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Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity

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ABSTRACT

Transcription *in vitro* of stringently controlled *Escherichia coli* genes by purified RNA polymerase holoenzyme is inhibited by guanosine tetraphosphate (ppGpp). In order to examine possible role of ω factor in this ppGpp sensitivity, RNA polymerases with or without the ω factor were reconstituted and tested for their ppGpp sensitivity using an *in vitro* mixed transcription system. RNA polymerase lacking the ω factor was found virtually insensitive to ppGpp but the addition of a purified ω factor restored the ppGpp sensitivity of this ω-free RNA polymerase. These results raise a possibility that the ω factor is a regulatory protein of RNA polymerase and is involved in the ppGpp-mediated alteration of the promoter selectivity.

INTRODUCTION

Level of the transcription initiation in *E. coli* is regulated by controlling the promoter activity through interaction with various trans-acting DNA-binding proteins (for a review, see ref. 1). In addition, the transcription regulation is achieved by controlling the promoter selectivity of RNA polymerase. Replacement of sigma subunit leads to enormous alteration of the promoter selection pattern of RNA polymerase (reviewed in ref. 2). In addition, modifications of RNA polymerase by phosphorylation or ADP-ribosylation and through physical associations with some protein or nucleotide factors are supposed to lead to alteration in the promoter selectivity of RNA polymerase (3). Guanosine tetraphosphate (ppGpp), the signaling molecule involved in the stringent control (4), is one of such RNA polymerase-binding transcription factors. A number of *in vitro* experiments have demonstrated direct effect of ppGpp on the promoter selectivity of RNA polymerase (5, 6, 7, 8, 9, 10, 11, 12). Transcription *in vitro* from stringently controlled promoters, *i.e.*, promoters of rRNA operons, ribosomal protein operons, and tRNA operons, was preferentially inhibited in the presence of ppGpp. This differential repression of

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transcription initiation by ppGpp was observed using purified preparations of RNA polymerase, suggesting that RNA polymerase is a target of ppGpp action. It is, however, not excluded yet that ppGpp exerts its effect by interaction with an unidentified accessory factor associated with purified RNA polymerase.

Omega (ω) factor of E. coli RNA polymerase is an accessory protein of 10 kDa (k represents 1,000), which tightly associates with both core enzyme and holoenzyme at a molar ratio of 0.5 to 2 per enzyme (13). This factor is, however, not required for RNA synthesis because RNA polymerase can be reconstituted from isolated subunits lacking ω (14), and because it can be removed from core enzyme or holoenzyme by treatment with low concentration of urea without disintegrating $\alpha_2\beta\beta'(\sigma)$ structure (15). These results raise possibilities that ω factor is either a specificity determinant of RNA polymerase or a protein endowed with a regulatory function. The gene (rpoZ) for ω factor has been cloned and sequenced (16). On the basis of the DNA sequence, it was found that the rpoZ gene is located next to spoT (17), the gene involved in ppGpp metabolism (4). This finding promoted us to examine whether ω factor is involved in the interaction with ppGpp.

In this study, we analyzed the ppGpp sensitivity of RNA polymerase with or without ω factor using the in vitro mixed transcription system (18, 19). The results suggest the involvement of ω factor in ppGpp-mediated alteration of the promoter selectivity of RNA polymerase.

MATERIALS AND METHODS

RNA polymerase and subunits

RNA polymerase was prepared from E. coli strain W3350 essentially according to the method of Fukuda et al. (20). Holoenzyme and core enzyme were separated each other by passing the purified RNA polymerase through a phosphocellulose column in the presence of 50% glycerol (21). σ^{70} was prepared from purified holoenzyme by the method of Lowe et al. (22) and further purified by HPLC with Waters Protein Pak G-DEAE and Protein Pak 300 columns. To obtain core enzyme devoid of ω factor, the core enzyme preparation was incubated in a 10 mM Tris-HCl buffer (pH 7.6) containing 1.5 M urea, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.5 M NaCl and 5% glycerol for 10 min at 37℃ and subjected to 15-35% glycerol density gradient centrifugation made in the same buffer. Centrifugation was carried out at 28,000 rpm and at 4℃ for 40 hr using a Beckman SW28 rotor. Core enzyme devoid of ω factor was recovered from upper part of the core enzyme

peak, dialyzed first against 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol, and then against the same buffer with 200 mM KCl and 50% glycerol, and stored at -20℃.

For the purification of ω factor, core enzyme was processed and centrifuged through glycerol gradient essentially as described above except that the concentration of urea was increased to 2.5 M. Fractions containing ω factor were pooled, dialyzed against 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA and 0.1 mM DTT, and applied to a Protein Pak G-DEAE column, which was eluted using a linear gradient of NaCl from 0 to 500 mM in 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, and 0.1 mM DTT. ω factor was eluted at about 120 mM NaCl.

The colorimetric method of Bradford (23) was employed for the determination of proteins except ω factor, which was determined by densitometry of Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gels using RNA polymerase α subunit as a standard.

To reconstitute holoenzyme with or without ω factor, mixtures of ω factor-free core enzyme and two-fold molar exess of σ^{70} were incubated in the presence or absence of various amounts of purified ω factor in 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, 25 μg/ml bovine serum albumin at 37℃ for 10 min.

In vitro mixed transcription

Plasmids used for the preparation of truncated DNA templates were as follows: pWT101 (24) for trp promoter, pJLO-2 (Fukuda, R. and Nagasawa, H. in preparation) for rplJ promoter, pSP261 (25) for $rpsA_{p1}$ promoter, pMZS11 (26) for metZ promoter, and pKB252 (27) for lacUV5 promoter. The strategies for the preparation of DNA fragments carrying these promoters were described previously (18,19).

In vitro mixed transcription was performed under the standard single-cycle reaction conditions (18,19) in the presence of 50 mM NaCl. Briefly, 35 μl of mixtures containing DNA templates and 10-fold molar excess of RNA polymerase were preincubated for 60 min at 37℃. Transcription was initiated by adding 15 μl of substrate mixture containing [α-^{32}P]UTP and heparin, and RNA synthesis was allowed for another 5 min. Final concentration of heparin was 0.2 mg/ml. The reaction was terminated by adding EDTA and RNA products were analyzed by gel electrophoresis and autoradiography as previously described (18,19). For quantitation of individual transcripts, autoradiograms were scanned with LKB ULTROSCAN XL laser densitometer.

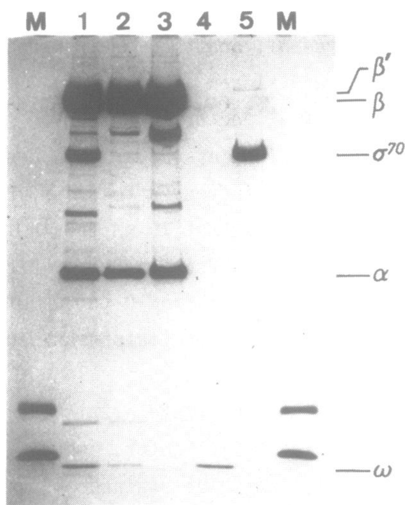

Figure 1. SDS-polyacrylamide gel analysis of purified RNA polymerase and subunits. Samples (20 pmol each) were analyzed by electrophoresis on a 10-20% polyacrylamide gradient gel. Lane 1, holoenzyme; lane 2, core enzyme; lane 3, ω factor-free core enzyme; lane 4, ω factor; lane 5, σ^{70} subunit; M, a mixture of molecular weight marker proteins, trypsin inhibitor (Mr=21,500) and cytochrome C (Mr=12,500). Electrophoresis was carried out in a Tris-glycine buffer (31) and the gel was stained with Coomassie brilliant blue.

RESULTS

Preparation of RNA polymerase holoenzyme lacking ω factor

Factor ω is associated with RNA polymerase even after dissociation of σ subunit by passing through phosphocellulose or phosphorylated agarose columns. Upon exposure to increasing concentrations of urea, core enzyme is stepwisely dissociated into subassemblies and individual subunits (reviewed in ref. 28). To dissociate ω factor, we treated core enzyme with various concentrations of urea and examined the subunit assembly by glycerol gradient centrifugation. Upon exposure to urea above 1.0 M, ω factor began to be dissociated without affecting the assembly of α, β and β' subunits. For preparation of ω factor-free RNA polymerase, core enzyme was treated with 1.5 M urea and subjected to glycerol gradient centrifugation. The ω-free core enzyme was recovered from the slowly sedimenting shoulder of core enzyme peak. This ω-free core enzyme showed a lower specific activity of σ subunit-independent poly(AU) synthesis than that of native core enzyme. This might be due to the presence of subassemblies generated

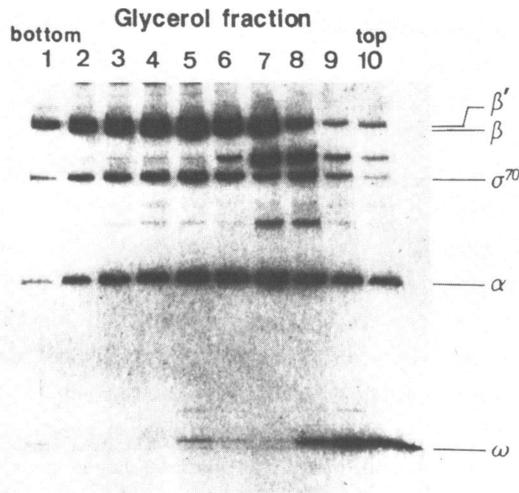

Figure 2. Glycerol gradient centrifugation of reconstituted holoenzyme. ω factor-free core enzyme (23.7 μg), σ^{70} subunit (4.4 μg), and ω factor (2.4 μg) were incubated for 10 min at 37℃ in the reaction buffer for *in vitro* mixed transcription and then layered on 2 ml of 15-35% glycerol gradient in the same buffer. The gradient was centrifuged at 4℃ for 4 hr at 55,000 rpm in Beckman TLS-55 rotor. Fractions of 200 μl were collected. Proteins were precipitated with trichloroacetic acid in the presence of deoxycholate as a carrier and analyzed by electrophoresis on a 10-20% polyacrylamide gradient gel.

by the urea treatment. When the assay was carried out using saturating amounts of the ω-free core enzyme over template DNA (enzyme saturation assay), its activity was comparable to that of native core enzyme, suggesting that the subassemblies did not interfere with transcription by intact enzyme.

For the preparation of ω factor, core enzyme was treated with 2.5 M urea. By this treatment, core enzyme was partially dissociated into various subunit complexes such as $\alpha\beta$, $\alpha\beta'$, $\alpha_2\beta$ and premature core ($\alpha_2\beta\beta'$) complexes (28). ω factor was purified to near homogeneity by glycerol gradient centrifugation and HPLC. SDS-polyacrylamide gel analysis of the enzymes and the subunits used in this study is shown in Fig. 1.

The ω factor content of the holoenzyme preparation used was 0.9 mole per holoenzyme but that of ω factor-free core enzyme used in this study was less than 0.1 mole per enzyme, as estimated from the densitometry of Coomasie brilliant blue-stained gels. However, the ω- free core enzyme was enriched with two species of polypeptides (approximate Mr, 110 kDa and 60

kDa). These polypeptides might be degradation products of β or β' which arose during urea-treatment but did not bind to core enzyme (for example, see Fig. 2). On the other hand, the purity of ω factor was greater than 95%.

To see whether the purified ω factor retained the activity of binding RNA polymerase, an excess amount of purified ω factor was incubated with a mixture of ω-free core enzyme and σ^{70}, and subjected to glycerol density gradient centrifugation in a microfuge. SDS-polyacrylamide gel analysis of glycerol fractions is shown in Fig. 2. Distribution of each subunit across fractions was somewhat broad. This was due to the low level resolution in a small volume of the glycerol gradient (2 ml) and the presence of subassemblies in the ω-free core enzyme preparation. ω factor formed two peaks: fast sedimenting RNA polymerase-bound peak (fraction #5); and slowly sedimenting free form. The peak fraction of holoenzyme contained at least 0.5 copy of ω subunit per holoenzyme. The two contaminating polypeptides in the ω-free core enzyme sedimented slower than the holoenzyme.

Effect of ω factor on the ppGpp sensitivity of RNA polymerase

The ppGpp sensitivity of native and reconstituted RNA polymerase holoenzyme was examined using the *in vitro* mixed transcription system. In this study, we used *rpsA*$_{p1}$, *rplJ*, and *metZ* promoters as the representative stringent promoters, and *trp* and *lac*UV5 promoters as the reference promoters. To test whether the depletion of ω factor influences the ppGpp sensitivity of RNA polymerase, RNA polymerase holoenzyme was reconstituted from ω-free core enzyme and σ^{70}, and in the presence of various amounts of ω factor. The level of RNA synthesis by the reconstituted holoenzymes was examined at various concentrations of ppGpp. Some representative autoradiograms are shown in Fig. 3.

In the absence of ppGpp, the addition of ω factor gave little effect on the transcription from all the promoters tested. On the other hand, when ppGpp was present at either 0.1 or 0.25 mM, transcription from the stringent promoters, *i.e.*, *rpsA*$_{p1}$, *rplJ*, and *metZ* promoters, decreased concomitantly with the increase of ω factor addition. In contrast, the transcription from the non-stringent reference promoters, *i.e.*, *trp* and *lacUV5* promoters, was little affected. Although the transcript from the *metZ* promoter was low in the given autoradiogram, we observed after prolonged exposure the same tendency of ppGpp-dependent decrease as those

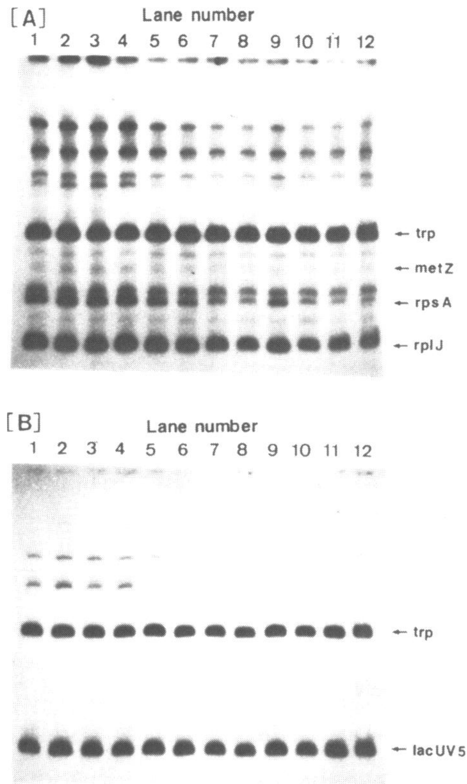

Figure 3. In vitro mixed transcription by the reconstituted holoenzymes. Reconstitution of holoenzyme was carried out from ω factor-free core enzyme and σ^{70} subunit in the presence of the following amounts of ω factor. ω factor/holoenzyme ratio during the reconstitution was: 0 (lanes 1, 5 and 9); 1 (lanes 2, 6 and 10); 2 (lanes 3, 7 and 11); and 4 (lanes 4, 8 and 12). In vitro mixed transcription was performed using either 3.2 pmol [A] or 2.0 pmol [B] of the reconstituted holoenzymes under the standard single-round reaction conditions. Mixtures of either 0.02 pmol of trp and 0.1 pmol each of $rpsA_{p1}$, rplJ, and metZ [A] or 0.1 pmol each of trp and lacUV5 [B] were used as templates; and the following concentrations of ppGpp were added throughout all the step reactions: 0 (lanes 1-4); 0.1 (lanes 5-8); and 0.25 mM (lanes 9-12).

from the $rpsA_{p1}$ and rplJ promoters. For quantitative comparison, each transcript was measured with a densitometer and the level of each transcription relative to trp RNA is summarized in Fig. 4.

The results in Fig. 4 (B) clearly indicate that ppGpp specifically repressed the transcription from $rpsA_{p1}$ and rplJ promoters, and that the

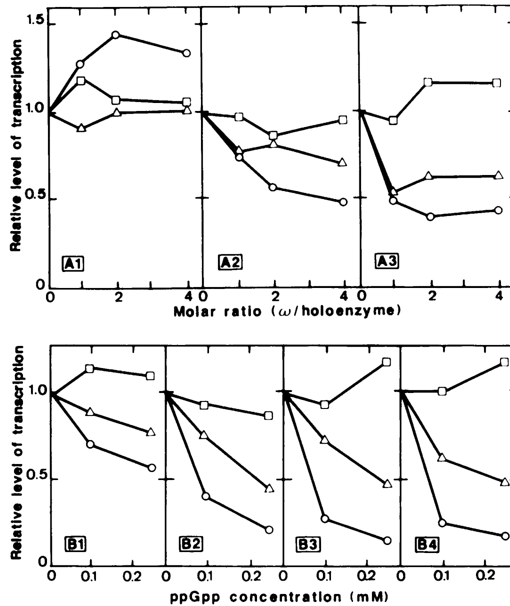

Figure 4. ppGpp sensitivity of the reconstituted holoenzymes. Transcription in vitro by the reconstituted holoenzymes was performed as described in Fig. 3 and transcripts were measured by densitometry of the autoradiograms. The level of each transcript was determined and normalized as a value relative to the level of trp transcript, and is plotted against the molar ratio of ω factor to holoenzyme [A] or against the concentration of ppGpp [B]. The concentration of ppGpp was: 0 (A1), 0.1 (A2) and 0.25 mM (A3). The ω factor/holoenzyme ratio was: 0 (B1), 1 (B2), 2 (B3) and 4 (B4). ○-○ ,$rpsA_{p1}$; △-△ ,rplJ; □-□ ,lacUV5.

extent of this inhibition was dependent on the addition of ω factor. The results in Fig. 4 (A) show that the ppGpp sensitivity became maximum when the reconstitution was carried out above two mole equivalents of ω factor per core enzyme. In contrast, the addition of ω factor gave no apparent effect on the transcription from lacUV5 promoter irrespective of the presence or absence of ppGpp, implying that ω factor did not give non-specific inhibition on RNA synthesis by the reconstituted RNA polymerase.

When we used native holoenzyme obtained after phosphocellulose column chromatography, which contained 0.9 copy of ω factor per enzyme (see Fig. 1), supplementation of ω factor did not give significant increase in the ppGpp sensitivity (Fig. 5). This result implies that the native holoenzyme was already saturated with ω factor, and that unassociated ω factor gave no effect on transcription.

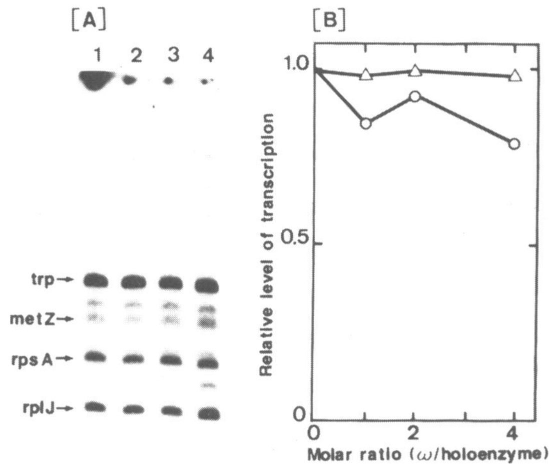

Figure 5. ppGpp sensitivity of native holoenzyme. [A] Various amounts of ω factor were incubated with native holoenzyme under the same conditions as described for the reconstitution of holoenzyme. The amount of ω factor was: 0 (lane 1), 1 (lane 2), 2 (lane 3) and 4 (lane 4). In vitro transcription was performed in the presence of 0.25 mM ppGpp. DNA templates used were 0.02 pmol of trp and 0.1 pmol each of $rpsA_{p1}$, rplJ and metZ. [B] Each transcript was quantitated as described in Fig. 4 and plotted against the molar ratio of ω factor to holoenzyme. O-O ,$rpsA_{p1}$; △-△ ,rplJ.

DISCUSSION

Reversible dissociation studies of E. coli RNA polymerase indicated that ω factor is not an essential component for RNA synthesis (14). In spite of the complete lack of knowledge on its function, ω factor has been anticipated to be involved in controlling yet unidentified function associated with RNA polymerase because it is tightly bound to RNA polymerase. The finding that the rpoZ gene coding for ω factor was located immediately next to the spoT gene (17) promoted us to examine the possibility if ω factor is necessary for the transcriptional inhibition of stringent promoters by ppGpp.

In this reconstitution study, we reached to a tentative conclusion that ω factor is required for the efficient inhibition of transcription by ppGpp. This conclusion is based on the following observations. First, the removal of ω factor rendered RNA polymerase less sensitive to ppGpp. Second, the addition of ω factor during the reconstitution restored the sensitivity to ppGpp. Amount of ω factor needed for this effect was within physiological range, because the addition of two to four fold molar

excess of ω factor over core RNA polymerase during the reconstitution was enough to achieve the saturating level of inhibition by ppGpp. The reconstituted ω-free holoenzyme was slightly inhibited by ppGpp (Fig. 4B). This slight inhibition might be due to the residual ω in the ω-free core enzyme preparation, or alternatively, ω-free enzyme might possess basal sensitivity to ppGpp.

Previously, it was found that certain E. coli mutants carrying mutations in the rpoB gene encoding RNA polymerase β subunit conferred to relaxed phenotype (29), and that RNA polymerase prepared from these mutants were insensitive to ppGpp in in vitro transcription from stringently controlled promoters (30). To meet these two observations, we propose two possible mechanisms: 1) ppGpp binds to ω factor, which interacts with the functional domain of β subunit for stringent control; 2) ω factor interacts with β subunit and thereby activates its binding site to ppGpp. To discriminate these two possibilities, direct identification of the target subunit of ppGpp is in progress using chemical cross-linking method.

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