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Transcriptional Control of *in Vitro* $tRNA^{Tyr}$ Synthesis†

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ABSTRACT: The *in vitro* transcription by purified *Escherichia coli* RNA polymerase of the $tRNA^{Tyr}_I$ gene carried by $\phi 80psu^+_3(0)$ phage DNA was studied as a function of transcription factors, ionic strength, and temperature. The synthesis of the $tRNA^{Tyr}$ was determined by competition with the hybridization of *E. coli* [^{32}P]tRNA on the $\phi 80psu^+_3$ (0) separated L-strand DNA. While little tRNA-like material was produced by the purified core polymerase, the σ factor promoted efficient transcription along the $tRNA^{Tyr}_I$ gene. The transcription was found to be salt sensitive, little tRNA synthesis occurring at high ionic strength. Addition of ρ factor enhanced the percentage of tRNA among the *in vitro* transcripts, probably by causing termination of RNA synthesis outside the $tRNA^{Tyr}_I$ gene. Maximal production of tRNA was obtained when transcription was performed by core polymerase + σ in the presence of ρ factor and about 0.05 M KCl. Initiation of $tRNA^{Tyr}_I$ transcription was shown to require a thermal activation, at 28° less tRNA being produced than at 38°. The presence of the protein elongation factors TuTs could not substitute for the thermal activation step.

Transducing bacteriophages carrying genes from bacterial origin have been used in order to study individual transcription units and their regulatory properties (Zubay *et al.,* 1970; Greenblatt and Schleif, 1971; Pannekoek and Pouwels, 1973). The regulation of biosyntheis of tRNA as well as of rRNA (stable RNAs) presents a problem of special interest. Though coded by a minute fraction of the bacterial chromosome (Yankofsky and Spiegelman, 1962; Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962), they constitute a large proportion of all the cellular RNA. The bacterial transcription mechanism must thus be able to discriminate among RNA promoters in favor of the stable RNA species.

We focused our attention on the su_3 gene, the structural gene for *E. coli* tyrosine $tRNA_1$ carried by the $\phi 80psu^+_3(0)$ phage (Andoh and Ozeki, 1968). In order to study the mechanism of tRNA biosynthesis, we transcribed $\phi 80psu^+_3(0)$ DNA by purified *E. coli* RNA polymerase. We have demonstrated (Daniel *et al.,* 1970; Littauer *et al.,* 1971) that the $\phi 80psu^+_3(0)$ DNA can serve as a template for *in vitro* transcription, leading to the synthesis of tRNA like molecules larger in size than 4 S (pre-tRNA). Several other laboratories have also studied $\phi 80psu^+_3$ DNA transcription *in vitro* (Ikeda, 1971; Zubay *et al.,* 1971). Using S_{30} preparations, Zubay *et al.* (1971) reported the successful synthesis of a biologically active $tRNA_{su^+3}$; however, due to the use of crude extracts, the mechanism of tRNA transcription was not investigated. In the present report we have studied the transcription of the transduced su_3 gene by purified *E. coli* RNA polymerase with respect to the effects of transcription factors, ionic strength, and temperature.

Experimental Section

Materials. Nucleoside triphosphates and ^{3}H-nucleoside triphosphates were obtained from Schwarz Bioresearch, Inc. Pancreatic ribonuclease (five times crystallized, A grade) was purchased from Calbiochem. DNase I, electrophoretically purified, was obtained from Worthington. Phage $\phi 80psu^+_3(0)$ was received from Dr. H. Ozeki, and grown on *E. coli* CA 274 (Hfr C Lac^-125_{Amber} $Trp^-{}_{Amber}su^-$) in λ broth agar (Matsushiro *et al.,* 1964). In this phage, transcription of the $tRNA^{Tyr}$gene is from the light (L) DNA strand (Daniel *et al.,* 1970).

Methods. $\phi 80psu^+_3(0)$ DNA preparation, DNA strand separation, and DNA–RNA hybridization were performed as previously described (Daniel *et al.,* 1970). [^{32}P]tRNA was prepared from *E. coli* B cells grown on Tris-glucose medium (Daniel *et al.,* 1969).

DNA-dependent RNA polymerase was purified from *E. coli* MRE-600 cells by the procedure of Chamberlin and Berg (1962). The enzyme preparation was then subjected to low salt glycerol gradient centrifugation (Burgess, 1969) and the core enzyme was separated from the σ subunit by phosphocellulose chromatography (Berg *et al.,* 1971). Termination factor ρ was prepared from *E. coli* MRE-600 cells by the procedure of Roberts (1969); it showed only one predominant band when exam-

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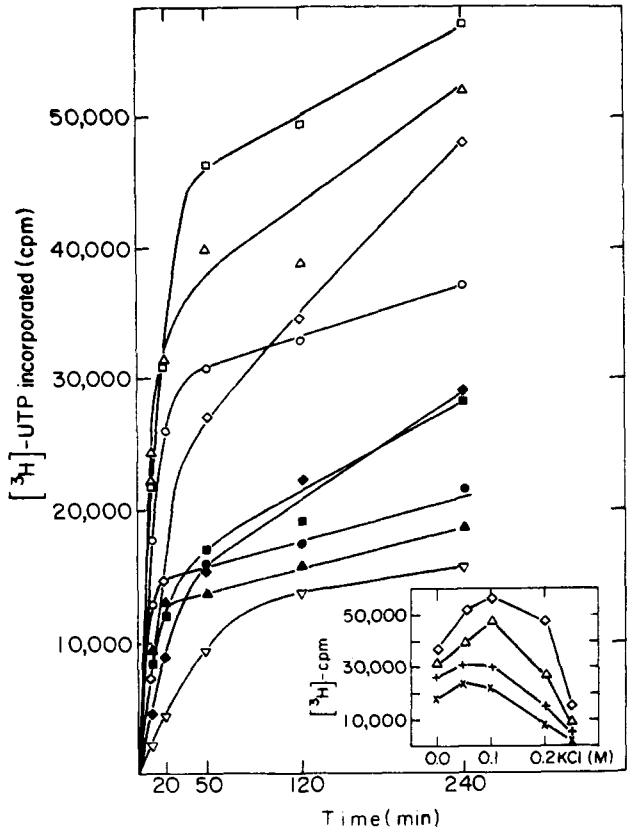

FIGURE 1: Transcription of ϕ80psu$^+_3$(0) DNA as a function of salt concentration. ϕ80psu$^+_3$(0) DNA transcription by core RNA polymerase + σ, with or without added ρ factor, was measured at 37° as a function of time of synthesis at several KCl concentrations (from 0 to 0.25 M KCl). The reaction mixture of 0.1 ml (Methods) contained [^{3}H]UTP (8×10^6 cpm μmol), 6 μg of DNA, 8 μg of core enzyme, 6 μg of σ, and 1.4 μg of ρ factor where indicated. Synthesis was initiated by addition of the four nucleoside triphosphates upon a prior 4 min long incubation at 37°. Samples of 15 μl were precipitated with trichloroacetic acid at the indicated times onto Whatman 3MM filter paper discs. Open symbols represent synthesis without ρ, closed symbols with ρ: (○, ●) no KCl addition; (△, ▲) 0.05 M KCl; (□, ■) 0.1 M KCl; (◇, ◆) 0.2 M KCl; (▽) 0.25 M KCl. Insert: [^{3}H]UTP incorporated after different incubation times in the absence of ρ are plotted as a function of KCl concentration: (x) 10 min of synthesis; (+) 20 min, (△) 50 min; (◇) 240 min.

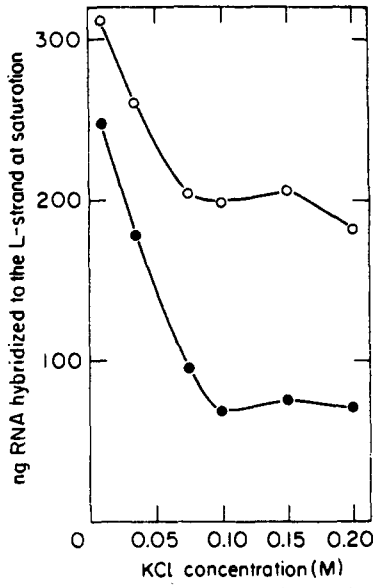

FIGURE 2: Amounts of RNA transcribed on the L strand of ϕ80psu$^+_3$(0) DNA as a function of salt concentration. Increasing concentrations of ϕ80psu$^+_3$(0) RNA, transcribed *in vitro* at 38° at different ionic strengths (as described in the legend to Table I), were hybridized to the separated L strand of ϕ80psu$^+_3$(0) DNA. The amounts (ng) of RNA annealed at saturation for each RNA preparation were calculated by extrapolation as follows: hybridization experiments were analyzed by reciprocal plots (x = 1/ng of input RNA; y = 1/ng of hybridized RNA). The value of $1/y$ at the intercept x = 0 represents the saturation value for this RNA preparation. Open symbols represent synthesis without ρ, closed symbols with ρ.

ined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (5% acrylamide and 0.1% sodium dodecyl sulfate). Incubation of *in vitro* synthesized RNA with the ρ preparation did not affect the size of the RNA molecules showing the absence of any contaminating nuclease activity (Beckmann, 1973). Transcription was carried out in a reaction mixture containing in 0.1 ml: 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM dithiothreitol, 75 mM KCl (or as indicated), 0.4 mM each of ATP, GTP, UTP, and CTP, [^{3}H]CTP or [^{3}H]UTP, DNA, RNA polymerase, and protein factors (σ, ρ, TuTs) as indicated. The incubation conditions are described in the respective legends to figures and tables. Reactions were stopped and processed as described elsewhere (Daniel *et al.*, 1970).

Results

Transcription of ϕ80psu$^+_3$(0) DNA. EFFECT OF IONIC STRENGTH. We have studied transcription of ϕ80psu$^+_3$(0) DNA by core enzyme + σ at different salt concentrations (Figure 1). For short incubation periods we found an optimum at 0.075 M KCl. The optimal salt concentration is found to vary with the duration of incubation: after long periods of synthesis it is shifted to higher salt concentrations (Figure 1, insert). This shift probably reflects termination and reinitiation events believed to be favored at higher ionic strength (Maitra and Barash, 1969; Richardson, 1970) and may explain why synthesis performed at high KCl does not level off after long incubation times.

In Figure 1 are also reported the results of transcription studies performed by core enzyme + σ in the presence of ρ termination factor. The effect of ρ is manifested, in this assay, by a reduction in total RNA synthesis. As can be seen, ρ factor seems to be effective in reducing the ϕ80psu$^+_3$ DNA transcription at all KCl concentrations tested (from 0 to 0.2 M), the extent of restriction on RNA synthesis being maximal around 0.05 M KCl and minimal in either 0.2 M KCl or in the absence of KCl. In addition, the presence of the ρ factor does not affect the salt sensitivity shown by the holoenzyme: the same optimal salt concentration (0.075 M KCl) is found for short time of synthesis, which is shifted to higher ionic strength upon long incubation times. In the presence of ρ, transcriptions performed at high salt do not level off upon long incubation periods (Figure 1).

Leftward Transcription of ϕ80psu$^+_3$(0) DNA. The products obtained upon transcription of ϕ80psu$^+_3$(0)DNA by core enzyme + σ or core enzyme + σ + ρ were hybridized on the separated L or H strands of ϕ80psu$^+_3$(0) DNA. Hybridization pattern of these two RNA preparations to the H strand was nearly unaffected by the presence of ρ during transcription (not shown). It was found, however, that hybridization of ϕ80psu$^+_3$(0) RNA with the L DNA strand was significantly lower when ρ factor was included in the transcription mixture (Figure 2). The hybridization experiments performed with RNA produced *in vitro* at different KCl concentrations indi-

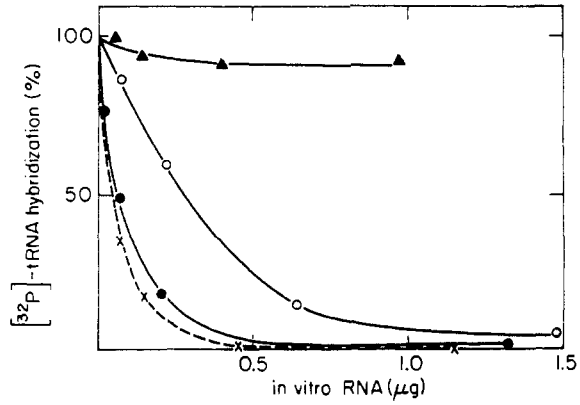

FIGURE 3: Competition between ϕ80psu$^+_3$(0) RNA and [^{32}P]tRNA for hybridization with ϕ80psu$^+_3$(0) L strand DNA. ϕ80psu$^+_3$(0) DNA (6 μg) was transcribed at 37° by the core enzyme (8 μg) (▲), core enzyme supplemented with 10 μg σ (○), or with either 10 or 3 μg σ and 1.4 μg ρ factor (● and and X, respectively) in a 100-μl reaction mixture containing 0.075 M KCl. The reaction mixture was preincubated for 4 min at 37° and RNA synthesis was started by the addition of the four nucleoside triphosphates. The RNAs were extracted and increasing amounts were assayed for competition-hybridization in reaction mixtures containing 0.8μg of [^{32}P]tRNA (1.4 × 10^6 cmp/μg) and 2 μg of purified L strand of ϕ80psu$^+_3$(0) DNA (Daniel *et al.*, 1970).

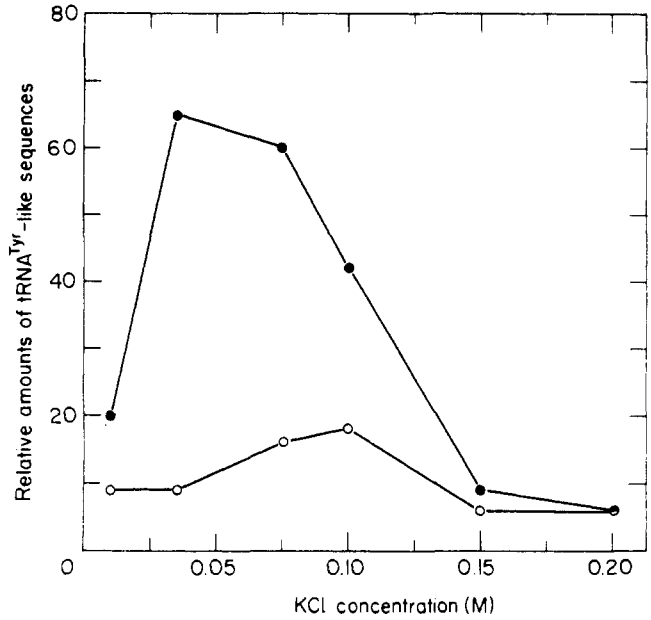

FIGURE 4: Relative amounts of tRNA^{Tyr} like sequences in ϕ80psu$^+_3$(0) RNA transcribed at different salt concentrations. ϕ80psu$^+_3$(0) RNA synthesized at 38°, at the indicated KCl concentrations, in the absence (open symbols) and in the presence (closed symbols) of ρ factor (see legend to Table I) was assayed for competition with [^{32}P]tRNA (10^6 cpm/μg) hybridization, on purified L strand of ϕ80psu$^+_3$(0) DNA (1 μg) (Daniel *et al.*, 1970). From each competition curve, the relative amounts of tRNA like material were computed. These are represented as a function of the KCl molarity in the transcription mixture.

cate that KCl also exerts a restriction on leftward transcription (Figure 2). In addition, ρ was effective in reducing RNA synthesis even at 0.2 M KCl. It was noted that low ionic strength transcripts contained relatively more L strand specific sequences, even when transcription was performed in the presence of ρ (Figure 2). We infer, therefore, that the magnitude of ρ effect on the L strand transcription is reduced when synthesis is conducted in low salt.

Transcription of the su$_3$ Gene. The transcription of the *su$_3$* gene was examined by competition-hybridization assay (Daniel *et al.*, 1970). The hybridization of [^{32}P]tRNA with the L strand ϕ80psu$^+_3$(0) DNA was challenged by increasing amounts of *in vitro* synthesized RNA products. From the competition curves, we estimated the percentage of tRNA-like material present among the total *in vitro* RNA population. The calculations are based upon the assumption that both [^{32}P]tRNA and tRNA^{Tyr} like chains have similar rates and efficiencies of hybridization. At 50% competition we should have equal amounts of [^{32}P]tRNA^{Tyr} and pre-tRNA^{Tyr} like molecules. The percentages so obtained are probably overestimated values (Beckmann and Daniel, 1974), but are useful, since they allow us to compare and relate different RNA preparations for the efficiency of *su$_3$* gene transcription.

EFFECT OF σ AND ρ ON *su$_3$* GENE TRANSCRIPTION. The RNA products synthesized by the core enzyme with or without σ and ρ factors were assayed for their ability to compete with [^{32}P]tRNA in the hybridization reaction (Figure 3). It is evident that the core enzyme directs very little transcription of the *su$_3$* gene. Upon addition of saturating amounts (10 μg) of σ, the holoenzyme can transcribe efficiently the tRNA gene (approximately 10% of the total RNA population is calculated to be tRNA like). Addition of ρ factor to the transcription system further augments the percentage of tRNA like material found among the *in vitro* transcripts (values between 40 and 60% were obtained from the curves in Figure 3). These results show that ρ improves the yield of tRNA gene transcription.

Competition curves for RNA produced by the core enzyme supplemented with ρ and two different quantities of σ factor (3 and 10 μg) are also shown in Figure 3. There is a two- to three-fold difference in the extent of stimulation of RNA synthesis by these two concentrations of σ factor. However, these two *in vitro* RNA products did not show significant differences in their ability to compete with [^{32}P]tRNA hybridization.

EFFECT OF IONIC STRENGTH ON *su$_3$* GENE TRANSCRIPTION. ϕ80psu$^+_3$(0) DNA was transcribed *in vitro* in the absence and presence of ρ, in reaction mixtures containing increasing amounts of KCl. The *in vitro* produced RNAs were tested for their tRNA like content by the competition-hybridization assay. From each competition curve (not shown) the percentage of tRNA like content was computed. These data are presented in Figure 4 which sums up the relative tRNA like contents in the RNAs synthesized by the holoenzyme, at different KCl concentrations, with or without ρ factor. It is clear that the presence of the termination factor ρ during transcription influences significantly the percentage of tRNA like material produced, this effect being largely affected by the salt concentration. It should be noted that the optimum KCl concentration for tRNA like production by the holoenzyme lies around 0.1 M KCl, while in the presence of ρ it is shifted to 0.05 M KCl. In addition, at high KCl (above 0.15 M) ρ factor has no effect on the yield of tRNA like molecules.

TEMPERATURE EFFECT ON *su$_3$* GENE TRANSCRIPTION. It has been proposed (Travers, 1973) that initiation of transcription of tRNA as well as of rRNA genes might be under one and the same control, exerted by the protein factor ψr and its putative cofactor ppGpp. According to this hypothesis the ψr factor, which is identical with the protein synthesis elongation factors TuTs, would allow the recognition of the tRNA or rRNA gene promoters, by melting locally the DNA, thereby promoting transcription (Travers *et al.*, 1973; Travers, 1973). This requirement for TuTs could be relieved by a preincubation of the template DNA at 38° upon which the rRNA pro-

TABLE I: Effect of Temperature on tRNA^{Tyr} Transcription.[a]

RNA Polymerase used in *in vitro* Transcription	Temp of Preincubation (°C)	Temp of Synthesis (°C)	[^{3}H]CTP Incorporated (cpm/2 μl)	Relative tRNA Like Content (%)
Holoenzyme	28	28	850	2
Holoenzyme + ρ	28	28	240	3.9
Holoenzyme	28	38	1200	3.8
Holoenzyme + ρ	28	38	440	11
Holoenzyme	38	28	885	2
Holoenzyme + ρ	38	28	280	3.9
Holoenzyme	38	38	1110	3.8
Holoenzyme + ρ	38	38	400	14
Holoenzyme	0	38	1100	1.1
Holoenzyme + ρ	0	38	360	3.5

[a] The RNA preparations were synthesized in 0.2-ml reaction mixtures (Methods) containing 0.1 M KCl, [^{3}H]CTP (4.2×10^6 cpm/μmole), RNA polymerase holoenzyme (21 μg), and ρ factor (8 μg). The complete reaction mixtures minus enzyme and ρ factor were preincubated for 15 min at the indicated temperatures. RNA synthesis was initiated by the addition of the missing proteins. After 15-min incubation at the listed temperature the reactions were stopped by the addition of DNase (to 10 μg/ml) and the RNAs extracted as previously described (Daniel *et al.*, 1970). The relative tRNA^{Tyr} content was determined by hybridization–competition (Daniel *et al.*, 1970).

TABLE II: Effect of TuTs on tRNA^{Tyr} Transcription.[a]

RNA Polymerase used in *in vitro* Transcription	Temp of Preincubation (°C)	Temp of Synthesis (°C)	[^{3}H]CTP Incorporated (cpm/3 μl)	Relative tRNA Like Content (%)
Holoenzyme + ρ	28	28	1030	24
Holoenzyme + ρ + TuTs (5 μg/ml)	28	28	1500	24
Holoenzyme + ρ + TuTs (250 μg/ml)	28	28	1830	24
Holoenzyme + ρ	38	38	1350	61
Holoenzyme + ρ + TuTs (5 μg/ml)	38	38	1250	61

[a] RNA synthesis was performed as described in legend to Table I except that the reaction mixture contained 0.075 M KCl and [^{3}H]CTP (2×10^7 cpm/μmol). Transcription was initiated by the addition of RNA polymerase, ρ factor, and the TuTs protein synthesis elongation factors, where indicated. The TuTs factors, a gift from Y. Groner, purified according to the procedure of Gordon *et al.* (1971) were active in the poly(U) directed polyphenylalanine synthesis.

moters would be opened and committed to rRNA transcription, independently of the temperature used for RNA synthesis (28 or 38°) (Travers, 1973).

To examine whether transcription of the su_3 gene is temperature dependent, ϕ80psu$^+{}_3$(0) DNA was preincubated for 15 min at 28 and 38° in the absence of enzyme and ρ factor. RNA synthesis was started by addition of RNA polymerase holoenzyme with or without ρ factor, in 0.1 M KCl, at either 28 or 38°. The synthesized RNAs were extracted and analyzed for their pre-tRNA content by the competition–hybridization test (Table I). There is a net influence of the temperature of transcription on the final yield in tRNA like molecules and this independently of the temperature of preincubation (28 or 38°). The ρ factor is found to increase the proportion of pre-tRNA at all temperatures. An effect of the preincubation temperature on the efficiency of tRNA gene transcription was observed by preincubating the reaction mixture at 0°, while performing transcription at 38° (the enzymes were added prior to the transfer at the higher temperature). The yield in pre-tRNA molecules was found to be three- to fourfold lower than the one obtained upon preincubation and synthesis at 38°.

Since we did observe a difference in the efficiency of su_3 gene transcription upon synthesis at 28 or 38° we performed the following experiments to test whether TuTs could control su_3 gene transcription *in vitro*. ϕ80psu$^+{}_3$(0) RNA was synthesized at 28 and 38°, in 0.075 M KCl, by the holoenzyme and ρ factor in the presence or absence of the TuTs protein synthesis elongation factors. When we tested these RNA products for their pre-tRNA content, we could not find any effect of TuTs on su_3 gene transcription (Table II). RNA produced at 28° contained the same amounts of tRNA like chains, whether TuTs were present or not during transcription.

Discussion

It has been established that transcription on various DNA templates presents different sensitivities to the salt concentration. While T4 DNA is a better template at high ionic strength, ϕ80 and λ DNA transcription are reduced at high KCl concentration (Maitra and Barash, 1969; Richardson, 1970. Okamoto *et al.*, 1970; Goldberg, 1970; Littauer *et al.*, 1971). *In vitro* transcription of ϕ80psu$^+{}_3$(0) DNA by purified RNA polymerase was shown to be optimal at 0.075 M KCl for short incubation times. For longer periods of incubation a shift in the optimal salt concentration to higher ionic strengths was observed. This shift in ionic strength requirement, which is not affected by termination factor ρ, may reflect the increased capacity for reinitiation of RNA synthesis at high salt concentrations. We have shown previously that ρ factor brings about termination of transcription on ϕ80psu$^+{}_3$(0) DNA (Daniel *et al.*, 1970). The findings of the present study show that although ρ directs termination of ϕ80psu$^+{}_3$(0) DNA transcription at all salt concentrations tested (up to 0.2 M KCl), the extent of the reduction in RNA synthesis varies with the ionic strength. Taking the ratio of the amount of RNA produced in the presence of ρ to that in its absence as a measure of the degree of termination, one observes that ρ termination is mostly effective between 0.05 and 0.1 M KCl (Figure 1). Controversial data on ρ-induced termination at high salt have been reported. At 0.2 M KCl, ρ restricted RNA synthesis on fd (Takanami *et al.*, 1971), ϕ80ptrp (Pannekoek and Pouwels, 1973), and ϕ80psu$^+{}_3$ (Figure 1) DNA templates. In contrast, transcription on λ, T4, and T7 DNA was unaffected by ρ at this high salt concentration (Roberts, 1969; Goldberg, 1970). This differential ability of ρ to restrict RNA synthesis at various ionic strengths may suggest the existence of more than one type of termination signals.

The specificity of the termination was studied by hybridizing RNA produced *in vitro* under different ionic strengths to the separated L strand of ϕ80psu$^+{}_3$(0) DNA (Figure 2). Relative-

ly more L strand specific RNA sequences were found to be transcribed by the holoenzyme at low than at high ionic strength. RNA sequences synthesized in the presence of ρ displayed considerably less L strand hybridization than RNA molecules produced in the absence of ρ, this effect of ρ being observed at all ionic strengths studied. We conclude therefore that both ρ factor and ionic strength restrict transcription along the L strand of $\phi 80psu^+_3(0)$ phage DNA.

Studies on *in vitro* transcription of the su_3 gene carried by the transducing bacteriophage have demonstrated that purified bacterial RNA polymerase can transcribe the tRNA gene (Daniel *et al.*, 1970; Littauer *et al.*, 1971; Ikeda, 1971). The presence of the σ-initiation factor was shown to be absolutely required for this transcription; in its absence core enzyme produced little tRNA like material (Figure 3). This suggests that the σ factor exerts a positive control on the tRNA transcription (and possibly on all stable RNA species) for proper and efficient initiation at the tRNA gene promoters. From our results it may be deduced that σ is not required in stoichiometric amounts for tRNA gene transcription, since the same proportion of tRNA like material was produced by a fully σ saturated or partially unsaturated holoenzyme. The presence of the termination factor ρ during transcription was shown to greatly enhance the relative production of pre-tRNA chains. This may suggest that ρ functions on $\phi 80psu^+_3(0)$ DNA transcription essentially by causing termination of RNA synthesis outside the su_3 gene.

Transcription of the su_3 gene was studied at different ionic strengths and temperatures. Maximal production of tRNA like material was found around 0.05 M KCl when transcription was performed in the presence of ρ and around 0.1 M KCl in its absence (Figure 4), the salt sensitivity of the tRNA gene transcription being better evidenced when RNA synthesis is conducted in the presence of ρ. These results imply that initiation of transcription at the tRNA gene promoters is much more salt sensitive than that of other phage genes. It was recently proposed (Travers, 1973) that transcription of rRNA and tRNA genes is under the control of the ψr factor (TuTs) which would function by allowing RNA polymerase to bind at the promoters of these RNA genes. In order to compensate for the absence of ψr, transcription could be performed *in vitro* after preincubation of the DNA at 38°, this temperature simulating the action of ψr factor. The authors further showed that the temperature of preincubation of the DNA template determined the extent of rRNA transcription (Travers *et al.*, 1973). In the transcription of the su_3 gene on $\phi 80psu^+_3(0)$ DNA we have observed that three to four times more tRNA like material was synthesized at 38 than at 28°. The relative amounts of tRNA like material were not affected by a preincubation of the DNA at 28 or 38°, thus showing that the activation of tRNA gene promoter between these two temperatures is a fast and reversible reaction. It should be noted that preincubation at 0°, followed by transcription at 38°, did lower the tRNA like production; this may reveal increased difficulties in the activation of promoters upon transfer from 0 to 38°. No effect on the production of tRNA like sequences could be detected, when TuTs was assayed on $\phi psu^+_3(0)$ DNA for its specific stimulation of the su_3 gene transcription (Table II).

In conclusion, RNA polymerase, containing the σ subunit, can efficiently transcribe the $tRNA^{Tyr}_1$ gene carried by the $\phi 80$ transducing bacteriophage. The presence of the termination factor ρ improves greatly the yield of pre-tRNA production. The sensitivity of the $tRNA^{Tyr}_1$ gene promoter to ionic strength and temperature distinguishes it from the other $\phi 80psu^+_3(0)$ gene promoters. Transcription of $\phi 80psu^+_3(0)$ DNA is little affected by changes in the temperature of synthesis between 28 and 38°. Initiation of transcription at the $tRNA^{Tyr}$ gene promoter requires a thermal activation.

Acknowledgments

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