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Promoter-dependent transcription of $tRNA_I^{Tyr}$ genes using DNA fragments produced by restriction enzymes

(promoter/initiation of transcription/RNA nucleotidyltransferase)

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ABSTRACT **Two DNA fragments prepared from the transducing bacteriophage strains $\phi 80ps u_{III}^+$ and $\phi 80hp su_{III}^{+,-}$ by digestion with restriction enzymes contain one tyrosine tRNA gene (su_{III}^+) and two tyrosine tRNA genes (su_{III}^+, su^-) in tandem, respectively, a single promoter in both cases, and some additional DNA regions at the two ends of both. Using these fragments, we have studied characteristics of the promoter-dependent transcription of the tyrosine tRNA genes. The promoter-dependent transcripts were shown to correspond to the expected tRNA precursors. Exposure of the transcript from the single gene fragment to an S100 extract from *Escherichia coli* gave, via intermediates, 4S material which was active in enzymatically accepting tyrosine and contained some modified bases.**

The two $tRNA_I^{Tyr}$ genes, which occur as a tandem duplication in *Escherichia coli*, have been integrated into the genome of the bacteriophage $\phi 80$ to give the nondefective transducing phage $\phi 80hps u_{III}^{+,-}$ ("doublet" phage). A second strain $\phi 80ps u_{III}^+$, carrying one functional $tRNA_I^{Tyr}$ gene ("singlet" phage) has been derived from the doublet phage by an unequal recombination event between homologous sequences such that one of the two $tRNA_I^{Tyr}$ genes (and any sequences between them) has been eliminated (1). The $tRNA_I^{Tyr}$ genes in this form have proved useful for genetic and biochemical analysis of the various steps in tRNA biosynthesis (2). Such studies have so far been carried out using the total $\phi 80ps u_{III}^+$ DNA (3–5) which includes a variety of other promoters. More recently, using restriction endonucleases it has been possible to prepare DNA fragments from both the doublet and the singlet strains which carry intact $tRNA_I^{Tyr}$ genes (6).

In the present work we show that transcription from these fragments is largely promoter-dependent, that the $tRNA_I^{Tyr}$ promoter has unusual properties, and that the transcription yields tRNA precursors which can be processed to active tRNA.

MATERIALS AND METHODS

Chemicals. Heparin and dinucleoside phosphates were purchased from Sigma Chemical Co. and rifampicin from Mann Research Laboratories. Nucleoside [α-^{32}P]triphosphates were purchased from New England Nuclear, [γ-^{32}P]CTP was prepared by phosphorylation of CDP with [γ-^{32}P]ATP using nucleoside diphosphate kinase, and [γ-^{32}P]GTP was prepared as described elsewhere (7).

Restriction Fragments C1a and B10a. These were isolated from the singlet phage (C1a, 410×10^3 daltons) and doublet phage (B10a, 560×10^3 daltons) by sequential digestions with endonucleases *Hin*cII and *Hin*dIII (6, 8). The structural portion of the tRNA genes within these fragments is skewed in such a manner as to leave approximately 400 nucleotides upstream of the promoter region and approximately 50 base pairs beyond the terminal C-C-A of the structural tRNA sequence.

Enzymes. RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) was prepared essentially according to Burgess (9) with an additional purification step utilizing DNA-cellulose chromatography (10). The enzyme was more than 95% pure as analyzed by sodium dodecyl sulfate gel electrophoresis. The σ factor was separated from the holoenzyme by chromatography on phosphocellulose (9). S100 Fraction from *E. coli* was prepared from the strain MRE 600 and was passed through a DEAE-cellulose column to remove tRNA.

RNA Synthesis. The standard assay mixture was 40 mM Tris·HCl, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM Na_3-EDTA, 20% (vol/vol) glycerol, 1 mM GTP, and 10 μM for the other nucleoside triphosphates. Amounts of the enzyme and DNA template used were as indicated in legends. The incubation temperature was 37°. The reactions were stopped by addition of electrophoresis buffer (50 μl/10 μl of reaction mixture) containing 7 M urea, 0.1% sodium dodecyl sulfate, and the dye markers.

Gel Electrophoresis was carried out under denaturing conditions as described elsewhere (20). For further analysis, the radioactive bands were eluted from the gel with 1 M NaCl, and after addition of carrier RNA the products were precipitated with 70% ethanol.

RESULTS

Transcription of the restriction fragment C1a

The kinetics and products of transcription were followed by polyacrylamide gel electrophoresis under denaturing conditions (Fig. 1). Ribonucleotide incorporation was linear for at least 2 hr and the proportions of different products remained constant throughout. As seen in Fig. 1A, in addition to the major product (indicated by arrow) a number of minor products of higher molecular weight were present. These results were obtained using the holoenzyme. In the next experiment, transcription was performed with the core RNA polymerase, in the presence and absence of sigma factor. As seen in Fig. 1B, a marked difference in the transcription products was observed. In the presence of the sigma factor (lane b), the major product again was the same as that obtained in Fig. 1A, while with the core enzyme different products were synthesized (lane a). Thus, the sigma factor directed the synthesis of the major product of Fig. 1A as well as inhibited the formation of the higher molecular weight products. It is therefore concluded that the synthesis of the major RNA band formed in the presence of the sigma factor is initiated by the specific promoter–holoenzyme interaction.

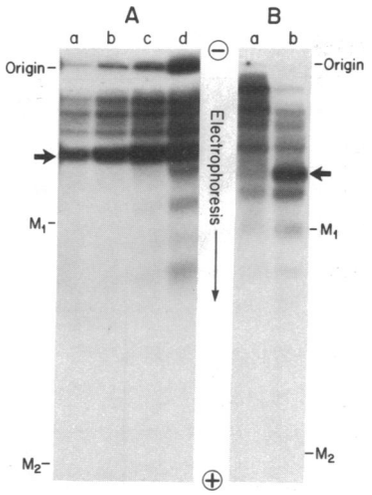

FIG. 1. Transcription products from the DNA fragment C1a. (A) *Kinetics. E. coli* RNA holo-polymerase (4 pmol) was incubated with 0.24 pmol of C1a fragment and the four ribonucleoside triphosphates (GTP and CTP, 500 μM; ATP and UTP, 10 μM, [α-^{32}P]UTP, 10,000 cpm/pmol) under standard conditions in a total volume of 40 μl. Aliquots of 10 μl were withdrawn and analyzed on a 6% polyacrylamide gel after (a) 15 min, (b) 30 min, (c) 60 min, and (d) 120 min. (B) *Effect of sigma factor.* The C1a fragment (0.1 pmol) was incubated with 1.0 pmol of the core RNA polymerase for 60 min under the standard conditions without (lane a) and with (lane b) 1.0 pmol of sigma factor. Analysis was on 6% polyacrylamide gel. M_1 and M_2 indicate the position of the dye markers xylene cyanol and bromphenol blue, respectively.

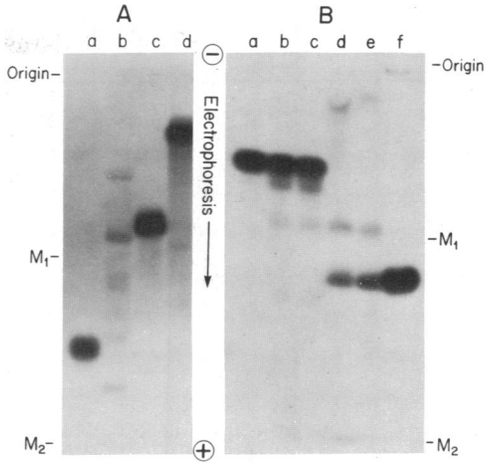

FIG. 2. (A) *Lengths of the transcription products from the B10a and C1a fragments.* The transcription products were electrophoresed on a 5% polyacrylamide gel along with the markers (a) $tRNA^{Tyr}$ and (b) B10a fragment digested with endonuclease *Hae* and treated with kinase. (c) Contained the transcript from C1a and (d) that from B10a. (B) *Processing of the transcript from C1a fragment.* The promoter-dependent transcription product from the C1a fragment was isolated from an acrylamide gel and digested with different amounts of S100 extract at 37° for 30 min (*Materials and Methods*): (a) control, (b) addition of 3 μl of 100-fold dilution, (c) addition of 2 μl of 10-fold dilution, (d) 1 μl, (e) 3 μl of S100 extract, and (f) $tRNA^{Tyr}$ marker. The incubation products were analyzed on a 6% polyacrylamide gel.

Characterization of the promoter-dependent transcripts from the singlet and doublet restriction fragments

The major RNA product described above (Fig. 1) corresponds to a length of about 175 nucleotides (Fig. 2A, lane c). Analysis of the transcript by fingerprinting of a T_1 nuclease digest is given in Fig. 3B. As described in the legend, all the oligonucleotides expected for the *in vivo* tyrosine tRNA precursor (11) are indeed present and, in addition, there are oligonucleotides which comprise an extra length of about 50 bases beyond the 3′-terminus of the above-mentioned precursor.

In vivo experiments (12) suggest that the tandem $tRNA_I^{Tyr}$ genes are transcribed as a single precursor molecule. The major product obtained from B10a in fact corresponded to a chain length of about 410 nucleotides (Fig. 2A, lane d). Fingerprinting of the T_1 ribonuclease digestion products gave the pattern shown in Fig. 3A. Oligonucleotides corresponding to the 41 nucleotides preceding the mature tRNA in the Altman-Smith precursor were again present, their amounts being 1 molar. All the oligonucleotides corresponding to the mature tyrosine tRNA sequence were present in 2 molar yields. Further, the oligonucleotides corresponding to the sequence beyond the C-C-A end and found above in the transcript from the C1a fragment were also present in this fingerprint but only in a 1 molar amount. In addition, a different large oligonucleotide present in 1 molar amount and representing the 3′-end was also present. These results indicate that the sequence immediately after the C-C-A end of the first $tRNA_I^{Tyr}$ gene is different from that after the second $tRNA_I^{Tyr}$ gene. The remaining spots in the fingerprint of the doublet transcript must originate from the intergenic space. The total results therefore indicate that the sequences adjoining the 5′- and 3′-ends of each of the two mature tRNAs are different from each other (cf. ref. 6 and T. Sekiya, unpublished results). The sum of the oligonucleotides assigned to the intergenic spacer region (approximately 150 bases) and to the region extending beyond the 3′-end of the mature tRNA (approximately 50 bases) agreed well with the estimates from restriction fragment mapping (A. Landy and W. Ross, unpublished results).

Characteristics of $tRNA_I^{Tyr}$ promoter

(*a*) Influence of Salt and Glycerol. The transcription was unusually salt-sensitive. Thus, even 0.1 M KCl caused more than 95% inhibition and at 0.15 M KCl no synthesis was detected. Since studies with several systems (e.g., ref. 13) have shown chain elongation to be relatively insensitive to high KCl, the inhibition observed is probably at the initiation step and is evidently a characteristic feature of the tyrosine tRNA promoter.

The $tRNA_I^{Tyr}$ promoter does, however, respond to glycerol like other promoters (14, 15). Optimal stimulation of promoter-dependent transcription was observed at 20% glycerol concentration. The effect of glycerol was more marked in the presence of salt. Thus at 0.002 M KCl the synthesis in the presence and absence of 20% glycerol was 100% and 62%, respectively. However, at 0.05 M KCl, when the synthesis in the absence of glycerol was only 18% of the above 100%, the presence of 20% glycerol increased the amount of synthesis to 75%. Therefore, 20% glycerol was introduced into the standard transcription experiments.

(*b*) Sensitivity to Heparin and Rifampicin. It has been generally observed that the complexes formed by interaction of promoters and RNA polymerases are resistant to heparin and are partially resistant to rifampicin (16, 17). In this study, preincubation of the reaction mixture even in the presence of the initiating triphosphate GTP did not result in resistance to heparin (20 μg/ml added 2 min ahead of the remaining triphosphates) or rifampicin (1 μg/ml added

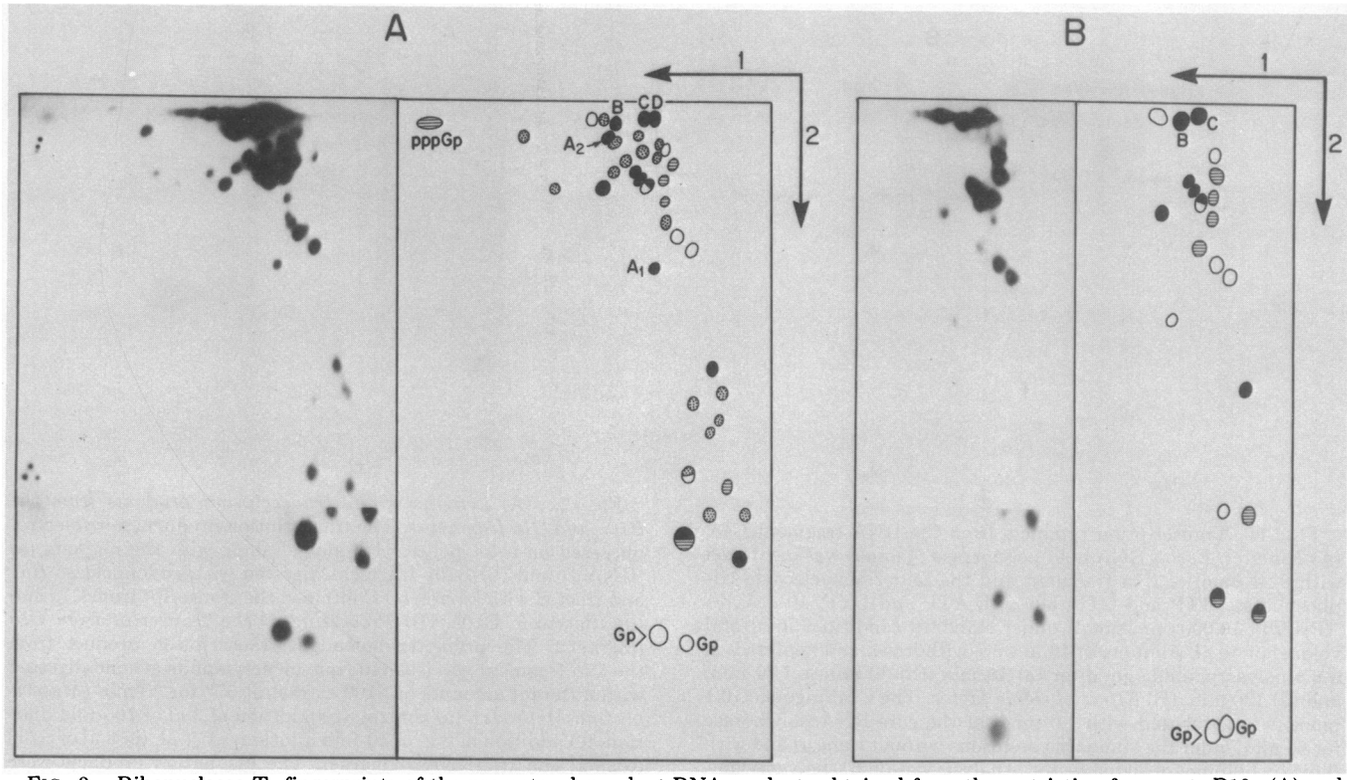

FIG. 3. Ribonuclease-T_1 fingerprints of the promoter-dependent RNA products obtained from the restriction fragments B10a (A) and C1a (B). The RNA products separated by polyacrylamide gels in 7 M urea were digested with ribonuclease T_1 and separated by electrophoresis on cellulose acetate paper at pH 3.5 in the first dimension and at pH 1.7 (7% formic acid) on DEAE-paper in the second dimension. The B10a RNA was labeled with [α-^{32}P]CTP and the C1a RNA with [α-^{32}P]CTP and [α-^{32}P]UTP. The schematic drawings to the right of each fingerprint show group identification of the different oligonucleotides: the oligonucleotides belonging to the 5′-precursor region are indicated by hatched spots (⊜), the oligonucleotides from the intergenic region by stippled spots (⊛), those from the tRNAs by solid spots (●) and those from the 3′-end region by hollow spots (○). In fingerprint A, the presence of *su*$^-$tRNA^{Tyr} was characterized by the anticodon oligonucleotides A_1 (A-C-U-Gp) and A_2 (U-A-A-A-U-C-U-Gp). The suppressor tRNA in both fingerprints A and B was identified by the oligonucleotide B (A-C-U-C-U-A-A-A-U-C-U-Gp). The oligonucleotides C (fingerprint A and B) and D (fingerprint A) are derived from the 3′-end regions of the two tRNAs. Since the RNA synthesis from C1a was primed by C-C, the initiating nucleotide, pppGp, which is present in fingerprint A, was absent and was replaced by C-C-Gp.

along with the remaining triphosphates). However, if the preincubation mixture also contained CTP, which corresponds to the second nucleotide of the $tRNA_I^{Tyr}$ precursor, so that the first inter-nucleotide bond could be formed, then the promoter-dependent transcription was resistant to both heparin and rifampicin. This sensitivity of promoter-dependent synthesis to rifampicin and heparin seems to be an unusual property of the $tRNA_I^{Tyr}$ promoter.

(c) Influence of Different Dinucleoside Phosphates and Ribonucleoside Triphosphates on Initiation. It has been shown previously that the requirement for high concentrations of the initiating triphosphate (18) can be satisfied by an appropriate dinucleoside monophosphate (19). Four different dinucleoside monophosphates were chosen on the basis of the sequence (20) surrounding the normal transcription initiation point (Fig. 4) and tested for their capacity to stimulate promoter-dependent transcription (Table 1). The only dinucleoside phosphate without effect was G-G. This is also the only dinucleoside phosphate with no corresponding template sequence in the immediate vicinity of the $tRNA_I^{Tyr}$ gene initiation site. Of the other dinucleoside phosphates, C-C was the most active in stimulating initiation. The precise sites utilized by C-C and G-C were determined by analyzing the T_1 digestion products of the transcripts made with [α-^{32}P]CTP. G-C initiated in the proper position (Fig. 4). Of the three potential sites for initiation by C-C (Fig. 4), the site-1 is preferred over site-2 by a factor of 10 and over site-3 by at least a factor of 100.

When C-C and GTP were present together at 500 μM each, the former was preferred over the latter for initiation by at least a factor of 10. Again, this was determined by T_1 fingerprint analysis of the gel-purified transcript which showed that the oligonucleotide C-C-Gp was present in 2 mole equivalents (it is normally present in 1 mole equivalent in $tRNA_I^{Tyr}$). Furthermore, pppGp could not be detected.

As expected from the findings of Anthony *et al.* (18), increasing the concentration of GTP, the normal initiator for promoter-dependent transcription, stimulated the synthesis. However, high concentrations of CTP also caused comparable stimulation (Table 1). Since transcription normally starts with purine nucleoside triphosphates, it was of interest to examine the basis of stimulation by CTP. Low to moderate concentrations of CTP (10–50 μM) stimulated the incorporation of GTP as the initiating nucleotide (Table 2). At higher concentration of CTP, however, the incorporation of [γ-^{32}P]GTP was inhibited while the extent of stimulation of RNA synthesis was unaffected. To confirm that this was due to the utilization of CTP as the initiating triphosphate, transcription was carried out using [γ-^{32}P]CTP as the labeled triphosphate (concentration, 100 μM; other triphosphates, 10 μM). Examination by gel electrophoresis showed the incorporation of radioactivity into a product with mobility corre-

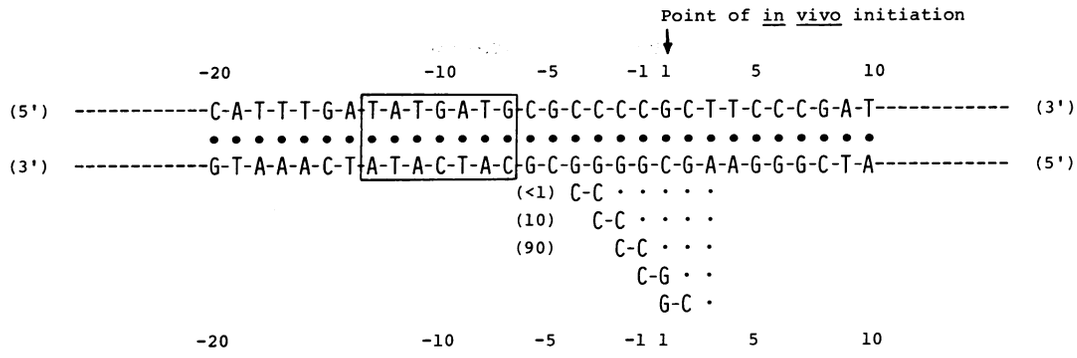

FIG. 4. DNA sequence in the pre- and post-initiation site (left and right, respectively) of the $tRNA_I^{Tyr}$ gene (20). Positions used by different dinucleoside phosphates for priming of initiation are shown. For C-C, relative efficiency of priming in three possible positions is indicated by numbers in parentheses. The heptanucleotide within the box shows the sequence common to all promoters.

sponding to that of the promoter-dependent transcript. Digestion with T_2 RNase followed by chromatography on PEI thin-layer chromatography plates showed the bulk of the radioactivity in an area expected for pppCp (mobility less than that of CTP). No radioactivity was found in the area of mononucleotides. These results showed that the radioactive label was due to the presence of [γ-^{32}P]CTP in the 5'-terminal position.

Processing of the transcript to functional tyrosine tRNA

Several laboratories have shown that the S100 fraction of an *E. coli* extract contains all of the enzymatic activities necessary to process the tRNA transcripts (21) and to produce a functional tRNA (3, 4). In the present system, it should be possible to directly follow the steps in the processing of the primary transcript. Fig. 2B shows the consequences of incubating the transcript from the C1a restriction fragment with various concentrations of the S100 fraction. Under these conditions three bands which are precursors to the mature tRNA are seen in the region of the gel between the initial transcript and the final 4S product.

These intermediates arise by specific endonucleolytic cleavages of the initial transcripts (22, 23). Further analysis of the 4S product showed that base modifications characteristic of tyrosine tRNA had occurred. Thus, in one experiment in which [α-^{32}P]CTP and [α-^{32}P]UTP were used for transcription, pseudouridine, 4-thiouridine, and ribosylthymine (67%, 7%, and 2% of theoretical, respectively) were formed.

[^{3}H]Tyrosine acceptance activity of the 4S product from C1a fragment was demonstrated. Thus, in one experiment 0.4 pmol of C1a fragment was incubated with 2 pmol of RNA polymerase according to the standard method. After incubation with S100 extract 0.04 pmol of [^{3}H]tyrosine was bound to the tRNA.

Table 1. Stimulation of promoter-dependent synthesis by GTP, CTP, and various dinucleoside phosphates

	Incorporation (cpm)	Stimulation (fold)
(a) *GTP*		
GTP, 20 μM	740	—
GTP, 140 μM	2056	2.7
GTP, 400 μM	5114	6.9
GTP, 1200 μM	4761	6.5
ATP, 450 μM	661	—
(b) *CTP*		
CTP, 10 μM	1045	—
CTP, 100 μM	4065	3.85
CTP, 300 μM	3818	3.65
CTP, 1800 μM	4516	4.3
(c) *Dinucleoside phosphates (500 μM)*		
None	1970	—
C-C	8646	4.3
C-G	7785	3.9
G-C	6323	3.2
G-G	1732	—

In 10 μl standard assay mixtures, 0.05 pmol of the fragment C1a was incubated with 0.8 pmol of RNA polymerase at 37° for 45 min. Except for GTP and CTP, whose concentrations are shown, other triphosphates were used at 10 μM concentration. [α-^{32}P]UTP, the radioactive triphosphate, had a specific activity of 10,000 cpm/pmol. The products were analyzed on 6% polyacrylamide gels, the promoter-dependent product being measured by Cerenkov counting of the excised gel bands.

DISCUSSION

The present study has demonstrated the usefulness of DNA fragments containing only one or two genes controlled by a single promoter in a definitive *in vitro* study of the promoter-dependent transcription. The following features of transcription products are noteworthy: (*1*) The singlet gene is transcribed to give mainly a precursor molecule which can be processed to 4S tyrosine acceptor tRNA. (*2*) The two tRNA genes in the doublet are also transcribed as a single product with the first of the two genes specifying the su_{III}^+ tRNA. (*3*) The distance between the two mature tRNA sequences in the primary doublet transcript is about 150 nucleotides. (*4*) The sequence preceding the mature tyrosine tRNA sequence in the second tRNA gene is very different from the known sequence of 41 nucleotides which precedes the mature $tRNA_I^{Tyr}$ sequence in the first gene. (*5*) Similarly, the sequences which immediately follow the C-C-A termini are different in the two $tRNA_I^{Tyr}$ genes. (*6*) Finally, neither the singlet nor the doublet fragments yielded termination of transcription close to the C-C-A terminus with or without the addition of rho factor (24). This could be due either to the absence of the necessary termination sequences on these fragments or the absence of a necessary termination factor. Partial processing of the transcripts with the *E. coli* S100 extract indicated a cleavage at six nucleotides beyond the terminal C-C-A (data not shown) and within a sequence which is capable of forming a 12-base hairpin structure (25). Bikoff and Gefter (23) have also shown that an endonucleo-

Table 2. Effect of CTP concentration on initiation of transcription by [γ-^{32}P]GTP

CTP (μM)	[γ-^{32}P]GTP incorporation (%)
10	100
30	155
50	230
100	90
200	115
500	46
900	30

In standard 10 μl reaction mixtures, 0.15 pmol of B10a fragment was incubated with 0.8 pmol of RNA polymerase for 45 min. Triphosphates other than CTP were at 10 μM [γ-^{32}P]GTP had specific activity of 90,000 cpm/pmol.

lytic cut is made within the first 12 bases following the C-C-A terminus.

The nucleotide sequence of $tRNA_I^{Tyr}$ gene in the promoter region is known (20), as are the corresponding sequences in seven other promoters. Pribnow (26) has pointed out that in these promoter sequences there is a largely conserved seven nucleotide sequence which is separated from the transcription-initiation point by an interval of six or seven nucleotides. This interval is a likely region for the melting of approximately seven base pairs (27) which is postulated to occur at the site of the DNA–enzyme complex prior to initiation (for recent reviews, see refs. 28 and 29). This region in the $tRNA_I^{Tyr}$ gene is composed entirely of G·C base pairs (Fig. 4). The unusual properties of the $tRNA_I^{Tyr}$ promoter reported here, namely the pronounced inhibition by low concentrations of KCl, the sensitivity of the binary complex to heparin, and the absence of any rifampicin-resistant complexes may all be due to the unusual base composition at the above site.

Although biological transcription of the $tRNA_I^{Tyr}$ gene begins with the sequence pppG-C-U- - -, the present experiments with dinucleoside phosphate primers show that there is a region of three to four nucleotides where initiation can occur (Fig. 4). In fact it was interesting that C-C was more efficient than G-C in initiating transcription. This could be due either to a general preference by polymerase for C-C or to a position effect within the promoter region. The nucleotide 10 from the center of the common hepta-nucleotide region seems to be the most favored as a starting point, and it may be significant that this is one full turn of the DNA helix. Further, it seems clear from our results (Table 2 and *text*) that with sufficiently high concentrations of CTP initiation can occur with this pyrimidine triphosphate in place of the normal purine triphosphate (GTP).

Finally, the experiments reported on the primary transcript from the singlet fragment showed (*1*) that it can be fully processed to a 4S tRNA which is capable of accepting tyrosine and (2) that it can undergo base modifications. Clearly, the present system provides unique opportunities for detailed studies of various aspects of tRNA biosynthesis.

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