Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3064–3067, September 1976
Biochemistry

Specific binding of formylated initiator-tRNA to *Escherichia coli* RNA polymerase*

(transcription/translation/σ-factor/RNA nucleotidyltransferase)

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Communicated by F. H. C. Crick, June 15, 1976

ABSTRACT ***E. coli*** **fMet-tRNA$_f^{Met}$ and *E. coli* RNA polymerase (RNA nucleotidyltransferase; EC 2.7.7.6; nucleoside-triphosphate:RNA nucleotidyltransferase) form a 1:1 complex with an apparent association constant of 9.0×10^6 M^{-1} at 37°. The affinity of polymerase to tRNA depends on the tRNA as well as the formyl methionine moiety. Core polymerase has a greatly reduced affinity for initiator tRNA. Optimal binding conditions are similar to those that are also optimal for binding initiator tRNA to ribosomes. Binding of initiator tRNA to polymerase stimulates the transcription of λ plac DNA, as determined in a crude cell-free system for β-galactosidase (EC 3.2.1.23; β-D-galactoside galactohydrolase) synthesis as well as in a highly purified transcription system.**

It is a common notion that transcription and translation in *Escherichia coli* are coupled. The underlying mechanisms at the molecular level are, however, still obscure. A coupling between transcription of rRNA genes and protein synthesis was suggested by the finding that rRNA synthesis *in vitro* is controlled by EF-T, a protein-synthesis elongation factor, and by ppGpp, a metabolic product of the ribosome (1, 2). Recent genetic studies have shown that the effect of *StrA* mutations on the propagation of phage T7 message is reversed by *rif* mutations (3). The data imply a direct link between the functioning of the ribosome and the RNA polymerase. During our own investigations of the molecular nature of such a link, we observed that ribosomes and RNA polymerase compete with each other for binding initiator tRNA. This observation prompted us to study the interaction of fMet-tRNA$_f^{Met}$ with RNA polymerase and its influence on transcription. The results of these studies are reported in this paper.

MATERIALS AND METHODS

E. coli tRNA$_f^{Met}$ and yeast tRNA^{Phe} were purchased from Boehringer, Mannheim, Germany. Pure initiation factor 2 of *E. coli* and *E. coli* tRNA$_m^{Met}$ were kindly provided to us by Dr. M. Grunberg-Manago, Institut Biochimie, Paris. *Bacillus stearothermophilus* tRNA$_f^{Met}$ and *B. stearothermophilus* tRNA$_m^{Met}$ were given to us by Dr. R. Mulvey, MRC Lab. Mol. Biol., Cambridge, England. Pure *E. coli* RNA polymerase (RNA nucleotidyltransferase; EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase) was generously made available to us by Dr. A. Travers, MRC Lab. Mol. Biol., Cambridge, England. L-[*Methyl*-^{3}H]Methionine (7.4 Ci/mmol), [^{14}C]phenylalanine (0.46 Ci/mmol), and [8-^{14}C]adenosine 5′-triphosphate (58 Ci/mol) were purchased from Amersham Buchler, Braunschweig, Germany.

tRNA$_f^{Met}$ and tRNA$_m^{Met}$ were charged with a crude synthetase preparation of *E. coli* as described (4). tRNA^{Phe} was charged with phenylalanine by a crude yeast synthetase preparation (5). *E. coli* tRNA$_f^{Met}$ was subsequently formylated using a transformylase/formyldonor system (6). *B. stearothermophilus* tRNA$_f^{Met}$ and tRNA$_m^{Met}$ were formylated chemically by a method similar to that of ref. 7. tRNA (0.5 mM) and 50 mM *N*-hydroxysuccinimide formyl ester were incubated in 20 mM $MgCl_2$, 0.2 M triethanolamine, pH 4.5 buffer, for 1 hr at room temperature. Thereafter, ethanol was added in the cold, and the precipitated tRNA was then redissolved in 1 mM $MgCl_2$, 2 mM potassium acetate, pH 6.0 buffer. *E. coli* RNA polymerase was isolated and purified (8). σ-Free *E. coli* RNA polymerase was prepared by passing polymerase through a DNA-agarose column (9). λ plac DNA was obtained by following the procedures described in ref. 10.

Binding of tRNA to polymerase was assayed by passing corresponding incubation mixtures through Millipore filters. tRNA and polymerase were incubated for 10 min at 37° under conditions specified in the figures and tables. Samples were then put on ice, diluted 20-fold with cold 10 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris·HCl (pH 7.8) buffer, and filtered. Filters were washed three times with 1.5 ml of dilution buffer and dried; radioactivity was measured in toluene-based scintillation fluid. RNA synthesis was measured by incubating 5 μg of λ plac DNA and 10 pmol of RNA polymerase in 10 mM $MgCl_2$, 0.1 mM EDTA, 150 mM KCl, 40 mM Tris·HCl (pH 7.8) buffer together with 0.15 mM CTP, 0.15 mM GTP, 0.15 mM UTP, 0.10 mM ATP, and 0.02 mM [^{14}C]ATP (58 Ci/mol) for 30 min at 37°. Cold 5% trichloroacetic acid (1.5 ml), containing 10 mM sodium pyrophosphate, was added. Samples were put on ice for 15 min, after which they were filtered through Millipore filters. The filters were washed three times with 10 mM sodium pyrophosphate/5% trichloroacetic acid and once with 3 ml of ethanol. After the filters were dried, their radioactivity was determined in toluene-based scintillation fluid. β-Galactosidase (EC 3.2.1.23; β-D-galactoside galactohydrolase) was synthesized *in vitro* essentially according to ref. 11, with 5 μg of λ plac DNA, 10 pmol of *E. coli* RNA polymerase, and 1 mM isopropylthiogalactopyranoside in the incubation mixtures. After 30 min at 37°, incubation mixtures were made 0.1 M in sodium phosphate (pH 7.3) and 0.14 M in 2-mercaptoethanol. Orthonitrophenylgalactoside (0.35 mg/ml) was added and incubations were continued for an additional 2–3 hr, in order to determine the amount of β-galactosidase that had been synthesized. After the addition of 1% acetic acid and subsequent removal of the resulting precipitate by centrifugation, samples were mixed with 1 M Na_2CO_3. Hydrolysis of orthonitrophenylgalactoside was then measured by reading the absorbance at 420 nm.

RESULTS

Under conditions that are optimal for the binding of initiator-tRNA to ribosomes, i.e., in 3–5 mM $MgCl_2$, 100 mM KCl, or 100 mM NH_4Cl and 50 mM Tris·HCl (pH 7.4–7.8) (12), initiator-

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Table 1. Binding of various tRNAs to *E. coli* RNA polymerase

tRNA	pmol bound
E. coli fMet-tRNA$_f^{Met}$	5.0
E. coli Met-tRNA$_f^{Met}$	1.0
E. coli Met-tRNA$_m^{Met}$	1.0
B. stearothermophilus fMet-tRNA$_f^{Met}$	2.2
B. stearothermophilus Met-tRNA$_f^{Met}$	0.9
B. stearothermophilus fMet-tRNA$_m^{Met}$	0.6
B. stearothermophilus Met-tRNA$_m^{Met}$	0.3
(Yeast) Phe-tRNA^{Phe}	0.8

Incubation mixtures contained in 0.08 ml: 4.5 mM $MgCl_2$, 125 mM KCl, 50 mM Tris·HCl (pH 7.8), 1 mM dithiothreitol, 6% (vol/vol) glycerol, 1.2 mM GTP, 0.2 μM *E. coli* RNA polymerase, and 0.3 μM tRNA as indicated. Incubation mixtures were kept for 10 min at 37°, then chilled on ice and diluted with 1.5 ml of cold 10 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris·HCl (pH 7.8) buffer. Binding was assayed by Millipore filtration.

tRNA also binds tightly to *E. coli* RNA polymerase (Table 1). Its association constant for binding to polymerase at 37° was in the order of 10^7 M^{-1}. After having obtained this unexpected result, we wondered if the binding of fMet-tRNA$_f^{Met}$ to polymerase was specific. The following experiments were accordingly chosen in order to characterize the specificity of this interaction.

If formylated initiator-tRNA is replaced by unformylated initiator-tRNA, the affinity to polymerase is considerably

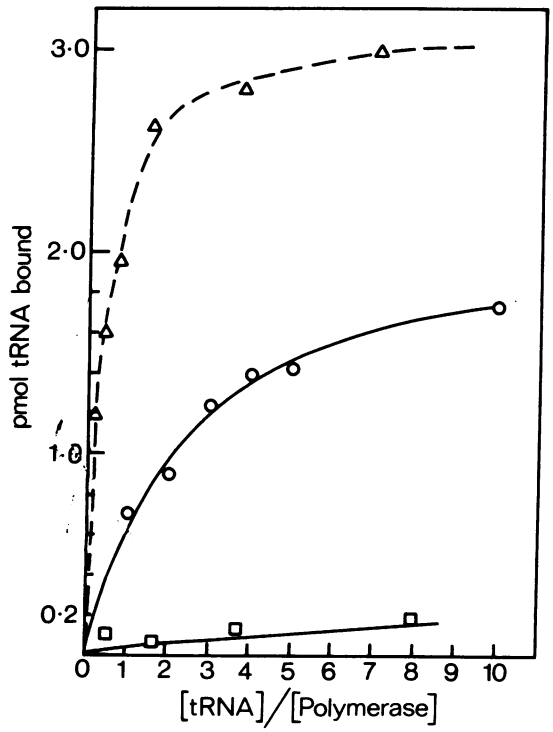

FIG. 1. Saturation curves of binding tRNA to *E. coli* RNA polymerase. Incubation mixtures contained in 0.08 ml: 4.5 mM $MgCl_2$, 125 mM KCl, 50 mM Tris·HCl (pH 7.8) buffer, 1 mM dithiothreitol, 6% (vol/vol) glycerol, 1.2 mM GTP, 16 pmol of RNA polymerase, and varying amounts of *B. stearothermophilus* fMet-tRNA$_f^{Met}$ (○) and *B. stearothermophilus* fMet-tRNA$_m^{Met}$ (□) (8–150 pmol), as indicated in the figure, or 8 pmol of RNA polymerase and varying amounts of *E. coli* fMet-tRNA$_f^{Met}$ (△). Incubations were for 10 min at 37°. Thereafter, binding was assayed as described in *Materials and Methods*.

Table 2. Apparent association constants of binding tRNA to *E. coli* RNA polymerase

tRNA	$K_{app.}$ (μM^{-1})
E. coli fMet-tRNA$_f^{Met}$	9.0
B. stearothermophilus fMet-tRNA$_f^{Met}$	0.5
B. stearothermophilus fMet-tRNA$_m^{Met}$	0.04

Association constants were calculated from the saturation curves (Fig. 1) with the assumption that all RNA polymerase molecules were equally active for binding tRNA and that they contained only one binding site.

lowered (Table 1). A comparable drop in affinity was observed if *E. coli* fMet-tRNA$_f^{Met}$ was replaced by *E. coli* Met-tRNA$_m^{Met}$ or by (yeast) Phe-tRNA^{Phe}. A comparison of the affinities of *B. stearothermophilus* fMet-tRNA$_f^{Met}$ and of *B. stearothermophilus* Met-tRNA$_m^{Met}$ revealed a similar pattern, i.e., *E. coli* RNA polymerase apparently preferentially binds initiator-tRNA. However, the data of Table 1 do not necessarily show that polymerase specifically recognizes the tRNA moiety that carries the formylated methionine. To distinguish whether RNA polymerase requires simply formylmethionine for recognition or whether the tRNA moiety is also necessary, we compared the saturation curves of binding of *E. coli* fMet-tRNA$_f^{Met}$, *B. stearothermophilus* fMet-tRNA$_f^{Met}$, and *B. stearothermophilus* fMet-tRNA$_m^{Met}$ to RNA polymerase (Fig. 1).

E. coli fMet-tRNA$_f^{Met}$ binding to RNA polymerase levels off at molar ratios of tRNA/polymerase above 1. The saturation curve, therefore, indicates that *E. coli* fMet-tRNA$_f^{Met}$ and *E. coli* RNA polymerase form a 1:1 complex. *B. stearothermophilus* fMet-tRNA$_f^{Met}$ binding to polymerase approaches a plateau at molar ratios of tRNA/polymerase above 5, whereas *B. stearothermophilus* fMet-tRNA$_m^{Met}$ binding is very low and does not reach a plateau under similar conditions.

These data suggest that the binding of *E. coli* fMet-tRNA$_f^{Met}$ to *E. coli* RNA polymerase is specific. Apparent binding constants were calculated from the saturation curves shown in Fig. 1. A comparison of the binding constants (Table 2) shows that RNA polymerase indeed recognizes initiator-tRNA as such. The affinity of *E. coli* fMet-tRNA$_f^{Met}$ for RNA polymerase is one order of magnitude higher than that of *B. stearothermophilus* fMet-tRNA$_f^{Met}$ and more than two orders of magnitude higher than that of *B. stearothermophilus* fMet-tRNA$_m^{Met}$. The data of Tables 1 and 2, therefore, indicate that the specificity of tRNA binding to RNA polymerase is determined by the tRNA and by the formylmethionine moiety.

At saturation, approximately 40% of all RNA polymerase molecules are complexed with *E. coli* initiator-tRNA (Fig. 1). It is not clear whether this low saturation level is due to experimental conditions of the tRNA binding measurements or whether it indicates that not all RNA polymerase molecules are active in binding tRNA. Removal of sigma factor from RNA polymerase reduces the affinity of initiator-tRNA to the enzyme (Table 3). The amount of initiator-tRNA bound to the core enzyme was 40% that bound to the holoenzyme. The data of Table 2 also show that a random polynucleotide like poly(A,U,G) cannot compete with initiator-tRNA binding to polymerase, even if the molar concentration of the polynucleotide is more than a hundred times higher than that of tRNA in the incubation mixtures. It was of interest to compare the formation of initiator tRNA–polymerase complex with that of initiation factor 2–initiator tRNA complex. Under identical incubation conditions, initiation factor 2 bound one-tenth the tRNA of RNA polymerase. This, somewhat surprisingly, indicated that RNA polymerase possesses a much higher affinity

Table 3. Influence of poly(A,U,G) and of σ-factor on the binding of fMet-tRNA$_f^{Met}$ to *E. coli* RNA-polymerase

RNA polymerase	Poly(A,U,G)	IF2	fMet-tRNA$_f^{Met}$ bound (pmol)
Core enzyme	–	–	5.0
Holoenzyme	–	–	12.5
Holoenzyme	+	–	12.2
—	+	+	1.5

Incubation mixtures contained in 0.08 ml: 6 mM $MgCl_2$, 100 mM NH_4Cl, 25 mM KCl, 50 mM Tris·HCl (pH 7.8), 1 mM dithiothreitol, 6% (vol/vol) glycerol, 1.2 mM GTP, 0.25 μM *E. coli* RNA polymerase or *E. coli* initiation factor 2 (IF2) as indicated, 0.6 μM *E. coli* fMet-tRNA$_f^{Met}$, and 30 mM poly(A,U,G), where indicated. Incubations were carried out for 10 min at 37°. Thereafter, 1.5 ml of cold 10 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris·HCl (pH 7.8) buffer was added and tRNA binding was assayed by Millipore filtration.

to initiator-tRNA than initiation factor 2, which mediates the binding of initiator-tRNA to ribosomes for initiating mRNA translation. Incubation of *E. coli* fMet-tRNA$_f^{Met}$ and RNA polymerase in the presence of initiation factor 2 did not significantly influence the formation of tRNA-polymerase complex (data not shown). It should also be noted that the presence or absence of GTP had no detectable effect on complex formation.

Optimum conditions for mRNA translation in the ribosomal system are achieved at low magnesium concentrations, i.e., at about 3–4 mM Mg^{2+} (12). On the other hand, RNA is generally synthesized *in vitro* at higher magnesium concentrations, i.e., at about 10 mM Mg^{2+} (11). Since all the binding assays so far were carried out at low magnesium concentrations, the dependence of tRNA binding to polymerase on the concentration of magnesium ions was investigated. The results of these studies are illustrated in Fig. 2. tRNA–polymerase complex formation is strongly dependent on magnesium concentration. Complex formation is best around 3–4 mM Mg^{2+}. At 10 mM Mg^{2+} it is about one-third that at 4 mM Mg^{2+}. Thus, the complex formation between initiator-tRNA and polymerase exhibits a dependence on magnesium concentration that is similar to that reported for the complex formation between mRNA, initiation factors, ribosomes, and initiator-tRNA (12). So far, the results show that the binding of initiator-tRNA to polymerase is specific and well defined. The question now was: does this complex formation have any influence on the transcriptional activities of RNA polymerase? Therefore, the influence of initiator-tRNA on mRNA synthesis was studied. In a crude cell-free system, λ plac DNA was used as a template for β-galactosidase synthesis *in vitro*. The results of these experiments, which are summarized in Table 4, show that the addition of *E. coli* fMet-tRNA$_f^{Met}$ to such an *in vitro* protein-synthesizing system stimulated β-galactosidase synthesis about 7-fold, whereas Met-tRNA$_m^{Met}$ and Phe-tRNA^{Phe} did not give any appreciable stimulation of β-galactosidase synthesis. The observed stimulatory effect of initiator-tRNA on the rate of β-galactosidase synthesis might arise from a variety of causes, perhaps even unrelated to the process of transcription. The results of Table 3, however, demonstrate that in a coupled transcription-translation system the concentration of initiator-tRNA in the incubation mixtures is of considerable importance.

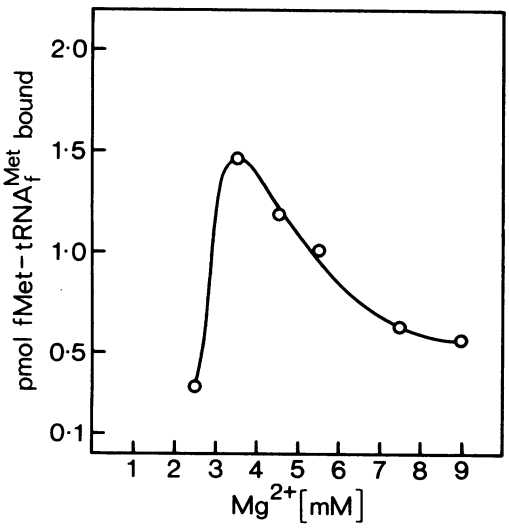

FIG. 2. Dependence of binding of fMet-tRNA$_f^{Met}$ to *E. coli* RNA polymerase on Mg^{2+} concentration. Incubation mixtures contained in 0.08 ml, 16 pmol of *E. coli* RNA polymerase and 24 pmol of *B. stearothermophilus* fMet-tRNA$_f^{Met}$. Buffer and incubation conditions were as described in the legend to Fig. 1, except that the $MgCl_2$ concentration was varied as indicated.

Table 4. Influence of fMet-tRNA$_f^{Met}$ on β-galactosidase synthesis

tRNA	β-Galactosidase synthesized (%)
—	100
E. coli fMet-tRNA$_f^{Met}$	690
E. coli Met-tRNA$_m^{Met}$	110
(Yeast) Phe-tRNA^{Phe}	150

β-Galactosidase was synthesized *in vitro* with 5 μg of λ plac DNA, 10 pmol of *E. coli* RNA polymerase, and 25 pmol of tRNA as indicated. For further details see *Materials and Methods*. β-Galactosidase activity (100%) corresponds to 4×10^{-3} units of enzyme.

To show that the observed enhancement of β-galactosidase synthesis could be due to a stimulatory effect of fMet-tRNA$_f^{Met}$ on transcription of λ plac DNA, we measured RNA synthesis in a highly purified transcription system. Fig. 3 shows that the addition of fMet-tRNA$_f^{Met}$ to RNA polymerase indeed stimulates RNA synthesis and that, as expected, the addition of Met-tRNA$_m^{Met}$ does not. The amount of stimulation is comparable to that which was observed in the complete *in vitro* λ plac DNA-dependent β-galactosidase-synthesizing system. The stability of the fMet-tRNA$_f^{Met}$ binding to RNA polymerase was investigated during the course of RNA synthesis. The results of these experiments, which are included in Fig. 3, show that fMet-tRNA$_f^{Met}$ strongly binds to RNA polymerase only during the onset of RNA synthesis. The binding to RNA polymerase is drastically reduced once mRNA synthesis has started, i.e., when chain propagation prevails over chain initiation. These data show that fMet-tRNA$_f^{Met}$ affects the activity of RNA polymerase, i.e., initiator-tRNA influences DNA transcription by binding to RNA polymerase.

DISCUSSION

Previously, a specific complex had been demonstrated between tRNA^{Trp} and avian myeloblastosis virus RNA-dependent DNA polymerase. It has been shown that the ability of reverse transcriptase to utilize tRNA^{Trp} as a primer for DNA synthesis involves a highly specific site on the enzyme (13). The data of this paper show that fMet-tRNA$_f^{Met}$ binds highly specifically to *E. coli* RNA polymerase and thereby stimulates the initiation of

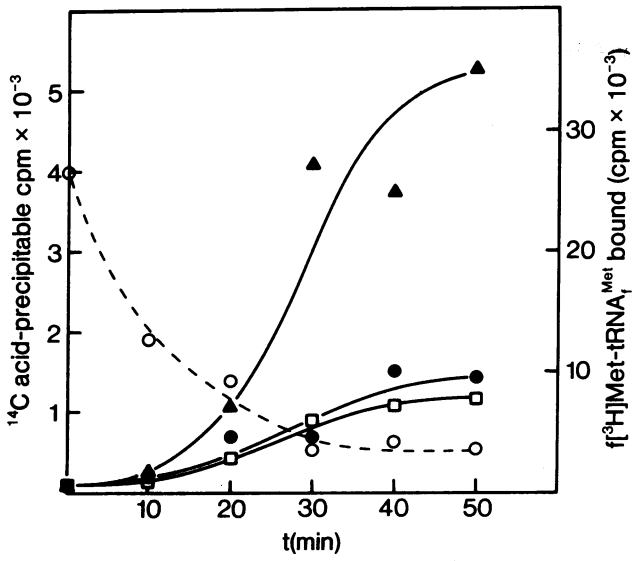

FIG. 3. λ plac DNA-dependent RNA synthesis by *E. coli* RNA polymerase. Time dependence of RNA synthesis was determined by measuring [^{14}C]ATP (58 Ci/mol) incorporation into trichloroacetic acid-precipitable material. Incubation mixtures contained in 0.08 ml, 5 μg of λ plac DNA and 10 pmol of *E. coli* RNA polymerase (□). Experimental conditions are described in *Materials and Methods*. (●) 25 pmol of Met-tRNA$_m^{Met}$ or (▲) 25 pmol of fMet-tRNA$_f^{Met}$ were added to the incubation mixtures. fMet-tRNA$_f^{Met}$ binding (○) was assayed at the times indicated by taking aliquots of the reaction mixtures, which then were diluted with 1.5 ml of cold 10 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris·HCl (pH 7.8) buffer and filtered over Millipore filters as described in the legend of Fig. 1.

RNA synthesis as measured with λ plac DNA as template. The biological significance of this finding, however, is far from understood. Transcription of the *lac* gene is regulated in a highly specific manner in the absence of fMet-tRNA$_f^{Met}$ (14). The effect of fMet-tRNA$_f^{Met}$ on the transcription of the *lac* operon may be an example of a coarse control arising from the modulation of RNA polymerase activity by the tRNA. As yet it is unclear whether the charged tRNA alters the quality of the transcript or merely changes the specific activity of the enzyme. Nevertheless, the effect of fMet-tRNA$_f^{Met}$ on the activity of RNA polymerase could well represent a cellular tool to couple the rate of mRNA production and mRNA translation. The formylation of Met-tRNA$_f^{Met}$ could act as a regulatory signal in *E. coli* and link the activity of RNA polymerase to the tetrahydrofolate cycle (15). Such a mechanism would ensure that many precursors required for the formation of DNA, RNA, and protein are in sufficient supply. Thus, the cell could exert a general positive metabolite control on transcription and translation in a concerted fashion, as has been actually observed. This control mechanism could complement the negative control that is imposed on rRNA synthesis in stringent *E. coli* cells by ppGpp, which is produced during an idling step of protein synthesis on the ribosome as a result of amino acid starvation (2).

In contrast to avian myeloblastosis virus RNA-dependent DNA polymerase, *E. coli* RNA polymerase apparently uses tRNA as an effector of RNA synthesis rather than as a primer. However, both polymerases possess the common property of a highly specific tRNA binding site. This suggests that interactions between tRNA and polymerase are not specific to reverse transcriptases of RNA tumor viruses, but appear to be a rather common feature of polymerases. It might be expected that future experiments will reveal many more such interactions in comparable systems.

We thank F. H. C. Crick and A. Travers for critical and stimulating discussions. This work was supported by a fellowship of Deutsche Forschungsgemeinschaft to O. P.

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