ESSENTIAL GENES IN THE RIF REGION OF THE ESCHERICHIA COLI CHROMOSOME

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF BIOCHEMISTRY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1989

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The University of British Columbia
Vancouver, Canada

Date 16 October 1989

DE-6 (2/88)

ABSTRACT

Regulation of the contiguous secE-nusG and rplKAJL-rpoBC operons, found in the rif region at 90 minutes on the Escherichia coli chromosome, was examined. SecE protein is important in protein export. NusG protein is involved in transcription antitermination. The rplKAJL-rpoBC gene cluster encodes, respectively, the four 50S subunit ribosomal proteins L11, L1, L10 and L12, and the β and β′ subunits of RNA polymerase.

The nucleotide sequences of the secE and nusG genes were determined and their transcripts were analyzed by primer extension and S1 nuclease mapping. The two genes are cotranscribed, with transcripts initiated at the P_{BG} promoter and terminated at the Rho-independent terminator overlapping the P_{L11} promoter. The majority of transcripts are processed in the 5′ untranslated leader region by RNaseIII and possibly by a second unidentified nuclease.

Transcripts from the rplKAJL-rpoBC gene cluster were quantitated by filter hybridization and their ends mapped by S1 nuclease protection. The most abundant transcript was the 2600 nucleotide tetracistronic L11-L1-L10-L12 mRNA initiated at the P_{L11} promoter and terminated at the attenuator in the L12-β intergenic space. Less abundant 1300 nucleotide L11-L1 and L10-L12 bicistronic transcripts were also observed. Two 5′ ends for the L10-L12 bicistronic mRNA were located, one at the P_{L10} promoter and the other 150 nucleotides downstream of P_{L10}, in a region where no promoter activity has been detected. About 80% of the transcripts were terminated at the attenuator; transcripts reading through the attenuator were partially processed by RNaseIII. No other major 5′ ends were observed in the L12-β intergenic region. During restriction of RNA polymerase activity, transcriptional disruption of rplKAJL and rpoBC results mainly from modulation in the frequency of initiation at P_{L11} and P_{L10} promoters, and termination and antitermination at the attenuator.

The rplJL transcript leader region is thought to mediate regulation of L10 and L12 synthesis by folding into a translationally closed or open secondary structure (T. Christensen, M. Johnsen, N.P. Fiil and J.D. Friesen (1984) EMBO J. 3, 1609-1612). Point mutants in the leader mRNA were created by site-directed mutagenesis and analyzed in an in vitro translation assay. Preliminary results suggest that alternative secondary or higher order RNA interactions may be involved.

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ABBREVIATIONS

A	adenosine
A_{460}	absorbance at 460 nm (or other wavelength)
ATP	adenosine triphosphate
bp	base pair(s)
C	cytosine
cpm	counts per minute
dCTP	deoxycytidine triphosphate
ddTTP	dideoxythymidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid
G	guanosine
GTP	guanosine triphosphate
h	hour(s)
KAN	kanamycin resistance cassette
kb	kilobases
L1, L2 ...	proteins from the large ribosomal subunit
m.o.i.	multiplicity of infection
mA	milliamperes
Met	methionine
min	minute(s)
mRNA	messenger RNA

N	λ-encoded antitermination factor
NAD	nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
ppGpp	guanosine 5′,3′-bis(diphosphate)
rif	rifampicin
r-protein	ribosomal protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit of sedimentation coefficient
s.a.	specific activity
S1, S2 ...	proteins from the small ribosomal subunit
SDS	sodium dodecyl sulphate
T	thymidine
TE	10 mM Tris-Cl (pH7.5), 1 mM EDTA
Tris	tris-(hydroxymethyl)amino methane
tRNA	transfer RNA
ts	temperature sensitive
U	uridine
V	volts
W	watts
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ACKNOWLEDGEMENTS

I wish to thank Deidre de Jong Wong for her technical assistance and Pat Dennis for providing me the opportunity to work in his laboratory. Thanks go to all my fellow students in the lab for discussions on everything; their friendship was appreciated. Special thanks go to Lawrence Shimmin for sharing, with great patience and generosity, his tremendous technical expertise. Also, his love for science is an inspiration in itself. It was a joy to work with Craig Newton; his joie de vivre was contagious. I also wish to thank Joan McPherson for her friendship.

My gratitude goes to P. Bonnard and R.H. van Rijn for reminding me what is important. Thanks also go to Ann Nelson and Trish Shwart for their friendship and for keeping the Muse alive.

Most of all, I wish to thank Rod, whose unconditional support has made all this possible.

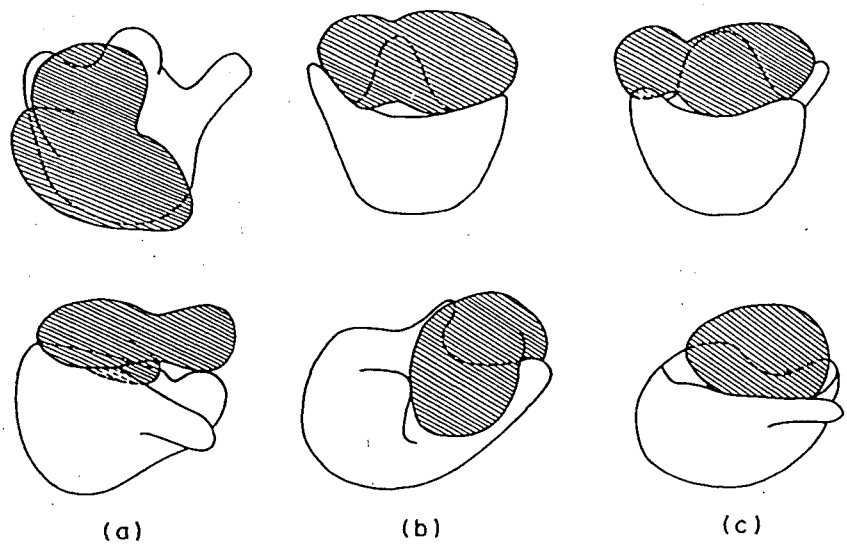
1.1 REGULATION OF RIBOSOME SYNTHESIS IN ESCHERICHIA COLI

1.1.1 BACKGROUND AND PERSPECTIVE

The Escherichia coli ribosome is a complex subcellular organelle comprised of three ribosomal RNA molecules (rRNA) and 52 different ribosomal proteins (r-proteins). A 70S particle, the ribosome is composed of a small 30S subunit which includes 21 ribosomal proteins and 16S rRNA, and a large 50S subunit which has 31 r-proteins and 23S and 5S rRNAs (fig. 1). Except for r-protein L12, which exists in four copies, each r-protein and each rRNA is found in one copy per ribosome.

Ribosomal protein genes, each found in a single copy per genome, are organized into at least 20 operons scattered throughout the E. coli chromosome. Some of these operons also contain genes essential for (i) DNA replication, such as dnaG (DNA primase), (ii) transcription, such as rpoA, rpoBC and rpoD encoding RNA polymerase subunits α, β, β' and σ_{70} respectively, (iii) translation, such as tufA (EF-TuA) and fusA (EF-G) and (iv) protein export, such as secY (secY) (fig. 2). There are seven operons for rRNA in E. coli. Each operon encodes a precursor transcript which upon processing generates 16S, 23S and 5S rRNA, as well as several tRNA species.

During exponential growth, the synthesis of ribosomal components is under growth rate-dependent control such that the cellular ribosome concentration increases with increasing growth rate and corresponds to the amount necessary to sustain a given level of translation.



.FIGURE 1. The Escherichia coli ribosome.

Three different arrangements of the E. coli ribosome from analysis of electron micrographs are shown. The hatched shape represents the 30S small subunit; the unmarked form represents the 50S large subunit. (a) The "UCLA" model (b) The "Nutley" model (c) The "Berlin" model. (Illustration taken from Liljas, 1982.)

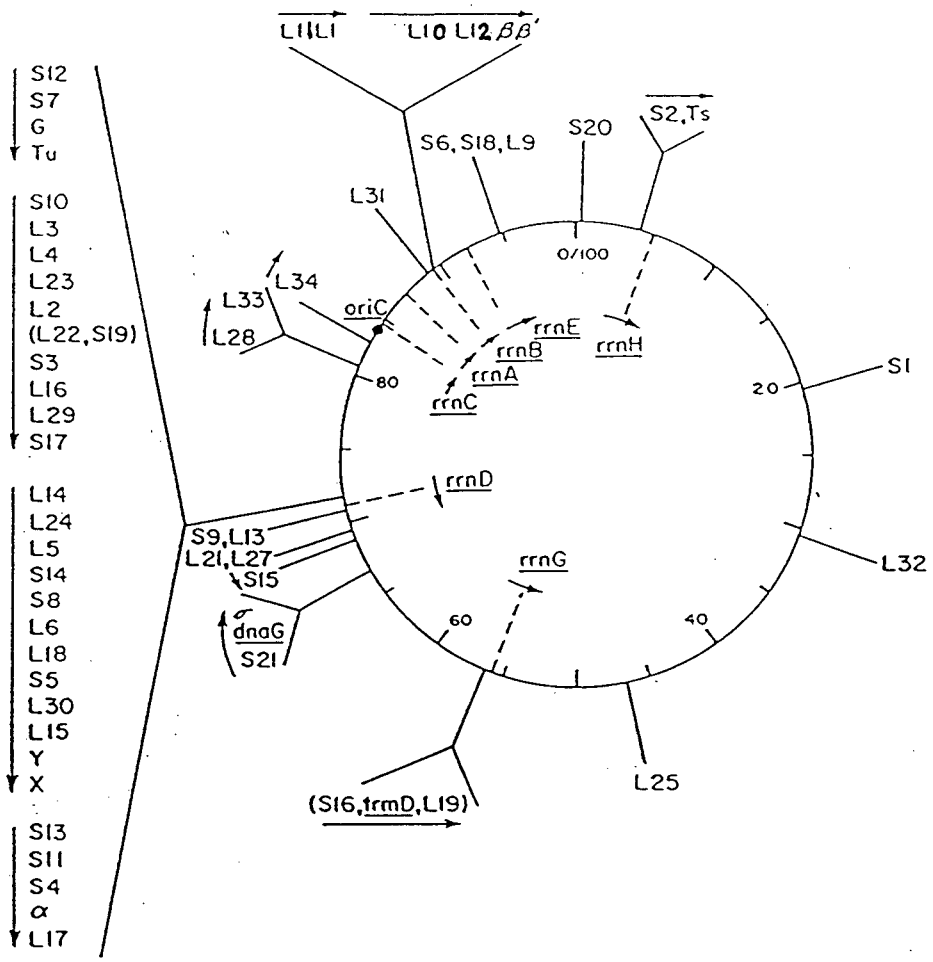


FIGURE 2. Location of rRNA and r-protein genes on the Escherichia coli genetic map.

Genes for r-proteins are represented by the protein product. The directions of transcription of the operons, when known, are indicated by arrows. The origin of replication (oriC) is situated at 84 minutes. (Figure taken from Nomura et al., 1984)

As well, the synthesis of most or all r-proteins are coordinately and stoichiometrically regulated to match the levels they represent in complete ribosomes, with little excess r-protein and rRNA production. Thus the control of ribosome synthesis in E. coli presents two major challenges : to coordinate the synthesis of the various ribosomal components and to balance the synthesis of these components with the growth requirements of the cell (Reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986).

1.1.2 REGULATION OF rRNA SYNTHESIS

Theories of transcriptional regulation of rRNA operons have been formulated in light of two classical regulatory phenomena : growth rate-dependent control and stringent control. During exponential growth, ribosome synthesis is correlated to growth rate; this relationship has been described above. Under this growth condition, the synthesis of the individual rRNA and r-protein components is coordinately controlled and there is no significant turnover of these components or build-up of pools of free components. However, during periods of amino acid starvation, the stringent response is evoked.

The stringent response involves a complex set of physiological changes in exponentially growing E. coli when aminoacylated tRNA becomes the limiting factor in protein synthesis. This starvation response can result from amino acid deprivation or inactivation of an aminoacyl-tRNA synthetase. The cellular changes are diverse and include a dramatic decrease in the synthesis of all ribosomal components and a concomitant accumulation of guanosine tetraphosphate (ppGpp) as well as other unusual nucleotides. There is growing evidence that ppGpp is the major regulatory signal during the stringent response and that its targets are at both the post-translational level and at the level of

transcription initiation. There appear to be two pathways of ppGpp synthesis. During amino acid starvation, formation of ppGpp is a relA-dependent reaction which occurs on idling ribosomes. Stringent relA$^+$ strains exhibit the stringent response whereas relaxed relA$^-$ strains continue to synthesize stable RNAs (rRNAs and tRNAs) and r-proteins under aminoacyl-tRNA starvation conditions. In addition, there is an ill-defined relA-independent pathway for ppGpp synthesis which occurs during exponential growth. The spoT gene product, responsible for the degradation of ppGpp, is thought to be part of this relA-independent pathway. The stringent response is probably integrated with other global responses (e.g. heat shock response) (review : Cashel and Rudd, 1987).

The ribosome feedback regulation model has been proposed by Nomura to explain the regulation of stable RNA synthesis under conditions of balanced growth. This model suggests that stable RNA synthesis is feedback regulated by non-translating or free ribosomes; however, there is no direct evidence that free ribosomes can interact with promoters to interfere with transcription. The concentration of free ribosomes is thought to be determined by the nutritional environment and thus can efficiently adjust the level of stable RNA (and hence ribosome) synthesis to environmental conditions. It is not known whether idle ribosomes act directly by blocking transcription or indirectly through the action of an effector (perhaps ppGpp), whose concentration reflects the level of free ribosomes (reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986).

During the stringent response, ppGpp has been suggested to be the effector in regulating stable RNA expression. The mechanism of its action is still unclear. RNA polymerase is a target for ppGpp (Glass et al., 1987) and its promoter selectivity for r-protein and stable RNA promoters is modulated by direct interaction with ppGpp (Ishihama

et al., 1987). Therefore, it appears that ppGpp interacts with RNA polymerase and alters the equilibrium between two (or more) forms of the enzyme, one which has a specificity for r-protein and stable RNA promoters and one which does not, as first proposed by Travers (1976), Travers et al. (1980). Also, Travers and co-workers have suggested that stringently regulated stable RNA promoters (and probably r-protein promoters; see below) are distinguished from non-stringent promoters by a GC-rich "stringent discriminator" sequence between the -10 Pribnow box and the initiating nucleotide (Travers 1980, 1984). However, questions concerning the significance of the discriminator sequence in stringent control have been raised by Yamagishi et al. (1987).

Alternatively, Bremer and colleagues have argued that the conventional distinction between growth rate control and stringent control is unfounded. The relationship between the rate of stable RNA synthesis relative to the rate of total RNA synthesis (r_s/r_t) and the concentration of ppGpp can be described by a single function which applies to exponential growth or amino acid deprivation conditions and which is independent of the relA allele (Ryals et al., 1982). This implies that ppGpp is the major, if not only, effector in regulation of stable RNA gene activity and that growth rate control and stringent control should be considered as one phenomenon differing only in extent (Cashel and Rudd, 1987).

Nucleotide ppGpp appears not to be determined directly by the growth medium but by the rate at which the cell can generate the substrates for protein synthesis. Since ribosomes starved of aminoacyl-tRNAs are the sites of ppGpp synthesis, one possibility is that the level of free ribosomes determines the level of ppGpp which then establishes the promoter preference of RNA polymerase by adjusting the equilibrium between the two forms of RNA polymerase (reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986).

1.1.3 REGULATION OF r-PROTEIN SYNTHESIS

1. Translational feedback regulation

Regulation of r-protein synthesis can be exercised at both the transcriptional and translational levels. At the translational level, much is now known about the molecular mechanisms involved in control of r-protein production, at least for the major r-protein gene clusters located at 73 and 90 minutes on the E. coli chromosome (fig. 2). To explain the phenomenon of coordinated and balanced synthesis of the various r-proteins, the translational feedback model was proposed. According to this model, the synthesis of r-proteins is coupled with the assembly of ribosomes. Briefly, each r-protein operon encodes a bifunctional regulatory protein; this ribosomal protein can either be incorporated into assembling ribosomes or, in the absence of an adequate supply of rRNA, can bind to a site on its own mRNA and prevent further translation (reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986). However, recent studies on the trmD operon have shown that r-protein synthesis from this operon is not translationally feedback regulated; Wikström et al. (1988) have proposed that r-protein operons which do not encode proteins that bind directly to rRNA are not under autogenous control. The control mechanisms for regulation of expression of these r-proteins have yet to be determined; some possibilities are protein degradation and metabolic regulation at the transcriptional level (Wikström et al., 1988).

Autogenous translational control was first described for the regulation of gene 32 expression in bacteriophage T4 (Lemaire et al., 1978). Subsequently, translational feedback regulation has been discovered to control the expression of a variety of E. coli genes

including secA (Schmidt and Oliver, 1989), thrS (threonyl-tRNA synthetase) (Springer et al., 1985) and ksgA (a methyltransferase) (van Gemen et al., 1989).

Many r-proteins have now been identified as "translational repressors" which selectively inhibit the synthesis of some or all of the r-proteins whose genes are in the same operon as the repressor r-protein. For example, r-proteins S7, L1, L10, S8 and S4 are the translational repressors of the str, L11 (rplKA), L10 (rplJL), spc and alpha operons, respectively (reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986).

The regulatory protein presumably interacts with its mRNA and the corresponding rRNA by recognizing similar regulatory sites predicted by sequence homologies (Nomura et al., 1980; Olins and Nomura, 1981; Johnsen et al., 1982; Deckman and Draper, 1985). Recent detailed studies on regulatory protein binding sites support this proposition (L11 : Thomas and Nomura, 1987, Said et al., 1988; S8 : Gregory et al.,1988 Cerretti et al., 1988; S20 : Parsons et al., 1988). The regulatory sites are usually situated in the leader region of the mRNA. However, in the str (Dean et al.,1981) and spc (Olins and Nomura, 1981) operons, the binding sites are located in an intergenic region. In most cases, the binding site on the messenger begins close to the initiation codon, but in the L10 operon it is found more than 100 nucleotides upstream (Johnsen et al., 1982).

Translational coregulation of genes in the same operon by r-protein binding at a single site is likely achieved by translational coupling, a phenomenon first demonstrated in the tryptophan operon by Oppenheim and Yanofsky (1980). When two genes are translationally coupled, efficient translation of a downstream gene requires prior translation of the preceding gene. Experimental evidence for this has been demonstrated for the L11

operon (Baughman and Nomura, 1983; Sor et al, 1987), the spc operon (Mattheakis and Nomura, 1988), the S10 operon (Lindahl et al., 1989) and the alpha operon (Thomas et al., 1987). Thus for certain r-protein operons, translational feedback regulation and translational coupling insure that r-protein synthesis is coordinated to each other and adjusted to rRNA synthesis.

2. Transcriptional regulation

As in the case of rRNA operons, transcriptional regulation of r-protein genes has been argued to be involved in growth rate (Little et al, 1981; Little and Bremer, 1984; Dennis, 1977a) and stringent control of r-protein synthesis (Dennis and Nomura, 1974; Maher and Dennis, 1977). Again, free ribosomes and/or ppGpp have been postulated as negative effectors of r-protein synthesis. The translation feedback loop may be superimposed on transcriptional control to determine the final rate of r-protein synthesis (reviews : Jinks-Robertson and Nomura, 1987; Nomura et al., 1984; Lindahl and Zengel, 1986).

There appears to be more than one strategy to achieve growth rate-dependence and stringent regulation of r-protein synthesis. Unique among E. coli r-protein operons, the synthesis of r-proteins in the S10 operon is controlled by feedback regulation by L4 both at the transcriptional (Freedman et al., 1987; Lindahl et al., 1983; Lindahl and Zengel, 1979) and translational levels (Freedman et al., 1987; Yates and Nomura, 1980). The binding of excess L4 not only stimulates transcription termination (attenuation) within the S10 mRNA leader, but also inhibits translation of the polycistronic mRNA. L4-mediated transcriptional and translational regulation share some sequence requirements but the two processes recognize different features of the S10 leader. Stringent control is accomplished at the level of transcription initiation only. However, growth medium-dependent control involves

regulation of both transcription initiation and transcription read-through at the attenuator (Freedman et al.,1985).

In contrast, studies involving r-protein promoter fusions to galK or lacZ indicate that the characteristic increase in r-protein synthesis with increasing growth rate is determined not by transcriptional processes but by post-transcriptional ones (Miura et al.,1981). As further support for this, Cole and Nomura (1986b) have argued that translational regulation is solely responsible for growth rate-dependent and stringent control of the synthesis of r-proteins L11 and L1. A base substitution mutation in the repressor binding site of the L11 operon abolishes not only autogenous translational control but also both growth rate regulation and stringent control.

Alternatively, as suggested by results of pulse-labelled RNA experiments, stringent regulation of r-protein synthesis was thought to function at the transcriptional level (Maher and Dennis, 1977). However, it is now known that translational feedback repression can cause selective inactivation of r-protein mRNA (Fallon et al., 1979; Singer and Nomura, 1985; Cole and Nomura, 1986a). Consequently, it is possible that the apparent change in r-protein mRNA synthesis rates in these experiments was due to a decrease in mRNA half-life caused by feedback repression. Hence, stringent control of r-protein synthesis may be an indirect effect mediated by the translational feedback process.

As in the case of rRNA genes, the effects of ppGpp on r-protein gene activity have been examined. The competitive template assays of Kajitani and Ishihama (1984) have revealed ppGpp sensitivity of promoters upstream of the rpsA and rplJ genes. Promoters of r-protein operons also contain the GC rich "stringent discriminator" sequence (see above), although its importance in stringent regulation is now in doubt (Yamagishi et al.,1987).

Attempts to examine ppGpp-dependent regulation of r-protein genes have produced unclear results because of other effects (e.g. polarity and RNA polymerase sink effects) which are superimposed on mRNA gene activities (Little and Bremer, 1984).

In summary, the role of transcription in the regulation of r-protein synthesis is still not clear. Unlike the translational feedback system which is now well characterized, the molecular mechanisms that control transcription of most rRNA and r-protein operons have not been elucidated. It may be too simplistic to assume that one or two sets of regulatory pathways can account for the regulation of all rRNA and r-protein operons under all physiological conditions.

1.2 PURPOSE OF THIS WORK - GENERAL OVERVIEW

Genes encoding 31 of the 52 r-proteins are found at two major loci at 73 and 90 minutes on the E. coli chromosome (fig. 2). The rif region at 90 minutes contains the following organization of genes essential for transcription, translation and protein export : tufB, secE-nusG, and rplKAJL-rpoBC. The tufB operon encodes four tRNAs and the translation elongation factor EF-TuB; the DNA sequence of this operon has been determined and its transcripts have been partially analyzed (An and Friesen, 1980a; Van Delft et al., 1987).

The secE and nusG genes are co-cistronic and both are essential for cell viability. The SecE protein is an integral membrane protein and is a component of the protein export apparatus (Schatz et al., 1989). The NusG protein is believed to be involved in transcription antitermination (J. Greenblatt, S. Sullivan and M. Gottesman, personal communication); its activity appears to be linked to those of other Nus proteins such as NusA, NusB and NusE. Because of the essential nature of secE and nusG in cell viability and because of their

grouping with other genes involved in translation and transcription, the first part of this thesis (chapter 3) is concerned with sequence and transcript analyses of this gene cluster as an initial effort to understand the regulation of expression of the secE-nusG operon.

Downstream, the rplKAJL-rpoBC gene cluster encodes, respectively, the four 50S subunit ribosomal proteins L11, L1, L10 and L12 , and the β and β′ subunits of RNA polymerase; the nucleotide sequence of this gene cluster has been determined previously (Post et al., 1979) and much work has been done on the regulation of this cluster (review: Jinks-Robertson and Nomura, 1987). However, several regulatory features in rplKAJL-rpoBC still require clarification, for a better understanding of the regulated synthesis of these ribosomal and RNA polymerase components. The second and greater part of this thesis (chapter 4) addresses regulatory mechanisms of the rplKAJL-rpoBC gene cluster.

Expanded introductions to the secE-nusG and rplKAJL-rpoBC operons are found in chapters three and four respectively.

2.1 BACTERIAL STRAINS AND PLASMID CONSTRUCTIONS

The bacterial strains and plasmid constructions used in this work are described in table 1.

2.2 MEDIA AND CULTURE CONDITIONS

Bacteria were grown exponentially either in YT media (5 g/l Bacto-yeast extract, 8 g/l Bacto-tryptone, 5 g/l NaCl, pH7.5) or in M9 minimal salts media (Miller, 1972) supplemented with glucose (0.2%), required amino acids (50 µg/ml), thiamine (0.5 µg/ml) and NAD (1 µg/ml) when required, in a rotary shaker bath or air shaker. Growth was at 37°C unless otherwise stipulated for temperature sensitive strains. Bacterial growth was monitored by measuring absorbance at 460 nm. When required, antibiotic concentrations used were : ampicillin (100 µg/ml), tetracycline (15 µg/ml) and kanamycin (50 µg/ml).

2.3 GENERAL TECHNIQUES OF MOLECULAR BIOLOGY

General recombinant DNA techniques were carried out according to Maniatis et al. (1982) unless otherwise specified.

2.3.1 PREPARATION OF PLASMID DNA

Small scale preparation of plasmid DNA was done by the alkaline lysis method (p.368, Maniatis et al., 1982). Large scale preparation was carried out according to the lysozyme-SDS lysis method (pp. 92-94, Maniatis et al., 1982).

Table 1

Bacterial strains and Plasmids

Strain	Description
C600	thr leu trp thi recA
XH56	F⁻ his thi metB strA lac rpoC(ts)
N2076	F⁻ thi argH1 nad84 lacY1 gal6 nalA1 λ^r xyl7 ara13 mtl2 str9 tonA2 rnc$^+$ (from D. Apirion)
N2077	F⁻ thi argH1 nad84 lacY1 gal6 nalA1 λ^r xyl7 ara13 mtl2 str9 tonA2 rnc105 (from D. Apirion)
NF536	leu valS(ts) relA$^+$
NF537	leu valS(ts) relA
N3431	HfrPO1 rel1 thi1 lacZ43 rne3071(ts) (from D. Apirion)
N3433	HfrPO1 rel1 thi1 lacZ43 rne$^+$ (from D. Apirion)
PD828	C600 / pBRU
PD858	C600 / pBRU::KAN

Plasmid	Description
pBRU	SmaI-EcoRI 2.1 kb fragment, containing the 3′ end of the tufB gene, the entire secE and nusG genes and the 5′ end of the rplK (L11) gene, cloned into the EcoRI and the blunt-ended ClaI sites of pBR322 (fig. 3)
pSS105	same as pBRU except the SmaI-EcoRI fragment was inserted, using an EcoRI linker at the SmaI end, into the EcoRI site of pBR322 (from S. Sullivan and M. Gottesman)
pBRU::KAN	pSS105 with a kanamycin cassette (from pUC4KISS; Pharmacia Inc.) inserted into the NruI site at nucleotide 347 within the secE gene (also known as pSS107, from S. Sullivan and M. Gottesman) (fig. 3)

2.3.2 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction enzymes used were purchased from Pharmacia Inc., Bethesda Research Laboratories (BRL) or New England Biolabs. Digests were carried out according to the instructions of the suppliers.

2.3.3 GEL ELECTROPHORESIS

Agarose slab gels (0.7% or 1.2%) were run in TAE buffer (40 mM Tris-acetate, pH 8.0, 20 mM sodium acetate, 1 mM EDTA) at 200 mA. The gels were run in the presence of 0.25 μg/ml ethidium bromide or were stained in ethidium bromide after electrophoresis. Genetic technology grade agarose (Schwarz/Mann Biotech) was used for preparative agarose gels.

Analytical (1 mm thick) or preparative (3 mm thick) 5% polyacrylamide gels were run in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 260 V.

2.3.4 DNA RESTRICTION FRAGMENT PREPARATION

Bands of restricted DNA, stained with ethidium bromide, were excised from agarose or polyacrylamide gels, placed in dialysis tubing and electroeluted in 0.5X TBE at 160 V for 1 h. The eluate was collected and purified by phenol/chloroform extraction and ethanol precipitation.

2.3.5 LIGATIONS

For sticky-end ligations, 40 fmoles of plasmid vector DNA and 1-3 fold molar excess of insert DNA were used. Incubation was at room temperature for 2 h or at 14-16°C, overnight.

For blunt-end ligations, the molar ratio of vector to insert DNA was 1:4. Incubation was at room temperature, overnight.

Total ligation volume was 20 µl. Half of the ligation mix was used per transformation.

2.3.6 TRANSFORMATIONS

E. coli host cells were made competent for DNA transformation by the $CaCl_2$ method (p. 250, Maniatis et al., 1982). Competent cells were gently mixed with 20-40 fmoles of DNA, left on ice for 40-60 min, heat shocked at 42°C for 2 min and plated directly on selective media. For tetracycline resistance, 1.0 ml YT medium was added to the cells after heat shock; the cells were incubated for 1 h at 37°C, centrifuged, resuspended in 0.1-0.2 ml YT media and plated.

2.3.7 BLUNT-ENDING RECESSED 3′ ENDS

When required, DNA restriction fragments with recessed 3′ ends were blunt-ended by using the Klenow fragment of E. coli polymerase I to fill in the recessed end. Each dNTP (0.25 mM) was used in a total volume of 20 µl (p. 113, Maniatis et al., 1982).

2.3.8 3′ END-LABELLING OF DNA FRAGMENTS

DNA restriction fragments containing recessed 3′ ends were end-labelled using Klenow enzyme and the appropriate [α-^{32}P]dNTP (s.a. 3000 Ci/mmol, 10 mCi/ml) (p. 115, Maniatis et al., 1982). The labelled fragment was purified by two successive ethanol precipitations. Radioactivity was measured by Cerenkov counting.

2.3.9 5′ END-LABELLING OF DNA FRAGMENTS

The 5′ ends of DNA restriction fragments were labelled with T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP (s.a. 3000 Ci/mmol, 10 mCi/ml) after dephosphorylation with calf intestinal alkaline phosphatase (p. 122, Maniatis et al., 1982).

2.3.10 5′ END-LABELLING OF OLIGONUCLEOTIDES

Oligodeoxyribonucleotides (250 ng) were 5′ end-labelled at 37°C for 40 min with 10 units of PNK and 100 µCi of [γ-^{32}P]ATP in 20 µl of ligase buffer (0.1 M Tris-Cl pH8.0, 5 mM DTT, 10 mM $MgCl_2$). The reaction was terminated by addition of 1 µl of 0.5 M EDTA (pH8.0), and incubation at 65°C for 5 min. Carrier tRNA (8 µg) was added and the reaction volume taken up to 100 µl with TE (10 mM Tris-Cl pH7.5, 1 mM EDTA). The labelled oligonucleotide was purified by two successive ethanol precipitations in the presence of 2.5 M ammonium acetate and redissolved in 20-50 µl TE.

2.3.11 LABELLING DNA FRAGMENTS BY NICK-TRANSLATION

High specific activity double-stranded DNA hybridization probes were prepared by the nick-translation method (Rigby et al., 1977; pp.109-112, Maniatis et al., 1982). Two different [α-^{32}P]dNTP's were used as radiolabels. The labelled probes were purified by two successive ethanol precipitations in the presence of 2.5 M ammonium acetate.

2.4 PREPARATION OF SINGLE-STRANDED DNA

2.4.1 FROM M13 PHAGE RECOMBINANTS

Single-stranded M13 phage DNA was prepared according to Sanger et al. (1980) and Messing (1983).

2.4.2 FROM pEMBL RECOMBINANTS

Single-stranded pEMBL DNA was prepared as described by Dente et al. (1983). Single colony pEMBL recombinants were grown overnight at 37°C in M9 minimal salts media with ampicillin. Two ml aliquots of YT + ampicillin were inoculated with 20 µl of

fresh overnight cultures and grown at 37°C to A_{600} ~ 0.2 (approximately 5 X 10^7 cells). The cells were superinfected with the f1 helper phage variant R408 (Russell et al., 1986) at a m.o.i. of 10:1 and then incubated at 37°C for 5-7 h. Phage particles and single-stranded DNAs were isolated as for M13 phage recombinants.

2.5 DNA SEQUENCING

Except for the sequencing ladders used to determine transcript ends in S1 nuclease mapping experiments, all DNA sequence determination was done by the dideoxynucleotide chain termination method (Sanger et al. 1977, 1980; Messing, 1983). Single-stranded DNA templates were prepared as described above. Double-stranded templates were denatured with alkali and precipitated with ethanol prior to sequencing (Hattori and Sakaki, 1986). Often the templates were first screened by using only the dTTP/ddTTP reactions to avoid sequencing redundant clones. Universal forward or reverse primers were used in most cases. Site-specific mutants in the L10 mRNA leader region were confirmed by sequencing, using oligonucleotide primer oPD28 (5′ - CAAGCTGAATAGCGACG - 3′); this oligonucleotide hybridizes to a position upstream of the mutated sites in the transcript leader (nucleotide position 1477 - 1493, Post et al., 1979). In the primer extension experiments, the oligonucleotide primer used for the sequencing ladder was the same as that used in the primer extension reaction, oWD32 or oWD33 (see section 2.7.5).

Sequencing ladders used for situating transcript ends in S1 nuclease protection experiments were prepared by the base modification procedure of Maxam and Gilbert (1980).

All sequencing reactions were analyzed on 8% and/or 6% polyacrylamide-urea gels (20 cm X 38.5 cm X .35 mm). Electrophoresis was done in 0.5X TBE at a maximum voltage of 1750 V and initial power of 35 W. The gels were dried onto Whatman 3mm filter paper and exposed to Kodak X-Omat RP film.

2.6 PREPARATION OF TOTAL CELLULAR RNA

2.6.1 GENERAL METHOD

Bacteria were grown at 37°C in 5-10 ml supplemented M9 minimal salts media, and the selective antibiotic when required, to early log phase, A_{460} = 0.3-0.4. Cells were poured over 5 ml of 40 mM NaN_3 at -70°C, centrifuged at 6000 rpm for 5 min and resuspended in 1 ml medium C (40 mM NH_4Cl, 40 mM Na_2HPO_4, 20 mM KH_2PO_4, 50 mM NaCl) and 10 mM NaN_3. This was added to 1 ml of SDS lysis mix (100 mM NaCl, 10 mM EDTA, 0.5% SDS) at 100°C and boiled for 15-30 sec. The lysate was immediately extracted with 2 ml phenol 3 times, followed once by 2 ml $CHCl_3$ and ethanol precipitated 3 times in the presence of 0.25 M NaCl. The RNA pellet was finally resuspended in 2-5 ml TE.

2.6.2 PREPARATION OF RNA OF TEMPERATURE-SENSITIVE MUTANTS

The bacterial strains of concern are : (i) XH56, (ii)NF536 (relA$^+$) and NF537 (relA) and (iii) N3433 (RNaseE$^+$) and N3431 (RNaseE$^-$) (table 1). Strain XH56 has a temperature-sensitive mutation in rpoC which is lethal to the cell at 42°C but semi-restrictive at 39°C. Strain NF536 (relA$^+$) has a temperature-sensitive valyl-tRNA synthetase which elicits the stringent response at the semi-restrictive temperature of 35.5-37°C; NF537 is the isogenic relA strain and exhibits the relaxed response (Dennis and Nomura, 1974; Maher and Dennis, 1977). Strains N3433 and N3431 are rne$^+$/rne isogenic strains; compared to N3433 grown at 30°C and 44°C, growth of N3431 is normal at 30°C but restricted at 44°C due to the temperature-sensitive mutation in rne.

Mutant bacterial cultures were grown exponentially in supplemented M9 minimal salts media at 30°C. When the cells reached A_{460} ~ 0.3-0.4, portions of the cultures were shifted to the appropriate semi-restrictive temperature. For strain XH56, the semi-restrictive temperature was 39°C for 15 min in all cases. For strains NF536 and NF537, the semi-

restrictive condition was 35.5°C for 15 min for the filter hybridization studies and, in order to maximize the stringent-relaxed responses, 37°C for 15 min for the S1 nuclease protection studies. For strains N3433 and N3431, the restrictive condition was incubation at 44°C for 15 or 30 min. Total cellular RNA was prepared from 5-10 ml culture aliquots at both 30°C and the semi-restrictive temperatures.

2.7 RNA ANALYSIS

2.7.1 SUCROSE GRADIENT FRACTIONATION

A 5 ml culture at an A_{460} of 0.35 was labelled with [^{3}H]uracil (25 µCi/ml; 0.09 µg of non-radioactive carrier uracil/ml) for 3 min. The cell lysate was prepared as described above (section 2.6.1) and layered directly onto a 12 ml 6% to 30% (w/v) sucrose gradient with NETS buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl (pH7.6). 0.2% SDS) and centrifuged for 4.5 h at 40,000 rpm in an SW41 rotor at 20°C. Fractions (0.3 ml) were collected and 20 µl was removed from each, precipitated with trichloroacetic acid, collected on a nitrocellulose filter and counted. A second portion (200 µl) was hybridized to DNA-containing filters (DNA in excess) as described below (section 2.7.2).

2.7.2 FILTER HYBRIDIZATION

DNA-RNA hybridizations were carried out as described (Dennis and Nomura, 1974; Dennis, 1977a, 1984). Cultures were labelled with [5,6-^{3}H]uracil (s.a. 42 Ci/mmol; 10 µCi/ml) for 1 min and RNA was prepared according to the protocol in section 2.6.1. Increasing amounts of total cellular RNA (12.5-50 µg) were hybridized to an excess of denatured M13, plasmid or λ phage DNA immobilized on nitrocellulose filters at 67°C for 16 h. Filters were washed in 2X SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH7.0), treated with RNaseA, and radioactivity was measured by scintillation counting.

Two separate hybridization series were carried out to measure either L11-L1 mRNA, β mRNA and λspc mRNA or L10-L12 mRNA and β′ mRNA. The λspc DNA was used as an external hybridization control; this DNA encodes 15 ribosomal proteins and the α subunit of RNA polymerase within a 9000 base region of the transducing phage (Jaskunas et al., 1975; Dennis, 1977a). The other DNA probes were as follows (Dennis, 1984). The L11-L1 DNA probe was a 617-base long EcoRI-BglII minus strand fragment cloned into M13mp9. The L10-L12 DNA probe was a 653-base long PstI-EcoRI minus strand fragment cloned into M13mp8. The β DNA probe was the 2.8 kb EcoRI fragment from the central region of the β subunit gene. The β′ DNA probe was the 2.6 kb EcoRI fragment from the central region of the β′ subunit gene.

2.7.3 RNA ELECTROPHORESIS AND NORTHERN HYBRIDIZATION ANALYSIS

Northern hybridization analysis was carried out according to Maniatis et al. (1982) with minor modification. Total RNA (10 μg) from exponentially growing cells was fractionated on a 1% agarose/formaldehyde gel. The gel was rinsed 3 times with distilled water. Without prior alkaline hydrolysis, the RNA species were transferred to Gene Screen (New England Nuclear) hybridization membranes by capillary transfer using 2X SSC. After baking under vacuum at 80°C for 2 h, the blots were prehybridized at 42°C for 9 h in a solution of 50% formamide, 5X SSC, 50 mM sodium phosphate (pH7.0), and 2X Denhardt's solution (Maniatis et al., 1982). Probes for hybridization were prepared by nick-translation of restriction fragments. The DNA probes used were the 617-nucleotide long EcoRI-BglII fragment spanning the L11-L1 region and the 290-nucleotide long HindIII-EcoRI fragment spanning the L10-L12 region. The probes were denatured in 200 μl of the hybridization buffer and approximately 10^7 dpm of probe in a final volume of about 5 ml was used for hybridization. Hybridization was at 42°C for 18 h. The membranes were washed twice with 2X SSC, 0.1% SDS for 5 min at room temperature, twice with the same solution for 15

min at 65°C and twice with 0.1X SSC, 0.1% SDS for 15 min at room temperature. The washed membranes were air-dried and exposed to X-ray film.

2.7.4 S1 NUCLEASE MAPPING

The 3′ and 5′ ends of *in vivo* mRNA transcripts were analyzed by S1 nuclease mapping as described by Berk and Sharp (1978) and as modified by Favaloro *et al.* (1980). Total *in vivo* RNA was prepared as described above; 5 µg of RNA was hybridized to approximately 10^4 to 10^5 dpm of denatured 5′ or 3′ end-labelled fragment (DNA in excess) at a temperature of 48-52°C for 3 h in 80% formamide hybridization buffer. Digestion with nuclease S1 (200-400 units/ml) was carried out at either 20 or 37°C for 30 min. Fragments of DNA protected from S1 nuclease digestion by complementary mRNA sequences were analyzed for length on 8% polyacrylamide DNA sequencing gels. Molecular length standards were *Msp*I fragments of pBR322, 3′ end-labelled with Klenow enzyme and [α-^{32}P]dCTP. In many experiments the G and A+G reaction products of Maxam-Gilbert sequencing of the 5′ or 3′ end-labelled probe were used as length standards (Maxam and Gilbert, 1980). These standards were assumed to run two nucleotides faster than the S1-protected fragments because they lack the terminal A or G nucleoside but retain the terminal phosphate group at the site of cleavage.

2.7.5 PRIMER EXTENSION

Transcript 5′ ends from the *secE-nusG* operon were analyzed by the primer extension method according to Newman (1987). Total RNA (10 µg) and 5′ end-labelled oligonucleotide primer (1 ng) were heated at 65°C for 5 min in 10 µl of 160 mM KCl, 40 mM Tris-Cl (pH8.5), 1 mM EDTA. The mixture was cooled gradually to 42°C and incubated at 42°C for 1 h. Five units each of AMV reverse transcriptase and RNase inhibitor were then added to each reaction with 10 µl of 10mM $MgCl_2$, 10 mM β-mercaptoethanol and 1 mM of each

dNTP. Incubation was continued at 42°C for 1 h. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH8.0) and 78 µl of TE. The products were precipitated with ethanol in the presence of 0.3 M sodium acetate. The pellet was dissolved in 5 µl of formamide sequencing dye mix and the radioactivity measured by Cerenkov counting. The reaction products were analyzed on 8% polyacrylamide-urea sequencing gels alongside a sequencing ladder generated by using an appropriate single-stranded template and the same primer (but unlabelled) as that used in the primer extension procedure. Two oligonucleotides were used as primers :

oWD32 5′ - GCAATCAGAATTACTACGGC - 3′

oWD33 5′ - CGGAAAACGCCTGAACGACG - 3′

Primer oWD32 is complementary to a sequence in the secE gene (position 378 - 397); oWD33 is complementary to a sequence in the proximal region of the nusG gene (position 654 - 673). The sequence numbering system is according to that used in chapter three.

Templates used for the corresponding sequencing ladders were (i) the SmaI(-684)-HpaI(419) 1.1 kb fragment cloned in the correct orientation into the SmaI site of M13mp19 with oWD32 as primer and (ii) the PstI(399)-PstI(967) 568 bp fragment cloned in the correct orientation into the PstI site of M13mp18 with oWD33 as primer.

2.7.6 mRNA STABILITY

Study of mRNA stability at the permissive and semi-restrictive temperatures was according to von Gabain et al. (1983) with some modification. Strain XH56 was grown at 30°C in 40 ml of supplemented M9 minimal salts media to A_{460} = 0.3 - 0.4. A 7 ml aliquot was taken for RNA preparation just before addition of rifampicin. Rifampicin was added to the remaining culture to a final concentration of 200 µg/ml. This was designated as time = 0 min. Successive 7 ml aliquots were taken for RNA preparations at 2, 4, 6 and 8 min. For studies of mRNA stability at the semi-restrictive temperature, the XH56 culture, when

it had reached a cell density of A_{460} = 0.4, was shifted from 30°C to 39°C for 25 min before rifampicin was added. Aliquots were taken at the same time points. Total RNA was prepared as described above. Levels of L11-L1, L10-L12 and β transcripts were analyzed, in duplicate, by S1 nuclease protection using 5 µg of total RNA. The following DNA restriction fragments, labelled at the 3′ end, were used as probes : (i) the 617 bp EcoRI-BglII fragment detects L11-L1 message, (ii) the 290 bp HindIII-EcoRI fragment detects L10-L12 message, (iii) the 584 bp SalI-EcoRI fragment detects β message and (iv) the 496 bp EcoRI-SalI fragment detects read-through transcripts as well as transcript 3′ ends in the L12-β intergenic region (fig. 15). For internal consistency, probes for L10-L12 and β transcripts were used together in the S1 nuclease protection study. Resultant autoradiogram bands were scanned by a video densitometer (BioRad model 620) and the data computer analyzed (BioRad 1-D analyst, version 2.01). Integrated areas of the appropriate peaks were used to calculate relative band intensities.

2.8 OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

2.8.1 CONSTRUCTION OF CLONING VECTOR pEMBL8⁺(BII)

The multiple cloning site (MCS) of pEMBL8⁺ (Dente et al., 1983) was replaced by a modified MCS containing a BglII restriction site derived from an altered pGEM4Z vector kindly provided by Jan St. Amand. The BglII recognition sequence was generated by the insertion of an XbaI 6mer linker into the XbaI site of pGEM4Z (Promega). The insertion is in frame and allows the usual colour selection with X-gal. The MCS of pEMBL8⁺(BII) has the following restriction sites surrounding the BglII site :

EcoRI - - - BamHI-XbaI-BglII-XbaI-SalI- - - HindIII

2.8.2 OLIGONUCLEOTIDES FOR MUTAGENESIS

The oligodeoxyribonucleotides were synthesized on an Applied Biosystem 380A DNA synthesizer and were deprotected and purified as described by Atkinson and Smith (1984). The crude DNA pellet was dissolved in 90 µl TE. Ammonium acetate (0.5 M) and magnesium acetate (10 mM) were added to a 15 µl aliquot in a final volume of 500 µl. The oligonucleotide solution was passed through a C_{18} SEP-PAK cartridge (Waters Scientific) which had been activated by washing with 10 ml of HPLC grade acetonitrile followed by 10 ml of distilled water. Contaminants were washed off the column with 4.5 ml of distilled water. The oligonucleotide was eluted off the column with 4.5 ml of 60% CH_3OH/40% distilled H_2O, the eluant being collected in three 1.5 ml fractions. The oligonucleotide concentration of each fraction was spectrophotometrically determined, with one A_{260} unit corresponding to 20 µg/ml. The oligonucleotide was frozen in dry ice, evaporated to dryness in a Savant Speed-Vac and dissolved in 50 µl of TE. The oligonucleotides used for mutagenesis are listed below; the single base mutation is underlined.

Name	Sequence	Nucleotide positions
oPD23	5′ - CCAGGCCT<u>T</u>CGTCGAAG - 3′	1529 - 1545
oPD24	5′ - ATATTCTG<u>A</u>CTTGTTTC - 3′	1615 - 1631
oPD25	5′ - GCTTGTTT<u>T</u>TGCTCACC - 3′	1623 - 1639
oPD26	5′ - TGCGTAGA<u>T</u>GGTGACAG - 3′	1578 - 1594

2.8.3 CONSTRUCTION OF MUTANT PLASMID DERIVATIVES

The mutagenesis method was according to Ner et al. (1988) and is a combination of the primer extension protocol of Zoller and Smith (1982) and the strand selection method of Kunkel (1985), and Kunkel et al. (1987). The 1.1 kb BglII-SmaI fragment from plasmids pNF1344, pNF1661 to pNF1664 were cloned into the BglII-SmaI site of pEMBL8$^+$(BII). This

BglII-SmaI fragment contains the 3′ end of the L1 gene, the untranslated L10 leader region and the 5′ end of the L10 gene. Competent dut⁻ung⁻ E. coli RZ1032 was transformed with the pEMBL8⁺(BII) recombinant plasmids. RZ1032 lacks the enzyme dUTPase (dut⁻) and the resulting elevated concentration of dUTP effectively competes with TTP for incorporation into DNA. RZ1032 also lacks the enzyme uracil N-glycosylase (ung⁻) which normally removes uracil from DNA. Thus in RZ1032, uracil is incorporated into DNA and is not removed (Kunkel, 1985; Kunkel et al., 1987).

Single-stranded, uracil-containing DNA was prepared as described above (section 2.4.2). In vitro mutagenesis was carried out as described by Ner et al. (1988). Oligonucleotides oPD23 to oPD26 were used to generate compensatory point mutations in the L10 leader regions of pNF1661 to pNF1664, respectively. Each oligonucleotide was also used to mutagenize the wild type plasmid pNF1344. Strong selection for the newly synthesized strand was accomplished by transforming the heteroduplex into the dut⁺ung⁺ host JM101. Transformants were screened for mutations initially by colony or DNA filter hybridization (Maniatis et al., 1982) and finally by dideoxy sequencing using oPD28 as the sequencing primer (see section 2.5). The mutagenized 1.1 kb BglII-SmaI fragment was excised from the pEMBL8⁺(BII) derivatives and inserted into the BglII-SmaI site of pNF1344, replacing the wild type sequence (fig. 17). The BglII-SmaI pNF1344 vector DNA was gel-purified away from its wild-type 1.1 kb BglII-SmaI fragment in order to increase the probability of obtaining mutant recombinants. Again, mutants were identified by the procedure described above. Mutant plasmids were designated as pNF1661′(23), pNF1344(23) etc. and are listed in table 2. Bacterial transformants carrying plasmids pNF1661′(23) and pNF1662′(24) were not viable. Partial sequences of the relevant DNA regions are shown in figure 18; the positions of these point mutations in the secondary structure of the L10 leader (Christensen et al., 1984) are illustrated in figure 19.

2.9 IN VITRO ASSAY OF MUTANT PLASMIDS

A prokaryotic, DNA-directed, in vitro translation system (Amersham) was used to assess the translational efficiency of the mutant plasmids. Procedure was as specified by the kit, with some modifications. Each assay consisted of :

1.2 - 1.5 µg	plasmid DNA template
10 units	RNase inhibitor (Pharmacia Inc)
1.5 µl	10 mM DTT
1.0 µl	[^{35}S]-Met (s.a.1200 Ci/mmol, 11.8 mCi/ml)
3.8 µl	supplement solution (kit)
1.5 µl	amino acid (minus Met) solution (kit)
2.5 µl	S-30 extract (kit)

in a total volume of 15 µl.

The assay was incubated at 37°C for 1 h and then chased with 2.5 µl of methionine chase solution (kit) at 37°C for 10 min. [^{35}S]-methionine incorporation was measured according to the supplied protocol. Each plasmid was assayed in duplicate. Translation products were electrophoresed in duplicate on SDS-polyacrylamide gels.

Mini SDS slab gels containing 15% polyacrylamide with a 4.5% stacking gel were prepared using the discontinuous buffer system of Laemmli (1970). A 1.5 µl aliquot of each assay was loaded per well. Molecular size standards were high range protein molecular weight standards from BRL. Electrophoresis was carried out at 100 V (maximum) and 15-30 mA with the bromophenol blue dye running 0.9-1X the gel length. The gel was stained and destained, dried and exposed to Kodak X-Omat RP film. Autoradiogram band intensities were analyzed by densitometry (section 2.7.6).

TABLE 2

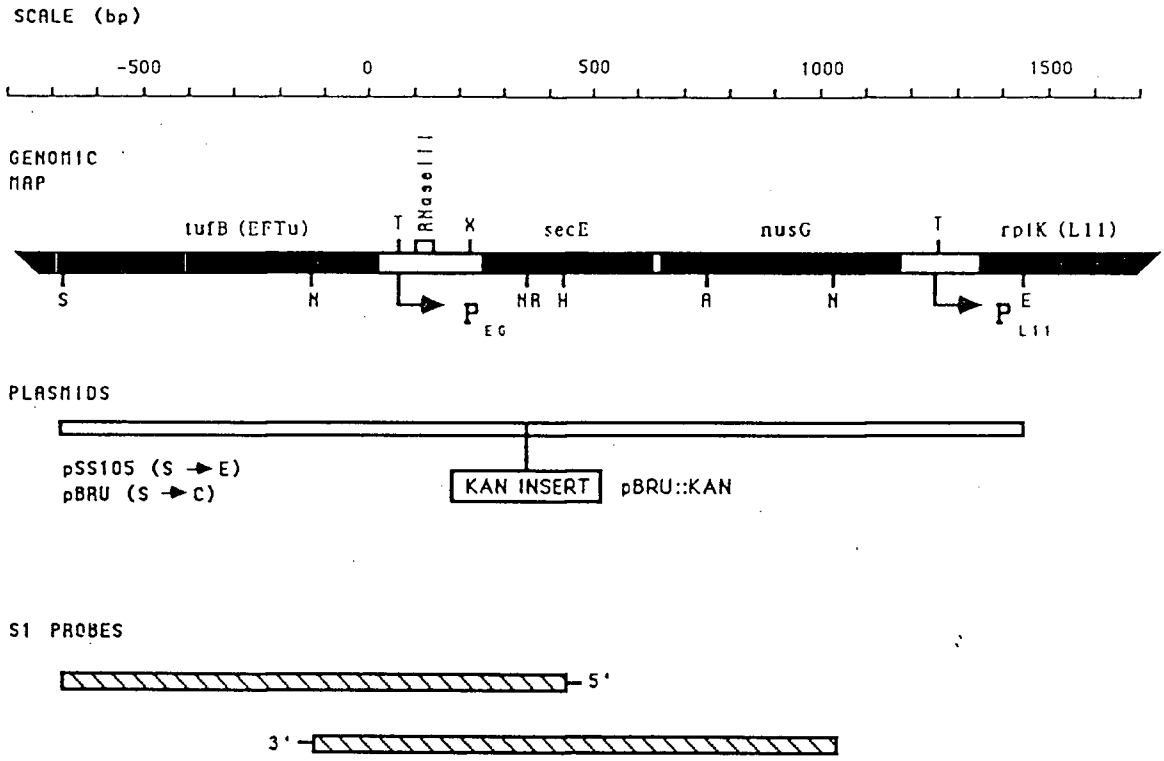
SINGLE AND DOUBLE BASE MUTANTS OF THE L10 LEADER REGION

Plasmid	Mutagenizing oligonucleotide	Mutation	Position (Post et al.,1979)
pNF1661'		G -> A	1516
pNF1344(23)	oPD23	C -> T	1537
pNF1661'(23)	oPD23	not viable	
pNF1662'		C -> T	1599
pNF1344(24)	oPD24	G -> A	1623
pNF1662'(24)	oPD24	not viable	
pNF1663'		G -> A	1594
pNF1344(25)	oPD25	C -> T	1631
pNF1663'(25)	oPD25	G -> A C -> T	1594 1631
pNF1664'		G -> A	1640
pNF1344(26)	oPD26	C -> T	1586
pNF1664'(26)	oPD26	G -> A C -> T	1640 1586

3.1 INTRODUCTION

The secE and nusG genes, whose products are essential for cell viability, are situated in the region between two well characterized operons, tufB and rplKAJL, around 90 minutes on the E. coli chromosome (fig. 3). The SecE protein has been shown to be an integral membrane protein and is an essential component of the protein translocation apparatus (Schatz et al., 1989). The other members of this protein export complex include proteins SecA, SecB, SecD and SecY (Review : Oliver, 1987). R-proteins S15 and L34, initially isolated as suppressors of mutations in secA, are also implicated in protein translocation. This possible involvement of r-proteins in protein secretion suggests an interaction between the protein export and translational machineries. Previous studies on the timing of protein secretion with respect to protein synthesis also provide evidence for the coupling of translation with translocation (Review : Oliver, 1987).

The Nus proteins, which include NusA, NusB, NusE and NusG, are factors that regulate transcription termination in E. coli. NusA, B and E were first identified as host genes necessary for N-mediated antitermination of λ transcription (Friedman and Gottesman, 1983). NusA and NusB proteins are also involved in the termination/antitermination process in several bacterial operons (Farnham et al., 1982; Kingston and Chamberlin, 1981; Ward and Gottesman, 1981; Kuroki et al., 1982; Sharrock et al., 1985). NusE, identified as r-protein S10, functions as a λ transcription antitermination factor; however, its effects on the expression of E. coli genes are not yet known. NusG is thought to be involved in regulating transcription antitermination since it is required, along with NusA, NusB and



.**FIGURE 3.** Genetic organization of the secE-nusG gene cluster.

The positions of the tufB (EF-Tu), secE, nusG, rplK (L11) genes are denoted by the filled rectangles. Selected restriction sites are indicated and their positions on the nucleotide scale are : SmaI (S, -684); NciI (N, -117, 1013); NruI (NR, 347); HpaI (H, 419); Asp718 (A, 753); EcoRI (E, 1438). The SmaI site at position -684 corresponds to the SmaI site at position 491 in the sequence numbering system of An and Friesen (1980a). Nucleotide 1158 and the EcoRI site at nucleotide 1438 correspond respectively to positions 1 and 280 in the sequencing numbering system of Post et al. (1979). The transcription start sites P_{EG} and P_{L11} (this work, section 4.2.2(2)) are at positions 60 and 1235. The terminators (T) for tufB and secE-nusG genes are located at positions 66-67 and 1238-1247, respectively. The RNaseIII processing sites (RNaseIII) are situated at nucleotides 96 and 129. A prominent 5′ transcript end which is located at nucleotide 216 is indicated by "X". Cloned derivatives of this chromosomal region are as follows. The SmaI-EcoRI 2.1 kb fragment was cloned , using an EcoRI linker at the SmaI end, into the EcoRI site of pBR322 to produce pSS105. Plasmid pBRU is identical to pSS105 except that the SmaI-EcoRI fragment was inserted into the EcoRI site and the blunt-ended ClaI site of pBR322. Plasmid pBRU::KAN was derived from pSS105 by insertion of a kanamycin (KAN) cassette into the NruI site at nucleotide 347. The probes used for S1 nuclease protection experiments were the 5′ end-labelled 1.1 kb SmaI-HpaI fragment and the 3′ end-labelled 1.1 kb NciI fragment (bottom).

NusE, for λ N-mediated antitermination in an in vitro transcription system (J. Greenblatt, personal communication). In addition, some mutations in nusG are able to suppress the E. coli nusA1 and nusE71 mutations, and restore N activity (S. Sullivan and M. Gottesman, personal communication).

Because of the essential nature of both the SecE and NusG proteins in cell viability, and because of their physical linkage to rplKAJL-rpoBC and their functional involvement with the translation and transcription apparati, the initial steps in understanding the regulation of their expression have been taken to sequence the secE-nusG gene cluster and to analyze the transcripts derived from this region.

3.2 RESULTS AND DISCUSSION

3.2.1 SEQUENCE ANALYSIS OF secE-nusG

A physical map of the 1318 nucleotide long region between the end of the tufB gene and the beginning of the rplK gene is depicted in figure 3. The complete nucleotide sequence of this region was determined using the SmaI-EcoRI 2.1 kb fragments obtained from both genomic DNA and from the transducing phage λrif^{d}18 (Kirshbaum and Konrad, 1973). The two sequences were identical and are presented in figure 4. The region contains two long open reading frames that have been designated secE and nusG. The nucleotide numbering system used in this chapter is different from that of An and Friesen (1980a) and Post et al. (1979); the terminal portion of the tufB sequence by An and Friesen extends to position 79 of this numbering system and nucleotide 1 of the rplKAJL sequence by Post et al. corresponds to position 1158.

.**FIGURE 4.** Nucleotide sequence of secE-nusG genes.

The predicted amino acid sequences of secE and nusG are given below the DNA sequence. The secE gene is located between nucleotides 240-620; the nusG gene is located between nucleotides 625-1167. The P_{EG} and P_{L11} transcription initiation sites are depicted by arrows (->) at position 59 and 1235 respectively. The -10 and -35 sequences associated with these 5′ transcript end sites are indicated. Other 5′ transcript ends that originate from processing or weak promoters are indicated by "X" for major and "x" for minor mRNA species. The sites of RNaseIII processing are noted. Sites of transcription termination of tufB mRNA and the secE-nusG mRNA are shown by filled circles at positions 66-67 and 1239-1241. Sequences exhibiting inverted repeat symmetry associated with termination are overlined and those associated with the stem structure recognized by RNaseIII are underlined. Oligonucleotides oWD32 and oWD33 used as primers for primer extension experiments are complementary to the indicated sequences. The kanamycin resistance cassette was inserted in the NruI site at position 347 (pBRU::KAN). The HpaI site (419) and the NciI site (1013) indicate respectively the ends of restriction fragments, 1.1 kb SmaI-HpaI and 1.1 kb NciI, which were used as probes for S1 nuclease mapping. The terminal portion of the nucleotide sequence of An and Friesen (1980a) extends to position 79. The two sequences are identical in the overlapping region with one exception; beginning at position 41, my nucleotide sequence has a run of four consecutive A's compared to a run of three consecutive A's in the sequence of An and Friesen (1980a).

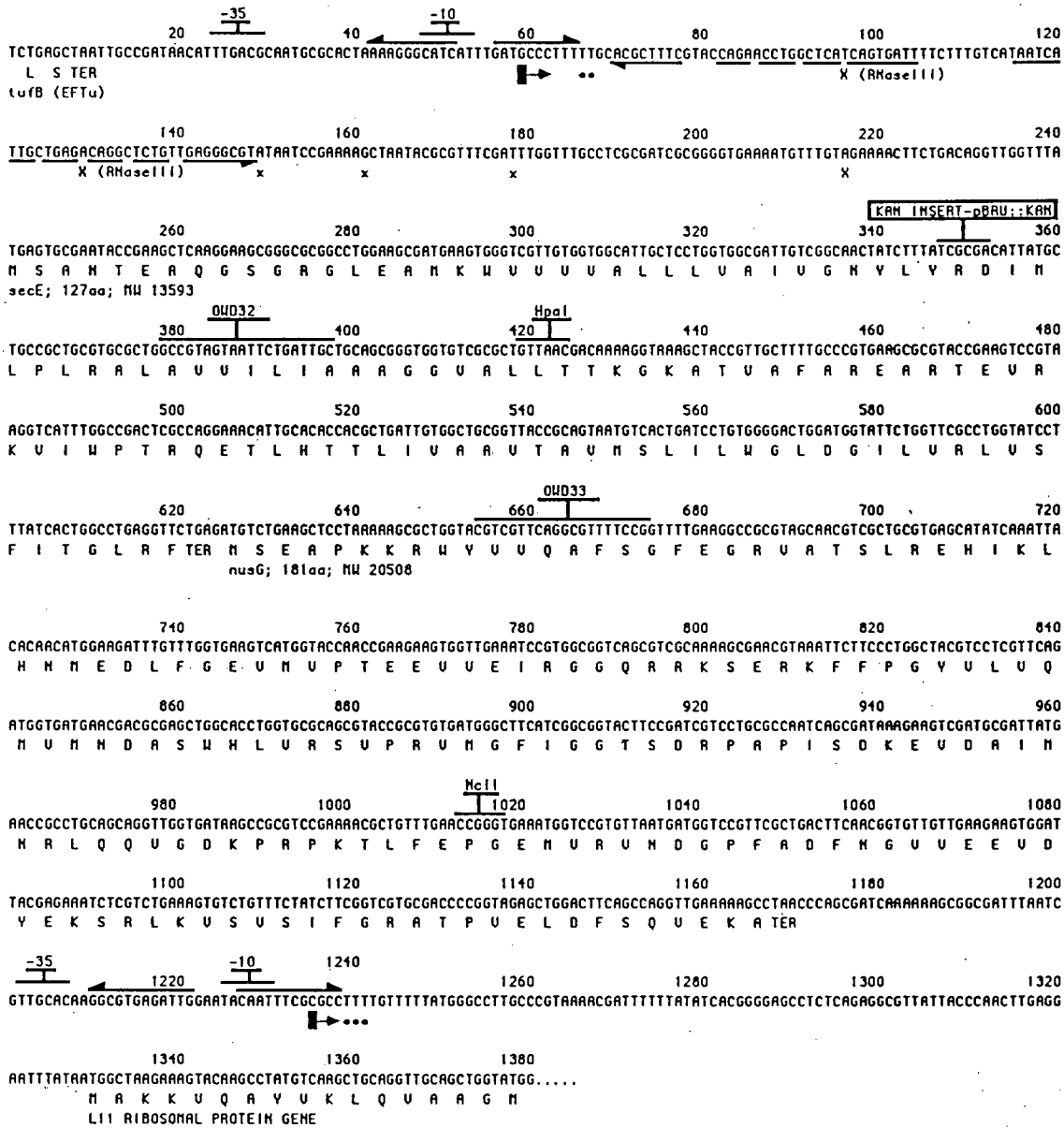

-35
-10
KAM INSERT-pBAV::KAM
OWD32
HpaI
OWD33
NcII
X (RNaseIII)
tufB (EFTu)
secE; 127aa; MW 13593
nusG; 181aa; MW 20508
L11 RIBOSOMAL PROTEIN GENE

FIGURE 4

The distance between the end of the tufB gene and the beginning of the secE open reading frame is 229 nucleotides (fig. 4). Overlapping sequences characteristic of a Rho-independent transcription terminator and an RNA polymerase promoter recognition sequence occur immediately after the tufB gene (between nucleotide positions 20-70) . If functional, the terminator would reduce or prevent extension of the abundant tufB transcripts into the secE-nusG region. Transcripts initiated at the promoter would contain a 5′ untranslated leader of approximately 180 nucleotides in length.

The secE gene (position 240-620) encodes a 127 amino acid long polypeptide that is rich in hydrophobic residues. Based on a number of different alkaline phosphatase (phoA) fusions to secE, Schatz et al. (1989) have shown that the secE gene product is an integral membrane protein containing three membrane-spanning domains. These domains, representing residues 19-36, 45-63 and 93-111, are 18 or 19 amino acids in length and are devoid of charged residues. The amino terminus of the protein is believed to be localized to the inside surface and the carboxy terminus to the outside surface of the cell membrane. The position of the initiating methionine codon at nucleotide 240 is supported by the isolation of a secE-phoA gene fusion with a junction immediately after the GAA glutamic acid codon at nucleotide 282. The initiation codon is preceded by a ribosome binding sequence at position 230-233. It has been shown that the secE gene is essential for cell viability and that its gene product is an important component of the bacterial protein translocation system (Schatz et al., 1989).

Only a single nucleotide separates secE from the open reading frame designated nusG. The nusG gene begins with an ATG methionine codon at position 625 and encodes a polypeptide of 181 amino acids in length. This protein contains a high proportion of acidic (14%) and basic (15%) residues and therefore is probably not an integral membrane

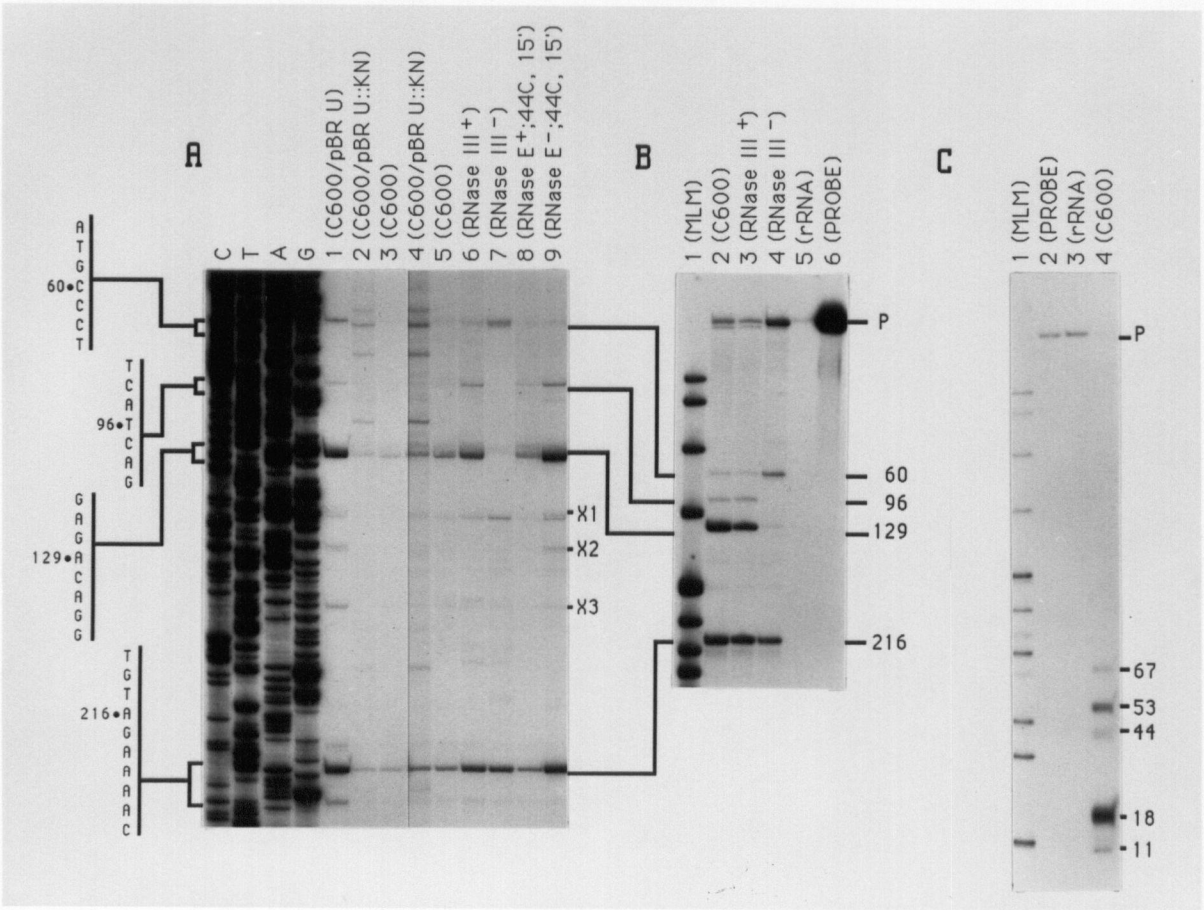
protein. Two fusions of alkaline phosphatase to nusG, at codons three and six, confirm the position of the initiation codon (Schatz et al., 1989). This conclusion has recently been substantiated by an N-terminal amino acid sequence of the purified NusG protein (J. Greenblatt, personal communication). The nusG gene has been shown to be essential for cell viability (Downing et al., 1989) and likely encodes a transcription termination factor (J. Greenblatt, S. Sullivan and M. Gottesman, personal communication).

The nusG-rplK intergenic space is 158 nucleotides long. This region contains the major promoter for transcription of the rplKAJL-rpoBC gene cluster which initiates at or near nucleotide 1235 (equivalent to nucleotide 77, Post et al., 1979; this work, section 4.2.2(2), fig. 10). This promoter region overlaps the terminator site for transcripts exiting the nusG gene.

3.2.2 TRANSCRIPT MAPPING

Plasmids pSS105 and pBRU contain the 2.1 kb SmaI-EcoRI fragment and are capable of complementing lethal mutations in the chromosomal secE (Schatz et al., 1989) and nusG genes (Downing et al., 1989). Neither plasmid contains the upstream tufB promoter, suggesting that secE and nusG are transcribed independently of tufB. In vivo transcripts derived from the secE-nusG region on the bacterial chromosome and the plasmid pBRU were characterized by primer extension and S1 nuclease protection analysis. For this purpose, two synthetic oligonucleotides, one complementary to a region in secE (oWD32) and the other complementary to a region in nusG (oWD33), were prepared.

The 5′ transcript end sites in the tufB-secE intergenic space were analyzed using oWD32 to prime reverse transcription using total RNA isolated from a number of different bacterial strains. A total of seven different 5′ end sites were evident using RNA from strain



.**FIGURE 5.** Transcript mapping by primer extension and S1 nuclease protection.

Panel A : Primer extension using oWD32 as primer. Reaction products from primer extension experiments were analyzed on a 8% polyacrylamide-urea sequencing gel alongside a sequencing ladder (G,A,T,C). The major 5′ transcript ends are located on the DNA sequence at positions 60, 96, 129 and 216. The minor 5′ mRNA ends are indicated by 'X1', 'X2', and 'X3', and correspond to nucleotide positions 149, 161 and 178, respectively. Ten micrograms of total cellular RNA, prepared from the following strains, were used for each reaction : lane 1, PD828 (C600/pBRU); lanes 2 and 4, PD858 (C600/pBRU::KAN); lanes 3 and 5, C600; lane 6, N2076 (rnc$^+$); lane 7, N2077 (rnc); lane 8, N3433 (rne$^+$); lane 9, N3431 (rne). Lanes 1-3 are short exposures (30 h) and lanes 4-9 are long exposures (2 weeks). **Panel B** : Nuclease S1 mapping of 5′ transcript ends derived from the tufB-secE intergenic region. The 5′ end-labelled 1.1 kb SmaI-HpaI restriction fragment was used as probe; five micrograms of RNA were used in each reaction. Lane designations are : lane 1, molecular length markers are 3′ end-labelled MspI fragments of pBR322 (their lengths are 623, 528, 405, 310, 243, 239, 218, 202, 191, 181, 161 nucleotides); lane 2, C600 RNA; lane 3, N2076 RNA (rnc$^+$); lane 4, N2077 RNA (rnc); lane 5, rRNA (control); lane 6, 5′ end-labelled 1.1 kb SmaI-HpaI probe. The predominant 5′ transcript ends are correlated with their respective primer extension counterparts. The probe (P) and the nucleotide positions of transcript termini are indicated. **Panel C** : Nuclease S1 mapping of 3′ transcript ends derived from the tufB-secE intergenic region. The 3′ end-labelled 1.1 kb NciI DNA fragment was used as probe (lane 2). The lane designations are similar to those in panel B. The 3′ transcript ends are situated on the DNA sequence at positions 11, 18, 44, 53 and 67.

C600 (fig. 5A, lanes 3 and 5); these sites are located at or near nucleotide positions 60, 96, 129, 149, 161, 178 and 216. Only the sites at positions 60 and 161 are preceded by easily recognizable and appropriately spaced -10 and -35 promoter consensus sequences. The intensities of the seven 5′ end sites were uniformly enhanced when RNA from strain C600 containing the pBRU plasmid (PD828) was used as template (fig. 5A, lane 1). This observation indicates that the transcripts derived from the secE-nusG region of the chromosome and the recombinant plasmid are identical and implies that transcription is not dependent on the upstream tufB promoter. The oligonucleotide oWD33, complementary to a region in nusG was also used to locate 5′ transcript ends. The 5′ end sites of the products generated with this primer correspond to those generated with oWD32 (data not shown).

Plasmid pBRU::KAN contains a kanamycin cassette inserted into the NruI site at nucleotide position 347 within the secE gene. When RNA from a strain carrying this plasmid (PD858) was used in the primer extension assay with oWD32 as primer, only the low level transcripts derived from the chromosomal secE-nusG region were detected (fig. 5A, lanes 2 and 4). In addition, S1 nuclease analysis clearly demonstrated that few, if any, transcripts exit from the kanamycin cassette (data not shown). Together, these results indicate that the secE and nusG genes are cotranscribed and that the kanamycin cassette in plasmid pBRU::KAN induces transcriptional polarity on the downstream nusG gene.

The two 5′ end sites at nucleotide positions 96 and 129 are located at opposite positions within a region of inverted repeat symmetry. The RNAs from an RNaseIII mutant strain (N2077) and its isogenic wild type parent (N2076) were examined by primer extension to determine if these sites were generated by RNaseIII cleavage (fig. 5A, lanes 6 and 7). In the mutant strain, the 5′ end sites at positions 96 and 129 were greatly reduced and the

intensity of the 5′ end site at position 60 was correspondingly increased. This result suggests that a precursor RNA with a 5′ end site at position 60 is either partially or slowly cleaved by RNaseIII at position 96 and/or 129 and that the site at position 60 probably represents the major transcription initiation site for the secE-nusG mRNA. As discussed below (section 4.2.2(2)), RNaseIII has a range of effects on the expression of many E. coli and bacteriophage genes. The function of RNaseIII processing in the secE-nusG leader sequence remains to be determined.

The 5′ transcript end at nucleotide 216 is of unknown origin. The sequence surrounding this anomalous but abundant end site exhibits some resemblance to the consensus recognition sequence for endonuclease RNaseE. In E. coli, RNaseE is an essential function required for the excision of precursor 5s rRNA from the nascent rRNA transcript and for the cleavage of RNAI, a transcript involved in replication of colEI plasmid DNA (Apirion and Lassar, 1978; Ghora and Apirion, 1979; Tomcsanyi and Apirion, 1985). To determine if RNaseE is responsible for generating the 5′ end site at position 216, total RNA was isolated from a temperature-sensitive RNaseE mutant strain that had been incubated for 15 or 30 min at the restrictive temperature of 44°C. Primer extension with oWD32 indicates that none of the extraneous 5′ end sites including the one at position 216 are produced by RNaseE cleavage (fig. 5A, lanes 8 and 9).

The major 5′ end site at nucleotide 216, while not generated by RNaseE processing, may be the result of an unidentified nuclease activity. The sequence in the vicinity of this 5′ end site is similar to the sequence in the region of the major 5′ transcript end in the L10-L12 leader (at nucleotide 1500; section 4.2.2(2), sequence numbering according to Post et al., 1979). It is possible that these sequences are processed by the same nuclease and hence may indicate a similar mode of regulation of these two operons.

```
secE-nusG leader      5'...CGGGGTGAAAATGTTTGTAGAA... 3'
                           |||   |||||  |||||
L10-L12 leader        5'...CGGATAGAAAAGATTTGTTCGT... 3'
                              |||||||  | ||   |
secE-nusG leader      5'...TTTGTAGAAAACTTCTGACAGG... 3'
```

(The 5′ end sites are underlined. Two alignments of the secE-nusG leader sequence are shown; in this region, nucleotide identities with the L10-L12 leader sequence are indicated.) The significance of this similarity in sequence and transcript processing requires further investigation.

As implied by their relative autoradiogram intensities, transcripts with 5′ termini at positions 129 (RNaseIII) and 216 are the predominant mRNA species (fig. 5A and B). If the mRNA 5′ end at position 216 is the result of nuclease activity, then it appears that the majority of transcripts initiated at position 60 are processed in the 5′ untranslated leader region; this post-transcriptional event may provide an additional level of regulation in the expression of these genes. RNA processing may be involved in the decay of transcripts or in the "unmasking" of the ribosome binding site for translation (King et al., 1986; Gegenheimer and Apirion, 1981).

The 5′ transcript ends detected by primer extension were confirmed by S1 nuclease protection experiments (fig. 5B). Total cellular RNAs isolated from E. coli C600 and rnc$^+$/rnc strains N2076 and N2077 were used to protect the 5′ end-labelled SmaI-HpaI 1.1 kb fragment spanning the tufB-secE intergenic region (fig. 3). The ends of fragments

protected from S1 nuclease digestion, correspond to the transcript ends observed in the primer extension experiments (fig. 5A and 5B).

Nuclease S1 protection experiments, using the 3′ end-labelled NciI-NciI 1.1 kb probe (fig. 3), indicate that transcripts exiting the tufB gene are efficiently terminated. The largest transcripts terminate at or near nucleotide position 66-67. This end site is probably a Rho-independent transcription terminator since it is within a tract of T residues that is preceded by inverted repeat symmetry. A number of other shorter but more abundant transcripts with 3′ end sites near nucleotide positions 11, 18, 44 and 53 were also detected (fig. 5C). All of these sites are beyond the tufB termination codon. It is unclear whether these 3′ end sites are generated by termination events or by nuclease cleavage in the 3′ untranslated portion of tufB mRNA.

The 3′ end of the secE-nusG transcript has been previously mapped to nucleotides 1238 to 1247 within a T-tract sequence that is preceded by inverted repeat symmetry (fig. 4) (Downing and Dennis, 1987; this work, section 4.2.2(2)). There is little if any transcription read-through into the downstream rplK gene. The major promoter for the rplKAJL-rpoBC gene cluster initiates transcription at or near nucleotide 1235. The overlap of this transcription start site with the secE-nusG termination site may permit some regulatory interaction between these two secE-nusG and rplKAJL gene clusters (Downing and Dennis, 1987; this work, section 4.2.2(2)).

IV. STUDIES OF THE rplKAJL-rpoBC GENE CLUSTER

4.1 INTRODUCTION

The rplKAJL-rpoBC gene cluster lies immediately downstream of the secE-nusG operon and encodes, respectively, the four 50S subunit ribosomal proteins L11, L1, L10 and L12, and the two large β and β′ subunits of RNA polymerase (fig. 6). The locations of these four r-proteins on the ribosome are known (fig. 7). Four copies of elongated r-protein L12 are found in the stalk of the large subunit. At the base of the stalk, they bind to a single copy of L10 protein (Strycharz et al., 1978; Petterson and Liljas, 1979) which, perhaps facilitated by the L11 protein, binds to the 23S rRNA (Dijk et al., 1979; Petterson, 1979). This complex forms part of the GTPase centre on the large subunit and is required for the binding of extrinsic translation factors (e.g. EF-Tu and EF-G) to the ribosome and the concomitant hydrolysis of GTP (reviews : Liljas, 1982). Protein L1 is found in the shoulder on the opposite side of the large subunit; it is involved in the interaction with peptidyl-tRNA at the P (peptidyl) site and indirectly with the GTPase centre (Subramanian and Dabbs, 1980; Lake and Strycharz, 1981; Sander, 1983).

Proteins β and β′ are components in the DNA-dependent RNA polymerase which is responsible for transcription of the bacterial genome. Found in two forms, the polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) initiates transcription at unique promoter sequences and the core enzyme ($\alpha_2\beta\beta'$) is responsible for RNA chain elongation. Transcription termination or pausing is mediated by ancillary factors such as Rho or Nus proteins. The β subunit is thought to be involved in binding the nucleoside triphosphate substrates and the β′ subunit appears to bind the DNA template (Zillig et al., 1976).

Regulation of the rplKAJL-rpoBC gene cluster is complex. Transcription is initiated

.**FIGURE 6.** Genetic organization of rplKAJL-rpoBC.

The positions of the nusG, rplK (L11), rplA (L1), rplJ (L10), rplL (L12), rpoB (β) and rpoC (β′) genes are indicated by the filled rectangles. The nucleotide numbering system is that of Post et al. (1979). The transcription start sites for the defined P_{L11} and P_{L10} promoters are, by S1 mapping, at nucleotides 77 and 1346, respectively. Other sites are: the terminator for nusG transcripts (T_{EG}) at about nucleotide 81; the translational control L1 binding site on the mRNA (L1 B) between nucleotides 130 and 200; the translational control L10 binding site on the mRNA (L10 B) between nucleotides 1510 and 1590; the transcription attenuator (ATT_β) at about nucleotide 2717, and the RNaseIII processing site (RNaseIII) at about nucleotide 2780. Relevant restriction sites and their nucleotide positions are as follows : AvaII (AII: -118); AvaI (AI: 228); EcoRI (R: 280, 2444, 3524, 6392, 7593, 10068); BglII (B: 897); HinfI (Hf: 1206, 1416, 1805, 2549, 3068); PstI (P: 1791); HindIII (H: 2154); HinPI (HP: 1293); Fnu4HI (F: 1404); HaeIII(Ha: 1513); DraI (D: 1728); NarI (Nr: 2730); AccI (Ac: 2941). The restriction fragments utilized as probes in the hybridization assays to quantify L11-L1, L10-L12, β and β′ mRNA sequences are illustrated above the genetic map; the 617 and 653 base probes are minus strand M13 clones, and the 2868 and 2475 base probes are pBR322 clones. The transcripts deduced from S1 protection experiments are illustrated below. The filled circles indicate 5′ ends corresponding to the sites of the defined P_{L11} and P_{L10} promoters; the open circle represents a 5′ end not associated with a previously recognized promoter. The open boxes correspond to 3′ end sites. The scissored interruption represents the site of RNaseIII processing of read-through transcripts.

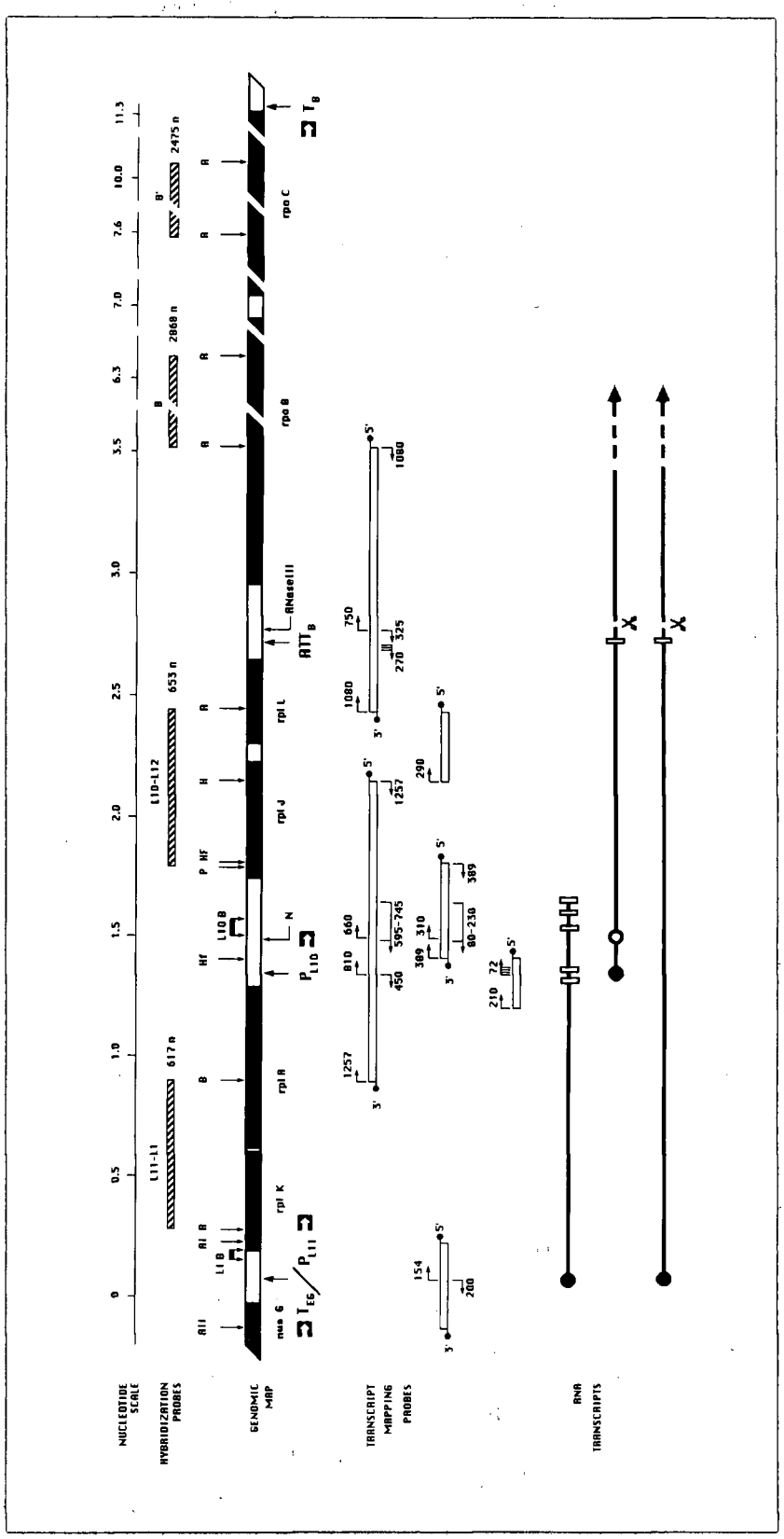
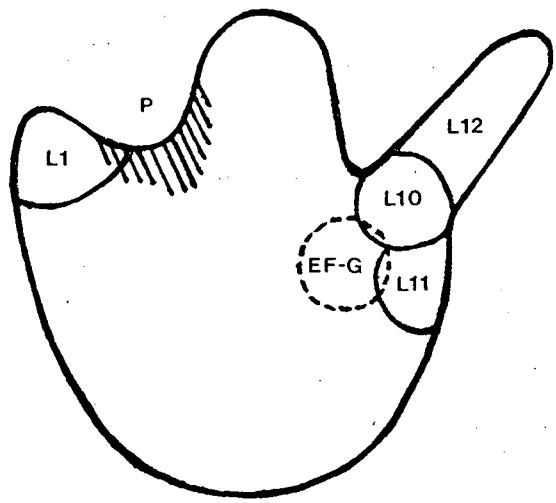

NUCLEOTIDE SCALE
HYBRIDIZATION PROBES
GENOMIC MAP
TRANSCRIPT MAPPING PROBES
RNA TRANSCRIPTS
rpl K
rpl A
rpl B
rpl J
rpl L
rpo B
rpo C
L11-L1
L10-L12
2475 n
2868 n
653 n
617 n
RNaseIII
ATT$_B$
T$_{ES}$/P$_{L11}$
P$_{L10}$
T$_B$

FIGURE 6



.**FIGURE 7.** Positions of r-proteins L11, L1, L10 and L12 on the E. coli large ribosomal subunit.

The positions of r-proteins on the ribosome have been determined by immune electron microscopy, neutron scattering and cross-linking studies. Four copies of L12 form the stalk of the large subunit. At the base of the stalk, they bind to L10 which, perhaps facilitated by L11, binds to the 23S rRNA; this complex forms part of the GTPase centre. The binding site for elongation factor EF-G is shown. Protein L1 is found on the shoulder of the large subunit near the peptidyl (P) site. (Adapted from Liljas (1982), and Noller and Nomura (1987))

at two major promoters, P_{L11} and P_{L10} (Post et al., 1979; Taylor and Burgess, 1979; Yamamoto and Nomura, 1978; Linn and Scaife, 1978) and 80% of the transcripts are terminated at the transcription attenuator in the L12-β intergenic space (Dennis, 1977a, 1984; Barry et al., 1979, 1980). However, no detailed analyses of in vivo transcripts from this genetic locus have been done. Several regulatory features in this gene cluster are of interest and require further investigation.

The stoichiometry of production of the four ribosomal proteins is coordinate but unequal; r-protein L12 is present in four copies per ribosome, whereas L11, L1 and L10 r-proteins are present in one copy per ribosome (Subramanian, 1975; Hardy, 1975). Bruckner and Matzura (1981) have concluded that the major transcript of this region is tetracistronic and encodes all four ribosomal proteins. Other investigators have suggested that the L10-L12 intergenic space contains an additional promoter required to enhance expression of the L12 gene (Newman et al., 1979; Ma et al., 1981).

The L12-β intergenic space contains a number of sequences which are important in regulating expression of the downstream β and β′ RNA polymerase genes (Barry et al., 1979, 1980; An and Friesen, 1980b). During balanced growth, transcription of the ββ′ genes is under the control of the L11 and L10 promoters and the transcription attenuator in the L12-β intergenic region which terminates about 80% of the transcripts reading through the upstream ribosomal protein genes (Dennis, 1977a, 1984). This results in reduced β and β′ gene expression and accounts for the five to one ratio of ribosomes to core RNA polymerase found in growing bacteria (Shephard et al., 1980). Downstream from the attenuator in the intergenic space is an RNaseIII processing site; processing per se at this site has little or no effect on ββ′ gene expression (Barry et al., 1980; Dennis, 1984). However, the sequences surrounding this site, as defined by deletion analysis, appear to be essential for efficient

translation of the downstream β and β′ transcripts. There may be a weak β promoter in the L12-β intergenic region but its contribution to ββ′ expression would be extremely minor (Barry et al., 1979; Yamamoto and Nomura, 1978; Linn and Scaife, 1978; Newman et al., 1979).

Although the ββ′ genes are co-transcribed with the upstream ribosomal protein genes, regulation of ββ′ synthesis is distinct. At the translational level, synthesis of β and β′ subunits is feedback regulated by RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) or the assembly intermediate $\alpha_2\beta$ (Bedwell and Nomura, 1986; Meek and Hayward, 1986; Dennis et al., 1985; Fukuda et al., 1978; Yang and Zubay, 1981).

Two physiological conditions elicit differentially controlled expression of these ribosomal protein and RNA polymerase genes. First, restrictions that limit RNA polymerase activity, mediated either by addition of the antibiotic rifampicin (Hayward and Fyfe, 1978; Morgan and Hayward, 1987) or by use of strains temperature sensitive in RNA polymerase activity (Dennis, 1977b; Little and Dennis, 1980), selectively stimulate transcription of ββ′ RNA polymerase genes relative to transcription of r-protein genes. Second, during amino acid deprivation, ribosomal proteins L11, L1, L10 and L12 are stringently regulated in relA$^+$ strains whereas RNA polymerase subunits are not (Blumenthal et al., 1976; Maher and Dennis, 1977; Reeh et al., 1976). It has been proposed that this differential transcriptional activity is a result of dynamic modulation of transcription initiation at the P_{L11} and P_{L10} promoters and termination at the attenuator (Dennis, 1977b; Little and Dennis, 1980).

Finally, as an additional level of control, the translation of L11-L1 mRNA and L10-L12 mRNA is regulated by their respective repressor proteins, L1 and L10 (or a complex

of L10-L12). The L1 binding site in the leader region of the L11-L1 transcript has been studied in great detail by using mutagenesis techniques; it is adjacent to, and overlapping with the L11 cistron translation initiation site (Baughman and Nomura, 1983; Thomas and Nomura, 1987; Said et al.,1988).

Unlike other r-protein binding sites, the L10 binding site in the leader region of the L10-L12 transcript is located about 140 nucleotides upstream from the L10 translation initiation site (Fiil et al., 1980; Johnsen et al., 1982). Point mutants and deletion mutants, located in or near the L10 binding site are translationally defective (Fiil et al., 1980; Friesen et al., 1983; Christensen et al., 1984). To account for the long range effect of these mutations on L10 and L12 synthesis, a model for translation regulation involving alternative secondary structures of the L10-L12 mRNA leader has been proposed by Christensen et al. (1984). Subsequently, a portion of this model was substantiated by Climie and Friesen (1987). However, the greater part of the proposed secondary structures still requires validation.

In conclusion, these questions regarding the regulation of expression of rplKAJL-rpoBC still remain : (i) the 4:1 stoichiometry of synthesis of r-protein L12 relative to other r-proteins, (ii) the differential transcription of rpoBC relative to rplKAJL and (iii) RNA secondary structure of the rplJL transcript leader region and translational regulation of r-protein L10, L12 synthesis. As an attempt to address these questions, transcripts derived from this gene cluster have been analyzed and characterization of the secondary structure of the L10-L12 mRNA leader region has been initiated.

TABLE 3

HYBRIDIZATION OF PULSE-LABELLED C600 RNA TO SPECIFIC DNA PROBES

Probe DNA [a]	Hybridiz.[b] (%)	% hybridiz per[c] nucleotideX10^4	relative transcriptional activity	percentage[d] termination
L11-L1(617n)	0.096	1.56	0.72	
L10-L12(653n)	0.128	1.96	0.91	
β(2860n)	0.100	0.350	0.16	82
spc(9000n)	1.94	2.16	1.00	

.TABLE 3. Hybridization of pulse-labelled C600 RNA to specific DNA probes.

(a) The various probes complementary to mRNA transcripts from ribosomal protein and RNA polymerase genes are described in figure 6 and in Materials and Methods (section 2.7.2). The length of each sequence complementary to mRNA is indicated in parentheses (n, nucleotides).

(b) The percentage of the input radioactivity in specific RNA-DNA hybrids is the average of 8 hybridizations (see Dennis, 1984). Input radioactivity was varied over a 4-fold range from 1.44 X 10^5 cpm (50 µl input RNA; about 12.5 µg) to 5.76 X 10^5 cpm (200 µl input RNA; about 50 µg).

(c) The percentage hybridization per nucleotide of complementary sequence in each of the DNA probes was calculated as the quotient of the percentage hybridization and the probe length . This value is an estimate of the transcriptional activity of each of these DNA sequences. The transcriptional activity of the spc gene was used as an external control and arbitrarily set at 1.00.

(d) The percentage of transcription termination at the attenuator in the L12-β intergenic space was determined as 1 minus the quotient of the transcriptional activities of the β and L10-L12 genes.

4.2 RESULTS AND DISCUSSION

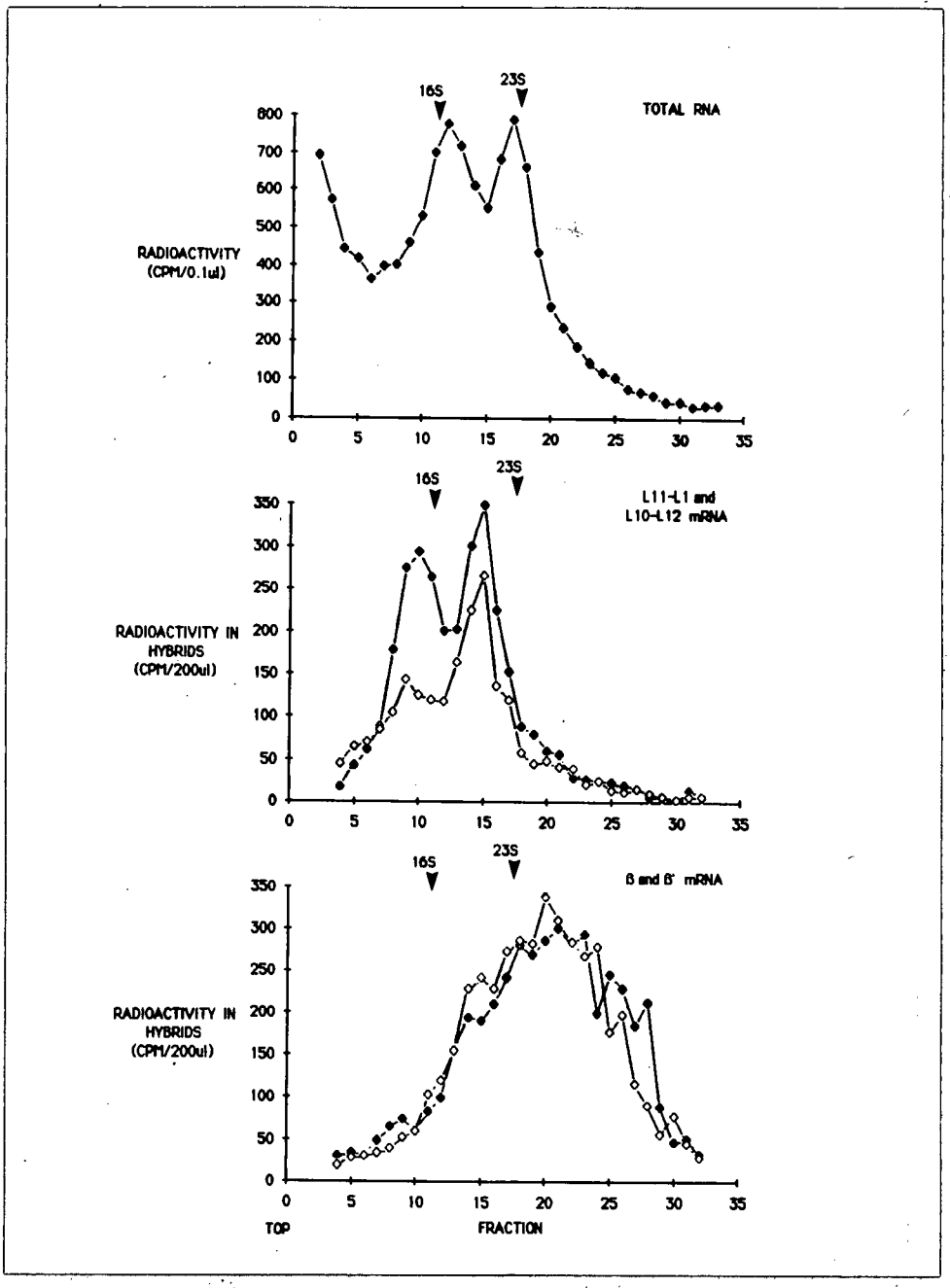
4.2.1 TRANSCRIPTIONAL PATTERN OF THE rplKAJL-rpoBC GENE CLUSTER

1. FILTER HYBRIDIZATION

Total cellular RNA, labelled with [^{3}H]uracil, was hybridized to a molar excess of the respective DNA probe immobilized on nitrocellulose filters. The fraction of input radioactivity hybridizing per nucleotide of probe DNA was determined (table 3). The results are as follows : (i) the L10-L12 genes were transcribed about 25% more frequently than the upstream L11-L1 genes (relative transcriptional activity of 0.91 versus 0.72) and (ii) the RNA polymerase genes, as represented by the β gene probe, were transcribed at about one-fifth the frequency of the upstream ribosomal protein genes. These results are consistent with previous suggestions of P_{L10} promoter activity in the L1-L10 intergenic space and a transcription attenuator in the L12-β intergenic region (Hui et al., 1982; Yamamoto and Nomura, 1978; Linn and Scaife, 1978; Taylor and Burgess, 1979; Barry et al., 1980). The attenuator terminates approximately 80% of the transcripts reading through the L12 gene and accounts, in part, for the reduced stoichiometry of RNA polymerase relative to ribosomes in growing bacteria (Dennis, 1977a, 1984; Shephard et al., 1980).

2. SIZE FRACTIONATION OF RNA TRANSCRIPTS

The size distribution of RNA transcripts from the rplKAJL-rpoBC gene cluster was analyzed by sucrose density-gradient centrifugation and Northern hybridization. Nascent RNA transcripts from an exponential culture were labelled with [^{3}H]uracil for 3 min and size-fractionated by sucrose density-gradient centrifugation. Fractions from the gradient



.FIGURE 8. Sedimentation analysis of total RNA.

A 5 ml bacterial culture was labelled for 3 min with [^{3}H]uracil, rapidly harvested and lysed, and was immediately sedimented through a 6 to 30% sucrose density-gradient. Fractions of 0.3 ml were collected. Upper panel: distribution of total radioactivity incorporated into RNA (cpm per 0.1 µl). Lower panels: distribution of mRNA sequences complementary to the four different DNA hybridization probes described in figure 6. Middle panel: L11-L1 mRNA (◊) and L10-L12 mRNA (♦). Lower panel: β mRNA (◊) and β′ mRNA (♦). The positions of mature 16S and 23S rRNA are indicated. There has been no correction of the hybridization data for the sizes of the different DNA probes.

were hybridized to specific DNA probes in order to estimate the relative amounts and molecular lengths of the L11-L1, L10-L12, β and β′ mRNA sequences (fig. 8).

The L11-L1 mRNA sequences were found in transcripts of about 2600 and 1300 nucleotides in length at a molar ratio of about 3 : 1. The L10-L12 mRNA sequences were also found in transcripts of about the same sizes but in a molar ratio of about 3 : 2. The major 2600 nucleotide transcript probably corresponds to the tetracistronic mRNA initiated at the P_{L11} promoter and terminated at the attenuator site in the L12-β intergenic space; this transcript hybridizes to both the L11-L1 and the L10-L12 probe DNAs. The shorter molecules of around 1300 nucleotides probably correspond to the separate bicistronic transcripts of the L11-L1 and the L10-L12 genes. The two shorter transcripts could arise by processing of the long tetracistronic transcripts. However, the molar excess of the downstream L10-L12 sequences suggests that at least some of these transcripts arise from promoter activity in the L1-L10 intergenic space.

The size distributions of the β and β′ mRNA transcripts were similar but heterogeneous with about two-thirds of the sequences in molecules greater than 3000 nucleotides long. The heterogeneous distribution probably results from the fact that the synthesis time of the intact 9000 nucleotide ββ′ mRNA molecule (about 3 min) is greater than the average half-life of mRNA (about 1.5 min). This means that many nascent molecules are simultaneously being elongated at their 3′ ends, degraded at their 5′ ends and translated by ribosomes in the region between the 3′ and 5′ ends. Few, if any, full-length 9000 nucleotide long molecules would be expected.

The size distribution of rplKAJL mRNA sequences observed in the sucrose density-gradient profile was confirmed by Northern hybridization analysis (fig. 9). Total in vivo

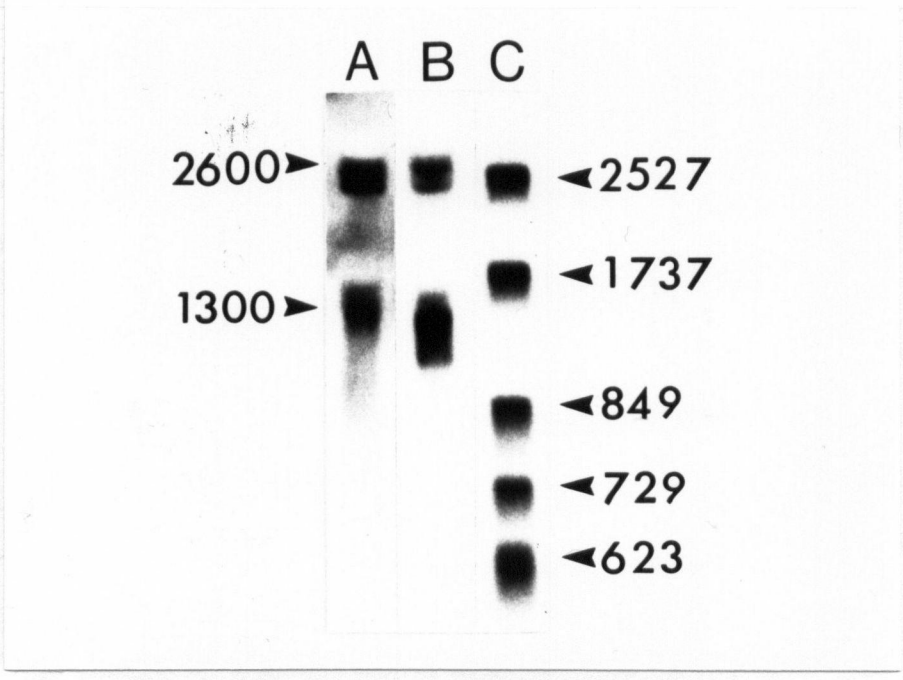


FIGURE 9. Northern hybridization analysis of L11-L1 and L10-L12 mRNA.

Total RNA (10 μg) was fractionated on an agarose/formaldehyde gel, transferred to nitrocellulose and probed with [^{32}P]-labelled, nick-translated restriction fragments. Lane A: the probe was the 617 nucleotide EcoRI-BglII fragment spanning the L11-L1 genes. Lane B: the probe was the 290 nucleotide HindIII-EcoRI fragment spanning the L10-L12 genes. Lane C: the molecular length markers were 5′ end-labelled HaeIII fragments of M13mp11 and are indicated in nucleotides.

RNA was fractionated on a denaturing agarose gel and probed with radioactive DNA fragments. The L11-L1 probe was the 617 bp EcoRI-BglII fragment (nucleotides 230 - 897; fig. 6) and the L10-L12 probe was the 290 bp HindIII-EcoRI fragment (nucleotides 2154 - 2444). Both probes hybridized to the 2600 nucleotide tetracistronic RNA transcript. Each of the probes hybridized also to smaller transcripts of about 1300 nucleotides, which represent the bicistronic L11-L1 and L10-L12 mRNA species, respectively. As observed in the sucrose density-gradient profile, the relative abundance of the bicistronic L10-L12 mRNA sequence appears to be greater than the L11-L1 mRNA sequences.

3. S1 NUCLEASE MAPPING

The 5′ and 3′ ends of RNA transcripts arise from transcription initiation or termination, from RNA processing, or from RNA degradation. The transcript ends derived from the rplKAJL-rpoBC gene cluster were located on the DNA sequence by S1 nuclease mapping. Appropriate sequencing ladders were used for resolution of transcript ends at the nucleotide level. The nucleotide numbering system in this chapter is according to Post et al. (1979). The results obtained are summarized in figure 6. These S1 nuclease transcript mapping experiments were carried out using RNA isolated from bacteria growing at rates of 2.00 (glucose plus casamino acids), 1.10 (glucose) and 0.83 (glycerol) doublings per hour. The results presented here used total in vivo RNA from the glucose-grown culture; the results with the other RNAs were qualitatively similar and no obvious differences were apparent.

(a) THE NusG-L11 INTERGENIC REGION

The NusG-L11 intergenic space contains the characteristic inverted repeat symmetry and T-rich sequence associated with Rho-independent terminators as well as the -10 and -35 recognition sequences of RNA polymerase (fig. 10). A 346 bp AvaII-AvaI fragment was used to locate the site of termination of nusG gene transcripts, to locate the site of initiation of P_{L11} transcripts and to determine the degree of transcription read-through from the nusG gene into the L11 ribosomal protein gene. Hybridization of the probe, 3′ end-labelled at the AvaII site in the nusG gene, to total RNA resulted in a protected fragment of about 200 nucleotides (fig. 10, lane E). This positions the 3′ end of the nusG gene transcripts at about nucleotides 82 to 87 on the DNA sequence that is preceded by inverted repeat symmetry.

Use of the same DNA probe, 5′ end-labelled at the AvaI site in the L11 gene resulted in a protected fragment 150 to 160 nucleotides long (fig. 10, lane D). This fragment was sized by electrophoresis alongside the Maxam-Gilbert G and A+G sequencing ladder. The length of the predominant fragment, about 157 nucleotides long (lane F), corresponds to the C residue at position 77; this site is preceded by the -10 and -35 RNA polymerase recognition signals of the P_{L11} promoter and is within one nucleotide of the point identified by in vitro transcription studies (Taylor and Burgess, 1979; Post et al., 1979). The negligible amount of protection of the full-length DNA probe in these experiments may be due to either reannealing of the double-stranded probe or to protection by a small amount of read-through transcript. Such read-through transcripts, if they exist, would contribute little to ribosomal protein gene transcription.

The transcription start point for the L11-L1 bi- and tetracistronic transcripts at nucleotide 77 is located within the second half of an inverted repeat symmetry that also

FIGURE 10. Nuclease S1 mapping of transcript ends derived from the NusG-L11 intergenic region.

The nucleotide sequence scale and the NusG-L11 intergenic region are illustrated (top). The open rectangle below represents the 346 bp AvaII-AvaI probe used to map transcript ends of transcripts in the intergenic space: the lengths of the 5′-protected fragments are illustrated above and the 3′-protected fragments below the open rectangle. Restriction site designation and positions are given in figure 6 (n, nucleotides). Autoradiograms of nuclease S1 protection products are shown (middle); T: top of gel, B: bottom of gel. The designations are: lane A, molecular length standards (3′ end-labelled MspI fragments of pBR322 with lengths of 623, 528, 405, 310, 243, 239, 218, 202, 191, 181, 161, 148, 123, 111, 91, 77 and 68 nucleotides); lane B, 5′ end-labelled 346 nucleotide AvaII-AvaI probe; lane C, 5′ end-labelled probe protected by rRNA; lane D, 5′ end-labelled probe protected by total RNA; lane E, 3′ end-labelled probe protected by total RNA; lane F, 5′ end-labelled probe protected by total RNA alongside the Maxam-Gilbert G and A+G reaction products. For lane F, the probe was 5′ end-labelled only at the AvaII site. The DNA sequence in the region surrounding the 3′ and 5′ transcript ends is illustrated (bottom). The positions of the 3′ and 5′ ends are indicated as well as putative secondary structures in the RNA transcript.

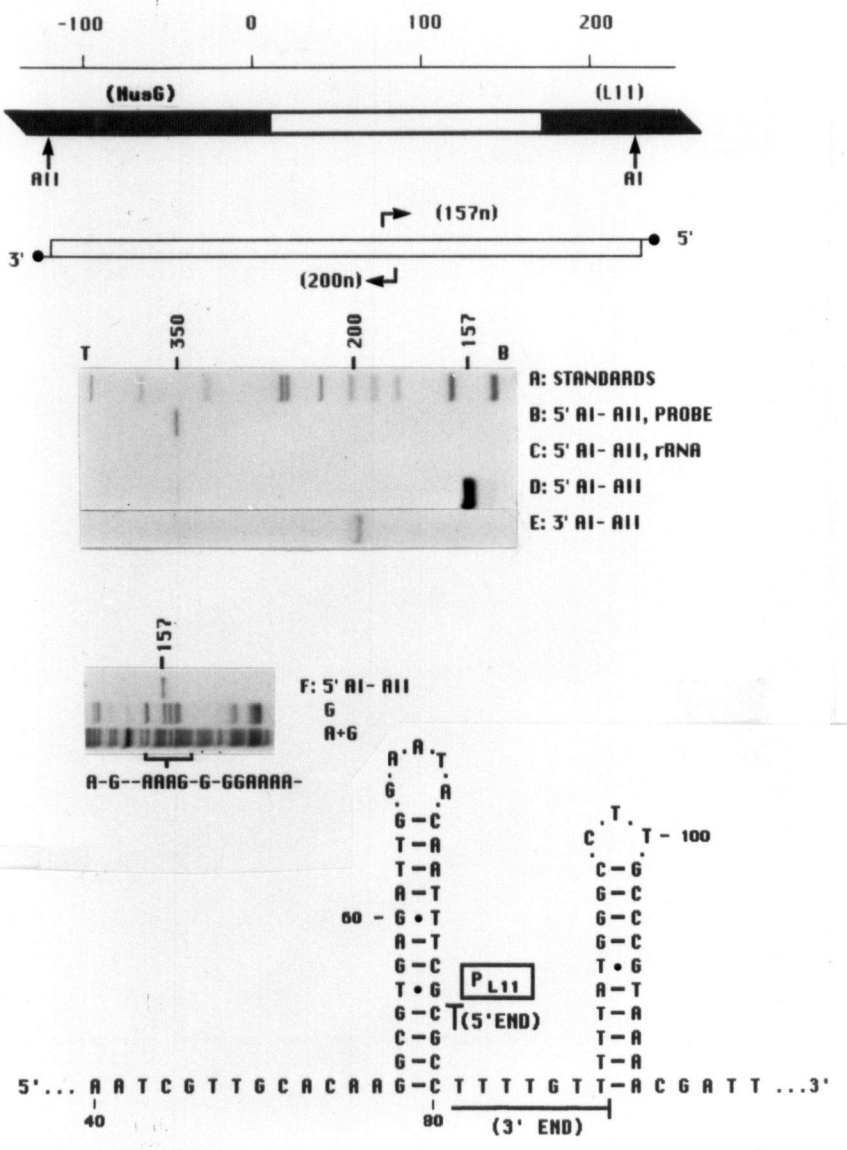

-100
0
100
200
(HusG)
(L11)
AII
AI
(157n)
3'
5'
(200n)
T
350
200
157
B
A: STANDARDS
B: 5' AI- AII, PROBE
C: 5' AI- AII, rRNA
D: 5' AI- AII
E: 3' AI- AII
157
F: 5' AI- AII
G
A+G
A-G--AAAG-G-GGAAAA-
T - 100
60 -
P L11
(5'END)
5'... A A T C G T T G C A C A A G - C T T T T G T T - A C G A T T ...3'
40
80
(3' END)

FIGURE 10

constitutes a portion of the termination signal for transcripts of the upstream nusG gene. Although there is little or no cotranscription of the nusG and the L11-L1 ribosomal protein genes, there may be some regulatory interaction between the overlapping terminator and promoter signals.

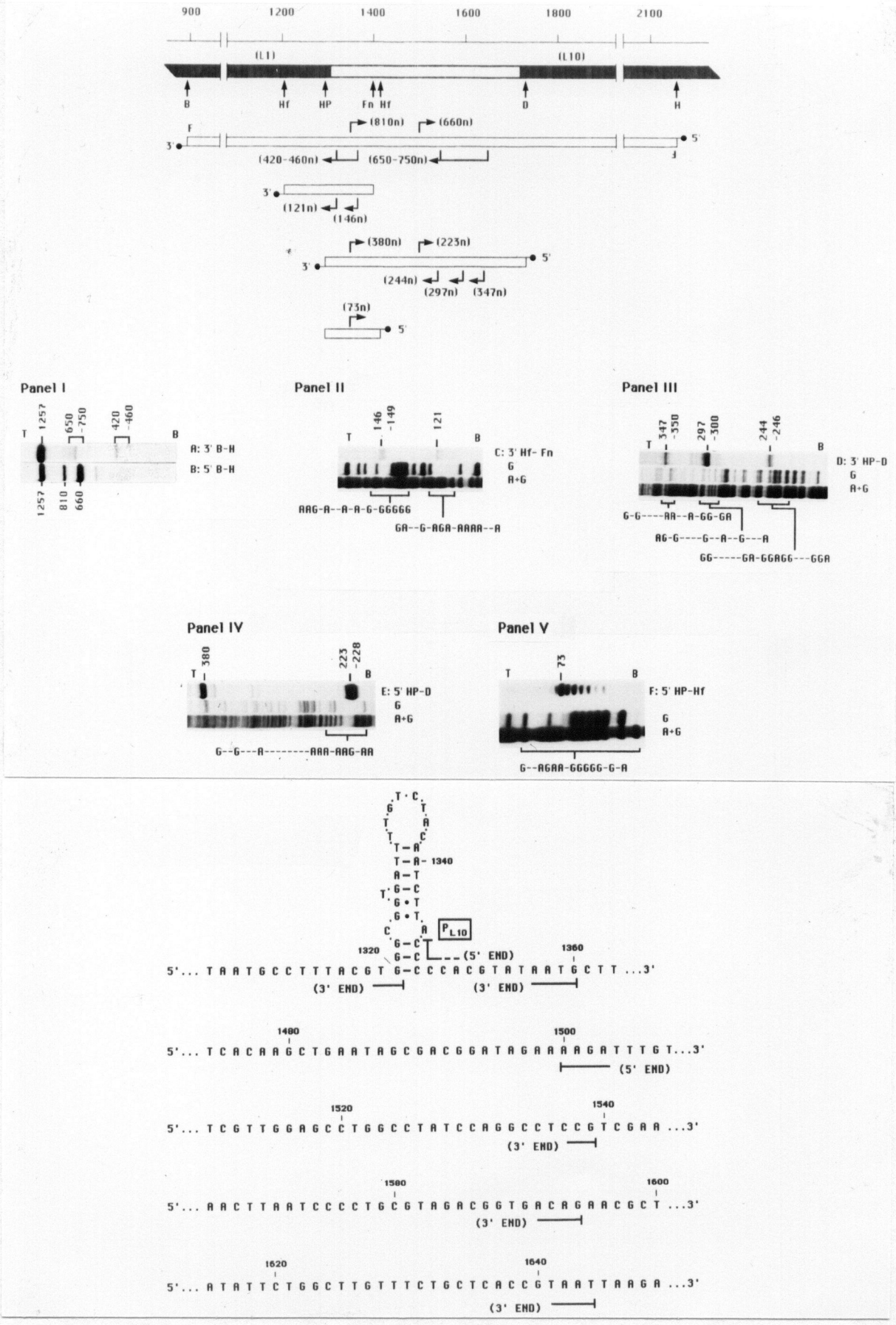
(b) THE L1-L10 INTERGENIC REGION

The L1-L10 intergenic space contains the -10 and -35 signals associated with the L10 promoter but lacks any recognizable Rho-independent signals (Platt, 1986; Yager and von Hippel, 1987) for terminating L11 operon bicistronic transcripts (fig. 11). The 1257 bp BglII-HindIII fragment was used to identify uninterrupted transcripts spanning the L1-L10 intergenic space as well as 3′ and 5′ transcript ends generated within this region. Both 5′ and 3′ end-labelled probes exhibited a 1257 nucleotide, full-length fragment as the major protection product (fig. 11: panel I, lanes A and B); these products were derived presumably from protection by the 2600 nucleotide tetracistronic mRNA observed in the sucrose gradient (fig. 8) and Northern analyses (fig. 9).

The less abundant bicistronic L11-L1 and L10-L12 mRNAs provided partial protection of the respective end-labelled BglII-HindIII probes. The probe, 3′ end-labelled at the BglII site in the L1 gene, resulted in fragments of about 420 to 460 nucleotides and a series of multiple fragments ranging in size from about 650 to 750 nucleotides (fig. 11: panel I, lane A). The set of shorter fragments corresponds to protection by a L11-L1 transcript with a 3′ end just beyond the L1 gene (nucleotides 1320 to 1360). The set of longer fragments result from protection by L11-L1 transcripts with 3′ termini in the region between nucleotides 1540 to 1650 in the DNA sequence.

.FIGURE 11. Nuclease S1 mapping of transcript ends derived from the L1-L10 intergenic region.

The nucleotide sequence scale and the L1-L10 intergenic region are illustrated (top). The open rectangles below represent various restriction fragments used to map transcript ends. These are: (i) a 1257 bp BglII-HindIII fragment labelled at either the 5′ or 3′ end; (ii) a 198 bp HinfI-Fnu4HI fragment 3′ end-labelled at the HinfI site; (iii) a 435 bp HinPI-DraI fragment labelled at either the 3′ end (HinPI site) or the 5′ end (DraI site) and (iv) a 121 HinPI-HinfI fragment 5′ end labelled at the HinfI site. The lengths of the 5′ and 3′ protected fragments are illustrated above and below each rectangle, respectively (n, nucleotides). The "F" at the ends of the BglII-HindIII fragments designates full protection by the tetracistronic read-through transcripts. Restriction site designations and positions are given in figure 6. Autoradiograms of nuclease S1 protection products are shown (middle); T: top of gel, B: bottom of gel. The DNA probes protected with total RNA are: **Panel I,** lane A, the 3′ end-labelled BglII-HindIII fragment; lane B, the 5′ end-labelled BglII-HindIII fragment; **Panel II,** lane C, the 3′ end-labelled HinfI-Fnu4HI fragment; **Panel III,** lane D, the 3′ end-labelled HinPI-DraI fragment; **Panel IV,** lane E, the 5′ end-labelled HinPI-DraI fragment and **Panel V,** lane F, the 5′ end-labelled HinPI-HinfI fragment. The probes in lanes C to F are labelled only in the minus strand, and the S1-protected products are electrophoresed alongside the Maxam-Gilbert A and A+G reaction products. The DNA sequence in the region surrounding the 3′ and 5′ transcript end sites are illustrated (bottom). The positions of the 3′ and 5′ ends are marked and a potential secondary structure in the mRNA is indicated.


900
1200
1400
1600
1800
2100
(L1)
(L10)
B
Hf
HP
Fn Hf
D
H
F
(810n)
(660n)
(420-460n)
(650-750n)
(121n)
(146n)
(380n)
(223n)
(244n)
(297n)
(347n)
(73n)
Panel I
A: 3' B-H
B: 5' B-H
1257
810
660
Panel II
C: 3' Hf- Fn
AAG-A--A-A-G-GGGGG
GA--G-AGA-AAAA--A
Panel III
D: 3' HP-D
G-G----AA--A-GG-GA
AG-G----G--A--G---A
GG-----GA-GGAGG---GGA
Panel IV
E: 5' HP-D
G--G---A--------AAA-AAG-AA
Panel V
F: 5' HP-Hf
G--AGAA-GGGGG-G-A
G
A+G
T
B

T-A- 1340
P L10
1320
1360
(5' END)
5'... T A A T G C C T T T A C G T G C C C A C G T A T A A T G C T T ...3'
(3' END)
(3' END)
1480
1500
5'... T C A C A A G C T G A A T A G C G A C G G A T A G A A A A G A T T T G T...3'
(5' END)
1520
1540
5'... T C G T T G G A G C C T G G C C T A T C C A G G C C T C C G T C G A A ...3'
(3' END)
1580
1600
5'... A A C T T A A T C C C C T G C G T A G A C G G T G A C A G A A C G C T ...3'
(3' END)
1620
1640
5'... A T A T T C T G G C T T G T T T C T G C T C A C C G T A A T T A A G A ...3'
(3' END)

FIGURE 11

The same DNA probe, when 5′ end-labelled at the HindIII site in the L10 gene, yielded fragments of about 810 and 660 nucleotides long (fig. 11: panel I, lane B). The 810 base fragment places the mRNA 5′ end near the P_{L10} promoter around nucleotide 1350. The major 660 base fragment protected by L10-L12 mRNA corresponds to a 5′ transcript end near position 1500, a region in which no promoter activity has been detected. More precise mapping of the positions of these 5′ and 3′ transcript ends was carried out using shorter restriction fragments and electrophoresing the protected fragments next to the Maxam-Gilbert G and A+G sequencing ladder.

The less prominent 3′ transcript ends in the region between nucleotides 1320 and 1360 were visualized using the 198 nucleotide HinfI-Fnu4HI fragment 3′ end-labelled only at the HinfI site at nucleotide 1209 (fig. 11: panel II, lane C). The protected fragments of 121 nucleotides and 146 to 149 nucleotides correspond to 3′ transcript ends at or near nucleotides 1320 and 1356 to 1360, respectively. Neither site exhibits identifiable termination-like sequences. The 3′ transcript ends in the region between nucleotides 1540 and 1650 were located by using a 435 nucleotide HinPI-DraI fragment 3′ end-labelled only at the HinPI site at nucleotide 1294 (fig. 11: panel III, lane D). Major protected fragments of 244 to 246, 297 to 300 and 347 to 350 nucleotides were apparent and correspond to 3′ transcript ends at positions 1537 to 1539, 1591 to 1594 and 1641 to 1644, respectively. Again, none of these regions exhibits identifiable termination-like sequences.

The 5′ transcript ends in the region between the L1 and L10 genes were precisely located using a 435 nucleotide HinPI-DraI fragment 5′ end-labelled only at the DraI site at nucleotide 1728 in the L10 coding sequence (fig. 11: panel IV, lane E). The major protected fragment of 223 to 228 nucleotides corresponds to a 5′ transcript end at nucleotides 1500 to

1503. No promoter activity has been detected in this region, and the sequences in the region show no similarity to promoter consensus signals.

A second, less prominent protected fragment of about 380 nucleotides long was apparent in lane E. The 5′ end of this second transcript was located by using a HinPI-HinfI fragment 5′ end-labelled only at the HinfI site at nucleotide 1419 (fig. 11: panel V, lane F). The protected products represent a graduated series. The largest protected fragment is 73 nucleotides long and corresponds to nucleotide position 1346; the series extends with decreasing intensity down the sequencing ladder. Similar results were obtained using different concentrations of S1 nuclease and digestion temperatures, and different DNA probes. The position of the longest 5′ transcript end at nucleotide 1346 is preceded by the -10 and -35 RNA polymerase recognition signal of the P_{L10} promoter.

The L1-L10 intergenic space contains five distinct 3′ end sites for L11-L1 bicistronic transcripts and two distinct 5′ end sites for L10-L12 bicistronic transcripts. There are no obvious termination signals associated with any of the five 3′ end sites or anywhere else in the intergenic region. Similarly, no corresponding 5′ end sites (the other product of an endonuclease cleavage) have been observed near or downstream from any of the major 3′ end sites, although such 5′ transcript ends could be less stable and escape detection by S1 analysis. The two L10-L12 mRNA 5′ transcript end sites are both located upstream from the three major L11-L1 mRNA 3′ transcript end sites. Consequently, none of these ends are likely the reciprocal products generated by an endonuclease cleavage of the tetracistronic transcript.

The most prevalent 3′ end site at nucleotide 1594 occurs immediately downstream of the binding site for the L10 translational control protein. This means that these L11-L1

bicistronic transcripts can potentially bind L1 protein near its 5′ end and L10 protein near its 3′ end. It is possible that binding of excess L10 protein to nascent L11-L1 transcripts elicits RNA polymerase pausing and transcription termination beyond this site. Such a mechanism could regulate, to some extent, the synthesis of L10-L12 mRNA sequences.

Insertion of transposon Tn5 into plasmids carrying this region of the bacterial chromosome have defined the limits of the P_{L10} promoter region between nucleotides 1282 and 1360; insertions at 1360 and beyond are polar on the expression of the L10, L12 and β genes in vivo (Hui et al., 1982). In vitro, RNA polymerase binding and transcription studies are in agreement with this result and position the major start site at 1347 to 1348 (Post et al., 1979; Taylor and Burgess, 1979). By S1 nuclease mapping, the 5′ end of putative P_{L10}-initiated in vivo transcripts is heterogeneous; the most prominent end corresponds to position 1346 with other ends appearing at one-nucleotide increments extending beyond position 1360. The heterogeneity may be due to processing at the 5′ end of the P_{L10}-initiated transcript or to artefacts caused by S1 nucleolytic activity at the end of the RNA-DNA hybrid. However, this result appears to be independent of the S1 concentration and the digestion temperature. Decay of transcripts in a net 5′ to 3′ direction in E. coli has been proposed by Cannistraro and Kennell (1985) and Portier et al. (1987). However, since no 5′ to 3′ exonuclease activity has been isolated, this decay may be due to 3′ to 5′ exonucleolytic processing following an initial endonucleolytic cleavage.

The second and more abundant 5′ end site for bicistronic L10-L12 transcripts is situated at nucleotide 1500. This end appears to be generated by RNA processing and not by transcription initiation since there are no recognizable -35 and -10 promoter consensus sequences in this region, and insertion of Tn5 140 nucleotides in front of this site is polar on downstream genes (Hui et al., 1982). Transcripts beginning at this site retain the intact

binding site for the L10 translational regulatory protein. The sequence at this 5′ transcript terminus bears some similarity to the sequence at a prominent 5′ transcript end in the leader region of the secE-nusG mRNA (see section 3.2.2). Whether or not this similarity signifies a common regulatory mechanism remains to be determined.

Finally, the generation of all transcript ends, both 5′ and 3′, within the L1-L10 intergenic space is not altered in the mutant strain defective in RNaseIII activity; if any of the transcript ends are generated by processing, RNaseIII most likely is not involved (data not shown).

(c) THE L10-L12 INTERGENIC REGION

The ribosome contains four copies of L12 protein and only single copies of all the other ribosomal proteins. To account for this stoichiometry, it has been suggested that the L10-L12 intergenic space contains a promoter which specifically enhances transcription and expression of the L12 gene (Newman et al., 1979; Ma et al., 1981; Ralling and Linn, 1984). A 290 nucleotide HindIII-EcoRI fragment, 5′ end-labelled at the EcoRI site within the L12 gene, was used to detect transcripts initiated in the L10-L12 intergenic space (data not shown). Only the full-length 290 base fragment resulting from protection by either the bicistronic L10-L12 mRNA or the tetracistronic mRNA was observed; no transcription initiation was detected in the L10-L12 intergenic space.

This suggests that the L12 message is translated more efficiently than the co-cistronic L10 message upstream, in order to account for the 4 : 1 molar ratio of L12 to L10 and to all other r-proteins. Noncoordinate expression, achieved solely at the translational level, has also been observed in the trmD operon; this operon is transcribed as one polycistronic

mRNA that encodes r-protein S16, an unknown 21K protein, a tRNA-methyltransferase and r-protein L19, in that order (Wikström and Björk, 1988). Under steady state conditions, the amount of r-proteins S16 and L19 is about 12 times higher than the amount of the 21K protein and about 40 times higher than the amount of the TrmD protein.

It has been suggested that codon usage in E. coli is modulated for gene expression, i.e. highly expressed genes contain few or no rare codons (review: de Boer and Kastelein, 1986). This relationship was demonstrated for the trmD operon (Wikström and Björk, 1988). However, in the rplJL operon, the increased translation of L12 relative to L10 is not likely due to biased codon usage since the codons used in both genes are those recognized efficiently by the most abundant tRNA species (Post et al., 1979).

There is some evidence that secondary or tertiary structure which may shield or expose the ribosome binding site (RBS) and the AUG initiation codon can be an important factor in controlling translation efficiency of mRNA (Kastelein et al., 1983; Queen and Rosenberg, 1981; Munson et al., 1984; Looman et al., 1986; Berkhout and van Fuin, 1985). The codon following the AUG initiation codon may also be involved in translational regulation, presumably by its effect on the RBS structure (Sherer et al., 1980; Looman et al., 1987). However, in the L10-L12 intergenic region, there are no obvious secondary structures which might enhance translation. Others have suggested that sequences 5′ to the RBS can affect translation without involving secondary structures (Boyen et al., 1982; Stanssens et al., 1985). Interestingly, the DNA sequences upstream from the initiation codon of the L19 gene and the L12 gene are both AT-rich; this has been suggested to be an important feature of the translation initiation region of some heavily translated mRNAs (McCarthy et al., 1985). Whether or not this is the mechanism of translational enhancement of L12 expression remains to be determined.

(d) THE L12-β INTERGENIC REGION

The L12-B intergenic region contains a transcription attenuation site that terminates about 80% of the transcripts entering the intergenic region (Dennis 1977a, 1984; Barry et al., 1979, 1980). Transcripts that read through the attenuator contain a potential downstream RNaseIII processing site (King et al., 1986; Gegenheimer and Apirion, 1981). Processing per se has no detectable effect on expression of the β and β′ RNA polymerase genes; however, a sequence in the vicinity of the processing site appears to be essential for efficient translation of the downstream mRNA sequences (Dennis, 1984). The 1080 nucleotide EcoRI fragment, either 5′ or 3′ end-labelled, was used to further characterize the activity of these two intergenic regulatory sites (fig. 12).

Using a 3′ end-labelled 1080 bp EcoRI fragment, three major fragments of 240 to 270 nucleotides were protected by total RNA (fig. 12, lanes A to C). The two minor bands of 325 and 1080 bases correspond to protection by RNaseIII processed mRNA and by unprocessed mRNA, respectively. Using total RNA from a RNaseIII⁻ mutant (N2077) (fig. 12, lane B), the 325 base protected fragment disappeared and the amount of the full-length 1080 base fragment was correspondingly increased. When the EcoRI probe was labelled at the 5′ end, protection products of 750 and again 1080 bases were observed (fig. 12, lane D). The 750 base product resulted from protection by the reciprocal portion of the RNaseIII processed transcript; in the RNaseIII⁻ mutant, this fragment also disappeared and the intensity of the full-length fragment accordingly increased (data not shown).

These 3′ mRNA ends were mapped more precisely by using a 395 base HinfI-AccI fragment 3′ end-labelled only at the HinfI site at nucleotide 2552 (fig. 12, lane E). Using

FIGURE 12. Nuclease S1 mapping of transcript ends derived from the L12-β intergenic space.

The nucleotide sequence scale the the L12-β intergenic region are shown (top). The open rectangles below represent various restriction fragments used to map transcript ends. These are: (i) a 1080 bp EcoRI fragment labelled at either the 3′ or the 5′ end; (ii) a 395 bp HinfI-AccI fragment 3′ end-labelled at the HinfI site and (iii) a 336 bp NarI-HinfI fragment 5′ end-labelled at the HinfI site. The lengths of the 5′ and 3′ protected fragments are illustrated above and below each rectangle, respectively (n, nucleotides). The "F" at the ends of the EcoRI fragment designates some full-length protection by read-through transcripts. Restriction site designation and positions are given in figure 6. Autoradiograms of nuclease S1 protection products are shown (middle); T: top of gel, B: bottom of gel. DNA probes protected with total RNA are: lanes A to C, the 3′ end-labelled EcoRI fragment; lane D, the 5′ end-labelled EcoRI fragment; lane E, the 3′ end-labelled HinfI-AccI fragment; lane F, the 5′ end-labelled NarI-HinfI fragment. The RNAs used for protection in lanes B and C were from isogenic rnc^- and rnc^+ strains, respectively. The probes in lanes E and F are labelled only in the minus strand, and the S1 reactions are electrophoresed alongside the Maxam-Gilbert A and A+G reaction products. The DNA sequence in the regions surrounding the 3′ and 5′ ends are shown and some potential secondary structures in the mRNAs are illustrated, i.e. the attenuator (upper) and the RNaseIII processing site (lower).

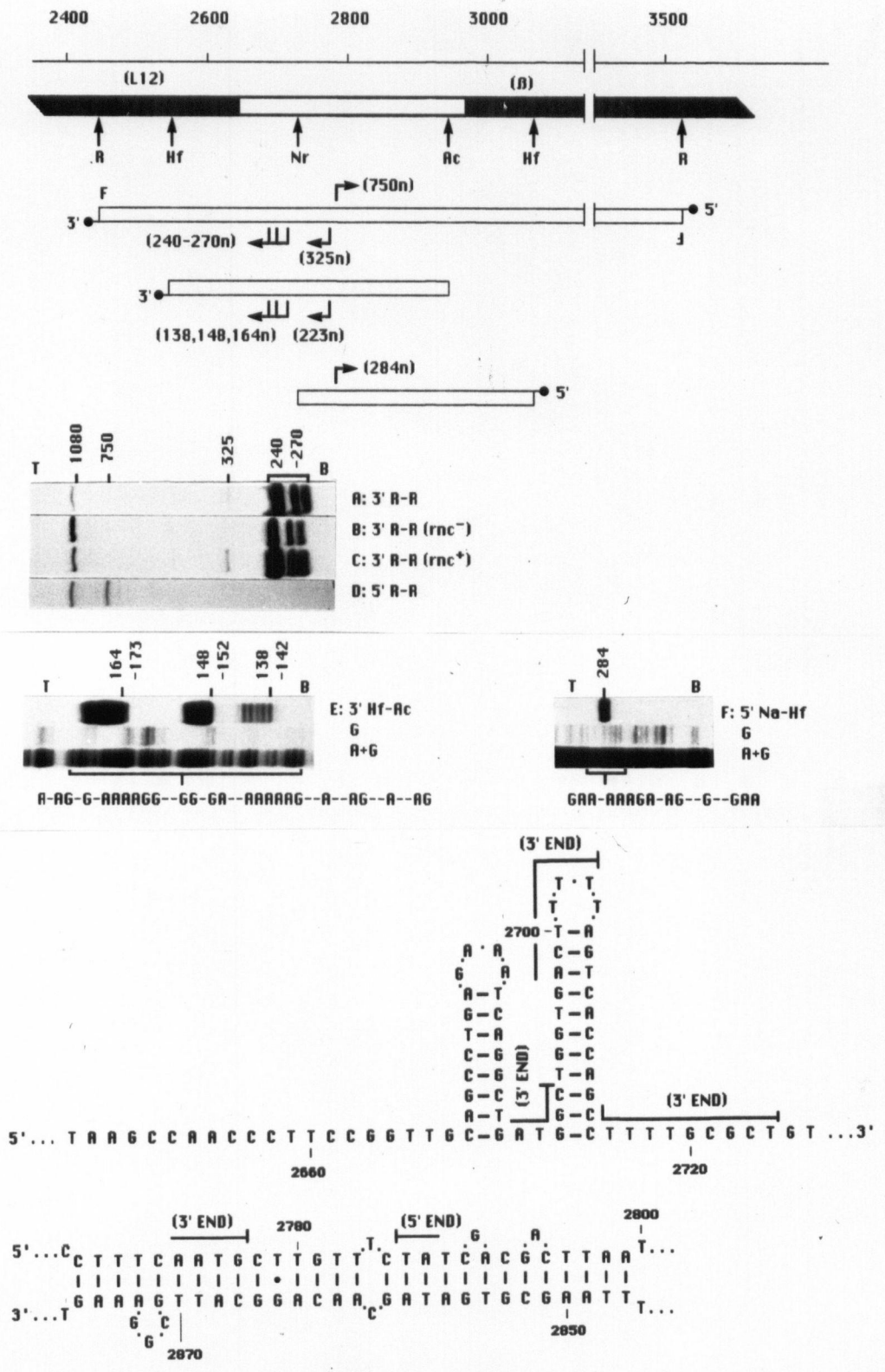

2400
2600
2800
3000
3500
(L12)
(ß)
R
Hf
Nr
Ac
Hf
R
F
(750n)
3'
5'
(240-270n)
(325n)
(138,148,164n)
(223n)
(284n)
1080
750
325
240
-270
T
B
A: 3' R-R
B: 3' R-R (rnc⁻)
C: 3' R-R (rnc⁺)
D: 5' R-R
164
-173
148
-152
138
-142
E: 3' Hf-Ac
G
A+G
A-AG-G-AAAAGG--GG-GA--AAAAAG--A--AG--A--AG
284
F: 5' Na-Hf
GAA-AAAGA-AG--G--GAA
(3' END)
2700
(3' END)
(3' END)
5'... T A A G C C A A C C C T T C C G G T T G C-G A T G-C T T T T G C G C T G T ...3'
2660
2720
(3' END)
2780
(5' END)
2800
5'...C C T T T C A A T G C T T G T T C T A T C A C G C T T A A T...
3'...T G A A A G T T A C G G A C A A G A T A G T G C G A A T T T...
2850
2870

FIGURE 12

this probe, the three major fragments were resolved as heterogeneous 3′ transcript ends ranging in length from 164 to 173 nucleotides, 148 to 152 nucleotides and 138 to 142 nucleotides. The longest set of fragments corresponds to the previously defined termination site, recognized as a transcription attenuator, at nucleotides 2716 to 2719 (Post et al., 1979; Barry et al., 1980; Ralling and Linn, 1987). The sequence at this site consists of four consecutive T residues and is preceded by a region of GC-rich inverted repeat symmetry characteristic of Rho-independent terminators (Reviews : Platt, 1986; Yager and von Hippel, 1987). The two shorter sets of fragments, 148 to 152 bases long and 138 to 142 bases long, correspond respectively to 3′ transcript ends within and preceding the inverted repeat sequence (fig. 12) at nucleotides 2700 to 2704 and at nucleotides 2690 to 2694: these fragments may be artifacts due to S1 nibbling at the ends of RNA-DNA hybrids. The potential for base interactions at the 3′ end of transcripts terminated near nucleotide 2717 could generate unusual structures in the RNA-DNA hybrids (i.e. cruciforms), which might be sensitive to S1 attack. Alternatively, these fragments might represent alternative termination sites that have not been resolved and identified. These multiple 3′ ends have been observed in all E. coli strains examined and are independent of S1 nuclease concentration and digestion temperature. This pattern of multiple ends was not observed by Barry et al. (1980), possibly because their method was less sensitive than that employed here. However, since completion of the transcript analysis presented here, Ralling and Linn (1987) have reported a similar pattern of 3′ transcript ends, with one exception, in the L12-β intergenic region; they did not observe the mRNA species whose 3′ terminus was situated at nucleotides 2690 to 2694.

Although the attenuator resembles a simple factor-independent terminator, the function of this structure appears to be more complex than that of a constitutive terminator. The attenuator is postulated to be a dynamic structure where the frequency of termination

can be modulated. For example, when RNA polymerase transcription capacity is inhibited in a temperature-sensitive E. coli mutant (XH56), increased transcription of the downstream β and β′ RNA polymerase genes is thought to result from a lower frequency of transcript termination at this site (Dennis, 1977b; Kirschbaum, 1978) (see below).

Other examples of attenuation in r-protein operons are seen in the S15, S21 and S10 operons (Regier and Portier, 1986; Burton et al., 1983; Freedman et al., 1987; Lindahl et al., 1983). Except for the S10 operon, transcription attenuation is used in these instances to down-regulate the transcription of non-ribosomal genes such as the polynucleotide phosphorylase gene (pnp) in the S15 operon, and the DNA primase (dnaG) and RNA polymerase σ subunit (rpoD) genes in the S21 operon. As described previously (section 1.1.3(2)), the S10 operon is unique among r-protein operons. The protein product of the third gene in this operon, protein L4, not only acts as the translational repressor but also causes transcription termination in the leader region of the operon (Freedman et al., 1987; Lindahl et al., 1983). No model explaining attenuation at this site has been proposed.

Transcription attenuation is also used to regulate amino acid biosynthetic operons in E. coli. In this case, the mechanism of attenuation has been elucidated : transcription termination is translationally regulated and occurs in the leader regions of these operons. Similar mechanisms of transcription attenuation regulate pyrimidine biosynthetic operons, the ampC operon and the tryptophanase operon among others (Review : Landick and Yanofsky, 1987).

However, the mechanism of modulation of transcription attenuation in the L12-β intergenic region is not known. Transcription termination in E. coli can be factor-independent or it can involve a number of different factors which interact with the RNA

polymerase core enzyme. These transcription termination factors include Rho protein, the Nus proteins (such as NusA, NusB, NusE and NusG) and the sfrB gene product (Review : Yager and von Hippel, 1987). Based on RNA filter hybridization results of various nus, rho and sfrB mutants, Ralling and Linn (1987) have suggested that Rho and NusA may regulate the frequency of transcription termination at the attenuator, even though the attenuator resembles a factor-independent terminator. In agreement with this hypothesis, Chamberlin et al. (1987) have identified two factors, Tau and NusA, which act at Rho-independent sites in vitro to reflect the accuracy and efficiency of termination in vivo. The specificity of these factors suggests that there may be several classes of Rho-independent terminators. Ralling and Linn (1987) have proposed that Rho normally increases the frequency of termination at the attenuator in the L12-β intergenic region and that NusA and the sfrB gene product decrease this frequency. However, it is conceivable that these termination factors act at a site distal to the attenuator since no obvious differences in the pattern of S1 protected fragments were seen between these mutants and the wild-type.

The 3′ end of the transcript created by RNaseIII processing protected about 223 nucleotides of the 395 base HinfI-AccI probe and corresponds to endonuclease cutting between positions 2775 and 2778. A much less prominent protected fragment about ten nucleotides longer and corresponding to cutting at nucleotides 2785 to 2788 was barely apparent (data not shown).

The 5′ end of the reciprocal RNaseIII processed transcript was localized using the 336 base NarI-HinfI fragment 5′ end-labelled only at the HinfI site at nucleotide 307 (fig. 12, lane F). The major protected fragment was 283 to 285 bases and corresponds to a 5′ end site at nucleotides 2786 to 2788. Both of the RNaseIII-dependent 3′ and 5′ transcript ends are located within the first half of an inverted repeat symmetry that is capable of forming

a duplex structure in the mRNA. Processing results in generation of a 3′ end and a 5′ end that are separated by about ten nucleotides. This implies either that RNaseIII cuts at two positions (separated by about one helical turn) or that one of the products of a single endonuclease cut is rapidly trimmed by about ten nucleotides. There does not appear to be any significant promoter activity in the L12-β intergenic region.

The role of RNaseIII processing of ββ′ mRNA transcripts remains unclear. The results reported here indicate that, at a given time, only about half of the transcripts spanning the 1080 bp EcoRI fragment are processed in an rnc$^+$ (RNaseIII$^+$) genetic background. This may mean that processing of the mRNA is either slow or incomplete. In an rnc$^-$ background, there was no detectable processing and no obvious effect on β and β′ gene expression. Possible functions of RNaseIII processing are discussed below (section 4.2.2(2)).

4.2.2 DIFFERENTIAL REGULATION OF rplKAJL AND rpoBC

Although the ββ′ genes are co-transcribed with the upstream ribosomal protein genes, regulation of ββ′ synthesis is distinct at both the translation level and the transcriptional level. It has been proposed that differences in transcriptional activities, during restriction of RNA polymerase activity or during the stringent response, can be attributed to modulation of transcription initiation at promoters P_{L11} and P_{L10} and termination at the attenuator (Dennis, 1977b; Little and Dennis, 1980). To clarify the mechanism of transcriptional regulation of rpoBC, transcripts produced under these restrictions were examined by S1 nuclease mapping.

Two sets of E. coli mutants were used. Strain XH56 has a temperature-sensitive mutation in rpoC which is initiation defective and thus lethal to the cell at 42°C but only semi-restrictive at 39°C. Strain NF536 (relA$^+$) has a temperature-sensitive valyl-tRNA synthetase which elicits the stringent response at the semi-restrictive temperature of 35.5 to 37°C; NF537 is the isogenic relA strain and exhibits the relaxed response (Dennis and Nomura, 1974; Maher and Dennis, 1977). In vivo transcripts produced by these strains at the permissive and semi-restrictive temperatures were analyzed by (i) filter hybridization to various DNA probes to measure relative transcriptional levels of the rplKAJL-rpoBC sequences and (ii) S1 nuclease mapping to locate 5′ and 3′ transcript ends. Observed differences in transcript levels of rplKAJL and rpoBC may be due to relative changes in transcript synthesis or degradation. In order to answer this question, the relative decay rates of rplKAJL-rpoBC transcripts from the strain XH56 were determined at the permissive and semi-restrictive temperatures.

1. FILTER HYBRIDIZATION

Total cellular RNA from exponential or semi-restricted cultures was analyzed by filter hybridization to DNA probes as described above (section 3.2.1). The percent of input radioactivity hybridizing per nucleotide of probe DNA was calculated (table 4).

A large change in transcript levels was observed with strain XH56. A temperature shift from 30 to 39°C resulted in almost a 5 fold increase in the transcription of β mRNA and only a 1.6 fold increase in the transcription of L10-L12 mRNA. The ratio of distal to proximal mRNA increased dramatically from 0.18 to 0.54. These results agree with previously published measurements (Dennis, 1977b).

TABLE 4. Filter hybridization of pulse-labelled RNAs isolated from strains XH56, NF536 and NF537.

(a) The various probes complementary to mRNA transcripts from ribosomal protein and RNA polymerase genes are described in figure 6 and in Methods and Materials (section 2.7.2). The length of each sequence complementary to mRNA is indicated in parentheses (n, nucleotides).

(b) The percentage of the input radioactivity in specific RNA-DNA hybrids is the average of 8 hybridizations (see Dennis, 1984). Input radioactivity was varied over a 4-fold range from 50 µl (about 12.5 µg) to 200 µl input RNA (about 50 µg).

XH56,	30°C	: from 1.32 X 10^5 to 5.28 X 10^5 cpm
	39°C	: from 4.65 X 10^4 to 1.86 X 10^5 cpm
NF536,	30°C	: from 1.49 X 10^5 to 5.96 X 10^5 cpm
	35.5°C	: from 5.50 X 10^4 to 2.20 X 10^5 cpm
NF537,	30°C	: from 1.34 X 10^5 to 5.36 X 10^5 cpm
	35.5°C	: from 2.27 X 10^5 to 9.08 X 10^5 cpm

(c) The percentage hybridization per nucleotide of complementary sequence in each of the DNA probes was calculated as the quotient of the percentage hybridization and the probe length . This value is an estimate of the transcriptional activity of each of these DNA sequences. The transcriptional activity of the spc gene at 30°C was used as an external control and arbitrarily set at 1.00.

(d) The ratio of β mRNA to L10-L12 mRNA was calculated as the quotient of the relative transcriptional activities of the β and the L10-L12 genes.

TABLE 4

FILTER HYBRIDIZATION OF PULSE-LABELLED RNAS ISOLATED FROM STRAINS XH56, NF536 AND NF537

Probe DNA[a]	hybridization[b] (%)		% hybridiz per[c] nucleotideX10[4]		relative transcriptional activity		ratio [d] β mRNA / L10-L12 mRNA	
	30°C	39°C	30°C	39°C	30°C	39°C	30°C	39°C
XH56 (rpoC[ts])								
L11-L1(617n)	0.112	0.170	1.81	2.76	0.73	1.10		
L10-L12(653n)	0.160	0.253	2.45	3.87	0.99	1.56		
β(2868n)	0.130	0.597	0.455	2.08	0.18	0.84	0.18	0.54
spc(9000n)	2.23	2.50	2.48	2.78	1.00	1.10		
α(700n)	0.127	0.169	1.81	2.41	0.73	0.97		
	30°C	35.5°C	30°C	35.5°C	30°C	35.5°C	30°C	35.5°C
NF536 (relA[+])								
L11-L1	0.079	0.040	1.28	0.64	0.59	0.29		
L10-L12	0.128	0.091	1.95	1.39	0.89	0.64		
β	0.089	0.103	0.31	0.36	0.14	0.17	0.16	0.27
spc	1.96	1.28	2.18	1.42	1.00	0.65		
α	0.109	0.081	1.55	1.16	0.71	0.53		
NF537 (relA)								
L11-L1	0.097	0.124	1.57	2.01	0.72	0.92		
L10-L12	0.135	0.178	2.07	2.72	0.95	1.25		
β	0.074	0.060	0.26	0.21	0.12	0.096	0.13	0.08
spc	1.96	2.48	2.18	2.76	1.00	1.27		
α	0.105	0.128	1.50	1.83	0.69	0.84		

In strain NF536 (relA$^+$), L11-L1, L10-L12 and control mRNA transcription decreased whereas β mRNA transcription increased slightly when the temperature was shifted from 30 to 35.5°C. The ratio of ββ′ mRNA sequences to L10-L12 mRNA sequences thus increased from 0.16 to 0.27. This was in contrast to hybridization results obtained using RNA from strain NF537 (relA); L11-L1, L10-L12 mRNA transcription increased, β mRNA transcription decreased and the distal to proximal mRNA ratio decreased from 0.13 to 0.08. Previous filter hybridization results indicate that the parental strain (NF314) at 30°C and 36°C displays transcription patterns identical to those of NF536 and NF537 at 30°C (Maher and Dennis, 1977). This similarity indicates that the differences in transcription patterns between these two mutant strains are not due to the temperature shift but to genetic differences in the relA gene.

Changes in the ratio of β mRNA to L10-L12 mRNA during these restrictions could result from modulation of transcription termination at the attenuator, activation of dormant or cryptic promoters in the L12-β intergenic region, changes in the rate of transcript degradation, or a combination of these possibilities.

2. S1 NUCLEASE MAPPING

To distinguish between the possible mechanisms of regulating transcription levels, 5′ and 3′ transcript ends generated during these restrictions were localized by S1 nuclease mapping. The DNA probes which were used were derived from the NusG-L11, the L1-L10 and the L10-L12 intergenic regions and have been described above. The patterns of transcript ends were identical to those for cultures in balanced growth; no new ends were detected in these P_{L11} and P_{L10} promoter regions although the quantity of ends fluctuated

as expected from the filter hybridization results during the temperature shifts. There were no transcript ends detected in the L10-L12 intergenic region under any of the conditions examined.

Autoradiograms of the S1 experiments using probes from the L12-β intergenic region are shown in figure 13. Using the 3′ end-labelled 1080 bp EcoRI probe and XH56 RNA, changes in relative intensities of major bands were apparent (fig. 13, lane A). As previously described (section 4.2.3(3d)), the fully protected 1080 nucleotide long fragment indicates read-through and unprocessed message. The other major 240-270 nucleotide long protected fragments represent transcripts terminated near the attenuator. The 325 base long band arises from RNaseIII processing. The second minor protection product, 420 nucleotides long, situates the transcript 3′ end near nucleotide 2860 which is in the second half of the stem-loop structure recognized by RNaseIII. This end could be an S1 artifact, the result of non-specific transcription termination or the result of an alternative RNaseIII endonuclease cleavage event. In the previous analysis using E. coli C600 RNA, this band was virtually undetectable (section 4.2.3(3d); fig. 12). For strain XH56 after the temperature shift to 39°C, fewer transcripts exiting L12 were terminated at the attenuator as indicated by the decrease in the relative amount of the 240-270 nucleotide long protected fragments and the increase in the relative amount of the 1080 nucleotide long protection product (fig. 13, lane A).

The 5′ transcript ends derived from the intergenic space were located by using the 5′ end-labelled 1080 bp EcoRI fragment as probe. The major protected fragments were 1080 and 750 nucleotides long and correspond to protection by unprocessed and RNaseIII processed transcripts respectively (fig. 13, lane B). These results indicate that the increase in the ratio of β mRNA to ribosomal protein mRNA following the temperature restriction

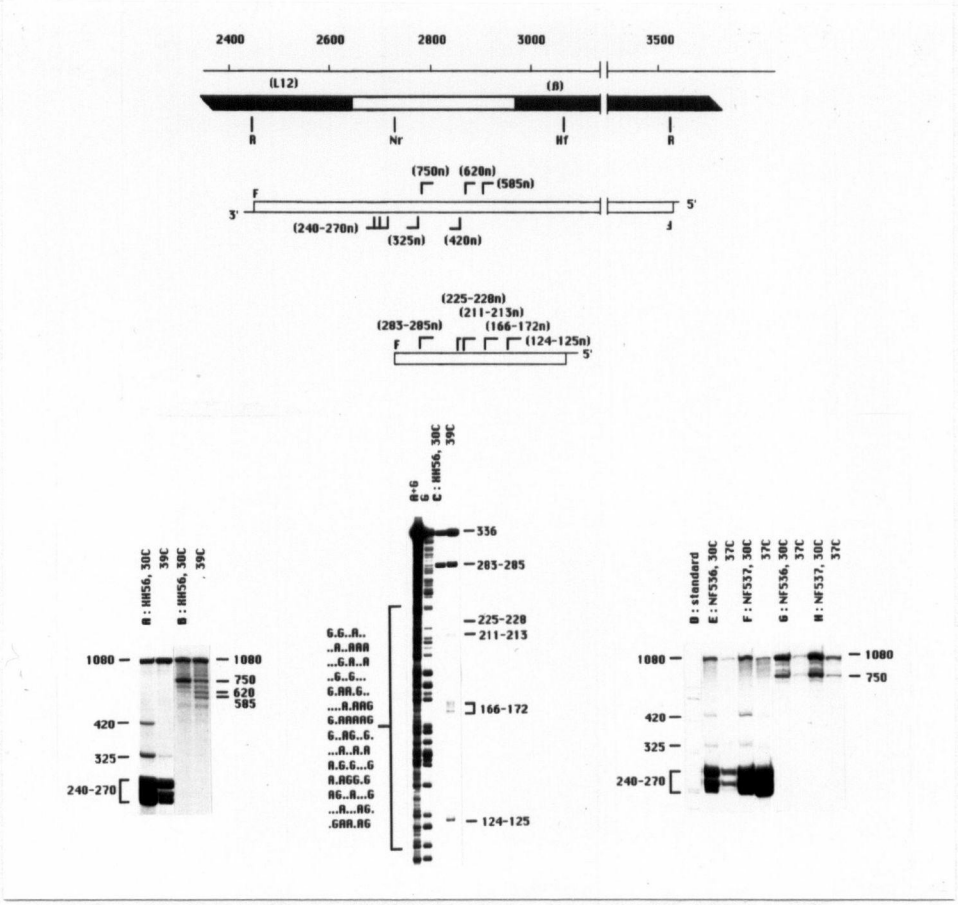
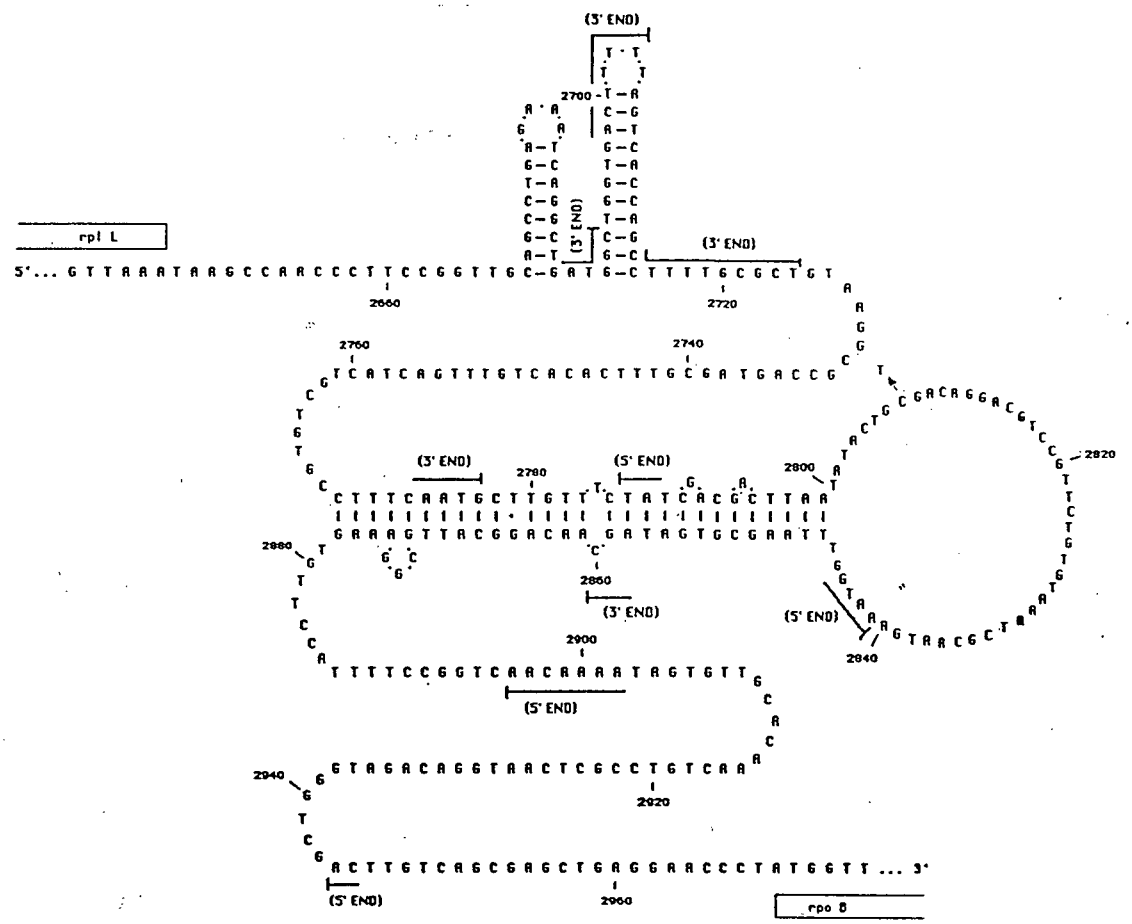


FIGURE 13. Differential transcriptional regulation of rplKAJL and rpoBC; nuclease mapping of transcript ends derived from the L12-β intergenic region.

The nucleotide sequence scale and the L12-β intergenic region are illustrated (top). The open rectangles below represent various restriction fragments used to map transcript ends. These are a 1080 bp EcoRI fragment labelled at either the 3′ or the 5′ end and a 336 bp NarI-HinfI 5′ end-labelled at the HinfI site. The lengths of the 5′ and 3′ protected fragments are shown above and below each rectangle, respectively (n, nucleotides). The "F" at the ends of the restriction fragments designates some full-length protection by read-through transcripts. Restriction site designation and positions are given in figure 6. Autoradiograms of nuclease S1 protection products are shown (bottom). DNA probes protected with total RNA are: lanes A, E and F, the 3′ end-labelled EcoRI fragment; lanes B, G and H, the 5′ end-labelled EcoRI fragment; lane C, the 5′ end-labelled NarI-HinfI fragment. The probe in lane C is labelled only in the minus strand and the S1-protected products are electrophoresed alongside the Maxam-Gilbert A and A+G reaction products. Lane D: MspI fragments of pBR322 used as size standards (fragment lengths are 623, 528, 405, 310, 243 and 239 nucleotides).



.**FIGURE 14.** Transcript end sites in the L12-β intergenic region.

The positions of 3′ and 5′ transcript end sites and some of the potential secondary structures in the RNA transcripts are shown. The secondary structure between nucleotide positions 2668 and 2715 is the transcription attenuator. The inverted repeat between nucleotides 2769 and 2878 is believed to be the substrate for RNaseIII processing; the major cleavage site generates a 3′ end at position 2777 and a 5′ end at position 2785 (section 4.2.1(3d)). The weak 5′ transcript end situated at nucleotide 2858 is not shown; it may be the reciprocal portion of the 3′ transcript end situated at approximately position 2860 produced as a result of endonucleolytic cleavage. The arrow at nucleotide 2807 indicates the single base change that resulted in an up mutation as reported by An and Friesen (1980b). The distal end of rplL and the proximal end of rpoB are denoted by the open boxes.

probably resulted from downward modulation of termination of ribosomal protein transcripts at the attenuator and not from activation of cryptic promoters in the L12-β intergenic space.

There are however at least two minor new or more intensified 5′ protected fragments, 620 and 585 nucleotides long, that are protected by the 39°C RNA. These 5′ transcript ends were more precisely located by using a 5′ end-labelled 336 base long NarI-HinfI probe and the protection products were electrophoresed alongside the Maxam-Gilbert G and A+G sequencing ladder (fig. 13, lane C). The major products 336 and 283-285 nucleotides long are derived respectively from protection by readthrough transcripts and RNaseIII processed transcripts. The remaining protection products are much less abundant. The 211-213 nucleotide long minor fragment seen at both 30°C and 39°C situates the 5′ transcript end at approximately nucleotide 2858 and may be derived by endonucleolytic cleavage from the transcript that gave rise to the 420 nucleotide long protected fragment using the 3′ end-labelled EcoRI probe (see above). The other 3 minor products appear to be unique to XH56 RNA at 39°C. The protected fragments 225-228, 166-172 and 124-125 nucleotides long position 5′ transcript ends at nucleotides 2841-2844, 2896-2902 and 2944-2945 respectively (fig. 14). These 3 minor transcripts have no obvious corresponding 3′ end sites and therefore may be transcription initiation products at weak cryptic promoters rather than products of an endonucleolytic cleavage event.

The new but minor 5′ transcript ends at positions 2841, 2896 and 2944 were analyzed for potential -35 and -10 (Pribnow box) RNA polymerase recognition sequences.

	-35	spacing	-10	spacing	5′ end position
consensus	TTGACA	(16-19n)	TATAAT	(5-7n)	
	GCGACA	(15n)	GTAAAT	(8n)	2841
	GTGATA	(22n)	TTCCAT	(9n)	2896
	TTGCAC	(18n)	ACAGAT	(6n)	2944

The homology of these putative cryptic promoter sequences with the consensus sequences is either absent or very poor; even where homology exists, some of the spacings between the -35 and -10 regions, the -10 region and 5′ transcript end vary substantially from the optimal. The putative promoter with the Pribnow sequence centred at nucleotide 2830 has been previously reported. An and Friesen (1980b) have described an up mutation in the -35 recognition region of this Pribnow sequence. In their mutant plasmid the wild type hexamer GCGACA centred at nucleotide 2808, is changed to GTGACA which now differs from the consensus sequence TTGACA by only one nucleotide. This substitution results in an activation of this cryptic promoter during balanced growth.

Although increased activity of cryptic promoters in the L12-β intergenic region may occur, it would make a minor contribution to the increased levels of ββ′ transcripts during periods of stress that require greater synthesis of RNA polymerase subunits relative to ribosomal proteins.

In XH56 39°C RNA, along with the decrease in transcript termination at the attenuator, there was a decrease in RNaseIII-processed mRNA relative to read-through message, as suggested by the decrease in intensity of the relevant protection products relative to the 1080 nucleotide fragment (fig. 13, lanes A, B, C). The decreased amount of processed transcripts could be due to a reduction in RNaseIII processing or to an

acceleration in the decay of processed transcripts. A study of decay rates of XH56 transcripts from the L12-β intergenic region showed that read-through and RNaseIII processed mRNAs have similar degradation profiles at both permissive and semi-restrictive temperatures (section 4.2.2(3); fig. 16). These results suggest that the relative decrease in the amount of RNaseIII processed transcripts was likely due to a decrease in RNaseIII processing. This reduction in RNaseIII activity may simply be the result of a limiting cellular concentration of RNaseIII or it may indicate a regulatory function of the enzyme.

The role of RNaseIII processing in the function of ββ′ mRNA is not known. RNaseIII processing appears to have various effects on gene expression in E. coli. RNaseIII is involved in the maturation of rRNAs from 30S precursor RNA transcripts and in the regulation of several E. coli and bacteriophage genes (Takiff et al., 1989, and references therein). RNaseIII has been shown to decrease λ int gene expression by processing at the 3′ end of the int transcript (Gottesman et al.,1982). As well, RNaseIII has been shown to process mRNA at the 5′ end of genes to either increase or decrease gene expression. Processing may enhance expression by removing base pairing which blocks the ribosome binding site as in T7 mRNAs (Dunn and Studier, 1975) or reduce expression by initiating the decay of transcripts downstream as in the polynucleotide phosphorylase (pnp) mRNA (Portier et al., 1987). Portier and co-workers (1987) have suggested that RNaseIII processing of ββ′ mRNA may have the same function as in the pnp message. In contrast, Morgan and Hayward (1987) have argued that ββ′ mRNA stability is not significantly affected by RNaseIII processing.

Deletion of nucleotides 2729 to 2890, which removes the putative RNaseIII processing stem, results in mRNA that is inefficient as a template for translation (Dennis, 1984). This suggests that sequences near or in the processing site, not processing per se, is important

for efficient translation of ββ′ mRNA; these sequences may be important for opening up the rpoB ribosome binding site which could otherwise be sequestered in an alternative secondary structure incompatible with translation initiation (Meek and Hayward, 1986). Similarly, Altuvia et al. (1987) have proposed that for the λ cIII gene, RNaseIII stimulates cIII translation by binding to a site in the mRNA leader region; this binding may expose the cIII ribosome binding site and hence stimulate translation. This stimulation does not involve RNA processing. The precise roles of RNA secondary structure and RNaseIII-mediated processing in determining translation efficiency of ββ′ mRNA remain to be determined.

Nuclease S1 protection experiments using total RNA from strains NF536 and NF537 produced results different from that seen for E. coli XH56. The 3′ end-labelled 1080 bp EcoRI probe showed that, for NF536 (relA$^+$) at the semi-restrictive temperature (37°C), there are fewer transcripts entering the L12-β intergenic region as a result of the stringent response (fig. 13, lane E). However, of these transcripts, there was no detectable change in the attenuated transcript level (based on densitometric measurements) as was anticipated from the filter hybridization results and previous transcript analysis of XH56 RNA. It is possible that the nuclease S1 protection technique is not sensitive enough to detect small changes at low transcript levels. In contrast, for strain NF537 (relA) at 37°C, there are more transcripts entering the intergenic space compared to NF536 at 37°C, but a much smaller fraction of these extend through the attenuator and into the β gene (fig. 13, lane F).

Using the 5′ end-labelled 1080 bp EcoRI probe, NF537 (relA) RNA revealed little change in relative intensities of read-through and RNaseIII processed mRNAs after the temperature shift (fig. 13, lane H). However, in NF536 (relA$^+$) (lane G), there appears to be an increase in the level of RNaseIII processed message relative to the amount of read-through message; this increase may partially explain the slight enhancement in the β to L10-

L12 mRNA ratio. Based on densitometry data, the ratio of read-through to processed mRNA was calculated to be 1 : 0.47 at 30°C and 1 : 0.9 at 37°C. There was no change in the relative level of the corresponding upstream fragment generated by RNaseIII processing (325 nucleotide long fragment in fig. 13, lane E; densitometry data not shown). Again, the low transcript levels may diminish the sensitivity of S1 nuclease protection analysis. It is possible that the elevated level of RNaseIII processed transcripts was due to an increased stability of this downstream fragment. The greater stability of β mRNA, if real, may be the result of increased translation of the β message which may be required during the stringent response; translating ribosomes have been shown to protect some mRNAs against decay (Schneider et al., 1978). Unlike XH56 at 39°C, no new or more intensified 5′ transcript ends were detected in the L12-β intergenic region for NF536 and NF537 (fig. 13, lanes G and H).

During the stringent response, the levels of L11-L1 and L10-L12 transcripts were reduced as compared to the level of β transcripts (filter hybridization and S1 results of strain NF536, 35.5°C or 37°C). Stringent control of r-protein synthesis was previously thought to act at the level of transcription (Maher and Dennis, 1977). However, translational repression is now known to cause an accelerated decay of some r-protein mRNAs (Singer and Nomura, 1985; Fallon et al., 1979), including L11-L1 mRNA (Cole and Nomura, 1986a). Also, Cole and Nomura (1986b) have demonstrated that translational regulation of r-proteins L11 and L1 can account for the stringent response of rplKA. Therefore, it is possible that the reduced levels of L11-L1 and L10-L12 transcripts were due to a decrease in mRNA half-lives caused by feedback inhibition during the stringent response. The initial results presented in this work are not sufficient to verify or to disprove this conclusion; further investigation on the decay rates of transcripts from the rplKAJL-rpoBC gene cluster under stringent conditions is required.

3. TRANSCRIPT STABILITY

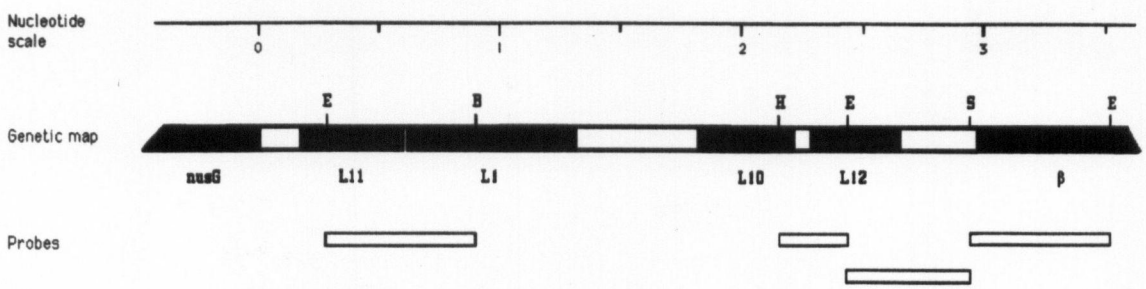
The steady state level of a mRNA reflects both its rate of synthesis and its rate of decay. Therefore, it is possible that the relative levels of rplKAJL and rpoBC transcripts, under the various restrictions examined above, were due to changes in mRNA degradation rates. As mentioned previously, it has been shown that for L11-L1 mRNA (Cole and Nomura, 1986a), S13-S11-S4 (alpha operon) mRNA (Singer and Nomura, 1985) and spc mRNA (Fallon et al., 1979), translational repression increases the decay rate of r-protein mRNA. However, the translational repression of the synthesis of r-protein S20 is not accompanied by an accelerated decay of its mRNA (Mackie, 1987).

In order to determine degradation rates of rplKAJL and rpoBC mRNAs, total cellular RNA from cultures of E. coli XH56 grown at 30°C and 39°C were analyzed by S1 nuclease protection according to von Gabain et al. (1983) with some modification. Transcription initiation was first blocked by the addition of rifampicin; total cellular RNA was then prepared from aliquots of cells taken at various time points. Levels of L11-L1, L10-L12 and β transcripts were probed respectively with 3′ end-labelled 617 bp EcoRI-BglII, 290 bp HindIII-EcoRI and 584 bp SalI-EcoRI restriction fragments (fig. 15). In addition, the 3′ end-labelled 496 bp EcoRI-SalI fragment was used to analyze amounts of attenuated and RNaseIII processed transcripts as well as uninterrupted transcripts in the L12-β intergenic region. Fragments protected from S1 nuclease activity were fractionated on a 8% polyacrylamide-urea gel; autoradiogram band intensities were quantified by densitometry as an estimate of transcript levels. For internal consistency, probes for L10-L12 and β transcripts were incubated in the same S1 nuclease reaction.

In general, the results show that the decay rates of the transcripts examined are

FIGURE 15. Nuclease S1 analysis of stability of rplKAJL-rpoBC transcripts in the mutant XH56.

Top : The nucleotide scale (Post et al., 1979) is shown in kilobases. The positions of genes are indicated. Relevant restriction sites and their nucleotide positions are : EcoRI (E: 280, 2444, 3524); BglII (B: 897); HindIII (H: 2154); SalI (S: 2940). The 3′ end-labelled restriction fragments used as probes were (i) the 617 bp EcoRI-BglII fragment to detect L11-L1 message, (ii) the 290 bp HindIII-EcoRI fragment to detect L10-L12 message, (iii) the 584 bp SalI-EcoRI fragment to detect β message and (iv) the 496 bp EcoRI-SalI fragment to detect transcript 3′ ends in the L12-β intergenic region as well as transcripts which read-through the region. Total cellular RNA was isolated 0, 2, 4, 6 and 8 min. after rifampicin treatment of E. coli XH56 at 30°C and 39°C. Five micrograms of RNA were used in each S1 nuclease protection assay. Autoradiograms of protection products are shown (middle and bottom). Probes for L10-L12 and β transcripts were used in the same nuclease S1 protection assay. Transcripts derived from RNaseIII processing (RNaseIII) and termination at the attenuator (ATT) in the L12-β intergenic region are indicated. Also see figure 16.


Nucleotide scale
0
1
2
3
Genetic map
E
B
H
E
S
E
nusG
L11
L1
L10
L12
β
Probes

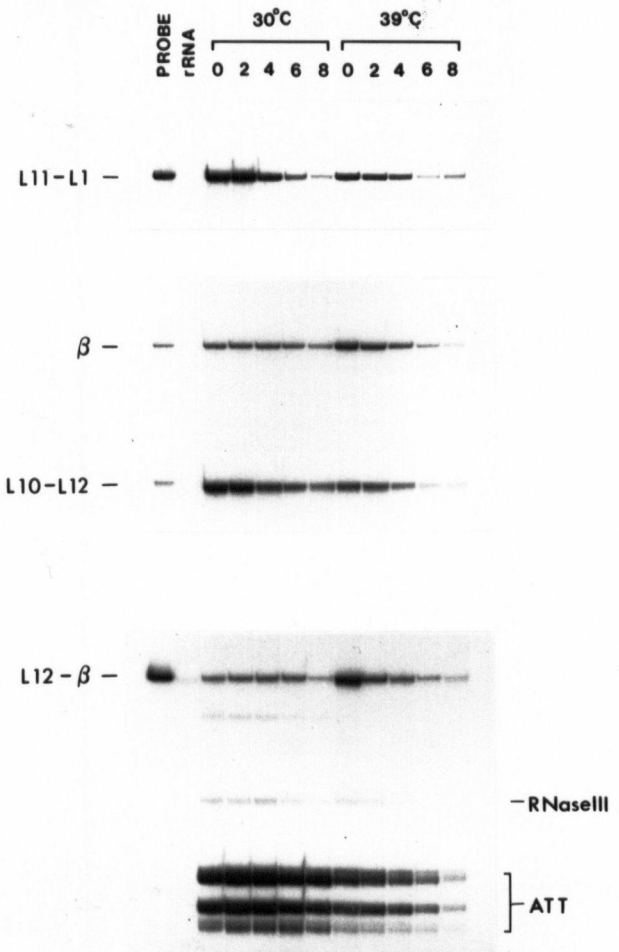

PROBE
rRNA
30°C
39°C
0 2 4 6 8 0 2 4 6 8
L11-L1
β
L10-L12
L12-β
RNaseIII
ATT

FIGURE 15

greater at 39°C than they are at 30°C (fig. 15 and 16). This may be a result of increased translational feedback inhibition, a result of the increase in temperature, or the consequence of an unknown event. Regardless of the cause, the increased decay rates cannot explain the elevated levels of L11-L1, L10-L12 and β transcripts under semi-restrictive conditions. This strongly suggests that the 1.5 to 1.6 fold increase in transcription of L11-L1 and L10-L12 at 39°C (table 4) was a consequence of increased transcription initiation at the P_{L11} and P_{L10} promoters, and not a result of changes in mRNA stability.

At the semi-restrictive temperature of 39°C, the level of β transcript increased five fold. If this increase was mainly due to a change in transcript stability, then the rate of degradation of β mRNA should decrease substantially at the semi-restrictive temperature. Again, the observed increase in rate of decay of β mRNA argues against this interpretation (fig. 15 and 16); transcript stabilities cannot account for the observed 5 fold increase in β mRNA as compared to the 1.5 to 1.6 fold increase in the upstream mRNAs. Consequently, increase in the level of β transcripts during these periods of stress is likely due to both increased transcription initiation at the P_{L11} and P_{L10} promoters and relaxation of transcription termination at the attenuator in the L12-β intergenic region. Modulation of attenuator activity has also been observed during rifampicin-mediated restriction of RNA polymerase activity (Morgan and Hayward, 1987). In vivo, rifampicin partially uncouples rpoBC from rplKAJL transcription by decreasing termination of mRNA at the attenuator. Alteration in mRNA stability and relaxation of post-transcriptional autogenous regulation were considered unlikely to be involved.

As noted previously, the decrease in RNaseIII processed transcripts relative to read-through message in XH56 RNA at the semi-restrictive temperature (fig. 13, lanes A, B, C; also section 4.2.2(2)) could be due to diminished RNaseIII activity in the L12-β intergenic

.**FIGURE 16.** Decay of rplKAJL-rpoBC transcripts in the mutant XH56.

Autoradiogram bands of nuclease S1 protected products (fig. 15) were scanned by a densitometer and relative band intensities were analyzed by computer. Logarithms of the band intensities (in arbitrary units) were plotted against time (in minutes). Degradation profiles of transcripts in the following intergenic regions were analyzed : (a) L11-L1, (b) L10-L12, (c) β and (d) L12-β. In all cases, the open circles and dashed lines represent readthrough message at 30°C; the filled circles and solid lines represent readthrough message at 39°C. In panel (d), the open triangles and dashed line represent RNaseIII processed transcripts at 30°C; the filled triangles and solid line represent RNaseIII processed transcripts at 39°C. Transcript stability studies were done in duplicate. The results shown here are from one experiment but are representative of the repeated findings.

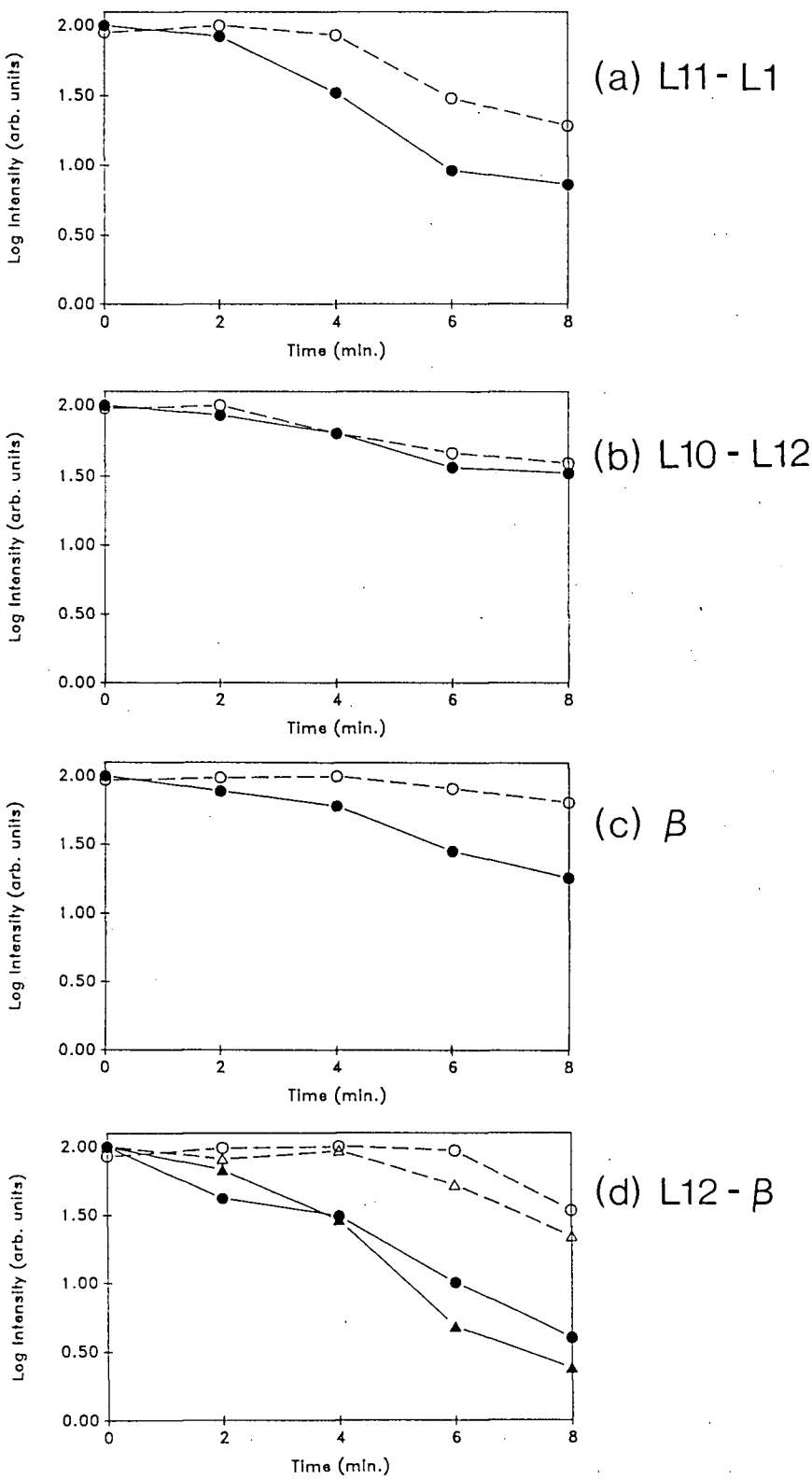

(a) L11 - L1
(b) L10 - L12
(c) β
(d) L12 - β
Log Intensity (arb. units)
Time (min.)
2.00
1.50
1.00
0.50
0.00
0
2
4
6
8

FIGURE 16

region or to intensified transcript degradation. The 3′ end-labelled 496 bp EcoRI-SalI fragment was used to analyze decay rates of RNaseIII processed transcripts (fig. 15). The results show that processed and read-through mRNAs have similar decay rates at 30°C and similar but accelerated decay rates at 39°C (fig. 16). This suggests that the relative decrease in processed transcripts at the semi-restrictive temperature was due to a reduction in RNaseIII processing.

4.2.3 TRANSLATIONAL POINT MUTANTS IN THE rplJL LEADER REGION

The translational feedback model has been proposed to explain the coordinated synthesis of r-proteins. Certain regulatory r-proteins are capable of binding to specific sequences on their own mRNA as well as related sequences on rRNA. A deficiency in rRNA results in protein binding to the mRNA and prevention of further translation. Repressor binding sites are usually located in the vicinity of the ribosome binding site and regulatory protein binding can directly block translation. Unique among r-protein operons, the L10-L12 repressor protein binding site (nucleotides 1523 -1579) is situated more than 100 nucleotides upstream of the L10 initiation codon (Johnsen et al., 1982). Point mutants and deletion mutants which are translationally defective have been isolated (Fiil et al., 1980; Friesen et al., 1983; Christensen et al., 1984); these mutations are localized some 80-200 bases upstream of the translation initiation site of rplJ.

Based on this evidence and on the identification of the repressor binding site, Christensen et al. (1984) have proposed a model for translation inhibition and RNA secondary structure of the L10-L12 mRNA leader sequence. The model involves two alternative configurations of this leader region (fig. 19). Normally, the portion of the leader between nucleotides 1505 and 1721 (the L10 start codon) exists in form I, in which the ribosome

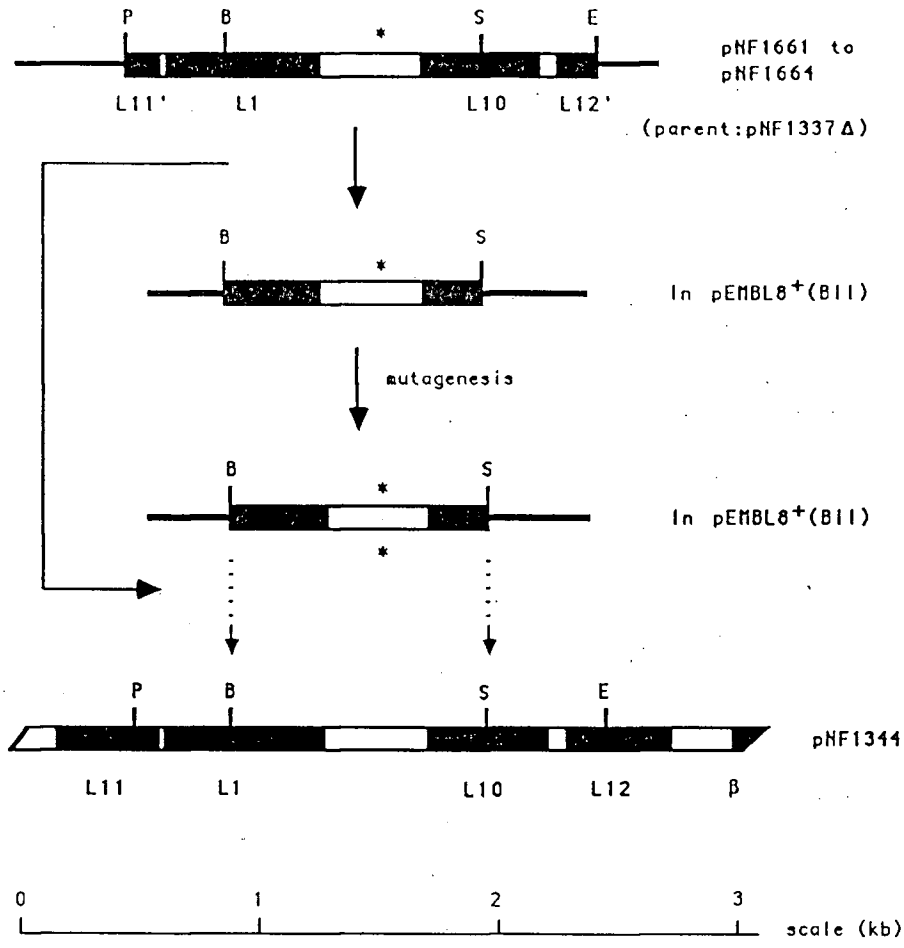
binding site is not base-paired and the transcript is thus open for translation. When there is an excess of L10-L12 , the repressor protein binds to its recognition structure, indicated in figure 19 as enclosed by the dotted box, and shifts the equilibrium to favour form II. In form II, the ribosome binding site is sequestered, and translation efficiency is reduced. In this way, binding of L10-L12 in the central region of the rplJL leader can exert its inhibitory effect, over a distance, on L10-L12 translation. Alternative mRNA structures have also been proposed as a way to regulate expression of drug resistance genes (Duvall et al., 1983; Mayford and Weisblum, 1985; Narayanan and Dubnau, 1987) and the λ cIII gene (Kornitzer et al., 1989).

While the assumption of secondary structure in the L10 leader sequence can explain many experimental observations, there was no evidence to confirm its existence. In order to test a segment of the secondary structures proposed by Christensen et al., Climie and Friesen (1987) have constructed a set of deletion, single base change, and double base change mutations in the leader region of a rplJ-lacZ fusion plasmid. The mutations are located in the stem loop A region (fig. 19) within the L10-L12 binding site. As well, the secondary structure in this region was examined by chemical modification. Results from these biological and chemical analyses define a region of secondary structure which is necessary for feedback regulation and which is in agreement with the predicted stem loop A structure. The results also indicate that the overall secondary structure, and not the primary sequence per se, is required for regulation.

In order to determine further the validity of other aspects of the proposed secondary structures in translational regulation of L10-L12 expression, regulatory mutants which were previously isolated (Fiil et al., 1980) were re-examined by site-specific mutagenesis. Designated as plasmids pNF1661 to pNF1666, these six original regulatory mutants were

derived from the parent plasmid pNF1337Δ. Plasmid pNF1337Δ is a pBR322 recombinant carrying the PstI(487)-EcoRI(2444) 1957 bp fragment which includes the 3′ end of the L11 gene, the entire L1 and L10 genes and the 5′ portion of the L12 gene (fig. 17). With this plasmid, expression of the plasmid-borne rplJ gene in the absence of an intact plasmid-borne rplL gene is detrimental to cell growth. However, the derivatives of pNF1337Δ which were isolated could overcome this detrimental effect. These regulatory mutants are transcriptionally normal but translationally defective. They were all determined to be point mutations in the central region of the rplJL leader, but outside of the L10-L12 binding site. According to the model of Christensen et al. (1984), each of these point mutations destabilizes form I and enhances form II; the result is concealment of the ribosome binding site and inhibition of L10-L12 translation. Four of these mutants, pNF1661 to pNF1664, were chosen for further study. The positions of these point mutations are indicated in figure 19, (a) to (d). One of these mutations (pNF1661) is situated in stem B while the remaining three all lie within stem C. The BglII-SmaI 1087 bp fragment containing the L10 leader sequence was subcloned from each of the mutants pNF1661 to pNF1664 into the BglII-SmaI site of pNF1344, replacing the analogous wild-type sequence. Plasmid pNF1344 contains the 3′ end of the nusG gene, the entire rplKAJL region and the 5′ end of the rpoB gene (fig. 17). Subcloning the mutant L10 leader region into pNF1344 permits the use of r-proteins L11 and L1 as internal standards in the translational assay of these mutants (see below). The mutant recombinants in pNF1344 are designated as plasmids pNF1661′ to pNF1664′.

To examine the putative RNA structures, a second point mutation thought to reestablish base-pairing in form I was introduced by site-directed mutagenesis into each of the plasmids pNF1661′ to pNF1664′ by using oligonucleotides oPD23 to oPD26, respectively. Theoretically, if the proposed structures, stem B and stem C, are the only structures involved in translation regulation, the double base changes should behave as pseudorevertants and



.FIGURE 17. Construction of plasmid derivatives carrying point mutations in the L10-L12 mRNA leader.

Top : single point mutants pNF1661 to pNF1664 were derived from the parent plasmid pNF1337Δ which is a pBR322 recombinant carrying the Pst(487)-EcoRI(2444) 2.0 kb fragment (Fiil et al., 1980). The asterisk indicates the general location of the point mutations. For one set of mutant derivatives, the 1.1 kb BglII(897)-SmaI(1984) fragment from pNF1661 to pNF1664 were cloned directly into the BglII and SmaI sites of pNF1344 to give pNF1661' to pNF1664'. In addition, this 1.1 kb fragment from the mutant plasmids or the wild type plasmid, pNF1344, were cloned into pEMBL8+(BII) for site-specific mutagenesis; only the mutant plasmids are illustrated. The mutagenized BglII-SmaI 1.1 kb fragments were then cloned into the BglII and SmaI sites of pNF1344 to yield pNF1344(25), pNF1663'(25) etc. Bottom : plasmid pNF1344 contains a 6.0 kb fragment from λrif^{d}18 DNA inserted into the PstI site of pBR322 (Hui et al., 1982); The relevant r-protein coding region of pNF1344 is illustrated. Pertinent restriction sites and their nucleotide positions are : PstI (P: 487); BglII (B: 897); SmaI (S: 1984); EcoRI (E: 2444) (Post et al., 1979). The nucleotide scale (in kb) is shown at the bottom.

FIGURE 18. Partial sequences of single base and double base point mutations in the L10-L12 mRNA leader region.

Plasmid pNF1344 is the wild type analog. * Plasmids pNF1661'(23) and pNF1662'(24) were not viable; their sequences shown here were derived from the pEMBL8$^+$(BII) recombinants carrying the corresponding 1.1 kb BglII-SmaI fragment used for mutagenesis. Arrows indicate the positions of the base mutations. For the nucleotide positions of these mutations, refer to table 2 and figure 19.

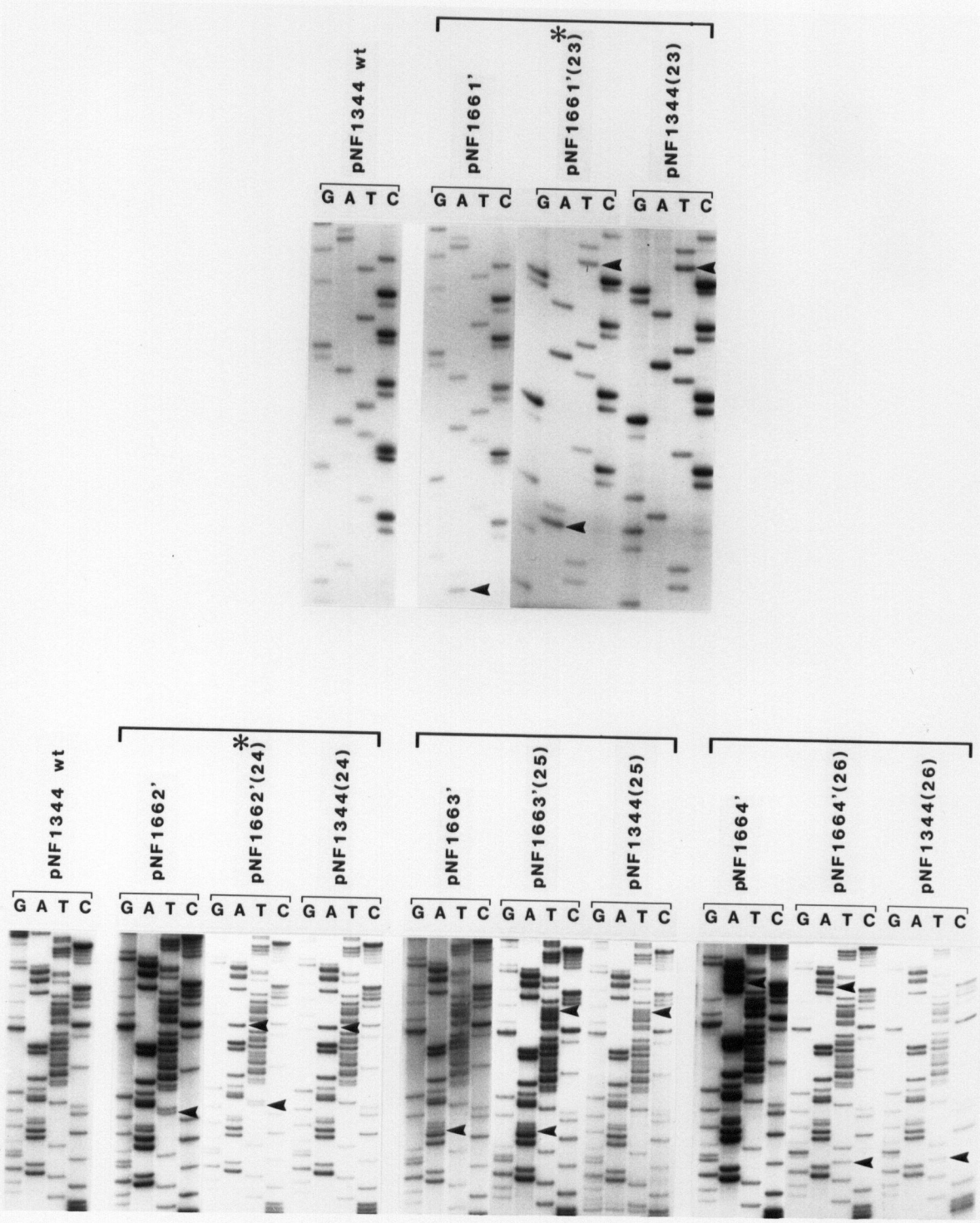

pNF1344 wt
pNF1662'
pNF1662'(24)*
pNF1344(24)
pNF1663'
pNF1663'(25)
pNF1344(25)
pNF1664'
pNF1664'(26)
pNF1344(26)
pNF1344 wt
pNF1661'
pNF1661'(23)*
pNF1344(23)
G A T C

FIGURE 18

restore L10-L12 translation. As a control, each oligonucleotide was also used to mutate the wild type plasmid pNF1344. The resultant mutants are designated as pNF1661'(23), pNF1344(23) etc. (table 2 and fig. 18). Plasmids pNF1661'(23) and pNF1662'(24) were not viable. Repeated attempts to clone these double mutant BglII-SmaI 1.1 kb fragments into the BglII and SmaI sites of pNF1344 failed. The integrity of the insert and vector DNA was tested in control experiments and the endonuclease restricted ends were found to be intact (i.e. able to be ligated and produce recombinants).

A prokaryotic, DNA-directed, in vitro translation system (Amersham) was used to assay the translation levels of plasmid-borne genes rplKAJL. Translation products were electrophoresed on a 15% SDS-polyacrylamide minigel using the discontinuous buffer system of Laemmli (1970) (fig. 20). Autoradiograms of the gels were analyzed by densitometry. Each plasmid was assayed in vitro twice, and each assay was analyzed by SDS-polyacrylamide gel electrophoresis and densitometry in duplicate.

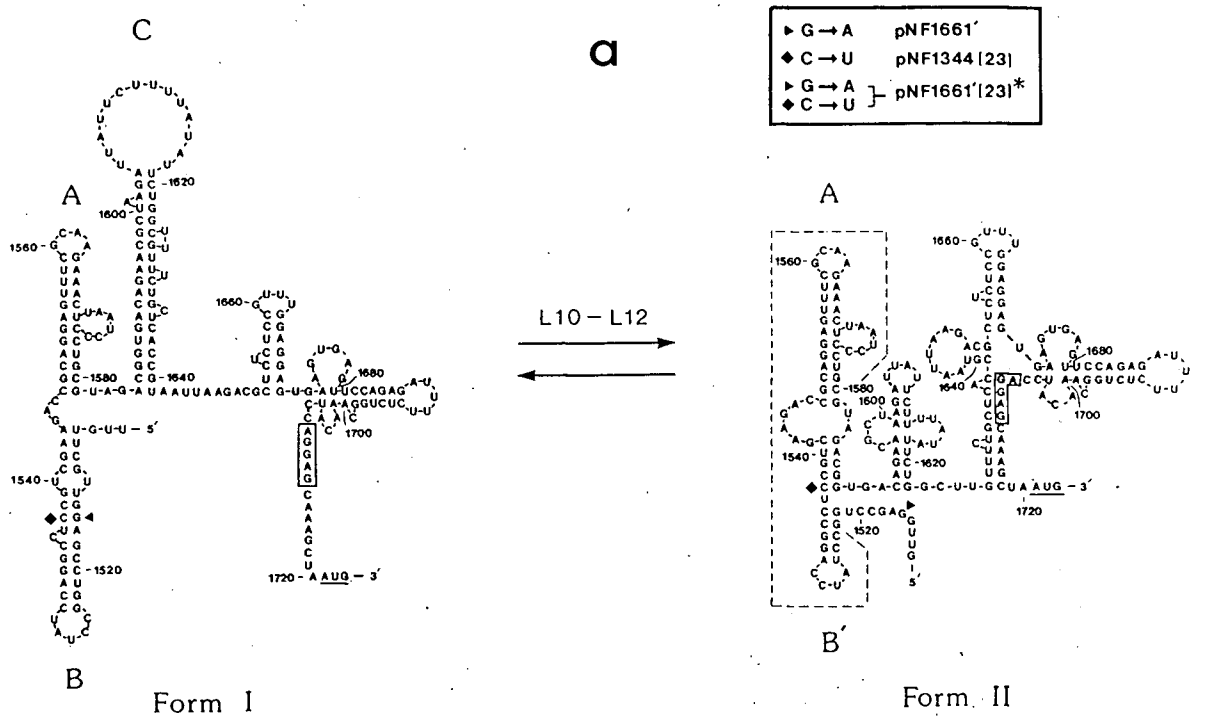
The results are summarized in table 5. After correcting for the number of methionine residues per protein, each densitometry measurement was first normalized to the arbitrary internal standard, r-protein L11 and second, normalized to each respective value calculated for the wild type plasmid pNF1344. RNase inhibitor was added to the in vitro translation system to insure that changes in protein levels were due to changes in translation and not due to changes in transcript stability.

Previous analysis of the in vivo expression of L10 from the original mutant plasmids pNF1661 to pNF1666 showed that translation is reduced by at least 20 fold relative to the expression of L10 from the wild type plasmid (Fiil et al., 1980). Also, these mutant plasmids demonstrated normal transcriptional activity but their L10-L12 mRNA was somewhat less

FIGURE 19. Positions of point mutations in the L10-L12 mRNA leader region; possible secondary structures as proposed by Christensen et al. (1984).

The point mutations carried by each of the mutant plasmids, depicted as the RNA transcripts, are indicated. The ribosome binding site is enclosed by a solid line. The dotted line in form II encloses the sequence that is protected in vitro by L10-L12 binding. Refer also to table 2 and figure 18.

* Bacterial transformants carrying plasmids pNF1661'(23) (in panel a) and pNF1662'(24) (in panel b) were not viable.


a
C
A
B
Form I
L10 – L12
▸ G → A pNF1661′
◆ C → U pNF1344 [23]
▸ G → A
◆ C → U pNF1661′[23]*
A
B′
Form II

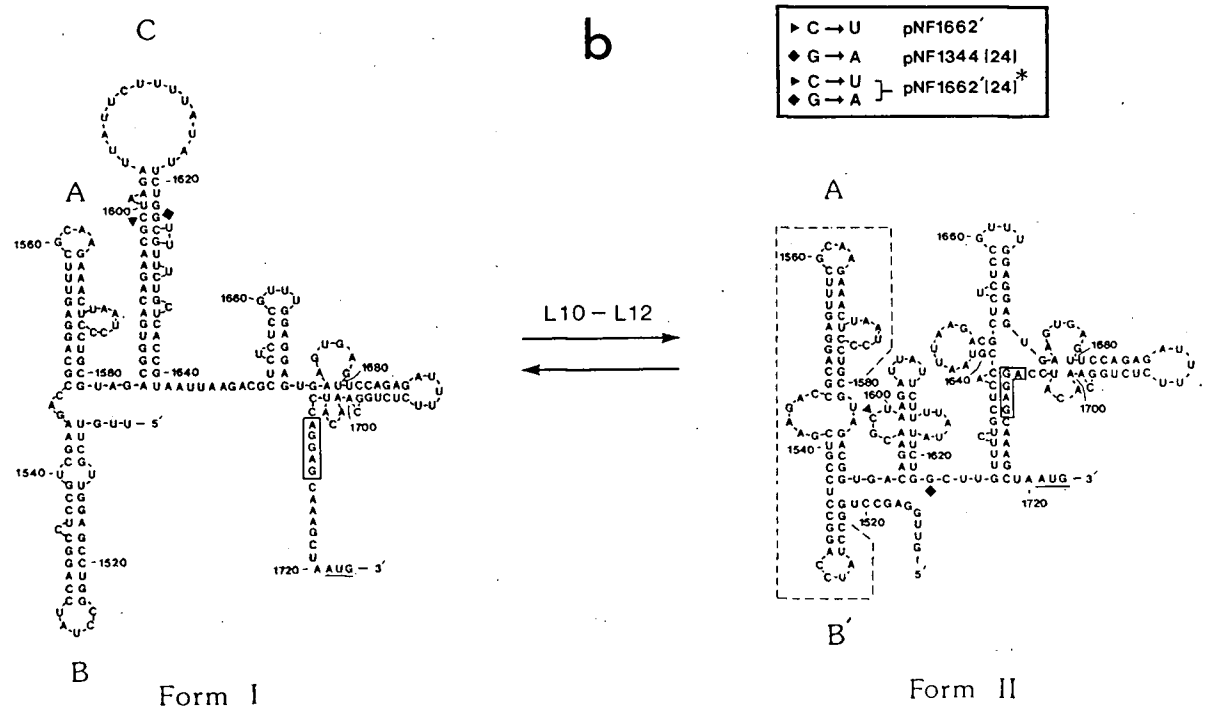
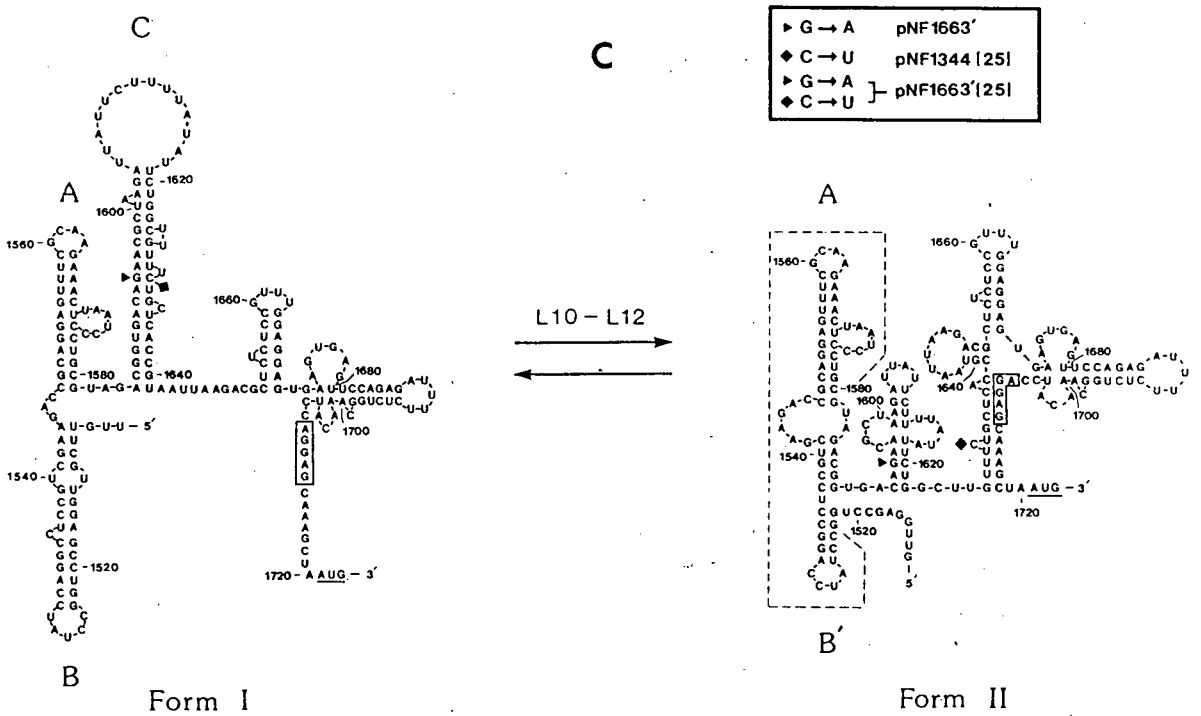

b
C
A
B
Form I
L10 – L12
▸ C → U pNF1662′
◆ G → A pNF1344 [24]
▸ C → U
◆ G → A pNF1662′[24]*
A
B′
Form II

FIGURE 19


C
A
B
Form I
c
L10 – L12
▸ G → A pNF1663′
◆ C → U pNF1344 [25]
▸ G → A
◆ C → U } pNF1663′[25]
A
B′
Form II

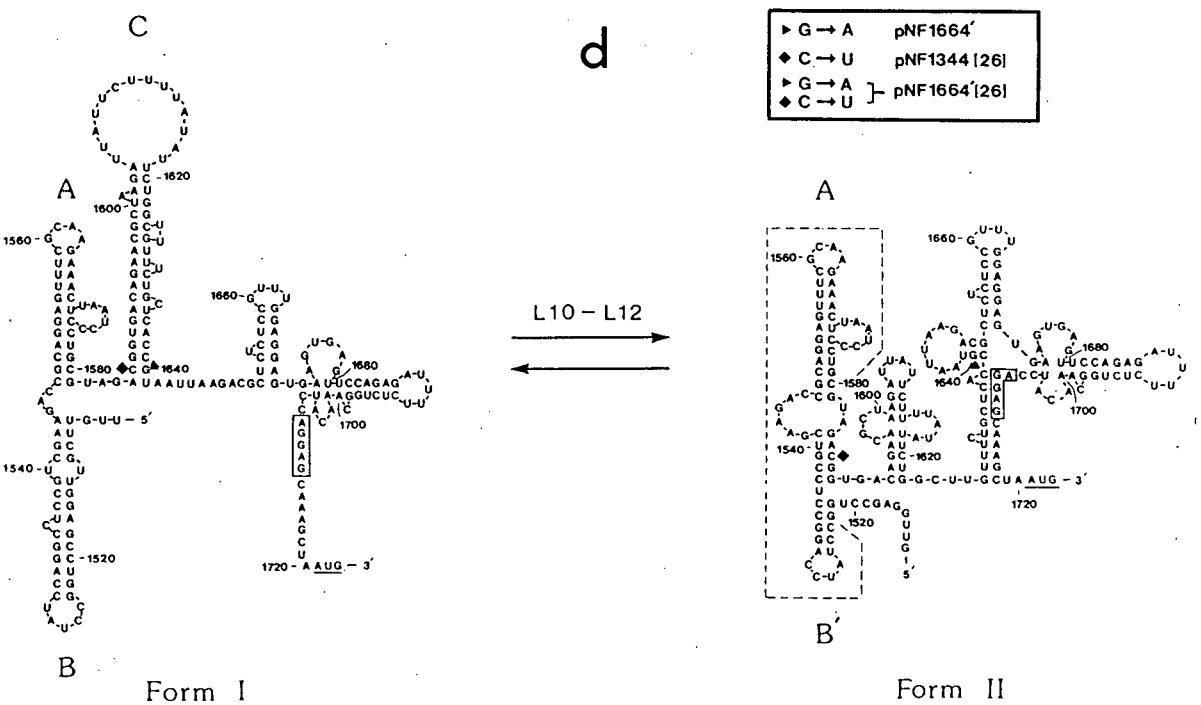

C
A
B
Form I
d
L10 – L12
▸ G → A pNF1664′
◆ C → U pNF1344 [26]
▸ G → A
◆ C → U } pNF1664′[26]
A
B′
Form II

FIGURE 19

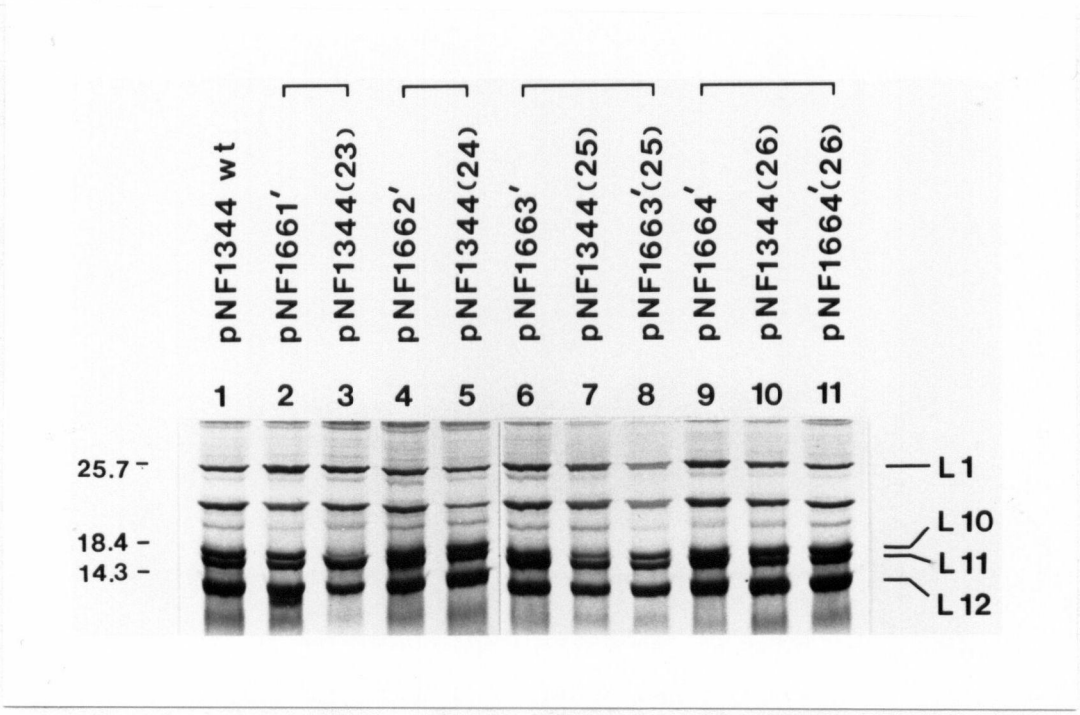


FIGURE 20. SDS-PAGE of in vitro translation products of mutant plasmids.

A prokaryotic in vitro translation system (Amersham) was used to assess the translational efficiency of the mutant plasmids. Translation products were electrophoresed on 15% SDS-polyacrylamide minigels using the discontinuous buffer system of Laemmli (1970). The protein molecular weight standards (in kilodaltons) are shown on the left. The positions of r-proteins L1, L10, L11 and L12 are indicated on the right. Refer also to table 5.

TABLE 5

IN VITRO TRANSLATION ASSAY OF MUTANT PLASMIDS : TRANSLATIONAL LEVELS OF rplKAJL

Plasmid	L11	L1	L10	L12	L12:L10*
pNF1344 (wt)	1.0	1.0	1.0	1.0	1.0
pNF1661′	1.0	1.3	0.7	0.8	1.1
pNF1344(23)	1.0	1.0	0.3	0.5	1.7
pNF1662′	1.0	1.1	0.7	0.7	1.0
pNF1344(24)	1.0	1.1	1.0	1.1	1.1
pNF1663′	1.0	1.0	0.6	0.6	1.0
pNF1344(25)	1.0	1.1	0.5	0.6	1.2
pNF1663′(25)	1.0	1.1	0.8	0.8	1.0
pNF1664′	1.0	1.0	0.7	0.7	1.0
pNF1344(26)	1.0	1.0	1.3	1.1	0.9
pNF1664′(26)	1.0	1.0	1.2	1.1	0.9

TABLE 5. In vitro translation assay of mutant plasmids : translational levels of rplKAJL.

In vitro translation products, derived from plasmids carrying point mutations in the L12 leader sequence, were fractionated on SDS-polyacrylamide minigels (fig. 20). Resultant autoradiograms were scanned by a densitometer (BioRad) and the band intensities quantitated (see Materials and Methods, section 2.9). Each densitometry measurement was (i) calculated as intensity (arbitrary units) per methionine residue, (ii) normalized to the value of the arbitrary internal standard, r-protein L11, and (iii) normalized to each respective value calculated for the wild type plasmid pNF1344.

* The ratio of L12:L10 was experimentally determined to be 4:1 for pNF1344; this value is set at 1.0 for the standard pNF1344.

stable than those in a non-plasmid control strain. The in vitro results of this work only partially reflect the previously reported in vivo results. Mutant plasmids pNF1661' to pNF1664' display levels of expression of r-proteins L10 and L12 that range from 60 to 80% of normal (i.e. relative to pNF1344; table 5). In vivo , the greater reduction in L10 r-protein synthesis may partially be due to the increased degradation of L10-L12 transcripts; the absence of loading ribosomes is associated with an increased mRNA decay rate (Schneider et al., 1978). RNase inhibitor added to the in vitro system prevents ribonuclease-mediated degradation of mRNAs and may indirectly cause the higher translation level of rplJL. Another difference is found in the plasmid construction; the original point mutants pNF1661 to pNF1664 contain only the P_{L10} promoter whereas the mutants of this study contain both P_{L11} and P_{L10} promoters. Also, the original mutants lack the attenuator stem-loop structure in the L12-β intergenic region, thus possibly destabilizing the L10-L12 transcripts in vivo. In addition, the two proposed RNA structures, forms I and II, may be further stabilized in vivo by cellular components which are absent or inactivated in vitro. Finally, the plasmid DNA concentration (100 μg/ml) used in the in vitro assay likely exceeded the saturating level for one particular plasmid; that is, in vivo, all other cellular mRNAs would be competing with rplKAJL transcripts for ribosomes and translational factors. This lack of competition in vitro may have had an effect on the translational efficiency of the plasmid-borne rplKAJL sequences. Some or all of these dissimilarities may explain the discrepancy between the in vitro and in vivo results.

Plasmid pNF1661' has a point mutation (G -> A) at position 1516 in the stem B structure. Synthesis of r-proteins L10 and L12 are 70-80% of normal. An attempt to introduce a second mutation (C -> U) at position 1537 to restore the putative stem B structure, and hence possibly to restore L10-L12 translation to normal levels, failed to yield viable recombinants. However, the singular C -> U mutation at nucleotide 1537

(pNF1344(23)) caused a reduction in L10 synthesis to 30% of normal and L12 synthesis to 50% of normal. Also, the molar ratio of L12 to L10, usually 4:1, increased 1.7 fold. Nucleotide position 1537 is located within the sequence protected by L10-L12 binding (fig. 19a) (Johnsen et al., 1982) and appears to be involved in the regulation of L10 and L12 synthesis. While the C -> U mutation apparently favours the closed form and inhibits translation, it also appears to disrupt the normal 4:1 stoichiometric synthesis of r-protein L12, perhaps via some complex tertiary interactions which have not been considered. The lethality of the double mutant also implies that other secondary or higher order RNA interactions may be involved in translational regulation of L10-L12 expression.

The remaining point mutants all lie within the putative stem C structure of form I (fig. 19b, c, d). Plasmids pNF1662' (C -> U mutation at nucleotide 1599) and pNF1344(24) (G -> A mutation at nucleotide 1623) display respectively, 70% and normal translation levels of r-proteins L10 and L12. Again, the double mutant was not viable. As predicted by the model, the C -> U mutation at position 1599 destabilizes the open form (form I) and favours the closed form (form II) (fig. 19b). However, the G -> A mutation alone (position 1623), which was expected to destabilize the stem C structure of form I and thus inhibit translation, does not appear to have an effect on the translation of rplJL. As before, this evidence as well as the lethal nature of the double mutant indicate that alternate secondary or tertiary RNA interactions may be involved.

Midway in the proposed stem C structure are the G -> A mutation (nucleotide 1594) of pNF1663' and across the stem structure, the C -> U mutation (nucleotide 1631) of pNF1344(25). Both single point mutations resulted in a decrease in r-protein L10 and L12 expression to approximately 50-60% of normal. In the double mutant pNF1663'(25), the synthesis of both L10 and L12 r-proteins were restored to 80% of normal levels. This

partial recovery of translational efficiency provides some evidence for the existence of the stem structure, at least in the general vicinity of nucleotides 1594 and 1631. It appears that the overall secondary structure, and not the nucleotide sequence, is more important in regulation. However, the incomplete translational recovery of the double mutant may indicate some significance in the sequence or involvement of these nucleotides in other secondary interactions. Alternatively, the decreased translation levels of the pseudorevertant may be a result of the destabilization of form I due to the GC (wild type) -> AT change.

The last set of mutants are situated at the base of stem C (fig. 19d). The G -> A mutation (position 1640) of pNF1664' caused a decrease in L10 and L12 r-protein synthesis to approximately 70% of wild type levels. However, the C -> U mutation at position 1586 in plasmid pNF1344(26) resulted in normal or slightly above normal expression of r-proteins L10 and L12. In this case, the mutant nucleotide U can still conceivably base pair with the opposite G residue in the putative stem structures in both forms I and II; thus, normal L10 and L12 production may be the net result. In the double mutant pNF1664'(26), synthesis levels of r-proteins L10 and L12 are similar to that seen for pNF1344(26). It is difficult to determine if the increase in L10 and L12 synthesis in the double mutant as compared to that in pNF1664' was a result of the second mutation reestablishing stem C in the open form since the second mutation alone (pNF1344(26)) can achieve the same level of L10-L12 expression.

These first attempts at characterizing the unusual rplKAJL mRNA leader region have provided inconclusive evidence for portions of the secondary structures proposed by Christensen et al. (1984); the translation assay was subject to shortcomings of an in vitro system. These initial results suggest that additional secondary or higher order RNA interactions may be involved in the translational regulation of rplKAJL. Certain RNAs are

thought to fold into unusual structures. For example, pseudoknot structures, in which the loop of a hairpin is base paired to sequences upstream or downstream of the hairpin, have been proposed for several RNAs including the alpha operon mRNA (C.K. Tang and D.E. Draper, 1989), 16S rRNA (Noller, 1984) and T4 gene 32 mRNA (McPheeters et al., 1988).

Procedures for the prediction of possible higher order structures are not yet available. The current methods used to estimate the stability of an RNA structure relate only to the secondary structure and only consider base pairs located in stem structures; demonstration of the strong influence of loop sequences on hairpin stability in some cases (Tuerk et al., 1988) reveals the limitations of these methods.

In conclusion, the nucleotide sequences of the secE and nusG genes have been determined. This completes the nucleotide sequence of the entire rif region at 90 minutes on the E. coli chromosome. The two genes are cotranscribed, with transcription initiation occurring at the P_{EG} promoter and termination occurring at the Rho-independent terminator in the vicinity of the P_{L11} promoter. The majority of transcripts are processed in the 5′ untranslated leader region by RNaseIII and possibly also by a second unidentified nuclease. These sites may be regulatory features involved in the expression of secE and nusG.

The SecE and NusG proteins are involved in seemingly unrelated cellular processes. However, the juxtaposition and co-transcription of a protein export factor and a transcription factor raise questions concerning a possible functional connection between these two processes. The regulation of this cistron may prove to be intriguing, in light of the present knowledge of regulation of other nus and sec genes. For instance, SecA protein expression is controlled by autogenous translational repression (Schmidt and Oliver, 1989). On the other hand, the expression of transcription termination factors Rho and NusA is known to be regulated by autogenous attenuation of transcription (Matsumoto et al., 1986; Plumbridge et al.,1985). It would be instructive to resolve the regulation of secE and nusG gene expression. The effect of SecE or NusG protein on transcription and translation of the chromosome-encoded secE-nusG operon can be examined in vivo by using an inducible expression vector. Also, sequences in the secE-nusG mRNA leader region which may be involved in regulation can be identified by the use of point mutants and deletion mutants of this region; effects of the mutations on gene expression can be assessed by using a reporter gene, such as lacZ.

Transcripts from the rplKAJL-rpoBC gene cluster were quantified and their ends mapped. The most abundant transcript was the 2600 nucleotide tetracistronic L11-L1-L10-L12 mRNA initiated at the P_{L11} promoter and terminated at the transcription attenuator in the L12-β intergenic space. Less abundant 1300 nucleotide L11-L1 and L10-L12 bicistronic transcripts were also observed. The 3′ ends of the L11-L1 transcripts were heterogeneous; most of the ends were localized to three sites within a 110 bp region in the L1-L10 intergenic space. This intergenic space also encodes the P_{L10} promoter and the mRNA binding site for the L10 translational control protein. Two 5′ ends were observed for L10-L12 bicistronic mRNA, one at the P_{L10} promoter and the other 150 nucleotides further downstream in a region in which no promoter activity has been detected; this second 5′ end may be generated by processing of the transcripts initiated at the P_{L10} promoter. No transcript initiation in the L10-L12 intergenic space was detected. About 80% of the transcripts reading through the L12 gene were terminated in the vicinity of the transcription attenuator that is responsible for the reduction in the expression of the downstream RNA polymerase genes. Transcripts reading through the attenuator were partially processed by RNaseIII. No other major 5′ ends were observed in the L12-β intergenic space. During restriction of RNA polymerase activity or during the stringent response, the normal balance between transcription of ribosomal protein genes and RNA polymerase genes is partially uncoupled. In the first situation, this transcriptional disruption results almost exclusively from modulation in the frequency of (i) initiation at P_{L11} and P_{L10} promoters and (ii) termination and antitermination at the attenuator. However, in the second situation, the results are not conclusive; during the stringent response, changes in the extremely reduced levels of rplKAJL transcripts are difficult to assess by the S1 nuclease protection method employed here. Further investigation is required to resolve this problem.

Finally, preliminary attempts at characterizing the unusual rplKAJL transcript leader region have given only inconclusive evidence for the secondary structures of this region as proposed by Christensen et al. (1984). The results, derived from in vitro translation assays of point mutants, suggest that alternative secondary or higher order RNA interactions may be involved in the translational regulation of rplKAJL. Structure mapping experiments of the L10 leader by chemical and enzymatic methods, in the presence and absence of L10 repressor protein, may help to define regions of secondary structure. In vivo studies of this region by using a rplJ-lacZ fusion plasmid should provide additional useful information. The fusion plasmid may also circumvent the problem of lethality of some of these double base mutants. Experimental verification of the proposed secondary structures awaits further investigation.

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