Pausing of RNA Polymerase during in Vitro Transcription of the Tryptophan Operon Leader Region†

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ABSTRACT: RNA polymerase molecules pause at a single site during in vitro transcription of the tryptophan (*trp*) operon leader region. Pausing was observed when DNA templates derived from *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella aerogenes* were used. Fingerprint analyses showed that the major RNA species produced by the transcriptional pause is 91 nucleotides long. A minor RNA species 90 nucleotides long was also detected. Single-round transcription experiments were used to study the kinetics of pausing. Time course, pulse-chase, and delayed-labeling experiments suggest that every RNA polymerase molecule transcribing the *trp* leader region pauses. At suboptimal ribonucleoside triphosphate concentrations, the half-life of paused-leader RNA was approximately 3 min at 22 °C and 0.7 min at 37 °C. At near-optimal ribonucleoside triphosphate concentrations, the half-life of the paused species dropped to about 0.3 min at 22 °C. The appearance and half-life of the paused species were unaffected by salt concentration, ρ factor, guanosine 3′,5′-bis(diphosphate), or point mutations in the *trp* attenuator region. It is postulated that transcriptional pausing may play a role in maintaining the synchronization of transcription and translation that is vital in the control of transcription termination at the *trp* operon attenuator.

Transcription of the structural genes of the tryptophan (*trp*) operon of *Escherichia coli* and other enteric bacteria is controlled by two independent mechanisms, repression and attenuation [for recent reviews, see Crawford & Stauffer (1980) and Yanofsky (1981)]. Repression involves exclusion of RNA polymerase (RNP)[1] molecules from the *trp* operon promoter by bound *trp* repressor. RNP molecules that escape exclusion by the *trp* repressor transcribe the leader region of the operon. Near the end of the leader region, RNP molecules either terminate transcription at a site called the attenuator and produce a 140-nucleotide transcript (TL-RNA) or continue transcription into the structural genes of the operon.

According to one model, the decision to terminate transcription at the attenuator or to read through into the structural genes of the *trp* operon is believed to be based on coupling of transcription with translation (Lee & Yanofsky, 1977; Zurawski et al., 1978; Oxender et al., 1979). The transcript of the *trp* leader region encodes a 14-residue peptide containing tandem tryptophans (Figure 1). The coupling of transcription termination with translation of the peptide-coding region of leader RNA is thought to be mediated by formation of mutally exclusive stem and loop structures in the RNA (Figure 1). When tryptophan is plentiful, translation of the leader peptide coding region presumably continues past the tandem Trp codons to the stop codon (Figure 1). A ribosome situated at the stop codon would prevent formation of the base-paired structures designated 1:2 and 2:3 (Figure 1) and would allow formation of the base-paired structure designated 3:4. Formation of the 3:4 structure is thought to signal transcription termination at the attenuator. In contrast, when tryptophan is limiting, the intracellular level of charged $tRNA^{Trp}$ drops, and ribosomes synthesizing the leader peptide presumably stall at either of the tandem Trp codons. A ribosme stalled at the Trp codons would prevent 1:2 pairing but would allow 2:3 pairing. Pairing of segment 2 with segment 3 would allow read-through, because 2:3 pairing prevents the 3:4 pairing necessary for transcription termination. Finally, in the absence of translation, the 1:2 structure presumably forms. Pairing of segment 1 with segment 2 excludes 2:3 pairing which in turn allows the 3:4 termination structure to form [see Yanofsky (1981)].

In the above model, synchronization of transcription and translation is essential in the regulation of transcription termination at the attenuator. The results presented in this paper show that RNP molecules pause during in vitro transcription of the *trp* leader region. Pausing could be involved in establishing the synchronization of transcription and translation that is crucial in the control of transcription termination at the *trp* attenuator.

Experimental Procedures

Materials. α-^{32}P-Labeled CTP, GTP, and UTP with specific activities of $\simeq$400 Ci/mmol were purchased from Amersham. The lithium salt of guanosine 3′, 5′-bis(diphosphate) (ppGpp) was purchased from P-L Biochemicals. Restriction fragments used as templates for transcription contained the entire *trp* promoter, leader, and attenuator (*trpPOL*) regions and the initial part of *trpE*. The *E. coli* $HpaII_{570}$ wild-type restriction fragment was prepared from plasmid pPS21 as previously described (Oxender et al., 1979). $HpaII_{570}$ restriction fragments carrying *trp* attenuator mutations were prepared from plasmid pVH153 derivatives following digestions with *Hpa*II and *Eco*RI (Stauffer et al., 1978; Zurawski et al., 1978; Zurawski & Yanofsky, 1980). The *Salmonella typhimurium* $HinfI_{500}$ restriction fragment was prepared from plasmid pKB5 (Lee & Yanofsky, 1977). An analogous restriction fragment from *Klebsiella aerogenes* was

† From the Department of Biological Sciences, Stanford University, Stanford, California 94305. *Received December 18, 1980.* This work was supported by grants from the U.S. Public Health Service (GM 09738), the National Science Foundation (PCM 77-24333), and the American Heart Association (69C-15). M.E.W. is a Helen Hay Whitney Postdoctoral Fellow. C.Y. is a Career Investigator of the American Heart Association.

[1] Abbreviations used: BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; PL-RNA, paused-leader transcript; ppGpp, guanosine 3′,5′-bis(diphosphate); RNP, RNA polymerase; RT-RNA, read-through transcript; TBE, 0.09 M Tris–borate (pH 8.3) and 2.5 mM EDTA buffer; TL-RNA, terminated-leader transcript; *trpPOL*, the *trp* operon promoter–operator–leader region.

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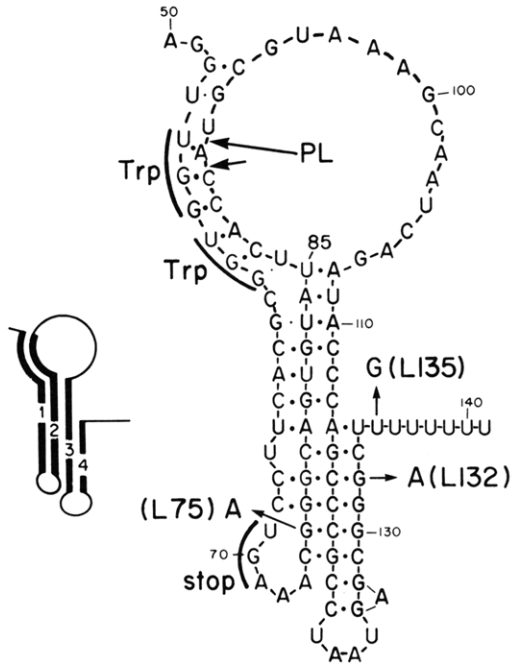

FIGURE 1: 3′ terminus of *E. coli* terminated *trp* leader RNA (TL-RNA). Nucleotide numbers are relative to the 5′ end of the transcript. The tandem Trp codons and stop codon for the peptide encoded by TL-RNA are indicated. Proposed alternate secondary structures are designated by numbering the RNA segments which participate in the hydrogen-bonded stems (i.e., 1:2 refers to the stem and loop structure in which segment 1 is hydrogen bonded to segment 2). The base changes in the *trpL75*, *trpL132*, and *trpL135* mutants are shown. "PL" indicates the two 3′ termini of the paused-leader RNA. Arrow size reflects the relative amounts of the paused species detected.

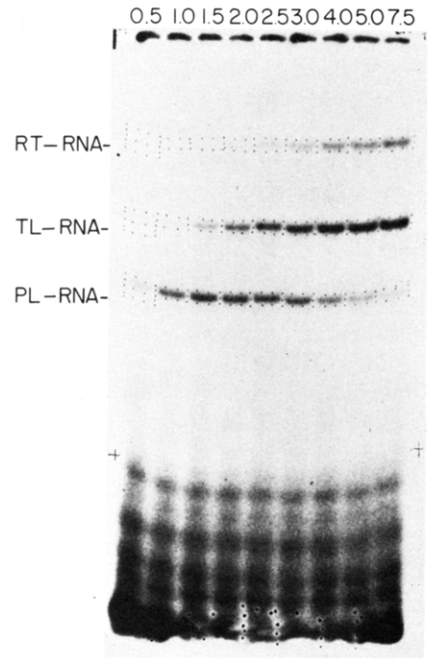

FIGURE 2: Gel electrophoresis of products synthesized in a single round of transcription under [α-^{32}P]GTP-labeling conditions. Incubation was at 22 °C with the *E. coli* wild-type *Hpa*II$_{570}$ restriction fragment as template. Additions to the standard reaction mixture (see Experimental Procedures) included 20 μM [α-^{32}P]GTP (50 μCi) and 150 μM each of ATP, CTP, and UTP. The final reaction volume was 100 μL. After the start of transcription, 10-μL aliquots of the reaction mixture were removed at the times indicated (in minutes) and added to stop solution. Transcription products were analyzed on an 8% TBE–urea denaturing gel (see Experimental Procedures). RT-RNA (260 nucleotides long), TL-RNA (140 nucleotides long), and PL-RNA (91 nucleotides long) are indicated.

provided by M. Blumenberg. Restriction fragment concentrations were estimated by absorbance at 260 nm assuming an extinction coefficient of 20 mg^{-1} cm^2. DNA concentrations were corrected for soluble acrylamide by subtracting the absorbance of a blank prepared by extracting gel slices that lacked DNA. RNA polymerase holoenzyme was prepared by the method of Gonzalez et al. (1977). ρ factor was prepared by the method of Finger and Richardson [referenced in Richardson & Conaway (1980)]. Both the RNP holoenzyme and ρ factor were homogeneous on NaDodSO$_4$ gel electrophoresis. Protein concentrations were measured by absorbance at 280 nm assuming an $E^{1\%}_{280nm}$ of 6.2 for RNP holoenzyme (Lowe et al., 1979) and an $E^{1\%}_{280nm}$ of 4 for ρ factor (Galluppi & Richardson, 1980). Activity of the ρ factor was demonstrated by measuring poly(C)-dependent ATPase (Lowery & Richardson, 1977) and transcription termination with bacteriophage λ DNA (Rosenbert et al., 1975).

Single-Round Transcription Experiments and Gel Electrophoresis. The standard transcription reaction mixture contained 36 mM Tris–acetate or HCl, 0.1 mM Na$_2$EDTA, 0.1 mM DTT, 4 mM MgCl$_2$, 150 mM KCl, 5% glycerol, 20 μg BSA/mL, and 5 μg of restriction fragment/mL. The pH of the standard reaction mixture was 7.8 at 22 °C, 7.5 at 31 °C, and 7.2 at 37 °C. In single-round transcription experiments, RNP was added to a standard reaction mixture at 4 °C which contained ATP and GTP as the only ribonucleoside triphosphates. The final RNP concentration was generally 7.2 μg/mL, which was nearly equimolar with the *Hpa*II$_{570}$ restriction fragment. The reaction mixture + RNP was transferred to a second tube containing freshly dried, labeled ribonucleoside triphosphate. Initiation complexes were allowed to form for 15 min at the reaction temperature (usually 22 °C). Single-round transcription was started by adding a prewarmed solution containing CTP, UTP, and rifampicin (10 μg/mL final concentration). Final ribonucleoside triphosphate concentrations, isotope concentrations, and reaction volumes are given in the figure legends. Thereafter, aliquots of the reaction mixture were removed at different times and added to an equal volume of stop solution (Oxender et al., 1979), heated for 3 min at 90 °C, and loaded directly on either 6% or 8% polyacrylamide–TBE–urea denaturing slab gels (Oxender et al., 1979). Transcription products were detected by autoradiography, cut from the gels, and counted directly by Cerenkov radiation. The efficiency of counting a gel slice was approximately 60%.

Fingerprint Analysis. Labeled RNA species were cut from gels, extracted, and digested to completion with RNase T1 as previously described (Squires et al., 1976). Oligonucleotides generated by RNase T1 digestion were resolved in two dimensions by using high voltage electrophoresis followed by homochromatography (Squires et al., 1976). Oligonucleotide identities were based on previous assignments (Squires et al., 1976; Oxender et al., 1979).

Results

Pausing at Suboptimal Ribonucleoside Triphosphate Concentrations. Single-round transcription experiments were performed in which transcripts of the *E. coli Hpa*II$_{570}$ restriction fragment were synthesized in the presence of 20 μM [α-^{32}P]GTP and 150 μM each of the other ribonucleoside triphosphates. Aliquots of this reaction mixture were removed at different times, and the transcription products were analyzed by gel electrophoresis (Figure 2). At early time points, the 140-nucleotide terminated-leader RNA (TL-RNA) and the 260-nucleotide read-through RNA (RT-RNA) (Lee & Yanofsky, 1977) were nearly absent. However, a third RNA

species, smaller than TL-RNA, was prominent (band PL-RNA). With time, PL-RNA accumulated and then disappeared. TL-RNA and RT-RNA accumulated in significant amounts only after the PL-RNA band decreased in intensity. This accumulation pattern suggests that RNP molecules pause during transcription of the *trp* leader region before reaching the attenuator. In the experiment depicted in Figure 2, the GTP concentration employed was most likely below the apparent, intrinsic Michaelis–Menten constant (K_s) for GTP in transcriptional elongation on native templates (see Discussion). Nevertheless, even at this suboptimal GTP concentration, only one major paused species appeared. PL-RNA was observed at both 50 mM KCl (data not shown) and 150 mM KCl (Figure 2). Farnham & Platt (1981) have independently noted the formation of PL-RNA in single-round transcription reactions.

Experiments analogous to the one shown in Figure 2 were performed with 20 μM [α-^{32}P]UTP and 150 μM each of ATP, CTP, and GTP (data not shown) instead of 20 μM [α-^{32}P]-GTP and 150 μM each of ATP, CTP, and UTP. A major paused species was present at the same position in the gel as the PL-RNA band in Figure 2. In addition, two minor paused species were detected. These minor species, which were not observed when 20 μM [α-^{32}P]GTP and 150 μM each of ATP, CTP, and UTP (Figure 2) were used, were smaller than PL-RNA and had considerably shorter half-lives.

Single-round transcription experiments similar to the one presented in Figure 2 were performed with *trpPOL*-containing restriction fragments derived from *Salmonella typhimurium* or *Klebsiella aerogenes* (data not shown). In each case, a single major paused species was present in the gel at the same relative position as the PL-RNA band observed with *E. coli* DNA (Figure 2). With the *S. typhimurium* and *K. aerogenes* templates, TL-RNA and RT-RNA also did not accumulate in significant amounts until the PL-RNA species began to disappear.

Sequence of E. coli PL-RNA. The sequence of PL-RNA was established by RNA fingerprint analysis. Fingerprints of RNase T1 digests of intact TL-RNA and PL-RNA labeled with [α-^{32}P]GTP are presented in Figure 3, A and B, respectively. The spots in Figure 3B form a subset of the spots in Figure 3A. In Figure 3B, spot 5 is present and spot 43 is absent. This indicates that PL-RNA extends from the normal 5′ terminus of the *trp* transcript to between nucleotides 81 and 93 (Figure 1).

Fingerprints of [α-^{32}P]UTP-labeled TL-RNA and PL-RNA are presented in Figure 3, C and D, respectively. Spots X and Y in Figure 3C correspond to the two TL-RNA 3′ termini, CU_7-OH and CU_8-OH, respectively (Bertrand et al., 1977). Figure 3D contains spot 13 but lacks spot 43. This indicates that the PL-RNA extends at least to nucleotide 83 (Figure 1). Additionally, two spots appear in the PL-RNA UTP fingerprint (spots a and b, Figure 3D) which are absent from the PL-RNA GTP fingerprint (Figure 3B). Therefore, spots a and b must correspond to the 3′-oligonucleotides of PL-RNA. Since 3′-oligonucleotides migrate according to size rather than base composition in the homochromatographic dimension (Bertrand et al., 1977), the mobilities of spots a and b relative to spots X and Y suggest that spot a is UAUUCACC-OH and spot b is UAUUCACCA-OH. Thus, the major PL-RNA species appears to be 91 nucleotides long, while a minor PL-RNA species is probably 90 nucleotides long (Figure 1). The ratio of the major PL-RNA species to the minor PL-RNA species estimated from spots a and b is about 3 to 1 (Figure 3D).

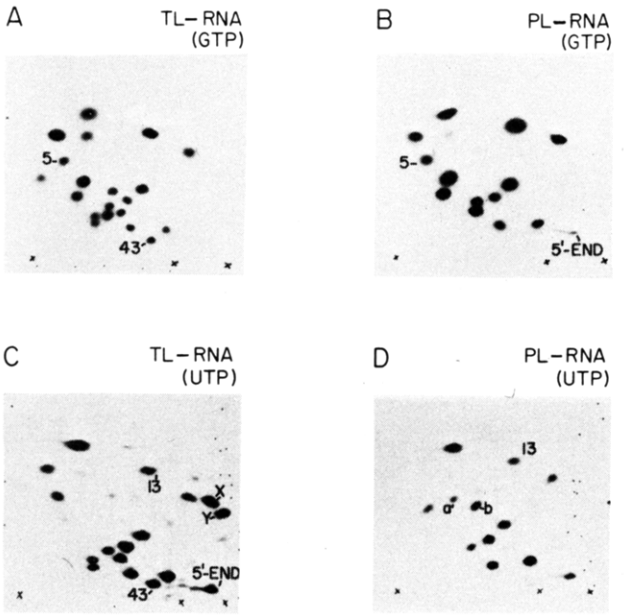

FIGURE 3: RNase T1 fingerprint analysis of TL-RNA and PL-RNA labeled separately with [α-^{32}P]GTP and [α-^{32}P]UTP. TL-RNA and PL-RNA were synthesized preparatively for 1 min in single-round transcription experiments like the one depicted in Figure 2. For the [α-^{32}P]UTP labeling, the reaction mixture contained 20 μM [α-^{32}P]UTP (50 μCi) and 150 μM each of ATP, CTP, and GTP. Spot assignments are discussed under Results. High-voltage electrophoresis was from left to right, and homochromatography was from bottom to top. (A) TL-RNA labeled with [α-^{32}P]GTP; (B) PL-RNA labeled with [α-^{32}P]GTP; (C) TL-RNA labeled with [α-^{32}P]UTP; (D) PL-RNA labeled with [α-^{32}P]UTP.

Pausing at Near-Optimal Ribonucleoside Triphosphate Concentrations. To determine whether the appearance of PL-RNA was caused by the use of suboptimal ribonucleoside triphosphate concentrations (see Discussion), single-round transcription experiments were performed at several ribonucleoside triphosphate concentrations. PL-RNA appeared when the transcription reaction contained 40 μM [α-^{32}P]GTP and 150 μM each of the other ribonucleoside triphosphates. However, attempts to detect PL-RNA in reactions containing 150 μM each of the four ribonucleoside triphosphates were unsuccessful, because contaminants in the [α-^{32}P]GTP, which comigrated with PL-RNA on gels, became too prominent at the high isotope concentrations required to maintain the [α-^{32}P]GTP specific activity in the transcription reactions. Nevertheless, pausing could be inferred from a delay in the appearance of TL-RNA and RT-RNA. Similar transcription experiments with 400 μM [α-^{32}P]UTP and 150 μM each of the other ribonucleoside triphosphates did produce a faint band on gels at a position corresponding to PL-RNA. At high isotope concentrations, contaminants in the [α-^{32}P]UTP also gave a heavy background on the gels. As with GTP labeling, accumulation of [α-^{32}P]UTP-labeled TL-RNA and RT-RNA was delayed. Single-round transcription experiments were also performed in the presence of 30 μM [α-^{32}P]CTP, 150 μM each of ATP and GTP, and 400 μM UTP (Figure 4). The apparent K_s for CTP in the transcriptional elongation reaction is considerably lower than the K_s values for the other ribonucleoside triphosphates (Chamberlin et al., 1976; Kingston et al., 1981; see Discussion). PL-RNA was clearly synthesized under these conditions, although it disappeared rapidly.

Accumulation and Disappearance of PL-RNA. Figure 5 presents a quantitative analysis of the accumulation and disappearance of the three *trp* RNA species synthesized in single-round transcription experiments performed at 22 °C

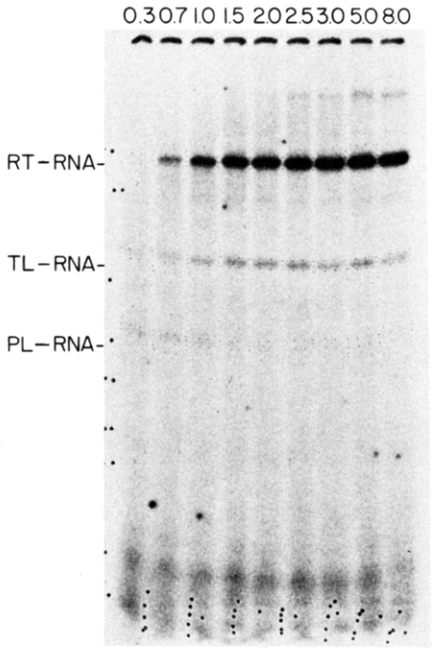

FIGURE 4: Gel electrophoresis of products synthesized in a single round of transcription using [α-^{32}P]CTP-labeling conditions. Incubation was at 22 °C with the *E. coli trpL75L135 HpaII*$_{570}$ restriction fragment as template. Additions to the standard reaction mixture (see Experimental Procedures) included 30 μM [α-^{32}P]CTP (50 μCi), 150 μM ATP, 150 μM GTP, and 400 μM UTP. The final reaction volume was 100 μL. Aliquots of 10 μL of the reaction mixture were sampled at the indicated times (in minutes) and analyzed by gel electrophoresis as detailed in the legend to Figure 2. RT-RNA, TL-RNA, and PL-RNA are indicated.

in the presence of 20 μM [α-^{32}P]GTP, 150 μM each of ATP, CTP, and UTP, and wild-type or mutant templates. In Figure 5A, the wild-type restriction fragment was the template, while in Figure 5B-D, restriction fragments containing *trp* attenuator mutations (see Figure 1) were transcribed. The *trpL132* mutation is a G → A change which destablizes the 3:4 paired structure and allows increased read-through transcription beyond the attenuator in vivo and in vitro (Stauffer et al., 1978). The *trpL75* mutation is a G → A change which both stabilizes 1:2 pairing by about −4 kcal and destabilizes 2:3 pairing (Zurawski et al., 1978). The *trpL75L135* template contains both the *trpL75* and *trpL135* mutations. The *trpL135* mutation is a T → G change in the A·T-rich region of the attenuator. The *trpL75L135* double mutant shows high-level read-through transcription in vivo and in vitro (Zurawski & Yanofsky, 1980).

Common patterns for the accumulation and disappearance of the *trp* RNA species can be seen in Figure 5. PL-RNA accumulated before either TL-RNA or RT-RNA. Significant accumulation of TL-RNA and RT-RNA did not occur until after PL-RNA had begun to disappear. PL-RNA decreased exponentially with an estimated half-life of 3.0 ± 0.2 min at 22 °C (Figure 5), 1.6 min at 31 °C, and 0.7 min at 37 °C (data not shown). None of the mutations studied affected the appearance or half-life of PL-RNA (Figure 5). In most cases, the maximum molar amount of PL-RNA, corrected for the early appearance of small amounts of TL-RNA and RT-RNA, approximated the sum of the final molar amounts of TL-RNA and RT-RNA. These observations suggest that every RNP molecule that transcribes the leader region pauses after synthesizing PL-RNA. Furthermore, accumulation of RT-RNA lagged slightly behind accumulation of TL-RNA. The lag was most reliably observed in experiments with fragments containing high read-through mutations (Figure 5B,D). This finding suggests that some RNP molecules may pause briefly at the attenuator before synthesizing RT-RNA.

Accumulation and disappearance of the RNA species were studied in experiments in which labeling was performed for only 30 s at the beginning of the transcription reaction period (pulse chase; Figure 6A) and in experiments in which the label was added at various times after beginning the transcription reaction (delayed labeling; Figure 6B). In pulse-chase experiments (Figure 6A), PL-RNA was still detected at the high ribonucleoside triphosphate concentrations present during the chase period. However, the half-life of PL-RNA was reduced appreciably (see Discussion). Labeled TL-RNA and RT-RNA did not accumulate in significant amounts until after the labeled PL-RNA disappeared (Figure 6A). Accumulation

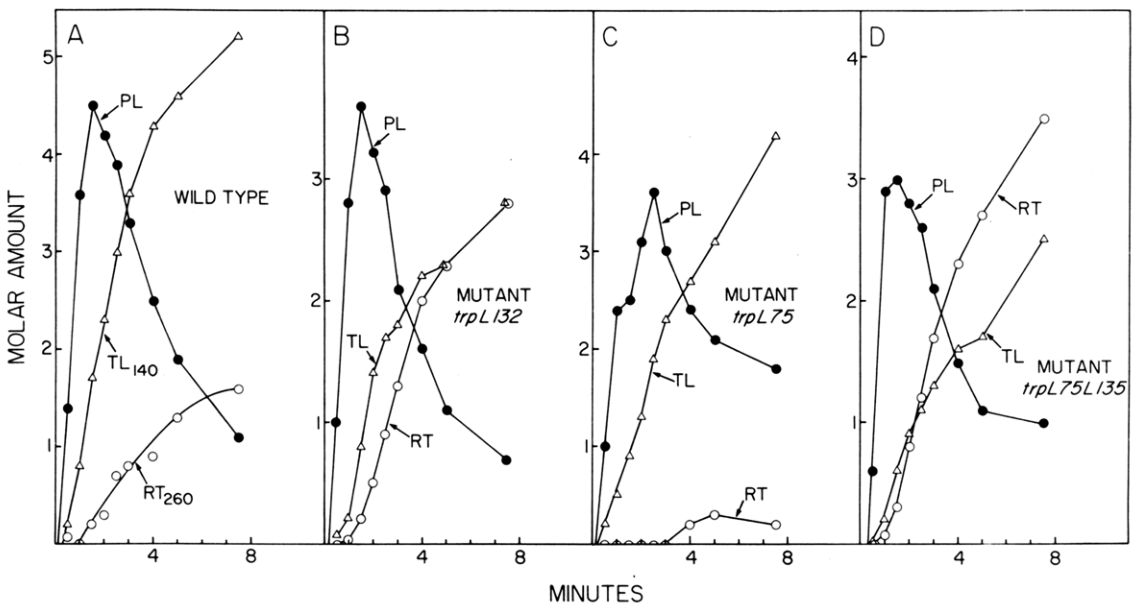

FIGURE 5: Time course of appearance and disappearance of RNA species labeled with [α-^{32}P]GTP in single-round transcription experiments. Experiments were performed exactly as described in the legend of Figure 2 with the following *E. coli HpaII*$_{570}$ resitriction fragments as templates: (A) wild type; (B) mutant *trpL132*; (C) mutant *trpL75*; (D) mutant *trpL75L135*. Transcription products were analyzed by gel electrophoresis and bands corresponding to PL-RNA (●), TL-RNA (△), and RT-RNA (○) were cut from the gels and counted. Data in (A) were taken from the gel shown in Figure 2. Multiplication of ordinate values by 3 × 10^{-17} gives actual molar amounts.

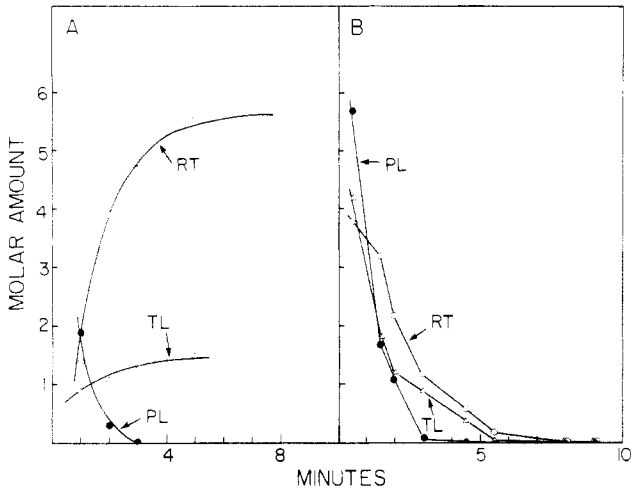

FIGURE 6: Pulse-chase (A) and delayed-labeling (B) single-round transcription experiments. Transcription products were analyzed by gel electrophoresis, cut from the gels, and counted. (A) Single-round transcription of the *E. coli trpL75L135 Hpa*II$_{570}$ restriction fragment was performed in a final volume of 200 μL at 22 °C. Additions to the standard reaction mixture (see Experimental Procedures) included 20 μM [α-^{32}P]GTP (160 μCi) and 150 μM each of ATP, CTP, and UTP. After 30 s, unlabeled GTP was added to 400 μM. Aliquots of 40 μL of the reaction mixture were removed to stop solution at the times indicated. (B) A single-round transcription reaction with the *trpL75L135* template was started at 22 °C in a final volume of 100 μL. Additions to the standard reaction mixture included 20 μM GTP and 150 μM each of ATP, CTP, and UTP. [α-^{32}P]GTP was initially omitted. At the times indicated, 12 μL of reaction were added to 10 μCi of [α-^{32}P]GTP. Incubation at 22 °C was continued 2 min longer before the reaction was stopped. The 2-min labeling period was less than the half-life of PL-RNA (see Results). (●) PL-RNA; (△) TL-RNA; (○) RT-RNA. Multiplication of ordinate values by 1.8 × 10^{-17} gives actual molar amounts.

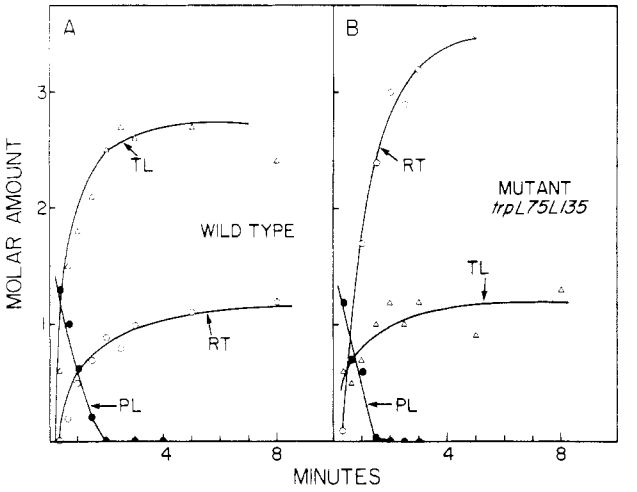

FIGURE 7: Time course of appearance and disappearance of RNA species labeled with [α-^{32}P]CTP in single-round transcription experiments. Experiments were performed exactly as detailed in the legend to Figure 4 with wild-type (A) and mutant *trpL75L135* (B) *Hpa*II$_{570}$ restriction fragments as templates. Data in (B) are taken from the gel shown in Figure 4. Bands were cut from gels and counted. Multiplication of the ordinate values by 4.4 × 10^{-17} gives actual molar amounts. (●) PL-RNA; (△) TL-RNA; (○) RT-RNA.

of RT-RNA again lagged slightly behind accumulation of TL-RNA. In delayed-labeling experiments (Figure 6B), there was very little synthesis of PL-RNA 2 min after the start of transcription. Two minutes corresponds to the approximate interval after which PL-RNA started to disappear in single-round transcription experiments (Figure 5D). In contrast, synthesis of TL-RNA and RT-RNA continued for nearly 5 min after the start of transcription. At the earliest point, the molar amount of RT-RNA was less than the molar amount of TL-RNA (Figure 6B).

Quantitation of accumulation and disappearance of *trp* RNA species in transcription experiments performed in the presence of 30 μM [α-^{32}P]CTP, 150 μM each of ATP and GTP, and 400 μM UTP is shown in Figure 7. In Figure 7, there is a direct relationship between disappearance of PL-RNA and accumulation of TL-RNA and RT-RNA. Accumulation of RT-RNA lagged slightly behind accumulation of TL-RNA. Compared to Figure 5, A and D, Figure 7, A and B, shows a more rapid accumulation of TL-RNA and RT-RNA and a shorter half-life for PL-RNA. In the transcription experiments shown in Figure 7, the half-life of PL-RNA was only about 0.3 min at 22 °C.

Accumulation and disappearance of PL-RNA were examined in the presence of factors known to affect transcriptional pausing in other systems (see Discussion). Termination factor ρ did not affect the appearance or half-life of PL-RNA in single-round transcription reactions completed at 31 °C in the presence of 20 μM [α-^{32}P]GTP, 400 μM ATP, 150 μM CTP, 150 μM UTP, and 50 mM KCl. ρ factor and RNP were each present at final concentrations of 8 μg/mL. Guanosine 3′,5′-bis(diphosphate) at a final concentration of 0.2 mM also did not affect the appearance or half-life of PL-RNA in experiments analogous to the one shown in Figure 2. ppGpp did, however, cause a general inhibition of in vitro transcription of the *trpPOL* template (data not shown).

Discussion

The experiments presented show that RNP molecules pause at a single site during in vitro transcription of the *trp* leader region. Time course (Figures 2, 4, 5, and 7) and pulse-chase (Figure 6A) analyses in single-round transcription experiments established a direct relationship between the disappearance of paused-leader RNA (PL-RNA) and the appearance of terminated-leader RNA (TL-RNA) and read-through RNA (RT-RNA). This pattern of accumulation and disappearance suggests that every transcribing RNP molecule pauses and that PL-RNA is an obligatory precursor of both TL-RNA and RT-RNA. The results of delayed-labeling experiments are consistent with this interpretation; synthesis of TL-RNA and RT-RNA continued for nearly 5 min after transcription was started (Figure 6B). Farnham & Platt (1981) have also observed the production of PL-RNA in single-round transcription experiments in vitro.

Two classes of transcriptional pause sites have been noted in studies with other systems. One class consists of short GC-rich regions in the DNA template [summarized in Gilbert (1976)]. These GC-rich regions are located immediately upstream from the 3′ termini of the paused transcripts. A second class of pause sites contains regions of dyad symmetry which do not have to be particularly GC rich. Like the GC-rich regions, these symmetrical sequences are located upstream from the 3′ termini of the paused transcripts. Regions of dyad symmetry are thought to mediate transcriptional pausing by allowing secondary structures to form either in the nascent RNA chains (Adhya & Gottesman, 1978; Rosenberg et al., 1978) or in the displaced DNA strand of the template (Kassavetis & Chamberlin, 1981).

Fingerprint analyses indicated that the major PL-RNA species is 91 nucleotides long while a minor PL-RNA species is 90 nucleotides long. The DNA region corresponding to the 3′ termini of PL-RNA is immediately preceded by both a GC-rich region and a region of dyad symmetry. This dyad symmetry corresponds to the 1:2 paired structure that can form

in the nascent *trp* RNA (Figure 1). It is not known whether the GC-rich region or the 1:2 paired structure or both are responsible for the pause in transcription in the *trp* leader region. The *trpL75* mutation is the only known change in this region of the template (Figure 1). This mutation reduces the G + C content of the GC-rich region and increases the stability of the 1:2 paired structure in the RNA by allowing an additional U·A hydrogen bond to form. The *trpL75* mutation did not affect the appearance or half-life of PL-RNA (Figures 5C,D and 7B). PL-RNA was observed in transcription experiments with the *K. aerogenes trpPOL* template despite the fact that the predicted stability of the 1:2 paired structure is considerably reduced in *K. aerogenes trp* RNA as compared to *E. coli trp* RNA (M. Blumenberg and C. Yanofsky, unpublished results). *E. coli* attenuator mutations *trpL132* and *trpL135*, which are distal to the PL-RNA termini, did not affect pausing (Figures 5B,D and 7B). Lastly, neither termination factor ρ nor ppGpp altered the accumulation or disappearance of PL-RNA under the conditions tested (see Results). ρ factor has been shown to cause termination and transcript release at pause sites (Rosenberg et al., 1978). However, not all pause sites are ρ release sites (Kassavetis & Chamberlin, 1981). Likewise, ppGpp enhances pausing at some, but not all, pause sites (Kingston et al., 1981).

It has been shown in other systems that the duration of pausing at specific sites is influenced by the concentrations of ribonucleoside triphosphates present during transcription (Maizels, 1973; Kassavetis & Chamberlin, 1981). Pause half-lives are generally lengthened in transcription experiments carried out at ribonucleoside triphosphate concentrations that are substantially below the K_s values for transcriptional elongation (Kassevetis & Chamberlin, 1981). The K_s values for RNA chain elongation on native templates have been estimated to be at least 150 μM for ATP, GTP, and UTP and about 50 μM for CTP (Kingston et al., 1981). Consistent with these observations and K_s values, the half-life of PL-RNA synthesized in the presence of 30 μM CTP and at least 150 μM each of the other ribonucleoside triphosphates (0.3 min; Figure 7) was 10-fold shorter than the half-life of PL-RNA synthesized in the presence of 20 μM GTP and 150 μM each of the other ribonucleoside triphosphates (3.0 min; Figure 5). Similarly, PL-RNA decayed rapidly during a chase in the presence of relatively high concentrations of all four ribonucleoside triphosphates (Figure 6A). Because of this marked dependence on ribonucleoside triphosphate concentration, changes in half-life of PL-RNA observed in transcription reactions containing normal substrates or base analogues must be examined as a function of ribonucleoside triphosphate concentration.

Conservation of a pause site at the same position in the leader regions of *E. coli*, *K. aerogenes*, and *S. typhimurium* suggests that pausing may play a role in *trp* operon regulation. Synchronization of transcription and translation is vital to the regulation of transcription termination at the *trp* attenuator (see introduction). A transcriptional pause after 91 nucleotides of the *trp* transcript are synthesized could ensure synchronization by allowing a ribosome sufficient time to bind to the leader transcript, initiate translation, approach the RNP molecule, and thereby participate in the decision to terminate transcription at or continue transcription beyond the *trp* attenuator.

The frequency of transcription initiation at the *trp* promoter has been estimated in bacterial cells which lack a functional *trp* repressor (*trpR*). In such bacteria growing at 37 °C in glucose-minimal medium, it has been estimated that RNP molecules initiate transcription on the *trp* promoter about once every 6 s (Bertrand et al., 1976). For pausing not to interfere with *trp* operon expression in vivo, the half-life expected for PL-RNA would have to be at most a few seconds. The half-life of PL-RNA measured in vitro is compatible with the short half-life anticipated in vivo. Since the half-life of PL-RNA is about 4.3-fold shorter at 37 °C than at 22 °C (see Results), the half-life for PL-RNA synthesized in vitro at near optimal ribonucleoside triphosphate concentrations would be expected to decrease from 18 s (0.3 min) at 22 °C (Figure 7) to approximately 4 s at 37 °C. Because of its short half-life and the fact that it does not accumulate, PL-RNA would be difficult to detect in vivo.

The data in Figures 5–7 suggest that those RNP molecules that do not terminate transcription at the attenuator may, however, pause briefly at this site. Appearance of RT-RNA always lagged behind appearance of TL-RNA by an interval of time that exceeds what would be expected for transcription of the 120-nucleotide difference in length between TL-RNA and RT-RNA. Accumulation of RT-RNA was similar in transcription reactions using templates containing either the *trpL132* mutation (Figure 5B), which destabilizes the 3:4 paired structure, or the *trpL135* mutation (Figure 5D), which might stabilize the pairing between the U-rich region of *trp* RNA and its complementary segment in the DNA template. Since aditional bands between PL-RNA and TL-RNA were not generally observed on gels, it seems likely that the second paused RNA species is about the same size as TL-RNA (140 nucleotides long). Delayed-labeling experiments were consistent with this interpretation, because the molar amount of RT-RNA was less than the molar amount of TL-RNA at the earliest time point taken (Figure 6B). Accumulation data (Figures 5, 6A, and 7) suggest that the presumed pause at position 140 would have a considerably shorter half-life than the pause at position 91. Transcription studies in vitro using a template containing a deletion of part of the A·T-rich region of the attenuator (*trpΔLC1419*) also indicate that RNP molecules pause briefly at position 140 during read-through transcription beyond the attenuator (Farnham & Platt, 1981).

Acknowledgments

We thank our colleagues in this laboratory for supplying bacterial strains and purified plasmids. We also thank them for useful discussions and critical reading of this paper. We appreciate the exchange of ideas and findings with Michael Chamberlin and his co-workers.

References

Adhya, S., & Gottesman, M. (1978) *Annu. Rev. Biochem. 47*, 967–996.

Bertrand, K., Squires, C., & Yanofsky, C. (1976) *J. Mol. Biol. 103*, 319–337.

Bertrand, K., Korn, L. J., Lee, F., & Yanofsky, C. (1977) *J. Mol. Biol. 117*, 227–247.

Chamberlin, M. J., Mangel, W., Rhodes, G., & Stahl, D. (1976) in *Control of Ribosome Synthesis—Alfred Benzon Symposium IX* (Kjeldgaard, N. C., & Maaloe, O., Eds.) pp 22–39, Academic Press, New York.

Crawford, I. P., & Stauffer, G. V. (1980) *Annu. Rev. Biochem. 49*, 163–195.

Farnham, P. J., & Platt, T. (1981) *Nucleic Acids Res. 9*, 563–577.

Galluppi, G., & Richardson, J. P. (1980) *J. Mol. Biol. 138*, 513–539.

Gilbert, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 193–205, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY.
Gonzalez, N., Wiggs, J., & Chamberlin, M. J. (1977) *Arch. Biochem. Biophys. 182*, 404-408.
Kassavetis, G. A., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2777-2786.
Kingston, R. E., Nierman, W. C., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2787-2797.
Lee, F., & Yanofsky, C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4365-4369.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344-1352.
Lowery, C., & Richardson, J. P. (1977) *J. Biol. Chem. 252*, 1375-1380.
Maizels, N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3585-3589.
Oxender, D. L., Zurawski, G., & Yanofsky, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5524-5528.
Richardson, J. P., & Conaway, R. (1980) *Biochemistry 19*, 4293-4299.
Rosenberg, M., Weissman, S., & de Crombrugghe, B. (1975) *J. Biol. Chem. 250*, 4755-4764.
Rosenberg, M., Court, D., Shimatake, H., Brady, C., & Wulff, D. L. (1978) *Nature (London) 272*, 414-423.
Squires, C., Lee, F., Bertrand, K., Squires, C. L., Bronson, M. J., & Yanofsky, C. (1976) *J. Mol. Biol. 103*, 351-381.
Stauffer, G. V., Zurawski, G., & Yanofsky, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4833-4837.
Yanofsky, C. (1981) *Nature (London) 289*, 751-758.
Zurawski, G., & Yanofsky, C. (1980) *J. Mol. Biol. 142*, 123-129.
Zurawski, G., Elseviers, D., Stauffer, G. V., & Yanofsky, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5988-5992.

Inhibition of the *Bam*HI Cleavage and Unwinding of pBR322 Deoxyribonucleic Acid by the Antitumor Drug *cis*-Dichlorodiammineplatinum(II)†

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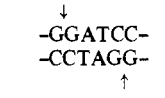
ABSTRACT: The antitumor drug *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) binds to pBR322 DNA and inhibits the cleavage of this circular DNA into a linear form by the restriction endonuclease *Bam*HI. The binding of platinum to DNA was monitored by agarose gel electrophoresis, and the amount of platinum bound per nucleotide (r_b) was measured by carbon rod atomic absorption spectroscopy. Electrophoretic mobility changes reflect a shortening and unwinding of the DNA duplex upon platinum binding as observed previously for the reaction of *cis*- and *trans*-DDP with pSM1 DNA [Cohen, G. L., Bauer, W. R., Barton, J. K., & Lippard, S. J. (1979) *Science (Washington, D.C.) 203*, 1014-1016]. The inhibition of *Bam*HI nuclease activity occurs at very low binding levels and is complete at r_b = 0.045. This value corresponds to the binding of one platinum atom within ±3 base pairs of the recognition sequence of the enzyme shown below. Treatment of the DNA with 0.2 M sodium cyanide after *Bam*HI cutting removes the platinum but does not alter the point at which *cis*-DDP inhibits the formation of the linear form III DNA. This result is in contrast with a previous report claiming that *Bam*HI could cut across a *cis*-DDP-induced GpG cross-link in DNA which could be subsequently revealed by cyanide reversal of platinum binding. When the platinum is removed by cyanide treatment, the drug-induced mobility changes are reversed and there is a pronounced sharpening of the bands in the gel. Quantitative study of the cyanide reversal shows the presence of a small amount of unremovable platinum tightly bound to the DNA at high ratios (~0.1) of bound platinum per nucleotide.



Although the site of cytotoxic action of the antitumor drug *cis*-DDP[1] (Rosenberg et al., 1969) is believed to be DNA (Roberts & Thomson, 1979), the exact nature of the platinum-induced lesion remains unknown. The antineoplastic activity of *cis*-DDP compared with the inactivity of the trans isomer suggests bifunctional coordination of the platinum drug to DNA utilizing its cis geometry: for example, intrastrand cross-linking of two adjacent guanine or cytosine bases (Roberts & Thomson, 1979; Kelman et al., 1977). DNA interstrand cross-links occur but at platinum binding levels greater than those required for cytotoxic action (Shooter et al., 1972; Munchausen, 1974).

We previously demonstrated (Cohen et al., 1979) that *cis*-DDP unwinds and shortens closed circular, supercoiled

† From the Department of Chemistry, Columbia University, New York, New York 10027. *Received September 11, 1980.* This work was supported by U.S. Public Health Service Grant CA-15826 and National Research Service Award (to T.D.T.) No. CA-06406, both from the National Cancer Institute, and by a National Institutes of Health National Research Service Award (to H.M.U.), Training Grant No. GM-07216.

[1] Abbreviations used: DDP, dichlorodiammineplatinum(II); form I DNA, covalently closed circular duplex DNA; form II DNA, nicked circular duplex DNA; form III DNA, linear duplex DNA; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TBE, 90 mM Tris base, 90 mM boric acid, and 2.2 mM EDTA, pH 8.3; BSA, bovine serum albumin; P_0, concentration of nucleotide phosphate determined at 260 nm by using 6600 M^{-1} cm^{-1} as the extinction coefficient; C_0, the initial concentration of platinum species in the reaction; C_b, the concentration of platinum bound to nucleic acid; r_f, ratio of C_0 to P_0; r_b, ratio of C_b to P_0; AAS, atomic absorption spectroscopy; DTT, dithiothreitol; EtdBr, ethidium bromide; σ_0, the superhelix density.

0006-2960/81/0420-3744$01.25/0