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STABILITY OF mRNAs PRODUCED FROM THE *ilvGMEDA* GENE CLUSTER OF *ESCHERICHIA COLI* K-12

by

CHENYI ZHOU

A dissertation submitted to Graduate Faculty in Biochemistry in partial fulfillment of the requirement of the degree of Doctor of Philosophy,

The City University of New York

1998

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Date	Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

STABILITY OF mRNAs PRODUCED FROM THE *ilvGMEDA* GENE CLUSTER OF *ESCHERICHIA COLI* K-12

by

CHENYI ZHOU

Adviser: Professor David H. Calhoun

Several lines of evidence revealed the presence of post-transcriptional processing pathways for the *ilvGMEDA*, *ilvEDA*, *ilvDA*, and *ilvE* transcripts produced by the *ilvGMEDA* gene cluster of *Escherichia coli* K-12. First, a mutation in endoribonuclease RNase E (*rne-3071*^{ts}) increased the stability of the *ilvGMEDA* and *ilvE* transcripts, and a mutation in endoribonuclease RNase P (*rnpA49*^{ts}) increased the stability of the *ilvEDA* and *ilvE* transcripts. Second, two min after inhibition of transcription initiation by rifampicin addition to a strain with an RNase E mutation, there was a marked decrease in the *ilvGMEDA* and *ilvEDA* transcripts that was simultaneous with a marked increase in the *ilvE* transcript, and there was also a simultaneous increase in partially processed intermediates present just above the *ilvE* mRNA. Third, the higher levels of the full length *ilvGMEDA* transcript in the Val^{R} compared to Val^{S} strains resulted in higher levels of the smaller *ilvEDA*, *ilvE*, and *ilvDA* transcripts in Val^{R} strains compared to the Val^{S} strain. And fourth, higher levels of the full length *ilvGMEDA* transcript in the Val^{S} strain

due to the presence of a Rho mutation also resulted in an increase in the smaller *ilvEDA*, *ilvE*, and *ilvDA* transcripts. These results indicated a processing of *ilvGMEDA* to *ilvEDA*, and *ilvEDA* to *ilvE* and *ilvDA*, with some fraction of the *ilvEDA* transcript formed directly from the constitutive internal *ilvEp* promoter. In the presence of a *rho-115* mutation, about half of the *ilvGMEDA* transcripts were degraded rather than processed to the smaller mRNAs. This result is related to the observation of Sozhamannan and Stitt (1997) that bulk mRNA levels decreased in the presence of some, but not all, *rho* mutations. This is the first example of a specific transcript that is less stable in the presence of a *rho* mutation. The participation of RNase E in the processing of the *ilvGMEDA* transcript was examined in more detail. RNase E was purified from an *E. coli* strain containing the gene on a high level expression plasmid. An RNA segment of 1,136 nt containing the *ilvE* - *ilvD* intercistronic region was synthesized in vitro. Treatment of this RNA segment with RNase E revealed the presence of several major and minor cleavage sites corresponding to RNase E. This in vitro result supports the interpretation of the in vivo experiments that the *ilvE* transcript is derived in part from the *ilvEDA* transcript by RNase E processing.

ACKNOWLEDGMENTS

The author wishes to express her gratitude to professor David H. Calhoun for his continuous guidance, patience and contribution in directing this endeavor. She would also like to thank Charlotte Russell, Sharon Cosloy, Rivka Rudner, Dani Mcbeth for serving on her thesis committee.

She would like to thank her family, particularly her parents for moral support and encouragement during the course of this work. Finally, thanks goes to her co-worker Libo Zhou and Yan-Yan Neal for friendship and kind help.

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ABBREVIATIONS

AHS I	Acetohydroxy acid synthase I
AHS II	Acetohydroxy acid synthase II
AHS III	Acetohydroxy acid synthase III
bp	Base pair
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
IHF	Integration host factor
kb	Kilobase(s)
kDa	Kilodalton(s)
MOPs	3-(N-morpholino-) propanesulfonic acid
NaAc	Sodium acetate
nt	Nucleotide
PCR	Polymerase chain reaction
pfu	Plaque forming units
PNPase	Polynucleotide phosphorylase (*pnp*)
Rho	Transcriptional termination protein
rif	Rifampicin
RNase E	Endoribonuclease E (*rne*)
RNase II	Exoribinuclease II (*rnb*)

RNase III	Endoribonuclease III (*rnc*)
RNase P	Endoribounuclease P (*rnpA*)
rRNA	Ribosomal RNA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
ts	Temperature sensitive
Val^R	Valine resistant
Val^S	Valine sensitive

CHAPTER 1

INTRODUCTION

The biosynthesis of isoleucine and valine in *Escherichia coli* K-12 occurs by parallel pathways and involves common precursors and shared bifunctional enzymes (Fig. 1-1, Baez et al., 1979). The genes encoding each of these enzymes are located at different positions on the chromosome. The *ilvHI* genes are located at 2 min, close to the leucine gene. The *ilvBN* genes are located at 82 min. The *ilvGMEDA* gene cluster is located at 85 min on the *E. coli* chromosome (Bachman and Low, 1980). In addition, the cluster also contains the *ilvC* gene and the positive control element that is needed for the *ilvC* expression, *ilvY* (Fig. 1-1, Umbarger, 1987). The regulation of gene expression and the allosteric regulation of enzyme activity in this pathway are complex, probably because the cells must coordinate the synthesis of each end product (Harms and Umbarger, 1991). The expression of the entire gene cluster is regulated by attenuation, internal promoters and terminators, translational coupling, binding of regulatory proteins, and possibly autoregulation by the *ilv*A gene product, threonine deaminase.

Gene-enzyme relationships in branched-chain amino acid biosynthesis pathway

Nutritional analysis of mutants of *E. coli* and *Salmonella typhimurium* found that many required both isoleucine and valine for growth. Also the growth of one of the commonly used strains of *E. coli*, strain K-12, is inhibited by valine and this inhibition is reversed by isoleucine (Umbarger, 1987). This effect was explained by the later finding that the biosynthetic pathways to valine and isoleucine are interrelated (Fig. 1-1).

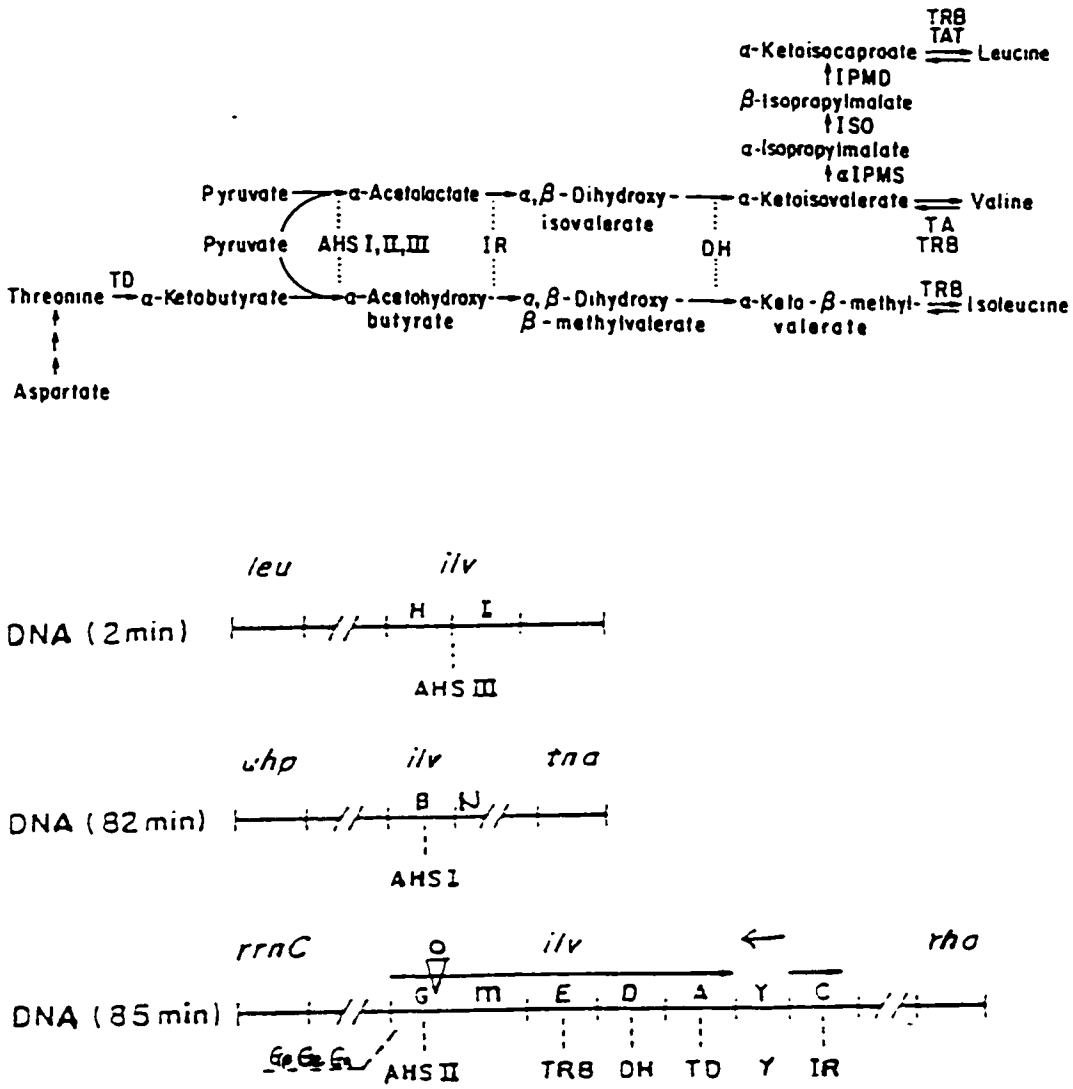


Fig. 1-1. Biosynthetic pathways of isoleucine and valine and the locations of the various *ilv* genes. The corresponding structural genes are indicated as follows: TD (*ilvA*), threonine deaminase; AHS I (*ilvBN*) and AHS III (*ilvIH*), valine sensitive acetohydroxy acid synthases; AHS II (*ilvGM*), valine resistant acetohydroxy acid synthase; IR (*ilvC*), acetohydroxy acid isomero reductase; DH (*ilvD*), dihydroxy acid dehydrase; TrB (*ilvE*), transaminase B; v (*ilvY*) is a positive control element required for substrate induction of the *ilvC* gene. The *ilvG* and *ilvM* gene are inactive in *E. coli* K-12, owing to a polar lesion in the *ilvO* region of the *ilvG* gene. Mutations in *ilvO* result in *ilvGM* function and increased downstream expression. Gp, Ge and Ga are the promoter, leader region, and attenuator, respectively, of the *ilvGMEDA* gene cluster (Baez *et al.*, 1979; Umbarger, 1987). The arrows above the genes at 85 min indicate the mRNAs produced and the the direction of transcription.

Four of the five steps in isoleucine formation are catalyzed by enzymes catalyzing the corresponding steps in valine formation. The sensitivity of the K-12 strain to valine is now understood in terms of enzymology and in terms of a kind of mutation that had occurred in a progenitor of the K-12 strain. As it is shown in the parallel pathways (Fig. 1-1), the first common feature in the two pathways is a reaction in which an acetyl group generated by decarboxylation of pyruvate is condensed with a second pyruvate or with α-ketobutyrate to lead to valine or isoleucine as the final products. For isoleucine, another specific step is required since α-ketobutyrate, required for acetohydroxybutyrate formation, is not an intermediate in the central metabolic route. For this function, both *E. coli* and *S. typhimurium* use threonine deaminase, the product of the *ilvA* gene.

The enzymes forming acetolactate and acetohydroxybutyrate present a complex picture. Three different isozymes of acetohydroxy acid synthase have been found *in S. typhimurium* and *E. coli* (Blatt and Umbarger, 1972; DeFelice et al., 1974; O'Neil and Freundlick, 1972; Shaw et al., 1980). The activities of acetohydroxy acid synthase I (AHS I, the product of the *ilvBN*) and acetohydroxy acid synthase III (AHS III, the product of the *ilvHI*) are inhibited allosterically by valine. Thus, they exhibit the pattern of end product sensitivity found so often among initial enzymes in biosynthesis pathways. The activity of the third isozyme, acetohydroxy acid synthase II (AHS II, the product of *ilvGM*), is not sensitive to inhibition by valine and this probably accounts for the fact that many bacteria overproduce valine and excrete it into the medium, particularly late in the growth cycle. AHS II, purified from *S. typhimurium*, consists of two kinds of subunits, one large [59.3 kilodaltons (kDa)] and one small (9.7 kDa; Schloss

et al., 1985). The gene for the larger subunit had been identified as *ilvG* gene by analysis of mutants and by nucleotide (nt) sequence determination (Favre et al., 1976; Lawther et al., 1981; Smith et al., 1976). The *ilvM* gene immediately downstream of the *ilvG* gene is shown to be the small subunit (Schloss et al., 1985). The *ilvM* gene product is essential for AHS II activity. No in vitro activity could be measured if plasmid carrying the *ilvG* gene alone.

The genes for AHS I were located on the opposite side of the chromosomal replication origin from the *ilv* gene cluster (Newman and Levinthal, 1979). The purification of AHS I by Eoyang and Silverman (1984) revealed the presence of the *ilvB* gene product (60 kDa) and a smaller protein (9.5 kDa). DNA sequence determination of the *ilvB* gene region has revealed an open reading frame downstream of the *ilvB* which could specify a subunit of 11.1 kDa (Friden et al., 1985). This gene has been designed *ilvN* and, with *ilvB*, constitutes a single operon. When an AHS I mutant strain of *E. coli* was transformed with a high-copy-number plasmid carrying the *ilvBN* operon from which most of the *ilvN* gene has been deleted, the activity of AHS I was reduced by about 90% and was quite resistant to valine inhibition. Thus, *ilvN* is important for AHS I activity and inhibition by valine.

The analysis of mutants led DeFelice (1974) to the discovery of AHS III in *E. coli*. The mutants that resulted in the loss of sensitivity of this isozyme to valine were identified as *ilvH* mutations, and subsequently mutations that resulted in loss of the activity itself were designated *ilvI* mutations. Nucleotide sequence determination has demonstrated an operon consisting of *ilvI* gene accounting for a 61.8 kDa subunit and the

ilvH gene accounting for a 17.5 kDa subunit (Squires et al., 1981). Furthermore, some homology between the *ilvH* and *ilvM* genes and between the *ilvI* and *ilvG* genes have been demonstrated by Squires et al. (1983). Very low levels of acetolacetate forming activity and insensitivity to valine was shown in clones containing an intact *ilvI* gene but only an incomplete *ilvH* gene (Squires et al., 1981). Thus, the small subunits of the three isozymes are important for maximal catalytic activity of the large subunit and contribute importantly to the regulation of the activity of the two valine-sensitive (Val^S) isozymes. The next step in each pathway is the conversion of acetolacetate and acetohydroxybutyrate to dihydroxyacids by the isomeroreductase, specified by the *ilvC* gene. The *ilvC* gene product functions as a tetramer of 53 kDa subunits. Mg2+ is required, but no prosthetic group is required. The dihydroxy acid precursors to the α-keto acids are converted by dihydroxyacid dehydrase, the product of the *ilvD* gene which is a dimer of 66 kDa. The final step in the biosynthesis of both isoleucine and valine is a transamination between the two α-keto acids and glutamate. Transaminase B is required for isoleucine biosynthesis. It is the product of the *ilvE* gene. The *ilvE* gene has been cloned and shown to specify a protein with a molecular weight of 23,960, including the N-terminal methionine, which is absent from the native protein (Kuramitsu et al., 1985). The enzyme recently was crystallized and a preliminary report of the molecular structure has been published (Kamitori et al., 1989). The crystal contained three subunits per asymmetric unit rather than the six found to make up the active enzyme (Adams et al., 1979; Lee-Peng et al., 1979; Lipscomb et al., 1971). The second route for valine formation from α-ketoisovalerate is via transaminase C (Fig. 1-1) which is the product of

the *uvt* gene (Szentirmai et al., 1968). Transaminase C accounts for the fact that *ilvE* mutants, lacking transaminase B, do not exhibit an absolute auxotrophy for valine, in contrast to the absolute requirement for isoleucine.

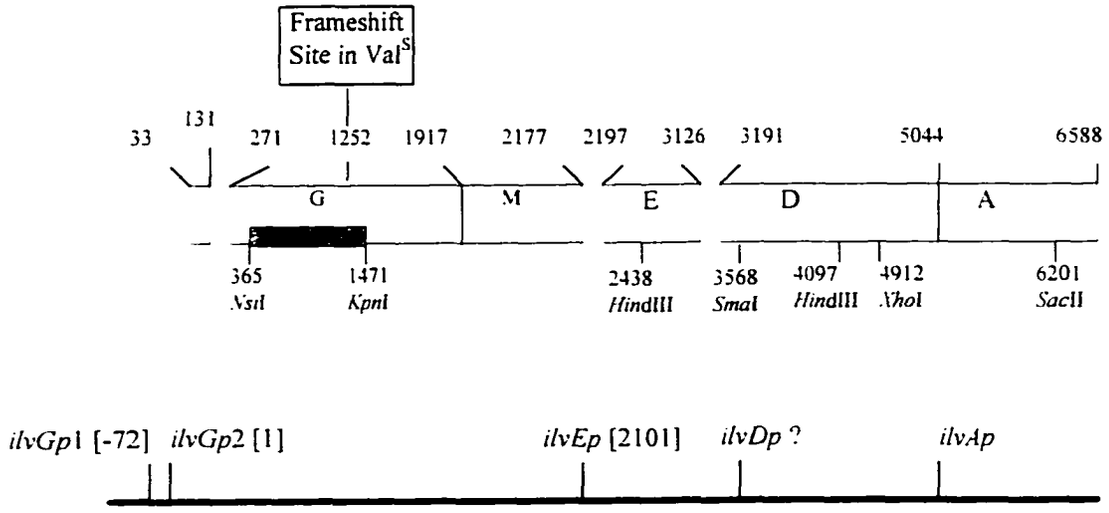
The Val^S and Val^R growth phenotype

It was reported by Tatum (1946) that the growth of *E. coli* K-12 is inhibited by valine and this inhibition is reversed by isoleucine. This phenomenon defines the valine sensitive (Val^S) growth phenotype.

Ramakrishnan and Adelberg (1965) characterized a class of mutations (Fig. 1-1) which they designed "*ilvO*" that conferred resistance to growth inhibition by valine (valine insensitive growth phenotype; Val^R). These *cis*-dominant mutants simultaneously lead to expression of the *ilvG* and derepression of the *ilvEDA*. The "*ilvO*" mutants produce a physically distinct isozyme of AHS (designated AHS II) whose activity is resistant to allosteric inhibition by valine (Gray et al., 1981; Lawther et al., 1981; 1982). Thus, this explains their valine resistant (Val^R) growth phenotype in *E. coli* K-12 mutants (Baez et al., 1979; Blatt et al., 1972; Defelice and Levinthal, 1977; Favre et al., 1976).

In the wild - type *E. coli* K-12 there is a polar frameshift site near the middle of the *ilvG* gene of the *ilvGMEDA* cluster (Figs. 1-2; 1-3; 1-4; Lawther et al., 1981; 1982). An inactive *ilvG* product is formed with no AHS II activity. The premature translation

A. Genes, Frameshift Site, Restriction Enzyme Sites, Promoter Regions and *ilvG* Internal Deletion



B. Transcripts and Rho Dependent Termination Sites in Val^S Strains

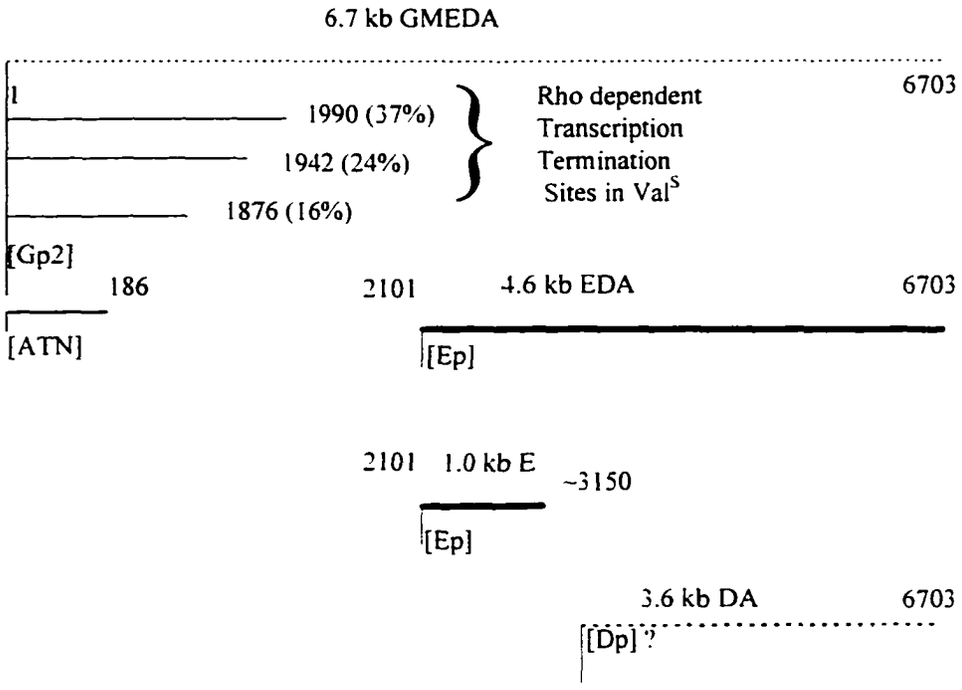


Fig. 1-2. The *ilvGMEDA* gene cluster, promoter regions and transcripts **(A)** Genes, locations of the coding regions for each polypeptide, frameshift site in the Val^S strains, locations of selected restriction enzyme sites, promoter regions and the *Nsi*I to *Kpn*I deletion (shaded bar) introduced in *ilvG* to remove the frameshift site in the construction of plasmid pCC17. These genes code for the large and small subunits of AHS II (*ilvGM*), transaminase B (*ilvE*), dihydroxyacid dehydrase (*ilvD*), and threonine deaminase (*ilvA*). The AHS II isozyme is produced in the Val^R strains, but not in the Val^S strains. The stop codons overlap the start codons for the *ilvGM* and *ilvDA* pairs, while there are untranslated segments of 20 and 65 bases between the *ilvM-ilvE* and *ilvE-ilvD* genes, respectively. A stable RNA stem loop structure is predicted for the *ilvE-ilvD* intercistronic region (Cox et al., 1987) that may block the action of 3'-specific exoribonucleases (Alifano et al., 1996; Belasco et al., 1993; Altman et al., 1993), and this may contribute to the relative stability of the *ilvE* transcript. **(B)** Relatively stable 4.6 kb *ilvEDA* and 1.0 kb *ilvE* transcripts (solid lines), relatively unstable and often undetectable 6.7 kb *ilvGMEDA* and 3.6 kb *ilvDA* transcripts (broken lines), and the Rho-dependent transcription termination sites following the frameshift site in the Val^S strains, with the percent of the transcripts that terminate at each site in parentheses. The frameshift site generates a UGA stop codon at positions 1252-1254, followed by Rho-dependent transcription termination sites in the *ilvGM* region centered at positions 1876, 1942, and 1990. The *ilvGp2* promoter is controlled by attenuation and the internal *ilvEp* is constitutively expressed. The first base of the *ilvGMEDA* mRNA directed by the *ilvGp2* promoter is designated position 1. The first base of the *ilvEp* is position 2101. The antisense RNA probe corresponds to the *Hind*III fragment from 2438 to 4097.

termination within the *ilvG* gene of wild-type K-12 causes the transcriptional polarity which decreases the expression of the distal *ilvEDA* genes. The "*ilvO*" mutations were found to be one base pair deletions or two base pair insertions in the *ilvG* gene (Lawther et al., 1981; 1982). DNA sequence (Fig. 1-4) shows that either a two-base pair (TA or GT) insertion (*ilvG2096*; *ilvG603*) or a one-base pair deletion (Δ*AilvG268*; Δ*TilvG671*; Δ*AilvG2096*; Δ*AilvG468*; Δ*AilvG269*, Lawther et al., 1982) were found within a stretch of only 10 base pairs preceding and including the AT base pairs at position 1254 of the *ilvG* gene (Lawther et al., 1982). This corrects the translational alignment (Fig. 1-3; Fig. 1-4) of the *ilvG* gene and removes the frameshift site, the *ilvG* gene is expressed, isozyme II (Val^R) is produced and the cell is phenotypically Val^R.

Regulatory regions of the *ilvGMEDA* gene cluster

Transcription promoters (Gp1p2, Ep, Dp, Ap)

The proximal promoter region of the *ilvGMEDA* gene cluster is presented in Fig. 1-2. In vitro, transcription of the *ilvGMEDA* regulatory region yields two RNA's, one of 186 nt and the other of 258 nt (Lawther et al., 1980; Adams et al., 1985). These transcripts initiate from two tandem promoters, *ilvGp1* [-71 base pair (bp) and *ilvGp2* (+1 bp)] and terminate at the attenuator (Fig. 1-2). In vivo, only the transcript from the *ilvGp2* can be detected (Adams et al., 1985; Ortuno, et al., 1987). Binding of integration host factor (IHF) to the *ilvGp1* promoter of the *ilvGMEDA* gene cluster inhibits the transcript initiated from the *ilvGp1* (Pereira et al., 1988).

In addition to transcriptional promoters at the beginning of the gene cluster, a

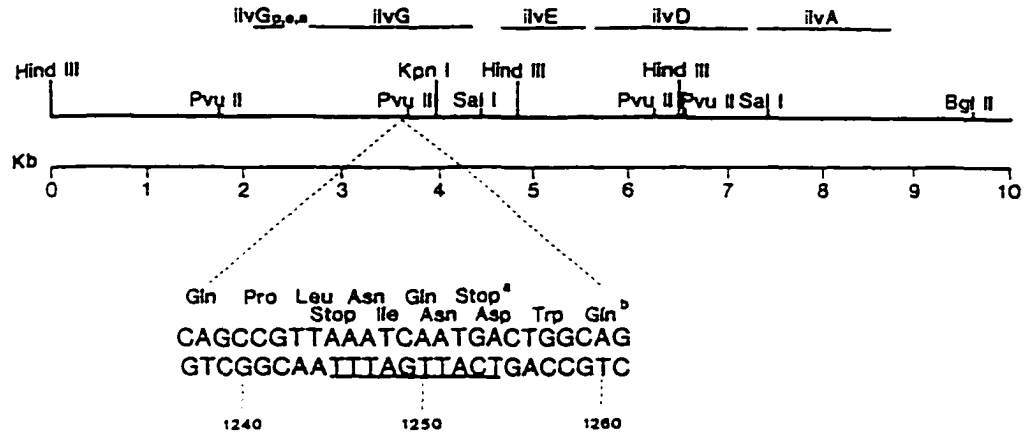


Fig. 1-3. DNA sequence of the region in which the ilvG$^-$ (IlvG$^-$) to ilvG (IlvG$^+$) mutation. (a) The amino acid sequence from the 5' terminus of ilvG. (b) The amino acid sequence from the 3' terminus of ilvG. The number beneath the sequence indicate the number of base pairs from the start of transcription. Kb. Kilobase pairs.

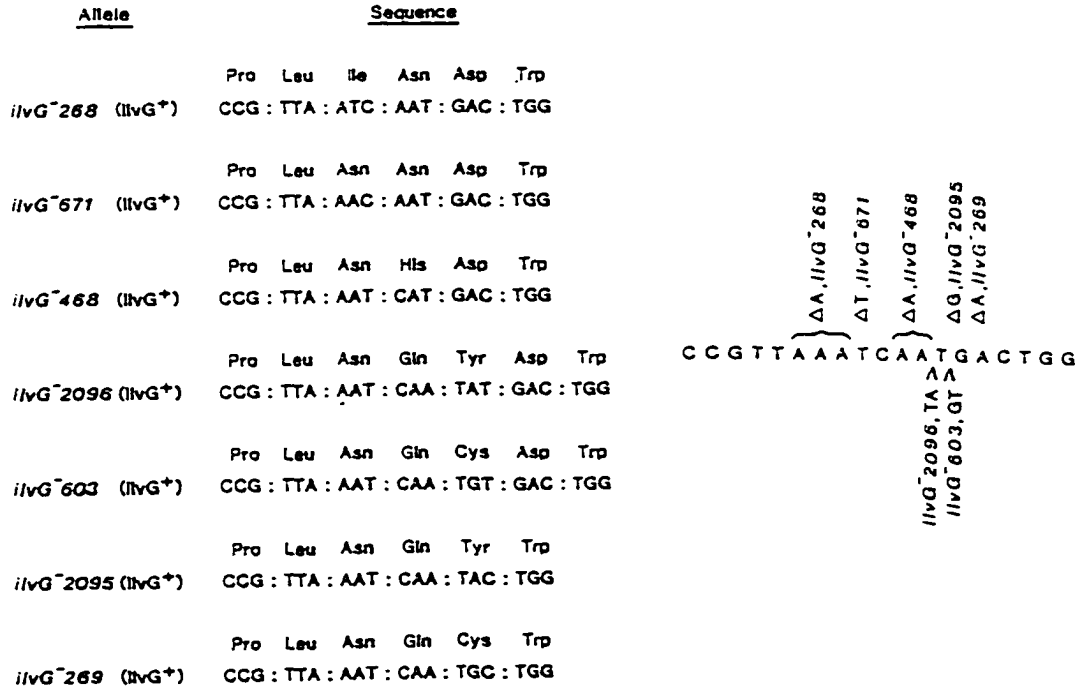


Fig. 1-4. The *ilvG* (IlvG$^-$) mutations and the amino acid sequence of the corresponding portion of the AHS II for each mutaiton.

secondary promoter (+2101 bp), *ilvEp*, is located in the distal portion of the *ilvM* (Fig. 1-2). The *ilvEp* promoter has been characterized in vivo and in vitro in both *E. coli* and *S. typhimurium*. This promoter is about fifty fold weaker than the maximum capacity (derepressed) of the 5'- *ilvGp1*, *Gp2* promoter (Calhoun et al., 1985; Wek and Hatfield, 1986; Lopes and Lawther, 1986). The internal promoter, *ilvEp*, was thought to be a weak promoter which functions to maintain a basal level of gene cluster. There are reports of other internal promoters, *ilvDp*, and *ilvAp* located upstream of the *ilvD* and *ilvA* gene of *E. coli*, respectively (Calhoun et al., 1985; Berg et al., 1979; Berg and Shaw, 1981; Blazey and Burns, 1979) using *galK* transcriptional fusion plasmids and in vitro transcription techniques.

Leader region and attenuation

A multivalent repression mechanism is found in the regulation of the *ilvGMEDA* gene cluster expression (Lawther et al., 1981; Nargancy et al., 1980). The expression of the *ilvGMEDA* gene products is reduced in the presence of all three of the branched chain amino acids (isoleucine, leucine, valine), but the rate of synthesis of these enzymes increases when growth of the cells is limited by the availability of any one of these amino acids. This inhibition is the result of controlling of the transcription into the structural genes by an attenuation of transcription at the end of the leader region.

The leader region is 186 nt RNA of the *ilvGMEDA* cluster which encodes a 32 residue polypeptide containing five isoleucine, five leucine and six valine amino acids. Based on predictions of secondary structure of the nt sequences of the leader RNA, the leader RNA is able to form alternative structures which control the level of transcription

into the *ilvGMEDA* structural genes (Lawther and Hatfield. 1980: Nargang et al.. 1980). The important codon is 21 (isoleucine) which is at the bottom of the 1:2 stem-loop (Fig. 1-5). A ribosome stalled at this isoleucine codon would favor the base pairing of unmasked downstream sequences leading to the formation of an antiterminator stem-loop 2:3. Under isoleucine limitation (Fig. 1-5), the antiterminator stem-loop 2:3 can be formed by ribosome binding on region 1 thus facilitated deattenuation. Otherwise, with a high concentration of isoleucine, the ribosome would bind to region 1 and 2, terminator stem-loop 3:4 would form, then release RNA polymerase and stop transcription.

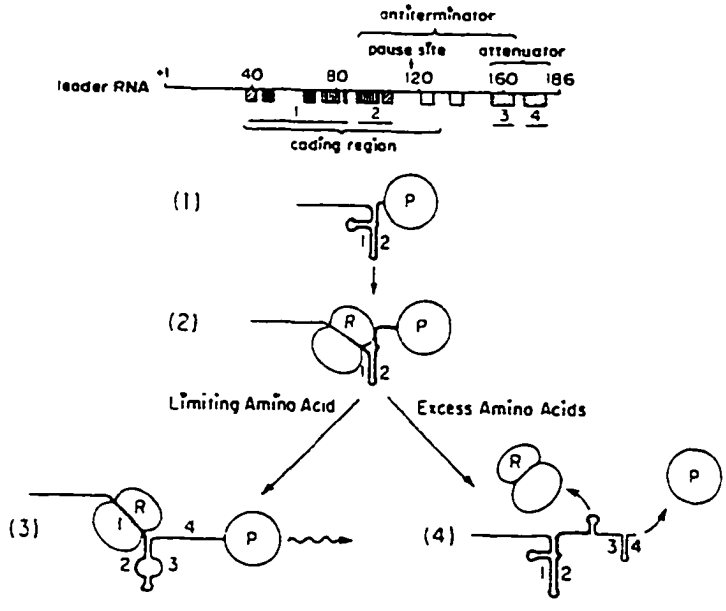


Fig. 1-5. Attenuator control of the *ilvGMEDA* gene cluster. Areas of pattern identity along the leader RNA represent regions of complementary sequences. The bold portion of the transcript depicts the leader polypeptide coding region. P represents an RNA polymerase and R represents a ribosome. 1, 2 and 3, 4 represent the regions involved in stem-loop (Lawther and Hatfield, 1980).

Rho-dependent or independent terminal sites

In prokaryotic systems transcriptional polarity is due to an uncoupling of translation and transcription which unmasks latent Rho-dependent termination sites in a polycistronic mRNA (Platt et al., 1986). In the Val^R stains, the specific activities of transaminase B (the distal *ilvE* gene product) and threonine deaminase (the distal *ilvA* gene product) respectively are elevated 3-4 fold compared to Val^S *E. coli* K-12 (Smith et al., 1976; Lawther and Hatfield, 1978). Distal gene (*ilvE, ilvA*) expression in isogenic strains containing *rho* mutations also increases as compared to the wild-*type E. coli*. K-12 (Smith et al., 1976; Lawther et al., 1978). Thus, these two types of mutations appear to relieve classical transcriptional polarity effects on distal gene expression in the *ilvGMEDA* gene cluster. However, the two mutations together do not have an additive effect. This means both mutations relieve a common transcriptional polarity event. Three tandem Rho-dependent terminal sites have been identified downstream of the frameshift site in the *ilvGM* gene region using in vitro transcription experiments (Fig. 1-2; Wek et al., 1987). Another Rho-dependent termination site, *ilvA*t', was also detected about 100 bp downstream of the Rho-independent termination site, *ilvA*.t, which is in the *ilvA* nontranscribed DNA strand, 116 base pair (bp) downstream of the *ilvA* translational stop codon (Platt, 1986; Sameshima et al., 1989). The presence of these two transcriptional terminators, one transcriptional termination protein (Rho)-dependent and one Rho-independent, at the 3'-terminus of the *ilvGMEDA* gene cluster appears to be similar to the *trp* operon (Wu et al., 1981), and the *rrnG* cluster (Albrechtsen et al, 1991) of *E. coli*. and the *leu* cluster of *S. typhimurium* (Friedberg et al., 1985).

Mutivalent control of the *ilvGMEDA* gene cluster expression

In the regulation of the isoleucine, valine biosynthetic pathway, control responding to branched-chain amino acid availability is considered to provide *ilv*-specific control, whereas factors that are independent of the branched-chain amino acids are considered non-*ilv*-specific factors.

In *ilv*-specific control, the regulation of biosynthetic enzymes is multivalent, with derepression occurring whenever one of the three branch-chain amino acids is limited, and repression requiring all three to be in excess (Umbarger, 1990). This expression control of the *ilvGMEDA* gene cluster in *E. coli* K-12 is considered to be due to attenuation of transcription. Mutations of the attenuator by deletion results in loss of the response to either excess or limiting levels of branched-chain amino acids (Bennett and Umbarger 1984; Chen et al., 1991). As summarized in Figs. 1-6 and 1-7, the repression of the *ilvGMEDA* genes requires all three branched-chain amino acids at a high concentration. In contrast, AHS I is derepressed only when either valine or leucine is limiting, it is not derepressed if isoleucine is limiting. AHS III formation is controlled by the level of leucine alone. Then, when valine is added to a minimal medium culture of the K-12 strain of *E. coli* (which contains only the valine-sensitive AHS I and AHS III), isoleucine is no longer formed and thus becomes relatively limiting. AHS II is derepressed when one of the three branched-chain amino acids is limiting. Acetohydroxy acid isomeroreductase, the product of the *ilvC* gene, is regulated by valine alone in the K-12 strain of *E. coli*. This is because isomeroreductase is induced by its substrates, which are formed by their acetohydroxy acid synthase (Ratzkin et al., 1972).

BRANCH POINT ENZYME	BRANCHLET	PRIMARY EFFECTORS	REMOTE EFFECTORS
L-Threonine Deaminase (TD)	ILE	ILE	LEU, VAL
α-Acetohydroxyacid Synthase (AHS) I	VAL	(I) VAL	LEU, ILE
α-Acetohydroxyacid Synthase (AHS) II	VAL	(II) ?(VAL^R)	LEU, ILE
α-Acetohydroxyacid Synthase (AHS) III	VAL	(III) VAL	LEU, ILE
α-Isopropylmalate Synthase (α-IPMS)	LEU	LEU	ILE

Fig. 1-6 The primary and remote effectors for the feedback regulated enzymes in each branchlet of the pathways (Hahn and Calhoun, 1978).

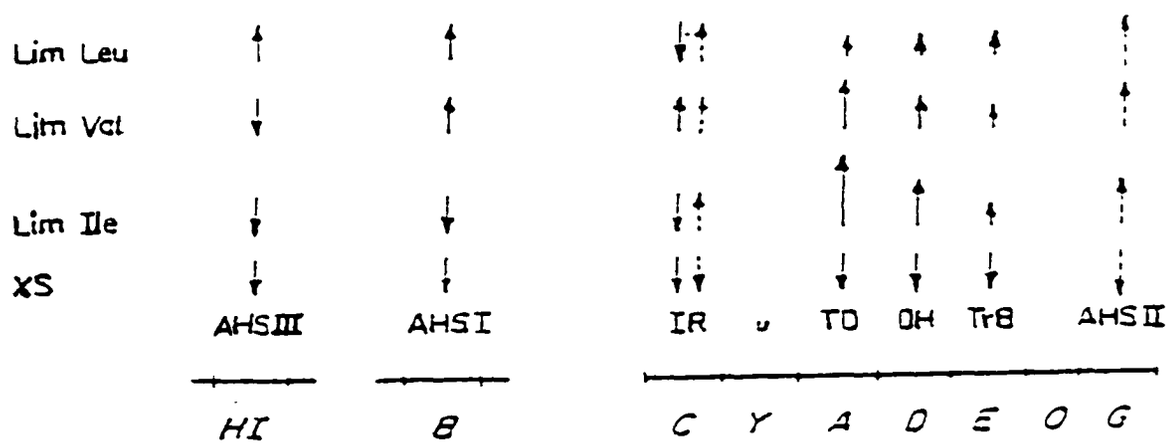


Fig. 1-7. Pattern of multivalent repression in isoleucine and valine biosynthesis. The arrows pointing upward indicate derepression under the indicated condition (limiting leucine, limiting valine, or limiting isoleucine). The arrows pointing downward indicate repression under the indicated condition. XS, excess branched chain amino acids in medium. Broken lines represent behavior in Val^R derivatives of *E. coli* K-12. Size of the upward-pointing arrow is a qualitative indicator of the derepression, relative to that of transaminase B, obtained upon growing cells under various derepression conditions . The *ilvO* site is now recognized to be the naturally occurring frameshift in the wild type *ilvG* gene. Also, we now know that AHS I has a small subunit encoded by *ilv B* and a large subunit encoded by *ilvN*, and AHS II has a small subunit encoded by *ilvM* and a large subunit encoded by *ilvG* (Umbarger, 1987).

Since *E. coli* K-12 has only the valine-sensitive isozymes, the inducer will be formed when valine is limiting.

As described below, there is also non-*ilv*-specific control including IHF, ppGpp, and possibly other effectors.

Differential expression of the *ilvGMEDA* gene cluster

Expression of the five genes in the *ilvGMEDA* cluster is coordinately controlled by attenuation. But there are two additional features resulting in discoordinated expression of the operon. First there are internal promoters designated *ilvEp* (Lopes et al., 1986; Wek et al., 1986), *ilvDp* (Berg et al., 1979) and *ilvAp* (Lopes and Lawther, 1989). These internal promoters express the three genetic subsets of the operon *ilvEDA*, *ilvDA* and *ilvA*. Second, in Val^S strains there are three tandem Rho dependent transcription termination sites in the *ilvGM* region downstream of the frameshift site in the *ilvG* gene. These transcriptional polarity site are responsible for terminating transcription of the genes upstream of *ilvEp* (Lawther et al., 1981; Wek et al., 1987; Smith et al., 1976; Lawther and Hatfield, 1978)). In addition, the expression of genes may depend on the rate of decay of their messenger RNA (Ehretsmann et al., 1992)

Internal promoters

The entire *ilvGMEDA* gene cluster can be transcribed as a unit from the promoter *ilvGp2* at the beginning of the operon, and there are internal sites for transcription initiation *ilvEp*, *ilvDp* and *ilvAp* promoters. Internal promoters have two functional roles. First, it can serve to regulate operon distal genes to effect discoordinate expression

of operon-encoded product (Valentin-Hansen et al., 1984). Second, it is unregulated and its constitutive activity is assumed to function to maintain a basal level of expression of distal genes (Horowitz and platt, 1983; Grisolia et al., 1983). The *ilvEp* promoter is located at the distal portion of the *ilvM* gene immediately upstream from the *ilvE* gene as determined by S1 nuclease protection analysis of in vivo and in vitro transcription (Wek and Hatfield, 1986). Transcription from the *ilvEp* was quantitated using a multicopy *galK* transcriptional fusion plasmid (Rosenberg et al., 1983) and plasmid copy number determination (Adams and Hatfield, 1984) or cloning at the upstream of *lacZ* gene coding for β-galactosidase (Calhoun et al., 1985). These analyses showed that the *ilvGp1p2* promoter is 52-fold stronger than the internal promoter, *ilvEp* (Wek et al., 1986), which is not regulated by the availability of branched-chain amino acids and a wide range of growth rates between 35 to 390 min (Harms and Umbarger, 1991).

The *ilvAp* promoter is located upstream at *ilvA* within the coding region of *ilvD* (Lopes and Lawther, 1989; Wek et al., 1986) although *ilvAp* promoter activity is very weak. Expression from the *ilvAp* promoter requires oxygen. Because insertion of Tn5 into the *ilvE* gene is only partially polar on expression of the downstream genes, this result indicates the possible existence of promoter *ilvDp* for expression of the *ilvDA* (Berg et al., 1979). The 5'- end of the *ilvD* transcript was also detected by primer extension (Huang et al., 1992). This is consistent with the existence of the *ilvDp* promoter if no processing event generates this 5'- end of mRNA.

Polarity of the *ilvGMEDA* gene cluster

In prokaryotic systems, transcription and translation are coupled. The premature

termination of translation of a polycistronic messenger RNA can reduce transcription of operon distal genes. It is called transcriptional polarity when the result of a mutation causes termination of translation within a distal gene of an operon (Wek et al., 1987).

There are a number of frameshift mutations found in the *ilvG* gene of wild type *E. coli* K-12, which were isolated as Val^R mutants that restore the translation frame of the distal half of the *ilvG* gene and concomitantly increase distal *ilvE, D, A* expression by 5 - or 10 - fold (Lawther et al., 1981). As described above, DNA sequence determination showed that either a two-base pair insertion or a one-base pair deletion were found within a stretch of only 10 base pairs preceding and including the AT base pairs at position 1254 of the *ilvG* gene (Figs. 1-3 and 1-4; Lawther et al., 1982). Each mutation allows for the full length expression of the *ilvG* gene product and therefore functional acetohydroxyacid synthase II activity.

There are three tandem Rho-dependent sites that have been located in the *ilvGM* gene region of wild type *E. coli* K-12 by in vitro transcription (Fig. 1-2; Panel B). Under steady state growth conditions, the specific activities of threonine deaminase, dihydroxy acid dehydrase and transaminase B were higher in a strain with a Rho mutation (Val^S, *rho221*) than in the wild-type strain (Val^S, *rho+*). Furthermore there was a nine-fold derepression of threonine deaminase, a two- to three -fold derepression of dihydroxy acid dehydrase and less than a two-fold derepression of transaminase B. This non-coordinate response was seen under both repressing and non-repressing growth condition (Smith et al., 1976). Thus, transcription polarity regulates the expression of the *ilvGMEDA* gene cluster.

Messenger RNA stability

In post-transcriptional regulation, mRNA stability affected the expression of mRNA. There were four transcripts identified (Huang, et al., 1992) from the *ilvGMEDA* gene cluster using multicopy plasmids. The *ilvEDA* and *ilvE* were ten to fifty fold more abundant than the *ilvGMEDA* and *ilvDA* transcripts, which were often either undetected or at the limit of detection, depending upon the strains tested and growth conditions. In *E. coli*, there are several endo- and exo- ribonucleases including endoribonuclease E (RNase E), endoribonuclease III (RNase III), and endoribonuclease P (RNase P), which are involved in the processing maturation and turnover of mRNA (Alifano et al., 1996; Belasco and Brawerman, 1993). It was not established prior to this work whether or not these ribonucleases were involved in the stabilities of the *ilvGMEDA* gene transcripts.

Control of the *ilvGMEDA* expression by regulatory factors:

Transfer RNA (tRNA)

The levels of aminoacylated $tRNA^{Ile}$, $tRNA^{Leu}$, and $tRNA^{Val}$ regulate the expression of the *ilvGMEDA* gene cluster of *E. coli* K-12 through an attenuation mechanism (Harris et al., 1983; Umbarger, 1990). The regulation region is a leader RNA which is messenger for a peptide rich in isoleucine, valine and leucine (Nargang et al., 1980; Lawther and Hatfield, 1980). Translation of the leader RNA in peptide region will be impeded and RNA polymerase will then transcribe the DNA containing the structural genes of the operon when the level of any or all of the aminoacylated regulatory tRNAs decreases. If the levels of aminoacyl-tRNA increases, the leader peptide is

translated, RNA polymerase will terminate transcription at the attenuation site prior to the structural gene. This result is supported by using *E coli* C6 which is a *relA*$^-$, *cys*$^-$, *met*$^-$ mutant (Harris et al., 1983). Under the conditions of cysteine starvation, this mutant accumulates thionucleotide-deficient tRNA. The levels of threonine deaminase, the *ilvA* gene product, and transaminase B, the *ilvE* gene product, were both lower. The effect was reversible by addition of the sulfur amino acid. This supports the facts which alters the state of aminoacylation of the regulatory tRNA may have an effect in the expression of the *ilvGMEDA*.

Mutation in *E. coli* K-12

There are several mutations in *E. coli* K-12 which increase the expression of the *ilv GMEDA*. The strain containing mutation in *ileR* (*avr-16*), localized between *trpR* and *thr* at min 99.9, constitutively expressed the *thr* and *ilv* operon and was resistant to the threonine analog DL-α-amino-β-hydroxyvaleric acid. The strain containing mutation allele *flr-9*, linked to *entA* at min 13, constitutively expressed *thr*, *leu* and *ilv* operon and was resistant to 5', 5', 5'-trifluoro-DL-leucine. The constitutive expression of the *thr*, *leu* and *ilv* operons in mutants *avr-16* and *flr-9* was partly reversed in cells harboring a plasmid, which lead to elevated levels of the *trp* aporepressor or which is able to express the *ileR* gene. In each case, expression was examined in a strain containing *lacZ* under the control of the *ilvG* or *thrA* promoter in the absence of the cognate attenuator regions. This indicated that *ileR* and *flr*-9 gene product functions are independent of attenuation. But no evidence was provided indicating that the repressing effect of *ileR* region was subject to any modulation. Another gene, *ilvR*, adjacent to *ileR* appears to both activate

and repress the *ilv* promoter (Johnson and Somerville, 1984). The experiments did not address whether their effects on the *ilvGMEDA* operon were at all subject to regulation. The *leuJ* locus was also identified as the site of a lesion conferring trifluoroleucine resistance and leading to elevated *ilvA* and *leuA* expression (Johnson and Somerville, 1983).

ppGpp stimulates in vitro transcription from the *ilvGp2* promoter (Umbarger, 1990). The Pribnow box and the discriminator region fit a pattern of ppGpp-activated promoters recognized by Riggs et. al. (1986). The stimulation by ppGpp would serve to amplify derepression that results from branched - chain amino acid limitation since ppGpp levels would rise as the growth rate was reduced owing to the amino acid limitation.

IHF Protein - Integration host factor

IHF protein, the gene products of the *himA* and *himD* genes, affect the expression of the *ilvGMEDA* gene cluster (Friedman, 1988). The *ilvG* has two binding sites for IHF (Pereira, et. al., 1988; Tsui et al., 1988). One of the binding sites overlaps with one of the two RNA polymerase-binding sites detected in vitro, designated *ilvGp1* (Pagel and Hatfield, 1991; Tsui and Freundlick, 1988; Winkelman and Hatfield, 1990). IHF binding at this site interferes with transcription from *ilvGp1* but activates transcription at the downstream promoter, *ilvGp2* (Pagel et al., 1991, and 1992). The second IHF binding site is downstream of the polymerase pause site of the leader region (Tsui and Freundlick, 1988). Binding at this site increases the polymerase pause period in an in vitro transcription experiment (Pagel and Hatfield, 1991). It was not possible to assay this

effect in vivo. A polymerase pause might not allow a ribosome to begin leader transcription in time. It may be that IHF ensures that RNA polymerase will remain at the pause site until the ribosome has initiated translation.

IHF binding to the DNA region of the *ilvGp* enhances bending of the DNA and results in activation of the *ilvGp2* promoter for 4-fold transcription from the *ilvGp2* (Pagel, et. al., 1992). This *cis* effect of the intrinsic DNA bending itself and the *trans* effect of IHF seem to be the factors affecting the *ilvGMEDA* operon expression independently of regulation related to branched-chain amino acid biosynthesis. IHF might activate transcription by severely bending the DNA helix in the upstream activating region and alter the local conformation of the DNA in the nearby *ilvGp2* promoter region in a way that facilitates a more favorable RNA polymerase-promoter interaction (Pagel, et. al., 1992). Thus, any factors regulating the amount of IHF might be recognized as global regulators of the *ilvGMEDA* expression as well as that of other operons that are affected by IHF.

Threonine deaminase

Threonine deaminase, the *ilvA* gene product, is the first enzyme of the isoleucine-valine biosynthetic pathways (Fig. 1-1) and is inhibited by isoleucine. The isoleucine inhibition is antagonized by valine, which functions as a positive effect. Evidence shows that a mutation (*ilvA538*), in which a C to T point mutation changes leucine-481 to phenylalanine in *ilvA* gene product (Taillon et al., 1988), results in lower than normal levels of isoleucyl, valyl - and leucyl - tRNA synthesis. Moreover, this regulatory

mutation decreases the level of expression of the *ilvGMEDA* gene cluster and causes a defect in *ilvC* induction (Calhoun et al., 1976; Singer et al., 1984; Levinthal et al., 1973). These regulatory effects are overcomed by a Val^R mutation in the *ilvH* or in the *ilvO* region of the *ilvG* (Calhoun et al., 1976; Singer et al., 1984). Analysis of the functional domains of threonine deaminase indicates that the C-terminal segment is presumably involved (i) in the binding or interaction with the allosteric effector isoleucine, or (ii) in subunit interactions (Taillon et al., 1988). Thus it was suggested that threonine deaminase might act as a negative control element of *ilvGMEDA* (Hatfield and Burns, 1970) and a positive control element of *ilvC* (Calhoun and Hatfield, 1973). A model was proposed (Singer et al., 1984): threonine deaminase and 2-ketobutyrate, the product of the threonine deaminase reaction, can form a high molecular weight complex which can reduce the activity of the isoleucyl and valyl-tRNA synthetase.

Ribonucleases, RNA processing, and RNA degradation

In order to determine if mRNA processing or Rho-dependent transcription termination participates in the formation of any of the *ilvGMEDA* coded transcripts, it would be useful to examine mRNA levels in isogenic strains with and without mutations in processing endoribonucleases, exoribonucleases, and transcription termination factor Rho. In the presence of specific RNase mutations, mRNA processing and decay occur more slowly, which allows for easier detection of unstable intermediates. In addition, it would be useful to examine chromosomal expression of these genes, since the potential exists for artifacts when these genes are expressed from plasmids as in our previous study

(Huang et al., 1992). Since the Val^S and Val^R strains are predicted to have different mRNA populations (Fig. 1-2), the comparisons should include both of these phenotypes.

In the present studies we compared the *ilvGMEDA* transcripts in strains with the frameshift site present (Val^S), and in strains with the frameshift site removed by (i) an in frame in vitro deletion (Fig. 2-1) that abolished activity of the *ilvG* gene product and therefore produced a Val^S phenotype, but translation of the *ilvGM* mRNA prevented the polar effects of the frameshift site, and (ii) a spontaneous mutation in *ilvG* (identified in a strain selected for the Val^R growth phenotype) that restored both the proper reading frame and enzyme activity, and eliminated the polar effects of the frameshift site.

We present reproducible effects of ribonucleases upon the *ilvGMEDA* transcripts using isogenic pairs of mutant hosts. Several ribonucleases have been implicated in the processing, maturation, and turnover of mRNAs, including endoribonucleases RNase E, RNase III, and RNase P, and the 3'-exoribonucleases PNPase and RNase II (Alifano et al., 1996; Belasco et al., 1993; Altman et al., 1993). RNase P was initially shown to cleave all tRNA precursors to generate the 5'-phosphoryl terminus of mature tRNA (Altman et al., 1993), but more recently a role for RNase P in mRNA processing was demonstrated (Alifano et al., 1996). The 7.3 kb *his* transcript of *S. typhimurium* is initially cleaved at specific sites by RNase E, and RNase P further cleaves the products generated by RNase E (Alifano et al., 1996). The results reported here reveal that a mutation in RNase E increased the stability of the *ilvGMEDA* and *ilvE* transcripts. A RNase P mutation increased the stability of the *ilvEDA* and *ilvE* transcripts. A mutation in RNase III had no apparent effects on the *ilv* transcripts. The *ilvGMEDA* transcripts

were more stable in strains with mutations in the 3'-exoribonucleses, PNPase and RNase II. Thus, we find a process similar to the *his* transcript of *S. typhimurium*, in that RNase E cleaves the full length *ilvGMEDA* transcript generating the *ilvEDA* transcript, which is further processed by RNase P.

We present a comparison of expression of transcripts from plasmids and from the chromosome. Detection of these transcripts at the lower levels resulting from single copy expression from the chromosome was not possible in the previous study (Huang et al., 1992) using [^{32}P]-labeled synthetic oligonucleotides or asymmetric PCR products as probes.

Finally, RNase E was partially purified from an *E. coli* strain that overproduces this enzyme. The partially purified RNase E was tested for activity using synthesized mRNA substrate corresponding to the boundary of the *ilvE* and *ilvD* genes in vitro. Major and minor cleavage sites were detected, which supports a role for RNase E in processing of the *ilv* transcripts.

CHAPTER 2

MATERIALS AND METHODS

Materials

RNA molecular weight marker, digoxigenin (DIG) RNA labeling kit and nucleic acid DNA detection kit were purchased from Boehringer Mannheim (Indianapolis, Ind.). Restriction enzymes, Calf intestine phosphatase and T4 DNA ligase, Protein Marker (broad range) were purchased from New England BioLabs, Inc. (Beverly, Mass.). RNasin was purchased from Promega Corp. (Madison, Wis.). The paired promoter SP6 system was purchased from Amersham (Arlington Heights, IL). Diethylpyrocarbonate (DEPC), 3-(N-morpholino)-propanesulfonic-acid (MOPS), formamide, formaldehyde, and rifampicin (rif) were from Sigma (St. Louis, MO). Lysozyme was purchased from Pharmacia (Piscataway, New Jersey).

Media

Cells were grown in M9 minimal medium (Miller, 1974) containing 0.2% glucose, 1 μg/ml thiamine, 2 mM of $MgSO_4$, 0.1 mM of $CaCl_2$, 50 μg/ml of ampicillin or 100 μg/ml of tetracycline (for plasmid containing strains) with supplemental amino acids. Concentrations of leucine, isoleucine, and valine were 0.3 mM. Other amino acids were used at concentrations of 50 μg/ml. Plates contained 1.5% agar (Difco) with 50 μg/ml of ampicillin or 100 μg/ml of tetracycline. LB broth contained, per liter: 10 g Bacto tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl and was adjusted to pH 7.0 with NaOH. This media was supplemented as follows: LBMg, 10 mM $MgSO_4$; LBCa plates

(R plate), 5 mM $CaCl_2$, and 1.5 % agar; LBCaCM plates, 5 mM $CaCl_2$, and 12.5 μg chloramphenicol/ml and 1.5 % agar; LB top agar, 0.5 % agar.

Bacterial strains and plasmids in this study

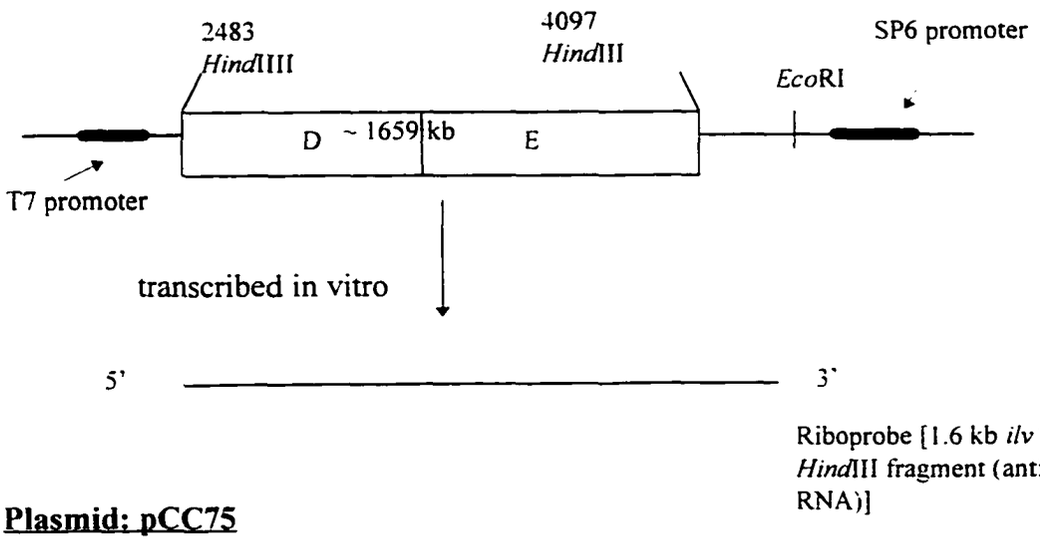
All strains and plasmids used in the experiments are listed in Table 2-1 and Table 2-2. Strains are derivatives of *E. coli* K-12.

Construction of plasmids pCC73 and pCC75

Plasmids pCC73 and pCC75 were generated by standard methodologies (Ausubel et al., 1991). Plasmids pAM18 and pAM19 were derived from plasmids pSP64 and pSP65 respectively (Amersham), by the insertion of a promoter for T7 RNA polymerase. In addition, two new cloning sites, *Sph*I and *Kph*I were introduced into their multiple cloning sites (MCS), and an internal *Sph*I site was deleted. The two vectors differ only in the orientation of the multiple cloning site relative to the SP6 and T7 promoters. These vectors allow the transcription of both coding and noncoding strands using either SP6 or T7 RNA polymerases, using the pair of vectors; or from the same vector using SP6 RNA polymerase to transcribe one strand, and T7 RNA polymerase to transcribe the other. Plasmid pCC73 was generated by using the 1.6 kb *ilv'ED' Hind*III fragment. This fragment was purified from low melting gel after *Hind*III digestion plasmid pJG17 (Table 2-2) and ligation to the *Hind*III cloning site of vector pAM18. Plasmids pCC75 and pCC73 differ only in the orientation of ~1.6 kb *ilv'ED' Hind*III fragment relative to the SP6 and T7 promoters. In vitro, antisense and sense mRNA is transcribed from plasmid pCC73 and pCC75,

respectively (Fig. 2-1).

Plasmid : pCC73



Plasmid: pCC75

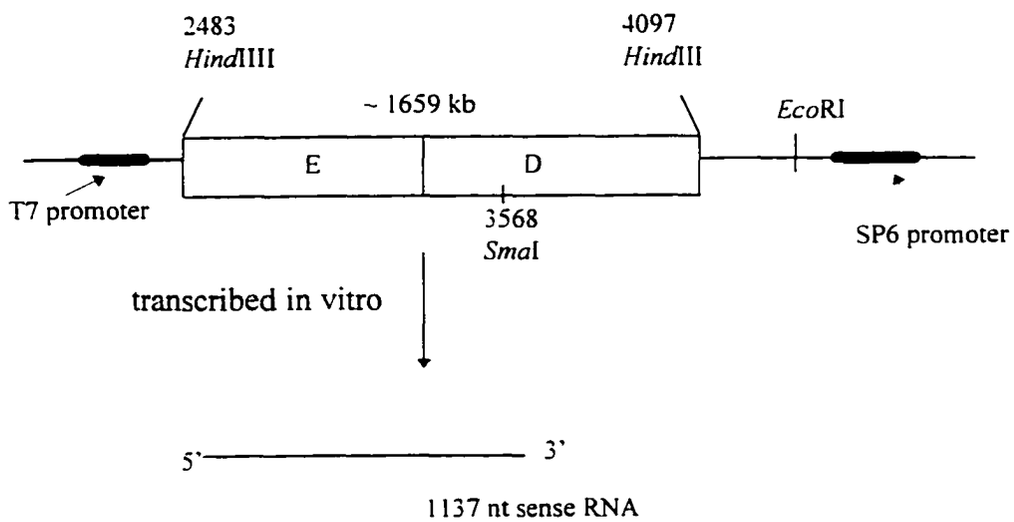


Fig. 2-1. Structures of plasmids used to synthesize antisense riboprobe and sense RNA for RNase E treatment. Structures of Plasmids pCC73 and pCC75; Riboprobe (1.6kb , *ilv'ED' Hind*III fragment) or RNase substrates (sense RNA) were transcribed by T7 polymerase from pCC73 and pCC75 respectively.

Growth rate of the mutant strains

Cells were grown overnight at 30°C in a rotary shaker (200 rpm), diluted 1/50 using prewarmed LB, and grown in a 250 ml Erlenmeyer flask at 30°C. Samples were taken by withdrawing 0.5 ml and immediately diluting with 0.5 ml chilled LB in a test tube. The sample (1 ml) was read at 600 nm in an Ultrospec II spectrophotometer (Model 4050). The temperature-sensitive mutant strains and their respective wild type isogenic strains were grown at 30°C to early log phase (A_{600}=0.3). Half of the culture was shifted to 43°C, and the other half was kept at 30°C. The curves were obtained by plotting the log of the optical density versus time (min).

Bacterial growth conditions

All strains used are derivatives of *E. coli* K-12. Cells were grown on a rotary shaker at 30°C or 37°C in M9 minimal medium (Miller, 1974), supplemented with 0.2% glucose and required nutrients to indicated stage and harvested for RNA isolation. Antibiotics were added at the following concentration: ampicillin, 50 μg/ml; tetracycline, 50 μg/ml; chloramphenicol, 10 μg/ml; and kanamycin, 50 μg/ml.

A Rho mutant.

Strains CC253 (*rho-115*) and CC256 (*rho*$^-$) were grown at 37°C in M9 minimal medium with 0.3 mM leucine, isoleucine and valine, and 0.05 mM thiamine to mid-log phage (OD_{600}=0.4), then stored in ice and harvested by centrifugation for isolation RNA.

Amino acid limitation

For amino acid limitation, the media and methods to grow cells under repressing and depressing condition were those described by Gayda et al. (1980). Cells were grown in repressing conditions with 50 μg each of L-isoleucine and L-leucine per ml and 100 μg of L-valine per ml in M9 minimal medium. When the OD_{600} reached 0.3, cells were washed using prewarmed M9 minimal medium once, and transferred to the same volume of fresh derepressing conditions medium supplemented with two branched-chain amino acids of repressing concentration and one at 1/20 of the repressing concentration (2.5 μg/ml of isoleucine, leucine; 5 μg/ml of valine). After 3 hours incubation at 37°C, strains were chilled in ice and harvested for RNA isolation by centrifugation at 4°C.

RNase E mutants

Derivatives of strain N3431 (*rne-3071*^{ts}) were constructed containing plasmids pJG51 (strain CC147) with the cryptic Val^{S} allele (frameshift^{+}) or pCC17 (strain CC148) with a deletion of the frameshift (Δ-frameshift). Cells were grown at 30°C in M9 minimal medium containing 0.05 M thiamine to early log phase (OD_{600} = 0.3), and 50 ml of culture was withdrawn for RNA isolation. The rest of culture (200 ml) was incubated at 43°C. After incubation for 15 min, rif was added to a final concentration of 0.2 mg/ml and then RNA was extracted from sample taken at various times.

RNase P mutants

The isogenic strains NHY312 (*rnp*$^{+}$, Val^{S}) and NHY322 (*rnpA49*^{ts}, Val^{S}) were grown at 30°C in M9 minimal medium containing 0.3 M proline to early log phase (A_{600}=0.3), and 50 ml of culture was withdrawn for RNA isolation. The rest of culture

(200 ml) was incubated at 43°C. After incubation for 15 min. rif was added to a final concentration of 0.2 mg/ml. and then RNA was extracted from sample taken at various times.

RNase III mutants

The isogenic strains BL321 (*rnc-150*, Val^S) and BL322 (*rnc+*, Val^S), strains CC513 (*rnc150*, Val^R) and CC515 (*rnc+*, Val^R), isogenic pair strains CC325 (BL321, pJG51) and CC326 (BL322, pJG51) were grown at 37°C in M9 minimal medium containing 0.3 M arginine to early log phase (A_{600}=0.3), and culture (50 ml) was withdrawn for RNA isolation. Rif was added to the rest of culture (200 ml) to a final concentration of 0.2 mg/ml and RNA was extracted from sample taken at various times.

Exoribonuclease II (RNase II), Polynucleotide phosphorylase (PNPase) mutants

Strain SK5003 (*pnp-7*, *rnb-500*^{ts}, Val^S), SK5004 (*pnp-7*, *rnb*$^-$, Val^S), SK5005 (*pnp*$^-$, *rnb-500*^{ts}, Val^S) and SK5006 (*pnp*$^-$, *rnb*$^-$, Val^S) were grown at 30°C in M9 minimal medium containing 0.3 M threonine, 0.3 M leucine to early log phase (A_{600}=0.3), and 50 ml of culture was withdrawn for RNA isolation. The rest of culture (200 ml) was shifted to 44°C. After incubation for 15 min. rif was added to a final concentration of 0.2 mg/ml and then RNA was extracted from sample taken at the indicated times.

Strains CC327 (SK5003, pJG51), CC328 (SK5004, pJG51), CC330 (SK5006, pJG51) were grown in the same condition as SK5003 (*pnp-7*, *rnb-500*^{ts}, Val^S), a single mutant strain SK5004 (*pnp-7*, *rnb*$^+$, Val^S), SK5005 (*pnp*$^+$, *rnb-500*^{ts}, Val^S) and wild-type

strain SK5006 (*pnp*$^{+}$, *rnb*$^{+}$, Val^{S}).

Construction of valine-resistant strains

Preparation of P1 lysates.

The temperature sensitive (ts) phage P1CM*clr100* (Rosner, 1972) was used for transduction. Phage lysates were prepared by confluent lysis method (Rosner, 1972): To 3 ml of a standing overnight, room-temperature culture of strain CU2501 (Val^{R}), 1.5 to 3.0 x 10^{6} phage were added and allowed to adsorb for 30 minutes at 37°C in the presence of 5 mM $CaCl_2$. Then 10 ml of LB Mg (10 mM $MgCl_2$ with LB) and 10 ml of LB top agar were added, equal volumes of this mixture were poured onto each of three LBCa plates (LB with 1.5% agar and 5 mM $CaCl_2$). The plates were incubated at 37°C until confluent lysis was observed (6-8 hours). The top agar layer was removed, triturated with a glass rod, and treated with 4 drops of $CHCl_3$ for 20 min at 30°C. The treated lysate was then collected by centrifugation for 5 min at 9,000 rpm in IEC B20 centrifuge with a #870 rotor. The supernatant which contained the P1 lysate was saved and titered by plaque assay. The lysate was stored in the cold in a sealed , sterile test tube over chloroform at 4°C. This procedure was repeated three times.

Titering P1CM*clr100* lysate

Serial dilutions of P1 lysate were prepared in LB containing 5 x 10^{-3} M $CaCl_2$. Each dilution (0.1 ml) was mixed with 5 drops of 1/50 diluted strain CU2501 overnight culture respectively and incubated in a 37°C water bath for 10 min. The top agar (2.5 ml) was added in, mixed and immediately poured into two LBCa plates. The plates were

incubated at 37°C for 18 hours. Lysates prepared in this way had titers of about 2 x 10^8 phages/ml.

Transduction procedure

The recipient bacteria were grown in LBMg (0.1 mM $MgCl_2$ with LB) at 30°C to a density of 2 x 10^8. The culture was stored in ice and $CaCl_2$ was added to a final concentration of 0.01 M. Then the culture (2.5 ml) was added to a 2.5 ml of phage P1 grown as described above on strain CU2501 (P1. CU2501) diluted in LBMg (0.1 mM $MgCl_2$ with LB) to a final concentration of 4 x 10^8 plaque forming units (pfu)/ml or 2.5 ml of LBMg instead for a negative control. The mixture was incubated in 30°C water-bath for 30 min. After the test tubes were iced and centrifuged at 3,200 rpm for 10 min in IEC B-20 centrifuge, the pellet was washed by 2 ml of ice cold phosphate-buffered-saline (PBS; 0.4% NaCl, 0.3% KH_2PO_4 and 0.7% Na_2HPO_4, pH 6.8), centrifuged at 3,200 rpm at 4°C for 10 min and resuspended in 1 ml of PBS. The number of valine-resistant (Val^R) cells was determined by plating a aliquots on M9 minimal plates containing valine (0.3 mM) and other required nutrients at 30°C for 2 days. Samples of the P1 lysates and recipient strain were also plated to verify their sterility. The plates were be kept at room temperature for 2 hours to minimize P1 growth before put them in the incubator.

Selection of cured cells

The thermoinducible lysogens were patched on the M9 minimal medium plates containing valine (0.3 mM) and other required nutrients. After overnight incubation of the plates at 42°C, the colonies were further three succession purified in M9 minimal

medium plate containing valine (0.3 mM) by single colony isolations at 42°C. Both 'smooth-edged' and 'nibbled' colonies were observed. The 'nibbled" colonies were discarded since they contained both cured cells and cells reinfected with P1 released by induction of the lysogens.

Selection of Val^R strains

For selection of Val^R derivatives from temperature-sensitive mutant RNase E strain N3431 and wild-type strain N3433, M9 minimal plate containing 1 mM valine were spreaded with 100 ul of strain N3431 or N3433 transduced by P1.CU2501 lysate and incubated at 30°C for 2 days. Only the Val^R colonies were selected. After curing at 42°C, Val^R derivatives were tested on M9 minimal plate containing 1 mM valine at 30°C. Strains CU4 and CU2501 were used as controls.

The Val^R derivatives from mutant RNase P or RNase III strains NHY312, or BL321 and their wild-type strains NHY322 or BL322 were selected by the same method as Val^R derivative selection from isogenic pair N3431 and N3433 except that RNase P and RNase III strains were grown in M9 plates containing 0.3 mM proline, 1 mM valine or 0.3 mM arginine, 1 mM valine respectively.

The Val^R derivatives from double mutant PNPase and RNase II strain SK5003, a single mutant PNPase or RNase II strain SK5004 or SK5005 and wild-type strain SK5006 were selected by M9 minimal plate containing 0.3 mM leucine, 0.3 mM threonine, and 1 mM valine.

Preparation of competent cells and transformation

Competent cells were prepared using the one-step protocol as described by Chung et al. (1989). A fresh overnight culture of bacteria was diluted 1:100 into 50 ml Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter pH 7.4) and incubated at 37°C with shaking (25 rpm) until the cells reach an OD_{600} of 0.3 to 0.4. The cells were collected by centrifugation 10 min at 1000 x g at 4°C and resuspended at one tenth of original volume in 1 x TSS [10% (wt/vol) polyethylene glycol 4000, 1 x LB, 50 mM $MgCl_2$, 5% (vol/vol) dimethyl sulfoxide, pH6.5]. For long-term storage, aliquots (0.5 ml) of the suspension were put in microcentrifuge tubes, then frozen in a dry ice/ethanol bath and stored at -70°C. Frozen cells were thawed on ice and used immediately in the transformation assay. Cells could be used for 6 months.

Transformation was performed as described by Chung et al. (1989). A 100 µl aliquot of competent cells were pipetted into a cold polypropylene tube containing 20 µl DNA (intact plasmid DNA or ligation mixture), swirling to mix, followed by incubation on ice for 30 min. LB medium (0.9 ml) containing 20 mM glucose was added followed by incubation at 37°C with shaking (225 rpm) for 1 hour to allow expression of the antibiotic resistance gene. An aliquot of transformed cells was spread on LB agar plates containing appropriate antibiotics (ampicillin at 50 µg/ml or tetracycline at 100 µg/ml) to select transformants.

Isolation of RNA

RNA was isolated from cells harvested in the log phase as previously described

(Huang et al., 1992). Cells (50 ml in a 250 ml side arm flask) were grown to midlog phase (OD_{600} = 0.4 to 0.5) at 37°C and 175 rpm in a gyratory shaker (Model R25 or G76, New Brunswick Scientific, Piscataway, NJ), placed in a 50 ml plastic tube (Falcon #2075), stored in an ice bucket for 5 min, and centrifuged at 3,500 rpm (2,400 x g) for 15 min at 4°C in a Sorvall Model RT6000B centrifuge (Norwalk, CT). The pellets were resuspended in 0.5 ml of protoplast buffer [10 mM tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 8.0, 0.45 M sucrose, 8 mM EDTA (ethylene-diaminete-traacetic acid)] and 160 µl of lysozyme solution (Pharmacia, Inc., Piscataway, NJ, cat. # 27-02667-01, 20,000 units/mg, 50 mg/ml) was added and incubated on ice for 15 min. The cells were centrifuged for 10 min at 4°C at 3,000 rpm (1,900 x g) in a Sorvall RT6000B centrifuge and resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM Na-citrate, 1.5% sodium dodecyl sulfate) and 15 µl of DEPC was added. The mixture was transferred to a microfuge tube (RNase free, cat.# D20033; Jersey Lab & Glove Supply, Livingston, NJ), incubated in a 37°C water bath for 5 min, 0.5 vol of saturated NaCl was added, the tube was put on ice for 10 min, and centrifuged at 4°C at 12,000 x g (13,750 rpm) for 15 min in a Fisher Model 235B Micro-Centrifuge (Fisher Scientific, Springfield, NJ). The supernatant was extracted twice with phenol:chloroform:isoamyl-alcohol (24:23:1) mixture (Jersey Lab & Glove Supply, cat. # 7883) and precipitated with 0.1 vol of 3.0 M NaAc (NaAc), pH 5.3, and 2.5 vol ethanol at -20°C overnight or -70°C for 30 min. The pellets were resuspended in 100 µl of DNase digestion buffer (80 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM $MgCl_2$, 10 mM 2-

mercaptoethanol) with 10 units of RQ1 RNase free DNase (Promega, cat # M610) and incubated at 37°C for 2 hours. The mixture was extracted twice with phenol:chloroform:isoamyl-alcohol (24:23:1) mixture (Jersey Lab & Glove Supply, cat.# 7883) and precipitated with 0.1 vol of 3.0 M NaAc (pH 5.3) and 2.5 vol ethanol at -70°C for 30 min. The precipitates were washed with 70% ethanol and resuspended in 30 µl of DEPC treated water and stored at -70°C.

Hybridization probe

The riboprobe used for Northern blots was prepared by in vitro transcription of plasmid pCC73 linearized with *Eco*RI to produce an antisense RNA that is complementary to segments of all four *ilvGMEDA* transcripts (Fig. 2-1). In vitro transcription used 1 µg of template with 40 units of T7 RNA polymerase at 37°C for 2 hours in buffer containing 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 1 mM each of ATP, GTP, CTP, and 0.7 mM DIG-UTP. RNA samples were incubated at 55°C for 15 min in the presence of 22.5% formamide and were fractionated on a 1% agarose gel (14 cm X 10 cm) in 10% of formaldehyde, 10% MOP buffer pH 7.0, for 2 hours at 70 mV (Fig. 2-2). The oliE oligonucleotide (5'-tgatctaaccagccccatttatc-3') complementary to 3091-3113 in *ilvE* is labeled with ^{32}P by using method described by Huang (1992). To label the oligonucleotide probes used for Northern blots with [^{32}P] using T_4 polynucleotide kinase, 30 pmoles of oligonucleotide was added to 30 µl of DEPC-treated H_2O, 5 µl of 10X kinase buffer [0.5 M

tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine)], 10 µl of (γ-^{32}P) dATP (New England Nuclear, specific activity 3000 Ci/mmol) and 1 µl of T_4 polynucleotide kinase (10 units/µl) and incubated at 37°C for 30 minutes. The reaction was stopped with the addition of 50 µl of STE [0.1 M NaCl, 10 mM Tris-HCl pH7.6, 1 mM ethylenediaminetetraacetic acid (EDTA)]. The labeled oligonucleotides were separated from unreacted ATP by spin-column chromatography using Sephadex G-50 as described by Maniatis et al. (1989).

Preparation of radioactive RNase E substrates and RNA ladder

RNase E substrates were prepared by method described by Cormack and Mackie (1992). Plasmid pCC75 linearized with *Sma*I was used as DNA template (Fig. 2-1). Plasmid pRC9S was linearized by *Acc*I. The reaction mixture for transcription contained 40 mM Tris-HCl (pH 7.2), 6 mM $MgCl_2$, 10 mM dithiothreitol (DTE), 4 mM spermidine, 0.5 mM-ATP, GTP and UTP, 0.1 mM CTP, 0.025 mg linear plasmid DNA/ml, 1 unit/µl RNA guard, (Pharmacia), 2 unit/µl of T7 or SP6 RNA polymerase, and 50 µCi of [^{32}P]CTP (NEN). After incubation of 90 min at 37°C, 5 to 10 units of RNase-free DNase I were added and the reaction was incubated for 10 min at 37°C. The reaction mixture was extracted with phenol:chloroform:isoamyl-alcohol (24:23:1) mixture (Jersey Lab & Glove Supply, cat.# 7883) twice at room temperature, precipitated from 3M NaAc (pH 5.3) with

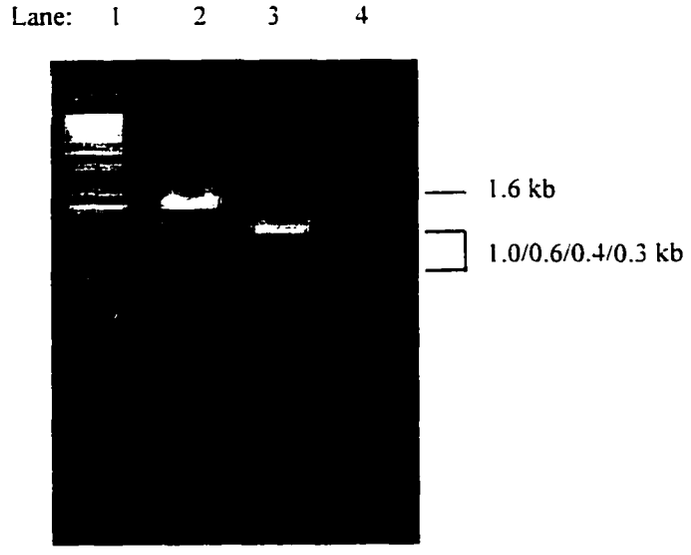


Fig. 2-2. Riboprobe with DIG-labeled transcribed in vitro. Samples (4 µl of transcription reaction mixture) were loaded on 1% formaldehyde agarose gel. The gel was run 1 hour at 70 mV and stained with ethidium bromide. Lane1: 1 kb ladder; Lane 2: 1.6 kb mRNA transcribed using pCC73 (1.6 kb *ilv'ED'* riboprobe); Lane 3: 760nt mRNA transcribed using control DNA from DIG-labeled kit; Lane 4. RNA ladder III (1.6\1.0\0.6\0.4\0.3 kb).

2.5 volume of ethanol at -70°C for 30 min, washed with 70% ethanol and dried in vacuum. The products were resuspended in 10 µl of DEPC-treated H_2O and stored at -70°C. Substrates were stable about 3 days. RNA ladder consists with four bands (1136\529\248 \119 nt). Bands (529 nt and 119nt) are created from plasmid pCC73 which is linearized with *Sam*I or *Pvu*II and transcribed in vitro by using T7 RNA polymerase. Bands (248nt and 1136 nt) are RNase E radioactive substrates. All bands are mixed and loaded with 2 µl as marker.

Purification of RNase E

RNase E was purified as described by the manufacturer (pET System Manual, Novagen). Strain CC523 (BL21; DE3, pFUS1500) was grown in 1000 ml of LB rich medium containing 100 µg/ml of ampicillin at 37°C until the OD_{600} had reached 0.6 and induced with 0.4 mM isopropyl β-D-thiogalacto-pyranoside (IPTG) for 3 hours. Cultures were stored on ice for 5 min and then harvested by centrifugation at 5000 x g for 5 min at 4°C. The supernatant was decanted and the cell pellet was allowed to drain as completely as possible. The cells were resuspended in 0.25 culture volume of cold binding buffer (20 mM Tris-HCl pH 7.9; 5 mM imidazole; 0.5 M NaCl), and sonicated (sonicator W-380, Heat System, INC.) until the sample was no longer viscous (about 2 min). The lysate was centrifued at 39,000 x g for 20 min at 4°C to remove debris. The binding buffer was allowed to drain to the top of non-denaturing immobilized metal affinity chromatography (IMAC) and the column was loaded with the extract. A flow rate of about 10 columns per hour was used for purification. The column was washed with 25 ml binding buffer and 15

ml of wash buffer (60 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl pH 7.9) and the bound protein was eluted with 15 ml of elution buffer (1 mM imidazole, 0.2 mM NaCl, 20 mM Tris-HCl). The partially purified RNase E (MW 180 kDa) was analyzed by 7% SDS-PAGE (Fig. 2-3). The fractions containing the RNase E were pooled and then dialyzed in 1 liter of dialysis buffer (50 mM Tris-HCl pH 7.9, 20% glycerol, 1 mM EDTA, 500 mM NaCl, 0.5% Triton X-100, 1 mM DTT) at 4°C overnight. After dialysis, the extract material was concentrated to about 300 µl by centrifugation at 200 x g at 4°C, for 2 hours using Centricon-10 concentrator (cat.# 4206, Amicon, Inc., Beverly, MA).

RNase E assay

RNase E activity was measured as described by McDowall et al (1995) and Cormack and Mackie (1992). Assays were performed at 30°C in of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 0.1% Triton-100, 0.1 mM DTT and 80 µg/ml of yeast tRNA (cat. # 5636, Sigma) in a total reaction volume of 10 µl. RNase E substrate (2 µl) was heated in this buffer for 2 min at 52°C water bath, 10 min at 37°C and then stored on ice. RNase E (0.36 µg) was added and reaction was followed by removal of portions at various times. The reactions were stopped and electrophoresised as described as Carpousis, (1994). To digestion product, 1 volume of stop buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.2% SDS, and 0.1 mg/ml proteinase K) was added and incubated at 52°C water bath for 10 min, and then denatured at 85°C for 10 min by addition of 1 volume of loading buffer (72% formamide, 4 M urea, 20 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), the sample were loaded on 4% polyacrylamide gel (acrylamide:

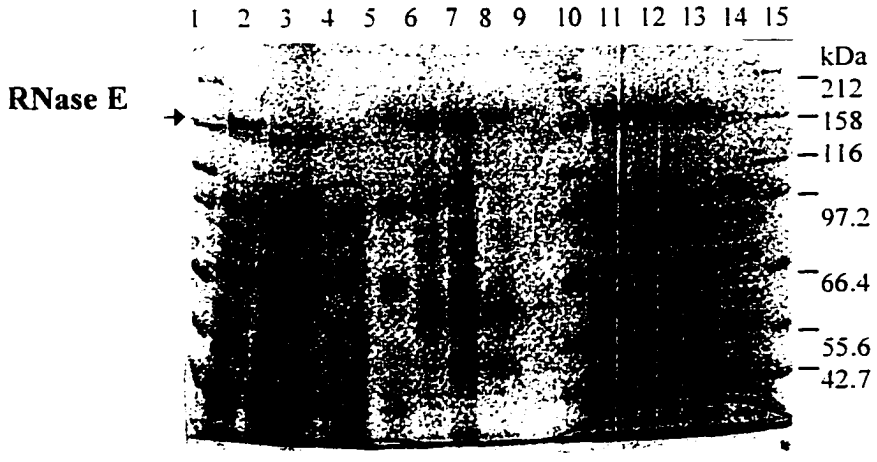


Fig. 2-3. Partial purification of RNase E. Partial purified RNase E were analyzed on 7% SDS-PAGE and stained by Coomassie blue. Lane1, 10, 15: protein marker. Lane 2: extract before purification. Lane 3: fraction of loading flow. Lane 4-5: two washes before elution (binding and wash buffer respectively). Lanes 6-9: different fractions of elution . Lanes 11-13: cells after induction by IPTG at 3, 2, 1 hour respectively. Lane 14: cells before induction by IPTG.

bisacrylamide: 40:2) containing 8 mM urea in Tris-borate-EDTA buffer (TBE) and run for 2 hours. The gel was directly to expose to X-ray film for 2 hours or fixed, dried and exposed to X-ray film.

Northern blots and image acquisitions

After electrophoresis of the RNA (15 μg) on 1% agarose gels, it was transferred by capillary action (Ausubel et al., 1991) in an Altec Labs (Boston, MA) apparatus to nylon membranes (Boehringer Mannheim, cat. # 1209272) with 10 x standard saline citrate (3 M NaCl, 0.3 M sodium citrate), and the membrane was dried in an oven (Blue M, model OV-18A) for 2 hours at 80°C. For all of the Northern Blots, duplicate RNA samples were electrophoresed in parallel lanes and stained with ethidium bromide to confirm that comparable levels of RNA were loaded in each lane. Prehybridization and hybridization with the [^{32}P] labeled oliE probe was described by Huang (1992). The filters were placed in sealable plastic bags (Kapak/Scotchpak cat. # 402) with aqueous hybridization solution (25 mM KPO_4 pH7.4, 5 X SSC, 5 X Denhardt's solution, 10 mM EDTA, 0.5% SDS, 100 μg/ml salmon testes DNA) and prehybridized for at least 4 hours at the 42°C. The prehybridization solution was then removed, the hybridization solution with the appropriate probe labeled with [^{32}P] radioactivity was added to the bag. The filters were incubated overnight at the same temperature as prehybridization, and washed according to the procedure of Gilman (1987). The probes used for the Northern blots was synthetic oligonucleotides derived from sequences encoding the *ilvE* gene product (nt 3113 to 3091; oliE). Prehybridization and hybridization with the riboprobe was

carried out at 68°C following the manufacturers instructions (Boehringer Mannheim, cat. # 1175025). Membranes were blocked at room temperature for 1 hours with 2% blocking reagent (Boehringer Mannheim, cat. # 1096176) and then incubated for 30 min with the anti-DIG-Fab fragments conjugated to alkaline phosphatase. The membranes were washed at room temperature for 30 min in two changes of wash solution (100 mM Tris-HCl, pH 7.0, 150 mM NaCl) , incubated with Lumi-Phos 530 (Boehringer Mannheim, cat. # 1413155) for 1 min and exposed to Kodak X-ray film (Kodak, cat. # 1651454). The X-ray films were scanned with a laser densitometer (Molecular Dynamics, Model PDSI-PC, Sunnyvale, California) and the images of all independent experiments were quantitated and linear exposure ranges were identified using the ImageQuantNT program (Molecular Dynamics). The membranes also can be incubated with color-substrate solution after two changes of wash solution (100 mM Tris-HCl, pH 7.0, 150 mM NaCl). The color-subtrate solution contains 45 µl of NBT-solution [Nitroblue tetrazolium salt, 75 mg/ml in dimethylformamide, 70% (v/v)] and 35 µl of X-phosphate solution (5-Bromo-4-Chloro-3-indolylphosphate toluidinium salt, 50 mg/ml in dimethylformamide) in 10 ml of buffer (10 mM Tris-HCl, 1 mM EDTA pH8.0). When the desired bands are detected, stop the reaction by washing the membrane for 5 min with 50 ml of buffer (10 mM Tris-HCl, 1 mM EDTA pH8.0) and membane may be dried in room temperature. Bands can not quantitated by this method.

TABLE 2-1. Bacterial strains used in experiments

Strain	Genotype	Plasmid(s)	Reference
BL21	*ompT*$r_B^-m_B^-$, F^-.	None	Studier and Moffatt, 1986
BL321	F^-, *thi-1, argH1, gal-6, lacY1, mtl-2, xyl-7, malA1, ara-13, str-9, tonA2,* λ^-, *supE44, rnc-105.*	None	Studier, 1975
BL322	F^-, *thi-1, argH1, gal-6, lacY1, mtl-2, xyl-7, malA1, ara-13, str-9, tonA2,* λ^-, *supE44,* rnc^+.	None	Studier, 1975
C600	F^-, *thi-1, thr-1, leuB6, lacY1, tonA21, supE44,* λ^-.	None	Appleyard, 1954
CC84	*rbs-301*::Tn5, Δ*ilvGMEDA732::Tn5-131.*	pJG51	This work
CC147	*lacZ43, relA1, spot1, thi-1,* $rne\text{-}3071^{ts}$ derived from strain N3431.	pJG51	Huang et al., 1992
CC148	*lacZ43, relA1, spot1, thi-1,* $rne\text{-}3071^{ts}$; derived from strain N3431.	pCC17	Huang et al., 1992
CC156	*lacZ43, relA1, spot1, thi-1,* rne^-, derived from strain N3433.	pJG51	Huang et al., 1992
CC221	*rbs-301*::Tn5, Δ*ilvGMEDA732*::Tn5-*131*, derived from strain FD1054.	pCC36	Huang et al., 1992
CC253	*araD139,* Δ(*ara leu*)*7697,* Δ*lacX74, galU, galK, rpsL, rho-115.*	pJG51	Calhoun et al., 1985
CC254	*araD139,* Δ(*ara leu*)*7697,* Δ*lacX74, galU, galK, rpsL, rho-115.*	pCC40	Calhoun et al., 1985
CC256	*araD139,* Δ(*ara leu*)*7697,* Δ*lacX74, galU, galK, rpsL,* rho^+.	pJG51	Huang et al., 1992

TABLE 2-1 (con't)

Strain	Genotype	Plasmid(s)	Reference
CC257	*araD139*, Δ(*ara leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *rpsL*, *rho*$^+$.	pCC40	Calhoun et al., 1985
CC283	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnp*$^+$; derived from NHY312.	pJG51	Calhoun et al., 1985
CC285	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnp*$^+$; derived from NHY312.	pCC40	Huang et al., 1992
CC287	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnpA49*^{ts}; derived from NHY322.	pJG51	Huang et al., 1992
CC289	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnpA49*^{ts}; derived from NHY322.	pCC40	Huang et al., 1992
CC325	F$^-$, *thi-1*, *argH1*, *gal-6*, *lacY1*, *mtl-2*, *xyl-7*, *malA1*, *ara-13*, *str-9*, *tonA2*, $^-$, *supE44*, *rnc-105*; transformant of strain BL321.	pJG51	Huang et al., 1992
CC326	F$^-$, *thi-1*, *argH1*, *gal-6*, *lacY1*, *mtl-2*, *xyl-7*, *malA1*, *ara-13*, *str-9*, *tonA2*, $^-$, *supE44*, *rnc*$^-$; transformant of strain BL322.	pJG51	Huang et al., 1992
CC327	*leu*$^-$, *thr*$^-$, Cam^r, *pnp-7*, *rnb-500*^{ts}; derived from SK5003.	pJG51	Huang et al., 1992
CC328	*leu*$^-$, *thr*$^-$, Cam^r, *pnp-7*, *rnb*$^-$. derived from SK5004.	pJG51	Huang et al., 1992
CC329	*leu*$^-$, *thr*$^-$, Cam^r, *pnp*$^-$, *rnb-500*^{ts}. derived from SK5005.	pJG51	Huang et al., 1992
CC330	*leu*$^-$, *thr*$^-$, Cam^r, *pnp*$^+$, *rnb*$^+$. derived from SK5006.	pJG51	Huang et al., 1992
CC475	A Val^R transductant (*ilvGMEDA*; Val^R) of strain N3431 (*lacZ43*, *relA1*, *spot1*, *thi-1*, *rne-3071*^{ts}) using P1.CU2501 (Val^R).	None	This work

TABLE 2-1 (con't)

Strain	Genotype	Plasmid(s)	Reference
CC479	A Val^R transductant (*ilvGMEDA*; Val^R) of strain N3433 (*lacZ43, relA1, spot1, thi-1, rne*$^+$) using P1.CU2501 (Val^R).	None	This work
CC500	A Val^R transductant (*ilvGMEDA*; Val^R) of strain NHY322 (Δ[*proB-lac*], *ara, gyrA, thi, zic-501*:: Tn10, *rnp*$^+$, *ilvGMEDA*; Val^R) by P1.CU2501 (Val^R) transduction.	None	This work
CC502	A Val^R transductant (*ilvGMEDA*; Val^R) of strain NHY322 (Δ[*proB-lac*], *ara, gyrA, thi, zic-501*:: Tn10, *rnp*A49^{ts}, *ilvGMEDA*; Val^R) by P1.CU2501 (Val^R) transduction.	None	This work
CC513	A Val^R transductant (*ilvGMEDA*; Val^R) of strain BL321 (*thi-1, argH1, gal-6, lacY1, mtl-2, xyl-7, malA1, ara-13, str-9, tonA2*, λ$^-$, *supE44, rnc-105, ilvGMEDA*; Val^R) by P1.CU2501 (Val^R) transduction.	None	This work
CC515	A Val^R transductant (*ilvGMEDA*; Val^R) of strain BL322 (*thi-1, argH1, gal-6, lacY1, mtl-2, xyl-7, malA1, ara-13, str-9, tonA2*, λ$^-$, *supE44, rnc*$^+$, *ilvGMEDA*; Val^R) by P1.CU2501 (Val^R) transduction, F$^-$.	None	This work
CC523	λDE3 lysogen of strain BL21.	pFUS1500	McDowall et al., 1995
CU2501	*rbs-200 ilvG468* (Val^R).	None	Vonder Haar et al., 1972
CU4	*galT12*, λ$^-$.	None	Calhoun, 1976

TABLE 2-1 (con't)

Strain	Genotype	Plasmid(s)	Reference
FD1054	*rbs-301*::Tn*5*, Δ*ilvGMEDA732*::Tn*5-131*.	None	Gayda et al., 1980
N3431	*lacZ43*, *relA1*, *spot1*, *thi-1*, *rne-3071*^{ts}.	None	Mudd et al., 1990a
N3433	*lacZ43*, *relA1*, *spot1*, *thi-1*, *rne*$^{-}$.	None	Mudd et al.,1990a
NHY312	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnp*$^{+}$.	None	Kirsebon et al., 1988
NHY322	Δ(*proB-lac*), *ara*, *gyrA*, *thi*, *zic-501*:: Tn10, *rnpA49*^{ts}.	None	Kirsebon et al., 1988
SK5003	*leu*$^{-}$, *thr*$^{-}$, Cam^{r}, *pnp-7*, *rnb-500*^{ts}.	None	Donovan et al., 1986
SK5004	*leu*$^{-}$, *thr*$^{-}$, Cam^{r}, *pnp-7*, *rnb*$^{-}$.	None	Donovan et al., 1986
SK5005	*leu*$^{-}$, *thr*$^{-}$, Cam^{r}, *pnp*$^{-}$, *rnb-500*^{ts}.	None	Donovan et al., 1986
SK5006	*leu*$^{-}$, *thr*$^{-}$, Cam^{r}, *pnp*$^{-}$, *rnb*$^{-}$.	None	Donovan et al., 1986

TABLE 2-2. Plasmids used and constructed in the experiments

Plasmid	Description	Reference
pAM18	cloning vector derived from pSP65 by the insertion of a promoter for T7 RNA polymerase and two new cloning sites (*Sph*I and *Kpn*I).	Amersham, Inc.
pCC73	*Hind*III fragment ('*ilvED*') from pJG51 cloned to plasmid pAM18, the opposite orientation as T7 promoter.	This work
pCC75	*Hind*III fragment ('*ilvED*') from pJG51 cloned to plasmid pAM18, the same orientation as T7 promoter.	This work
pJG16	*Eco*RI segment from *ilv581* (Val^R) cloned to pBR322, *ilvGMEDA* (Val^R).	Gray et al., 1982
pJG51	tandem *Eco*RI segments from λ*c112* cloned to pBR322, *ilvGMEDAYC* (Val^S).	Gray et al., 1982
pCC17	pJG51 with *Nsi*I-*Kpn*I deletion in *ilvG* (Val^R).	Huang, 1992
pCC36	*ilvGMEDAYC* (Val^R) from a Val^R transductant of strain CC84 [pJG51 (Val^S) in strain FD1054] with P1.CU2501 (*ilvG468*, Val^R)	Huang, 1992
pCC40	replace *Nsi*I-*Kpn*I fragment in pJG51 (Val^S) with same fragment from pJG16, *ilvGMEDAYC* (Val^R).	Huang et al., 1992
pFUS1500	*rne* was cloned into translation pET-16b vector which contains the highly efficient ribosome binding site from the phage T7 maior capsid protein and cloning site adjacent to the His-Tag sequence.	McDowall et al., 1995

TABLE 2-2 (con't)

Plasmid	Description	Reference
pRC9S	the PCR product corresponding to the 9S sequence from *rrn*B operon with *Hind*III and *Acc*I was subcloned into the vector pSP65 within the multiple cloning sites.	Cormack and Mackie. 1992

CHAPTER 3

RESULTS

Detection of all four *ilvGMEDA* transcripts with a single DIG-labeled riboprobe: Plasmid pCC36 (Val^R) expression

Several different [^{32}P]-labeled synthetic oligonucleotides and asymmetric polymerase chain reaction (PCR) products were previously used as gene specific probes to detect and identify the two major and two minor *ilvGMEDA* transcripts expressed from multicopy plasmids (Huang, 1992). Since none of the previously used gene specific probes hybridized to all four transcripts we selected an internal *ilv'ED' Hind*III fragment as a probe that would be complementary to all four transcripts. Also, in the present experiments an antisense RNA probe coupled to an enzymatic amplification protocol increased the sensitivity and permitted detection of transcripts from single copy *ilvGMEDA* chromosomal genes. Using these methods to monitor expression, we detected similar levels of the same four transcripts previously reported (Huang, 1992) from the single chromosomal copy of the *ilvGMEDA* genes (Figs. 3-3; 3-4; 3-5; 3-9; 3-14; 3-15; 3-17; 3-20) and multicopy plasmids (Figs. 3-1; 3-2; 3-6; 3-7; 3-8; 3-10; 3-11; 3-16; 3-18). We also confirmed the previous reports (Huang, 1992, Huang et al., 1992) that the *ilvEDA* and *ilvE* transcripts are usually detected, but the *ilvGMEDA* and *ilvDA* transcripts are often not detected due to their low abundance. Also, in a direct comparison, the RNA probe was found to be a more sensitive method than the use of [^{32}P] labeled oliE probe (Fig. 3-1). The DIG- labeled probe can detect the signal about 10 min by addition of color-substrates (Fig. 3-1, lanes 3 and 4). But the [^{32}P] labeled

oliE probe can detect signal by exposure to X-ray film about 65 hours with two screen (lanes 5 an 6).

Effects of end product amino acids upon the *ilvGMEDA* transcripts:

Multicopy plasmids with the Val^S or Val^R allele

The *ilvGMEDA* gene cluster of *E. coli* K-12 are multivalently regulated by the percent aminoacylation of $tRNA^{Val}$, $tRNA^{Ile}$ and $tRNA^{Leu}$ (Smith et al., 1979). The expression of the entire operon is specifically regulated by the presence of all or the absence of any one of the branched-chain amino acid via translational control of transcription termination at an attenuator site preceding *ilvG*, the first structural gene of the operon (Gayda et al., 1980; Harms and Umbarger, 1991; Lawther et al., 1980). In order to determine the mRNA level of the *ilvGMEDA* gene cluster under the various repression growth conditions, strain FD1054 derivatives (with the chromosomal *ilvGMEDA* genes deleted), harboring pJG51 containing Val^S allele or pCC36 containing Val^R allele were used. RNA (15 μg) was isolated from strains CC84 (strain FD1054 with plasmid pJG51) and CC221 (strain FD1054 with plasmid pCC36) grown in either minimal medium containing no branched chain amino acids (Fig. 3-2, lane M), or two branched-chain amino acids in repressing concentration and one in limiting concentrations of the three end products (Fig. 3-2, lane L, I, V.). The mRNA level was analyzed by using Northern blots. For all of the Northern Blots, duplicate RNA samples were electrophoresed in parallel lanes and stained with ethidium bromide to confirm that comparable levels of RNA were loaded in each lane (e. g., Fig. 3-2; Panel C). The levels

of mRNAs in strain CC221 (pCC36, Val^R) was five-fold to ten-fold higher than it in Val^S strain CC84 (pJG51, Val^S; Fig. 3-2). With wild-type CC84 (pJG51, Val^S), the *ilv*-specific attenuation control was observed. About five-fold depression was observed when isoleucine (Fig. 3-2, lane 8) and three amino acid were limiting (Fig 3-2, lane 6). A two-fold derepression was observed when valine was limiting (Fig. 3-2, lane 10). Leucine-mediated derepression was low. These results indicate that end-product amino acids affect the transcription initiated from the *ilvGp2* promoter. In strain CC221, derepression was observed when isoleucine was limiting (Fig. 3-2, lane 3).

Single chromosomal gene cluster with the Val^S or Val^R allele

Strains CU4 and CU2501 were used to monitor effects of supplementation with branched-chain amino acids on *ilv* transcripts from the single copy chromosomal gene cluster (Fig. 3-3). Similar results were observed as when using [^{32}P]-labeled oligo was used as probe (Huang, 1992). In the wild type strain CU4 (Val^S), there was the highest, intermediate, lower level of mRNAs in the isoleucine limiting, absence of three end products and a limiting concentration of leucine, valine, respectively (Fig. 3-3). In the mutant strain CU2501, the mRNA levels were higher than those in the Val^S strain except isoleucine limiting (Fig. 3-3, Lane 3). The results also indicated there was highest level of mRNA in the absence of the three end products (Fig. 3-3, lane 6), intermediate levels under limiting concentration of leucine or valine (Fig. 3-3, lane 9, 10), and lower levels in excess end products (Fig. 3-3, lane 7) and under a limiting concentration of isoleucine (Fig.3-3, lane 8).

Effects of a RNase E on the *ilvGMEDA* transcripts:

Single chromosomal gene cluster with the Val^R allele

The effects of RNase E on *ilv* transcripts was tested in Val^R strains CC475 (*rne-3071^{ts}*) and CC479 (*rne$^+$*), which have a single copy of the *ilvGMEDA* gene cluster with the Val^R allele on chromosome were used (Fig. 3-4). Four transcripts were detected in mutant *rne-3071^{ts}* and in wild type *rne$^+$* strains just before addition of rif (Fig. 3-4, lane 2 and lane 7). The *ilvE* transcript had a longer half-life in *rne-3071^{ts}* strain than it in *rne$^+$* strain (Fig. 3-4, compare lanes 1-5 with lanes 6-10).

RNase E: Single chromosomal gene cluster with the Val^S allele

To study the effects of RNase E on *ilv* transcripts in Val^S isogenic strains N3431 (*rne-3071^{ts}*) and N3433 (*rne$^+$*) which carried a single copy of the *ilvGMEDA* gene cluster with the Val^S allele on the chromosome were also examined. The expected results were seen: Expression of the *ilvGMEDA* gene cluster was lower than in the Val^R strain (compare Fig. 3-4 and Fig. 3-5). The *ilv* mRNA levels were higher in the *rne-3071^{ts}* strain after the temperature shift before addition of rif (Fig. 3-5: compare lanes 2 and 7), but the mRNA levels were too low to detect in either strain after rif addition.

RNase E: Multicopy plasmids with the Val^R allele

Strains CC261 and CC263 were grown at 30°C to logarithmic-phase and shifted to 43°C for 15 min and rif was added. The total RNA was extracted from aliquots withdrawn at various times after addition of rif to 43°C cell cultures and analyzed using

Northern blots (Fig. 3-6; Panel A). The *ilvE* (1.1 kb) was more stable in *rne-3071*^{ts} than in *rne*$^{+}$ strains. However there was an *ilvE* transcript accumulation in mutant strain *rne-3071*^{ts} (Fig. 3-6; Panel A, compare lane 1 and lane 2) which also was found in mutant strain CC148 (*rne-3071*^{ts}, pCC17) (Fig. 3-8, lane 5 and lane 6).

RNase E: Multicopy plasmids with the Val^{S} allele

The temperature-sensitive mutation in *rne-3071*^{ts}, was used to test effects of RNase E on the stability of the *ilvGMEDA* transcripts. Both mutant strain N3431 (*rne-3071*^{ts}) and wild type strain N3433 (*rne*$^{+}$) containing pJG51 were grown at 30°C to logarithmic-phase and shifted to 43°C for 15 min, and rif was added. Total RNA was extracted from aliquots withdrawn at various time after addition of rif and analyzed using Northern blots (Fig. 3-7; Panel A). RNAs were hybridized with the riboprobe and bands were quantified using a densitometric scanner (Fig. 3-7; Panel B). Four transcripts were detected on both strains at 0 min (Fig. 3-7, lane 1 and lane 5). The *ilvE* (1.1 kb) transcript was more stable in *rne-3071*^{ts} strain than in the *rne*$^{+}$ strain (Fig. 3-7, compare lanes 1-2 to lanes 5-6). Similar results were obtained using [^{32}P]-labeled probe (Huang, 1992). It is concluded that RNase E is involved in stabilization of the *ilvE* transcript. The *ilvGMEDA* (6.7 kb), *ilvEDA* (4.5 kb) and *ilvDA* (3.5 kb) transcripts in both strains were undetectable after 5 min (Fig. 3-7, lane 2 and lane 6).

RNase E: Multicopy plasmids with Val^{S} or Δframeshift alleles

Monitoring the reduction of transcript levels after rifampicin addition to growing

cultures provides an estimate of mRNA stability. In addition, if a precursor to product relationship exists and one of the larger transcripts is processed to a smaller transcript, the smaller transcript may exhibit a longer apparent half life, or even a transient increase in abundance, after rif addition. Fig. 3-8 shows the fate of the *ilvGMEDA* transcripts following rif addition to the RNase E mutants containing plasmid pCC17 (which has the polar frameshift site in *ilvG* removed by an in frame 1106 bp *Nsi*I to *Kpn*I deletion within the *ilvG* gene; see Fig. 1-2) and plasmid pJG51 (with a Val^S allele). As expected, all transcripts were present at lower levels in the Val^S (Fig. 3-8, lanes 1-4) compared to the frameshift deletion (Fig. 3-8, lanes 5-8). An increase (3.3-fold; Fig. 3-8, Panel B) in the *ilvE* transcript is apparent at 5 min after rif addition (Fig. 3-8, compare lanes 5 and 6; 5 min exposure) for the strain with plasmid pCC17, while the larger transcripts have decreased in intensity. The *ilvE* transcript appears to be more stable than the other three transcripts, and we interpret this to be due, at least in part, to continued formation of the transcript by processing of larger precursor(s) after rif addition. The *ilvE* transcript could be produced from the *ilvEDA* transcript by (i) endonucleolytic cleavage to generate a stable 5' *ilvE* segment and unstable 3' *ilvDA* segment, or by (ii) 3' exonucleolytic removal of the *ilvDA* segment with termination of exonucleolytic processing at the postulated stem loop structure (Cox et al., 1987) between *ilvE* and *ilvD*.

Effects of RNase P on *ilvGMEDA* transcripts in vivo:

Single chromosomal gene cluster with the Val^S or Val^R allele

RNase P holoenzyme in *E. coli*, which was first identified in the processing of

precursor tRNA to generate the 5' terminal of mature tRNA by cleavage of 5' terminal extra nt, consists of a basic protein C5 (molecular weight 13,800) and an RNA, M1RNA, which contains 377 nucleotides (Kirseborn et al., 1988). RNase P involvement in mRNA degradation was first reported in the processing of polycistronic mRNA of the histidine operon of *E. coli* (Alifano et al, 1994). Expression was monitored from a single chromosomal copy of the *ilvGMEDA* genes containing either the Val^S or Val^R alleles (Fig. 3-9, lanes 1-10 and 11-20, respectively) in isogenic hosts with or without the temperature sensitive RNase P allele (*rnpA49*^{ts}).

As expected, transcript levels were higher when the frameshift site was eliminated by mutation in the Val^R (Fig. 3-9, lanes 1-10) compared to the Val^S (Fig. 3-9, lanes 11-20). The RNase P allele had two effects upon the *ilvGMEDA* coded transcripts. The first was an overall decrease in *ilv* transcript levels that was not predicted (Fig. 3-9, compare lanes 1-5 to lanes 6-10, and lanes 11-15 to lanes 16-20), and is due to pleiotropic effects of the mutation upon growth. This temperature sensitive allele does affect the growth rate even at 30°C, with doubling times of 36 and 24 min for the mutant and wild type, respectively, under these growth conditions (Fig. 3-13). The second effect of the RNase P mutation was an increased stability of *ilv* transcripts detected after rif addition. For the Val^R strain with the *rnpA49*^{ts} allele, the shift from 30°C to 43°C leads to a two- to three-fold decrease in levels of the *ilvGMEDA*, *ilvEDA*, and *ilvDA* transcripts, but the *ilvE* transcript levels increased about 2.5-fold (Fig. 3-9, compare lanes 6 and 7). A transient increase in abundance of the *ilvE* transcript was detected two minutes after rif addition for the Val^S strain with the RNase P mutation (Fig. 3-9, lane 17 and lane 18). These results

suggest a role for RNase P in *ilv* transcript formation, and indicate that the increased levels of the *ilvE* transcript formed after rif addition is due at least in part to an RNase P dependent processing event.

RNase P: Multicopy plasmids with the Val^R allele

The isogenic strains NHY322 (*rnpA49^{ts}*) and NHY321 (*rnp$^-$*), harboring the pCC40 (Val^R) plasmid were used to examine the effect of RNase P on the stability of the *ilvGMEDA* coded transcripts (Fig. 3-10). Growth condition and assays were the same as in Fig 3-11. The *ilvE* transcript was more stable in mutant (*rnpA49^{ts}*) strain than in wild type (*rnp$^+$*) strain (Fig. 3-10. lanes 5-8 and lanes 1-4). The *ilvEDA* transcript could not be detected in either strains 5 min after addition of rif.

RNase P: Multicopy plasmids with the Val^S allele

Plasmid pJG51 was transformed into mutant strain NHY322 (*rnpA49^{ts}*) and isogenic strain NHY321 (*rnp$^-$*) to generate strains CC287 and CC283, respectively. Cells were grown to log phase at 30°C, and shifted to 43°C for 10 min before addition rif. Cells were harvested at different time after addition rif. Total RNA was isolated and 15 μg is used for Northern blots (Fig. 3-11; Panel A). Riboprobe was used in hybridization. Four transcripts were detected in both strains (Fig. 3-11, lane 1 and lane 5). The *ilvE* and *ilvEDA* had longer half-lives in the mutant strain than in the wild-type strain (Fig. 3-11, compare lanes 1-3 with lanes 5-7). This results confirmed that RNase P is involved in the stability of the *ilvE* and *ilvEDA* transcripts. Similar results had been seen by using a

[^{32}P]-labeled oligo probe (Huang, 1992).

Growth rates of *rne* mutant and *rnp* mutant strains

Strains CC475 (*rne-3071^{ts}*, Val^{R}) or CC479 (*rne^{+}*, Val^{R}) were grown under same conditions as N3431 (*rne-3071^{ts}*, Val^{S}) and N3433 (*rne^{+}*, Val^{S}) (Fig. 3-12). Similar doubling times were found in Table 3-1 in strain CC475 (*rne-3071^{ts}*, Val^{R}) and N3431 (*rnp-3071^{ts}*, Val^{S}) or strain CC479 (*rne^{+}*, Val^{R}) and N3433 (*rne^{+}*, Val^{S}).

Strains CC502 (*rnpA49^{ts}*, Val^{R}) and CC500 (*rnp^{+}*, Val^{R}), were grown under same conditions as the isogenic pair of strains NHY322 (*rnpA49^{ts}*, Val^{S}) and NHY312 (*rnp^{+}*, Val^{S}) (Fig. 3-13). Similar growth rate was found in Table 3-1 in transductant strain CC502 (*rnpA49^{ts}*, Val^{R}) and strain NHY322 (*rnpA49^{ts}*, Val^{S}) or strain CC500 (*rnp^{+}*, Val^{R}) and parental NHY312 (*rnp^{+}*, Val^{S}).

Table 3-1. List of doubling time of strains

	30°C	43°C
NHY312 (*rnp^{+}*, Val^{S})	24 min	16 min
NHY322 (*rnpA49^{ts}*, Val^{S})	36 min	60 min
CC500 (*rnp+*, Val$^{R)}$	24 min	18 min
CC502 (*rnpA49^{ts}*, Val^{R})	36 min	66 min
N3433 (*rne+*, Val^{S})	41 min	54 min
N3431 (*rne-3071^{ts}*, Val^{S})	45 min	105 min
CC479 (*rne+*, Val^{R})	64 min	42 min
CC475 (*rne-3071^{ts}*, Val^{R})	65 min	108 min

As expected, the temperature sensitive mutants grew more slowly when shifted to the higher non-permissive temperature, while the isogenic wild type strains grew more rapidly at the higher temperature. At 30°C, the temperature sensitive strains grew more slowly (*rnpA49*^{ts}) or at about the same rate (*rne-3071*^{ts}) compared to the isogenic wild type, indicating some affect of the mutation on cell metabolism even at 30°C.

Effects of RNase III on *ilvGMEDA* transcripts in vivo:

Single chromosomal gene cluster with the Val^R allele

RNase III, the *rnc* gene product of *E. coli*, was initially reported to specifically degrade double-stranded ribonucleic acid (Studier, 1975). It was later reported to affect specific mRNAs (Bardwell et al., 1989; Portier et al., 1987; Schmeissner et al., 1984). More recently, more bacterial mRNAs have been found that are also cleaved by RNase III, and this affects mRNA half-life time and the level of gene expression (Court, 1993) by controlling of messenger RNA stability (Belasco and Brawerman, 1993). Strain BL321 (*rnc*-150) and its parent strain BL322 (*rnc*$^+$) were constructed by transduction using phage P1 grown on strain CU2501 which contains the Val^R allele in the *ilvGMEDA* gene cluster. They were designated CC513 (*rnc*-150) and CC515 (*rnc*$^+$). There were no effects of RNase III on the *ilvGMEDA* transcripts stability directed by a single chromosomal gene cluster with the Val^R allele (Fig. 3-14). A pleiotrophic effect of the *rnc-105* mutation caused a decrease in the *ilvGMEDA* coded transcripts (Fig. 3-14, compare lanes 1 and 5). This pleiotropic effect results from defects in ribosomal RNA

processing due to the RNase III defect.

RNase III: Single chromosomal gene cluster with the Val^S allele

In order to further examine potential effects of RNase III on *ilv* transcripts stability directed by a single chromosomal gene cluster with the Val^S allele, BL321 (*rnc-150*) and its parent strain BL322 (*rnc*$^+$) were used (Fig. 3-15). The results indicated an absence of an effect of an RNase III on the *ilvGMEDA* transcripts stability directed by a single chromosomal gene cluster with the Val^S allele. The pleiotropic effect of the mutation on the *ilvGMEDA* transcript level was also observed as in Fig. 3-14.

RNase III: Multicopy plasmids with the Val^S allele

A RNase III mutation (*rnc-105*) had no apparent effect upon the *ilvGMEDA* transcripts stability as monitored after transcription initiation was blocked by rif addition to strains with a plasmid containing the Val^S allele (Fig. 3-16, compare lanes 1-4 to lanes 5-8). Detectable levels of the *ilvE* and *ilvEDA* transcripts were seen at 5 min after rif addition in the wild type (Fig. 3-16, lane 6), but not the mutant (Fig. 3-16, lane 2). We interpret the difference to be due to the much lower levels of *ilv* transcripts in the mutant (Fig. 3-16, lane 1) compared to the wild type (Fig. 3-16, lane 5). RNase III was observed to have no effect on the *ilvGMEDA* transcripts expressed from a plasmid containing a Val^S allele using the radioactive DNA probes (Huang, 1992).

Effects of RNase II and PNPase on *ilvGMEDA* transcripts in vivo:

Single chromosomal gene cluster with the Val^S allele

RNase II and PNPase are two major 3' to 5' exonucleases in *E. coli*. They degrade RNA processively to mononucleotides by attacking the 3' end of RNAs (Grunberg-Manago, 1963; Spahr and Schlessinger, 1964). In vivo, the PNPase and RNase II can substitute for each other to degrade the same mRNAs (Donovan and Kusher, 1986). This probably explains why the *pnp* and the *rnb* single mutant strains are viable while the thermosensitive *pnp rnb* double mutant is lethal at non-permissive temperature (Donovan and Kusher, 1986). Strain SK5003, with mutations in RNase II (*rnb-500*^{ts}) and PNPase (*pnp-7*), had transcripts with longer apparent stability compared to the isogenic wild type strain SK5006 (Fig. 3-17; Panel A, compare 0 min and 2 min time points for the wild type and mutant), as seen for these Val^S strains monitored from the single chromosomal copy of the *ilvGMEDA* genes. Higher levels of the transcripts prior to the temperature shift were present in the strain containing the defects in RNase II and PNPase (Fig. 3-17, compare -15 min for the mutant and wild type). This accumulation of transcripts in the mutant during log phase growth is in accord with the longer apparent stability in the presence of these mutations seen after rif addition.

RNase II and PNPase: Multicopy plasmids with the Val^S allele

Double mutant SK5003 (*pnp-7*, *rnb-500*^{ts}), a single mutant SK5004 (*pnp-7*) and wild-type SK5006 (*pnp*$^+$, *rnb*$^+$) strains, which harboring pJG51, were used to examine the stability of the *ilvGMEDA* transcripts. Cells were grown to log phase at 30°C, and

shifted to 44°C for 10 min before addition rif. In the double mutant strain CC327 (pJG51, *rnb-500*^{ts}, *pnp-7*), the *ilv* transcripts were more stable compared to the isogenic strain CC330 (pJG51, *rnb*$^{+}$, *pnp*$^{+}$) (Fig 3-18, lanes 2-5 and lanes 12-15). The mRNA levels were slightly higher in a single *pnp-7* mutant strain than in the wild-type (*pnp*$^{+}$, *rnb*$^{+}$) strain (Fig 3-18, lanes 7-10 and lanes 12-15). The mRNA levels in a single *rnb-500*^{ts} mutant strain and wild-type (*pnp*$^{+}$, *rnb*$^{+}$) strain were almost the same (data not shown). These results indicate that PNPase or PNPase/RNase II are involved in degradation of the *ilvGMEDA* transcripts. Note that the *ilvEDA* transcript level increases two min after rif addition in the double mutant (Fig. 3-18, lanes 2-3). This result is consistent with the formation of some fraction of the *ilvEDA* transcripts from a *ilvGMEDA* precursor. This result also implicates RNase II/PNPase in the degradation of the *ilvEDA* transcript.

Effects of RNase E on the *ilv'ED'* mRNA in vitro

The in vivo data suggested a role for RNase E in the stability of the *ilvGMEDA* coded transcripts (Figs. 3-4; 3-5; 3-6; 3-7 and 3-8). Therefore, we decided to examine the role of RNase E in vitro. We tested the 1136 nt *ilv'ED'* *Hind*III and *Sma*I fragment as substrates for RNase E. RNase E which was purified from *E. coli* by Ni^{+} affinity chromatography (Fig. 2-3) cleaved the 1136 nucleotides *ilv'ED'* *Hind*III and *Sma*I fragment (Fig. 3-19, lane 7), The positive control is 9S rRNA (Fig. 3-19, lane 3), which is known to be a substrate of RNase E (Cormack and Mackie, 1992). This result agreed with the previous results obtained in vivo (Figs. 3-4; 3-5; 3-6; 3-7 and 3-8), and confirms

RNase E is important in the processing the *ilvGMEDA* transcripts.

Effects of a Rho protein on the *ilvGMEDA* transcripts: multicopy plasmids with the Val^S or Val^R allele

Transcript levels were compared in isogenic strains containing plasmids with the Val^S or Val^R alleles in the presence and absence of the *rho-115* allele to test the prediction (Fig. 1-2) that the full length *ilvGMEDA* transcript is (i) less abundant in the Val^S than in the Val^R, and (ii) reduced by Rho-dependent transcription termination in the Val^S strain. As predicted, the level of the *ilvGMEDA* transcript was higher in the presence of the Val^R allele (Fig. 3-20, lane 4) than for the Val^S allele, where it was below the limit of detection (Fig. 3-20, lane 2). As noted above, it had not been anticipated that the smaller *ilv* transcripts levels would be less abundant with the Val^S allele (Fig. 3-20, lane 2) compared to the Val^R allele (Fig. 3-20, lane 4). Instead, it was only predicted that the Val^R allele would lead to higher levels of the full length *ilvGMEDA* transcripts compared to the Val^S allele. Therefore, the increased levels of the smaller transcripts with the Val^R compared to the Val^S allele (Fig. 3-20, compare lanes 2 and 4) must be due to processing of the more abundant full length *ilvGMEDA* precursor to the smaller transcripts in the Val^R strain.

Partially processed *ilvGMEDA* transcripts were detected between the positions of the full length *ilvGMEDA* transcript and *ilvEDA* transcript in the presence, but not is the absence, of the Rho mutation in the Val^S strain (Fig. 3-20, compare lanes 1-2). This increase in partially processed *ilvGMEDA* fragments reflects an increase in *ilvGMEDA*

formation, as predicted due to the presence of the three Rho-dependent transcription termination sites (Fig. 1-2) in the Val^S strain. The increase in *ilvEDA*, *ilvE*, and *ilvDA* transcript levels due to the presence of the Rho mutation in the Val^S strain (Fig. 3-20, compare lanes 1 and 2) must be indirect, and resulted from the increase in *ilvGMEDA* transcript levels due to reduced Rho-dependent transcription termination in the *ilvGM* region. Therefore, this result confirmed that the *ilvGMEDA* transcripts were precursors to the smaller transcripts.

The *ilvDA* transcript was a prominent band in the Val^R strain (Fig. 3-20, lane 4) compared to the Val^S strain (Fig. 3-20, lane 2), where it was not detected. In contrast, the *ilvEDA* and *ilvE* transcripts were detected, but at lower levels, in the Val^S strain (Fig. 3-20, compare lanes 2 and lane 4). These differences in transcript levels explain, in part, the downstream amplification effects, in which the change in expression ratios comparing the Val^R to the Val^S strains is greater for promoter distal than for promoter proximal genes. This difference in the *ilvDA* transcript levels does not, however, account for the greater change in expression of the *ilvA* gene compared to the *ilvD* gene. We have carried out Western blots to monitor levels of the *ilvEDA* gene products under various growth conditions (L.-B. Zhou and D. H. Calhoun, unpublished). These studies revealed that the *ilvD* gene product (dihydroxyacid dehydrase), but not the *ilvE* and *ilvA* gene products (transaminase B and threonine deaminase, respectively) was relatively unstable in vivo, and a significant fraction of the enzyme was present as partially degraded, catalytically inactive polypeptide fragments. This result indicates that the *ilvDA* transcript produces more active enzyme product from the *ilvA* gene than from the *ilvD*

gene. Thus, the higher levels of the *ilvDA* transcript and the instability in vivo of dihydroxyacid dehydrase together account for the downstream amplification effects in which the relative expression ratios comparing the Val^R to the Val^S strains is *ilvA>ilvD>ilvE.*

Unexpectedly, the *ilvGMEDA* transcript was dramatically reduced to in the presence of the Rho mutation in the Val^R strain (Fig. 3-20, compare lanes 3-4). There are no reports of a Rho-dependent step in the formation of the *ilvGMEDA* transcript. The presence of the Rho mutation also led to a simultaneous reduction in the levels of the *ilvEDA*, *ilvDA*, and *ilvE* transcripts in the Val^R strain (Fig. 3-20, compare lanes 3-4), as expected if the *ilvGMEDA* transcripts were a precursor to the smaller transcripts. The presence of the Rho mutation led to the appearance of additional degradation intermediates (Fig. 3-20, lane 3, noted by arrows to the right) that were not detected in the absence of the Rho mutation (Fig. 3-20, lane 4). These results indicate that in the presence of the mutated Rho factor, some fraction of the full length *ilvGMEDA* transcript was diverted to a pathway leading to degradation rather than processing to the smaller transcripts. The best estimate of the relative rates of processing and degradation of the *ilvGMEDA* transcripts in the presence of the *rho-115* mutation is based on a comparison of the levels of the smaller *ilvEDA*, *ilvDA*, and *ilvE* transcripts (Fig. 3-20, compare lanes 3-4). The levels of the smaller transcripts were clearly reduced in the presence of the *rho-115* mutation (Fig. 3-20, compare lanes 3-4), but they are still higher than in the Val^S strain (Fig. 3-20, compare lanes 2 and 3). Thus, the levels of the smaller transcripts in the Val^R , *rho-115* strain (Fig. 3-20, lane 3) were intermediate between the Val^S , *rho*$^+$

strain (Fig. 3-20, lane 2) and the Val^R , *rho*$^+$ strain (Fig. 3-20, lane 4), indicating that about half of the *ilvGMEDA* transcripts were processed, and half were degraded.

In the presence of the Rho mutation the appearance of the *ilvE* transcript was altered in both the Val^R and Val^S strains (Fig. 3-20, compare lanes 1 to 2 and 3 to 4). The more diffuse appearance of the *ilvE* mRNA and the presence of more rapidly migrating transcripts in the *ilvE* band in the presence of the Rho mutation (Fig. 3-20, compare lane 1 to 2 and 3 to 4) was due to the presence of partially degraded transcripts, indicating that the Rho mutation has subtle effects upon the stability of this mRNA.

Note that the presence of the Rho mutation had opposite effects in the Val^S strain (Fig. 3-20, compare lanes 1 and 2), and the Val^R strain (Fig. 3-20, compare lanes 3-4). The levels of all of the transcripts increased in the Val^S strain due to relief of polarity, and decreased in the Val^R strain due to reduced stability of *ilvGMEDA* precursor. Since the net effect of the Rho mutation was to increase transcript levels in the Val^S strain, it is apparent that the relief of polarity was quantitatively greater than the decrease in stability of the *ilvGMEDA* transcript in the Val^S strain. The interactions of the mutated Rho factor at the frameshift sites in the Val^S strain do not occur in the Val^R strain, and this could alter the destabilizing effects of the altered Rho factor upon the *ilvGMEDA* transcript.

In Val^S strain, the mRNA level of *ilvE* and *ilvEDA* are about 21% and 79% with Rho mutation or 47% and 53% without mutation respectively. In Val^R strain, the mRNA level of *ilvE* and *ilvEDA* are about 17% and 48% with Rho mutation or 26% and 22% without Rho mutation respectively. The mRNA leavel of *ilvDA*, *ilvGMEDA* are also

detected and about 29% and 23% without Rho mutation in Val^R strain respectively.

The *rho-115* allele is defective in RNA binding, including both the high and low affinity sites (Pereira et al., 1995). It is possible that binding of Rho factor to specific or non-specific sites in some transcripts (e.g., *ilvGMEDA*) may protect them from RNase attack. It was reported by Sozhamannan and Stitt (1997) that bulk mRNA stability decreased in some Rho mutants, but specific transcripts were not examined. These authors determined that it was the RNA binding ability of Rho rather than its transcription termination function that affected bulk mRNA lifetime. Several models of mRNA stability propose that transcripts may be protected from nucleolytic degradation by proteins that bind to specific sites (Belasco et al., 1993). It was recently postulated that factors affecting mRNA turnover, including poly (A) polymerase I (Ingle et al., 1996), are present in a multiprotein complex associated with polysomes. Rho factor would also be expected to be present in the polysomes associated with nascent transcripts.

Fig. 3-1. Detection of all four *ilvGMEDA* transcripts with a single DIG-labeled riboprobe: Plasmid pCC36 (Val^R) expression. RNA was isolated from strain CC221 and analyzed on 1% formaldehyde agarose gel. RNA was stained with ethidium bromide (lane 2) or transferred to membrane for hybridization with DIG-labeled *ilv 'ED'* riboprobe and detection with color-substrate solution (lanes 3 and 4) or [^{32}P]-labeled oliE probe and detection by exposing to X-ray film (lanes 5 and 6). All lanes have 15 μg of RNA except lane 1 (1 kb DNA ladder). Hybridization pattern is shown underneath the picture. The dashed arrows indicated the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membranewith ribosomal RNA).

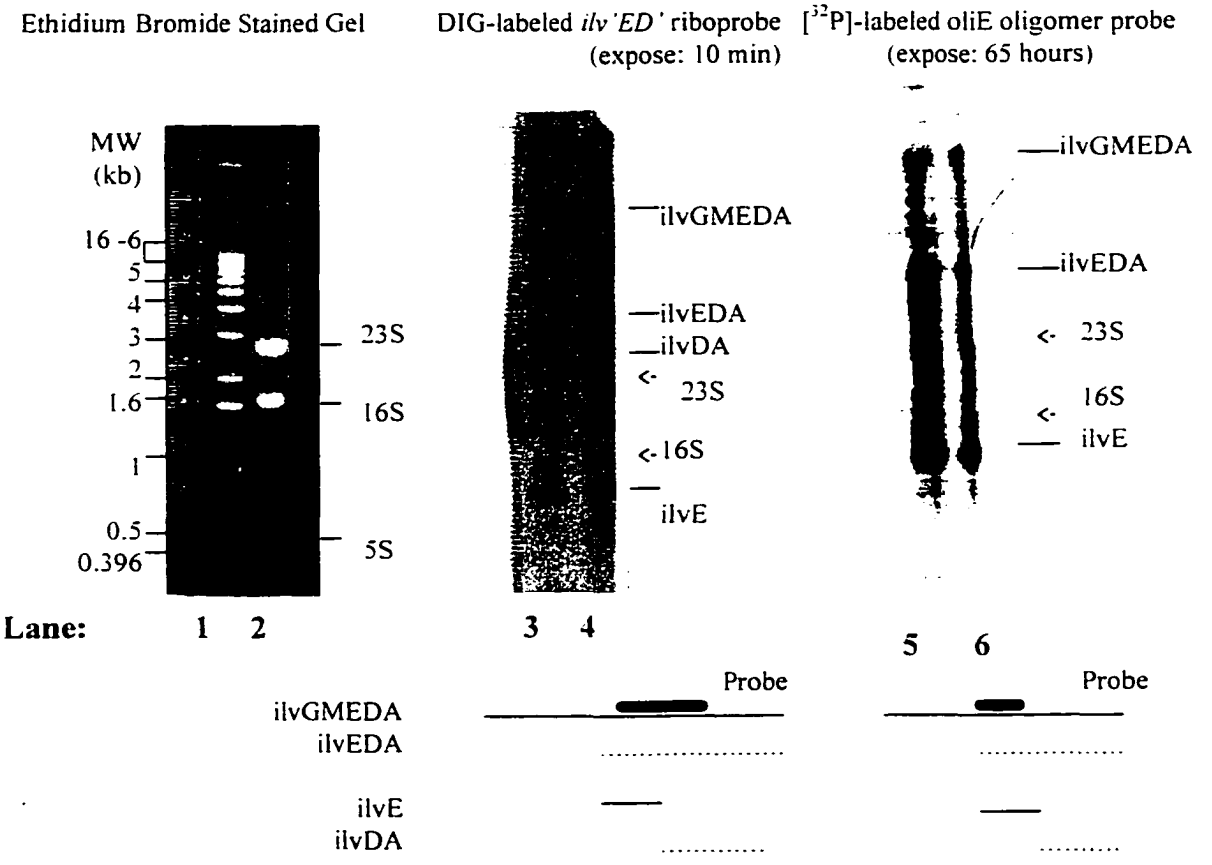
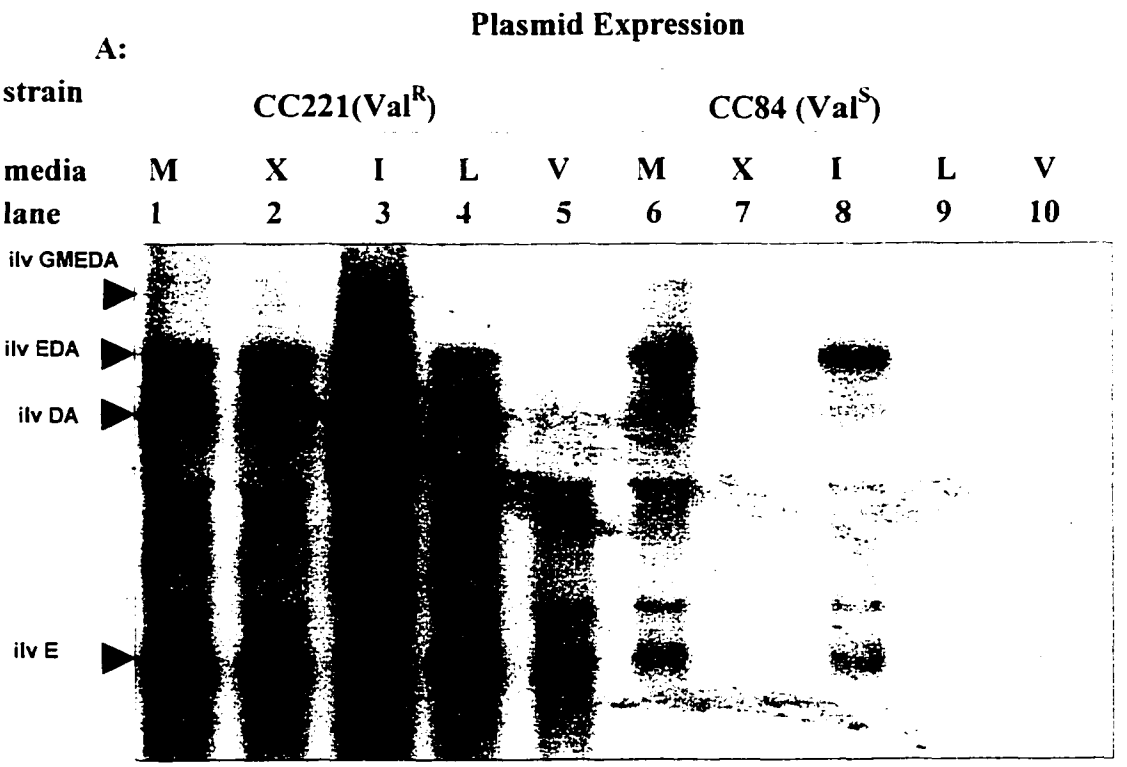
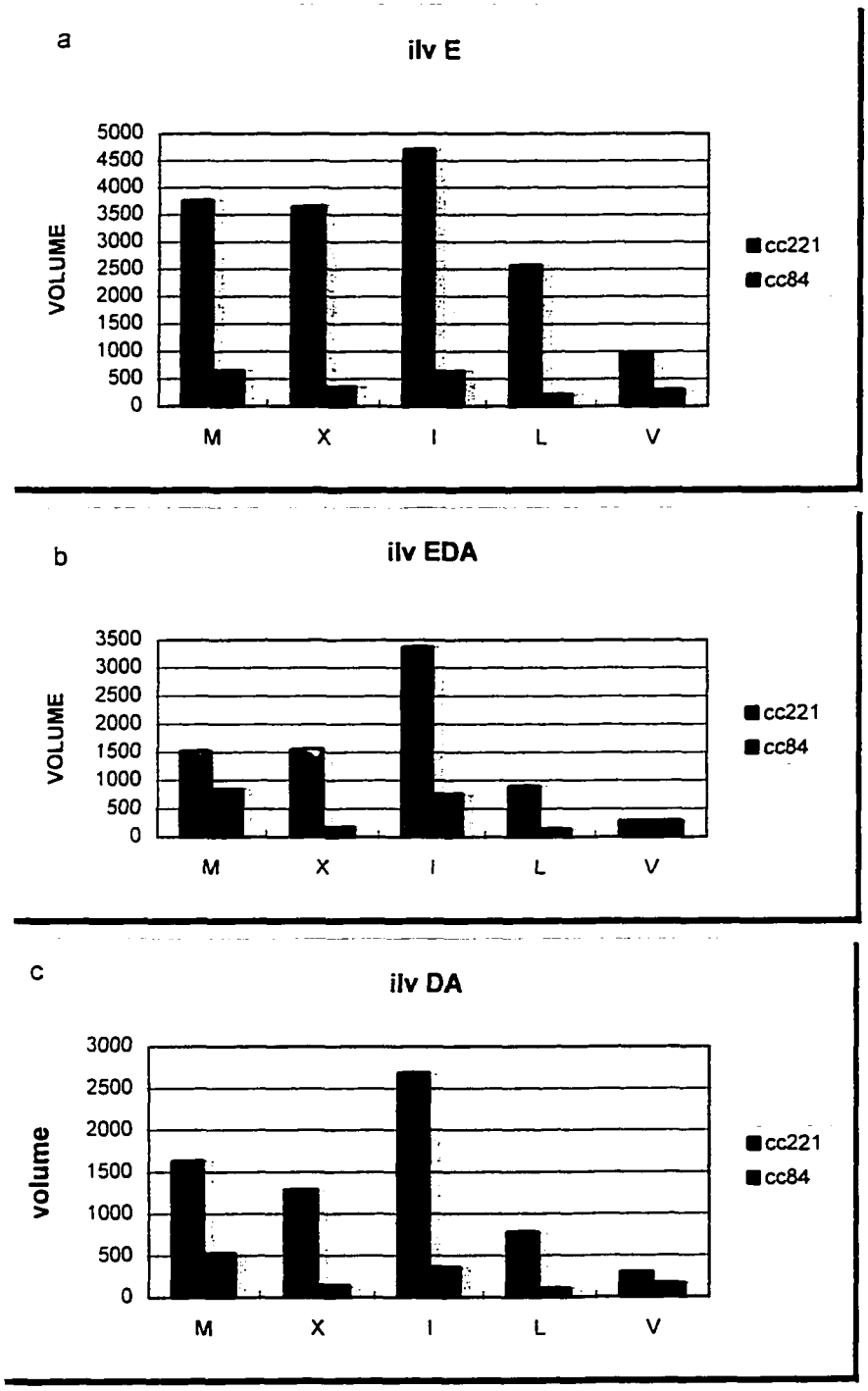


Fig. 3-2. Effects of end product amino acids on *ilvGMEDA* transcripts: Multicopy plasmids with the Val^S or Val^R allele. (A). CC84 (FD1054, pJG51, Val^S) and CC221 (FD1054, pCC36, Val^R) were grown in repression concentration at 37°C until OD_{600} had reached 0.3. Cells were washed by prewarmed minimal medium once and grown at 37°C for 3 hr in different media (**M**, minimal medium without addition any end products amino acids; **X**, with addition of excess three end product amino acids (isoleucine, leucine, valine); **I**, with limitated isoleucine and excess valine, leucine; **L**,with limitated leucine; **V**, with limitated valine). 15 micrograms of total RNA extracted from cells were analyzed by using Northern blots. Riboprobe was used in hybridization. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA .** (a,b,c) The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4. kb), *ilvDA* (3.5 kb) mRMA in CC84, CC221 grown in different media (**M** minimal medium; **X**, excess isoleucine, leucine, valine ; **I** , **L** and **V**, limited isoleucine, leucine and valine respectively) were quantified by densitometer scanner. **(C).** Ethidium bromide stained gel of RNA preparation that was a duplicate gel run in parallel with the Nothern blots.



B:



C:

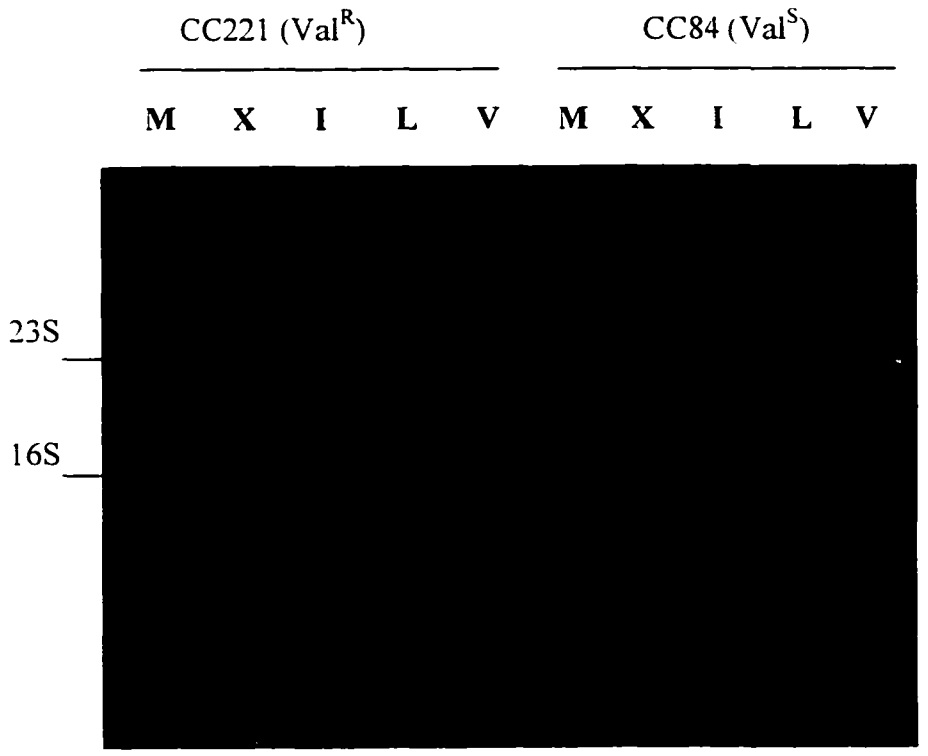
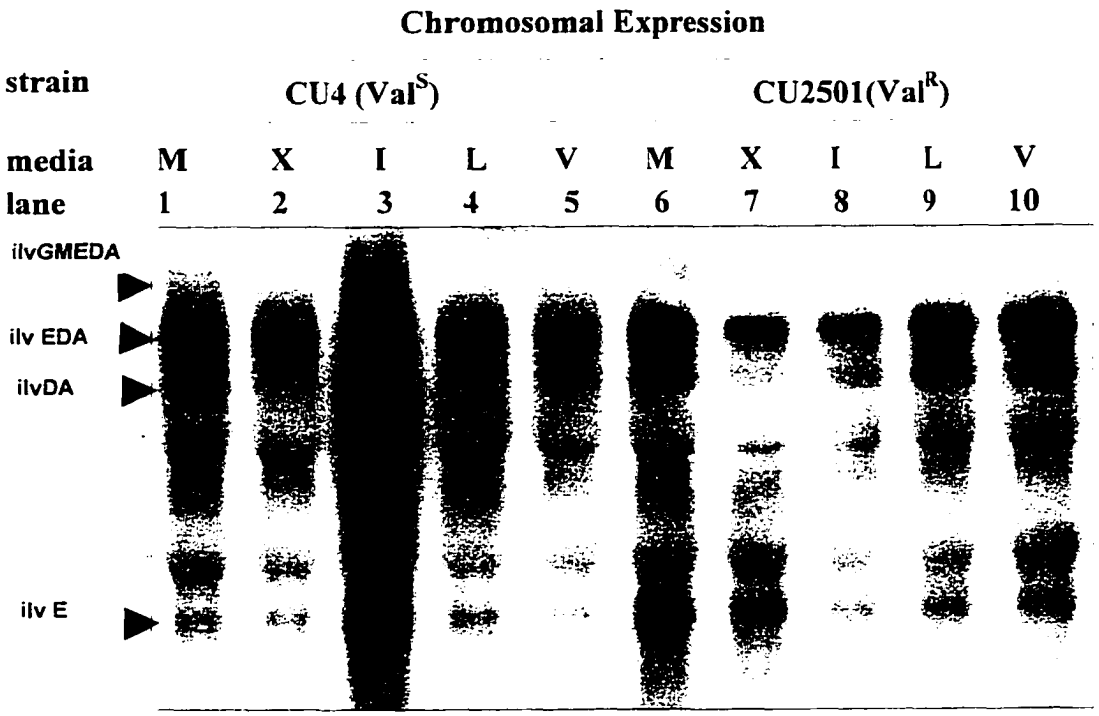


Fig. 3-3. Effects of end product amino acids on *ilvGMEDA* transcripts: Single chromosomal gene cluster with the Val^S or Val^R allele. (A). Strains CU4 (Val^S) and CU2501 (Val^R) were grown and analyzed described as Fig. 3-2. **(B). Quantity of mRNA.** (a, b, c) The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb), *ilvDA* (3.5 kb) mRNA in CU4, CU2501 were quantified as described in Fig. 3-2.

A:



B:

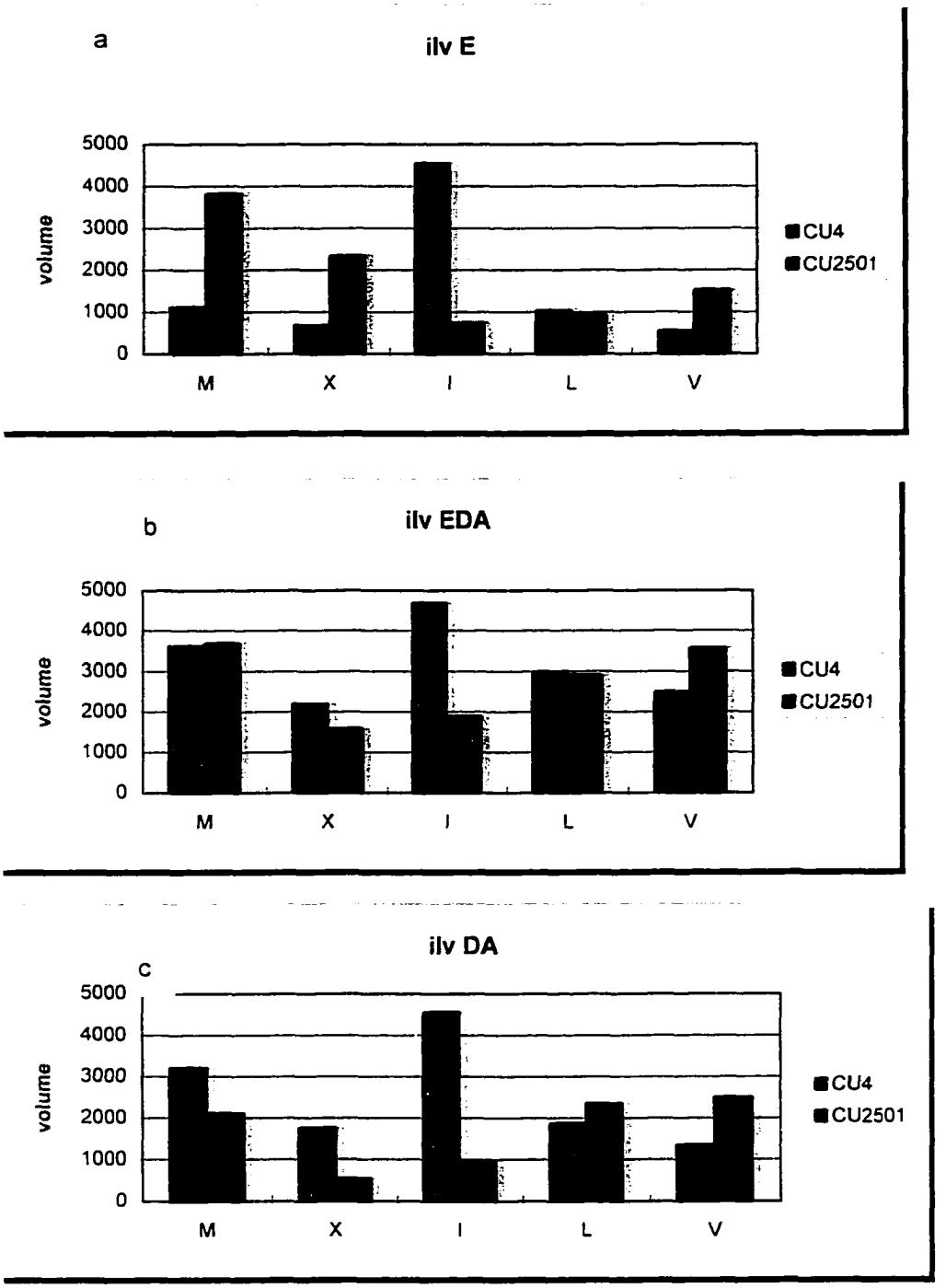
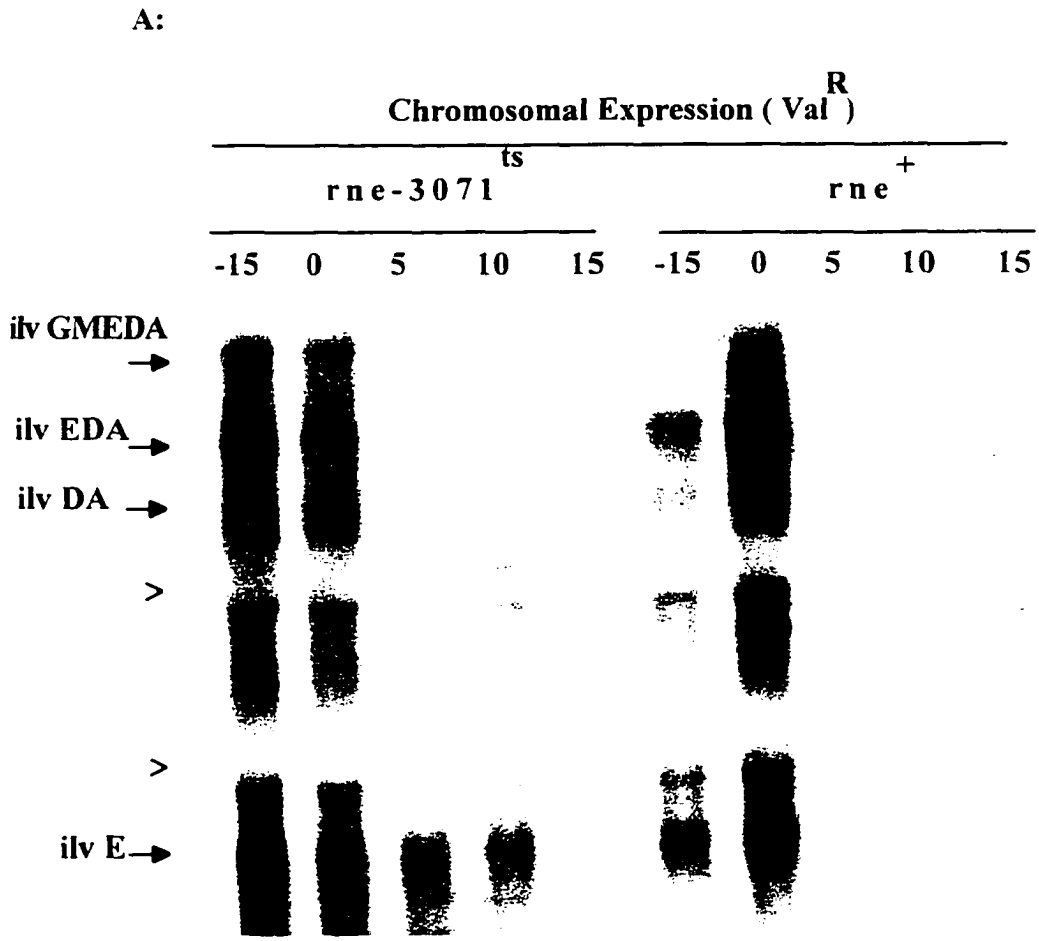


Fig. 3-4. RNase E: Single chromosomal gene cluster with the Val^R allele. (A). Strains CC475 (*rne-3071*^{ts}) and CC479 (*rne*$^+$) were grown at 30°C until midlog, transferred to a 43°C water bath for 15 min, and rifampicin was added (at 0 min). Samples withdrawn at the indicated times were used for Northern analysis . The solid arrows indicate the *ilvGMEDA* (6.7 kb), *ilvEDA* (4.6 kb), *ilvDA* (3.6 kb) and *ilvE* (1.1 kb) transcripts. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). Partially degraded *ilv* mRNA detected by the *ilv* specific probe accumulates as an electrophoretic artifact ahead of these shadows (Huang, 1992). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb), *ilvDA* (3.5 kb) mRNA were quantified as described in Fig. 3-2.



B:

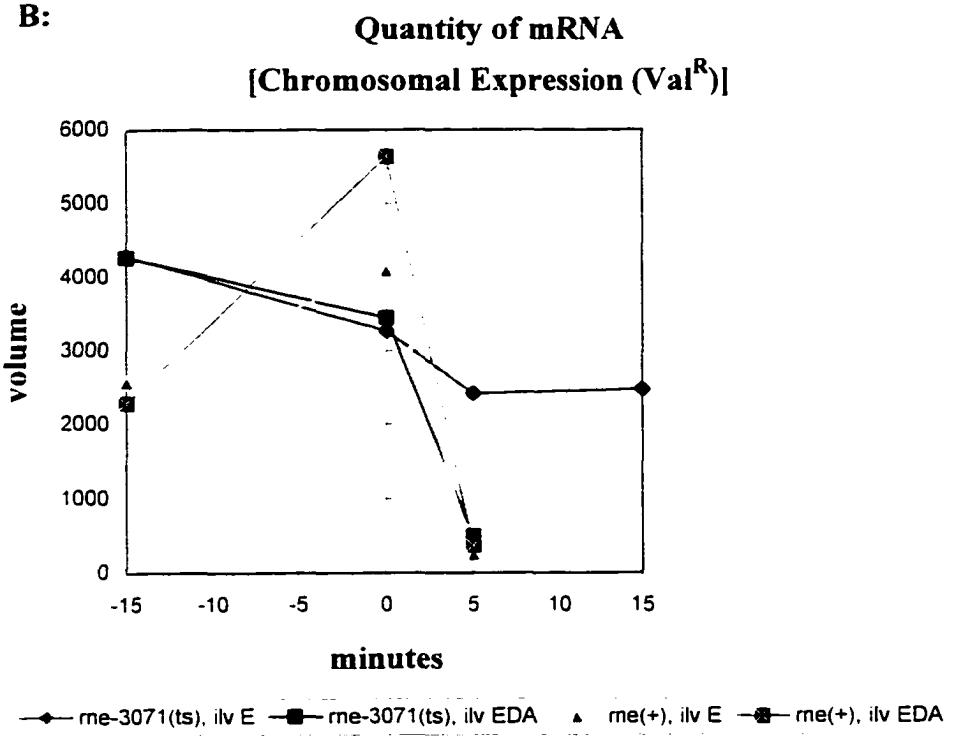
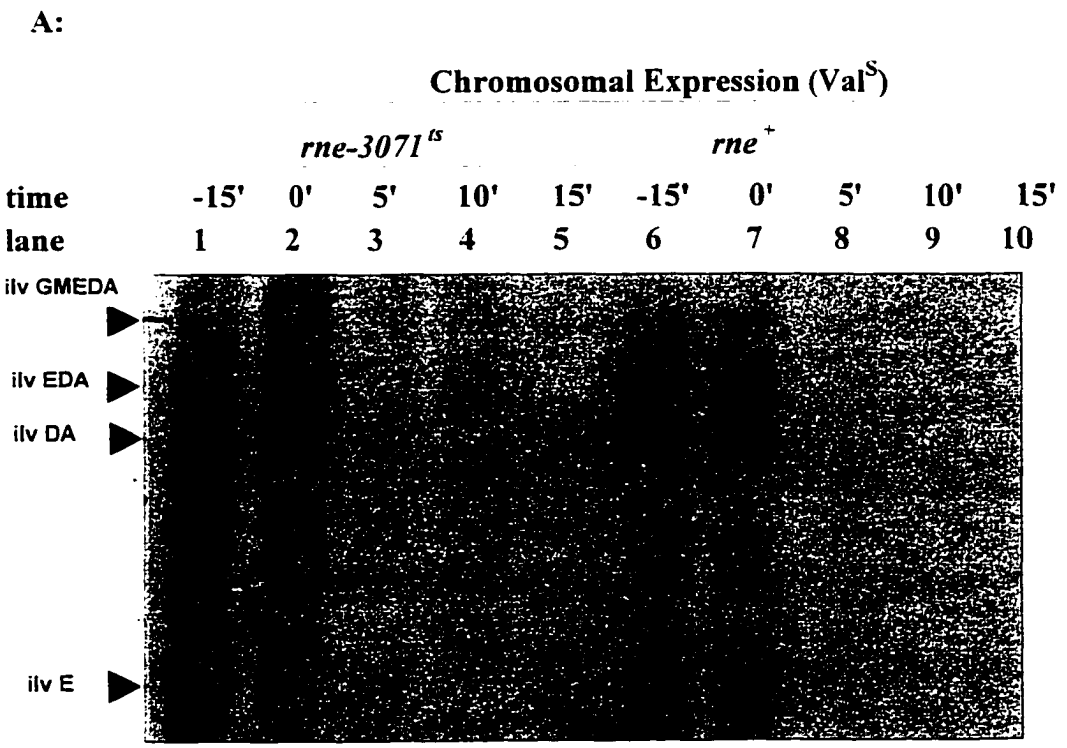


Fig. 3-5. RNase E: Single chromosomal gene cluster with the Val^S allele. **(A).** Strains N3431 (*rne-3071^{ts}*) and N3433 (*rne$^+$*) were grown at 30°C until the OD_{600} had reached 0.4. Rif was added to a final concentration 0.2 mg/ml after cells were shifted to 43°C for 15 min. 15 microgram of total RNA extracted from aliquots withdrawn at the different times which indicated on top of each lane following addition of rif were analyzed on Northern blots. Riboprobe was used as probe. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRMA in N3431 (*rne-3071^{ts}*) and N3433 (*rne$^+$*) were quantified by densitometer scanner.



B:

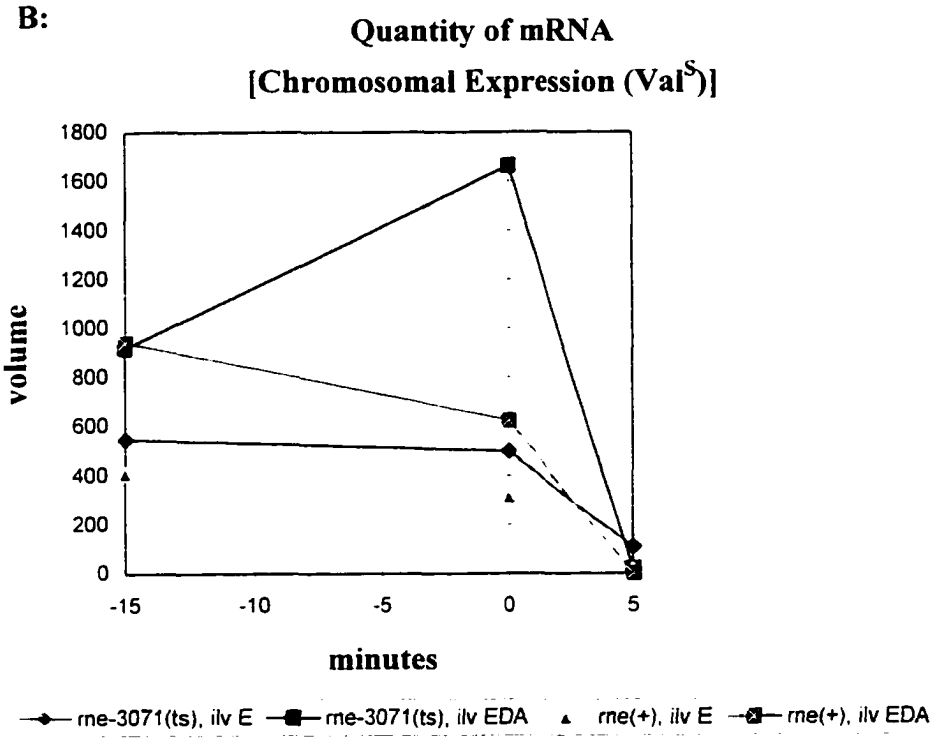
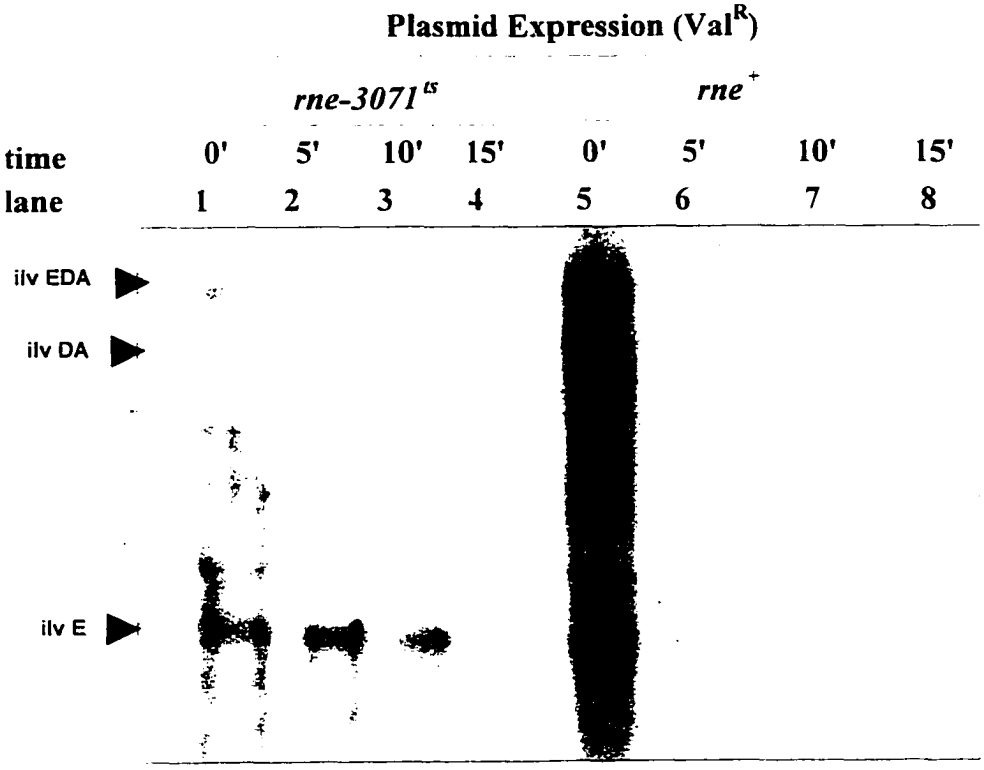


Fig. 3-6. RNase E: Multicopy plasmids with the Val^R allele. (A). Strains N3433 (*rne-3071^{ts}*) and N3431 (*rne$^+$*) transformed with plasmid pCC40 (Val^R) were used to examine the transcript stability. Cells were grown and analyzed as descrided in Fig. 3-7. **(B).Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRMA in N3431 (*rne-3071^{ts}*) and N3433 (*rne$^-$*) containing pCC40 were quantified by densitometer scanner.

A:



B:

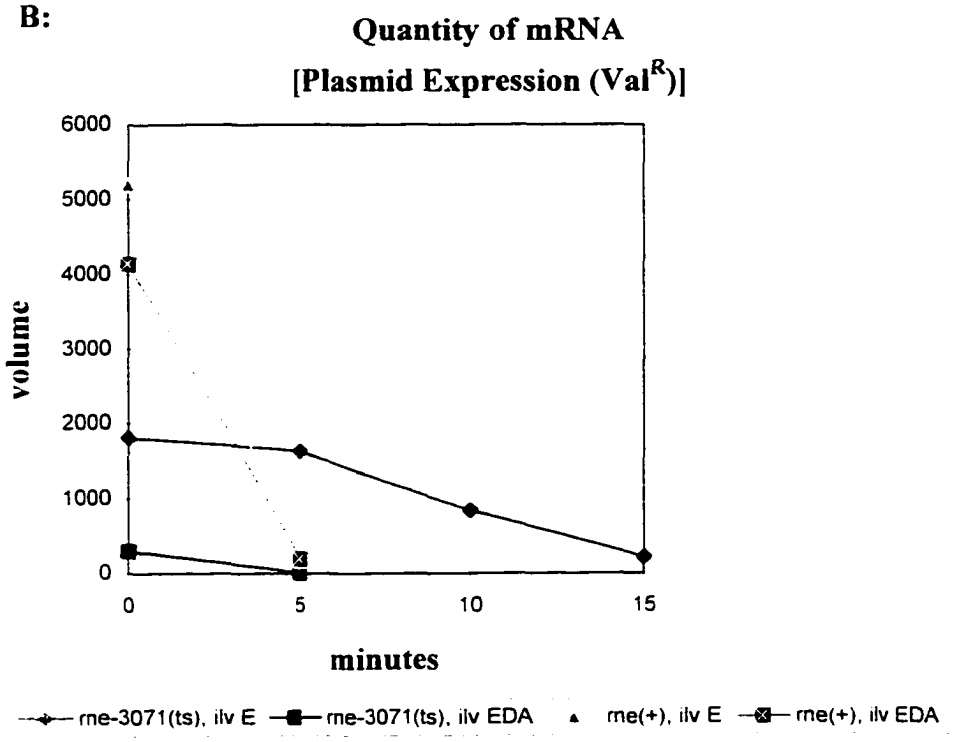
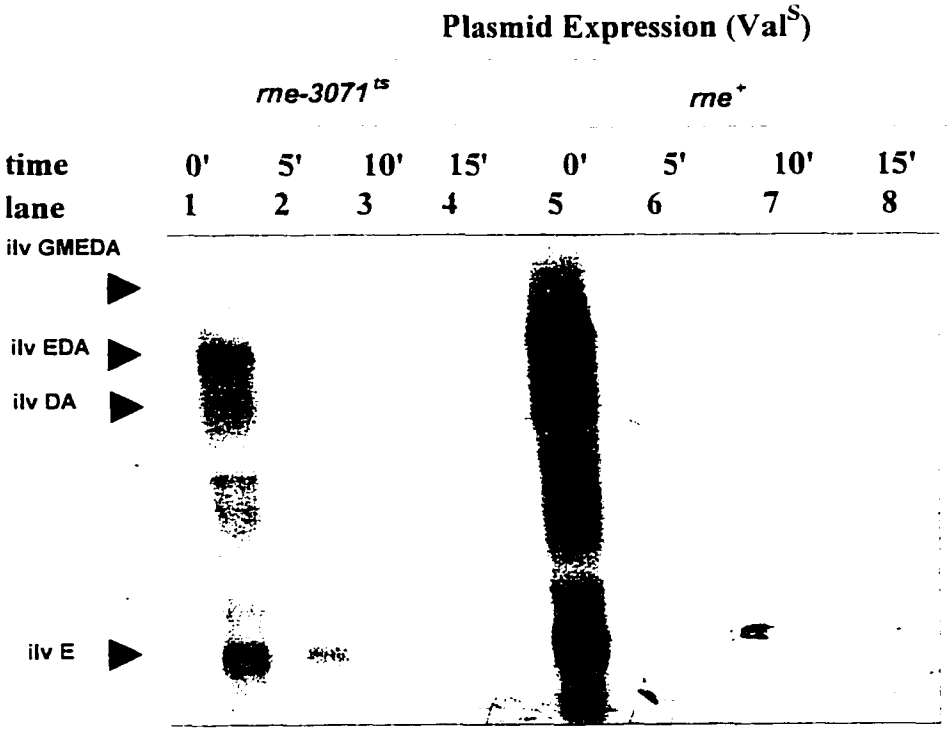


Fig. 3-7. RNase E: Multicopy plasmids with the Val^S allele. **(A).** Strains N3431(*rne-3071^{ts}*) and N3433 (*rne$^+$*) transformed with pJG51(Val^S) were used to examine transcript stability. Cells were grown in M9 medium with thiamine at 30°C until the OD_{600} had reached 0.4. Rif was added to a final concentration 0.2 mg/ml after cells were shifted to 43°C for 15 min. 15 microgram of total RNA extracted from aliquots withdrawn at the different times which indicated on top of each lane following were analyzed on Northern blots. Riboprobe was used as probe. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA .** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRMA in N3431 (*rne-3071^{ts}*) and N3433 (*rne$^+$*) containing pJG51 were quantified by densitometer scanner.

A:



B:

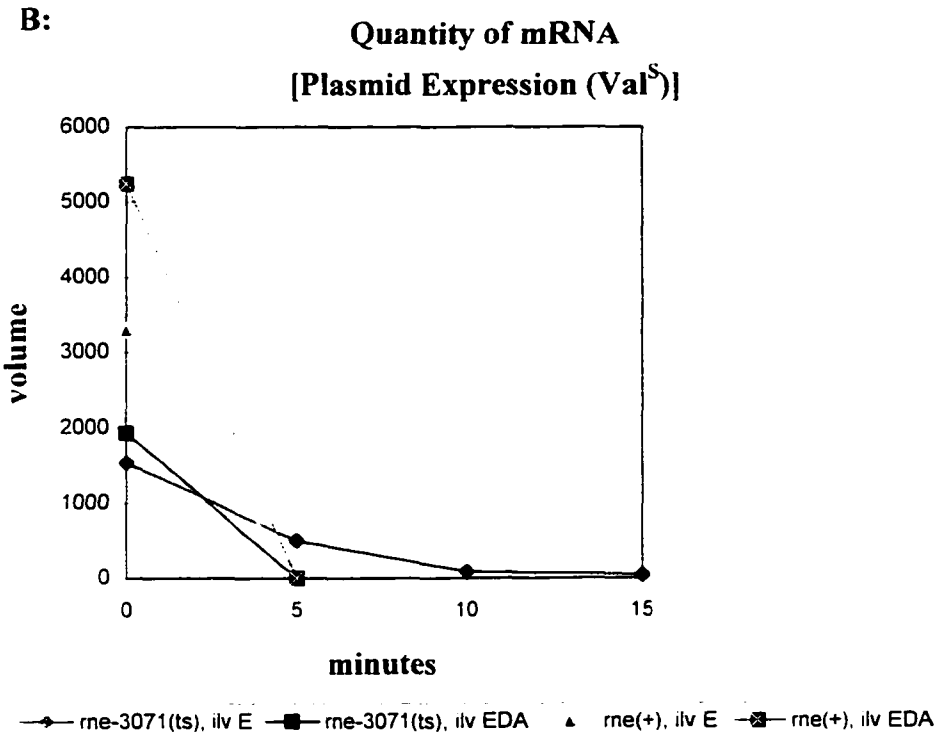
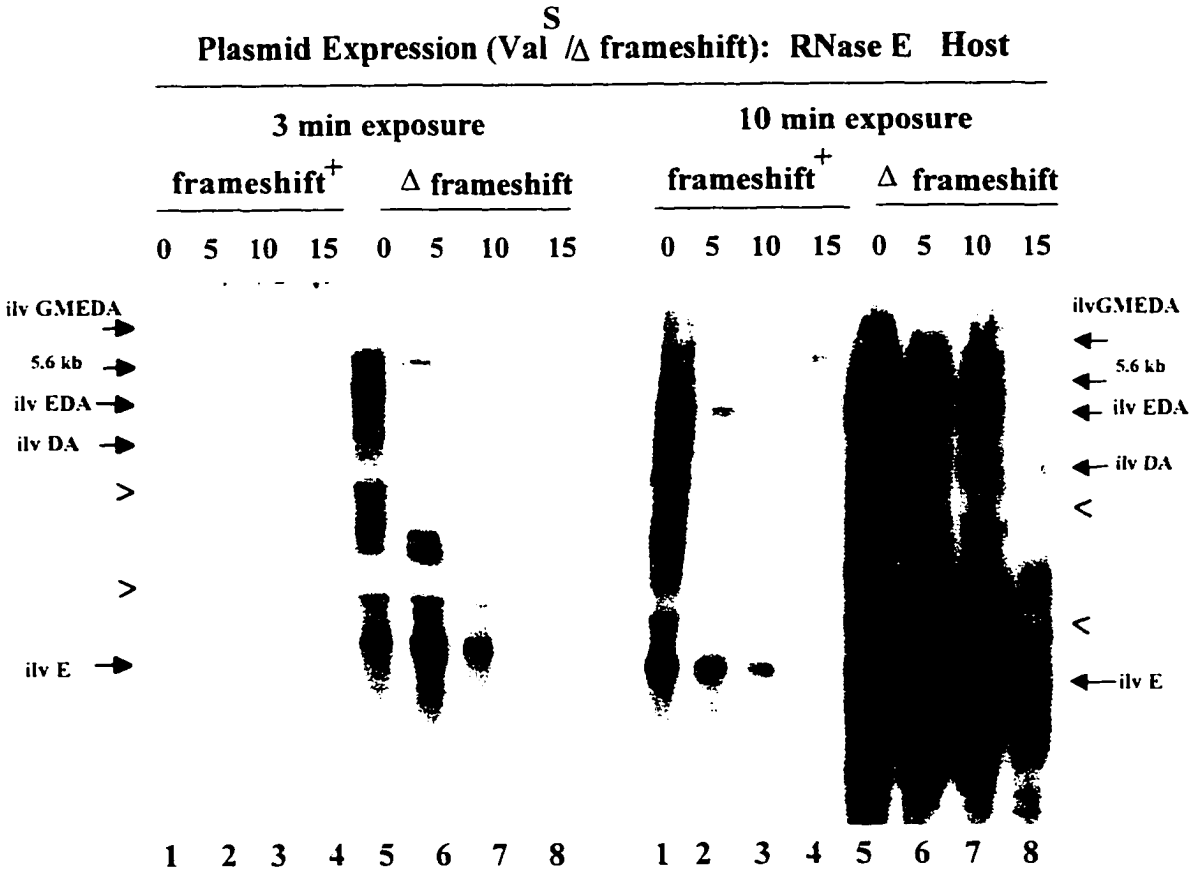


Fig. 3-8. RNase E: Multicopy plasmids with the Val^S or Δfraneshift allele. (A). Derivatives of strain N3431 (*rne-3071*^{ts}) were constructed containing plasmid pJG51 (strain CC147) with the cryptic Val^S allele (frameshift $^-$) or pCC17 (strain CC148) with a deletion of the frameshift (Δ-frameshift). Strains CC148 (lanes 1-4) and CC147 (lanes 5-8) were grown as described in Fig. 3-4. The panels on the left and right were exposed for 3 min and 10 min. respectively. Plasmid pCC17 has a 1.1 kb in frame deletion in the *ilvG* gene and the 6.7 kb transcript is reduced in size to 5.6 kb. Expression of the *ilvGMEDA* genes from plasmid pCC17 (lanes 5-8) is the highest in this study, which accounts for the high sensitivity of transcript detection. The decrease in the *ilvGMEDA* and *ilvEDA* transcripts after rif addition that is simultaneous with the increase in *ilvE* transcript levels, and the presence of processing intermediates just above the *ilvE* transcript (compare lanes 5-6) reveals that some fraction of the *ilvE* transcripts had been derived by post-transcriptional processing from the larger precursors. **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), . *ilvEDA* (4.6 kb), *ilvDA* (3.5 kb) mRNA were quantified as described in Fig. 3-2.



B:

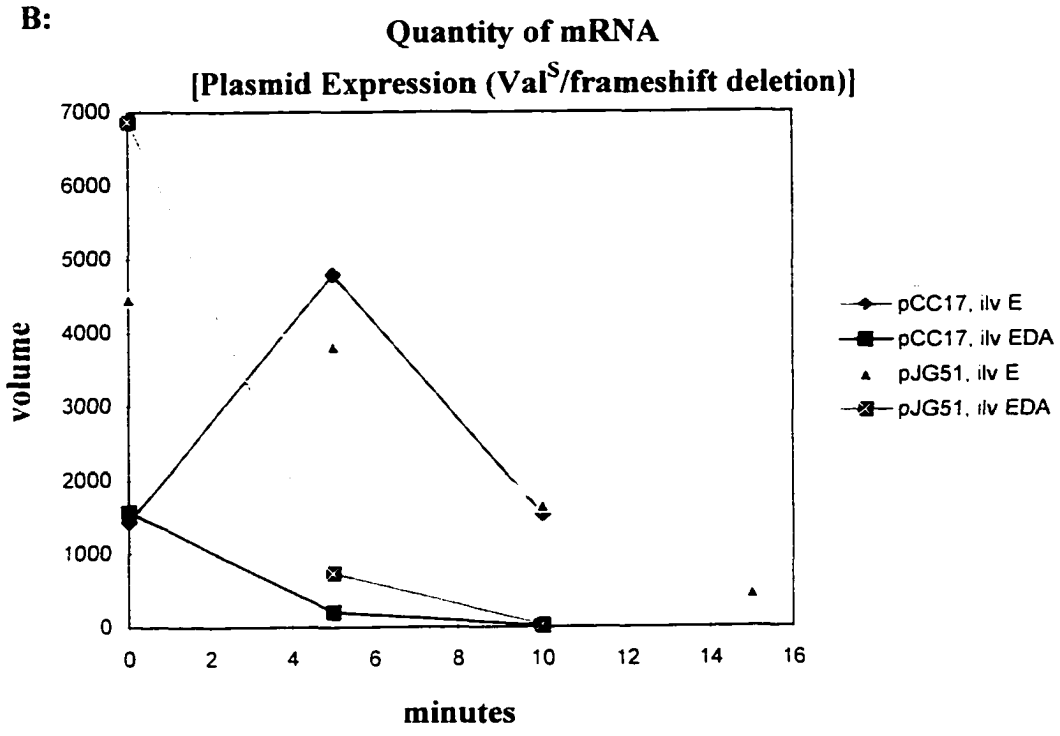
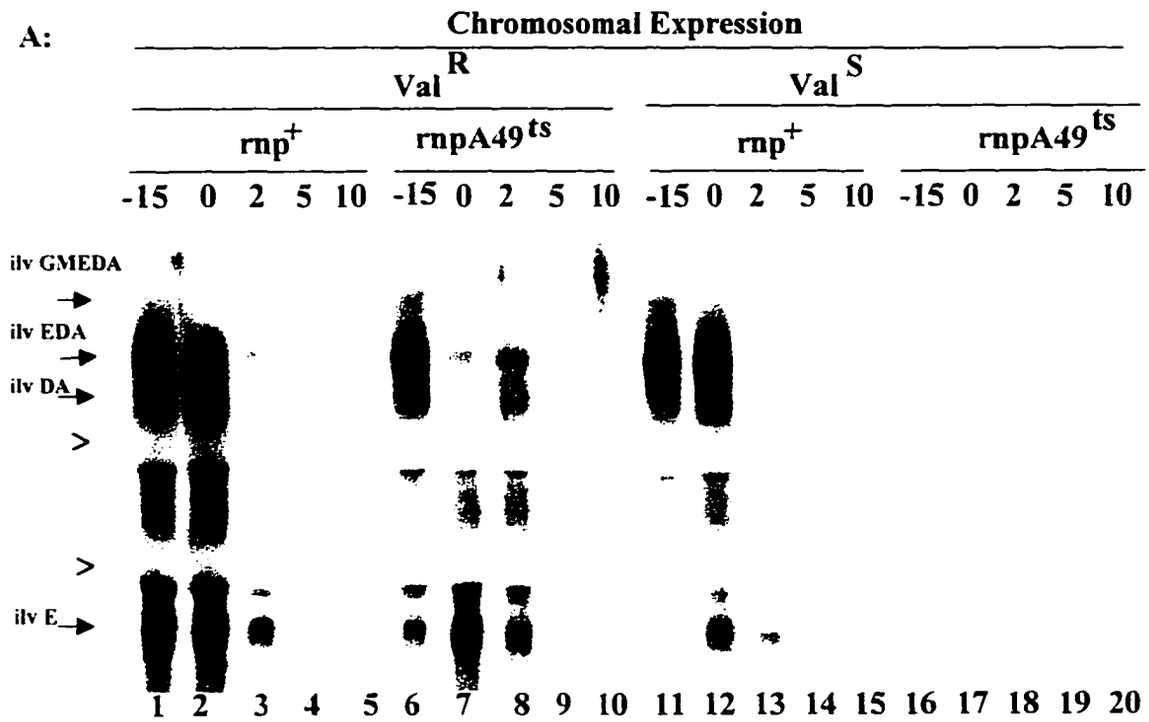
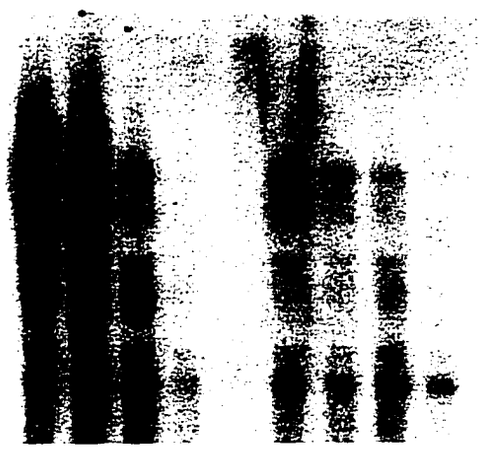
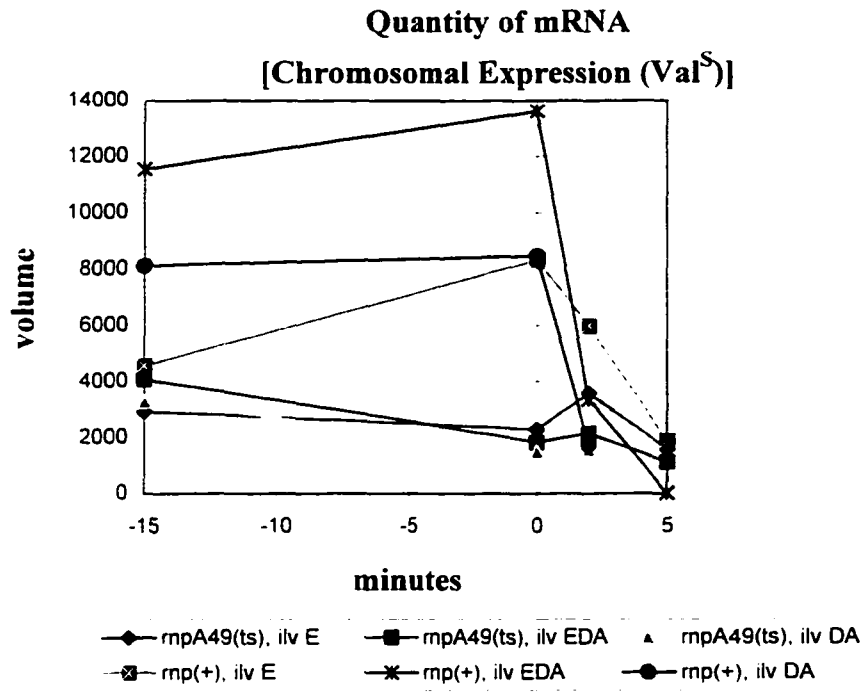


Fig. 3-9. RNase P: Single chromosomal gene cluster with the Val^S or the Val^R allele. **(A).** Strains CC500 (*rnpA*$^+$, Val^R, lanes 1-5), CC502 (*rnpA49*^{ts}, Val^R, lanes 6-10), NHY312 (*rnpA*$^+$, Val^S, lanes 11-15), and NHY322 (*rnpA49*^{ts}, Val^S, lanes 16-20) were grown as described in Fig. 3-11. The image of lanes 11-20 was enhanced in the lower panel so that the lower levels of expression in the Val^S strains can be compared to the Val^R strain (lanes 1-10). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb), *ilvDA* (3.5 kb) mRNA were quantified described as Fig. 3-2.





[Enhanced Lanes 11 - 20]



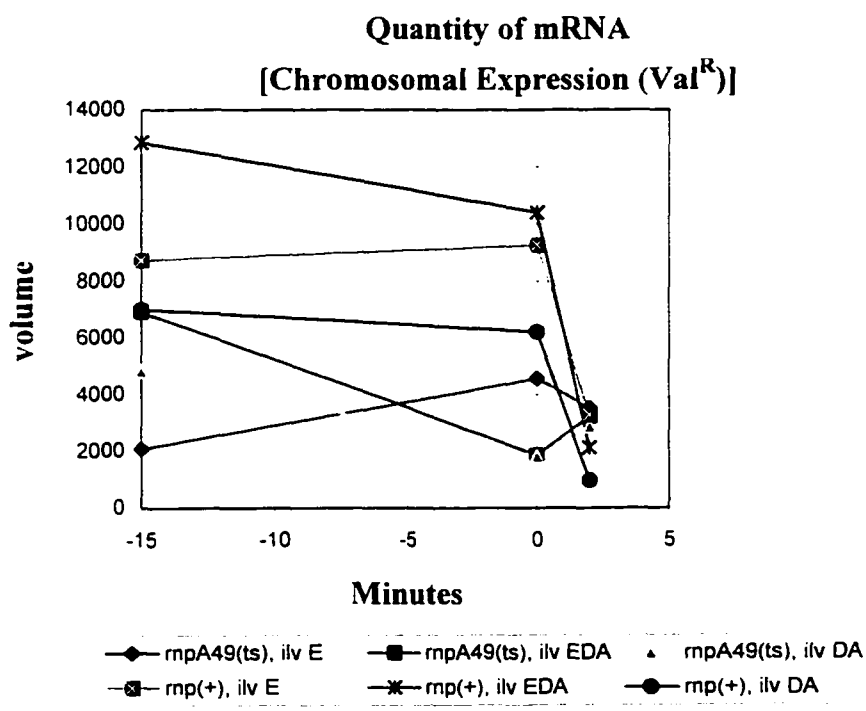
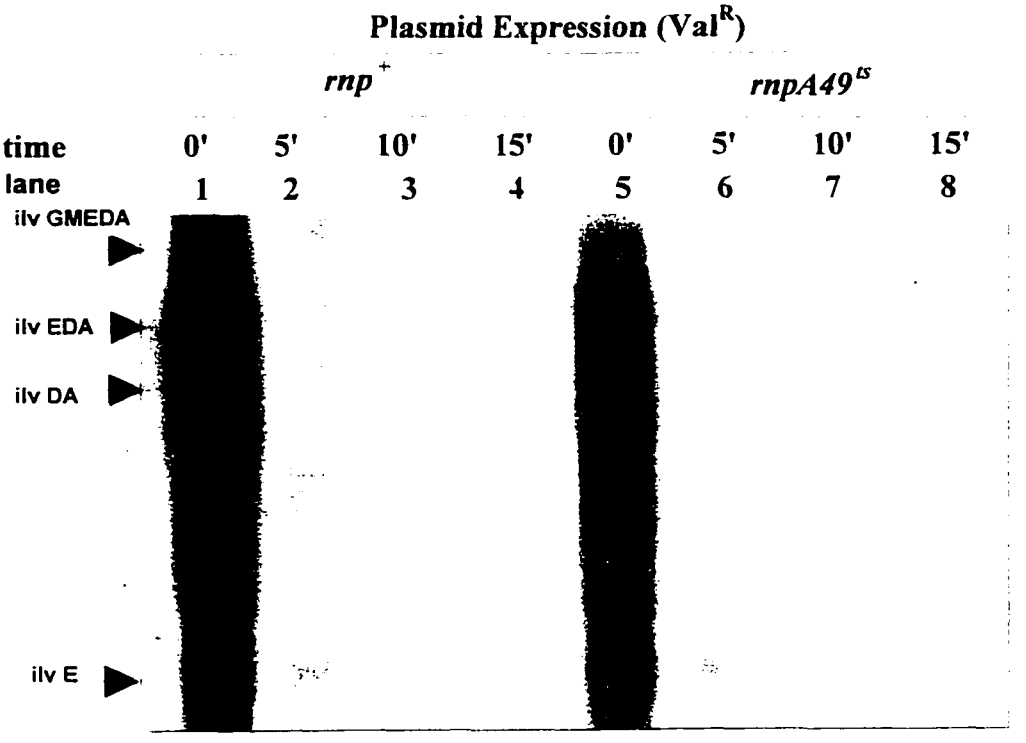


Fig. 3-10. RNase P: Multicopy plasmids with the Val^R allele. (A). Strains CC289 (*rnpA49^{ts}*, pCC40) and CC285(*rnp$^+$*, pCC40) were grown and analyzed as described in Fig. 3-11. **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRNA in CC289 (*rnpA49^{ts}*, pCC40) and CC285(*rnp$^+$*, pCC40) were quantified by densitometer scanner.

A:



B:

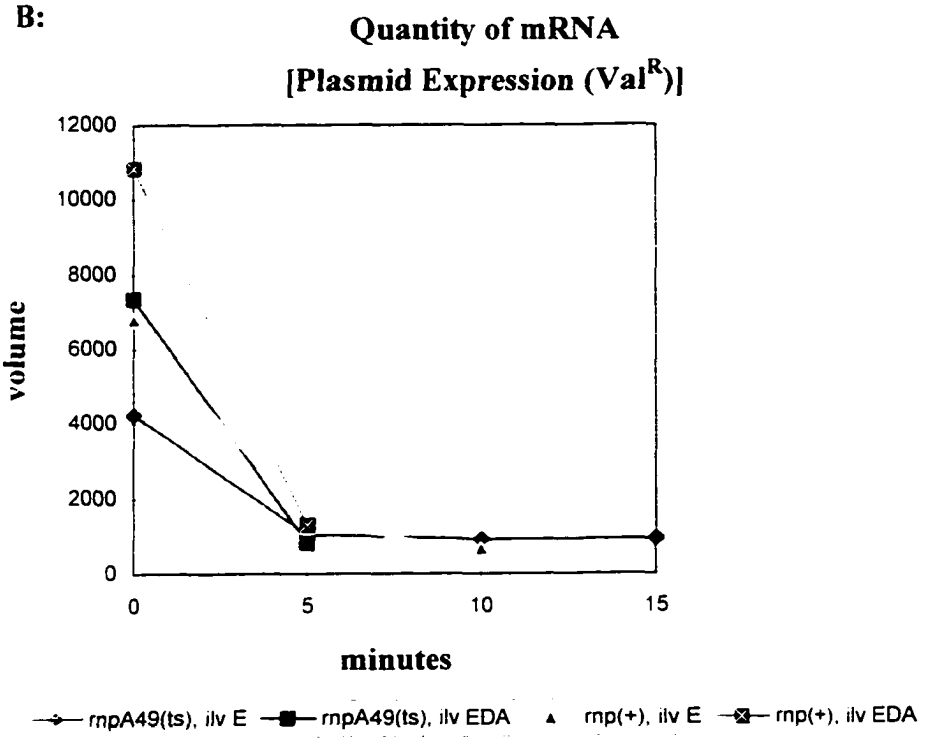
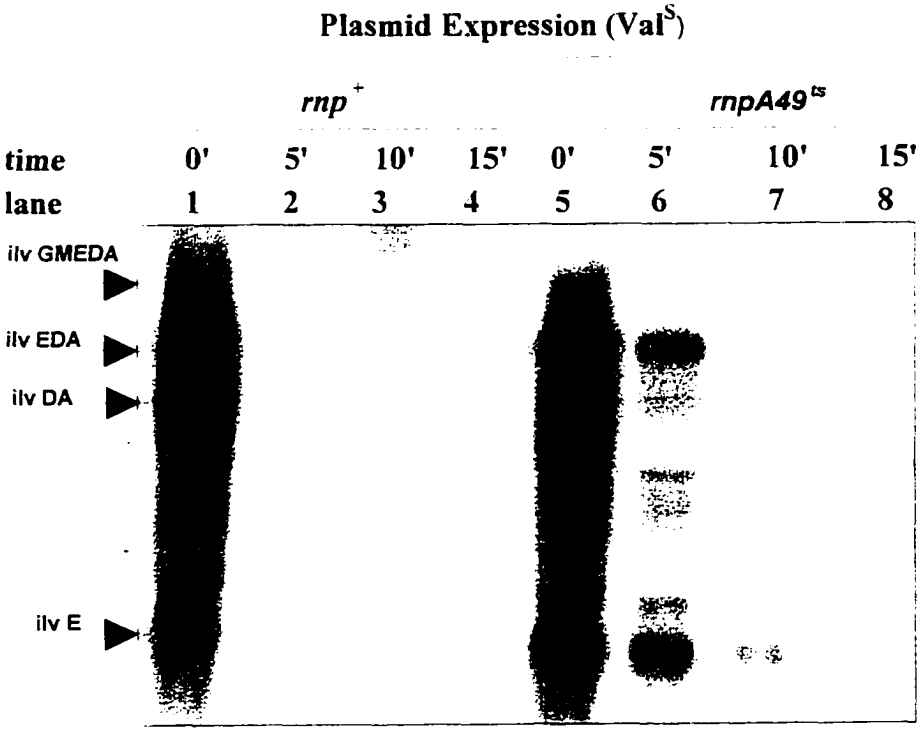


Fig. 3-11. RNase P: Multicopy plasmids with the Val^S allele. **(A).** Strain CC287(*rnpA49^{ts}*, pJG51) and CC283(*rnp$^+$*, pJG51) were grown at 30°C until the OD_{600} had reached 0.4. Rif was added to a final concentration 0.2 mg/ml after cells were shifted to 43°C for 15 min. 15 microgram of total RNA extracted from aliquots withdrawn at the different times which indicated on top of each lane following addition of rif were analyzed on Northern blots. Riboprobe was used as probe. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRNA in CC287 (*rnpA49^{ts}*, pJG51) and CC283 (*rnp$^-$*, pJG51) were quantified by densitometer scanner.

A:



B:

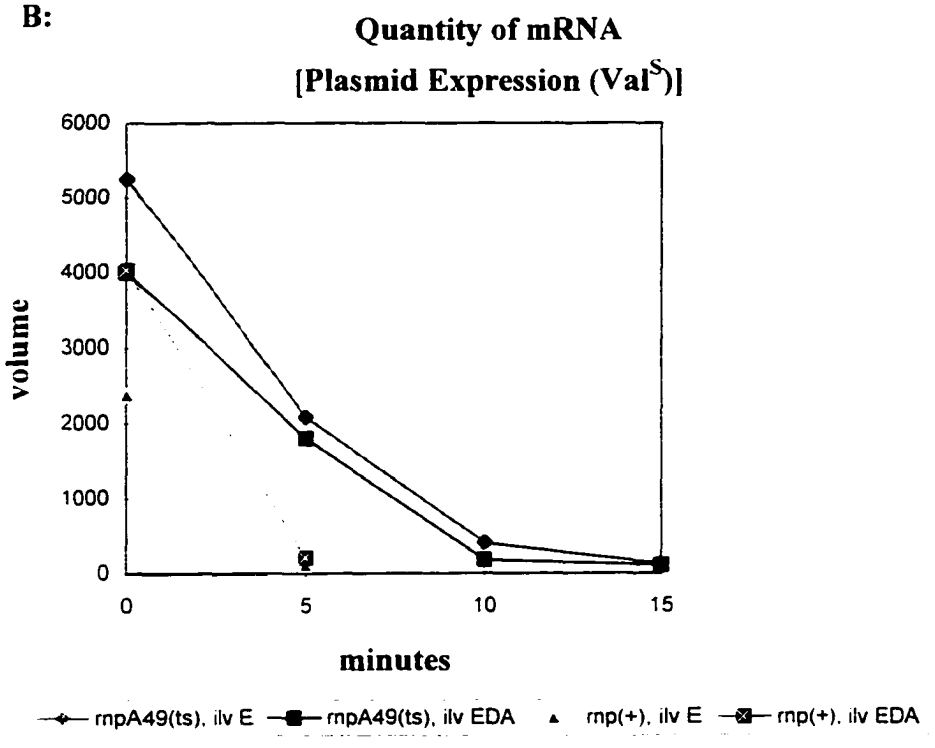
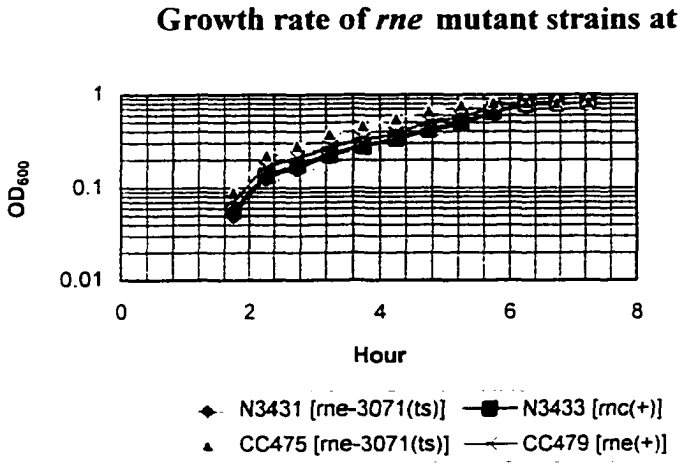


Fig. 3-12. Growth rates of *rne* mutant and wild type strains at 30°C and 43°C. Mutant (*rne-3071*^{ts}) and wild type (*rne*$^{+}$) strains were grown at 30°C (a) and 43°C (b) respectively.



b

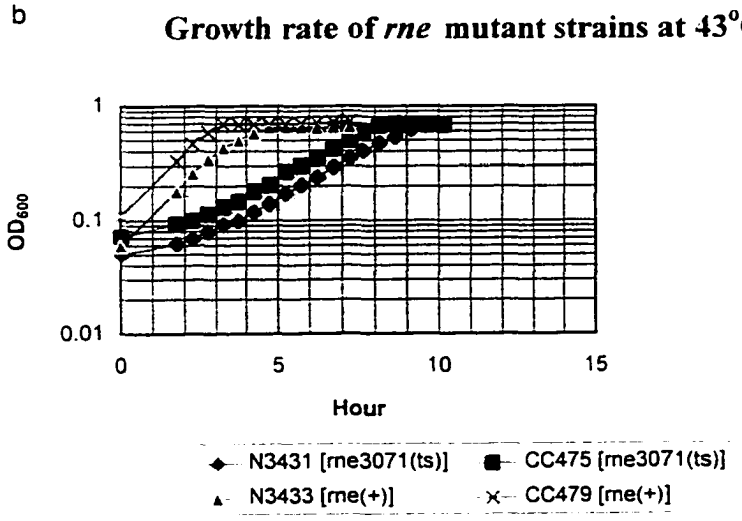
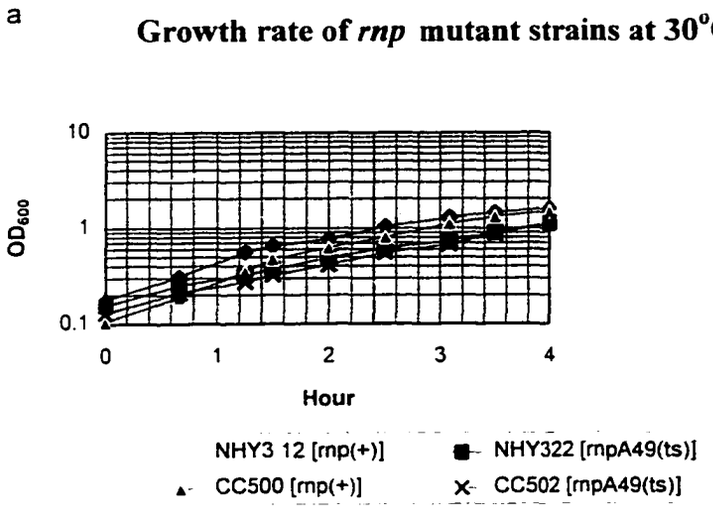


Fig. 3-13. Growth rates of *rnp* mutant and wild type strains at 30°C and 43°C. Mutant (*rnpA49*^{ts}) and wild type (*rnp*$^{+}$)strains were grown at 30^{0}C (a) and 43°C (b) respectively.

a



b

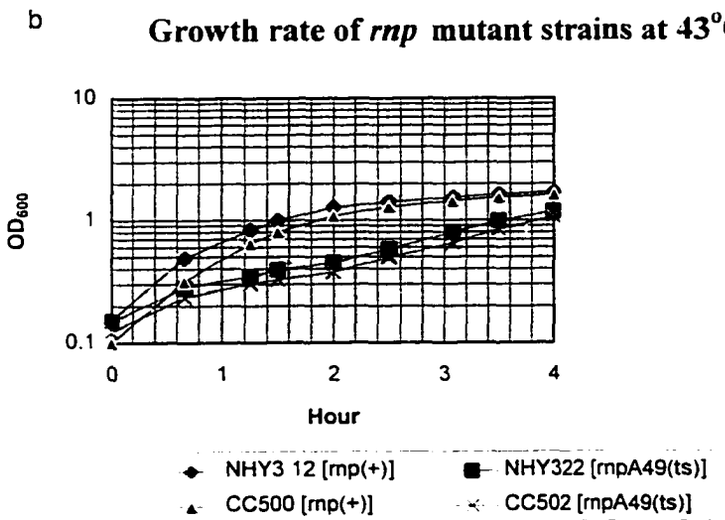
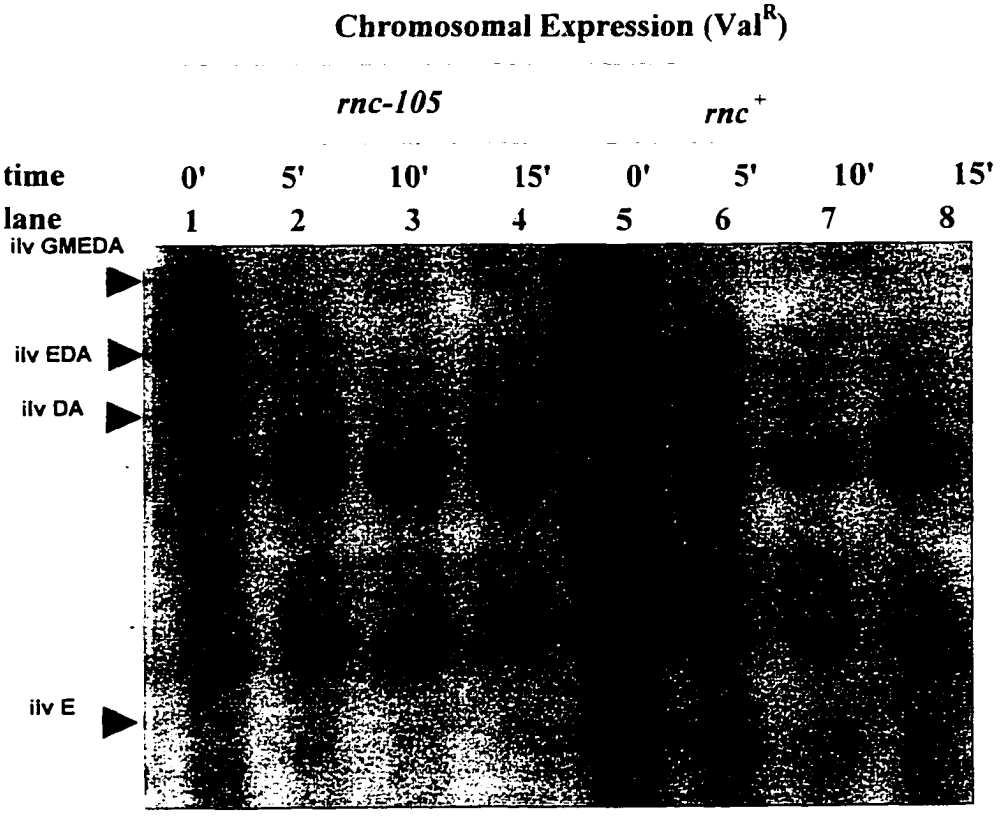


Fig. 3-14. RNase III: Single chromosomal gene cluster with the Val^R allele. (A). Strains CC513 (*rnc-105*) and CC515 (*rnc*$^+$) were grown and analyzed as described in Fig. 3-15. **(B). Quantity of mRNA.** The relative amouts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRNA in Strains CC513 (*rnc-105*) and CC515 (*rnc*+) were quantified by densitometer scanner

A:



B:

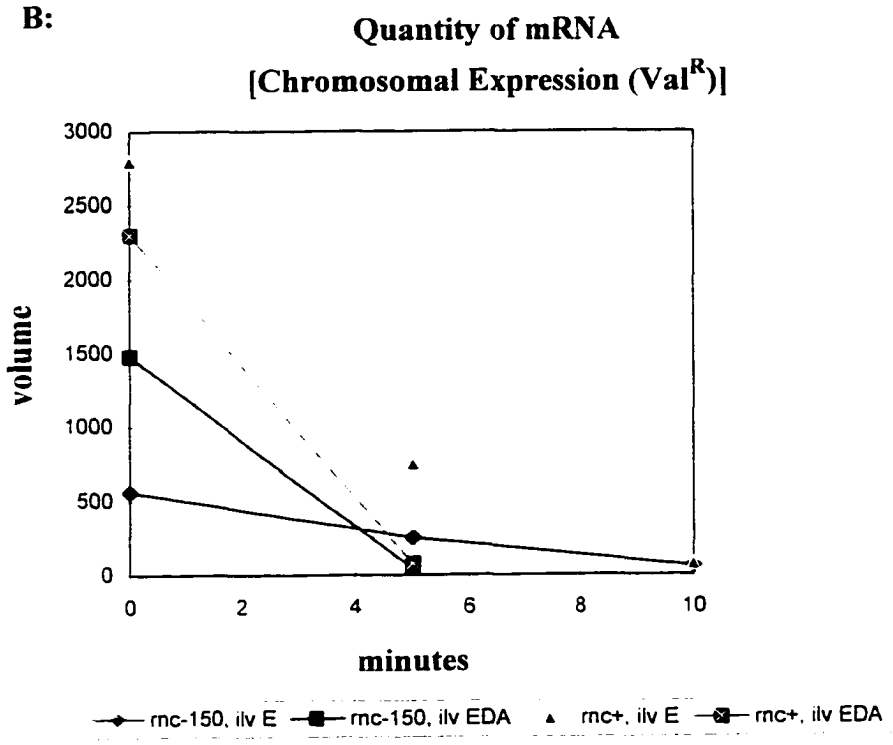
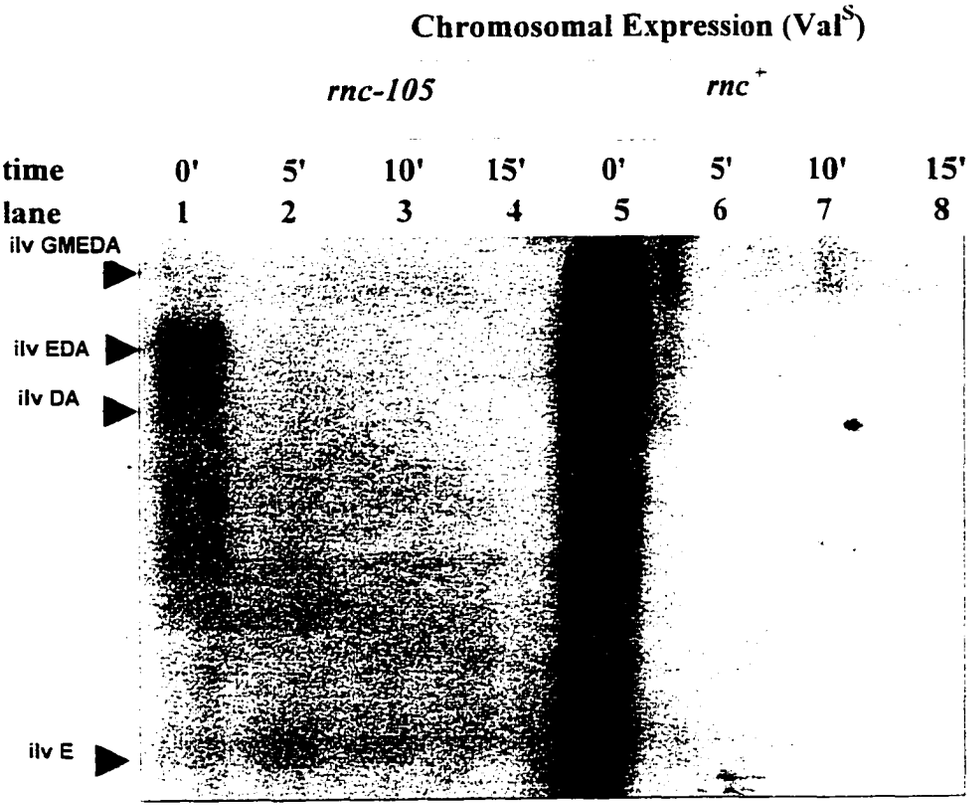


Fig. 3-15. RNase III: Single chromosomal gene cluster with the Val^S allele. **(A).** Strains BL321(*rnc-150*) and BL322 (*rnc*$^+$) were grown at 37°C until the OD_{600} had reached 0.4. Rif was added to a final concentration 0.2 mg/ml. 15 microgram of total RNA extracted from aliquots withdrawn at the different times which indicated on top of each lane following the addition of rif were analyzed on Northern blots. Riboprobe was used as probe. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb). *ilvEDA* (4.6 kb) mRNA in BL321(*rnc-150*) and BL322 (*rnc*+) were quantified by densitometer scanner.

A:



B:

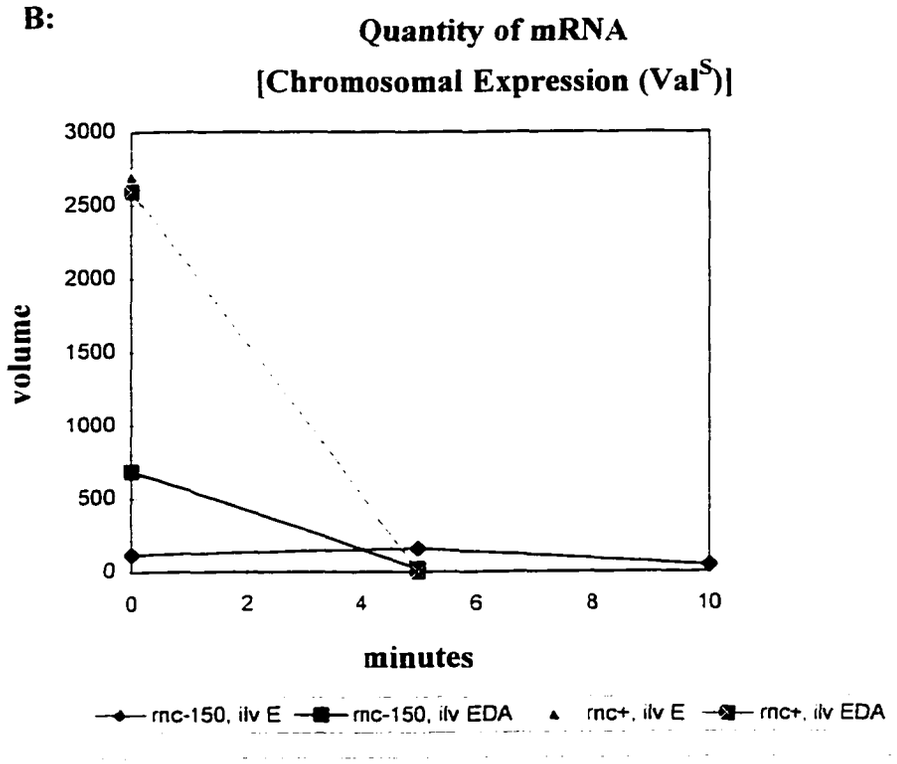
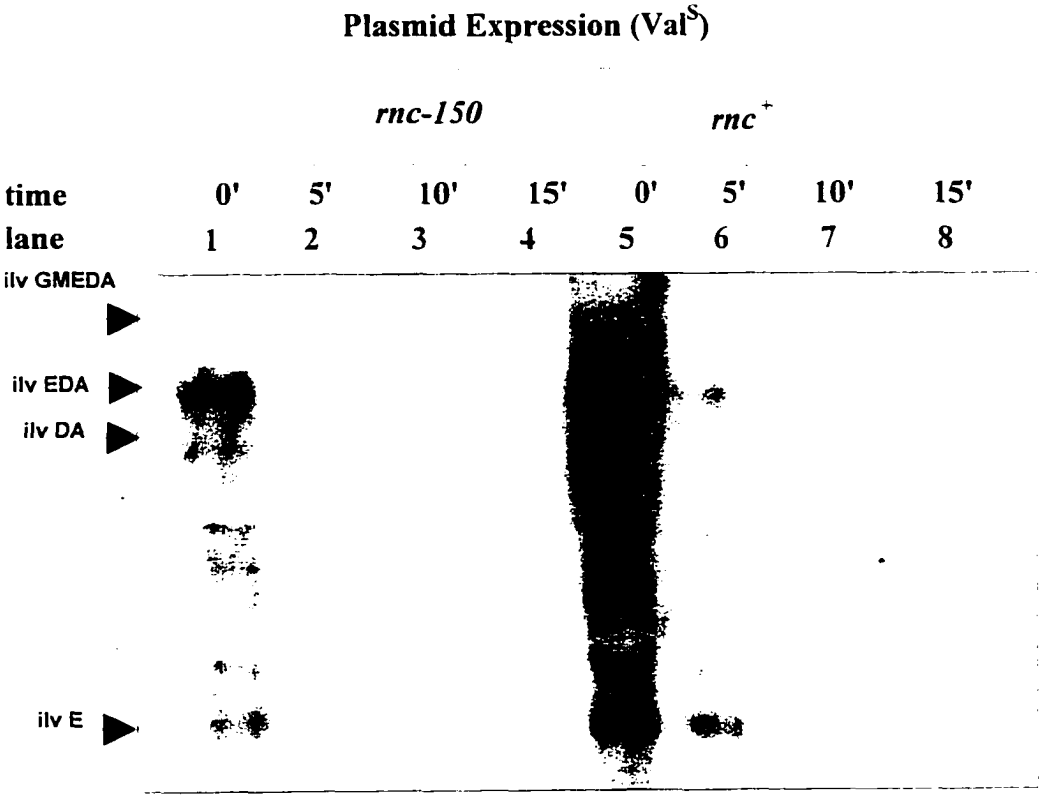


Fig. 3-16. RNase III: Multicopy plasmids of with the Val^S allele. **(A).** Strains CC325 (*rnc-150*, pJG51) and CC326 (*rnc*$^+$, pJG51) were grown and analyzed as described in Fig. 3-15. **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRNA in CC325 (*rnc-150*, pJG51) and CC326 (*rnc*$^+$, pJG51) were quantified by densitometer scanner.

A:



B:

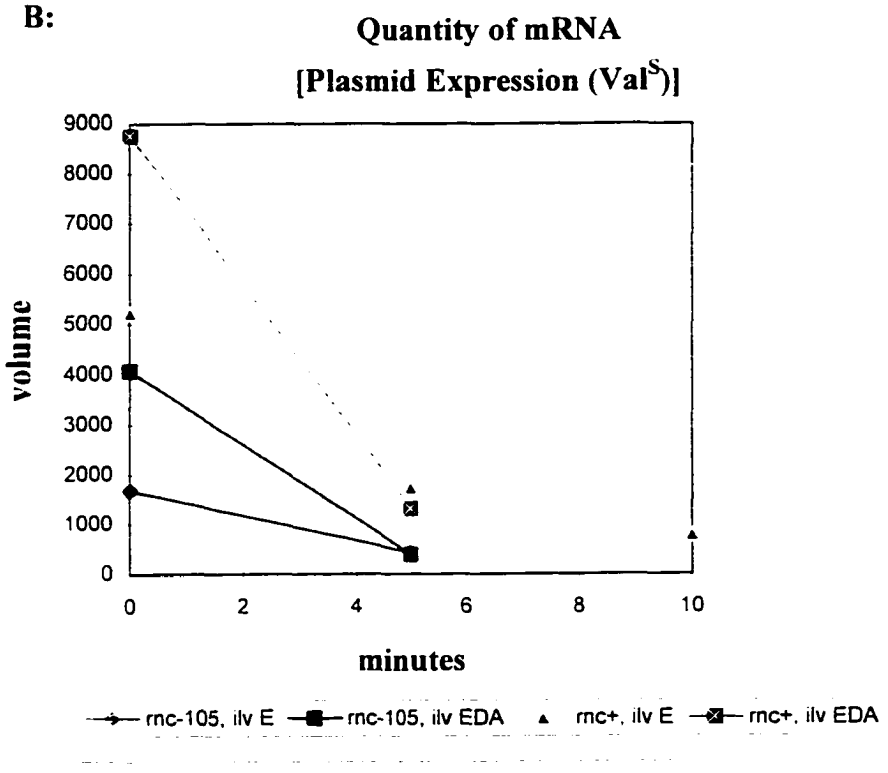
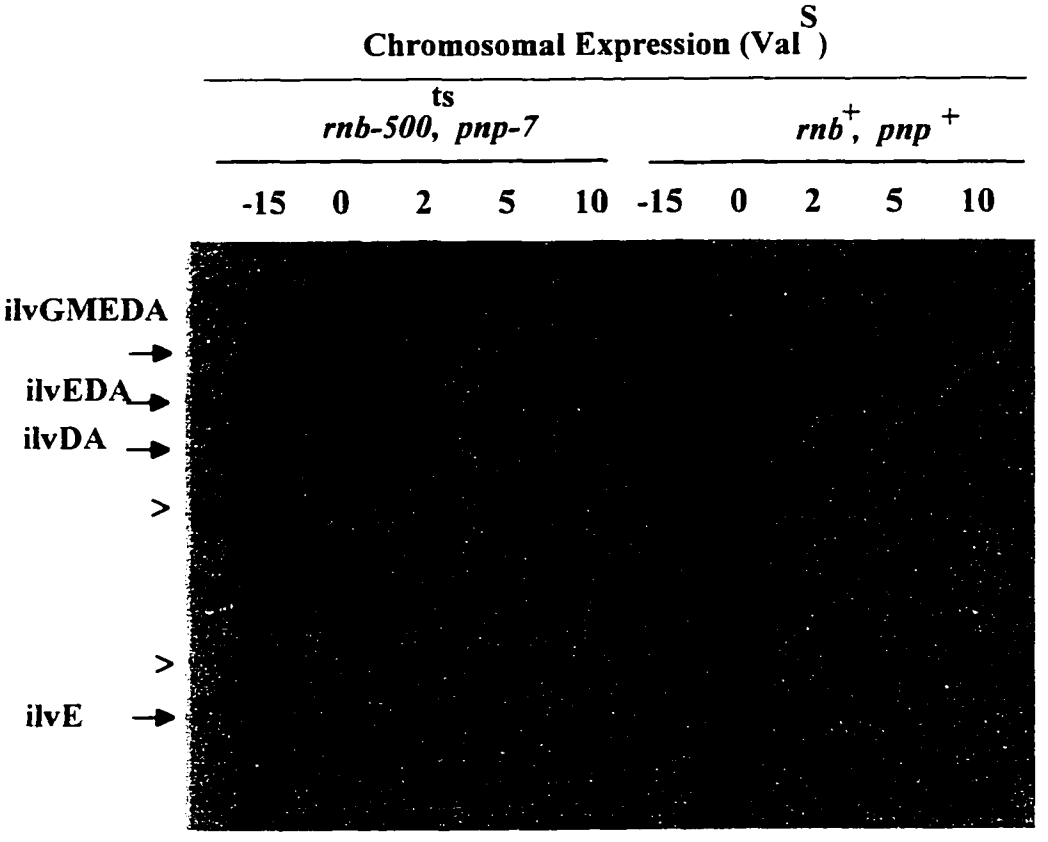


Fig. 3-17. RNase II and PNPase: Single chromosomal gene cluster with the Val^S allele. (A). Strains SK5003 (*pnp-7*, *rnb-500*^{ts}) and SK5006 (*pnp*$^+$, *rnb*$^+$) were analyzed as described in Fig. 3-9. **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilv DA* (3.5 kb) and *ilvEDA* (4.6 kb) mRMA in SK5003 (*pnp-7*, *rnb-500*^{ts}) and SK5006 (*pnp*$^+$, *rnb*$^+$) were quantified by densitometer scanner.



B:

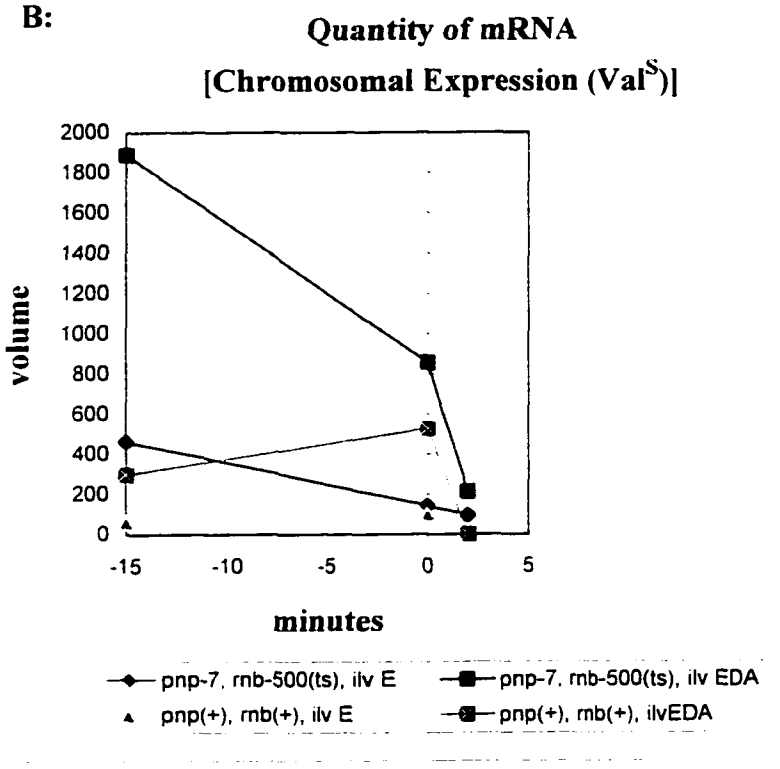
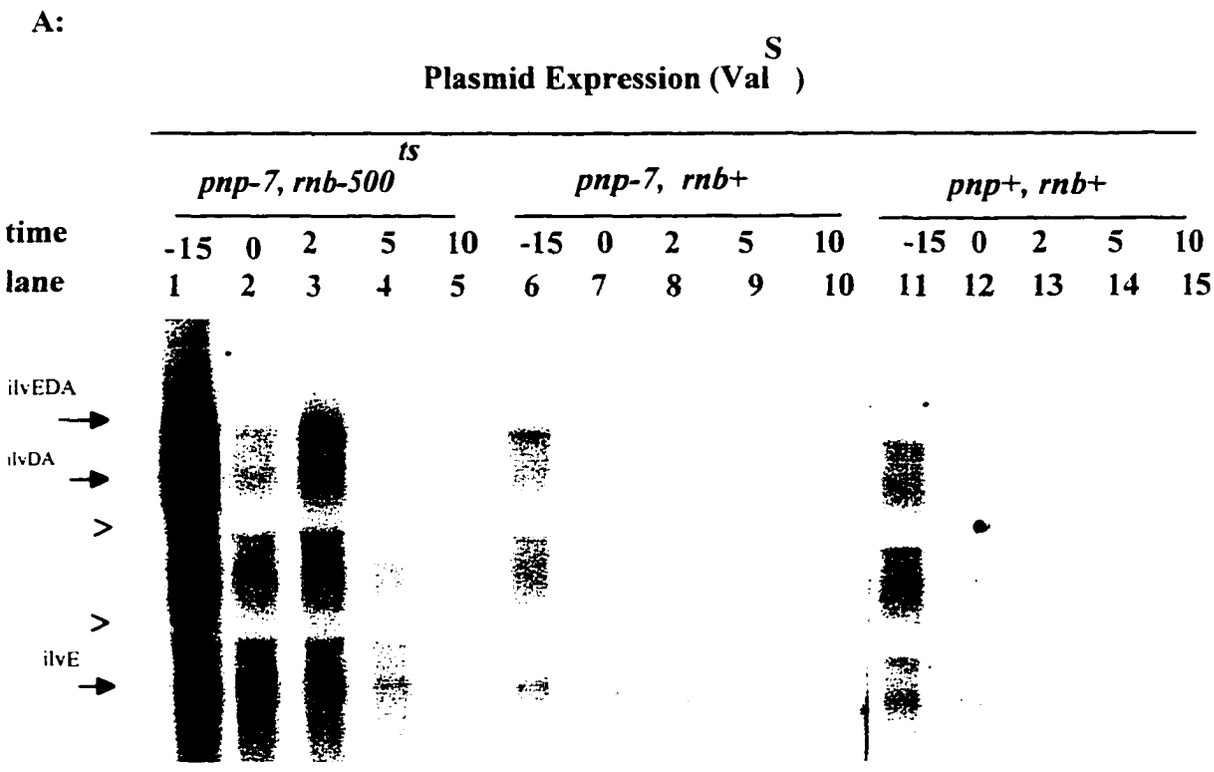


Fig. 3-18. Effects of RNase II and PNPase: Multicopy plasmids with the Val^S allele. (A). Strains CC327 (*pnp-7, rnb-500^{ts}*), CC328 *(pnp-7, rnb$^+$*)and CC330 (*pnp$^+$, rnb$^+$*) containing pJG51 were grown at 30°C until the OD_{600} had reached 0.4. Rif was added to a final concentration 0.2 mg/ml after cells were shifted to 44°C for 15 min. 15 microgram of total RNA extracted from aliquots withdrawn at the different times which indicated on top of each lane following addition of rif were analyzed on Northern blots. Riboprobe was used as probe. The dashed arrows indicate the 16S (lower) and 23S (upper) ribosomal RNA shadows (caused by saturation of the nylon membrane with non-radioactive ribosomal RNA). **(B). Quantity of mRNA.** The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb) mRMA in CC327, CC328 and CC330 were quantified by densitometer scanner.



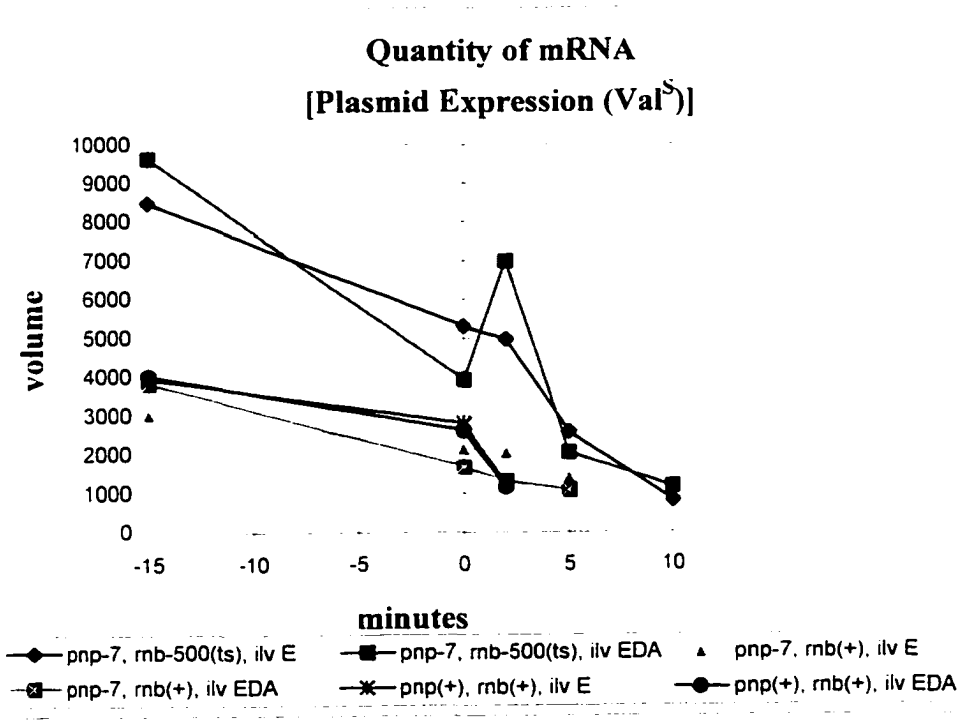


Fig. 3-19. Effects of RNase E on the *ilv'ED'* mRNA in vitro. Sense RNA (1136 nt) was transcribed in vitro by using plasmid pCC75. 9S (248 nt) was transcribed by using plasmid pRC9S . Reaction mixtures were run on 4% polyacrylamide gel and exposed to X-ray film. RNase E concentration in reaction was 10 fold higher (360 ng) in lanes 2-3 or lanes 6-7, than in lanes 4-5 or lanes 8-9. M: RNA ladder (lane1). No RNase E was present in reaction of lane 10.

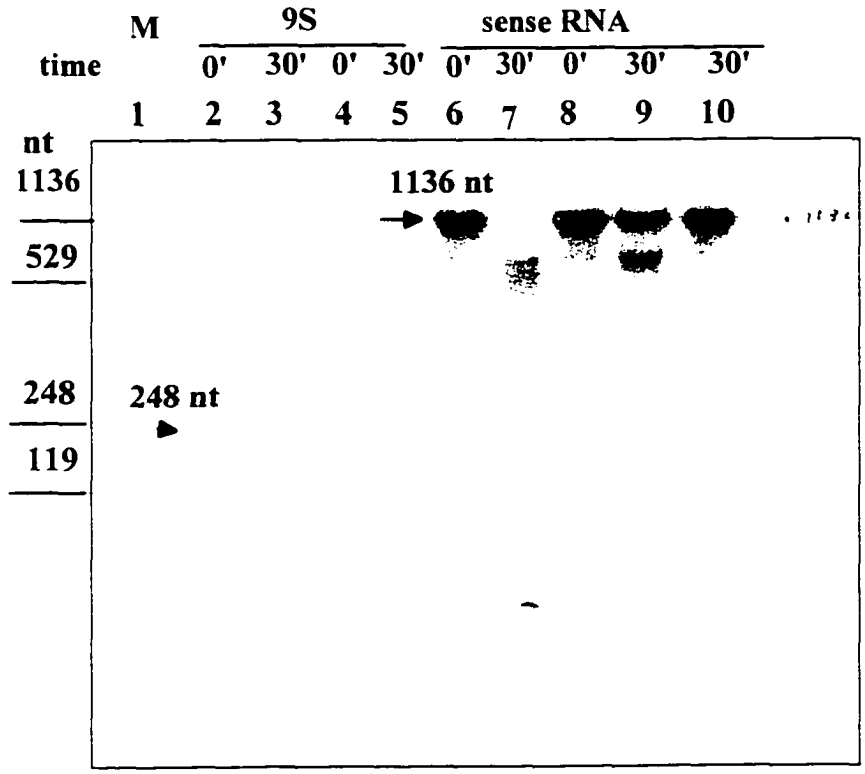
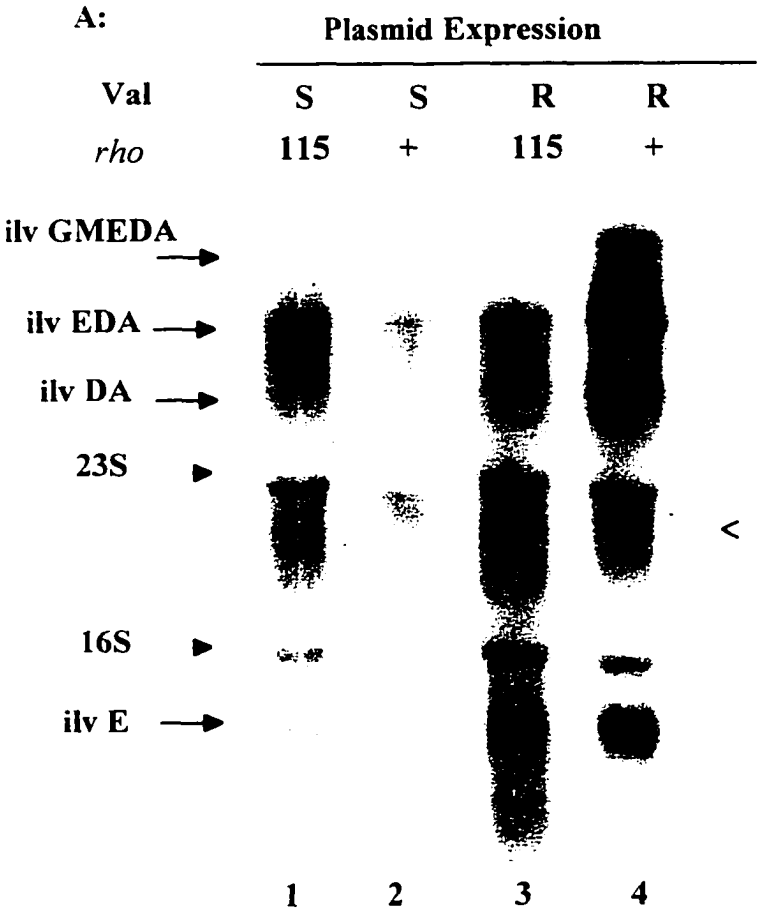
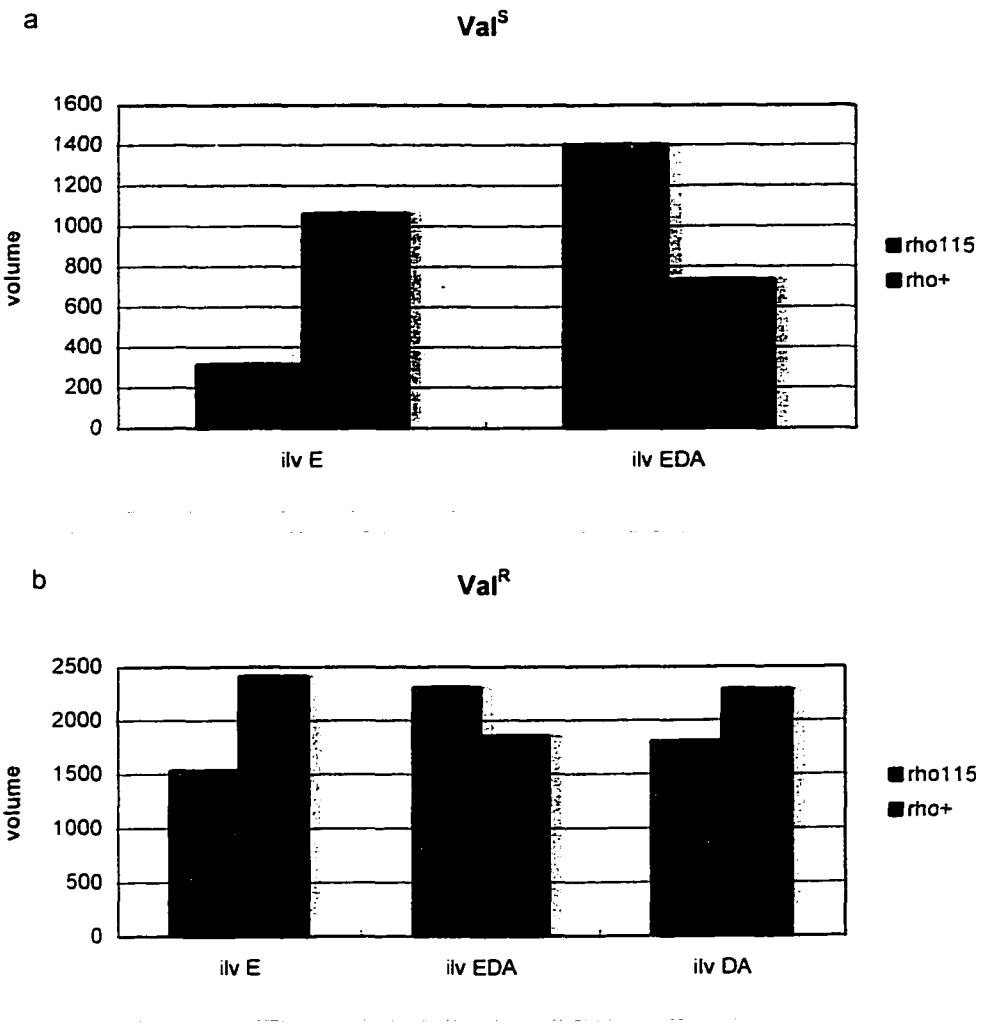


Fig.3-20. Effects of a Rho protein on *ilvGMEDA* transcripts: Multicopy plasmids with the Va^S or Val^R allele. **(A).** RNA was isolated from strains CC253 (*rho-115*, pJG51 [Val^S]), CC256 (*rho*$^+$, pJG51 [Val^S]), CC254 (*rho-115*, pCC40 [Val^R]), and CC257 (*rho*$^+$, pCC40 [Val^R]) in lanes 1-4, respectively, that were grown to midlog phase and hybridized to the riboprobe. The arrow on the right indicates additional degradation products between the *ilvDA* and *ilvE* transcripts seen in the presence of the *rho-115* mutation (lane 3) compared to the *rho*$^+$ (lane 4). **(B). Quantity of mRNA .** (a. b) The relative amounts of *ilvE* (1.1 kb), *ilvEDA* (4.6 kb), *ilvDA* (3.5 kb) mRNA were quantified described as Fig. 3-2.



B:



CHAPTER 4

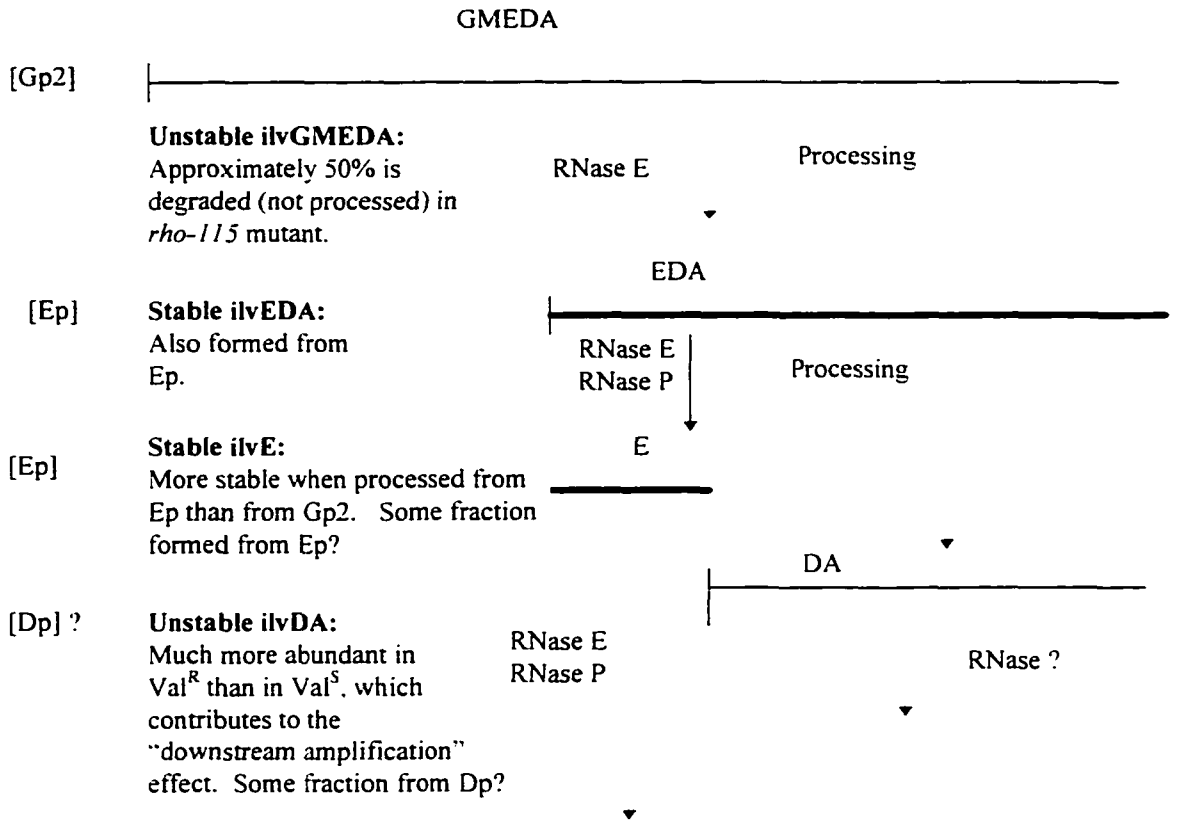
DISCUSSION

RNase E, RNase P and PNPase, RNase II but not RNase III affect the ilvGMEDA transcripts

We used a sensitive method that permitted detection of transcripts produced from the single chromosomal copy of the *ilvGMEDA* genes. While the level of expression from plasmids was higher than from the single chromosomal copy of the *ilvGMEDA* genes, there were similar ratios of the same four transcripts. A RNase E mutation increased the stability of the *ilvGMEDA* and *ilvE* mRNAs. A RNase P mutation increased the stability of the *ilvEDA* and *ilvE* transcripts. A RNase III mutation had no apparent effect upon *ilvGMEDA* transcript stability. Strains with mutations in RNase II and PNPase had more stable *ilvEDA* and *ilvE* transcripts compared to the isogenic wild type strain. The levels of the *ilvEDA*, *ilvDA*, and *ilvE* transcripts were higher when the levels of the full length *ilvGMEDA* transcripts were higher in the Val^R and frameshift deletion strains compared to the Val^S strains. After inhibition of transcription initiation by rif addition there was a post-transcriptional increase in the *ilvE* transcript levels in strains with RNase E or RNase P mutations, and a post-transcriptional increase in *ilvEDA* levels in a strain with a RNase P mutation and in the RNase II/PNPase double mutant. The results revealed a processing pathway (Fig. 4-1) in which some or all of the unstable *ilvGMEDA* transcripts were converted to the to the relatively stable *ilvEDA* transcripts, while some fraction of

Fig. 4-1. Formation, processing, and stability of the *ilvGMEDA* transcripts. The upstream attenuation regulated *ilvGp2* promoter generates the very unstable *ilvGMEDA* transcript that is a precursor to the smaller mRNAs. About half of these transcripts were diverted from the processing pathway to a pathway leading to degradation in the presence of the *rho-115* mutation. The *ilvGMEDA* transcript is a substrate for RNase E, which participates in its processing to the more stable *ilvEDA* transcript. The *ilvEDA* transcript can also be formed from the internal constitutive *ilvEp* promoter. The *ilvEDA* transcript is a substrate for RNase P, which participates in its processing to the stable *ilvE* and unstable *ilvDA* transcripts. The *ilvE* transcript is a substrate for RNase E and RNase P, which participate in its degradation. It remains to be determined how much of the *ilvE* transcript is derived from initiation at *ilvEp* and termination at the postulated Rho-dependent terminator after the *ilvE* gene. A potential RNA stem loop structure after the *ilvE* gene was predicted from examination of the DNA sequence, and this predicted RNA stem loop may protect the *ilvE* mRNA from 3'-exoribonucleases. The *ilvDA* transcript is dramatically more abundant in the Val^R compared to the Val^S strains. This increased abundance of the *ilvDA* transcript, along with the post-translational instability of the *ilvD*-coded dihydroxyacid dehydrase, accounts for the "downstream amplification" effect, in which the change in levels of gene expression for the *ilvEDA* genes is greater for the promoter distal genes than for promoter proximal genes comparing the Val^R to the Val^S strains. The participation of RNase E in the processing of the *ilvGMEDA* transcript was examined in more detail. RNase E was purified from an *E. coli* strain containing the gene on a high level expression plasmid. A RNA segment of 1,136 nt containing the *ilvE* - *ilvD* intercistronic region was synthesized in vitro. Treatment of this RNA segment with RNase E revealed the presence of several major and minor cleavage sites for RNase E. This in vitro result supports the interpretation of the in vivo experiments that the *ilvE* transcript is derived in part from the *ilvEDA* transcript by RNase E processing.

Formation, Processing, and Stability of the *ilvGMEDA* Transcripts



the *ilvEDA* transcript originated directly from the internal *ilvEp* promoter. Some or all of the *ilvGMEDA* and/or *ilvEDA* transcripts were processed to the *ilvE* and *ilvDA* transcripts.

A Rho mutation reduces polarity and leads to increased degradation, rather than processing, of the ilvGMEDA transcript

A *rho* mutation increased the levels of the full length *ilvGMEDA* transcripts in the presence of the Val^S allele as predicted due to the presence of the Rho-dependent transcription termination sites in the *ilvGM* region in the Val^S strains. This *rho-115*-dependent increase in the levels of the full length *ilvGMEDA* transcript in the Val^S strain also led to an increase in the levels of the smaller transcripts. In the presence of a *rho-115* mutation, about half of the *ilvGMEDA* transcripts were degraded rather than processed to the smaller mRNAs. This result was not predicted, but is in accord with the report (Sozhamannan and Stitt, 1997) that some, but not all, *rho* mutations reduce the stability of bulk mRNA, and provides the first example of a specific transcript that is less stable in a *rho* mutant.

The ilvE transcript was more stable when processed from the more stable ilvEDA mRNA derived de novo from the internal ilvEp promoter

There were differences in the stability and pattern of decay of the *ilvE* transcript in the Val^S strain compared to the Val^R and the frameshift deletion strains. The *ilvE*

transcript showed a more gradual decay and longer relative stability in the Val^S strains compared to (i) the strain with the frameshift deletion in the presence of the RNase E mutation (Fig. 3-8, compare lanes 1-4, 10 min exposure to lanes 5-8, 3 min exposure), and (ii) the Val^R strain in the presence of the RNase P mutation (Fig. 3-9, compare lanes 16-20 enhanced to lanes 6-10). For the strains with the frameshift deletion (Fig. 3-8, lanes 5-8, 3 min exposure) and the Val^R allele (Fig. 3-9, lanes 6-10) the *ilvE* transcript went through a distinct rise in abundance and then fell to very low or undetectable levels. In contrast, in the Val^S strains the *ilvE* transcript level decreased gradually and was still detected at the last (15 min) time point (Fig. 3-9, lanes 1-4, 10 min exposure) or stayed at approximately the same level with a slight increase 2 min after rif addition (Fig. 3-9 lanes 6-20, enhanced). A comparison of Fig 3-10 and Fig. 3-11 reveals a similar results, with a gradual decay of the *ilvE* transcript in the Val^S strain (Fig. 3-11, lanes 5-8) and an abrupt drop in *ilvE* levels in the Val^R (Fig. 3-10, lanes 5-8)

Because the *ilvGp2* promoter initiates 5-10-fold more transcripts than the internal *ilvEp* under these growth conditions, most of the *ilvE* transcript was derived from the *ilvGMEDA* precursor in the Val^R and frameshift deletion strains. In contrast, due to the presence of the frameshift site in the Val^S strains, most of the *ilvE* transcript in the Val^S strains came from an *ilvEDA* transcript generated by the internal *ilvEp* promoter. Thus, the comparison of the Val^S to the frameshift deletion and the Val^R strains makes it possible to monitor the separate fates of the *ilvGMEDA* transcript derived from the upstream *ilvGp2* promoter compared to the *ilvEDA* transcript derived from the internal *ilvEp* promoter.

The abrupt rise and fall of the *ilvE* transcript in the Val^R (Fig. 3-9, lanes 6-10) and frameshift deletion (Fig.3-8, lanes 5-8, 3 min exposure) strains was due to the transient presence of the more abundant but less stable *ilvE* transcript processed from the *ilvGp2*-derived *ilvGMEDA* precursor. The more gradual decay of the *ilvE* transcript in the Val^S strain (Fig. 3-8, lanes 1-4, 10 min exposure; Fig. 3-9, lanes 16-20, enhanced) was due to a less abundant but more stable *ilvE* transcript processed from an *ilvEp*-derived primary *ilvEDA* precursor. Since the *ilvGMEDA* precursor is itself very unstable, it may have signals that lead to more rapid turnover of itself, as well as to the *ilvE* transcript derived from it.

The *ilvE* transcripts derived from the relatively stable de novo *ilvEDA* transcripts initiated at the constitutive internal *ilvEp* promoter were more stable than the *ilvE* transcripts derived from the very unstable *ilvGMEDA* transcripts initiated at the upstream attenuation controlled *ilvGp2* promoter. The dramatically higher levels of the *ilvDA* transcript in the Val^R compared to the Val^S strain, together with the greater post-translational stability of the *ilvA* gene product compared to the *ilvD* gene product, accounts for the "downstream amplification" effect in which the relative change in gene expression is *ilvA>ilvD>ilvE*.

Similar results were found monitoring expression from plasmids and single chromosomal copies, and using DIG and radioactive probes

It is significant that the experiments reported here gave similar results monitoring expression from plasmids and from single chromosomal copies. In addition, similar

results were independently observed using the DIG probe from single copy chromosomal genes and multicopy plasmids and from multicopy plasmids using radioactive probes. This independent corroboration using two different methods adds credence to the interpretation.

CHAPTER 5

CONCLUSION

1. RNase E, RNase P and PNPase, RNase II but not RNase III affect the ilvGMEDA transcripts
2. A Rho mutation reduces polarity and leads to increased degradation, rather than processing, of the ilvGMEDA transcript
3. The ilvE transcript was more stable when processed from the more stable ilvEDA mRNA derived de novo from the internal ilvEp promoter
4. Similar results were found monitoring expression from plasmids and single chromosomal copies, and using DIG and radioactive probes

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IMAGE EVALUATION
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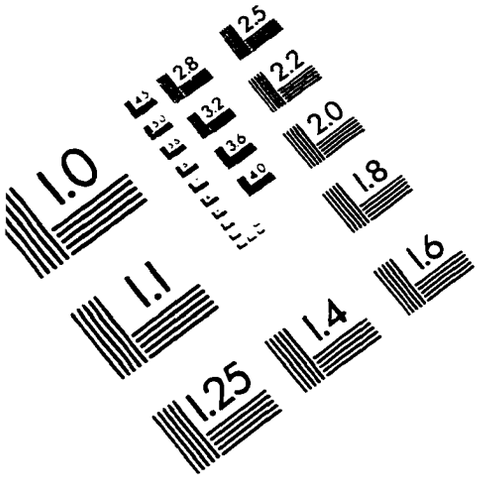

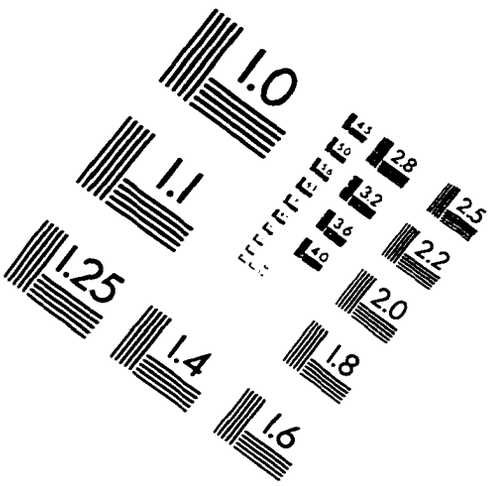

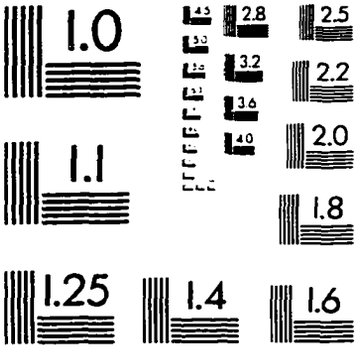

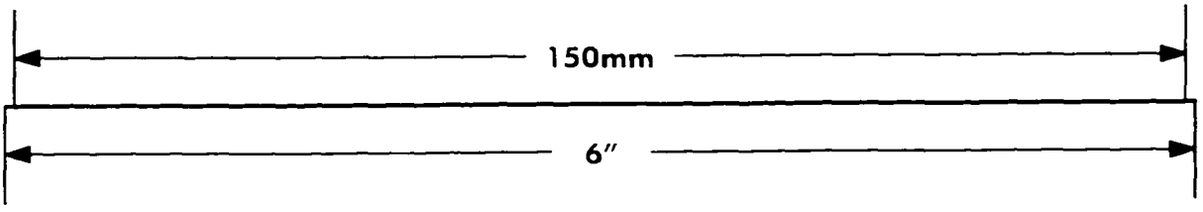

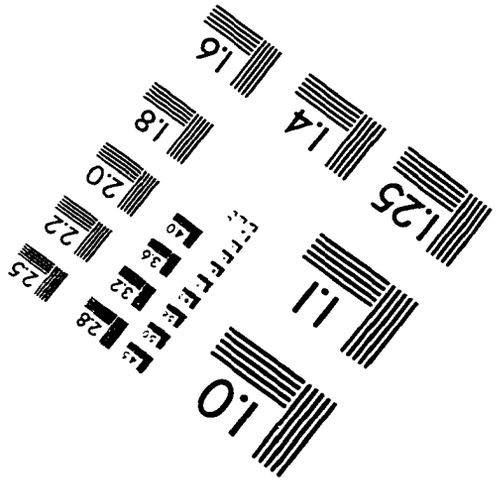

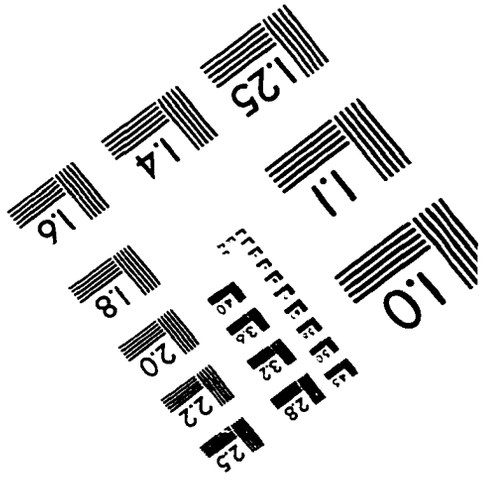

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989