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The Translation Start Signal Region of TEM β-Lactamase mRNA Is Responsible for Heat Shock-Induced Repression of *amp* Gene Expression in *Escherichia coli*

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pBR322 contains the *amp* gene encoding β-lactamase. When *Escherichia coli* carrying this plasmid is exposed to heat shock, β-lactamase synthesis is repressed transiently at the translational level. To identify the DNA element responsible for this translational repression, DNA segments containing the translation start region of the *amp* gene were excised from pAT153 and fused in frame with the *lacZ* reading frame in the open reading frame vector pORF1. These constructs were introduced into *E. coli*, and the effect of heat shock of the cells on the synthesis of β-galactosidase starting from the *amp* start codon was examined. As is the case for pBR322-encoded synthesis of β-lactamase, the synthesis of β-galactosidase encoded by the fused genes also ceased transiently upon heat shock. It is concluded that the heat shock-induced repression of the *amp* gene occurs at the initiation step of translation. As far as the present study is concerned, the minimum DNA segment responsible for the repression is AT TGA AAA AGG AAG AGT ATG AG, which includes the Shine-Dalgarno sequence (AAGGA) and the initiation codon (ATG).

Previous studies have shown that, in *Escherichia coli* harboring pBR322 or related plasmids that contain the *amp* gene, the synthesis of β-lactamase encoded by this gene is transiently repressed by a temperature shift-up, e.g., from 30 to 42°C (4; unpublished results). This arrest of synthesis is followed by an adjustment of the cellular level of β-lactamase to a new steady state (4). Thus, in *E. coli* as in eucaryotic cells (12, 14), heat shock leads not only to transient overproduction of a specific set of (heat shock) proteins (10, 11), but also to transient repression of the synthesis of certain proteins (4, 7). Since the synthesis of β-lactamase mRNA is not repressed by temperature shift-up (4), it is thought that the repression of β-lactamase synthesis occurs at the translation level. In fact, the translation of β-lactamase mRNA in vitro has been shown to be specifically repressed at higher temperature, such as 45°C (unpublished results).

The purpose of the present study is to identify the region of β-lactamase mRNA that is responsible for this repression. For this purpose, DNA fragments corresponding to the 5′ untranslated leader sequence of β-lactamase mRNA were inserted between the *ompF* and *lacZ* genes (in frame with the latter gene) in the open reading frame vector pORF1 (17). These constructs were then introduced into *E. coli*, and the effect of heat shock on the expression of the *lacZ* gene starting from the initiation codon of the *amp* gene was examined. The results obtained indicate that the heat shock-induced repression of β-lactamase synthesis takes place at the initiation step of the translation.

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated: restriction endonucleases were from New England BioLabs and Nippon Gene; Klenow fragment of DNA polymerase I and T4 DNA polymerase were from Boehringer Mannheim; T4 DNA ligase was from Takara Shuzo; *o*-nitrophenyl-β-D-galactropyranoside (ONPG) was from Sigma Chemical Co.; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Wako Chemical.

Bacterial strains and plasmids. *E. coli* JM109 [*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* (F′ *traB36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)] was used as a host for constructing recombinant plasmids and for gene expression studies. Strains MC4100 [F$^-$ *araD139* Δ (*argF-lac*)*U169 rpsL150*(Str$^+$) *relA1 flb5301 deoC1 ptsF25*] and MH3000 [*araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 galU galK rpsL* (Str$^+$) *ompR101*] were used as hosts for plasmids pAT153 (16) and pORF1 (17), respectively. These plasmids were prepared by the method described before (8) and purified by equilibrium centrifugation in a $CsCl_2$ gradient (8).

Medium. L-broth (8) was used. When necessary, ampicillin and X-Gal dissolved in *N*,*N*-dimethylformamide (10 mg/ml) were added to L-broth agar at concentrations of 100 and 20 μg/ml, respectively.

Construction of recombinant plasmids. All manipulations of DNA were carried out by standard methods (8). The procedures used for construction of pNO5 and pNO2 are outlined in Fig. 1. pAT153 was digested with *Apa*LI and *Eco*RI, and the small *Apa*LI-*Eco*RI fragment (326 bases) produced was isolated by agarose gel (1.4%) electrophoresis and electroelution. This fragment possessing two *Mbo*II sites was then digested with *Mbo*II, resulting in the production of an *Mbo*II-*Mbo*II fragment (196 base pairs [bp]), the downstream region from the *amp* second codon, and 5′ noncoding region upstream from one of the *Mbo*II sites. The *Mbo*II-*Mbo*II fragment was isolated by agarose gel (1.7%) electrophoresis. The overhanging 1 nucleotide at the 3′ ends was removed by digestion with T4 DNA polymerase in the presence of four deoxyribonucleoside triphosphates. Prior to insertion of this blunt-ended fragment (195 bp) into the *Sma*I site of pORF1, this fragment was treated as follows. To make the *amp* start codon (present in the blunt-ended fragment) in frame with the *lacZ* reading frame in pORF1, the fragment was ligated with *Hin*dIII linker (CCAAGCT TGG) and then was digested with *Hin*dIII. The recessed

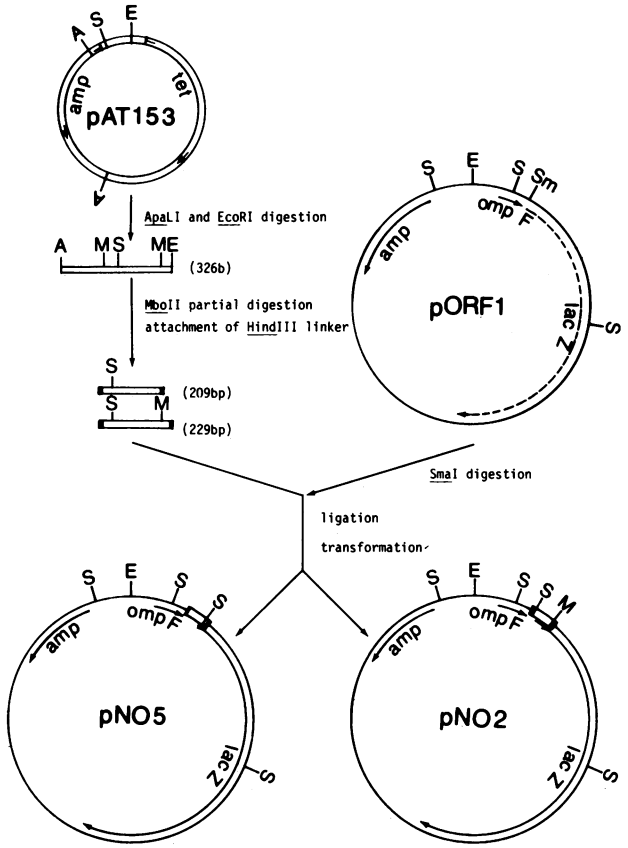

FIG. 1. Construction of the fused *ompF-amp-lacZ* gene. The procedure is described in the text. Thin lines represent the open reading frame vector pORF1. pAT153 DNA and the *Hin*dIII linkers are shown as open and solid boxes, respectively. The restriction enzyme *Apa*LI (A), *Ssp*I (S), *Eco*RI (E), *Sma*I (Sm), and *Mbo*II (M) sites are shown. Only the *Mbo*II site present in the inserted DNA fragments is shown in this figure.

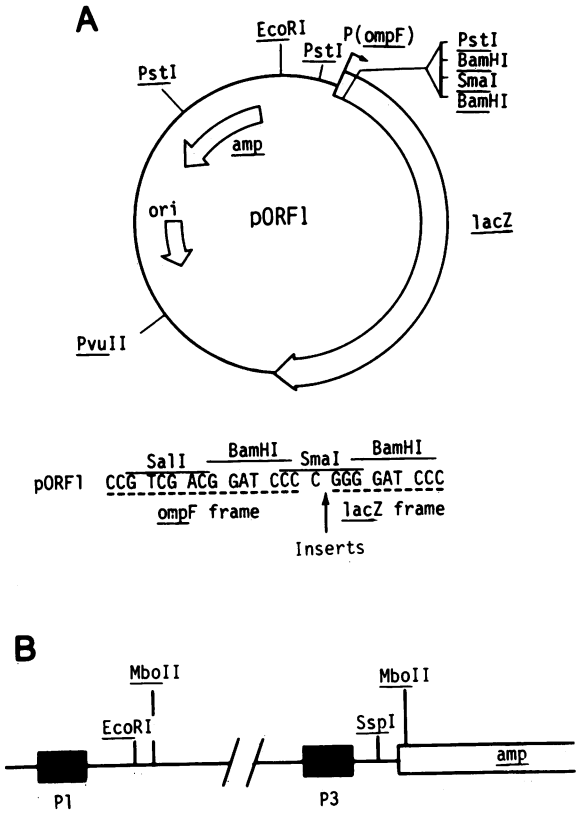

FIG. 2. (A) Open reading frame expression vector pORF1 and the nucleotide sequence of the *lacZ* and *ompF* fusion joint. DNA fragments derived from pAT153 are inserted into the *Sma*I site, which is indicated by an arrow. The direction of transcription from the *ompF* promoter [P(*ompF*)] is shown with an arrow on pORF1. (B) Organization of the promoter-translation start signal region of the *amp* gene in pAT153. Two promoters (P1 and P3) are indicated by solid boxes. The *amp* reading frame is shown by an open box. The locations of *Eco*RI, *Ssp*I, and *Mbo*II sites are indicated.

ends created by this digestion were filled in with Klenow fragment in the presence of four deoxyribonucleoside triphosphates. The *Mbo*II-*Mbo*II fragment with AGCTTGG at the 5' end and CCAAGCT at the 3' end (209 bp) was inserted into the *Sma*I site of pORF1 by blunt-end ligation with T4 DNA ligase. The recombinant plasmids constructed were introduced into *E. coli* JM109 (8). The transformants showing the Ap^r and $LacZ^+$ phenotypes on L-broth agar plates containing ampicillin and X-Gal were isolated. Plasmids were prepared from the screened transformants and characterized by restriction mapping. To confirm the nucleotide sequence of the inserts and the fusion regions, the *Bam*HI-*Bam*HI fragments derived from the plasmids were subcloned onto M13 phage and then sequenced by the dideoxy chain termination sequencing method (13). The recombinant plasmid that carried the intact insert in the desired orientation was termed pNO5.

During the course of characterization of the plasmids, another (pNO2) was found in the isolated plasmids. In plasmid pNO2, the *Mbo*II-*Eco*RI fragment having the *Hin*dIII linkers at the ends (total, 229 bp) was inserted in the *Sma*I site of pORF1 in the opposite orientation to that of the insert in pNO5. This indicates that the digestion of the *Apa*LI-*Eco*RI fragment with *Mbo*II was incomplete and the *Mbo*II-*Mbo*II fragment isolated was contaminated with the *Mbo*II-*Eco*RI fragment.

The *Mbo*II-*Mbo*II fragment contains one (P3) of the two *amp* promoters upstream of the *Ssp*I site, and the translation start signal was thought to be located downstream of the *Ssp*I site (Fig. 2B). It was therefore of interest to construct a recombinant plasmid carrying the *Ssp*I-*Mbo*II fragment. To this end, the *Mbo*II-*Mbo*II fragment having the *Hin*dIII linkers at the ends was digested with *Ssp*I. The products were dephosphorylated and inserted into the *Sma*I site of pORF1 by blunt-end ligation. The plasmids constructed were introduced into *E. coli* JM109, and transformants having the Ap^r and $LacZ^+$ phenotypes were isolated. The plasmids were characterized by restriction mapping and DNA sequencing. It was thus found that a plasmid, called pNO18, contained the small *Ssp*I-*Mbo*II fragment in the desired orientation at the *Sma*I site of pORF1.

DNA sequencing. Sequencing of DNA was performed by the method of Sanger et al. (13) according to standard M13 protocols (Amersham).

Assay of β-galactosidase. The β-galactosidase synthesized in *E. coli* under direction of the recombinant plasmids was assayed by the method of Miller (9). A portion (0.5 ml) of culture was withdrawn and mixed with 0.5 ml of Z-buffer containing 1 drop of toluene. After the toluene was removed by evaporation, an appropriate amount of the toluene-treated cell suspension in 1.0 ml of Z-buffer was incubated at 28°C with 0.2 ml of ONPG (4 mg/ml) as a substrate for

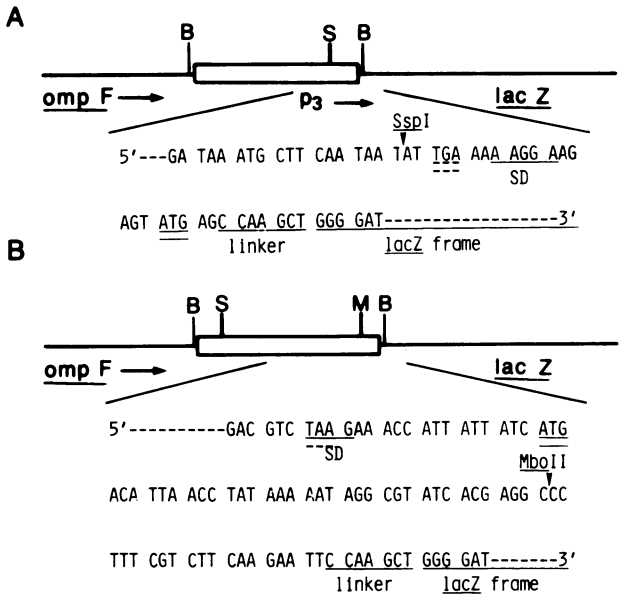

FIG. 3. Nucleotide sequences in the regions of the *amp-lacZ* fusion joint. A and B represent the fusion joints on plasmids pNO5 and pNO2, respectively. The thin lines represent the regions of pORF1, and the open boxes indicate the regions of the inserts. The restriction enzyme *Ssp*I (S), *Mbo*II (M), and *Bam*HI (B) sites, the Shine-Dalgarno sequence (SD), the *amp* start codon (double solid underline), and the stop codon that is in frame with the *lacZ* reading frame and is the nearest to the *amp* start codon (double broken underline) are shown. The arrows show the direction of transcription from the *ompF* and *amp* promoters (P3) on the plasmid constructs.

β-galactosidase. The reaction was stopped by adding 0.5 ml of 1.0 M Na_2CO_3 solution, and the increase in A_{420} was measured in a Beckman model 25 spectrophotometer. One unit of activity is defined as that causing 0.001 unit of increase in the A_{420} per min at 28°C.

RESULTS

Construction of the *lacZ* gene fused with the *amp* translation start signal. To elucidate whether the translation start signal of *amp* mRNA is responsible for the transient arrest of β-lactamase synthesis upon exposure of *E. coli* carrying pBR322 to heat shock, DNA segments containing the *amp* translation start signal were excised from pAT153 and inserted into the *Sma*I site of the open reading frame vector pORF1 (17). The *Sma*I site of pORF1 is located in the polylinker region and is followed by the *lacZ* reading frame. This vector contains the *ompF* promoter and the segment encoding the amino-terminal portion of OmpF protein, which is not in frame with the *lacZ* sequence. In the present study, three recombinant plasmids, in which the *amp* translation start signal is inserted into pORF1, were constructed and termed pNO5, pNO18, and pNO2.

In pNO5, the *Mbo*II-*Mbo*II fragment (of pAT153) containing the *amp* promoter (P3) and the *amp* translation start signal (Fig. 2A) is inserted into the *Sma*I site of pORF1 in the proper direction (Fig. 3A). The transcription of the *lacZ* sequence in this construct can start from both the *ompF* and *amp* promoters. Both the *amp* start codon inserted and the *ompF* start codon originally present in pORF1 are in frame with the *lacZ* reading frame. However, translation of the

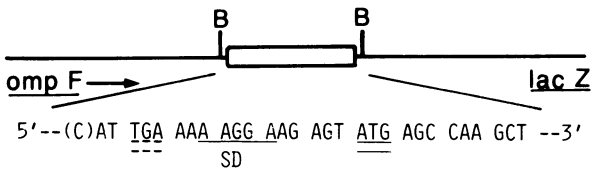

FIG. 4. Nucleotide sequence of the small *Ssp*I-*Mbo*II fragment inserted into the *Sma*I site of pORF1. The *Ssp*I-*Mbo*II fragment with CCAAGCT at the ends (open box), pORF1 (thin lines), and *Bam*HI (B) sites on the thin lines are indicated. (A) Nucleotide sequence of the *Ssp*I-*Mbo*II small fragment, which was inserted into the *Sma*I site of pORF1 in the desired orientation. The Shine-Dalgarno sequence (SD), the *amp* start codon (double solid underline), and a stop codon (double broken underline) on the insert are indicated. The orientation of the insert in panel B is opposite to that in panel A.

transcript starting from the *ompF* promoter should be terminated before reaching the *lacZ* reading region, because stop codons that are in frame with the *lacZ* reading frame have been also introduced upstream of the *amp* start codon during construction (Fig. 3A). Therefore, expression of the *lacZ* gene in this construct can be achieved only from the *amp* initiation codon.

In pNO18, the small *Ssp*I-*Mbo*II fragment, which lacks the *amp* promoter (P3) (Fig. 2B), is inserted into the *Sma*I site of pORF1 in the same direction as the pNO5 insert (Fig. 4A). In this construct, therefore, transcription of the *lacZ* gene starts only from the *ompF* promoter, but translation of the transcript starts from the *amp* start codon, as in pNO5. Insertion of this *Ssp*I-*Mbo*II fragment in the opposite direction also produces a construct on which the *ompF* start codon is in frame with the *lacZ* reading frame (Fig. 4B). This recombinant plasmid could, however, not be isolated, probably because the overproduction of the hybrid protein encoded by the fused gene, like that of OmpF–β-galactosidase fusion protein (17), is lethal to *E. coli* JM109.

As mentioned above, in pNO2, the *Mbo*II-*Eco*RI fragment is inserted into the *Sma*I site of pORF1 in the opposite direction to that of the pNO5 insert. As shown in Fig. 3B, in this construct a Shine-Dalgarno sequence (TAAG) and a start codon (ATG) that is in frame with the *lacZ* reading frame were located 77 and 60 nucleotides, respectively, upstream from the first nucleotide of the *lacZ* sequence. This putative false translation start signal may be active, because the high A content in this region is favorable for translation initiation (1). A termination codon (TAA), which is in frame with the *lacZ* reading frame, was also seen in the Shine-Dalgarno sequence of this fragment. Since the *ompF* start codon in pNO2 is not in frame with the *lacZ* reading frame, it is likely that the translation of the *lacZ* gene initiates from the aforementioned putative translation start signal. To determine the start site of the *lacZ* translation, however, amino-terminal sequencing of the *lacZ* product is necessary. In any case, pNO2 can serve as a control in examination of the effect of heat shock on β-galactosidase synthesis, because it lacks the *amp* translation start signal.

Level of β-galactosidase in *E. coli* harboring the recombinant plasmids. Table 1 shows the levels of β-galactosidase in *E. coli* harboring pNO2, pNO5, and pNO18. The cellular levels of β-galactosidase varied depending on the construct introduced into *E. coli*. This difference was not due to the difference in gene copy number, because the copy number was practically the same in all the cells (data not shown).

TABLE 1. Levels of β-galactosidase in *E. coli* JM109 carrying recombinant plasmids derived from pORF1[a]

Plasmid	β-Galactosidase activity (U/OD_{600})	
	30°C	42°C
pORF1	0	0
pNO2	976	1,200
pNO5	288	74
pNO18	240	52

[a] Cells carrying the plasmids were grown in 20 ml of L-broth with shaking at 30 and 42°C. In the mid-logarithmic phase of growth, 0.5-ml portions of the cultures were withdrawn to examine the cellular content of β-galactosidase by the method of Miller (9).

The cells harboring pNO2 showed by far the highest activity. However, as discussed above, it is likely that the β-galactosidase encoded by pNO2 has an amino-terminal sequence that is different from the enzyme encoded by the other two plasmids, and it is unknown whether the two types of the enzyme have the same specific activity. This raises the possibility that the difference in β-galactosidase levels between the cells with pNO2 and those with pNO5 and pNO18 does not indicate a difference in the extent of the expression of the *lacZ* gene in the two types of cells. The levels of β-galactosidase expressed in cells carrying pNO5 and those carrying pNO18 were not significantly different. In pNO5, transcription of the *lacZ* gene is expected to initiate from both the *ompF* and *amp* promoters, whereas the *lacZ* gene in pNO18 seems to be transcribed only from the *ompF* promoter. These results indicate that the *amp* promoter located between the *ompF* promoter and the *amp* translation start signal does not apparently affect the expression of *lacZ* gene. It is likely that the *amp* translation start signal, but not the *amp* promoter, is the determinant of the cellular level of β-galactosidase. Another possibility is that the *amp* promoter is occluded by the *ompF* promoter and thus has no influence on the level of β-galactosidase encoded by pNO5 and pNO18. When β-galactosidase synthesis was initiated from the *amp* start codon, i.e., that taking place in cells harboring pNO5 or pNO18, the β-galactosidase level in cells growing at 42°C was only about 25% of that in cells growing at 30°C, as in the case of β-lactamase synthesis in cells carrying pBR322 (4). In contrast, cells harboring pNO2 produced about 1.2-fold more β-galactosidase at 42 than at 30°C.

Transient arrest of β-galactosidase synthesis upon heat shock. *E. coli* carrying the recombinant plasmids was grown at 30°C, and then the temperature was shifted up to 42°C. Samples of culture were withdrawn at 5-min intervals before and after the temperature shift-up for measurement of β-galactosidase activity. As shown in Fig. 5, β-galactosidase synthesis in cells carrying pNO2 was not inhibited by the temperature shift-up, but rather accelerated. This acceleration was certainly due to the high temperature. In contrast, this synthesis in cells carrying pNO5 and pNO18 was arrested upon the temperature shift-up. This arrest persisted for about 15 min, and then synthesis was gradually restored to a new steady-state level. It can therefore be concluded that the synthesis of β-galactosidase starting from the *amp* initiation codon (encoded by pNO5 and pNO18) is transiently arrested upon heat shock. It is to be noted that the extent and pattern of heat shock-induced arrest of β-galactosidase synthesis encoded by pNO18 were practically identical with those of the enzyme encoded by pNO5, even though the insert of the former plasmid lacks the *amp*

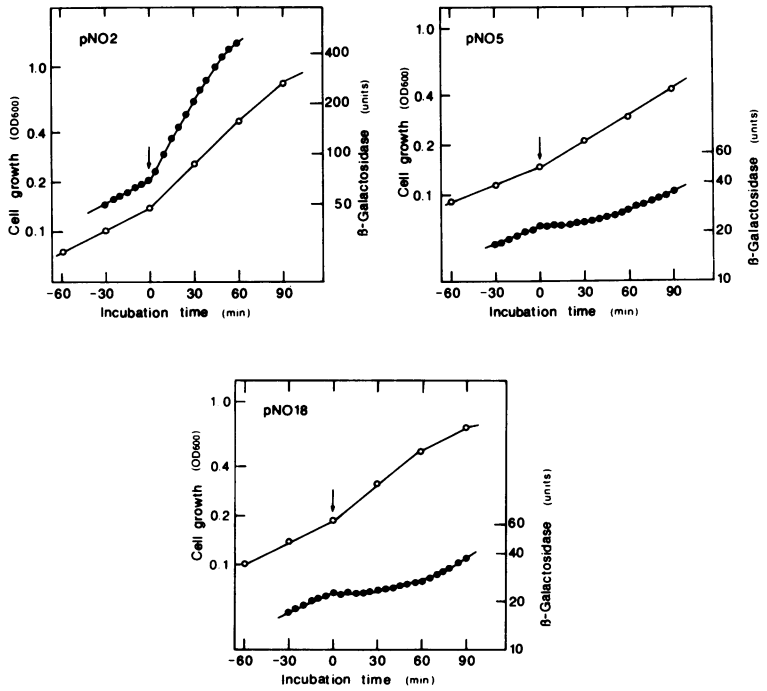

FIG. 5. Effects of temperature shift-up on increases in β-galactosidase in cells carrying plasmid pNO2, pNO5, or pNO18. Cells carrying the constructed plasmids were cultured at 30°C with shaking in 50 ml of L-broth, and then the temperature was shifted up to 42°C. The time of temperature shift-up is indicated by an arrow and represented as time zero. Samples (0.5 ml) of the cultures were withdrawn at 5-min intervals before and after temperature shift-up for measurement of β-galactosidase by the method of Miller (9). Increase in cell mass (○) was followed by measurement of the OD_{600}. β-Galactosidase activities in cells (●) are expressed as units per milliliter of culture.

promoter. It is thus clear that the *amp* promoter is not required for the observed transient arrest of β-galactosidase synthesis. These results lead to the conclusion that the translation start signal of the *amp* mRNA is responsible for the observed heat shock-induced repression of the *amp* gene expression, although the involvement of other regions cannot be ruled out.

DISCUSSION

The results described above provide evidence that a short segment of the *amp* gene, including the Shine-Dalgarno sequence and the initiation codon, is responsible for the heat shock-induced arrest of pBR322-encoded synthesis of β-lactamase in *E. coli*, an event which takes place at the translational level. This conclusion was reached by finding that an inframe fusion of this segment with the *lacZ* gene in the open reading frame vector pORF1 resulted in the transient arrest of β-galactosidase synthesis upon heat shock. The lack of heat shock-induced arrest of β-galactosidase synthesis encoded by pNO2, which lacks the responsible element, also supports this conclusion. It is to be noted that the results shown in Table 1 and Fig. 5 can also be accounted for by assuming that the *ompF-amp-lacZ* and *amp-lacZ* fusion mRNA became extremely labile upon the temperature shift-up. This possibility is, however, unlikely because in these fusion mRNAs the contribution of *ompF* and *amp* moieties are small compared with that of the *lacZ* moiety. An attempt to check this possibility experimentally was not successful.

As far as the present study is concerned, the minimum size of the segment responsible for the heat shock-induced repression is a docosanucleotide (ATTGAAAAAGGAA GAGTATGAG), in which the Shine-Dalgarno sequence (AAGGA) (15) and the start codon (ATG) are connected by a spacer pentanucleotide (AGAGT). This region is flanked by a 5′ untranslated heptanucleotide sequence (ATTGAA) and a dinucleotide (AG) following the initiation codon. This docosanucleotide segment has a high A content and no C, but otherwise shows no special characteristics. An examination of the nucleotide sequences in the translation start signal region of other genes whose expression is arrested by heat shock at the translational level is necessary for deducing the consensus sequence required for this function.

In the recombinant plasmids actually constructed in this study, a heptanucleotide, CCAAGTC, was ligated to the 3′ end of the docosanucleotide in order to match the reading frame of the *amp* start codon with that of the *lacZ* sequence. In the plasmids constructed (pNO5 and pNO18), therefore, the sequence from the start codon is ATGAGCCAAGCT---, which is different from that of corresponding region of the original *amp* gene (ATGAGTATTCAA---). In pNO5 and pNO18, the sequences upstream of the docosanucleotide segment are ---AAATGCTTCAATAAT and ---CGTCGACG CATCCCC, respectively. Despite this difference, the β-galactosidase synthesis encoded by both pNO5 and pNO18 was arrested upon temperature shift-up. These facts indicate that the 5′- and 3′-flanking regions of the docosanucleotide segment are not required for heat shock-induced repression. In other words, the element responsible for this phenomenon is present within the docosanucleotide segment.

I have previously shown that expression of the *amp* gene in vitro is enhanced by a heat-labile protein(s), termed HLF, which is present in *E. coli* extracts (3, 5). I have also found that HLF actually stimulates the translation of *amp* mRNA in vitro (unpublished results). Since the HLF activity cannot be detected in extracts from *E. coli* cells exposed to heat shock (5), it is highly likely that the heat shock-induced inactivation of HLF is at least a cause of the arrest of *amp* mRNA translation. Combined with the conclusion reached in the present study, this leads to the possibility that HLF interacts with the translation start signal region (the docosanucleotide segment) of the *amp* mRNA (or ribosomes) and renders the mRNA accessible to ribosomes at the initial stage of translation. Several studies have demonstrated that accessibility of the translation start signal of an mRNA to ribosomes is an important factor for efficient translation of mRNA (2). Another possibility is that HLF is required for the formation of the initiation complex consisting of *amp* mRNA and a ribosome, to which fMet-tRNA can bind to start β-lactamase synthesis. In both cases, it is expected that the inactivation of HLF will lead to the repression of *amp* gene expression at the initiation step of translation.

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