Volume 14 Number 17 1986 Nucleic Acids Research

Promoter selectivity of *Escherichia coli* RNA polymerase: alteration by fMet-tRNA$_f^{Met}$

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Received 17 June 1986; Revised and Accepted 12 August 1986

ABSTRACT

An in vitro mixed transcription system was employed to examine the possible alteration of the promoter selectivity of Escherichia coli RNA polymerase by specific tRNAs. Transcription in vitro was inhibited by most of the tRNAs examined, although the extent of the inhibition differed with the tRNA species. The inhibition by tRNAs was due to competition with DNA for binding RNA polymerase. This inhibitory effect remained after charging of the tRNAs with amino acids. The charging of tRNA$_f^{Met}$ with fMet, but not with Met, abolished its inhibitory effect, and instead gave a stimulatory effect on the transcription from some promoters. These observations suggest that fMet-tRNA$_f^{Met}$ plays a specific regulatory role in the coupling of transcription to translation.

INTRODUCTION

The rate of overall RNA synthesis in Escherichia coli is related to the level of the intracellular amino acid pool (1). To explain this relationship, it was hypothesized that tRNAs are the regulatory factors involved in RNA synthesis, the regulatory activity being controlled through charging with amino acids (1). Later, it was observed that the inhibition of the formylation of Met-tRNA$_f^{Met}$ reduced not only the rate of protein synthesis but also that of RNA synthesis (2,3). On this line, Travers (4) proposed that fMet-tRNA$_f^{Met}$ is a specific regulatory factor which mediates the coupling between transcription and translation.

Detailed and systematic studies are needed on the effects of various specific tRNAs and their aminoacylated forms on the promoter selectivity of E. coli RNA polymerase. For this purpose, we employed an in vitro mixed transcription system (5,6), in which transcription was performed with mixtures of various E. coli DNA fragments, each carrying a specific promoter(s), and the products were separately determined by polyacrylamide gel electrophoresis and autoradiography. This system was shown to be useful for the analysis of altered promoter selection by mutant RNA polymerases (7),

and for search of transcriptional factors (8) or conditions (9) affecting the transcription of specific genes.

In this report, we show that both the charged and uncharged forms of tRNAs inhibit RNA polymerase by competing with DNA templates. Although $tRNA_f^{Met}$ and Met-$tRNA_f^{Met}$ exhibit similar inhibitory effects on RNA polymerase, the formylation of Met-$tRNA_f^{Met}$ abolishes this inhibition, and instead stimulates the transcription from some promoters. These results support the notion that $tRNA_f^{Met}$ plays a regulatory role not only in translation but also in transcription.

MATERIALS AND METHODS

Preparation of Truncated DNA Templates --- The plasmids used in this study are listed in Table 1. DNA was prepared from transformed *E. coli* cells essentially according to the procedure of Birnboin (21). DNA fragments carrying specific promoters were prepared as follows.

Plasmid 48 containing *alaS* promoter (10,11) was digested with *Bam*HI and *Kpn*I, and the resulting 287 bp fragment was used as an *alaS* template (Fig. 1). Plasmid pYY105 containing *glnS* promoter (12,13) was digestd with *Bgl*II and the resulting 198 bp fragment was used as a *glnS* template (Fig. 1). Plasmid pSY343 (14) carries the 4.2 kbp *Hind*III fragment of λpsu°6, which contains *supP* promoter. The 1.1 kbp *Ava*I fragment of pSY343 was subcloned

Table 1.
Plasmids used for the Preparation of Promoters.

Plasmids	Genes	Gene products	References
p48	*alaS*	Alanyl-tRNA synthetase	10,11
pYY105	*glnS*	Glutamyl-tRNA synthetase	12,13
pSY343	*supP*	$tRNA^{Leu}$	14
pKU1	*nusA*	NusA protein	15
pSP621	*rpsA*	Ribosomal protein S1	16
pMM5	*dnaQ*	DNA polymerase III ε subunit	17,18
	rnh	Ribonuclease H	
pJLO-2	*rplJ*	Ribosomal protein L10	R. Fukuda[1]
pTM-2	*recA*	RecA protein	19
pRP-1	*rrnE*	rRNA	A. Muto[2]
pKB252	*lac*UV5	β-Galactosidase	20

1. R. Fukuda, personal communication.
2. A. Muto, personal communication.

to pBR322 at the AvaI site, and the resulting plasmid, pSu°6, was used to prepare a supP promoter fragment. The 1.1 kbp AvaI fragment was digested with HpaII and the resulting 320 bp fragment was used as a supP template (Fig. 1). pKU1 (15) containing the nusA operon was digested first with PstI and then the resulting 1,886 bp fragment was digested with FokI to generate a 449 bp fragment. This 449 bp fragment was further digested with HinfI, and the resulting 172 bp fragment was used as a nusA templater (Fig. 1). Plasmid pSP621 (16) containing rpsA promoters was digested with SalI and EcoRI. The resulting 877 bp fragment was further digested with HinfI to generate a 391 bp fragment containing both the P1 and P2 promoters of the rpsA gene (Fig. 1). DNA fragments containing the rplJ, recA, rrnE P1 and P2 promoters were prepared as described previously (5,6). The DNA fragment containing both the dnaQ and rnh promoters was prepared according Nomura et al. (9,17).

Preparation of tRNA and Aminoacyl-tRNA --- Amino acid-specific tRNAs from E. coli were purchased from Sigma Corporation. Aminoacyl tRNA synthetase was prepared from E. coli DH1 as described by Kung et al. (22). In brief, cells were sonicated with a Bronson sonifier and then extracted with a buffer containing 20 mM Tris-HCl, pH 7.8, 10 mM Mg acetate, 1 mM DTT and 0.5 mM EDTA. Cell debris was removed by centrifugation at 30,000 g for 20 min. The supernatant was then centrifuged for 16 hrs at 30,000 rpm. The resulting supernatant was dialyzed against 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT and 0.5 mM EDTA and then loaded on a DE52 column equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT, 1 mM $MgCl_2$ and 10% glycerol. The column was eluted with 0.25 M potassium phosphate buffer, pH 6.5, containing 2 mM DTT, 1 mM $MgCl_2$ and 10% glycerol. The eluate was dialyzed against 85% ammonium sulfate solution, and the resulting precipitate was dissolved in a buffer comprising 10 mM Tris-acetate, pH 8.2, 14 mM Mg acetate, 60 mM potassium acetate and 1 mM DTT, followed by dialysis against the same buffer.

Aminoacylation of tRNA was carried out by the method of Caillet et al. (23). The reaction mixture contained in 50 µl: 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM or 5 mM ATP, enzyme, 25 µg tRNA and [^{14}C] protein hydrolysate or [^{35}S] methionine.

Formylation was carried out under the same conditions as described above except for the addition of 1 mM 10-formyltetrahydrofolate. After incubation at 37°C for 30 min, the mixture was extracted with phenol and chloroform, and then the tRNA was precipitated with ethanol. The precipitate was dissolved in 5 mM Na acetate, pH 5. 10-Formyltetrahydrofolate was prepared from folinic

acid (Ca salt) as described by Dubnoff and Maitra (24). The aminoacylation of tRNAs was examined by measuring the incorporation of labeled amino acids into the acid insoluble fraction. Formylation of Met-$tRNA_f^{Met}$ was tested by the ethylacetate extraction method described by Leder and Bursztyn (25).

In Vitro Transcription --- In vitro mixed transcription was carried out under the standard reaction conditions (5,6). In brief, 35 µl of a preincubation mixture containing 0.1 pmol each of the test templates and a 10-fold molar excess of the RNA polymerase holoenzyme, in 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM Mg acetate, 0.1 mM EDTA, 0.1 mM DTT, 25 µg/ml nuclease-free BSA, 50 mM NaCl and tRNA at various concentrations, was incubated for 60 min at 37°C. Transcription was initiated by adding 15 µl of a prewarmed mixture on substrates and heparin in the same buffer. The final concentrations were 0.16 mM for ATP, GTP and CTP, 0.05 mM for [α-^{32}P]UTP (2 µCi per reaction) and 200 µg/ml for heparin, respectively. RNA synthesis was allowed to proceed for 5 min and then terminated by adding 50 µl of a stopping solution containing 40 mM EDTA and 10 µg E. coli rRNA. The RNA products were precipitated with ethanol and then analyzed by 7% polyacrylamide gel electrophoresis in the presence of 8 M urea. The gels were exposed to X-ray films and then the RNA products were quantitated by tracing the autoradiograms with a Joyce-Loebl microdensitometer.

RNA polymerase was purified from E. coli strain W3350 essentially according to the method of Fukuda et al. (26). The holoenzyme was obtained by passing the purified RNA polymerase through a phosphocellulose column in the presence of 50% glycerol (27).

Chemicals and Enzymes --- Restriction endonucleases were obtained from Takara Shuzo, Japan. [α-^{32}P]UTP, [^{14}C] protein hydrolysate and [^{35}S] methionine were purchased from Amersham, England. Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals, USA. DE52 was a product of Whatman Ltd., England.

RESULTS

Inhibition of RNA Polymerase by tRNA

The effects of various amino acid-specific tRNAs on the transcription of specific genes were examined using an in vitro mixed transcription system. In this study, we used various E. coli DNA fragments as templates, each carrying specific promoters of cloned E. coli genes, shown in Table 1. The structures of truncated DNA fragments and of RNAs transcribed from these templates are illustrated in Fig. 1.

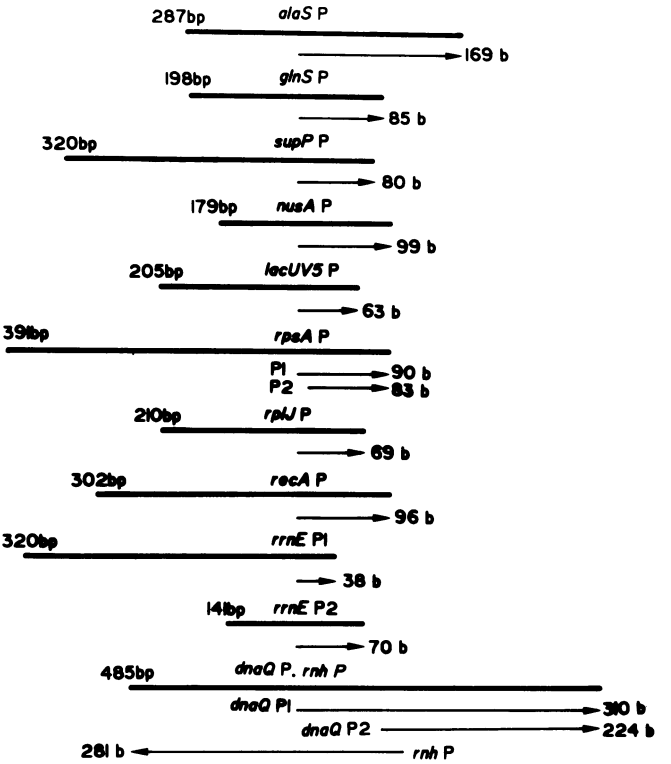

Fig. 1. Structures of truncated DNA templates.
Truncated DNA templates, each carrying a specific promoter(s), were prepared as described in MATERIALS AND METHODS from the plasmids listed in Table 1. Filled bars indicate the DNA fragments, while arrows represent transcripts directed by the respective DNA fragments. The numbers represent the nucleotide lengths of template DNAs and RNA transcripts.

The amino acid-specific tRNAs tested were glutamic acid, tyrosine, formylmethionine, valine, phenylalanine and serine specific tRNAs. All these six tRNAs exhibited general inhibitor activity toward transcription directed by the alaS, recA, glnS and rplJ templates (Fig. 2). The inhibition was observed even after further purification of tRNA by treatment with phenol-chloroform but no more when tRNA was degraded with pancreatic ribonuclease A.

Among the six tRNAs, the phenylalanine-specific tRNA was the most powerful inhibitor. With increasing concentrations of these tRNAs, differential inhibition was observed for transcription from various promoters. For example, the transcription was almost completely abolished

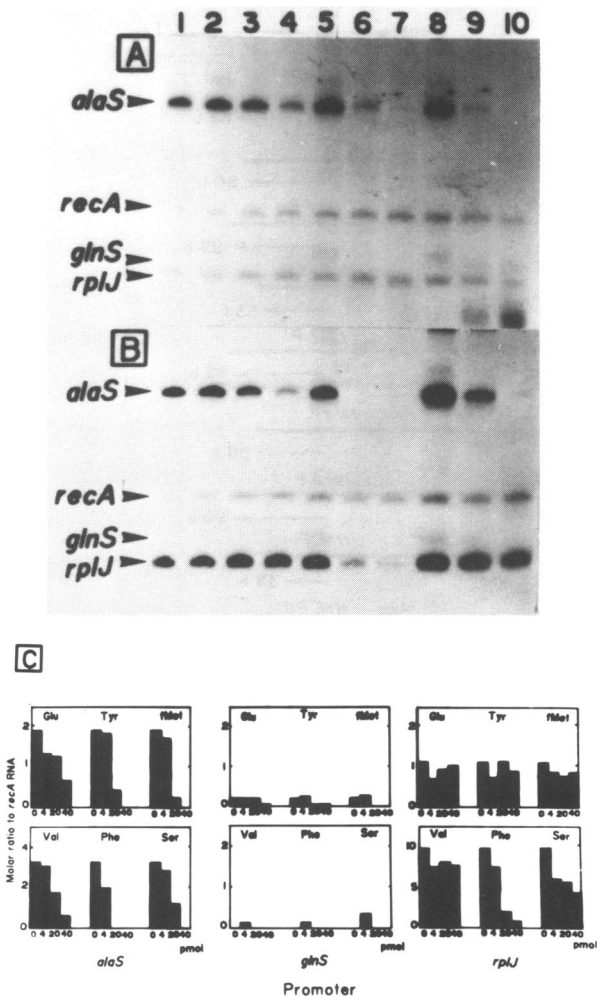

Fig. 2. Effects of various amino acid-specific tRNAs on transcription.
[A] The preincubation mixtures (35 µl) containing 0.1 pmol each of the alaS, recA, glnS, rplJ DNA fragments, 4 pmol of the RNA polymerase holoenzyme and various tRNAs were incubated at 37°C for 60 min, and then RNA synthesis was initiated by adding 15 µl of substrates and heparin mixture. After 5 min incubation, RNA products were fractionated by electrophoresis on 7% polyacrylamide gel. The tRNAs used were :$tRNA^{Glu}$, 0, 4, 20 and 40 pmol (lanes 1-4); $tRNA^{Tyr}$, 4, 20 and 40 pmol (lanes 5-7); $tRNA_f^{Met}$, 4, 20 and 40 pmol (lanes 8-10). [B] $tRNA^{Val}$, 0, 4, 20 and 40 pmol (lane 1-4); $tRNA^{Phe}$, 4, 20 and 40 pmol (lane 5-7); $tRNA^{Ser}$, 4, 20 and 40 pmol (lanes 8-10). [C] The autoradiograms were traced and the amounts of RNA products were determined after correction of U contents. The molar ratios of alaS, glnS and rplJ RNAs relative to recA RNA were plotted.

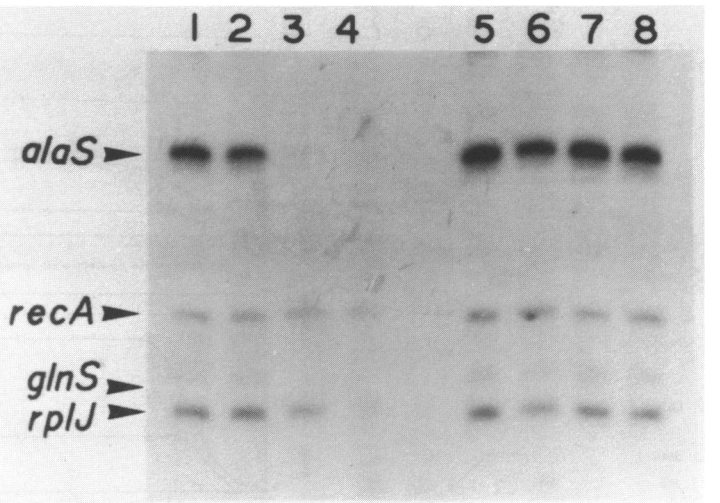

Fig. 3. Inhibition of the RNA polymerase reaction by $tRNA^{Phe}$.
Lanes 1-4: RNA polymerase and various amounts of $tRNA^{Phe}$ (lane 1, 0; lane 2, 4; lane 3, 10; and lane 4, 20 pmol) were preincubated for 10 min at 37°C and then RNA synthesis was initiated by the simultaneous addition of alaS, recA, glnS and rplJ DNAs, and substrates. Lanes 5-8: DNAs and RNA polymerase were preincubated for 10 min at 37°C and then RNA synthesis was initiated by the addition of substrates and various amounts of $tRNA^{Phe}$ (lane 5, 0; lane 6, 4; lane 7, 10; and lane 8, 20 pmol). Other experimental details are the same as in Fig. 2.

by the addition of a 5-fold molar excess of $tRNA^{Phe}$ over RNA polymerase except for recA RNA synthesis (Fig. 2, lane 6). The recA (an internal control) promoter was the most resistant to the inhibitory activity of tRNA (therefore, in Fig. 2 A, B and C, the molar ratios to recA RNA are plotted). The rplJ promoter exhibited intermediate resistance. The alaS and glnS promoters are the most sensitive to the inhibition by all the tRNA species tested. The bands migrating faster than rplJ RNA were transcripts of tRNA because these bands were identified for the reactions lacking DNA templates (for example, the fast migrating bands in lanes 9-10 were transcripts of $tRNA_f^{Met}$).

In another experiment, the other four promoters, nusA, supP, rrnE (P2) and lacUV5, were tested, and it was found that the transcription from the rrnE P2 promoter was the most sensitive, followed by that from nusA and supP promoters. The lacP(UV5) promoter (another reference promoter) was the most resistant in this group of promoters (data not shown).

To determine the step at which tRNA causes inhibition of transcription,

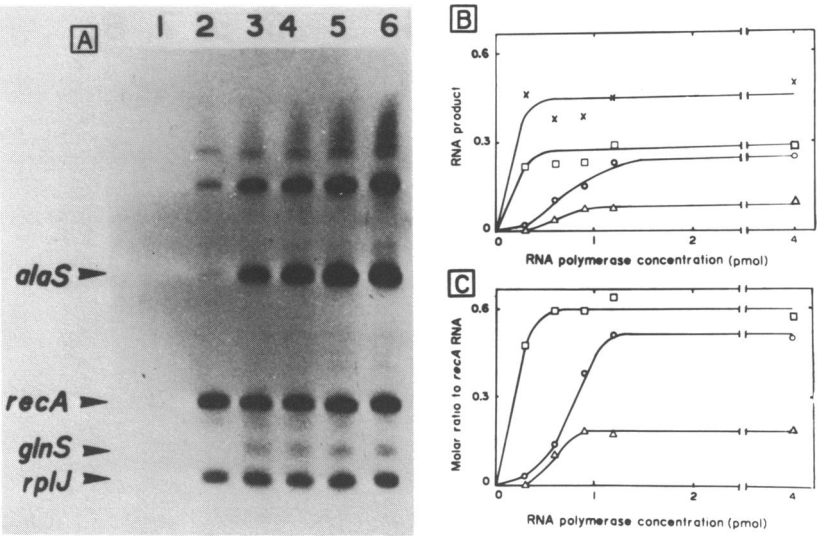

Fig. 4. Effect of the RNA polymerase concentration on the selection of the alaS, recA, glnS and rplJ promoters.

[A] In vitro transcription was performed using the alaS, recA, glnS and rplJ DNA fragments as templates, as described in Fig. 2, except that the amount of RNA polymerase was varied as follows: lane 1, 0; lane 2, 0.3; lane 3, 0.6; lane 4, 0.9; lane 5, 1.2; and lane 6, 4 pmol. [B] The autoradiogram was traced and the amounts of individual RNA species were determined after correction of U contents. [C] The amounts of alaS, glnS and rplJ RNAs were replotted as values relative to recA RNA. X—X , recA RNA; O—O , alaS RNA; Δ—Δ , glnS RNA; □—□ , rplJ RNA.

tRNAs were added at various times during in vitro transcription. Figure 3 shows one such experiment, in which RNA polymerase was preincubated with either $tRNA^{Phe}$ (lanes 1-4) or a DNA mixture (lanes 5-8) for 10 min at 37°C. RNA synthesis was initiated by the simultaneous addition of the counterparts and substrates. When RNA polymerase and $tRNA^{Phe}$ were preincubated (lanes 1-4), significant inhibition was observed for the transcription from the alaS, glnS and rplJ promoters. When DNA and RNA polymerase were preincubated (lanes 5-8), inhibition was not observed at all. Furthermore, when the tRNA was added to the open RNA polymerase-DNA complexes at 10 min before the addition of substrates, no significant inhibition was observed. These results clearly showed that the inhibition of transcription by tRNAs takes place before the formation of an open complex, due to a decrease in the concentration of functional RNA polymerase on the complex formation with tRNA.

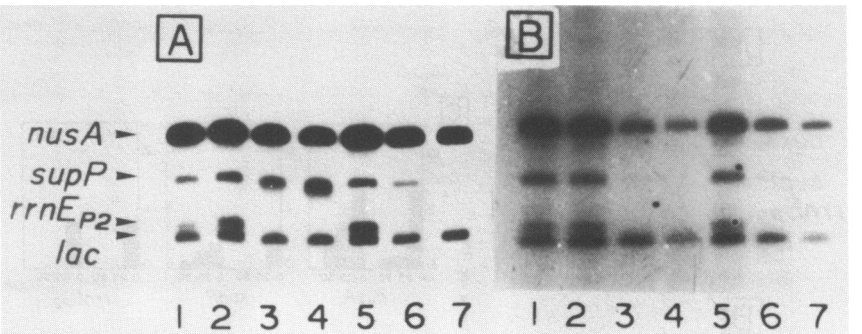

Fig. 5. Effects of various aminoacyl-tRNAs on transcription.
[A] In vitro transcription was performed using the nusA, supP, rrnE P1, P2 and lac DNA fragments as templates in the presence of either total uncharged tRNAs (lane 1, 0; lane 2, 5; lane 3, 25; and lane 4, 50 pmol) or aminoacylated tRNAs (lane 5, 5; lane 6, 25; and lane 7, 50 pmol). [B] Transcription was performed in the presence of either uncharged $tRNA^{Phe}$ (lane 1, 0; lane 2, 5; lane 3, 25; and lane 4, 50 pmol) or phe-$tRNA^{Phe}$ (lane 5, 5; lane 6, 25; and lane 7, 50 pmol).

To confirm this hypothesis, we examined the promoter strengths by varying the DNA/RNA polymerase ratios. Figure 4 shows the results of an in vitro transcription experiment using the alaS, recA, glnS and rplJ DNA templates. At a low RNA polymerase concentration, the recA and rplJ promoters are stronger than the others (Fig. 4A), indicating that a limited amount of RNA polymerase is preferentially utilized by these two promoters. This result is consistent with the transcription pattern in the presence of high concentrations of tRNAs (see Fig. 2). The transcription from the rplJ promoter was little affected by the decrease in the functional RNA polymerase concentration due to tRNA. Taking the above results together, it was concluded that the inhibition of transcription by tRNAs is due to competition with DNA for binding RNA polymerase. In fact, the level of tRNA transcription increased concomittantly with the decrease of DNA transcription.

Effect of Aminoacyl tRNA on the Transcription

Next we examined the effect of the aminoacylation of total tRNAs and phenylalanine-specific tRNA on the inhibitory activity toward transcription. For this purpose, tRNAs were charged in vitro with amino acids using partially purified aminoacyl-tRNA synthetase. In vitro transcription directed by the nusA, supP, rrnE(P2), lac(UV5) and rrnE(P1) promoters was performed in the presence of either tRNAs or aminoacyl-tRNAs. As shown in Figure 5,

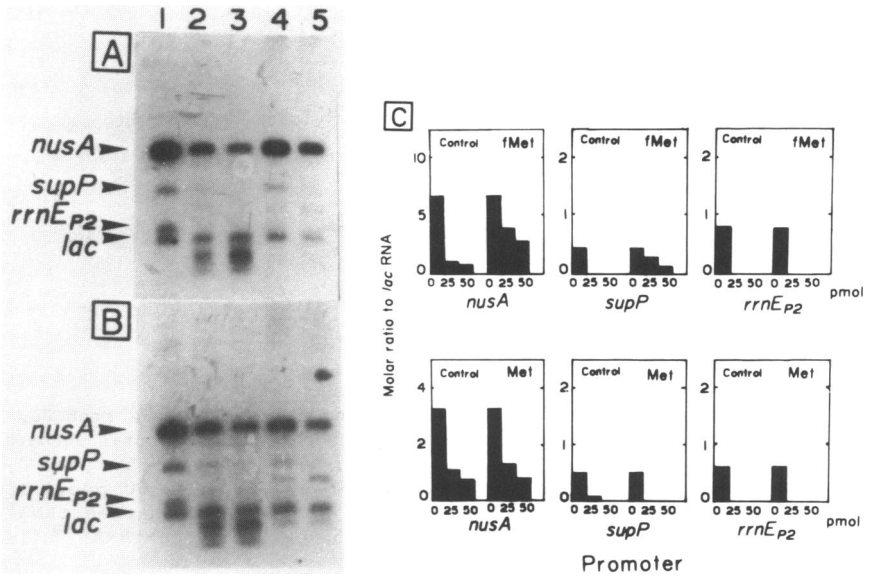

Fig. 6. Effects of Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ on transcription.
[A] In vitro transcription was performed using the nusA, supP, rrnE P1, P2 and lac DNA fragments as templates in the presence of either uncharged tRNA$_f^{Met}$ (lane 1, 0; lane 2, 25; and lane 3, 50 pmol) or fMet-tRNA$_f^{Met}$ (lane 4, 25; and lane 5, 50 pmol). [B] Transcription was performed in the presence of either uncharged tRNA$_f^{Met}$ (lane 1, 0; lane 2, 25; and lane 3, 50 pmol) or Met-tRNA$_f^{Met}$ (lane 4, 25; and lane 5, 50 pmol). [C] The autoradiograms were traced and the amounts of individual RNA species were determined after correction of U contents. The molar ratios of nusA, supP, rrnE P2 RNAs relative to lac RNA are plotted.

aminoacylation of tRNAs did not abolish their inhibitory effect on transcription.

Finally, we examined the effect of formylation on the inhibitor activity of Met-tRNA$_f^{Met}$. Formylation of Met-tRNA$_f^{Met}$ was performed in vitro using a partially purified enzyme and 10-formyltetrahydrofolate as a formyl donor. The results are shown in Figures 6 and 7. Met-tRNA$_f^{Met}$ inhibited the transcription as well as tRNA$_f^{Met}$. Formylation of Met-tRNA$_f^{Met}$, however, abolished its inhibitory effect on the transcription from some promoters. Inhibition of transcription from the nusA, supP, alaS and glnS promoters was suppressed; transcription of the rplJ promoter being rather enhanced. Transcripts of tRNA$_f^{Met}$ migrated below lac RNA (lanes 2-3), the level of which decreased by charging tRNA with either Met or fMet (lanes 4-5).

The results of another in vitro transcription experiment indicated that transcription from the dnaQ, rnh and rpsA promoters also became insensitive

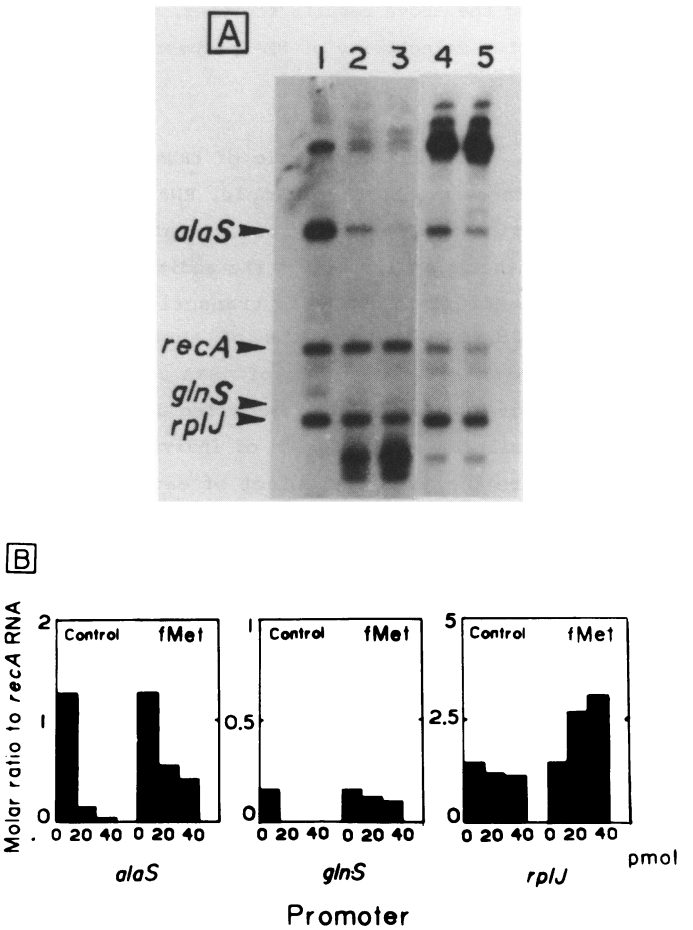

Fig. 7. Effect of fMet-tRNA$_f^{Met}$ on transcription.
[A] In vitro transcription was performed using the alaS, recA, glnS, and rplJ DNA fragments as templates in the presence of either uncharged tRNA$_f^{Met}$ (lane 1, 0; lane 2, 20; and lane 3, 40 pmol) or fMet-tRNA$_f^{Met}$ (lane 4, 20; and lane 5, 40 pmol). [B] The autoradiogram was traced and the amounts of individual RNA species were determined after correction of U contents. The molar ratios of alaS, glnS, and rplJ RNAs relative to recA RNA are plotted.

to the inhibition (data not shown). In this experiment, we noticed that the synthesis of template-sized end-to-end transcripts was also enhanced by the addition of fMet-tRNA$_f^{Met}$. Since the formylated tRNA$_f^{Met}$ preparation used in these experiments was a mixture of fMet-tRNA$_f^{Met}$ and Met-tRNA$_f^{Met}$, the loss of its inhibitory activity or the appearance of its enhancing activity might be more pronounced provided that all the tRNA$_f^{Met}$ molecules are charged with

formylmethionine. Taking the above results together, we concluded that fMet-$tRNA_f^{Met}$ alters the promoter selectivity of RNA polymerase.

DISCUSSION

The initial proposal of a regulatory role of tRNAs in RNA synthesis was based on physiological observations. For example, RNA synthesis requires a continuous supply of amino acids. When protein synthesis is inhibited by chloramphenicol, RNA synthesis is induced on the addition of amino acids (1). With this in mind, the effect of tRNAs on transcription was examined in several laboratores (28,29). The results of such studies raised the possibility that tRNAs inhibit the action of RNA polymerase but charged tRNAs do not. These studies were, however, carried out with phage or total bacterial DNA as the template, and the levels of individual products were not determined. We therefore analyzed the effect of each tRNA species on the transcription from specific promoters, using an *in vitro* mixed transcription system.

The present study demonstrated that transcription is generally inhibited when RNA polymerase is titrated after binding with either charged or uncharged tRNAs. We failed to obtain relief of the inhibition by tRNAs even after charging with amino acids. This is apparently inconsistent with the previous proposal (28,29). The discrepancy might be due to that such an inhibitory effect depends on either the tRNA species or the DNA template. For example, aminoacylation only inactivates some tRNA species, others remaining active, as to the inhibitory effect on transcription. Otherwise, aminoacylated tRNAs do not exert an inhibitory effect on the transcription from certain promoters other than those examined in this study.

In contrast, however, we found that formylation of Met-$tRNA_f^{Met}$ abolished the inhibition of transcription. Debenham *et al* (30) reported that fMet-$tRNA_f^{Met}$ inhibits the synthesis of suIII tRNA and rRNA, but stimulates *lac* RNA synthesis. In this study, we found that the transcription from the *nusA*, *supP*, *alaS* and *glnS* promoters became insensitive to the inhibition by $tRNA_f^{Met}$ after charging with fMet but not with Met. Furthermore, the transcription from the ribosomal protein L10 (*rplJ*) promoter was rather enhanced in the presence of fMet-$tRNA_f^{Met}$.

As to the mechanism of the action of fMet-$tRNA_f^{Met}$, specific binding of fMet-$tRNA_f^{Met}$ to the RNA polymerase holoenzyme has been elucidated (31). The results was, however, challenged by Spassky *et al* (32). The results of our preliminary experiment demonstrated that fMet-$tRNA_f^{Met}$ associates more

predominantly with the RNA polymerase holoenzyme than Met-$tRNA_f^{Met}$ does (data not shown). For detailed understanding of the specific influence of fMet-$tRNA_f^{Met}$ on transcription, it is needed to locate the binding sites on RNA polymerase for fMet-$tRNA_f^{Met}$ and other tRNAs. On this line, we are searching for mutant RNA polymerases defective as to the interaction with fMet-$tRNA_f^{Met}$ as one means of elucidating this mechanism. The idea that the level of fMet-$tRNA_f^{Met}$, which is one of the essential components of the protein synthesis machinery, regulates not only the level of translation initiation but also that of transcription of at least some genes explains the coordination of transcription and translation in prokaryotes.

Acknowledgements: We thank Drs. F. Imamoto, H. Ozeki, F. Yamao and P. Schimmel for the gifts of the plasmids. This study was supported by grants from the Ministry of Education, Science and Culture of Japan, the Uehara Memorial Foundation and the Toray Science Foundation.

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