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Requirement of a Heat-Labile Factor(s) for In Vitro Expression of the *amp* Gene of pBR322

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The *amp* gene of pBR328 and pBR322 was expressed less efficiently at 45 than at 30°C in an in vitro coupled transcription-translation system of *Escherichia coli*. Preincubation of S30 extract at 45°C reduced specifically its ability to express the *amp* gene at 30°C, indicating inactivation of a factor(s) required for efficient expression of the *amp* gene.

I have recently reported that a shift-up of temperature from 30 to 42°C caused reversible repression of synthesis of pBR322-encoded β-lactamase in *Escherichia coli* but did not affect the level of β-lactamase mRNAs. It was concluded that the temperature shift-up repressed β-lactamase synthesis at the translational level (4), in contrast to the characteristics of heat shock proteins, the syntheses of which are regulated by sigma 32 as the *rpoH* gene product at the transcriptional step (2, 6, 8, 12–15). The repression of β-lactamase synthesis by heat shock seems to be an example of the heat shock response of *E. coli* and should be useful for the study of the regulation of gene expression at the translational level in *E. coli*. Here I show that incubation of an in vitro gene expression system at 45°C inactivated a factor(s) essential for efficient expression of the *amp* gene and thus decreased the synthesis of β-lactamase.

To study the effect of temperature on the in vitro synthesis of β-lactamase, pBR328-directed (11) protein synthesis was performed at 30 and 45°C with a procaryotic DNA-directed translation kit (obtained from Amersham Corp.). The incorporation of [^{35}S]methionine into protein products, estimated in precipitates with hot 5% trichloroacetic acid, was 3.8-fold faster at 45 than at 30°C. A portion of the radioactive products was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5) and subsequent fluorography (7). The products synthesized at 30°C gave two major bands that corresponded to the molecular weights expected for chloramphenicol acetyltransferase and pre-β-lactamase, respectively. On the fluorograph of the products synthesized at 45°C, however, chloramphenicol acetyltransferase was detected only as one major band (Fig. 1A). Similarly, when pBR322 (1) was used as a template instead of pBR3288 (11), the products at 45°C gave a much weaker band of pre-β-lactamase than those at 30°C (data not shown). Therefore, expression of the *amp* gene on the both plasmids was concluded to be efficient in the in vitro coupled transcription-translation system at 30°C but to be repressed at 45°C.

For examination of whether the inefficiency of *amp* gene expression at 45°C was reversible, a reaction mixture without [^{35}S]methionine and template DNA was incubated at 45°C for 6 min and then was shifted to 30°C, and protein synthesis was examined by adding [^{35}S]methionine and pBR328 as a template. After this preincubation, the incorporation of [^{35}S]methionine into the acid precipitate was reduced by about 30%. Analysis of the radioactive products by SDS-PAGE (5) and fluorography (7) showed that the incubation at 45°C before protein synthesis decreased markedly the ability to synthesize pre-β-lactamase at 30°C, but that this treatment hardly affected the synthesis of chloramphenicol acetyltransferase in the same reaction mixture (Fig. 1B). This effect of the preincubation at 45°C was further examined in more detail, and it was found that the activity to synthesize pre-β-lactamase decreased with a half-life of 3.5 min that was two- to threefold shorter than that for total protein synthesis in the in vitro system (data not shown). The result suggests that the in vitro expression of the *amp* gene requires some factor(s) that is more heat labile than the factors and enzymes involved in gene expression in general.

To study the distribution of this putative heat-labile factor(s) (HLF) in subcellular fractions, I separated S30 into ribosome and soluble protein fractions by ultracentrifugation and examined the distribution of HLF in the two fractions. After incubation at 0 and 45°C for 6 min, the mixtures containing S30 were centrifuged at 150,000 × *g* for 120 min at 2°C. The supernatants (S150) were separated from the resultant precipitates, which consisted mainly of crude ribosomes, and in vitro systems for gene expression were reconstructed with various combinations of the S150 and ribosomes obtained. Then pBR328-directed protein synthesis in the reconstructed in vitro system was conducted at 30°C, and the reaction products were analyzed. The results in Fig. 2 show that in the reaction mixture with the S150 from the mixture incubated at 45°C the synthesis of chloramphenicol acetyltransferase was also reduced by about half, but that the synthesis of pre-β-lactamase was reduced much more. The ratio of pre-β-lactamase to chloramphenicol acetyltransferase synthesized with the S150 heated at 45°C was less than 1/15 of that with unheated S150. The ability of ribosomes to support protein synthesis was not affected by the heat treatment, indicating that HLF was located in S150. The efficiency of chloramphenicol acetyltransferase synthesis was not reduced in reaction mixture with the supernatant and ribosomes that had been preincubated at 45°C but not separated by ultracentrifugation. The observed decrease in the synthesis of chloramphenicol acetyltransferase seemed, for some unknown reason, to be due to ultracentrifugation of the mixture after the preincubation.

The results presented above indicate that the in vitro synthesis of pre-β-lactamase encoded by plasmids pBR322 (1) and pBR328 (11) requires HLF. Preincubation for 6 min at 45°C decreased the ability of HLF to support the synthesis of pre-β-lactamase but did not affect the synthesis of chloramphenicol acetyltransferase, another polypeptide encoded by pBR328 and synthesized simultaneously with pre-β-lactamase in the in vitro system (9). HLF seems unlikely to

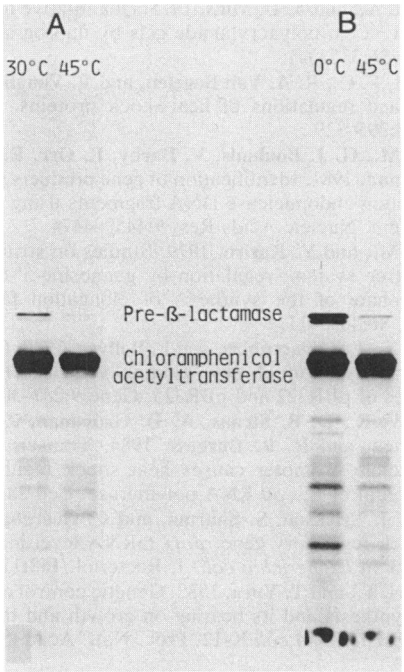

FIG. 1. Effect of temperature on the in vitro synthesis of pre-β-lactamase and chloramphenicol acetyltransferase encoded by pBR328. (A) Portions (65,600 cpm) of the radioactive products synthesized in 15 min at 30°C and in 6 min at 45°C were analyzed by SDS-PAGE (12.5% polyacrylamide) (5) and fluorography (7). (B) Reaction mixtures (22 μl) without [^{35}S]methionine and DNA as a template were incubated for 6 min at 0 or 45°C. After subsequent incubation at 30°C for 2 min, 3 μl of [^{35}S]methionine (15 μCi/μl) and 5 μl of pBR328 (1 mg/ml) as a template were added and in vitro protein synthesis was done for 15 min at 30°C. Portions (54,500 cpm) of the acid precipitate synthesized were analyzed as described for panel A.

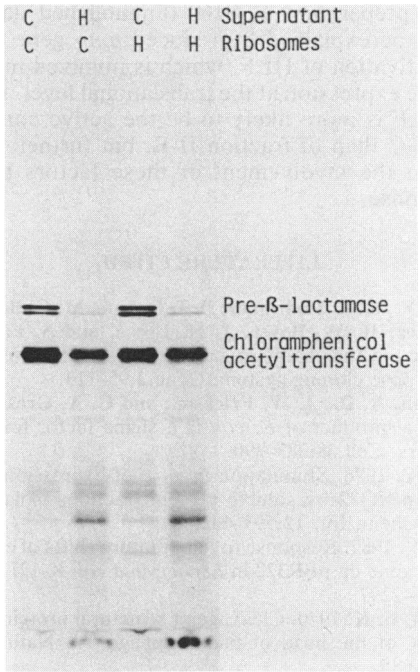

FIG. 2. Localization of HLF in S150. The reconstructed mixture (11 μl) was incubated with 2.5 μl of pBR328 (1 mg/μl) and 1.5 μl of [^{35}S]methionine (15 μCi/μl) at 30°C for 15 min. Portions (21,600 cpm) of the acid precipitate were analyzed by SDS-PAGE (12.5% gel) (5) and fluorography (7). C and H indicate components that were derived from the mixtures incubated at 0 and 45°C, respectively.

be one of the factors or enzymes that have been reported to be essential elements for expression of genes in general, because its effect was specific for expression of the *amp* gene. Recently, I reported that an in vitro system for gene expression reconstructed with unwashed ribosomes and a polyethylene glycol-treated S30 extract from *E. coli* (10) required two additional factors (fractions II-B and III-C) for efficient expression of the *amp* gene in pBR322 (3). Both factors that were isolated from a supernatant fraction (S160) (centrifuged at 160,000 × *g*) of an *E. coli* extract stimulated pBR322-directed synthesis of pre-β-lactamase in this in vitro system, but only one of the factors (II-B) stimulated pBR322-directed RNA synthesis. Although HLF has not yet been purified and characterized, it may be the active component in either fraction II-B or fraction III-C since, like HLF, both factors were heat labile under the experimental conditions (unpublished observation). On the other hand, the in vivo synthesis of β-lactamase was arrested transiently on shift-up of a temperature from 30 to 42°C and during culture at 42°C their level of β-lactamase decreased (4). This repression of β-lactamase synthesis took place at the translational level. As shown in Fig. 3, the repression of *amp* gene expression occurred in a similar temperature range in in vivo and in vitro systems. S160 that was prepared from *E. coli* grown at 30°C stimulated preferentially the decreased synthesis of pre-β-lactamase. However, this activity of S160 was markedly reduced when cells were exposed to 42°C

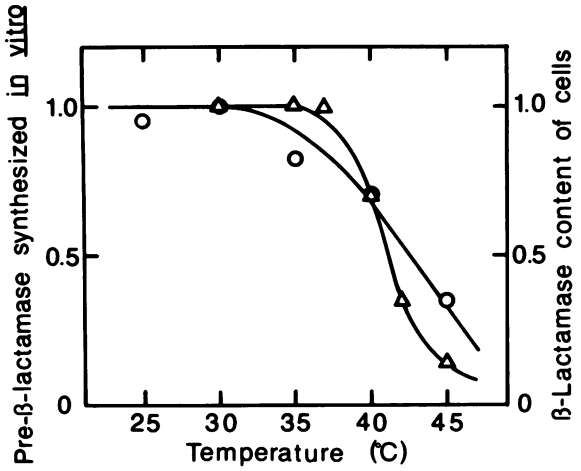

FIG. 3. Comparison of the effect of temperature on the in vitro synthesis of pre-β-lactamase with that on the level of β-lactamase in cells. *E. coli* 4100 carrying pBR322 (1) was grown in LB medium (20 ml) at the indicated temperatures. Cells at logarithmic phase were harvested and broken by sonication. β-Lactamase in the sonicate was assayed with 50 μM [1-(thienyl-2-acetamido)]-3-[2-(4-*N*,*N*-dimethylamino phenylazo)pridium methyl]-3-cepem-4-carboxylic acid as a substrate in 10 mM sodium phosphate (pH 7.0) (4). In vitro synthesis of pre-β-lactamase under direction of pBR322 was also conducted at the various temperatures indicated. Portions (53,300 cpm) of the radioactive products were subjected to SDS-PAGE (5) and fluorography (7), and then synthesized pre-β-lactamase was determined by measurement of the intensity of bands corresponding to the position of pre-β-lactamase on fluorographs (2). The data are expressed as relative to data obtained at 30°C. Symbols: ○, pre-β-lactamase synthesized in vitro, △, level of β-lactamase in cells.

before the preparation of S160 (unpublished data). These results can be explained by reduced *amp* gene expression due to inactivation of HLF, which is involved in regulation of *amp* gene expression at the translational level. From these results, HLF is more likely to be the active component of fraction III-C than of fraction II-B, but further studies are required on the involvement of these factors in the heat shock response.

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