transcript ratio, indicating a relative increase in rplJL transcripts. Strain SA2243, carrying a lesion in sfrB, an anti-termination protein affecting tra operon expression (Beutin and Achtman 1979; Beutin et al. 1981), shows an increased ratio, again consistent with the anti-termination function of the wild type protein. Finally, the rplJL/rpoB transcript ratio was measured in a strain, TGL177 (Table 1), carrying a multi-copy plasmid pRJ1. This plasmid carries a DNA sequence extending from the EcoRI site in rplK to the first EcoRI site in rpoB. As a multi-copy plasmid carrying rplJp and downstream sequences to the first EcoRI site in rpoB, it should over-express transcripts from this region. That this is the case is demonstrated by the approximately 10-fold increase in the percentage of counts bound to specific probes for this region (data not shown). The effect of this overproduction on the rplJL/rpoB ratio was determined by

using the same probe for rplJL but a shorter 5' proximal rpoB sequence probe (Fig 10), specific for the sequences downstream of atn and carried on pRJ1 (Fig 9a). The transcript ratio in this experiment was seen to decrease to a value of 3.6, suggesting that the overproduction of an rplJL/rpoB transcript may alter the efficiency of termination at atn or, alternatively, the shorter rpoB probe may hybridize with a greater efficiency (see Discussion 4.1.3).

3.5 S1 MAPPING OF rplL-rpoB TRANSCRIPTS

3.5.1 Sizing transcripts

The location of atn, which terminates transcripts in the intercistronic space between rplL and rpoB, had been mapped by Barry et al. (1980) using both an S1 mapping technique and RNA sequencing. Working in a strain carrying a multi-copy plasmid to amplify rplJLrpoBC transcripts, these workers visualized S1-digested transcripts hybridizing to a 1.1 kb probe by separating the RNA/DNA heteroduplex fragments on polyacrylamide gels, staining with ethidium bromide and sizing the fragments by comparison with double-stranded DNA restriction fragment size markers. To determine more accurately the length of transcripts derived only from the chromosome, a shorter 503 bp probe from pGR9 (Fig 9b) which allows better separation on gels, was used for the present study. Transcripts from a wild type *E. coli* chromosome were sized by separating the single stranded probe fragments surviving S1 digestion beside a set of single stranded size markers of the same probe sequence (Fig 14). The predominant fragment resolved was estimated to be 275 bp long, demonstrating most transcription through the intercistronic space is terminated about 72 bp downstream of rplL (Fig 15), agreeing with the results of Barry et al. (1980). A smaller, much fainter, band at 255 bp coincides with a second terminator-like structure which can be inferred from the sequence of this region (Barry et al. 1980). Of the longer, read-through transcripts, one band at 503 bp indicates some full length transcripts are detected. The two bands resolved at 325 and 340 bp are the RNaseIII processed transcripts (Fig 16a), which again were originally described and sized by Barry et al. (1980). However, these gels indicate lengths of 325 and 340 bp, placing the 3' ends of the

FIGURE 14: **Sizing S1 digested rplL-rpoB transcript-probe hybrids.** S1 digested hybrids of total RNA hybridized to the 3' end-labeled 503 bp probe (Fig 10) were denatured and electrophoresed at 55°C on urea-polyacrylamide gels to resolve ssDNA probe fragments(lane B). For use as size standards, φX174 DNA was digested with HinfI, end-labeled in the same fashion as the probe, and run alongside the S1 digest (lane A). To more accurately size S1 fragments, the 503 bp probe was purified and subject to limited digestion at guanines (Maxam and Gilbert 1980) to produce labeled fragments (lane C) of identical sequence to the S1 fragments. The larger print numbers on the left show the sizes assigned to the S1 fragments.

A B C

713

427

417-18

275

144

FIGURE 15: Alignment of S1 transcripts with the DNA sequence.

The probe used for the S1 experiments was derived from pGR8 (Fig 9), cut with EcoRI, the 3' termini labeled with ^{35}S-dTTP, and then re-cut with SalI. This yielded a 503 bp probe, end-labeled on the DNA strand complementary to the rplL transcript. When hybridized to total RNA and then digested with S1 nuclease, only the probe/RNA hybrids survive. These fragments can be visualized and sized on polyacrylamide gels (Figs 16 and 17) or denatured and the DNA portion alone resolved on denaturing polyacrylamide gels (Fig 14). By sizing such fragments, the position at which the transcript is processed or terminated can be determined.

The figure opposite illustrates the most probable sites that the fragments, sized from gels shown in Fig 14, terminate. The major band of about 275 bp is of the correct size to terminate at the end of a stretch of 5 uridines. This is the basis for the contention that atn is about 72 bp downstream of rplL. A minor band estimated to be 255 bp suggests that a small portion of transcripts may terminate before atn within another stretch of 5 uridines. Fragments estimated to be 340 and 325 bp are derived from transcripts that read through atn and are processed by RNaseIII, probably at the sites shown (positions 2774 and 2786). The assignment of these lengths to the RNaseIII processed transcipts, about 80 bp upstream of the sites reported by Barry et al. (1980), suggests that processing does not remove the translational control sequences located by sequence analysis to begin at position 2811 (ibid) or by deletion analysis to be in the vicinity of the RNaseIII site between positions 2789 and 2890 (Dennis 1984).

503
340
325
275
255

2790-C-G-2856
U-A
A-U
U-A
C-G
U-A
2783-U-A
G-C
U-A
U-A
C-G
G-C
U-A
A-U
A-U
C-G
2773-U-A
U-A
U-A

U-A
C-G
A-U
G-C
U-A
G-C
G-C
U-A
C-G
G-C

2675
2720
2750
2890

GCCUGAGAAAUCAGGCUGAUG-CUUUUUGCGCUGUAAGGCGCCAGUAGCGUUUCACACUGUUUGACUACUGCUGUGCC-GUGUUCCAUUUUCCGGUCAAGAA

rplL
rpoB
2444
2944
EcoRI
2647
SalI
2973

transcripts about 127 and 142 bp downstream of rplL (Fig 15), whereas they were mapped about 210 and 225 bp downstream of rplL by Barry et al. (1980). The translational control features for rpoB have been located between positions 2789 and 2890 (Dennis 1984). The location of the RNaseIII sites 5' to the translational control region of the rpoB transcript corroborates the observation that RNaseIII processing does not affect the expression of rpoB and rpoC (Dennis 1984).

3.5.2. Transcripts from mutant strains

Several proteins were implicated in the attenuation process by examining the ratio of rplJL and rpoB transcripts. If these proteins affect the frequency of termination at atn, mutant strains carrying lesions in rho, nusA, rnc and sfrB should show reduced or increased levels of transcripts terminating before or after atn and such changes might be confirmed by the direct visualization of transcripts using an S1 transcript mapping technique. Furthermore, an altered pattern of transcript bands may be resolved on gels if termination is not occurring normally in the presence of a termination mutation. Both native and denaturing gels were run for this work, but only on the native gels were the intensities of the bands sufficient to allow adequate reproduction. Therefore it is the native gels that are reproduced here. The results seen on the denaturing gels were identical to those seen on the native gels.

It has already been demonstrated that transcripts that read-through atn are processed by RNaseIII and that in the presence of an rnc mutation, the level of processed transcripts is reduced and that of full length transcripts is increased (Barry et al. 1980), an observation confirmed by the S1 mapping protocol employed in the present study (Fig 16a and b). Similarly, S1 transcripts from nusA (Fig 16c and d), rho (Fig 17) and sfrB (not shown) mutants were visualized both on native and denaturing gels. The banding pattern of the S1 transcripts seen with the termination mutant strains and their isogenic parents did not differ. The relative intensity of the S1 transcript bands remained the same in all mutants and parents, except in the case of strain X10015 (Fig 17b) carrying a rho mutation and compensating rpoB mutation (Table 1). In this strain, the intensity of the processed transcript bands increased six-fold as determined by laser

FIGURE 16: S1 transcripts from rnc and nus mutants.

S1 digested RNA/DNA hybrids were run on both denaturing and non-denaturing gels to examine the relative size and intensities of the resolved bands. The non-denaturing gels showed much better contrast and are reproduced here. The results however did not differ. The most prominent band in lanes a-d is about 275 bp long and results from hybridization of the labelled probe to atn terminated fragments. The markers on either side of the figure are end-labeled HinfI fragments of X174 DNA but are not a good indication of size since they are dsDNA and do not run to the same position as RNA/DNA hybrids of the same size. They are shown to allow the comparison of the relative sizes of S1 fragments on different gels. **(a)** N2076, the isogenic parent of N2077. **(b)** N2077 is rnc (RNaseIII$^-$) and shows a diminished level of processed transcripts which resolve directly above the atn terminated transcript band. On this gel, the two processed transcript bands resolve as a single band. **(c)** N99, the isogenic parent of C205. **(d)** C205 is nusA1, showing a decreased JL/B transcript ratio (Table 7). The pattern and intensity of the bands is the same in both lanes.

99

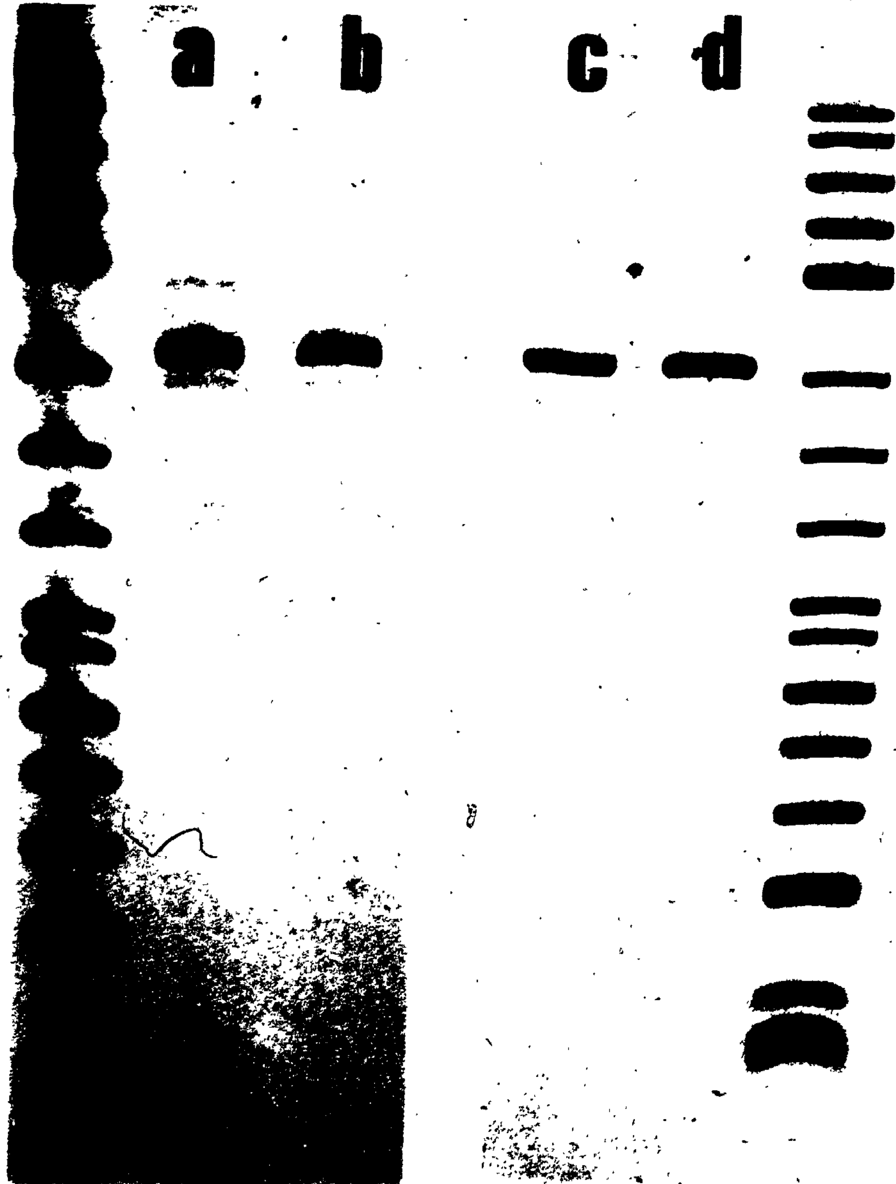
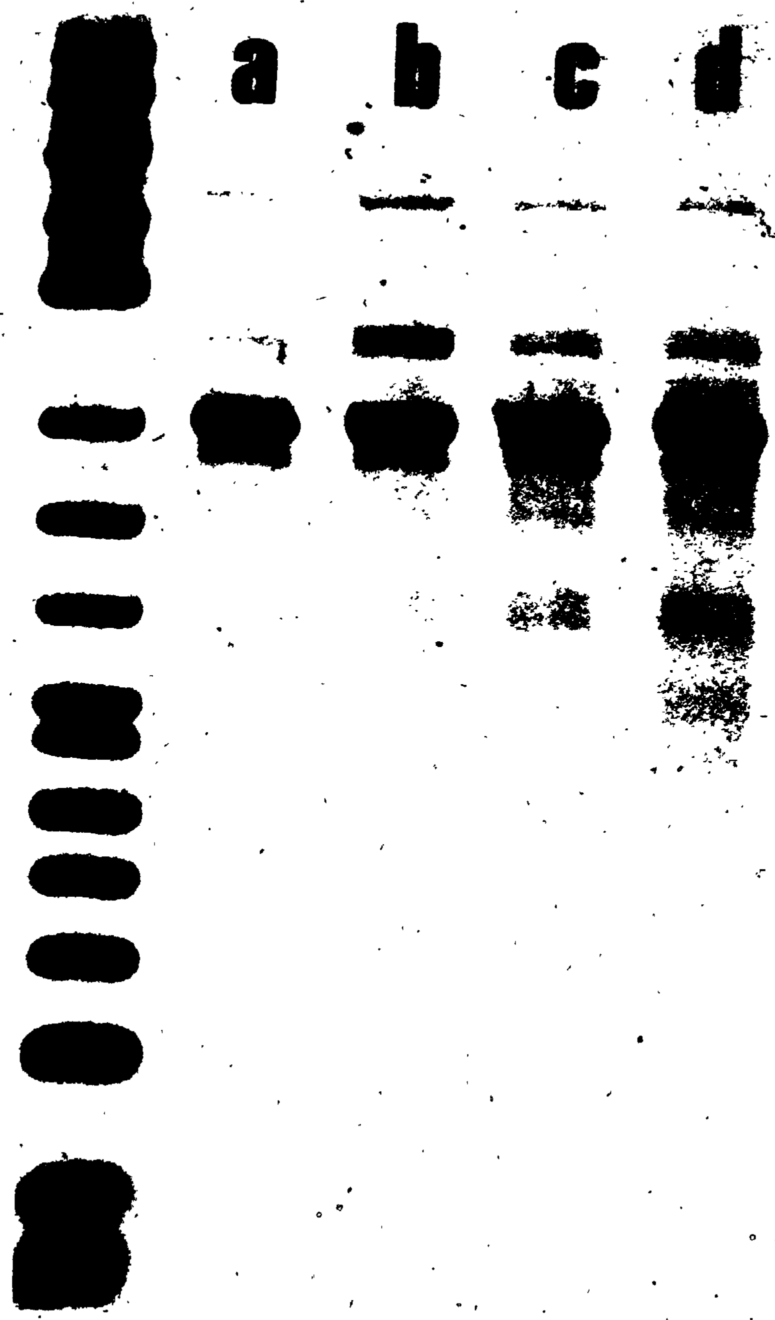


FIGURE 17: **S1 transcripts from rho mutants.**

This figure is similar to Figure 16 except here rho strain S1 transcripts are shown. **(a)** SA1030, the isogenic parent of AD1600 and AD1900. **(b)** X10015 is rho201 rpoB203, lacking Rho, but with a compensating mutation that restores termination but not necessarily at the Rho-dependent terminator (Guarente and Beckwith 1978; Platt 1981). In this strain transcription appears to terminate at a new site beyond the RNaseIII processing site since the intensity of the processed transcript band increases. This strain functions as something of a positive control on the S1 mapping technique. **(c)** AD1600 is rho15 and showed a decreased JL/B transcript ratio (Table 8). The bands appear to be the same as in the parent strain. **(d)** AD1919 is rho112 and showed decreased JL/B transcript ratio (Table 8). Again, no apparent intensity or size difference is apparent in the bands when compared to the parent strain.


a
b
c
d

densitometry tracings (data not shown). Although the other lanes in this gel and other gels were scanned by laser densitometry, the contrast of the autoradiograms and the sensitivity of the technique were not sufficient to detect any quantitative differences. Therefore the effects of termination proteins on termination at atn which had been suggested by the hybridization experiments could not be directly confirmed with the S1 mapping technique.

DISCUSSION

4.1 ASSESSING rplKAJLrpoBC TRANSCRIPTION BY GENE FUSION

4.1.1 Relative activities of rplKAJLrpoBC promoters

Previous experiments had suggested that the transcriptional control of rplJLrpoBC would be complex. Although a number of groups had examined transcription in rplJLrpoBC, none had attempted a comprehensive analysis of the entire gene cluster using a single approach and the confusing picture that emerged from those studies may have resulted from their disparate approaches. As previously described, earlier workers had employed a UV-irradiated cell system to assess rplJp and rplLp (Linn and Scaife 1978; Yamamoto and Nomura 1978). Others had examined some of the regulatory features of rplKAJLrpoBC by fusing restriction fragments between arap and lacZ on a multi-copy plasmid and in this way had identified atn and assessed its efficiency (Barry et al. 1979). Another study compared rplJp and rplLp by fusing rplJ or rplL-rpoB to trpB on a lambda vector in such a way as to create a fusion protein under the transcriptional and translational control of the upstream cloned sequences (Holowachuck et al. 1980). Finally, in vitro studies which measured the level of L7/12 synthesis, directed by plasmid-cloned rplKAJLrpoBC fragments, had suggested rplLrpoBC were together in an operon under the control of a strong promoter (Goldberg et al. 1979) carried downstream of rplJp. This was partially corroborated by still another multi-copy plasmid fusion study (Fiil et al. 1979), but which placed the promoter for rplL-rpoBC further upstream. Therefore, one of the strengths of the present study derives from its comparison of all of the previously identified regulatory sites under identical conditions, allowing a direct comparison of each site's transcriptional activities.

The relative activities assigned to the three promoters, rplKp, rplJp and rpLp, based on measurements made in the host strain GR50-7, expressed in terms of rplJp are respectively 135, 100 and 8. For comparison, the derepressed lac promoter is assigned by this work a relative strength of 68. The initial estimate of the relative strength of rplKp was not entirely corroborated in the host

strain MG4. In this strain, rplKp and rplJp are the same strength except at low growth rates where rplJp is slightly stronger.

Previous assessments of rplJp agree that it is an efficient promoter both under non-physiological conditions in a UV-irradiated cell system (Linn and Scaife 1978; Yamamoto and Nomura 1978) or when carried on a multi-copy vector fused to lacZ or tet (Howe et al. 1982; Hui et al. 1982; An and Friesen 1980). Those workers that compared rplJp to rplKp suggested that rplKp had a relative strength of 160 (An and Friesen 1980) or 110 (Howe et al. 1982). The values reported here are therefore comparable to estimates made by others.

Similarly, several groups had estimated the strength of a minor promoter downstream of rplJp. Relative to rplJp in a UV-irradiated cell system, rplLp had a strength of 2 (Ma et al. 1981); relative to rplJp on a phage vector, transcription initiated downstream of rplJp, presumably from rplLp, had a value of 10 (Holowachuck et al. 1980). Again, the value reported here of 8 is comparable to estimates made by others. Finally, a third promoter, rpoBp, was estimated from the data of others to be about the same strength as rplLp (Barry et al. 1979; Ma et al. 1981), in agreement with a semi-quantitative approach (An and Friesen 1980). These last workers precisely located this promoter by selecting up mutations and determined the sequence change responsible. However the present study finds no evidence for such a promoter.

4.1.2 Relative efficiency of termination at atn

The efficiency of termination at atn was estimated by comparing β-gal levels expressed by lysogens of fusions carrying either rplJp alone (λGR1) or with rplJp upstream of atn (λGR3). The first measurements, made with GR50-7 lysogens, suggested that 68 percent of transcripts initiated at rplJp terminated at atn (Table 4). This value incorporated the same growth rate induced error as the initial estimates of promoter strength: that is, lysogens of λGR1 grew slower than lysogens of λGR3 and therefore were inappropriate for comparison. However, the data presented in Fig 11 allow comparison of GR50-8 and GR50-10 β-gal values at comparable growth rates. This has the effect of reducing estimates of the efficiency of atn to about

60 percent. Similarly the efficiency of atn can be estimated from the MG4 lysogen data (Fig 12 and 13) to be about 60 percent. Previous estimates of the efficiency of atn made with arap suggested that between 83 and 98 percent of transcripts initiated at arap are terminated by atn (Barry et al. 1979; Howe et al. 1982). With arap upstream of both rplKp, rplJp and atn, the efficiency of atn was measured to be 89 percent (Howe et al. 1982). The estimates from the present study are the only ones reported using either rplJp fused in its normal orientation to atn or both rplKp and rplJp fused in their normal orientations with respect to atn. Both constructions suggest the efficiency of termination at atn is about 60 percent. Expressed as a ratio of transcription initiated at rplJp to transcription that reads through atn, a value of 2.5:1 is seen.

4.1.3 Fusion vs. hybridization estimates of rplKAJLrpoBC transcription

A previous study had examined the rate of transcription of rplJL relative to the rate of transcription of rpoBC by a hybridization technique (Dennis 1977). This work used two overlapping DNA probes to hybridize rplKAJLrpoB transcripts out of a sample of total RNA pulse-labeled with ^{3}H-Uracil and estimated their relative levels by subtraction. Similar experiments have been done in this laboratory but with probes specific for rplJL and rpoB (Ralling et al. 1985). Both sets of data suggest that rplJL is transcribed at 5 times the frequency of rpoB. This estimate suggests that termination at atn is 80 percent efficient. Transcriptional fusions suggest that rplJL is transcribed 2.5 times as frequently as rpoB with a termination efficiency at atn of 60 percent. What is the reason for the difference between these estimates and which approach is likely to yield a more reliable result?

Hybridization experiments done with pulse-labeled RNA attempt to measure the rate at which a given transcript is synthesized. Because the rate of elongation of RNA is constant, the frequency with which the specific transcript is transcribed is being estimated. To compare the frequency of transcription of two different transcripts, several assumptions are made. The efficiency that each hybridizes to its respective probe is assumed to be constant and the

rate at which each is degraded is assumed to be the same. To minimize the amount of degradation, the length of the pulse is kept to a minimum, in this case, 50 seconds is the practical lower limit. Given that procaryotic transcripts have half lives in the order of one to two minutes, there is undoubtedly some degradation occurring during this interval.

If one of the two transcripts is more stable than the other, a hybridization experiment will over-estimate the frequency with which the more stable of the two is transcribed. There is reason to believe that the rplL portion of the transcript might be quite stable. The protein translated from rplL is found at four times the level of other ribosomal proteins. Since rplL is not transcribed at a substantially higher level than rplJ, implied is that the transcript is translated more frequently, and consequently, is likely to be more stable. This may in turn lead to an over-estimation of the rplJL/rpoB transcript ratio.

The hybridization experiments cited both use probes that are of quite different lengths to compare the frequency of transcription of rplJL to rpoBC. It has been suggested that hybridization efficiency may be affected by the length of the probe used and may especially be a problem when trying to compare probes less than 1.0 kb with probes greater than 1.0 kb (Dennis 1984). The probe used for rplJL transcripts is 658 bp long so as to remain specific for rplJL, but the rpoB probe is 2873 bp long. A shorter probe would not hybridize enough counts to give a meaningful result. Therefore, both assumptions that must be made about the behavior of the transcripts to allow the comparison of their transcription frequencies may be faulty in the case of the experiments that examine rplJL and rpoB transcripts.

In an attempt to avoid the difference in probe lengths but still incorporate enough counts to be meaningful, a short rpoB probe was cloned comparable in size to the rplJL probe (587 bp; Fig 10), and the rplJL/rpoB ratio measured in a strain carrying a plasmid, pRJ1 (Fig 9), that over-expresses transcripts from rplKAJLrpoB by a factor of about 10. The resulting transcript ratio of 3.6 (Table 7) suggested that the efficiency of termination was 72 percent. This

supports a contention that the hybridization ratios may be in error rather than the fusion ratios. Half life experiments to determine the rate of degradation of these messages are probably not feasible, since the hybridization technique is already at the limit of its resolving power with these low level transcripts (Dennis 1984).

A transcriptional fusion approach avoids having to make assumptions about the behaviour of different transcripts, since the level of the same message lacZ is always being compared. Provided the assay conditions are the same, it seems safe to assume that translation, mediated by the lacZ ribosome binding site, will be of the same efficiency for all the fusion transcripts. The protein product of lacZ, β-galactosidase, is a very stable enzyme whose activity does not diminish for at least several hours after the growth of the culture has been arrested. Therefore, it would seem that such an approach should yield a reliable comparison of the frequency of transcription directed by different sequences.

For the reasons outlined above, it was felt that transcriptional fusions could provide a more accurate estimate of the transcriptional frequency of rplKAJLrpoBC. Dennis (1977) had reported that the transcriptional frequency of rplKAJL vs. rpoB is about 5:1 and estimates from this laboratory using probes specific for rplJL and rpoB agree (Table 7; Ralling et al. 1985). These measurements imply an attenuation efficiency of 80 percent whereas β-gal levels from the transcription fusions suggest rplJL vs. rpoB transcriptional frequency is 2.5:1, implying a termination efficiency of 60 percent. Dennis (1977) pointed out the similarity between the ratio of rplJL and rpoB transcripts that he measured by hybridization (5:1) and the stoichiometry of ribosomal and RNA polymerase proteins established by others to be about 6:1 (see Chapter 1). However, this coincidence is probably specious. The expression of r-proteins is apparently controlled post-transcriptionally and the frequency with which rplJL is transcribed does not correspond precisely to the level at which it is expressed. RNA polymerase expression on the other hand now appears to remain constant (Ralling et al. 1985). Therefore, the level of expression of proteins from these genes need bear little resemblance to the level at

which they are transcribed. Witness to this is the four-fold higher level of L7/12 with respect to L10, which is virtually without a trancriptional basis; there being no evidence to suggest L10 is transcribed significantly more frequently than rplJ.

The only hybridization study which compares the frequency of transcription of rplKA to rplJL reported that rplKA was transcribed with about two-thirds the frequency of rplJL (Dennis et al. 1985). In contrast, the present study finds rplKp to initiate transcription at the same level or more frequently than rplJp, suggesting rplKA must be transcribed at least as frequently as rplJL. Again, assumptions about the hybridization efficiencies of different probes or the rate at which the two messages are degraded are perhaps in error.

4.1.4 Which is the real promoter for rplJLrpoBC?

S1 mapping studies have reported that transcripts initiated at rplKp continue into rplJL (Bruckner and Matzura 1981) and others have alluded to similar results (Ma et al. 1981). Still others have used an S1 approach to look for transcripts which terminate after rplA and not found them (Friesen - personal communication). These observations suggest that transcription from rplKp continues into rplJL defining an operon rplKAJL. The present study does not deal with this question directly but does make some observations of relevance. The promoter, rplJp, is a strong promoter under all conditions used in this study. It initiates transcription which reads through atn in the absence of rplKp upstream, and its pattern of transcription initiation in response to growth rate reflects that of rplKp. It is difficult to explain the presence of a promoter with such characteristics if it is not functional. In lysogens of GR50-7 (Table 4), it appeared that initiation from rplKp was substantially greater than initiation at rplJp (3986 vs. 2983). Since transcription downstream of rplJp was the same whether or not rplKp was in its normal position upstream, the argument could be made that at least some transcripts initiated at rplKp terminate before rplJp. The results from MG4 lysogens suggest that these two promoters are the same strength and do not permit such an argument to be made. Post et al. (1979) argued that since there did not appear to be a consensus termination sequence after rplA, transcription might read through into

rplJ. Rho-dependent terminators are still not apparent from sequence data, so this argument is equivocal.

4.2 TRANSCRIPTION OF rplKAJLrpoBC AS A FUNCTION OF GROWTH RATE

4.2.1 Objectives of this study

The pattern of expression of ribosomal proteins and RNA polymerase proteins in response to the rate of growth had been well documented at the outset of the present study. Ribosomal proteins are synthesized relative to total protein ($\alpha_{(r)}$) at a rate which increases in direct proportion to the growth rate. The proteins expressed from rplJL, L10 and L7/12 conform to this pattern (Dennis 1974b). In contrast, the synthesis of RNA polymerase proteins relative to the synthesis of total protein ($\alpha_{(p)}$), although reported to increase in response to growth rate increases, did so at a lower rate (Shepherd et al. 1980). More recent work in this laboratory has shown that $\alpha_{(p)}$ does not change in the range of growth rate from .5 to 2.5 doublings per hour (Ralling et al. 1985).

If the control of expression of proteins from rplJLrpoBC is exerted transcriptionally, the frequency with which rplJL and rpoBC are transcribed should reflect these previously reported levels of expression. Already established is a mechanism whereby the expression of ribosomal proteins is controlled, at least in part, autogenously. This mechanism is known to function in both rplKA (Dean and Nomura 1980) and in rplJL (Yates et al. 1981). Such a mechanism does not preclude the action of transcriptional controls that might contribute to the overall control of expression but does establish that transcriptional controls are not the only mechanism for regulating gene expression.

The transcriptional control of rplKAJLrpoBC has been shown to be complex, with at least one minor promoter and an attenuator functioning downstream of a strong promoter. It has been demonstrated that not only is the expression of the ribosomal proteins and RNA polymerase genes not always coordinate, but that the transcription of rplJL can be uncoupled from that of rpoBC under some conditions (Maher and Dennis 1977; Blumenthal and Dennis 1980). The

objective of the present study was to determine how transcription of rplKAJLrpoBC contributes to the expression of the proteins derived from these genes. If transcriptional controls were entirely responsible for expression, one would predict that initiation from rplKp and rplJp increase in proportion to the growth rate. One might also predict that the transcription of rpoBC decrease relative to that of rplJL. This latter, relative decrease, could result from an increase in the efficiency of termination at atn or possibly from a decrease in initiation specific for rpoBC from a minor promoter. Even assuming the expression of some or all of the proteins from rplKAJLrpoBC is affected by post-transcriptional mechanisms, transcriptional controls could still play a major rôle in controlling the expression of these genes. The present growth rate study wished to examine the transcription of rplKAJLrpoBC to determine what was the nature and magnitude of the contribution of transcriptional controls to the control of rplKAJLrpoBC expression as the growth rate increased.

4.2.2 The role of minor promoters and attenuation

As outlined in the previous section, minor promoters or a decreased efficiency in termination at atn could reduce the transcription of rpoBC and hence expression of β and β', and perhaps explain the reduction in $\alpha_{(p)}$ relative to $\alpha_{(r)}$. To examine the possibility that the activity of a minor promoter might change, lysogens of transcriptional fusions carrying rplLp or rpoBp were grown at different rates and β-gal measurements made. As presented in the previous chapter (Fig 11 and 13), the expression of β-gal directed by these minor promoters does not change and in any case is only at very low levels. Therefore, a decreasing level of initiation at either is ruled out as an explanation for the decreasing level of β and β' relative to ribosomal proteins. Similarly, the behaviour of the attenuator was assessed by comparing the level of β-gal expressed by lysogens of fusions carrying rplJp and rplJp+atn (Fig 11 and 13) or comparing rplKp and rplKp+rplJP+atn (Fig 12 and 13). Because the β-gal levels from these pairs of fusions maintain a constant ratio, it is concluded that efficiency of termination at atn remains constant. This conclusion is supported by two hybridization studies which have examined the level of rplJL transcripts relative to rpoB transcripts and found their ratio to be

constant at all rates of growth examined (Dennis 1977; Ralling et al. 1985). This conclusion is the same, irrespective of whether rplKp or rplJp is the promoter normally used for rplJL transcription. The results of these experiments suggest that post-transcriptional controls must be responsible for the discoordinate expression of L10 and L7/12 with respect to β and β'.

4.2.3 Transcription frequency from rplKp or rplJp

The results presented in Chapter 3 support the contention that the transcription of rplKAJLrpoBC does not increase as the growth rate increases, but remains at a constant level relative to total protein at a growth rate above 0.8 doublings per hour (Fig 13). This implies that transcriptional controls play no part in controlling the expression of these ribosomal and polymerase proteins. However, the relative rate of synthesis of ribosomal proteins has been unequivocally shown to increase directly in proportion to the growth rate. This dichotomy can be resolved by invoking a model of post-transcriptional regulation of ribosomal protein expression (Nomura et al. 1984). Miura et al. (1981), in a similar set of experiments, utilizing r-protein operon promoters fused to lacZ or galK, concluded that r-protein transcription must be constant and at a high enough rate to support the highest rate of r-protein expression. According to the model developed principally by Nomura and his co-workers, excess transcripts of r-protein operons are inactivated by the binding of regulatory ribosomal proteins and degraded rapidly. There is abundant evidence to support both these latter aspects of the model (Nomura et al. 1984). However, the conclusion, drawn by Miura et al. (1981) and implied by this study, that r-protein operons are always transcribed at a frequency high enough to support maximum levels of expression of r-proteins, is unexpected. This observation implies that transcriptional regulation plays little role in controlling the expression of ribosomal proteins, a conclusion that has attracted some criticism (Ingraham et al. 1983). Because the conclusion is unexpected and perhaps a little controversial, it bears further examination.

Previous studies employing hybridization techniques had examined the rate of synthesis and the steady state amount of r-protein mRNA in cells grown at different rates and had suggested

that the amount, but not the rate, of synthesis of these messages increased (Dennis and Nomura 1975; Dennis 1977; Gausing 1977). The most comprehensive of these (Gausing) found that a group of r-protein gene transcripts comprised a constant fraction of total RNA synthesis, but the author argued that because mRNA comprised a diminishing fraction of total RNA synthesis, the level of r-protein transcription was an increasing fraction of mRNA. However, her estimates of total mRNA synthesis are derived from subtracting rRNA and an assumed amount of tRNA from total RNA to arrive at a fraction of total RNA that was mRNA. Miura et al. (1981) argued that this calculation is in error.

When attempting to measure the level of a specific procaryotic transcript, it is only appropriate to measure the level of that transcript relative to another, in order to correct for differences in the recovery of RNA. One cannot assign an absolute value to such a measurement since the total amount of transcription is increasing but the behaviour of the various components of total transcription, rRNA, tRNA and mRNA cannot be precisely determined. To avoid this difficulty, it would be desirable to relate the transcription of a specific gene to a parameter that can be accurately and easily determined, and which ideally comprises a constant fraction of the cell at any growth rate. Transcriptional fusions allow this, since the frequency of transcription of a given gene is indicated by the level of an easily assayed and stable protein which can be measured in terms of several parameters.

Mass (dry weight) as estimated by O.D. is the easiest parameter to determine and the early measurements of β-gal made for this study are normalized to mass. However, it may not be valid to assume that the rate of mass increase maintains a direct relationship to growth rate at low growth rates (Ingraham et al. 1983: Chapter 6). A more appropriate parameter to use in this work may be total protein. The values $\alpha_{(r)}$ and $\alpha_{(p)}$ are estimates of the rates of synthesis of ribosomal and RNA polymerase proteins with respect to the rate of synthesis of total protein. By using transcriptional fusions, the rate of transcription of <u>rplKAJLrpoBC</u> can be related to this same parameter. The final growth rate data (Fig 13) normalized β-gal

levels to total protein. In this way, the frequency of transcription of an r-protein operon, estimated by gene fusion, could be related directly to the rate of total protein synthesis, in effect a measure of total mRNA levels.

A technical problem may arise at low growth rates. All of the lysogenized strains used in this work are recA, a lesion which causes cell division to lag behind growth. When examined microscopically, recA strains of E. coli appear to increase in length, becoming snake-like as the growth rate increases above about one doubling per hour (unpublished observations). Estimating the mass of bacteria in suspension by O.D. measurements is based on the observation that "dilute suspensions of most bacteria independently of cell size have nearly the same absorbance per unit dry weight concentration" (Koch 1981). However, the amount of light scattered by a particulate suspension is proportional to the ratio of particle size to the wavelength of incident light (Ingraham et al. 1983: Chapter 5) and is affected to some extent by the conformation of the particles (Koch 1981). Therefore it may not be valid to assume that the O.D. of the cultures grown for this study can be directly compared without correction for the growth rate. Such a correction is impracticable. A simpler solution is to measure a parameter that can be quantitated directly and protein determinations satisfy this requirment.

Since neither approach suggests that the frequency of transcription of rplKAJLrpoBC increases as a function of growth rate, the relative advantage of using mass or protein to normalize β-gal values is perhaps moot. However, a decrease in the level of transcription would be extremely difficult to resolve with protein levels. Obviously β-gal values do appear to decrease when normalized to mass, and decrease when normalized to protein at growth rates less than 0.8 doublings per hour. Ribosomes and ribosomal proteins increase in direct proportion to the increase in growth rate except at very low growth rates (<0.6 doublings per hr), where ribosomes are found in a relative excess (Maaloe 1979). Therefore, r-protein transcription may in fact increase at low growth rates. Alternatively, the relative increase in the level of ribosomes may simply result in a relatively more efficient translation of the lacZ message.

4.2.4 - The problem of a positive control

As already discussed it is perhaps more reliable to compare the frequency with which two genes are transcribed using transcriptional fusions and thereby avoid making erroneous assumptions about the behaviour of the transcripts in a hybridization experiment. To use a fusion approach to compare transcriptional activities at different growth rates requires that assumptions be made about the behaviour of the lacZ transcript that are hard to substantiate. Assumed is that the lacZ transcript has the same half-life at all growth rates and that it is always translated at a constant efficiency. At least the first of these is cast into doubt by recent evidence which suggests that growth rate can have a dramatic effect on the rate at which a given transcript is degraded (Nilsson et al. 1984), although no information is available to suggest that lacZ is subject to such an effect. The second assumption is challenged by the observations reported here that the expression of β-gal, directed by some of the fusions increases, contrary to expectation, at low growth rates. It has been argued that this may be due to an increase in the efficiency of translation of these transcripts when the level of ribosomes increases at low growth rates. Good controls would provide evidence that these assumptions are valid but such controls have not been forthcoming.

Miura et al. (1981), performing similar experiments to those reported here, had a very good positive control. Those workers fused a ribosomal RNA promoter, rrnEp, to lacZ and found that such a fusion directed an increasing level of β-gal synthesis with respect to growth rate that reflected the increase in rRNA transcription as a function of growth rate. Similar such stable RNA promoter fusions had been reported by several other groups (Ota et al. 1979; Friesen et al. 1980; Glaser et al. 1980; Duester et al. 1982). At least two of these groups reported that such fusions behaved in a growth rate dependent fashion (Ota et al. 1979; Duester et al. 1982). At the outset of the present study, it was assumed that such a positive control would be easy to make. However, many unsuccessful attempts to clone rrnBp and rrnEp were made. Finally, a single phage was isolated which carried a fragment of an appropriate size (Fig 6d),

derived from λmetA20 (Fig 2), to carry rrnEp and which, when examined as a lysogen (MG14), expressed high levels of β-gal that increased marginally at a high growth rate (Fig 12b). If β-gal values expressed by MG14 are normalized to total protein, β-gal does increase modestly as a function of growth rate (data not shown).

Because of the extreme difficulty encountered in cloning this fragment, one questions the nature of the resulting construction. It has been known for some time that extremely strong promoters are difficult or impossible to clone without an appropriate downstream terminator (Gentz et al. 1981). Recently, it has been reported that rrn promoters cannot be cloned without downstream terminators (Sarmientos et al. 1983), an observation not surprising in light of recent evidence that an anti-termination mechanism functions in rrn operons. This leaves some doubt as to the nature of the previously reported positive controls, without which some doubt is cast on the conclusions made here with respect to the growth rate control of transcription in rplKAJLrpoBC.

4.2.5 A model for the control of expression of rplKAJLrpoBC

The transcriptional fusion data presented here suggest that the frequency of transcription of rplKAJLrpoBC does not increase as a function of growth rate, in contrast to the increased rate of synthesis of ribosomal proteins in direct proportion to the growth rate. This observation, although not substantiated by positive controls, is supported indirectly by hybridization experiments performed in this laboratory which measured the ratio of rrn transcripts to rplJL transcripts at increasing rates of growth. Such experiments found this ratio to increase in a fashion similar to the relative rate of r-protein synthesis (Ralling et al. 1985). Thus, different mechanisms must control the rates of transcription of rrn genes and rplJL. This thesis contends then that rplKAJL is transcribed at a constant rate. The increasing rate of ribosomal protein synthesis is exclusively a function of post-translational controls.

By the same logic, rpoBC is also seen to be transcribed at a constant frequency. The relationship of β and β' synthesis to total protein synthesis $\alpha_{(\beta\beta')}$, as a function of growth rate,

was not well established at the outset of this work (Shepherd et al. 1980). Measurements of $\alpha_{(\beta\beta')}$, made with MG4, employing a technique to directly measure β and β' synthesis (Ralling et al. 1985), suggest that these proteins are synthesized at a constant rate relative to total protein synthesis (ie. $\alpha_{(p)}$ is constant). This is reflected by the constant frequency of transcription of rpoBC, indicated by the transcriptional fusion work. On this basis, the simplest model for the growth rate controlled expression of RNA polymerase is one that suggests that the synthesis of β and β' is controlled at the transcriptional level in contrast to the co-transcribed ribosomal protein genes.

4.3 NATURE AND FUNCTION OF atn

4.3.1 Is atn a simple terminator that leaks?

The presence of a terminator between structural genes of an operon is highly unusual. In rplJLrpoBC, atn serves to allow the reduced expression of the two promoter distal genes rpoBC. However, if atn is simply a leaky terminator functioning downstream of a promoter that initiates transcription at a constant frequency, it seems a gratuitous complication of the transcriptional control of rpoBC. Why is rpoBC not simply in its own operon under the control of a relatively weak promoter? Surely the arrangement of RNA polymerase genes downstream of ribosomal protein genes under the transcriptional control of a shared promoter implies these genes are coordinately expressed, at least under some conditions. Not only is some coordination implied by the arrangement of these genes in an operon, the presence of atn implies the need to uncouple the expression of RNA polymerase from ribosomes under some conditions.

The above logic, and previous hybridization experiments that described the uncoupling of transcription or expression of rplJL from rpoBC, lead to an expectation that such an uncoupling would be seen when lysogens of transcriptional fusions were assayed for β-galactosidase under appropriate conditions. The mechanism directing the uncoupling of rplJL from rpoBC expression could be examined by comparing the behaviour of selected lysogens. For instance, transcription of rpoBC had been reported to increase relative to that of

rplJL under some conditions (see Introduction). If this is due to a relaxation of attenuation, the level of β-gal expressed by a lysogen carrying rplJp should not change, whereas that expressed by a lysogen carrying rplJp+atn should increase. Such an uncoupling was not seen to function when the effect of growth rate on the frequency of transcription of rplJL and rpoBC was examined using transcriptional fusions.

The growth rate experiments show a constant termination efficiency at growth above 0.8 doublings per hour, even though the expression of ribosomes and RNA polymerase diverge markedly over this range of growth rate. Because it is difficult to interpret the results of experiments done with transcriptional fusions at very low growth rates (see previous), there was little point in measuring β-gal expression in lysogens grown very slowly, even though it may be at extremely low rates of growth that transcriptional controls become significant in ribosomal protein and RNA polymerase genes. Unfortunately, it is also difficult to measure the transcription of these genes in slow growing cultures with current hybridization techniques. A constant efficiency of atn mediated termination at rates of growth between 0.5 and 2.5 doublings per hour is indicated by both the transcriptional fusion experiments described here and the hybridization experiments reported elsewhere (Ralling et al. 1985; Dennis 1977). However there is no evidence to suggest that atn is anything but a leaky terminator under normal growth conditions.

4.3.2 Evidence that atn is Rho-dependent

The presence of a transcriptional attenuator between genes has to date been confirmed in only two *E. coli* operons. One of course is in rplJLrpoBC, the other is in the macromolecular synthesis operon which expresses rpsU, dnaG, and rpoD, the attenuator being found between rpsU, and the promoter distal genes dnaG and rpoD (Lupski et al. 1983). A model for the regulation of transcription of dnaG and rpoD requiring anti-termination at the upstream attenuator was proposed by the authors based on the identification, from sequence data, of a sequence between the promoter rpsUp and the attenuator, of a site homologous to the λ nut site. Although no direct evidence to support this contention has been forthcoming, it is reasonable to

presume, as discussed in Chapter 1, that anti-termination mechanism(s) function in E. coli. A computer assisted examination of the DNA sequence of rplKAJLrpoBC revealed no nut-like sequence, but did suggest that a good boxA sequence (Friedman and Olson 1983) was to be found within rplA (7/8: position 756-763). The very presence of atn and the suggestion of a appropriate boxA sequence are enough to provoke speculation that an anti-termination mechanism functions in rplKAJLrpoBC, even though the fusion and hybridization studies do not support such a hypothesis. Therefore attempts were made to identify proteins that affect the efficiency of termination at atn.

One of the original justifications for directing so much effort to the construction of the rplKAJLrpoBC transcriptional fusions was to allow the easy screening of mutant strains to assess the effect of specific mutations on rplKAJLproBC transcription by simply lysogenizing the mutant strain with selected transcriptional fusion phages. This approach proved to be unworkable for termination mutants for two reasons. First of all, many termination mutants were first identified by their effects on phage lambda metabolism and some of these mutants, such as rho, are impossible to lysogenize (Fassler et al. 1985). Secondly, some of the mutants of interest, such as nusA, may have an effect on the transcription of lacZ (Greenblatt 1984). The difficulty lysogenizing rho mutants was overcome by introducing a rho mutation, by P1 transduction, into lysogens which carried a transcriptional fusion. This of course is a cumbersome approach requiring a genetic construction for each lysogen and is made more difficult by the necessary presence in each lysogen of a recA mutation. The recA mutation is required to keep the spontaneous induction frequency of the lysogen down to levels which do not interfere with the β-gal assay. Although the rho112 lesion was successfully introduced into lysogens of transcriptional fusions which carried either rplJp alone or in combination with atn (Table 6), and the results interpreted to implicate Rho in the efficiency of termination at atn, caution must be exercised before drawing strong conclusions from these data. The strain which carried both a lesion in rho, and λGR1 with rplJp fused to trpB, was a very sick strain. In a medium which can support growth rates of 1.5 to 2.0 doublings per hour, GR50-8-1919 grew at 0.2

doublings per hour. The rho lesion could not be crossed into GR50-10, so another lysogen of the same phage λGR3 was used for comparison. This means the comparison of the effect of a rho lesion on rplJp and rplJp+atn is not being made in an isogenic pair of strains.

For the reasons outlined above, strains with lesions in genes that could have an effect on the function of atn were examined by measuring the ratio of their rplJL transcripts to their rpoB transcripts. This was done by hybridizing a fixed amount of pulse-labeled RNA to ssDNA probes specific for either rplJL or rpoB. The relative level of rplJL/rpoB transcripts is constant in wild type E. coli and such a measurement is independent of the growth rate of the strain (Ralling et al. 1985). This approach implicated the Rho, NusA, and SfrB proteins in termination at atn, by virtue of their effects on the transcript ratio (Table 7). Because rho mutants have a lower rplJL/rpoB transcript ratio, it is concluded that Rho normally promotes transcription termination at atn. Because both nusA and sfrB lesions raise the value of this same ratio, they normally function to anti-terminate transcription at atn. The attenuator atn is designated as Rho-dependent because of the effect of Rho, a designation that is also supported by the implication of NusA (see Chapter 1). Does the hybridization evidence really support such a conclusion?

The known effect of Rho on transcription is to promote termination at Rho sensitive sites. Therefore a reduction in Rho activity is expected to decrease termination at Rho-dependent sites which, in the case of atn would serve to increase the transcription of rpoBC. This would result in an decrease in the ratio of rplJL/rpoB transcripts which of course is the change seen in the transcript ratio in the presence of a rho lesion. Of the two rho lesions used, rho15 appears to be the tighter, having much more pronounced phenotypic effects. The rplJL/rpoB ratio is reduced to 1.6 with rho15 and to 2.5 with rho112, changes consistent with the phenotypic effects of the two mutations. The double mutant X10015 has a rho lesion and a compensating mutation in rpoB and was originally isolated as a pseudo-revertant of a rho mutation which restores more efficient termination at Rho-dependent sites than is seen in the wild type parent (Guarente and Beckwith 1978). Therefore a prediction that the

rplJL/rpoB transcript ratio should increase in X10015 is fulfilled by the observation that this ratio more than doubles in this strain (Table 7). Although admittedly weak evidence by itself, the result derived from crossing a rho lesion into a lysogen carrying rplJp+atn, and which resulted in the doubling of β-gal levels, also supports a conclusion that Rho is involved in termination at atn

Similar arguments can be made to support the conclusions that both NusA and SfrB proteins are involved in the normal read-through of atn. Not only do the ratios of rplJL/rpoB transcripts change in the presence of lesions in the genes for these proteins, but they change in a direction consistent with the reported activities of these proteins. One would also predict from what is known of other Rho-dependent termination sites that if atn is Rho-dependent, NusA should also be involved. Again this prediction is satisfied by the increase in the rplJL/rpoB transcript ratio, observed with the hybridization experiment on the nusA mutant.

The only puzzling result with the hybridization approach was seen in the transcript ratio determined in an rnc mutant. This mutant does not process transcripts correctly, due to a lesion in the gene encoding RNaseIII (Apirion and Watson 1975). Such a mutation can have an effect on the anti-termination of some lambda phage transcripts (Lozeron et al. 1983). The rplJL/rpoB ratio increased in the presence of rnc, suggesting a similar mechanism may affect the transcription of rpoBC. However it has been reported that a lack of RNaseIII processing does not affect the expression of β and β' (Dennis et al. 1985). To resolve these observations, a post-transcriptional mechanism to regulate the expression of rpoBC would have to be proposed.

4.4 CONFIRMATION OF THE NATURE OF atn BY S1 MAPPING

In an attempt to directly confirm the action of Rho and NusA at atn, transcripts that terminated in the rplL-rpoB intercistronic region were visualized by an S1 mapping procedure. The intention of this work was, firstly, to determine if transcription terminated at normal sites in mutant strains carrying lesions in termination protein genes and, secondly, was to directly confirm that the altered transcript ratios

suggested by the hybridization study were a result of increased or decreased termination at atn. The first of these objectives could be realized by examining the pattern of bands seen on autoradiographs of gels that visualized the S1 fragments of transcripts from termination mutants and their isogenic wild-type parents. The presence of a new band or the absence of a previously visualized one would suggest that termination sites were altered in the absence of normal termination proteins. This might help distinguish a Rho-dependent from a Rho-independent site, if both types of termination sites functioned in this region. No altered patterns were seen in any of the mutants examined, except in the mutants carrying the rnc or rpoB203 lesions. The rnc strain was really a positive control, since an altered pattern of transcripts should be seen in this processing mutant (Fig 16). Although the rpoB203 mutation restores termination at Rho-dependent sites, it does not necessarily terminate at exactly the same site (Platt 1981). In the mutant X10013, the new termination site is downstream of the processing sites, since the intensity of these two bands increases 6-fold, but upstream of the SalI site, since the intensity of the probe length band does not increase (laser densitometer data not shown, but see Fig 17). Therefore, although no banding patterns were seen to change in the termination protein mutants, clear differences could be seen in other strains.

Disappointingly, the contrast between the S1 transcript bands which were visualized by autoradiography was not sufficient to permit their quantification and a comparison of their relative intensities. It had been hoped that by comparing the intensity of the atn terminated transcript with that of the read-through transcripts in rho strains and in the nusA strain, the effect of each of these mutations on atn could be corroborated. However, the read-through transcripts are not only at a much decreased level due to attenuation, they are processed to further distribute them over three bands. Therefore one is trying to compare bands of intensities that are different by a factor of perhaps 15 or 20.

4.5 SUMMARY

The work described in this thesis was designed to identify the transcriptional controls that regulate the transcription of the rplKAJLrpoBC cluster of genes. Two strong promoters and an attenuator are active in these genes and are the primary transcriptional regulatory features. A weak promoter directs transcription of rplL but is of dubious physiological significance. The behaviour of these regulatory sites is not modulated in response to changes in the growth rate.

The attenuator atn terminates 60 percent of all transcription initiated upstream and functions at a constant efficiency at growth rates between 0.5 and 2.5 doubling per hour. Transcriptional controls apparently are not responsible for the changing level of synthesis of the ribosomal proteins encoded by rplKAJL seen by others to increase as a direct function of growth rate increases.

However, that atn may be active in modulating the transcription of rpoBC is not ruled out by this work. The ratios of rplJL/rpoB transcripts, determined by hybridization in mutants with lesions in rho and nusA genes, suggest that termination at atn may be subject to an anti-termination process and, as such, the efficiency of termination at atn may be modulated. S1 mapping experiments do not confirm directly the involvement of Rho or NusA. Such a confirmation may have to come from in vitro experiments.

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FIGURE A1: **Physical map of pBR325 showing restriction sites used in this work.** All of the plasmids constructed for this work are based on pBR325 (Bolivar 1978), a 5995 bp multicopy ColE1 plasmid. The cloning sites used in this work and the genes they interrupt are shown. The HindIII sequence does not cut within the *tet* gene, but severs the *tet* gene promoter. Therefore, fragments cloned in this site may show degrees of tetracycline resistance if the fragment carries an appropriately directed promoter.

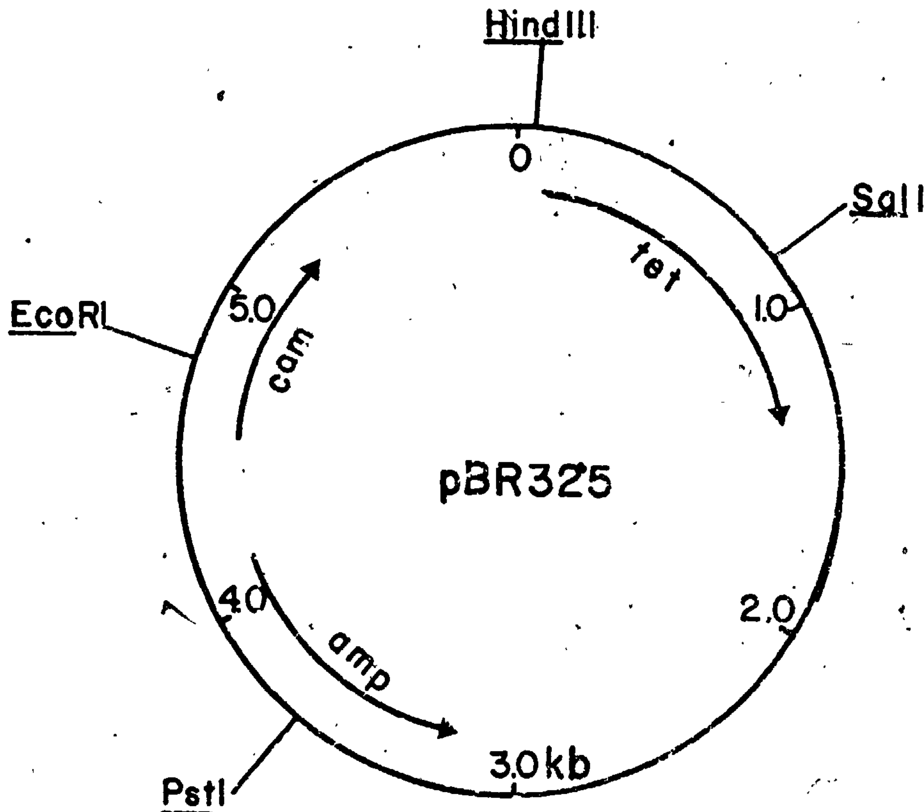
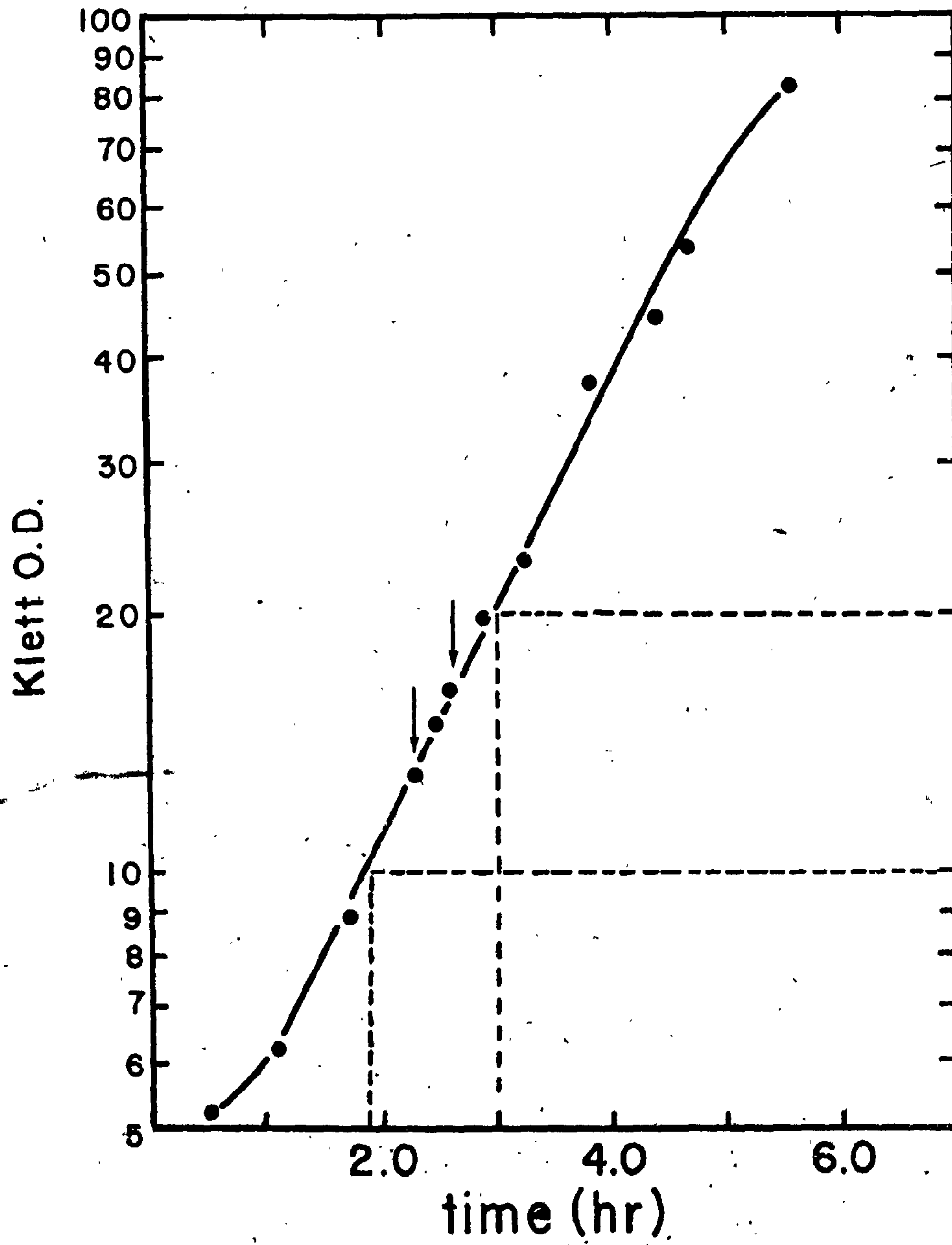

HindIII
0
SalI
tet
1.0
EcoRI
5.0
cam
pBR325
4.0
amp
2.0
PstI
3.0kb

FIGURE A2: Sample growth rate plot for β-galactosidase assay.

Growth of cultures was monitored as described (2.5.3) with a Klett colourimeter holding a red filter transmitting light in the 640 to 700 nm range. When grown under steady state conditions, a culture will increase in mass, that is in O.D., in an exponential fashion. When the log of such O.D. values are plotted, a straight line will result.

Growth rate as referred to in this work is expressed as the number of generations per hr. This is the reciprocal of the doubling time, which is derived in the plot opposite by reading the time points from the abscissa, corresponding to any doubling in the O.D., read from the ordinate axis. For example, time at Klett O.D. = 10 is 1 hr 50 min; at Klett O.D. = 20, time is 3 hr. If the doubling time is 70 min, the doublings per hr or generations per hr is 0.86.



Klett O.D.
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
2.0
4.0
6.0
time (hr)

FIGURE A3: Saturation curve for hybridization experiments.

In order to determine the amount of DNA probe sufficient to hybridize all homologous transcripts in a 2 μg sample of RNA, the saturation curve opposite was constructed. Based on this curve, made using MP8-658 (Fig 10), all hybridization experiments (2.28) were performed with 0.2 μg or greater amounts of DNA.

140

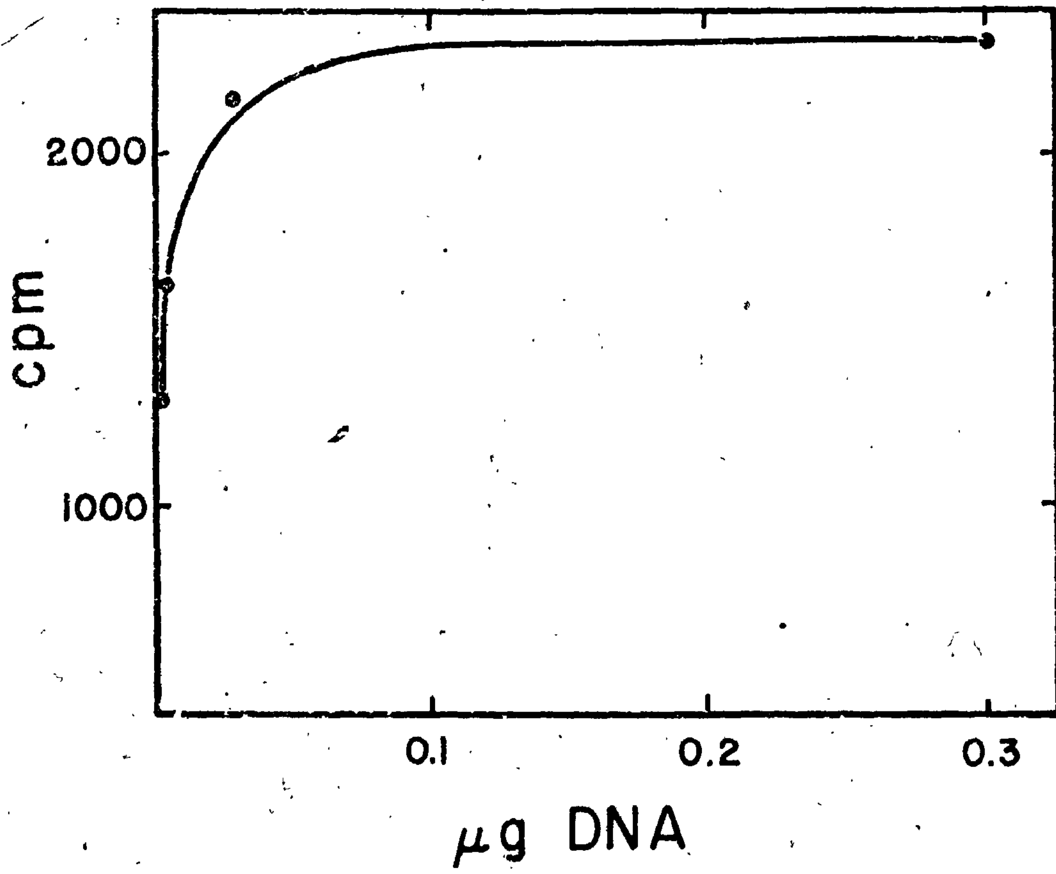


TABLE A1: Hybridization data used to calculate rplJL/rpoB transcript ratios. Shown are the raw data upon which the rplJL/rpoB ratio calculation is based. Only the relevant genotype is listed. The complete genotype of each strain is described in Table 1. Each of the cpm values is the average of 3 values obtained from independently hybridized RNA samples.

STRAIN	GENOTYPE	CPM ADDED x 10^{-5}	CPM JL PROBE	CPM B PROBE	CPM MP8	CPM CORRECTED JL PROBE	CPM CORRECTED B PROBE	JL/B RATIO (x 3.57)*
SA1030	w.t.	6.26	1983	1766	1128	855	638	4.8
SA1600	rho15	3.60	1468	1904	1121	347	783	1.6
AD1919	rho112	4.80	1687	1953	1028	659	925	2.5
X10015	rho201,rpoB203	1.14	411	319	272	139	47	10.5
N99	w.t.	4.24	3224	2796	1628	1596	1168	4.9
C205	nusA1	5.20	13[illegible]7	686	140	1187	546	7.8
C347	nusB5	3.60	1127	918	142	985	776	4.5
K556	nusE71	5.90	937	758	268	669	490	4.9
N2076	w.t.	9.40	3217	2839	1839	1378	1000	4.9
N2077	rnc105	2.90	977	769	529	448	240	6.7
SA2244	w.t.	11.60	876	746	98	778	648	4.3
SA2243	sfrB	9.80	687	366	52	635	314	7.2
TGL177	pRJ1	0.76	1747	550	156	1591	394	3.6+

* The JL probe MP8-JL (Fig 10) has 173 uridines in the cloned fragment, the B probe MP8-B has 617. 617 ÷ 173 = 3.57.

+ The B probe in this case is MP8-587 with 156 uridines in the cloned fragment. The correction in this case is 0.9 (156 ÷ 173).

END

08 04 87

FIN