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Translational Efficiency of φX174 Lysis Gene *E* Is Unaffected by Upstream Translation of the Overlapping Gene *D* Reading Frame

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The lysis gene *E* of bacteriophage φX174 is entirely embedded in gene *D*. Expression studies of genes *D* and *E* in *Escherichia coli* minicells and lysis times obtained in the presence or absence of *D* translation showed that the simultaneous expression of gene *D* does not affect protein E production. Thus, unlike other overlapping gene pairs, gene *E* expression is independent from the upstream translation of gene *D*. *lacZ* fusion studies and primer extension inhibition analysis (toeprinting) revealed an intrinsically weak *E* ribosome-binding site, which seems to be the major factor determining the low expression rate of the gene and thus proper scheduling of cell lysis.

Bacteriophage φX174 has a single lysis gene, *E* (26). The expression of gene *E*, either from the vegetative genome or from a plasmid, is necessary and sufficient to cause lysis of *Escherichia coli* (24, 42). Gene product E (gpE) is a membrane-bound polypeptide with an M_r of 10,500 (1, 6). Sodium dodecyl sulfate-resistant multimeric forms of gpE have been detected in extracts of envelope fractions, and it has been proposed that it forms an oligomeric structure penetrating the inner and outer membranes (8). In this model, lysis is considered to be the result of a sudden release of the osmotic pressure to the environment. It is not known whether the formation of the lysis pore requires anything more than accumulation of a certain level of gpE. Recessive mutations in a host gene *slyD*, mapping at 72.3 min on the *E. coli* chromosome, block the lethal effect of gpE, even from very strong promoters (32), and may define an intracellular target for the lysis protein. In any case, the absolute level of gpE at the time of lysis is in the order of 100 to 1,000 molecules (32). In the φX174 life cycle, gene *E* expression therefore must be strictly limited to prevent premature lysis of the host.

Inspection of the translational start region of *E* reveals a consensus Shine-Dalgarno sequence, GAGG, at nucleotide positions 555 to 558 (see Fig. 2). The importance of this sequence for translational initiation of gene *E* was first demonstrated by using site-directed mutagenesis to change GAGG to GAAG without affecting the overlapping gene *D* reading frame (20). This phage mutant showed a very delayed lysis phenotype, indicating that *E* translation was drastically reduced.

Transcription of φX174 mRNA is principally driven by three main constitutive promoters, designated promoters A, B, and D, as a result of their location upstream of the corresponding genes (13). Since each φX174 transcript includes the *E* cistron (23), transcriptional control is not an option in regulation of lysis gene expression. Thus, control of gene *E* expression should occur posttranscriptionally. Gene *E* is entirely embedded within gene *D* (39), the product of which acts as the major scaffolding protein (19). The consequence of an overlapping gene organization on gene *E* expression has been discussed (2, 18), and it was predicted that the upstream translation of gene *D* would severely limit gene *E* initiation. Moreover, in studies recently reported by Buckley and Hayashi (11), gene *E* expression appeared to depend on a −1 frameshift within gene *D* when *D* is being translated simultaneously. According to their model, a frameshift would lead to chain termination events just 5′ of the *E* start and thus would allow initiation of *E*.

In this study, we examined the effect of different factors that could affect gene *E* expression on a translational level. The influence of upstream translation of gene *D*, the strength of the *D* and *E* ribosome-binding sites (RBS), the mRNA structure around the *E* RBS, and the possibility of occurrence of a frameshift event within the *D* reading frame were investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12ΔH1Δtrp Sm^r *lacZ*(Am) Δ(*bio-uvrB*) Δ*trpEA2* (λ *N*am7 *N*am53 *c*I857 ΔH1) and plasmid pLc236 (35) were provided by E. Remaut, University of Ghent, Ghent, Belgium. *E. coli* MC1061 *araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsdR rpsL* and plasmid pMC1403 (12) were provided by M. J. Casadaban. The genotypes of *E. coli* CQ21 (34), DS410 (7), CJ236 (29), and pop2135 (10) are described elsewhere. Plasmids pUR222 (37), pcI857 (36), pSB1 (9), pBH20 (27), and pSB12 (7) have also been described previously. Cells were routinely grown at 28°C in LB broth (33) in the presence of 100 μg of ampicillin per ml. Cell growth and lysis were monitored by measuring the optical density at 600 nm.

Construction of plasmids. Both φX174 genes *D* and *E* were placed under control of the lambda p_L promoter in plasmid pSB35 (Fig. 1). Replicative-form intermediate DNA of φX174 was prepared as described previously (42) and digested with *Hpa*I. The 1,264-base-pair (bp) *Hpa*I fragment containing the complete φX174 genes *C*, *D*, *E*, *J*, and *K* and part of *F* was subsequently cleaved with *Sfa*NI. The *Sfa*NI cleavage removed the φX174 promoter located directly upstream of the *D* start site (Fig. 1). The resulting 927-bp fragment corresponds to nucleotides 368 to 1294 of the φX174 DNA and carried the complete φX174 genes *D*, *E*, and *J*. After filling in the 3′ of recessed ends created by *Sfa*NI, the fragment was cloned under control of the lambda p_L promoter into the filled-in *Eco*RI site of plasmid pLc236.

Plasmid pSB36 is isogenic to pSB35. As described below, a T → A change was performed by site-directed mutagenesis

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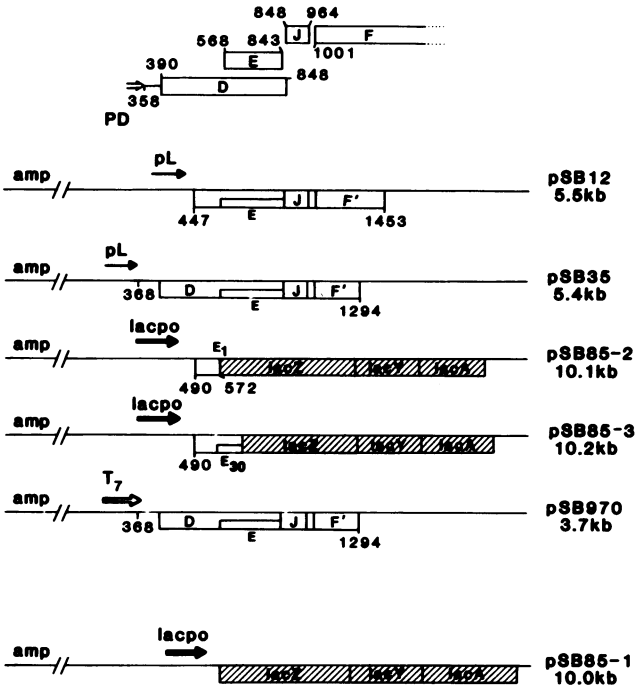

FIG. 1. Schematic diagrams of plasmids pSB12, pSB35, pSB85-1, pSB85-2, pSB85-3, and pSB970. The top section shows the ϕX174 genome organization at the *D-E* overlap. The nucleotide numbers are taken from Sanger et al. (39). PD, ϕX174 promoter *D*. Lysis gene *E* is translated in the +1 reading frame relative to gene *D*. Symbols: ▭, Genetic information of ϕX174; ▨, *lac* operon genes. Capital letters signify ϕX174 genes. Details of plasmid constructions are outlined in Materials and Methods. Direction of transcription from the p_L, *lac* promoter-operator (lacpo), or T7 promoter is shown by an arrow.

at position 554 in gene *D* (Fig. 2). The ϕX174 genes were then isolated from the M13mp18 construct on an *Eco*RI-*Bam*HI fragment and cloned into plasmid pLc236.

Two ϕX174 gene *E* (*E*ϕ)-*lacZ* fusions were constructed. The restriction fragments containing the translational start site of gene *E* were derived from plasmid pSB1. Plasmid pSB1 was subjected to *Pvu*II-*Rsa*I and *Pvu*II-*Hin*dII digestion, respectively. The generated fragments of 271 bp (*Pvu*II-*Rsa*I fragment) and 355 bp (*Pvu*II-*Hin*dII fragment) contained both the *lac* promoter-operator region and the translational start sites of *lacZ* and gene *E*. Both fragments were cloned into the filled *Eco*RI site of plasmid pMC1403, giving rise to plasmids pSB85-2 and pSB85-3, encoding an E_1ϕ-*lacZ* and an E_{30}ϕ-*lacZ* fusion, respectively (Fig. 1). The *lacZ* transcriptional and translational control signals were reconstituted in the control plasmid pSB85-1 (Fig. 1) by inserting the 198-bp *Pvu*II-*Bam*HI fragment of plasmid pUR222 into the *Sma*I-*Bam*HI sites of pMC1403.

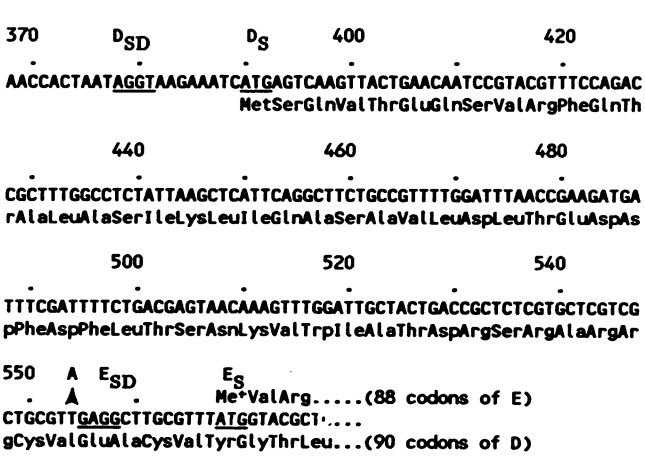

FIG. 2. Translational start regions of ϕX174 genes *D* and *E*. The DNA sequence is taken from Sanger et al. (39). The Shine-Dalgarno elements (D_{SD} and E_{SD}) and the corresponding start codons (D_S and E_S) are underlined. The T → A mutation (nucleotide 554) present in plasmid pSB36 is depicted.

Plasmid pSB970 is a derivative of plasmid pGEM3Z (Promega Biotec) carrying genes *D* and *E* under transcriptional control of a T7 promoter. The filled-in 927-bp *Sfa*NI-*Hpa*I fragment used in the construction of plasmid pSB35 (see above) was first cloned into the *Sma*I site of M13mp18. This provided suitable ends for insertion of genes *D* and *E* as a 970-bp *Eco*RI-*Pst*I fragment into pGEM3Z.

Plasmids pSB13-1 and pSB14-1 are derivatives of pBH20. As described below, an *Eco*RI site was created by site-directed mutagenesis at positions 546 and 550, respectively, 5′ of gene *E*. The resulting *Eco*RI-*Bam*HI fragments obtained from the M13mp10 construct were then each inserted into the *Eco*RI-*Bam*HI sites of plasmid pBH20.

Site-directed mutagenesis. Site-directed mutagenesis was carried out as described by Kunkel et al. (29). The oligonucleotide 5′-CGCAAGCCTCTACGCAGC-3′ (T → A at position 554) was used on the constructed single-stranded M13mp18 template carrying the 927-bp ϕX174 insert comprising genes *D*, *E*, and *J*. The oligonucleotides 5′-CCT CAACGCAGGAATTCGCACGAGAGC-3′ (*Eco*RI site at position 546) and 5′-GCAAGCCTCAACGAATTCACGAG CACG-3′ (*Eco*RI site at position 550) were used on a single-stranded M13mp10 template containing gene *E* on an *Eco*RI-*Bam*HI fragment derived from plasmid pKY117 (42).

In vitro transcription and toeprint analysis. To obtain a runoff transcript carrying ϕX174 genes *D* and *E*, 5 μg of plasmid pSB970 (Fig. 1) was restricted with *Bst*NI, which cuts at nucleotide position 881 within gene *J*. In vitro transcription was carried out by using T7 polymerase and the Riboprobe Gemini System II (Promega Biotec) as recommended by the manufacturer. A 0.5-μg amount of *Bst*NI-restricted pSB970 was used as a template for marker RNA synthesis in the presence of [α-^{32}P]UTP. The transcribed *D-E* mRNA was purified on an 8% polyacrylamide-urea gel (31), suspended in 30 μl of 0.1 mM EDTA, and used for toeprinting. A 10-pmol amount of the synthetic oligonucleotide 3′-CTCAAATAACGACGGCAGTA-5′, complementary to nucleotides 615 to 634 of the ϕX174 sequence, was labeled with 10 pmol of [γ-^{32}P]ATP. Annealing of the primer to purified mRNA, sequencing reactions, and primer extension inhibition analysis (toeprinting) were performed exactly as recently described (10, 22).

T_1 digestion. The same concentration of mRNA annealing mix used for toeprinting was subjected to RNase T_1 digestion. The T_1 reactions contained 2 μl of annealing mix, 1 μl of a 3.75 mM deoxynucleotide triphosphate mix, 1 μl of RNase T_1 (5 or 11 U), and 5 μl of standard buffer (10). The reactions were kept on ice for 10 min. Then 0.3 U of avian myeblastosis virus reverse transcriptase was added, and the reactions were incubated at 48°C for 15 min. Reactions were terminated by heating to 95°C and addition of 10 μl of loading dye.

Expression of plasmid pSB12- and pSB35-encoded proteins

TABLE 1. Lysis times of constructs used

Plasmid[a]	D upstream translation	E. coli strain	Lysis time[b] (min)
pSB12	−	K12ΔH1Δtrp	7
pSB35	+	K12ΔH1Δtrp	7
pSB12	−	pop2135	13
pSB35	+	pop2135	13
pSB36	+	pop2135	13

[a] A map of plasmids pSB12 and pSB35 is shown in Fig. 1.
[b] Cultures were grown at 30°C to an optical density at 600 nm of 0.3 and then shifted to 42°C. Lysis time is defined as the time in minutes between shift to 42°C and onset of lysis.

in minicells. *E. coli* DS410 minicells harboring either plasmids pSB12 and pcI857 or plasmids pSB35 and pcI857 were purified, labeled, and further processed as reported previously (7). The samples were analyzed on a 14 to 20% polyacrylamide-sodium dodecyl sulfate gel, and the labeled polypeptides were visualized by autoradiography.

β-Galactosidase assay. Since the *Eφ-lacZ* fusion protein encoded by plasmid pSB85-3 carries the first putative membrane insertion sequence of gpE (42), the whole extract of lysozyme-EDTA-disrupted cells carrying plasmids pSB85-1, pSB85-2, and pSB85-3, respectively, was used to measure β-galactosidase activity. *O*-Nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis was monitored at A_{420} in the supernatant after centrifugation (33).

RESULTS AND DISCUSSION

Simultaneous translation of gene *D* does not affect lysis timing and protein E synthesis. There are precedents of overlapping gene pairs in which the downstream gene is negatively coupled to translation of the upstream reading frame (14, 25, 40). A common phenomenon is that the absence of upstream translation leads to an increased translation of the downstream gene. In contrast, upstream translation of the MS2 coat gene is required for translational initiation of the overlapping lysis gene *L* (28). However, this does not apply for the *D-E* gene pair. It has been shown that translation of *D* is not necessary for protein E synthesis (7, 11, 24, 42).

To test whether upstream translation of gene *D* affects the translational efficiency of gene *E*, genes *D* and *E* or gene *E* were expressed by using the phage lambda p_L promoter on plasmid pSB35 or pSB12, respectively. The plasmids differ mainly in the presence of the translational start for *D* in pSB35 but not in pSB12 (Fig. 1). The use of these plasmids made it possible to evaluate the effect of *D* upstream translation on *E* initiation directly in the φX174 sequence context. It has been shown that onset of lysis mediated by cloned gene *E* to some extent depends on the *E. coli* strains used (U. Bläsi, unpublished data). Therefore, we compared the lysis time mediated by plasmid pSB12 or pSB35 in two different *E. coli* strains. Lysis with *E. coli* K-12ΔHIΔtrp carrying either plasmid pSB12 or pSB35 was observed 7 min after induction, whereas induction of gene *E* in pop2135 (pSB12) and pop2135(pSB35) resulted in lysis 13 min after induction (Table 1). This result showed that for a certain *E. coli* strain, the threshold level of gpE necessary to induce lysis is reached at the same time in the presence or absence of *D* translation.

In addition, we used *E. coli* minicells, which are not lysed by gpE, to assess the synthesis of *D* and *E* directly. Repression of the lambda p_L promoter on plasmids pSB12 and pSB35 was ensured by the presence of the compatible plasmid pcI857, carrying the temperature-sensitive *c*I857 repressor allele. As described earlier (7), the position of the monomeric form of gpE was identified by using an *E*-negative control (not shown). No difference in the amount of gpE produced from plasmids pSB35 and pSB12 was observed (Fig. 3, lanes 1 and 2). The percentage of gpE in both samples was approximately 2% of the total of labeled protein. Taken together, these data clearly demonstrated that protein E production was not detectably affected by the upstream translation of gene *D*.

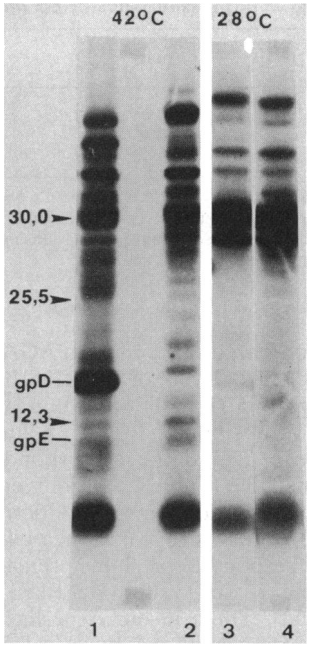

FIG. 3. Synthesis of protein E in the presence and absence of *D* translation. Lanes: 1 and 2, polypeptides synthesized at 42°C in thermally induced *E. coli* DS410 minicells carrying plasmids pSB35 and pcI857 or plasmids pSB12 and pcI857, respectively; 3 and 4, protein patterns of DS410(pSB35, pcI857) and DS410(pSB12, pcI857) minicells, respectively, obtained under uninduced conditions at 28°C. The proteins were labeled with [^{35}S]methionine, and 110,000 cpm per sample was resolved on a 14 to 20% sodium dodecyl sulfate-polyacrylamide gel. The positions and molecular masses (in kilodaltons) of marker proteins and of gpD and gpE are shown at the left.

On the basis of a gene fusion study, it has been proposed (11) that mistranslation of alanine codons in the *D* gene, upstream of the *E* start, acts as a frameshift-inducing signal, which leads to premature termination at nearby stop codons located in the −1 frame. These terminations appeared to be necessary for *E* initiation (11). Frameshifting is usually a low-probability event (15) but can reach a rate of 50% (16). Even if the proposed frameshifting within the *D* reading frame occurred with a high frequency, one would anticipate a delayed lysis time with plasmid pSB35 (5′ translation of *D*) in comparison with pSB12 (no 5′ translation). However, this was not the case (Table 1).

A TGA stop codon in the −1 frame at positions 554 to 556, which overlaps with the *E* Shine-Dalgarno sequence (Fig. 2), has especially been implicated in premature termination of *D* translation (11). According to Buckley and Hayashi (11), terminating ribosomes can then reinitiate at the *E* RBS, just downstream. To test directly whether this TGA stop codon is important for *D* chain termination, and consequently for *E*

TABLE 2. Expression rates of *Eϕ-lacZ* fusions

Plasmid	Fusion	β-Galactosidase activity[a]
pSB85-1		6,052
pSB85-2	E_1ϕ-*lacZ*	124
pSB85-3	E_{30}ϕ-*lacZ*	366

[a] *E. coli* MC1061 carrying plasmids pSB85-1, pSB85-2, and pSB85-3, respectively, was grown in LB broth. Triplicate samples of each culture were taken, and the β-galactosidase activity was determined as described in Materials and Methods.

initiation, we converted this codon to AGA (Fig. 2) by using in vitro mutagenesis. Lysis of pop2135 harboring the isogenic plasmid pSB35 (Fig. 1) or pSB36, which carries the T → A change at position 554 (Fig. 2), started 13 min after induction of gene *E*, and the corresponding lysis profiles were indistinguishable (Table 1). Moreover, in vitro translation of the *D*- and *E*-containing mRNA derived from plasmid pSB970 (Fig. 1) did not reveal truncated D polypeptides, which could correspond to products prematurely terminated upstream of the *E* start site (U. Bläsi and R. Young, unpublished data). Thus, in our experiments we found no indication that a frameshift event within *D* is necessary for *E* initiation while genes *D* and *E* are translated simultaneously. How can we account for the discrepancies between our results and the findings of Buckley and Hayashi (11)? As discussed below, it is possible that the use of the very strong *cro* translational initiation site (41) and its 5′ coding sequences for their studies as well as moving these sequences close to the *E* initiation site account for the different findings. Our observation is paralleled by the recent reexamination of MS2 lysis gene *L* activation. Berkhout et al. (5) have demonstrated that *L* initiation does not, as formerly believed (28), depend on frameshift-induced premature termination of the coat gene, upstream of the *L* start.

Gene *E* has a weak RBS. Since protein E production was unaffected by upstream translation of gene *D*, we asked whether the strength of the *E* RBS could determine its low expression rate. For that purpose, the translational initiation signals of gene *E* were used to direct expression of the *lacZ* gene. Two different *Eϕ-lacZ* fusions were constructed: E_1ϕ-*lacZ* (plasmid pSB85-2) and E_{30}ϕ-*lacZ* (plasmid pSB85-3). Compared with the β-galactosidase activity produced by the control plasmid pSB85-1, β-galactosidase was poorly expressed from plasmids pSB85-2 and pSB85-3 (Table 2), indicating a weak RBS for gene *E*.

In addition, the strength of the *E* RBS was evaluated by primer extension inhibition analysis (toeprinting). Toeprinting has been used to reveal the site of 30S ribosomal subunits bound in vitro in ternary complexes (10, 22). Briefly, in the presence of $tRNA^{fMet}$, 30S particles bind to mRNA at the translational start and terminate elongation by reverse transcriptase of a downstream primed cDNA. Primer extension is stopped at the 3′ edge of the 30S subunit, usually 15 nucleotides downstream of the adenosine of the initiation codon, AUG. Toeprinting was carried out with a transcript derived from plasmid pSB970 (Fig. 1), carrying the translational start sites of genes *D*, *E*, and *J*. The oligonucleotide primer used was complementary to positions 615 to 634 of the ϕX174 sequence. Thus, toeprint signals could be obtained only for the translational initiation sites of genes *D* and *E* located at positions 379 to 392 and 555 to 570, respectively (Fig. 2). A relatively strong toeprint signal was obtained 15 nucleotides downstream of the adenosine of the

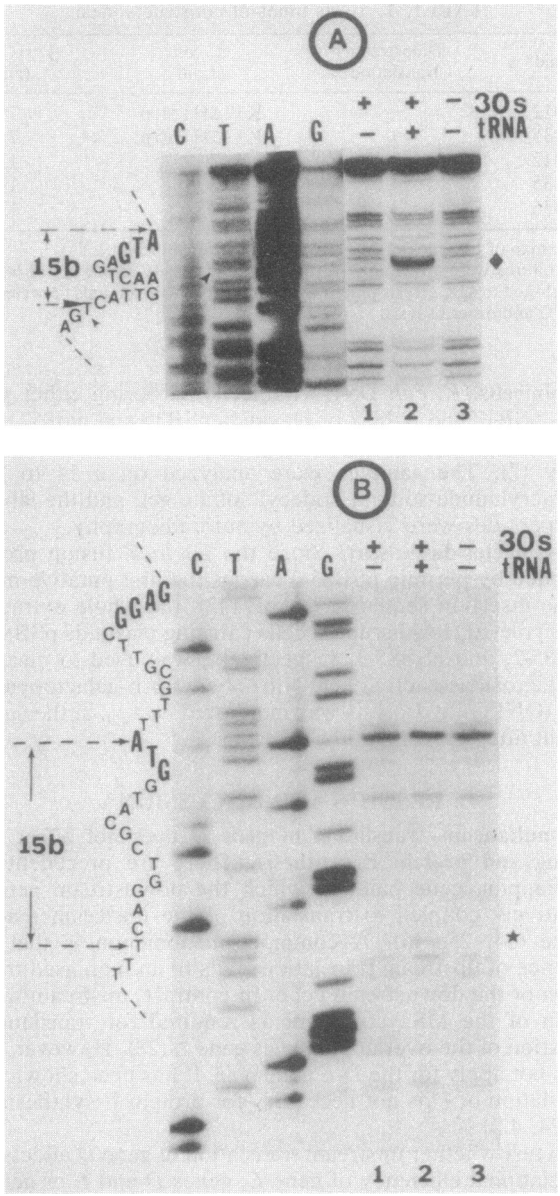

FIG. 4. Toeprint analysis of the RBS of genes *D* and *E*. The toeprint and sequencing reactions performed on pSB970-derived mRNA containing the *D* and *E* start sites were analyzed in parallel on an 8% polyacrylamide-urea gel. Only the relevant sections of the autoradiogram displaying the toeprint signals obtained for the *D* start (A; ◆) and the *E* (B; ∗) start are shown. The DNA sequence of the initial coding regions of genes *D* (A) and *E* (B) are given at the left. b, Bases. Lanes: 1, primer extension reactions without addition of $tRNA^{fMet}$ but with addition of 30S ribosomes; 2, toeprint signals obtained for the *D* (◆) and E (∗) start sites, obtained in the presence of $tRNA^{fMet}$ and 30S ribosomes; 3, primer extension reactions carried out in the absence of $tRNA^{fMet}$ and 30S ribosomes.

gene *D* start codon (Fig. 4A, lane 2). The corresponding toeprint signal for the gene *E* translational initiation site, however, was less intense (Fig. 4B, lane 2). Densitometric analysis of the two signals showed that ternary complex formation at the *D* RBS was favored over starts at the *E* initiation site by 5:1.

Our data suggest that the expression of *E* is limited by an intrinsically weak RBS. What accounts for the relative

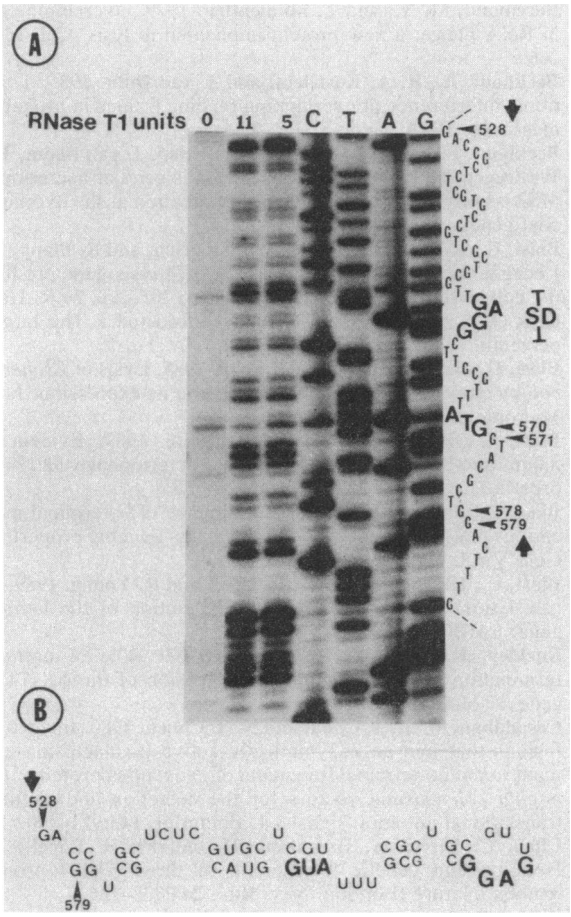

FIG. 5. Structural mapping of the *E* initiation region. (A) RNase T_1 (5 or 11 U) was added to pSB970-derived mRNA, and the cleavage sites were then mapped by using primer extension as described in Materials and Methods. The T_1 digestions were analyzed together with sequencing reactions as described for Fig. 4. The nucleotide numbers are according to reference 39. The gene *E* Shine-Dalgarno sequence (SD) and the *E* start codon are in bold. (B) Possible secondary structure of the *E* initiation region. The φX174 transcript spanning nucleotides 358 to 991 was analyzed by using the computer algorithms developed by Zuker and Stiegler. The *E* start codon and RBS are in bold.

weakness of the *E* start region despite the fact that gene *E* has a canonical Shine-Dalgarno sequence? The *E* RBS lacks A nucleotides downstream of the Shine-Dalgarno element, which are an indicator for a good initiation site (17, 30). Moreover, the *E* RBS is G+C rich, which is considered to be unfavorable since secondary structures can lead to sequestration of the *E* RBS (17, 21). RNase T_1 protection experiments indeed suggest that the weak E RBS is due to a local secondary structure. Exploitation of an RNA folding program developed by Zuker and Stiegler (43) revealed a potential mRNA secondary structure comprising nucleotides 530 to 579 of the φX174 sequence (Fig. 5B). The free energy of formation is predicted to be −18.6 kcal (ca. −77.8 kJ)/mol (38). To test whether this structure might occlude the *E* RBS, the transcript derived from plasmid pSB970 (Fig. 1) was digested with RNase T_1, and the cleavage sites were then mapped by using primer extension. Only weak cleavage was observed between positions 530 and 571 on the transcript (Fig. 5A), indicating that this region is substantially double stranded. Moreover, T_1 cleavage observed at G-571, immediately downstream of the gene *E* start codon, was much less intense than, for instance, at G-585. The G nucleotides in the gene *E* Shine-Dalgarno region and initiation codon are also weakly accessible to RNase T_1. These data suggest that the translational initiation region of gene *E* is at least partially sequestered by a local secondary structure.

In contrast, the *D* initiation region is strikingly rich in A (Fig. 2), which should keep it unstructured (30). However, the *D* Shine-Dalgarno domain shows, compared with the *E* Shine-Dalgarno sequence, a shorter complementarity to the 3′ end of the 16S RNA (Fig. 2). It might explain why the *D* RBS is only fivefold stronger than that of *E*. This is consistent with in vitro translation experiments, which showed that the *D*/*E* ratio is somewhere between 5:1 and 10:1 (B. Henrich, Ph.D. thesis, University of Kaiserslautern, Kaiserslautern, Federal Republic of Germany). The moderate

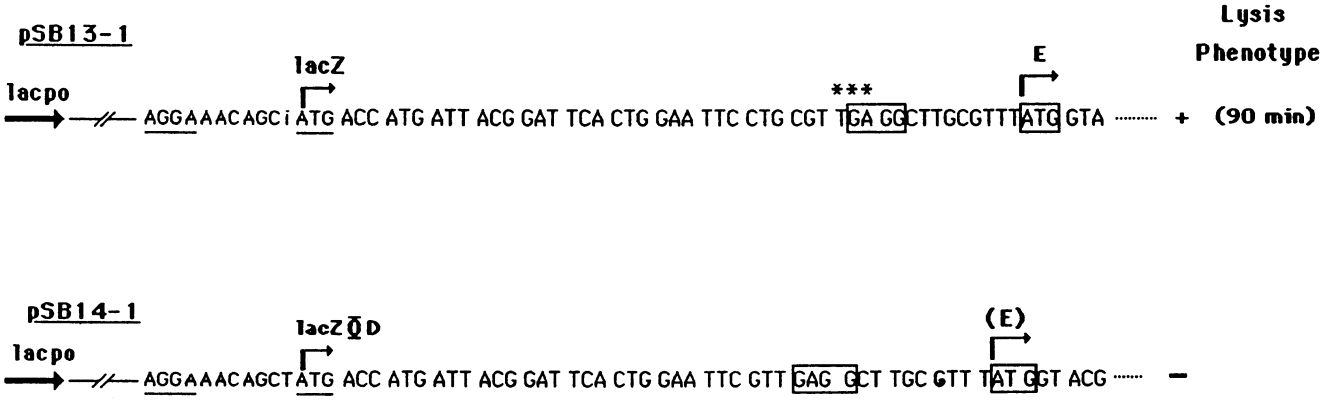

FIG. 6. Translational start region of gene *E* in plasmids pSB13-1 and pSB14-1. The gene *E* Shine-Dalgarno sequence and start codon are boxed. Transcription of gene *E* in both plasmids is driven by the *lac* promoter (lacpo). Synthesis of the *lacZ* polypeptide in pSB13-1 and the LacZ-φD fusion protein in pSB14-1 is directed by the underlined translational initiation signals of *lacZ*. The TGA stop codon terminating translation of the 13-amino-acid LacZ peptide is marked by asterisks. *E. coli* CQ21 harboring plasmid pSB13-1 or pSB14-1 was grown in LB broth to an optical density at 600 nm of 0.2, when synthesis of the *lac* promoter-controlled genes was induced with 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Lysis time is defined as the time between addition of IPTG and onset of lysis.

expression rate of *D* might also explain our observation that *E* initiation is unaffected by *D* translation. We suggest that because of the relatively low translational frequency of *D*, the inhibitory effect on the downstream *E* start is negligible. In other words, the *E* RBS is equally accessible in the presence or absence of normal levels of *D* translation.

To test the idea that the previously described effects (11) of *D* translation on *E* expression were due to excessive translation of the *D* reading frame, two plasmids were constructed in which gene *E* expression is controlled by the *lac* promoter. In plasmid pSB13-1, the first eight 5′ codons of *lacZ* are fused to φX174 reading frame −1 relative to gene *D* at position −21 relative to the adenosine of the *E* initiation codon, leading to expression of a 13-amino-acid polypeptide that is terminated by the TGA codon at positions 554 to 556 (Fig. 2 and 6). In plasmid pSB14-1, the same *lacZ* sequence is fused to the *D* reading frame at position −17 relative to the *E* start codon (Fig. 6), leading to expression of a LacZ-φD protein. Induction of plasmid pSB13-1, which directs termination of *lacZ* translation 5′ of the *E* start, resulted in lysis. In contrast, no lysis occurred with plasmid pSB14-1 under conditions in which translation of the *lacZ*-φ*D* gene progresses through the *E* start (Fig. 6). This finding shows that strong upstream translation, as obtained by the use of the *lacZ* translational initiation signals, as well as positioning of the upstream RBS close to the *E* RBS, can silence *E* initiation. Findings concerning MS2 lysis gene *L* expression support our hypothesis. Berkhout et al. (4) have shown that under conditions in which the *L* gene can be independently translated from the overlapping coat gene, cell lysis does not occur (i.e., protein L is not produced in sufficient amounts) when upstream translation of the coat gene occurs simultaneously. However, when in the same constructs translation of the upstream coat gene was decreased, cell lysis and thus protein L production increased with decreasing translation of the coat gene (4). Under wild-type conditions, the coat protein and L protein are synthesized with a ratio of 25:1 (3), at least 1 to 2 orders of magnitude higher than the D/E ratio. In this respect, the result obtained with plasmid pSB14-1 is consistent with the proposal of Berkhout et al. (4) that only an efficiently decoded upstream message inhibits access of ribosomes to a downstream RBS.

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