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In vitro transcription of E. coli tRNA genes

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ABSTRACT

Transcription of tRNA genes carried by transducing bacteriophages Ø80psu$_3^+$ (tRNA$_1^{Tyr}$) and λh80T (tRNA$_2^{Tyr}$, tRNA$_2^{Gly}$su$_{36}^+$, tRNA$_3^{Thr}$) was studied in vitro in a system consisting of whole bacteriophage DNA and purified RNA polymerase. In contrast to unusual requirements for tRNA$_1^{Tyr}$ gene transcription from DNA fragments, the transcription on whole bacteriophage DNA was found to be relatively not salt sensitive, did not require glycerol and rifampicin-resistant complexes with RNA polymerase were formed in the absence of nucleoside triphosphates. Termination factor ρ stimulated the transcription of the tRNA genes as well as that of a 4S RNA on λh80T DNA template. The stimulatory effect of ρ was abolished by rifampicin and seems to be due to the release of RNA polymerase and reinitiation of transcription.

INTRODUCTION

The tRNA genes carried by the DNA of bacteriophages Ø80psu$_3^+$ (tRNA$_1^{Tyr}$) and λh80T (tRNA$_2^{Tyr}$, tRNA$_2^{Gly}$su$_{36}^+$, tRNA$_3^{Thr}$) have been efficiently transcribed in vitro by purified E. coli RNA polymerase to produce high molecular weight tRNA precursors (1, 2). The tRNA precursors were processed by S-100 E. coli extracts to mature size tRNA molecules (3) and the ability of the synthesized tRNA to undergo base modifications and aminoacylations was demonstrated (4). These studies have shown that the in vitro system is appropriate for the study of various aspects of tRNA biosynthesis. The properties of the tRNA$_1^{Tyr}$ gene promoter have been studied in vitro using as a template the whole Ø80psu$_3^+$ DNA (5), or restriction endonuclease-derived DNA fragments (6). Kupper et al. (6) have studied the transcription of the tRNA$_1^{Tyr}$ gene on isolated DNA fragments. They found the promoter of the tRNA$_1^{Tyr}$ gene to have rather unusual properties, being inhibited by low salt concentrations, requiring glycerol for efficient transcription and being unable to form any rifampicin resistant complexes with RNA polymerase. In the present study we have examined the properties of tRNA gene transcription from whole transducing bacteriophage DNA templates. We find that the promoter-dependent transcription is relatively not salt sensitive, does not require glycerol and rifampicin-

resistant complexes with RNA polymerase can be formed in the absence of initiation of RNA synthesis. These findings suggest that the transcriptional properties of a promoter may vary according to whether it is located on isolated DNA fragments or complex DNA structures.

MATERIALS AND METHODS

DNA from Ø80psu$_3$$^+$ (7) and λh80T (8) was transcribed in vitro by E. coli RNA polymerase in reaction mixtures containing 50 mM Tris-HCl buffer pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, and 0.5 mM each of ATP, CTP, GTP, UTP, one of which was [α-^{32}P] labeled (Amersham, England) as previously described (3). The RNA polymerase was used in limiting amounts as determined by transcription of Ø80psu$_3$$^+$ DNA with increasing amounts of enzyme (Fig. 1), and ρ factor was in excess. The synthesized RNA was processed by S-100 extracts (3) and fractionated by acrylamide gel electrophoresis on 40 x 20 cm 5% gels (20:1 acrylamide-bis-acrylamide) containing 7 M urea in Tris-borate buffer (9) at 175 V for 16-19 h. DNA dependent RNA polymerase, termination factor ρ and S-100 extracts were prepared from E. coli MRE-600 cells (3). Restriction enzymes Hind II+III were obtained from New England Biolabs (Beverley Ma.) and Ø80psu$_3$$^+$ DNA digest was prepared as described by Landy et al. (12).

RESULTS

Influence of ρ factor, glycerol and salt

As seen in Fig. 2, transcription in vitro of Ø80psu$_3$$^+$ DNA by E. coli polymerase

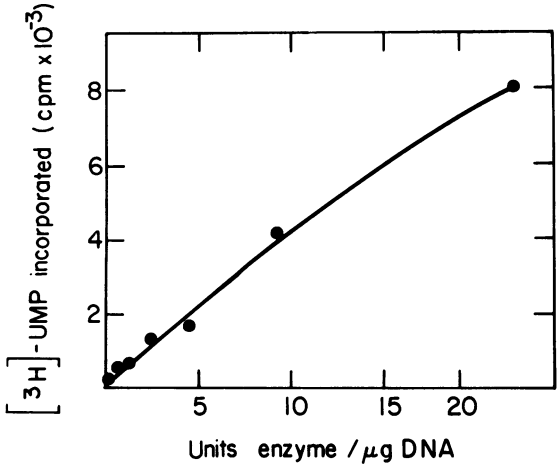


Fig. 1: Transcription of Ø80psu$_3$$^+$ DNA by increasing amounts of RNA polymerase.
Reaction mixtures (50 μl) as described in Materials and Methods containing 2.8 μg Ø80psu$_3$$^+$ DNA and increasing amounts of RNA polymerase were preincubated for 10 min at 37°. RNA synthesis was initiated by addition of nucleoside triphosphates together with rifampicin (12 μg/ml). After incubation for 10 min at 37° the incorporated radioactivity was measured by trichloroacetic acid precipitation.

in the absence or presence of ρ factor yields an RNA product of about 9S (band A) which was identified as the precursor to $tRNA_1^{Tyr}$ (3). Rho appears to stimulate the synthesis of the $tRNA_1^{Tyr}$ precursor without affecting its size (Fig. 2). The stimulation of tRNA synthesis by ρ can also be seen after digestion of the primary transcription products of Ø80psu$_3$+ and λh80T DNA by S-100 E. coli extracts to form mature size tRNA molecules (Fig. 3A and 3B gel 2). This suggests that the increase in tRNA synthesis reflects an increase in the number of initiations at the tRNA gene promoters and not a ρ-mediated suppression of read-through transcription. The possibility of an effect of glycerol, which is present in our ρ preparation, was ruled out since glycerol at concentrations as high as 20% has no effect on tRNA gene transcription (Fig. 3A and 3B, gels 3 and 4).

Rifampicin (12 μg/ml) was added along with the four nucleoside triphosphates to a preincubated reaction mixture containing the respective DNAs and RNA polymerase in the absence or presence of ρ factor. Fig. 3A and 3B (gels 5 and 6) show that the stimulating effect of ρ on tRNA synthesis was abolished by rifampicin indicating that the effect is due to an enhanced reinitiation of tRNA gene transcription. It should be observed that the presence of ρ either in the preincubation mixture before addition of rifampicin and nucleoside tri-

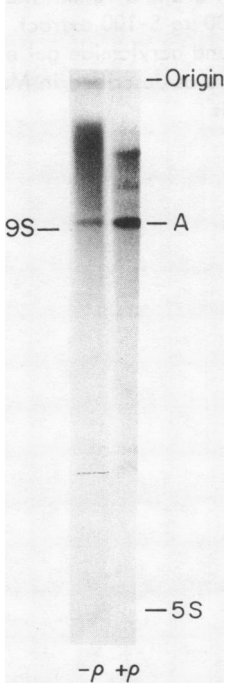


Fig. 2: Effect of ρ on $tRNA_1^{Tyr}$ precursor synthesis
2.8 μg Ø80psu$_3$+ DNA in 50 μl reaction mixtures (Material and Methods) were transcribed by 14 units RNA polymerase in the absence or presence of ρ factor for 30 min at 37°. The RNA products were extracted by phenol, ethanol precipitated and fractionated by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. RNA markers: E. coli 5 S RNA and rabbit 9 S globin mRNA

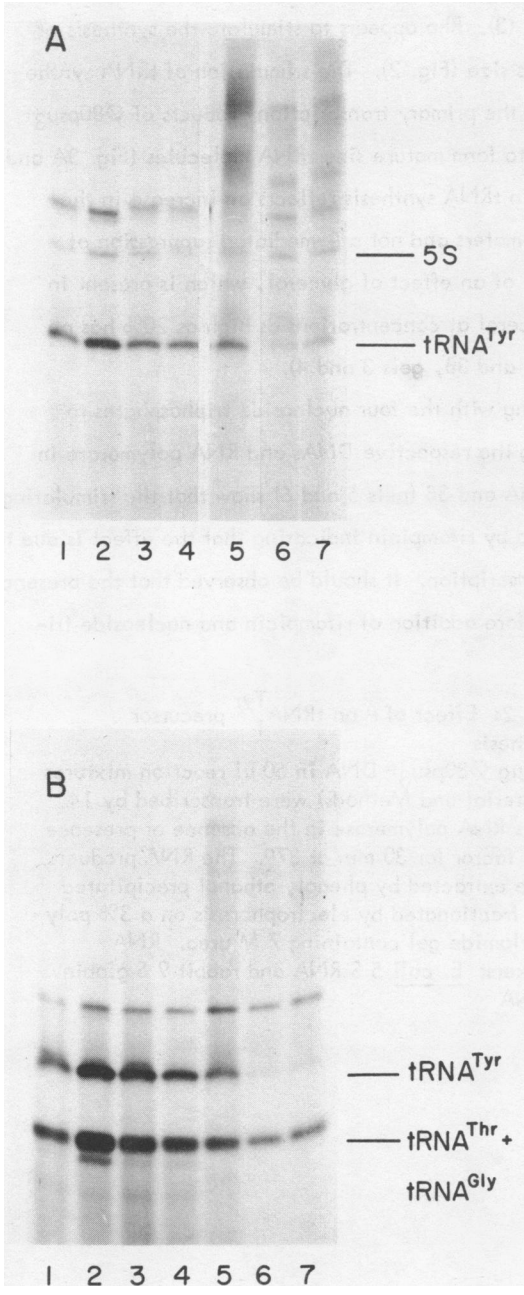


Fig. 3: Effect of ρ factor, glycerol and rifampicin on tRNA gene transcription. Reaction mixtures (90 μl) as described in Materials and Methods contained, 7 μg Ø80psu3$^+$ (A) or 10 μg λh80T (B) DNA, 120 units E. coli RNA polymerase (8000 u/mg) and 7.5 μCi [α^{32}P] UTP (30 μM). Reaction mixtures 2 and 6 contained also 8 μl ρ factor and reaction mixtures 3 and 4 contained 5 and 20% glycerol respectively. After 10 min at 37° transcription was initiated by the addition of the nucleoside triphosphates. To reaction mixtures 5, 6 and 7 rifampicin (to 12 μg/ml) was added together with the nucleoside triphosphates, which contained 12.5 μCi (α-^{32}P) UTP (30 μM). To reaction mixture 7, rho factor was added immediately after rifampicin and nucleoside triphosphates. Reaction mixtures were incubated for 30 min, followed by a 10 min incubation in the presence of 1.5 μg DNase and 3 μg actinomycin D and a 90 min incubation with 600 μg S-100 extract. RNA extraction and acrylamide gel electrophoresis were as described in Materials and Methods.

phosphates (Fig. 3A and 3B, gel 6) or immediately after (Fig. 3A and 3B gel 7) results actually in a reduced production of both $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ and has no effect on $tRNA^{Gly}$ and $tRNA^{Thr}$ synthesis. Further evidence that ρ factor may affect transcription by making available the RNA polymerase molecules for reinitiation was obtained by comparing the effect of ρ on the transcription of $\emptyset 80psu_3^+$ DNA with that on $[\gamma\text{-}^{32}P]$GTP incorporation. Fig. 4A shows that the addition of increasing amounts of ρ. while reducing the amount of

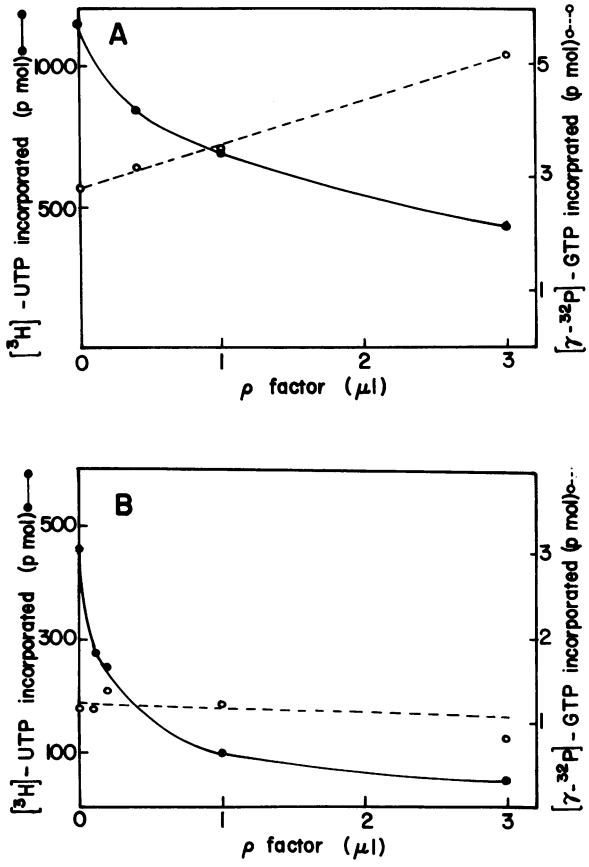
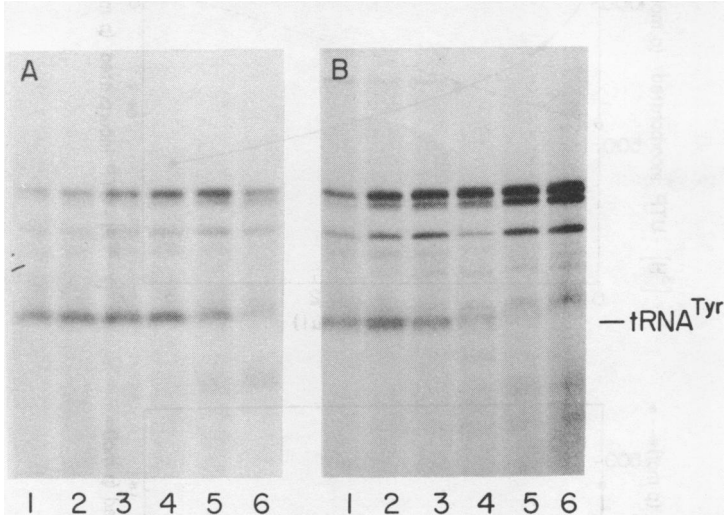


Fig. 4: Effect of rho factor on total RNA synthesis and RNA chain initiation. Reaction mixtures (50 μl) as described in Materials and Methods contained 3 μg $\emptyset 80psu_3^+$ DNA, 60 units RNA polymerase, [3H] UTP (10 Ci/mmol), $[\gamma\text{-}^{32}P]$ GTP (0.8 Ci/mmol) and increasing amounts of ρ factor. After 10 min preincubation at 37° transcription was initiated by the addition of the four nucleoside triphosphates alone (Fig. 2A) or together with 0.2 μg rifampicin (Fig. 2B) followed by 30 min incubation at 37°. The reaction was stopped by 5% trichloroacetic acid, the RNA was collected on millipore filters washed and the incorporated radioactivity counted in a scintillation spectrophotometer.

RNA synthesis, brings about an increased RNA chain initiation. The enhanced [γ-^{32}P]GTP incorporation observed in the presence of ρ was abolished when rifampicin was added along with the nucleoside triphosphates (Fig. 4B).

The transcription of $tRNA_1^{Tyr}$ genes on isolated DNA fragments was reported to be unusually salt sensitive (6). Fig. 5A shows that the transcription of the $tRNA_1^{Tyr}$, genes on whole phage Ø80psu3+ DNA does not seem to be inhibited by salt and takes place within a broad range of salt concentrations (0 to 0.15 M KCl). A shift to a lower optimal salt concentration (0.05 M KCl) is observed when ρ is present during the transcription of $tRNA_1^{Tyr}$



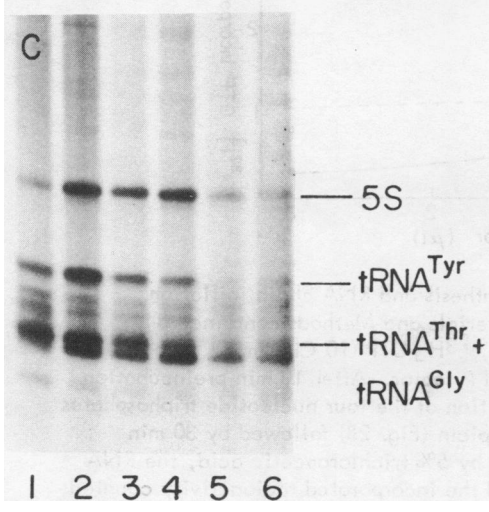


Fig. 5: Effect of salt on tRNA gene transcription.
Ø80psu3+ DNA was transcribed in the absence (A) and presence (B) of ρ factor and λh80T DNA was transcribed in the presence of ρ factor (C) at the following KCl concentrations: 1 (no salt), 2 (0.05 M) 3 (0.075 M), 4 (0.10 M), 5 (0.15 M), 6 (0.2 M).
[α-^{32}P]UTP (5 Ci/mmol), [α-^{32}P]CTP, (6 Ci/mmol) and (6.7 Ci/mmol) were used in experiments A, B and C respectively. RNA processing, isolation and fractionation were as described in Materials and Methods.

(Fig. 5B) and $tRNA_2^{Tyr}$, $tRNA^{Thr}$, $tRNA^{Gly}$ (fig. 5C) genes

Stability of tRNA gene promoter-RNA polymerase complexes

The experiments presented in Fig. 3A and 3B (gels 5 to 7) show the formation of rifampicin resistant complexes between the promoters to the tRNA genes and RNA polymerase. Preincubation of $\emptyset 80psu_3^+$ or λh80T DNA and RNA polymerase in the presence of GTP, the initiating nucleotide for $tRNA_1^{Tyr}$, did not enhance the amount of the synthesized $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ while a preincubation with both GTP and CTP resulted in a 2 to 4 fold increased synthesis (Fig. 6A and 6B). The resistance to rifampicin of the complex between RNA polymerase and the promoter $tRNA^{Gly}$, $tRNA^{Thr}$ genes seems to be less affected by the presence of GTP and CTP (Fig. 6B).

Transcription of restriction DNA fragments

$\emptyset 80psu_3^+$ DNA was digested with Hind II+III enzymes and the mixture of fragments was used as a template for *in vitro* tRNA gene transcription. Fig. 7 shows that using this template the $tRNA_1^{Tyr}$ synthesis becomes stimulated by 20% glycerol and inhibited by 0.1 M

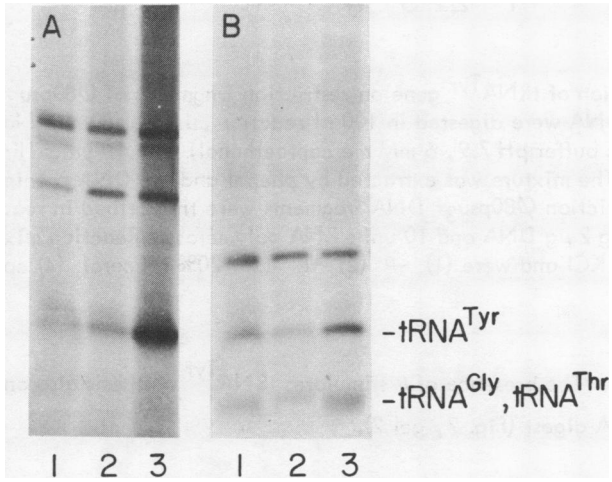


Fig. 6: Stability of tRNA gene promoter - RNA polymerase complex to rifampicin $\emptyset 80psu_3^+$ (A) and λh80T (B) DNA were preincubated with RNA polymerase for 10 min at 37° in a reaction mixture containing 50 mM KCl in the absence of nucleoside triphosphates (1) in the presence of 500 μM GTP (2) or 500 μM GTP+20 μM CTP (3). RNA synthesis was initiated by the addition of the missing nucleoside triphosphates (one α-^{32}P-labeled) and rifampicin (16 μg/ml final concentration) followed by 20 min incubation at 37°. RNA processing, isolation and fractionation were as described in Materials and Methods.

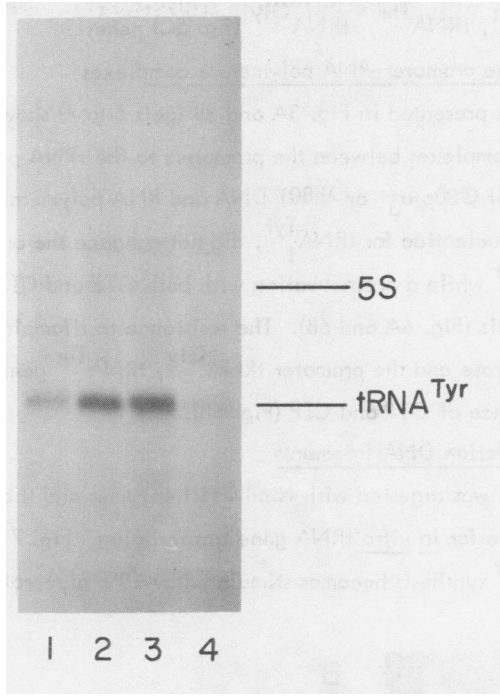


Fig. 7: Transcription of $tRNA^{Tyr}$ gene on restriction fragments of Ø80psu3+ DNA. 20 μg Ø80psu3+ DNA were digested in 100 μl reaction mixture (60 mM NaCl, 6 mM $MgCl_2$, 6 mM Tris buffer pH 7.9, 6 mM mercaptoethanol) with 10 units Hind II+III for 12 hours at 37°. The mixture was extracted by phenol and the DNA precipitated by ethanol. The restriction Ø80psu3+ DNA fragments were transcribed in reaction mixtures of 50 μl containing 2 μg DNA and 10 units RNA polymerase. Reaction mixtures 1 to 3 contained 0.05 M KCl and were (1) -ρ (2) +ρ (3) +20% glycerol (4) contained 0.1 M KCl.

KCl (gels 3 and 4). The presence of ρ stimulates $tRNA_1^{Tyr}$ synthesis also on the unfractionated Ø80psu3+ DNA digest (Fig. 7, gel 2).

DISCUSSION

In the present study the transcription in vitro of tRNA genes was carried out using whole transducing bacteriophage DNA (Ø80psu3+, and λh80T) and limiting amounts of RNA polymerase. In such a system, we have previously observed that the addition of an excess of ρ factor (titrated against RNA polymerase) resulted in an increase in the amounts of tRNA (4, 5) as well as tRNA precursor (10) produced. Fig. 2 shows that the increase in the amount of the synthesized tRNA precursor is not accompanied by a change in its size. These observations have led us to assume that ρ factor may not have a termination effect on tRNA

gene transcription and that the stimulation of tRNA synthesis is due to a release of RNA polymerase and reinitiation of transcription. This interpretation is supported by the present finding that rifampicin abolishes the ρ-stimulation of tRNA genetranscription (fig. 3A and 3B). The lack of effect of ρ after rifampicin addition, even when ρ was preincubated with RNA polymerase and DNA, rules out an effect of ρ to enhance the affinity of RNA polymerase for tRNA gene promoters.

Fig. 3B gel 2 shows that the presence of ρ in λh80T DNA transcription stimulated the synthesis of a 4 S RNA molecule smaller than $tRNA^{Thr}$ and $tRNA^{Gly}$. This finding is similar to that reported by Rosenberg et al. (11) who observed the ρ-stimulated production of a 4S RNA upon in vitro transcription of λDNA. It should be observed that, unlike Rosenberg et at. (11), we have processed the λh80T DNA transcription products by S-100 extracts and 4 S RNA appears to be resistant to nuclease attack. Were the 4S RNA synthesized in the absence of ρ part of a large size RNA molecule, it would have been detected by the digestion with S-100 extract. The increased formation of 4S RNA by ρ seems therefore to be due not to an enhanced termination of this molecule, but rather to a reinitiation of transcription similar to that observed for the tRNA genes. This interpretation is supported by the abolishing effect of rifampicin on the ρ-stimulated synthesis of 4S RNA (Fig. 3B, gels 6, 7). Recently, Howard et al. (13) came also to the conclusion that ρ factor stimulates the synthesis of 4S RNA on λDNA by facilitating the release of RNA polymerase from the termination site.

Our findings on the effect of salt and glycerol on tRNA gene transcription and the stability to rifampicin of the promoter-RNA polymerase complexes indicate that these promoters are not as "weak" as previously claimed (6). The transcription takes place at a broad range of salt concentrations, does not require glycerol, and the promoter-RNA polymerase complexes formed by preincubation in the absence of nucleoside triphosphates are relatively resistant to rifampicin. The discrepancy between our results and those reported by Kupper et al. (6) seems to reside in the DNA template used. The promoter to the $tRNA_1^{Tyr}$ gene appears to be "weak" only when studied on unfractionated (Fig. 7) or isolated (6) Hind II+III restriction-DNA fragments. The transcription of small DNA fragments obtained by digestion of $\emptyset 80psu_3^+$ DNA with Hind II+III enzymes and consequently containing 5' single stranded ends is complicated by the competition of the terminal ends with the true promoter for binding of RNA polymerase. We do not know how this affects the properties of the promoter but it seems that the transcription of the $tRNA_1^{Tyr}$ gene on the restriction fragments is not efficient. Only 0.04 pmole tyrosine accepting $tRNA^{Tyr}$ is synthesized on

0.4 pmole single gene containing DNA fragment (6) as compared with the synthesis of 5.6 and 19.6 pmole $tRNA^{Tyr}$ on 1.4 pmoles $tRNA^{Tyr}$ gene-DNA on a whole phage DNA template (4).

It can always be argued that the transcription of tRNA genes on whole phage DNA and on restriction fragments does not start from the same promoters. This possibility seems improbable in view of the fact that a mixture of GTP and CTP is found to increase the resistance of the complex $tRNA^{Tyr}$ promoter-RNA polymerase to rifampicin on both Ø80psu3+ and λh80T DNA (Fig. 6A and 6B) as it does for the $tRNA_1^{Tyr}$ gene on the restriction DNA fragments (6). More studies are necessary to understand the interaction of RNA polymerase with tRNA gene promoter on multipromoter-containing DNA structures and the factors which control the transcription of tRNA genes.

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