# Transcription of single-copy hybrid *lacZ* genes by T7 RNA polymerase in *Escherichia coli*: mRNA synthesis and degradation can be uncoupled from translation

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## ABSTRACT

**In *Escherichia coli* transcription of individual genes generally requires concomitant translation, and thus the decay of mRNAs cannot be studied without the complication of translation. Here we have used T7 RNA polymerase to transcribe *in vivo lacZ* genes carrying ribosome binding sites of variable efficiency. We show that neither cell viability nor growth rate is affected by the T7-driven transcription of these genes, provided that they are present as single chromosomal copy. Furthermore, transcription is now completely uncoupled from translation, allowing large amounts of even completely untranslated mRNAs to be synthesized. Taking advantage of these features, we discuss the influence of the frequency of translation upon the processing and degradation of the *lac* message.**

## INTRODUCTION

Bacterial mRNAs vary widely in their stabilities, and this feature contributes largely to the uneven expression of bacterial genes (1). However, those properties of mRNAs which are responsible for their variable lifetimes are quite obscure. A major difficulty, which confuses the interpretation of many experiments, arises from the tight coupling which exists *in vivo* between the translation and the transcription of many genes. Thus, a reduced translation will increase the probability that transcription stops before reaching the end of the gene (polarity; see ref. 2–5). It follows that untranslated mRNAs are generally not synthesized to the end, and therefore it is difficult to study the mechanisms responsible for mRNA decay without the complication of concomitant translation (6).

In an effort to bypass the polarity problem, Stanssens et al. introduced into the cell an antiterminator from phage lambda, the protein N (4). When a binding site for N (*nut*L) is present within the gene of interest, polarity is effectively eliminated. However, the requirement for a *nut* site places constraints on the transcribed sequence; moreover, the N protein seems somewhat toxic to *Escherichia coli* (4). An alternative approach consists of using an heterologous, polarity-insensitive, RNA polymerase to transcribe the target gene. Bacteriophage T7 RNA polymerase seems to be promising in this respect (7,8). Unfortunately, the enzyme is so active that the cell will generally not support the presence of a multicopy plasmid bearing a functional T7 promoter, and therefore the usefulness of this system for uncoupling transcription from translation has not been fully exploited (but see (9)).

In this work, we have introduced a single copy of the large, polarity-sensitive, *lacZ* gene, bracketed by a promoter and a terminator specific for T7 RNA polymerase, into the chromosome of an *E. coli* strain which produces this enzyme. We show that, in this case, transcription of the *lacZ* gene is not harmful to the cell, and that balanced growth is easily achieved. Using a collection of *lacZ* genes which differ only by point mutations within the ribosome binding site (RBS; see (10)) we further show that, in this system, transcription is fully independent of translation, so that large amounts of mRNA can be detected even when the gene is not translated at all. We use this system to investigate the relationship between *lacZ* translation and *lac* mRNA maturation and degradation, and we discuss the results in view of current models on mRNA degradation in *E. coli*.

## MATERIALS AND METHODS

### Bacterial strains

*E. coli* strain JM101 (Δ(*lac pro*) *thi supE* F′ *traD36 proAB lacI*$^q$ *lacZ*ΔM15) was routinely used for genetic constructions. C600 (*supE44 thi1 thr1 leuB6 lacY1 tonA21*) was the recipient host for single-copy transfers into the chromosome (11). Strain BL21(λDE3) (*hsdS gal*) was obtained from Dr. M. Uzan, with permission from the authors (7). This strain carries a single copy of the bacteriophage T7 gene 1 coding for the RNA polymerase, under the control of the IPTG-inducible *lac*UV5 promoter.

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### Plasmid construction

We started from pSB118, a pUC18 derivative which carries a second *Eco*RI site downstream from the *Hind*III site (courtesy of Dr. P. Stragier). To avoid homologous recombination between the *lacZ* α-peptide sequence of pSB118 and the *lacZ* gene to be introduced later, we first deleted the *Eco*RI-*Nar*I fragment encompassing the α-peptide coding sequence as well as the polylinker, and subsequently reintroduced the polylinker (which was fortuitously inverted at this stage) into the reconstituted *Eco*RI site. Moreover, the unique *Pst*I site was removed with T4 DNA polymerase. As outlined in Fig. 1, we then introduced successively the following fragments into the *Bam*HI site, which remained unique throughout the procedure: the *Bgl*II-*Bgl*II fragment of plasmid pET-3 encompassing the T7 gene 10 promoter and terminator (12), a 4 kbp fragment from plasmid pMC1396 (13) which encompasses the *lac* sequence from the eighth codon of *lacZ* to the middle of *lacY*, and finally a *Bam*HI-*Bgl*I fragment from plasmid pEMBLΔ46 (14). The latter provides two nearby *Bam*HI and *Pst*I sites for convenient in-phase cloning of fragments carrying ribosome binding sites in front of *lacZ*. The resulting plasmid is named pMAMA0 (Fig. 1).

We have recently reported the isolation of a 71 bp *Bam*HI-*Pst*I fragment carrying the *galE* RBS (fragment named 113−10 in Ref. 14). Three mutated alleles of this fragment, carrying the simple or multiple mutations *l8l9*, *l17l18l22*, and *l29* which were formerly reported to depress translation of the *galE* gene (15; 16; Fig.1) were constructed by oligonucleotide-directed mutagenesis. Similarly, a 51 bp *Bam*HI-*Pst*I fragment carrying the RBS of *lamB* was synthesized, and the mutations *701*, *708* and *713* previously shown to depress the translation of this gene were introduced into it by site-directed mutagenesis (17; Fig. 1).

The fragments carrying the wild-type or mutated alleles of the *galE* and *lamB* RBSs were then cloned into pMAMA0. The resulting plasmids are named pMAMA1 and pMAMA5, respectively, followed by the name of the relevant mutation (Fig. 1).

### Transfer of the target gene onto the chromosome of the T7 polymerase-producing strain

The transfers were performed as described by Raibaud et al. (11,18), except that pOM43 (see below) was used instead of the originally described pOM41. The latter is a pBR322 derivative which carries two large inserts from the *malA* region of the chromosome separated by a unique *Eco*RI site, into which genes to be transferred are cloned. This plasmid is designed so as to allow an easy selection of the Mal$^-$ Amp$^s$ recombinants resulting from double homologous recombination between the plasmid and the chromosome, followed by plasmid segregation (11). The polylinker from pSB118 was inserted into pOM41 with the orientation *malT-Eco*RI-*Kpn*I-*Xba*I-*Eco*RI-*malPQ*, yielding pOM43, and the *Kpn*I-*Xba*I inserts from the pMAMA series were unidirectionally cloned in this vector and subsequently transferred into the *malA* region of the chromosome of C600, a strain which does not produce T7 RNA polymerase.

From BL21(λDE3), we constructed the derivative MO00, which harbors a Tn*10* insertion within its endogenous *lacZ* gene, and two selectable markers (*glpD* and *aroB*) on each side of the *malA* region. *lacZ*::Tn*10* was introduced by transducing BL21(λDE3) with a P1 lysate grown on Hfr3000 *lacZ*::Tn*10* and selecting for tetracycline resistance. *aroB* and *glpD* were simultaneously introduced into the resulting strain by isolating a spontaneous *malT* derivative and transducing it for the Mal$^+$Aro$^-$Gly$^-$ phenotype, using a P1 lysate grown on pop3653 (19). Finally, the target genes were introduced into MO00 by transduction, using P1 lysates made on the C600 derivatives bearing the *Kpn*I-*Xba*I inserts from the pMAMA0, pMAMA1, pMAMA5 and the mutated versions of the latter two, and selecting on minimal plates containing glycerol as the sole source of carbon (Gly$^+$Aro$^+$ transductants). The correctness of the final strains was ascertained by checking their Mal$^-$ character, by Southern blot analysis and by direct sequencing of the promoter/RBS region after PCR amplification. The strains are designated MO0 (no RBS), MO1 (wild-type *galE* RBS), MO3 to MO6 (wild-type, *701*, *708*, and *713* alleles of the *lamB* RBS), and MO8 to MO10 (*l8l9*, *l17l18l22*, and *l29* alleles of the *galE* RBS, respectively).

We have recently constructed a strain (HfrG6Δlac12) designed to allow the exact replacement of the *lacZ* RBS by various cloned RBSs within the genuine chromosomal *lac* operon (14). In order to record the effect of the RBS mutations upon transcription when the *E.coli*, rather than T7, RNA polymerase is used, the *galE* RBS and its mutated *l8l9* allele were transferred in front of *lacZ* in HfrG6Δlac12.

### Enzyme assays

For enzyme assays, bacteria were grown in either MOPS medium with 0.2% w/v glycerol (20) or in minimal M63B1 medium with 0.4% w/v glycerol (21), as indicated, and in the presence of 0.7 to 1 mM IPTG, a concentration large enough to fully induce the *lac* promoter even in *lacY* strains. Cells were harvested in exponential phase, disrupted by sonication, centrifuged, and β-galactosidase, lactose transacetylase, or amylomaltase activities were assayed in the supernatant (21−23). β-galactosidase activities are expressed in nmole ONPG hydrolyzed per min and per mg of protein. The total protein concentration was estimated according to Lowry et al. (24).

### Northern blots

RNA was extracted as described in Aiba et al. (25) from cells growing exponentially in MOPS-glycerol medium. RNA (5 μg) was electrophoresed on a 1% agarose-formaldehyde gel and capillary blotted onto a Hybond nylon membrane. To probe the *lacZ* transcripts, we used the 17-mer universal sequence primer (complementary to nt. 59 to 75 of the *lac* message) which was 5′ end-labelled with $^{32}$P (26). The membranes were subsequently washed and reprobed successively with the *Kpn*I-*Xba*I fragment from pMAMA0 (full-length probe; see Fig. 1), and with the *Bam*HI-*Bgl*II fragment from plasmid pET-3, encompassing the T7 terminator (12). Both fragments were $^{32}$P-labelled by random priming, as recommended by BRL.

## RESULTS AND DISCUSSION

### Construction and properties of strains carrying a single copy *lacZ* gene under the control of T7 RNA polymerase

Figure 1 describes the construction of a cassette carrying the *lac* sequence extending from the eighth codon of *lacZ* to the middle of *lacY*, bracketed by the T7 gene 10 promoter and terminator (12). This construct lacks the natural *lacZ* ribosome binding site (RBS), which is replaced by unique *Bam*HI and *Pst*I sites into which fragments harbouring various other RBSs can be conveniently introduced (Fig. 1). Since, for the present work, it was desirable to compare mRNAs which differ vastly in their translational efficiencies but yet are as alike as possible so as

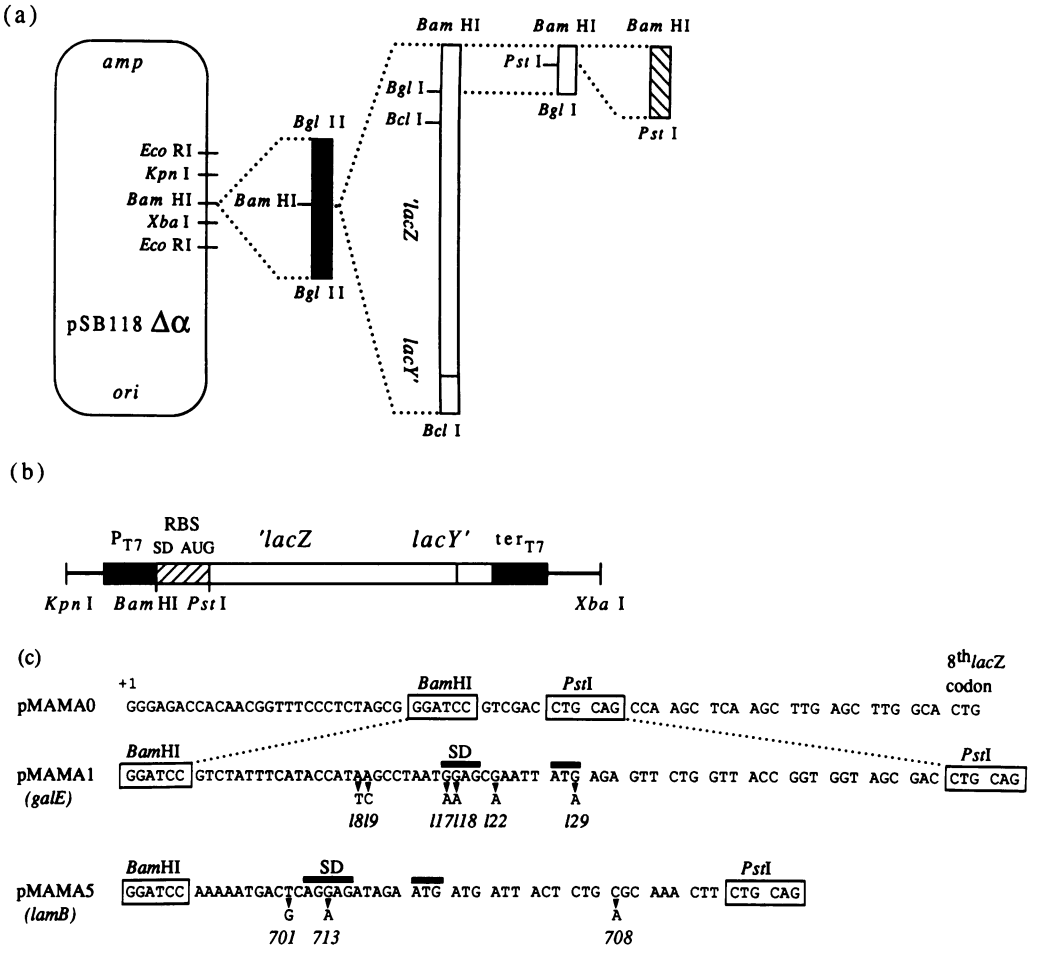


**Figure 1.** Construction of a *lacZ* gene placed under the control of the T7 gene 10 promoter, and carrying different RBSs. (a) general outline of the cloning strategy. Black box: T7 gene 10 promoter and terminator from plasmid pET3 (12). Open box: *lacZ* fragments from pMC1396 (13) and pEMBLΔ46 (14). Hatched box: RBS-containing fragments (14). (b) Schematic drawing of the resulting *lac* hybrid gene. (c) Sequence of the 5′ end of the hybrid mRNAs, up to the 8th *lacZ* codon. Nucleotides 1 to 26 originate from the leader sequence of the T7 gene 10.

to minimize differences unrelated to translation, we decided to start from well characterized RBSs and to introduce into them point mutations that depress their activity; we then compared the *lacZ* genes carrying either the wild-type or the mutated RBSs.

Many such mutations have been formerly characterized in the RBSs of the *galE* and *lamB* genes, which encode the enzyme UDP-galactose epimerase and the phage lambda receptor, respectively. In particular, Schwartz et al.. have isolated three nearby mutations affecting the translation of the *lamB* gene. One of these mutations (*713*) altered the Shine-Dalgarno (SD) element, whereas the other two (*701* and *708*), resulted in similar inhibitory secondary structures (17; Fig. 1). Concerning the *galE* RBS, Busby and Dreyfus (16) isolated a triple mutant, denoted *l17l18l22*, in which the SD sequence had been destroyed, and a simple mutant (*l29*) in which the initiator AUG had been converted into AUA. In addition, Bingham et al (15) described a double mutation (*l8l9*), which presumably inhibits translation through the formation of a stable hairpin sequestrating the initiation codon and the SD element (Fig.1). Eight DNA fragments carrying either the wild-type *galE* and *lamB* RBSs, or their mutated alleles, were prepared and inserted into the cassette, in front of *lacZ* (Fig.1). These constructs, as well as the parent construct lacking a RBS, were then transferred into the *malA* region of the chromosome of MO00, a *lacZ*::Tn*10* derivative of BL21(λDE3) (Fig. 2). The latter *E. coli* B strain harbors the T7 gene *1*, coding for the RNA polymerase, under the control of the IPTG-inducible *lac*UV5 promoter (7). The two-step transfer procedure which is outlined in Materials and Methods was chosen to avoid exposing the T7-polymerase producing strain to multiple copies of the target *lacZ* gene.

Contrasting with those strains which harbour T7-driven genes on multicopy plasmids, the final strains were stable and grew identically whether IPTG was present or not. As expected, the synthesis of β-galactosidase was IPTG-inducible, and all mutations introduced into the *lamB* and *galE* RBSs decreased expression compared to wild-type, as in the original *galE* and *lamB* genes. Altogether, the β-galactosidase activities spanned three orders of magnitude, being highest for the wild-type *lamB* RBS, and lowest for the *l8l9* allele of the *galE* RBS (Table I and Fig. 3). However, even in this latter case it was still 10-fold higher than in the complete absence of an RBS (compare MO0 and MO8 in Table I).

IPTG stimulated the synthesis of β-galactosidase about 60-fold (Table I); interestingly, the synthesis of the T7 RNA polymerase, which is under the control of the *lac*UV5 promoter, is not expected to be induced to that extent (27). This suggests that the T7 gene *10* promoter may not be saturated with polymerase, even when IPTG is present. Yet, the amount of β-galactosidase

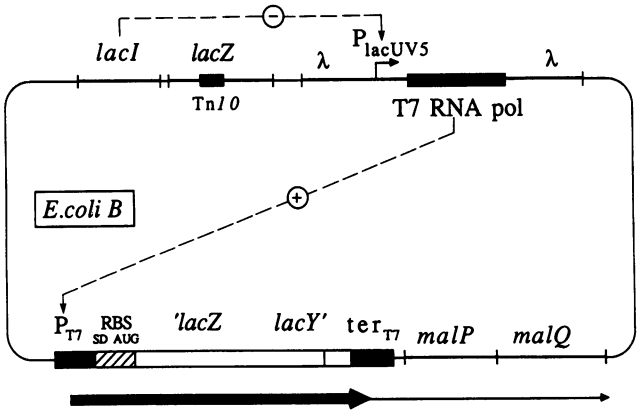


**Figure 2.** Structure of *E. coli* B strains carrying a single-copy *lacZ* gene transcribed by T7 RNA polymerase. The *KpnI-XbaI* fragments from pMAMA0, pMAMA1, pMAMA5 and the mutated derivatives of the latter two (Fig. 1) were inserted into the *malA* region of the chromosome of MO00 (see Materials and Methods). Black rectangles represent genetic elements from bacteriophage T7. The symbols + and − stand for positive and negative controls by the T7 polymerase and *lac* repressor, respectively.

**Table I.** β-galactosidase and amylomaltase levels in strains carrying various RBSs in front of *lacZ*.

| Strain | RBS | IPTG (1mM) | β-galactosidase units/mg | amylomaltase units/mg |
|---|---|---|---|---|
| MO0 | none | + | 2 | 410 |
| MO8 | *galE l8l9* | + | 30 | 450 |
| MO1 | *galE* | + | 17,000 | 375 |
| MO1 | *galE* | − | 280 | 10 |

For description of strains, see legend of Fig. 2. Cells were grown in M63 glycerol medium. Activities are averaged from three independent measurements.

synthesized from the highest producers was already impressive, especially given that the *lacZ* gene is single copied. Indeed, on PAGE-SDS gels, the level of synthesis of β-galactosidase driven by the wild-type *lamB* RBS appeared much higher than that of any other cellular proteins, amounting to c.a. 13−15% of total protein synthesis (J. Guillerez, unpublished observations). In most systems designed so far for high expression of foreign genes in *E.coli*, the target genes are placed on multicopy plasmids, the stability of which is often problematic. We believe that the use of single copy chromosomal genes, transcribed by the very active T7 RNA polymerase, may be seriously considered as a possible alternative.

## Transcription of the *lac* mRNA by T7 polymerase is uncoupled from translation

In order to test whether in this system transcription is actually uncoupled from translation, we used the two strains that harbour either the wild-type or the *l8l9 galE* RBS: the *l8l9* double mutation resulted in a 600-fold drop in β-galactosidase synthesis (Table I). For completeness, the strain lacking a RBS was also included in the comparison.

To check that transcription and translation are effectively uncoupled, we exploited the leakiness of the T7 gene *10* terminator (7). Indeed, the downstream *malQ* gene (Fig. 2), coding for amylomaltase, was substantially expressed when the cassette was transcribed. β-galactosidase and amylomaltase activities were induced to the same extent by IPTG in the case of the *galE* RBS, showing that the two genes are cotranscribed by T7 RNA polymerase. Strikingly, however, the synthesis of amylomaltase was the same, independent of which RBS was located in front of *lacZ* (Table I). A control experiment showed that, in contrast, the *l8l9* mutation caused considerable polarity when the *lacZ* gene was transcribed by the *E.coli* RNA polymerase. To this end, we used a recently described technique which allows the replacement of the genuine *lacZ* RBS by cloned RBS from other genes, within the chromosomal *lac* operon (14). This was done for the *galE* RBS and its mutated allele. In this context, the *l8l9* mutation caused a 20-fold drop in the expression of the promoter distal gene, *lacA*, which encodes lactose transacetylase (Jacques et al., in preparation).

Thus, the activity of T7 RNA polymerase is insensitive to accompanying translation. Studier already reported that amber mutations in bacteriophage T7 were nonpolar (28), and, more recently, Lindahl et al. noted that, when the bicistronic S10-L3 operon was transcribed by the T7 RNA polymerase, the transcription of the downstream gene was insensitive to the translation of the upstream one (9). Here we have shown that T7 polymerase will pass through the very long, polarity-sensitive *lacZ* gene, whether translated or not. The reason for this insensitivity presumably lies in the rapid elongation rate of the enzyme (29), which runs far ahead of accompanying ribosomes and therefore is unaffected by their eventual absence. In contrast, transcription by *E.coli* RNA polymerase is known to be tightly coupled to translation: any factor which increases the distance between the polymerase and the leading ribosome—premature termination of translation, or reduction in the translation initiation frequency or elongation rate—elicits efficient pausing or termination of transcription (2−5). In particular, this holds true for the *lacZ* gene (30). Transcription-translation coupling in bacterial genes probably serves to cut off the wasteful transcription of untranslatable mRNAs. It is also exploited in some delicate regulatory loops, such as attenuation in biosynthetic operons (31). Apparently, bacteriophage T7 does well without these subtleties.

## Degradation and processing of the *lac* message as a function of translation

As pointed out above, it is technically difficult to study *in vivo* the decay of mRNAs without the complication of interfering translation (6). Moreover, it is often not easy to distinguish between those species resulting from premature termination of transcription, and those which are processed from longer transcripts. The use of T7 RNA polymerase should help to sort out these issues, since normal *E. coli* terminators are inefficiently recognized by this enzyme (32) and since transcription is now uncoupled from translation.

The steady-state pattern of translated or non-translated *lac* mRNA was examined by Northern blot analysis. For these experiments, RNAs extracted from strains harbouring the wild-type or mutated alleles of the *galE* and *lamB* RBSs, or no RBS, were probed with an oligonucleotide complementary to nt. 59 to 75 of the *lac* mRNA. This analysis disclosed interesting differences between untranslated and translated mRNA. In the former case, two prominent bands, corresponding to the full-length transcript (4.2 kb) and to an abundant 5'-terminal fragment (0.24 kb), were detected (Fig. 3 A and B, lane 0 RBS). A short transcript was also seen when the mRNA was translated, and it was shifted upwards by c.a. 60 nt., as expected from the insertion of the RBS. In addition, a new band corresponding to

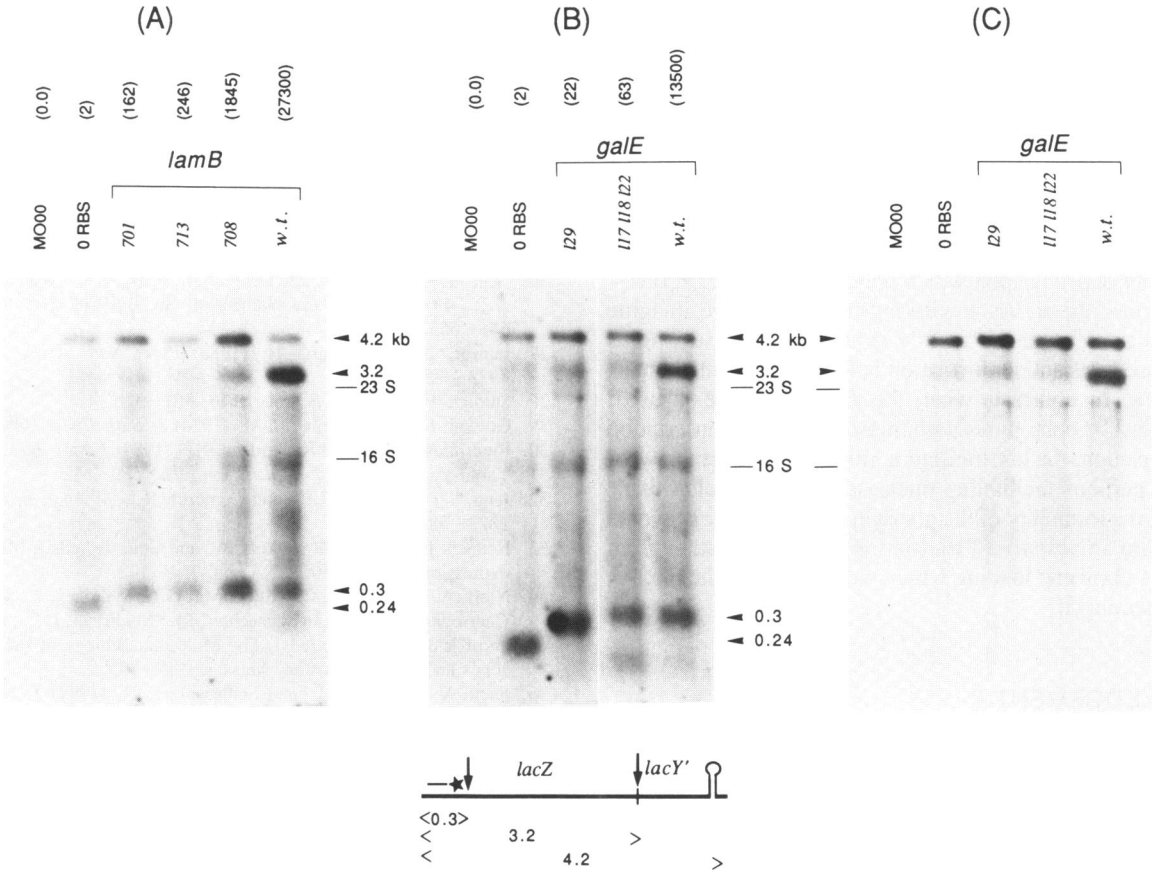


**Figure 3.** Northern blot analysis of RNAs (5μg) extracted from derivatives of strain MO00 carrying either the *lac* cassette without a RBS (0 RBS), or the *lac* cassette with the wild-type or mutated *galE* and *lamB* RBSs, as indicated (cf. Fig. 1 and 2). Cultures were grown in MOPS-glycerol medium, and the corresponding β-galactosidase activities are given in parenthese above each lane. In A and B, the blots were probed with the universal sequence primer (complementary to nt. 59 to 75 of the *lac* message). In C, the same membrane as in B was reprobed with the randomly labelled *Kpn*I-*Xba*I fragment of pMAMA0, which encompasses the whole *lac* cassette. A schematic drawing showing the 3′ end of the processed transcripts (arrows) and the position of the oligonucleotide probe (starred bar) is also shown.

a 3.2 kb species was observed for the highest β-galactosidase producers (Fig. 3A and 3B, lanes *lamB 708*, *galE wt* and *lamB wt*). The accumulation of this species was dependent upon the level of β-galactosidase synthesis, but apparently not upon the exact nature of the RBS. Essentially the same picture was observed when the membranes were probed with a fragment corresponding to the whole length of the transcript (fragment *Kpn*I-*Xba*I in Fig. 1), indicating that species lacking the 5′ end of the message do not accumulate (Fig. 3C; the 0.24−0.3 kb species only became visible upon longer exposures). Finally, as expected, neither the 0.24−0.3 kb species, nor the 3.2 kb species, were seen with a probe encompassing the T7 terminator only. Aside from the 4.2 kb full-length species, this 3′ probe detected only a 0.2 kb species, which corresponds to sequences outside the *lacZ* gene. Consistent with the insensivity of the T7 RNA polymerase to polarity, the intensity of this band was the same irrespective of *lacZ* translation (not shown).

The 3.2 kb mRNA species has been observed as a major product of *lac* transcription by *E.coli* RNA polymerase (33, 34; I. Iost and M.D., unpublished observations), and it has been attributed either to processing of the full-length message between *lacZ* and *lacY* (33), or to termination of transcription in the intergenic region (34). That the same species is observed with a transcript synthesized by T7 RNA polymerase indicates that it results at least in part from processing. Moreover, our observations indicate that the accumulation of this processed transcript requires translation. Conceivably, translation might either protect this species against degradation, or accelerate the processing of the full-length transcript, or both. Since the emergence of this species is accompanied by at most a modest fading of the 4.2kb species, the second hypothesis requires that the processed species is much longer-lived than the unprocessed one, so that a small amount of extra processing causes a visible accumulation of the processed species. The stabilities of these different species is currently being investigated..

As for the 3.2 kb. species, the 0.24 kb species, which has been observed during transcription of the *lac* operon by the *E.coli* enzyme (4, 30) must be processed from a longer transcript. When transcribed by the *E.coli* enzyme this species was particularly apparent when *lacZ* was under the control of inefficient RBSs (4), or when cells were aminoacid-starved, i.e. when ribosomes lag behind polymerase (30). We confirm here that this species is quite abundant in the absence of transcription/translation coupling (Fig. 3). It is not clear at present whether the 0.24 kb species (as well as the 3.2 kb. species) results from a primary endonucleolytic hit (35) or from exonuclease trimming of longer products.

The mechanism of degradation of the *lacZ* message has been extensively studied. Kennell et al. (reviewed in 33) have observed that the lifetime of this mRNA is very short when ribosomes are denied access to it. Therefore, they proposed that the degradation of the message keeps up with the last translating ribosome, with

segments of the naked message being chopped off endonucleolytically and rapidly trimmed by 3'-5' exonucleases. Here, we have observed that the full-length transcript, as well as its 0.24 kb. processed fragment, accumulate in considerable amounts even when the message is not translated at all (compare the intensity of the 4.2 kb band in the lanes 0 RBS of Fig. 3A and 3B, with the summed intensities of the 4.2 and 3.2 kb bands in lanes *lamB wt* and *galE wt*, respectively). This would suggest that the naked *lac* mRNA is not *intrinsically* unstable, but rather that its stability depends upon which polymerase—T7 or *E.coli*—is used to transcribe it. As a working hypothesis, we imagine that in the former case, the rapidly synthesized message folds into a nuclease-resistant conformation before coming into contact with RNAses. In contrast, when the *E.coli* enzyme is used, translation must be contiguous with transcription for transcription to go to completion: the last translating ribosome will then unwind the mRNA, perhaps facilitating nuclease action. Should this be correct, then the instability of the poorly translated message would not result from an *absence* of translation *per se*, but rather from an *inefficient* ribosome loading which would unwind the mRNA without protecting it.

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