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Formylmethionyl-tRNA alters RNA polymerase specificity

(promoter recognition/transcription–translation coupling/guanosine 3′-diphosphate 5′-diphosphate)

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ABSTRACT *Escherichia coli* fMet-tRNA$_f^{Met}$ alters the pattern of promoter selection of *E. coli* RNA polymerase (RNA nucleotidyltransferase, nucleosidetriphosphate:RNA nucleotidyltransferase; EC 2.7.7.6), affecting RNA synthesis from the rRNA, su_{III}^+ tRNA, and *lac* promoters in different ways. The *in vitro* synthesis of the stable RNA species is selectively decreased, whereas that of *lac* RNA from both the wild-type and mutant UV5 promoters is selectively increased at high ionic strength. The functional effect of fMet-tRNA$_f^{Met}$ resembles that of the nucleotide guanosine 3′-diphosphate 5′-diphosphate (ppGpp). This nucleotide competes with the binding of fMet-tRNA$_f^{Met}$ to RNA polymerase.

Formylmethionyl-tRNA, fMet-tRNA$_f^{Met}$, plays a key role in the initiation of bacterial protein synthesis, acting as the donor of the NH_2-terminal amino acid of the nascent polypeptide chain (1–3). A regulatory function for this tRNA was recently suggested by the observation of its binding to *Escherichia coli* RNA polymerase holoenzyme; this is a specific interaction that requires both the formyl and tRNA moieties (4). In this paper we examine the functional consequences of the binding of fMet-tRNA$_f^{Met}$ to RNA polymerase (RNA nucleotidyltransferase, nucleosidetriphosphate:RNA nucleotidyltransferase; EC 2.7.7.6) and show that the polynucleotide decreases the initiation of stable RNA synthesis *in vitro* while concomitantly stimulating the synthesis of *lac* mRNA from either the mutant UV5 promoter or the wild-type promoter. Thus, fMet-tRNA$_f^{Met}$ functions as a macromolecular effector of RNA polymerase eliciting a response similar to the low molecular weight effector guanosine tetraphosphate (ppGpp). *In vivo* such a response to an accumulation of the initiator tRNA would be in accord with homeostatic models of transcription–translation coupling.

MATERIALS AND METHODS

Materials. *E. coli* tRNA$_f^{Met}$ was purchased from Boehringer Mannheim and was charged and formylated as described (4). *E. coli* RNA polymerase holoenzyme was prepared by the methods of Burgess and Travers (5) and Burgess and Jendrisak (6). Preparations were >95% pure as judged by 0.1% NaDodSO$_4$/polyacrylamide gel electrophoresis and contained at least 0.75 mol of σ subunit per 2 mol of α subunit. *Lac* repressor was the generous gift of N. Geisler and K. Weber, ϕpsu_{III}^+ and ϕ80 *plac* DNA were prepared by gentle phenol extraction of purified phages. A ColE1–rDNA chimera, pER24 (7), including the 5.7-kilobase *Eco*R1 restriction fragment from λ *rif* d18 DNA containing the rRNA promoter region and the proximal portion of the 16S rRNA cistron (8), was prepared from cultures of carrier bacteria that were kept overnight in 2 liters of tryptone/yeast extract broth containing chloramphenicol at 200 μg/ml. The plasmid DNA was extracted and purified by the method of Clewell and Helinski (9). The Cla restriction fragment (10) containing the su_{III}^+ tRNA gene was prepared from ϕ80 psu_{III}^+ DNA electrophoresis of 200 μg of a mixed *Hin*dII and *Hin*dIII nuclease digest in a 5% acrylamide slab gel with Tris glycine buffer (11, 12). The gel was stained with ethidium bromide and the Cla fragment was identified, cut out under UV light, and eluted by the method of Robertson *et al.* (13) with the modification that the aqueous phase was not precipitated but was further extracted with isobutanol, ether, and chloroform/phenol to remove any soluble acrylamide. This fragment was finally dialyzed against and sorted in 10 mM Tris·HCl, pH 7.9/25 mM KCl/0.1 mM EDTA. A DNA restriction fragment containing the *lac* UV5 promoter was purified from an *Eco*RI digest of pOP1 (14) by the same procedure, except that the preparative gel contained 10% acrylamide.

***In Vitro* Transcription.** The reaction mixtures (200 μl) for RNA synthesis contained, unless otherwise stated, 0.04 M Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 250 μM each of ATP, CTP, and GTP, 4 μM [^{3}H]UTP (specific activity 23 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), KCl, and DNA as indicated. The reaction mixture was preincubated for 5 min at 30°C, and RNA synthesis was started by the addition of RNA polymerase to a final concentration of 40–80 nM and was allowed to proceed for 15–30 min at 30°C. When necessary, fMet-tRNA$_f^{Met}$ was preincubated in equimolar proportion with RNA polymerase for 5 min at 0°C in the same buffer solution as the reaction mixture prior to starting the reaction. Under these preincubation conditions, the maximal effect of fMet-tRNA$_f^{Met}$ on rRNA synthesis was attained at a molar ratio of the charged tRNA to polymerase holoenzyme of >0.7. In a typical experiment the concentrations of RNA polymerase and fMet-tRNA$_f^{Met}$ in the preincubation mixture were 3 and 3.2 μM, respectively. To determine preinitiation complex, a reaction mixture (1 ml) lacking nucleoside triphosphates and containing 1.5 nM form II pER24 DNA and 0.075 M KCl was preincubated for 5 min at 30°C. RNA polymerase was then added to 50 nM and the incubation was continued for a further 10 min at 30°C. The reaction mixture was then divided into two 300-μl aliquots; 100 nM fMet-tRNA$_f^{Met}$ was added to one aliquot. From each aliquot, 90-μl samples were withdrawn after various times at 30°C and added to 10 μl of a mixture containing heparin at 4 mg/ml and the nucleoside triphosphates including 100 μM [^{3}H]UTP (specific activity, 12.5 Ci/mmol), and RNA synthesis was allowed to continue for 20 min at 30°C. After a 30-min incubation under standard reaction conditions, approximately 20% of the fMet-tRNA$_f^{Met}$ was discharged.

Analysis of *In Vitro* Transcript. su_{III}^+ tRNA synthesis, rRNA synthesis, and *lac* RNA synthesis were analyzed as described (15–17). Transcription from the DNA restriction fragments

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Abbreviation: ppGpp, guanosine 3′-diphosphate 5′-diphosphate.

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containing the *lac* and su^+_{III} tRNA promoters was analyzed by electrophoresis on an 11% acrylamide gel in Tris glycine buffer. The gel was dried and fluorographed (18) and the individual bands on the fluorograph were quantitated by densitometry on a Joyce–Loebl Mark III densitometer.

Binding of tRNA to Polymerase. Binding of fMet-tRNA$_f^{Met}$ to polymerase holoenzyme was determined as described (4), except that the KCl and $MgCl_2$ concentrations were 0.05 M and 0.01 M, respectively.

RESULTS

fMet-tRNA$_f^{Met}$ Alters Transcriptional Specificity. The highly specific binding of fMet-tRNA$_f^{Met}$ to RNA polymerase requires the presence of both the formyl group and the initiator tRNA moiety (4). To test the functional effect of this binding, RNA polymerase was first preincubated with the charged tRNA and then its ability to transcribe various DNA templates was determined as a function of the KCl concentration. This parameter affects both the nonspecific binding of polymerase to DNA (19) and the regulation of transcription by ppGpp (16, 20). Pongs and Ulbrich (4) reported that, in a crude cell free system lacking cAMP, fMet-tRNA$_f^{Met}$ increased by 4-fold the rate of RNA synthesis from λ *plac* 5 DNA. Because the charged tRNA inhibits transcription of both φ80 and λ DNA (data not shown), this result suggests that this polynucleotide increases preferentially transcription of *lac*-specific sequences.

To test whether fMet-tRNA$_f^{Met}$ alters transcriptional selectivity, a mixture of two restriction fragments, each containing a single promoter, was used as template for *in vitro* transcription. These fragments, Cla and *lac* 205, contain, respectively, the su^+_{III} tRNA and *lac* UV5 promoters (10, 14). Their transcription by polymerase holoenzyme yields in each case a single major RNA product ≈150 and ≈65 nucleotides, respectively, long (21, 22). With enzyme alone the salt optimum for the transcription of the 150-nucleotide RNA from Cla DNA was lower than that of the 65-nucleotide *lac* RNA (Fig. 1). Addition of fMet-tRNA$_f^{Met}$ inhibited Cla transcription by 50–70% at KCl concentrations <100 mM. By contrast, the charged tRNA *increased lac* transcription by 40–50% except at the lowest salt concentration tested. Thus, at 25–75 mM KCl, fMet-tRNA$_f^{Met}$ increased the extent of *lac* relative to su^+_{III} transcription by 3- to 3.5-fold. We conclude that the charged tRNA alters transcriptional selectivity *in vitro*.

This change in the pattern of *in vitro* transcription is observed in a situation of template competition. To determine whether fMet-tRNA$_f^{Met}$ would effect similar changes with only one species of active promoter present, each restriction fragment was tested alone. In these experiments total transcription was determined, this being an accurate reflection of the extent of synthesis of the individual major RNA species from each fragment (unpublished results). With Cla DNA as template, fMet-tRNA$_f^{Met}$ again inhibited transcription at low, but not at high, salt concentrations (Fig. 2*a*). However, in this case the inhibition was substantially less, being at most 20–30%. Under similar conditions, uncharged tRNA$_f^{Met}$ did not significantly influence Cla transcription (Fig. 2*b*). With *lac* 205 DNA alone as template, the response to fMet-tRNA$_f^{Met}$ was qualitatively different from that observed with both restriction fragments present. Instead of stimulating *lac* RNA synthesis, the charged tRNA inhibited transcription over the entire range of KCl concentrations tested (Fig. 2*c*). Therefore, in the absence of template competition, the effect of fMet-tRNA$_f^{Met}$ on transcription is without significant selectivity in this system.

One possible reason for the loss of selectivity might be the increase in absolute DNA concentration on mixing the two templates. To test this possibility, the effect of fMet-tRNA$_f^{Met}$

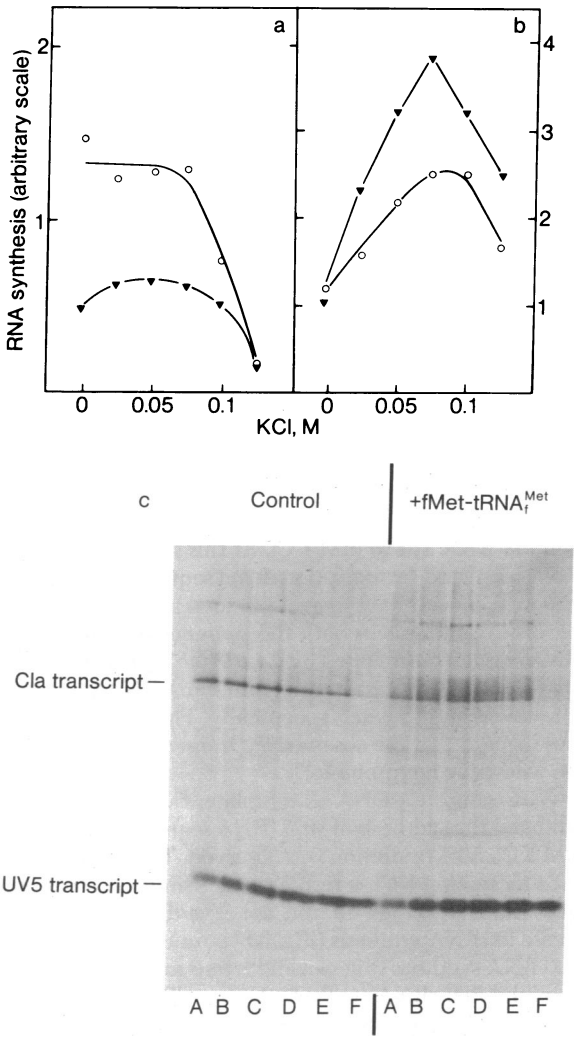

FIG. 1. Effect of fMet-tRNA$_f^{Met}$ on *in vitro* transcription from a mixture of Cla and *lac* 205 DNA restriction fragments. The reaction mixture contained 32 nM RNA polymerase, 0.67 nM Cla DNA, and 1.3 nM *lac* DNA. (*a*) Synthesis of 150-nucleotide RNA species from Cla fragment. (*b*) Synthesis of 65-nucleotide RNA species from *lac* 205 fragment. ○, Polymerase alone; ▼, polymerase with fMet-tRNA$_f^{Met}$. The arbitrary scale for the ordinate is derived from densitometry tracings of the autoradiography of the gel shown in *c*. The band at the top of the gel corresponds to a full-length transcript of *lac* 205 DNA. The densitometry measurement for Cla transcription also includes the region immediately above the 150-nucleotide RNA species. Lanes A–F indicate increasing KCl concentration from 0 to 150 mM in 25 mM steps.

on *lac* 205 DNA transcription was determined as a function of increasing DNA concentration. At a low DNA concentration (<1 nM) the charged tRNA was without effect, but at high concentrations RNA synthesis was substantially inhibited (Fig. 2*d*). Because an increase in the concentration of *lac* 205 DNA alone results in the increased *inhibition* of *lac* RNA synthesis, the observed *stimulation* in the presence of fMet-tRNA$_f^{Met}$ cannot be attributed to a simple increase in DNA concentration. Rather it appears that the presence of Cla DNA modifies control of *lac* RNA synthesis.

To investigate further the effect of fMet-tRNA$_f^{Met}$ on transcriptional selectivity, transducing phage DNA species were used as templates. With φ80*plac* DNA containing a wild-type *lac* promoter, cAMP-independent *lac* transcription by polymerase alone had a KCl optimum of 50 mM (Fig. 3*b*). By contrast, in the presence of fMet-tRNA$_f^{Met}$, the enzyme synthesized

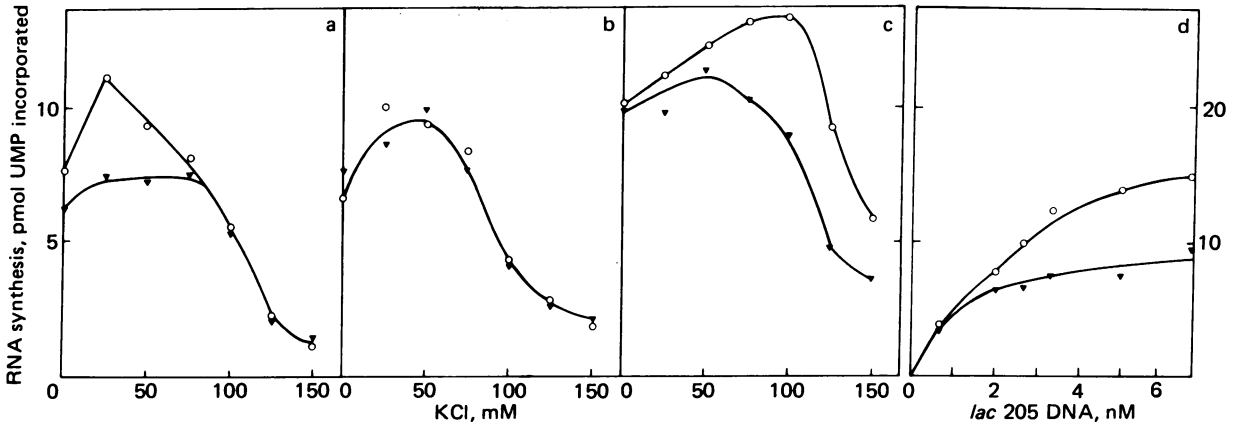

FIG. 2. Effect of fMet-tRNA$_f^{Met}$ on *in vitro* transcription of DNA restriction fragments. (*a*) Cla DNA and fMet-tRNA$_f^{Met}$. (*b*) Cla and tRNA$_f^{Met}$. (*c* and *d*) *lac* 205 DNA and tRNA$_f^{Met}$. For all panels polymerase concentration was 32 nM and template concentrations were 0.70, 0.70, and 1.3 nM for *a*, *b*, and *c*, respectively. For *d* KCl concentration was 75 mM. O, Polymerase alone; ▼, polymerase with fMet-tRNA$_f^{Met}$.

most *lac* RNA at 150 mM KCl. At this salt concentration, the tRNA increased transcription of *lac* sequences by up to 5-fold without a concomitant increase in total transcription (Fig. 3*a*). Lac RNA synthesis in both the presence and absence of the tRNA was ≈90% inhibited by 0.1 μM *lac* repressor. Under these conditions, the repressor fails to inhibit λ RNA synthesis (data not shown) and thus acts specifically. The extent of repression thus suggests that the observed *lac* transcription originates from the wild-type *lac* promoter.

With $\phi 80 psu^+_{III}$ DNA as template, fMet-tRNA$_f^{Met}$ strongly inhibited the synthesis of su^+_{III} tRNA sequences (Fig. 3*d*). At 50 mM KCl, 50% inhibition was observed. This value diminished with increasing KCl, a response very similar to that observed with Cla DNA (Figs. 1*a* and 2*a*). The data show that the decrease in tRNA synthesis (Fig. 3*d*) is much greater than that of $\phi 80$ RNA synthesis (Fig. 3*c*) and is thus selective. fMet-tRNA$_f^{Met}$ also inhibited rRNA synthesis. With pER24 DNA as template, rRNA synthesis was preferentially inhibited over the entire salt range tested (Fig. 3 *e* and *f*), the inhibition in this case increasing with increasing KCl concentration. Again we conclude that for each template tested the charged tRNA alters transcriptional selectivity and fMet-tRNA$_f^{Met}$ acts prior to initiation.

The differential response of *lac* and su^+_{III} tRNA transcription of fMet-tRNA$_f^{Met}$ suggests that the tRNA might act by altering the capacity of RNA polymerase to form preinitiation complexes at different promoters. To test this hypothesis RNA polymerase was first preincubated with form II pER24 DNA in the absence of nucleoside triphosphates to allow the formation of such complexes. These complexes were then challenged with fMet-tRNA$_f^{Met}$ and after various times the extent of complex formation was assayed by the simultaneous addition of nucleoside triphosphates and heparin, a polyanion that sequesters polymerase molecules that are free or weakly bound to DNA (23, 24). When fMet-tRNA$_f^{Met}$ was added together with heparin, it had no significant effect on the extent of subsequent rRNA production (Fig. 4). However, on incubation with the charged tRNA in the absence of heparin, the number of polymerase–promoter complexes competent to initiate rRNA synthesis declined to ≈20% of the control within 5 min and then remained at that value. This decrease in rRNA synthesis is greater than that observed under similar conditions with continuous initiation (Fig. 3*f*). This presumably reflects the difference between equilibrium and nonequilibrium conditions of complex formation. We conclude that fMet-tRNA$_f^{Met}$ inhibits rRNA production by altering the position of the equilibrium for the formation of rRNA promoter–polymerase complexes and that therefore the charged tRNA acts prior to the initiation of transcription.

ppGpp Competes with fMet-tRNA$_f^{Met}$ Binding to RNA Polymerase. fMet-tRNA$_f^{Met}$ binds to RNA polymerase and alters the pattern of promoter selection by the enzyme. In these respects the tRNA is functionally equivalent to the RNA polymerase effector ppGpp. Does this effector influence the interaction between fMet-tRNA$_f^{Met}$ and the enzyme? To test this possibility the binding of the charged tRNA to RNA polymerase was determined over a range of KCl concentrations. In the absence of ppGpp this binding was strongly salt dependent, significant binding being detected only below 100 mM KCl at 10 mM Mg^{2+} (Fig. 5*a*). At higher KCl concentrations, the amount of fMet-tRNA$_f^{Met}$ retained by the enzyme was decreased to levels similar to those observed (4) for nonspecific binding. ppGpp at 200 μM, a concentration required for maximal inhibition of *in vitro* rRNA synthesis (15, 20), decreased the extent of fMet-tRNA$_f^{Met}$ binding at low salt concentrations to the level characteristic of high salt concentrations. With 0.2 μM fMet-tRNA$_f^{Met}$ present, 10 μM ppGpp was sufficient to decrease the level of tRNA binding to half the maximum level (Fig. 5*b*). This competition was dependent on the presence of the 3′-pyrophosphate group, guanosine 5′-diphosphate being without effect on fMet-tRNA$_f^{Met}$ binding at concentrations up to 100 μM.

DISCUSSION

What is the functional significance of the specific binding of fMet-tRNA$_f^{Met}$ to RNA polymerase holoenzyme? In this paper we have shown that *in vitro* this polynucleotide inhibits the transcription of su^+_{III} tRNA from both intact $\phi 80 psu^+_{III}$ DNA and from a DNA restriction fragment containing the su^+_{III} tRNA promoter. Similarly, rRNA transcription from a ColE1–rDNA chimera is also strongly inhibited. By contrast, at high ionic strength fMet-tRNA$_f^{Met}$ stimulates *lac* mRNA transcription from both intact $\phi 80$ *plac* DNA and from a DNA restriction fragment containing the mutant *lac* UV5 promoter. The different salt profiles of RNA synthesis from the wild-type and mutant *lac* promoters (of Figs. 1*b* and 3*b*) may be explained in part by the tighter binding of RNA polymerase to the mutant promoter (25). The quantitative and specific alterations in the pattern of transcription elicited by fMet-tRNA$_f^{Met}$ require that this effector be present prior to the initiation of transcription, a result that is consistent with the observed interaction of fMet-tRNA$_f^{Met}$ with free polymerase and with the effect of the charged tRNA polymerase–RNA promoter complexes.

The alterations in transcriptional selectivity induced by

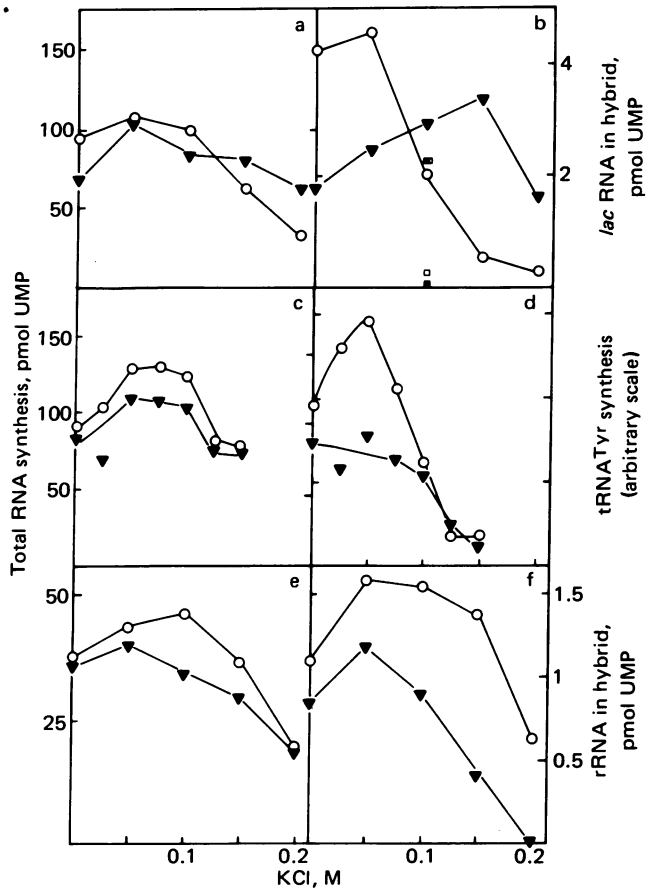

FIG. 3. Effect of fMet-tRNA$_f^{Met}$ on *in vitro* transcription. (*a* and *b*) Total and *lac* RNA synthesis, respectively, from ϕ80 *plac* DNA. (*c* and *d*) Total and su_{III}^+ tRNA synthesis, respectively, from ϕ80 psu_{III}^+ DNA. (*e* and *f*) Total and rRNA synthesis from pER24 DNA. Polymerase and template concentrations were, respectively, for *a* and *b*, 40 nM and 0.7 nM; *c* and *d*, 46 nM and 2 nM; *e* and *f*, 64 nM and 1.5 nM. All data points relate to 25-μl aliquots of the reaction mixtures. ○, Polymerase alone; ▼, polymerase with fMet-tRNA$_f^{Met}$ (or tRNA$_f^{Met}$). In *b*: *lac* RNA synthesis in the presence of 0.1 μM *lac* repressor without (□) and with (■) fMet-tRNA$_f^{Met}$; □, *lac* RNA synthesis in the presence of 0.1 μM *lac* repressor and 0.5 mM isopropylthiogalactoside for reaction mixtures both with and without fMet-tRNA$_f^{Met}$.

fMet-tRNA$_f^{Met}$ are paralleled by a heterogeneity in RNA polymerase with respect to its capacity to bind the initiator tRNA (26). When sedimented on a glycerol gradient, RNA polymerase is separated into populations of enzyme molecules that differ in this property. Those polymerase molecules that bind the effector to the greatest extent do not transcribe efficiently either rRNA or su_{III}^+ tRNA genes but do transcribe the *lac* 205 fragment. Conversely, those polymerase molecules with the lowest capacity for fMet-tRNA$_f^{Met}$ transcribe efficiently both the rRNA and su_{III}^+ tRNA genes but have a low activity on *lac* 205 DNA. Of particular interest is the further requirement for the presence of an alternative DNA species for the fMet-tRNA$_f^{Met}$-mediated stimulation of *lac* RNA synthesis from the isolated restriction fragment. Because the inhibition of Cla DNA transcription by the charged tRNA is concomitantly increased, this stimulation is unlikely to be a trivial consequence of the removal of a specific inhibitor. Rather the results suggest that the characteristics of *in vitro* transcription from a particular promoter depend not only on the intrinsic properties of that promoter but also on the nature of other DNA sequences present. We suggest elsewhere (17, 27) that this phenomenon

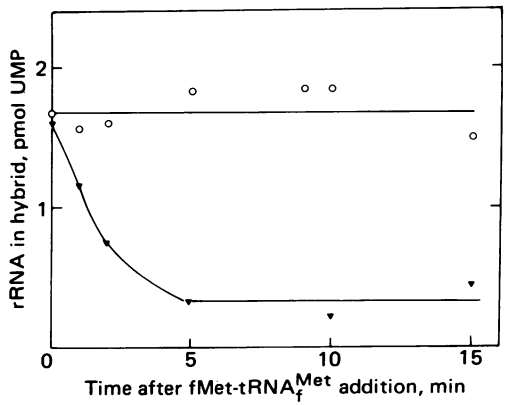

FIG. 4. Effect of fMet-tRNA$_f^{Met}$ on polymerase–promoter preinitiation complexes on pER24 DNA. KCl, 75 mM. ○, Polymerase alone; ▼, polymerase with fMet-tRNA$_f^{Met}$. Data points are for 90-μl samples of reaction mixtures.

is a consequence of different forms of RNA polymerase possessing unequal affinities for a given promoter. By this model with only one restriction fragment present, all forms of the enzyme will bind to the promoter but different forms will initiate at different rates. The mixing of two dissimilar promoters that are preferentially utilized by different structural forms of the enzyme would result in one promoter sequestering some or all of the forms that bind weakly to the other promoter and *vice versa*. Thus, mixing of restriction fragments changes the subset of polymerase molecules that initiate at a particular promoter. Because different forms of the enzyme differ in their ability to bind fMet-tRNA$_f^{Met}$, such a change would also alter the promoter-specific response of transcription to fMet-tRNA$_f^{Met}$.

Whereas tight binding of fMet-tRNA$_f^{Met}$ to RNA polymerase is only detectable at low salt (KCl < 0.1 M), the specific effects of the tRNA on transcription of *lac* RNA and rRNA are apparent over the entire salt range tested (0–0.2 M KCl). A possible explanation for this apparent paradox is that, whereas in the binding experiments the concentrations of fMet-tRNA$_f^{Met}$ and RNA polymerase are $\approx$0.1 μM, in the preincubation prior to addition to a transcription reaction mixture the concentrations are $\approx$1 μM. Because the calculated maximum association constant of the charged tRNA and enzyme is 9.0 μM^{-1} (4) the amount of fMet-tRNA$_f^{Met}$ bound to polymerase under binding assay conditions will be more sensitive to any salt-dependent

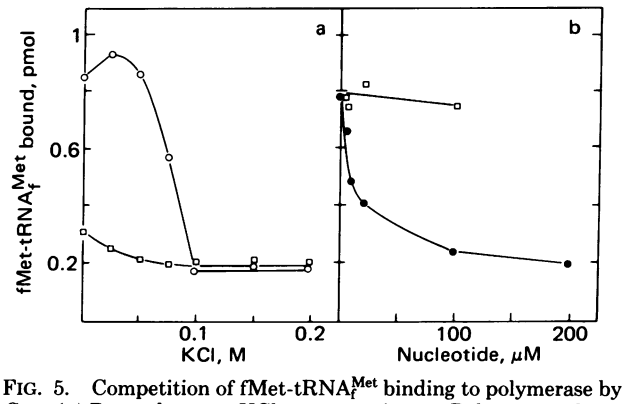

FIG. 5. Competition of fMet-tRNA$_f^{Met}$ binding to polymerase by ppGpp. (*a*) Dependence on KCl concentration. ○, Polymerase alone; □, polymerase plus 200 μM ppGpp. (*b*) Dependence on guanine nucleotide concentration. ●, Polymerase plus ppGpp; □, polymerase plus ppG. Reaction mixture (100 μl) contained 0.02 nmol of [*methyl*-^{3}H]methionine-labeled fMet-tRNA$_f^{Met}$ (5000 cpm/pmol) and 8 pmol of RNA polymerase.

variation of this constant than that bound under preincubation conditions.

The alteration of the pattern of *in vitro* transcription observed in the presence of fMet-tRNA$_f^{Met}$ is very similar to that effected at high concentrations—i.e., ≈100–200 μM ppGpp, a regulatory nucleotide that binds to RNA polymerase and directly modulates its initiation specificity (15, 20, 28). Both effectors strongly inhibit the synthesis of stable RNA species while substantially increasing that of *lac* mRNA (26). Furthermore, both effectors destabilize the polymerase–rRNA promoter complex. These functional parallels suggest that fMet-tRNA$_f^{Met}$ and ppGpp may act by a similar mechanism.

What might be the *in vivo* relevance of the existence of two effectors, ppGpp and fMet-tRNA$_f^{Met}$, selectively inhibiting stable RNA synthesis *in vitro*? In general there is a good correlation between a rapid increase in intracellular levels of ppGpp and a preferential decrease in the initiation of stable RNA chains. However, a shutoff of stable RNA synthesis can occur without concomitant accumulation of ppGpp (29, 30), suggesting that other mechanisms analogous to that of ppGpp may control RNA synthesis within the cell. However, there is as yet no direct *in vivo* evidence implicating fMet-tRNA$_f^{Met}$ in such control, although a link between one-carbon metabolism and RNA polymerase has been suggested by the selection of mutants in the enzyme apparently able to overcome growth restrictions imposed by a folate antagonist (31). Similarly, inhibition of formylation by trimethoprim *in vivo* can result in the production of substantially more stable RNA than in untreated cells growing at an identical rate (32). Despite the lack of *in vivo* evidence, the effect of fMet-tRNA$_f^{Met}$ on transcription *in vitro* could be fully compatible with a role in which an accumulation of this tRNA would signal a lack of mRNA and a consequent excess of ribosomes. This physiological condition should not trigger ppGpp accumulation, a response elicited by uncharged tRNA (33, 34). Thus, ppGpp and fMet-tRNA$_f^{Met}$ can be envisaged as mediating independent but complementary homeostatic controls.

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