In Vitro Studies of Transcript Initiation by *Escherichia coli* RNA Polymerase. 2. Formation and Characterization of Two Distinct Classes of Initial Transcribing Complexes†

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ABSTRACT: By following the kinetics of abortive and productive synthesis in single-round transcription assays, we confirm the existence of two general classes of initial transcribing complexes (ITCs), which we term "productive ITC" and "unproductive ITC". The productive ITCs are able to escape from the promoter rapidly to produce full-length transcripts, but only after carrying out an obligate series of abortive initiation steps. The unproductive ITCs were found to synthesize mostly abortive transcripts of 2−3 nucleotides and escape from the promoter extremely slowly, if at all. Formation of the unproductive ITC is not due to the inactive RNA polymerase. Instead, RNA polymerase molecules recovered from both the productive and unproductive ITC fractions were shown to carry out abortive and productive synthesis with both the partitioning tendency and transcription kinetics similar to those of the original enzyme. Our results suggest that early transcription complexes are partitioned into the productive and unproductive ITCs most likely during the formation of open promoter complexes. The extent of partitioning varies with individual promoter sequences and is dependent on the nature and concentration of the initiating nucleotide. Thus, multiple classes of ITCs can be formed during promoter binding and transcript initiation.

The conversion of an open promoter complex into a stable elongation complex completes the initiation phase of the transcription cycle. This phase, now known as the promoter escape phase, is complex and, in turn, contains two major processes: abortive initiation and the promoter escape transition. During abortive initiation, 2−15 nt[1] RNA transcripts are synthesized and released from the initial transcribing complexes (*1*−*8*). Abortive initiation appears to be a universally conserved process carried out by all RNA polymerases examined *in vitro* (*9*−*15*). During the promoter escape transition, RNA polymerase translocates downstream along the template DNA after it has successfully synthesized ∼8−15 nt RNA without aborting these transcripts. Translocation is now thought to involve scrunching of downstream DNA into the enzyme interior, generating strain that ultimately leads to the displacement of σ contacts on promoter DNA and resulting in the formation of a stable elongation complex in which the transcript is tightly bound in the RNA exit channel (*15*−*18*). Whereas the promoter escape event was thought to occur coincidentally with the release of σ factor (*4*−*6*, *19*−*21*), recent evidence questions the obligatory release of σ factor at the initiation−elongation transition (*22*, *23*). Instead, better criteria for the completion of the initiation phase might be the cessation of abortive product formation (indicative of the discontinuance of repetitive reinitiation) and, also, the shrinkage of the DNase I footprint of enzyme−promoter complexes (indicating the relinquishment of σ contacts to the core promoter elements) (see the discussion in ref *15*).

It has been generally assumed that transcript initiation is a sequential process in which each RNA polymerase goes through many rounds of abortive cycling before escaping from the promoter (*2*, *5*, *12*, *13*). This assumption is based in part on the observation that abortive RNAs are synthesized in large molar excess over productive RNAs (*2*, *3*, *7*, *13*). Because of the unique ability of RNA polymerases to initiate phosphodiester bond formation *de novo*, abortive initiation has been viewed as a prerequisite for producing a tightly bound RNA primer for subsequent RNA chain elongation, a process analogous to the "primase" reaction in DNA replication (*2*, *4*). Were this view correct, abortive initiation would have a *bona fide* role in transcription and can serve as a potential target for regulation during which transcription can be either aborted or maintained, depending on the regulatory cues.

The assumptions that the transcript initiation process is sequential and that abortive initiation is a natural process of transcription have been called into question in light of the study of Kubori and Shimamoto (*24*). By following the kinetics of abortive and productive RNA synthesis from

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[1] Abbreviations: ITCs, initial transcribing complexes; nt, nucleotide(s); ECs, elongation ternary complexes; FLB, formamide loading buffer; EP_o, enzyme−promoter open complex.

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single-round transcription at the *lacUV5* and a modified lambda P_R promoters, these authors have demonstrated the existence of two classes of ITCs. One class contains the so-called "moribund" complexes that are capable of continuously synthesizing and releasing abortive RNA without escaping the promoter. The other class contains the "productive ITCs" which are capable of escaping the promoter to make productive transcripts. Whether the productive ITCs undergo abortive initiation as a prelude to escape is not clear from the study of Kubori and Shimamoto (*24*). These authors further stipulate that abortive initiation might be a reaction carried out by inactivated ITCs, the inactivation being induced during the *in vitro* transcription reactions. Thus, abortive initiation might simply be an artifact of *in vitro* transcription rather than a natural process of transcription.

To resolve the many uncertainties regarding the role of abortive initiation in transcription, we performed kinetic assays to test the various models of transcript initiation. Our results confirm the existence of two distinct classes of ITCs: productive and unproductive. The unproductive ITCs remain at the promoter and carry out abortive synthesis. The productive ITCs are able to escape from the promoter, after a requisite series of abortive initiation. We performed further tests to show that the unproductive ITCs identified in our study are unlike the moribund complexes (*25*); their formation is not due to inactivated RNA polymerase but rather is dictated by the promoter sequence and the nature and concentration of the initiating nucleotide. These results provide strong evidence against the idea that abortive initiation is merely an artifact of *in vitro* transcription.

MATERIALS AND METHODS

RNA Polymerase. Escherichia coli DNA-dependent RNA polymerase was purified from *E. coli* DG156 by the method of Gonzalez et al. (*26*); preparations used in this study contained 18−65% active molecules as determined by the method of Chamberlin et al. (*27*). The *E. coli* RNA polymerase holoenzyme with a hexahistidine (His_6) tag at the C-terminus of the β' subunit was purified from strain RL712 (R. Landick) by the method of Uptain (*28*); His_6-tagged holoenzyme preparations used in this study contained 40−75% active molecules at the time of use (*27*).

Buffers. Transcription buffer III contains 40 mM Tris-HCl (pH 8), 150 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10 μg/mL acetylated BSA. Buffer W, used for washing Ni^{2+}−NTA agarose and streptavidin-conjugated magnetic beads (Dynal), contains 20 mM Tris-HCl (pH 8), 50 mM KCl, 4 mM $MgCl_2$, 2% glycerol, and 40 μg/mL acetylated BSA. Formamide loading buffer (FLB) contains 80% (v/v) deionized formamide and 0.025% (w/v) xylene cyanol (XC) in TBE buffer.

Promoter Templates. Single-promoter fragment templates, usually spanning positions −150 to 60 relative to the transcriptional start site of +1, were prepared by polymerase chain reaction (PCR; *29*, *30*). Sequences of the promoters used in this study are shown in Figure 2.

Gel Electrophoresis. RNA products from all transcription reactions were resolved on 23% polyacrylamide (38:2)−7 M urea gels and electrophoresed using the salt gradient buffer system (*31*). Gels were scanned using a Molecular Dynamics Storm phosphorimager, and RNA signals were quantitated using ImageQuant software.

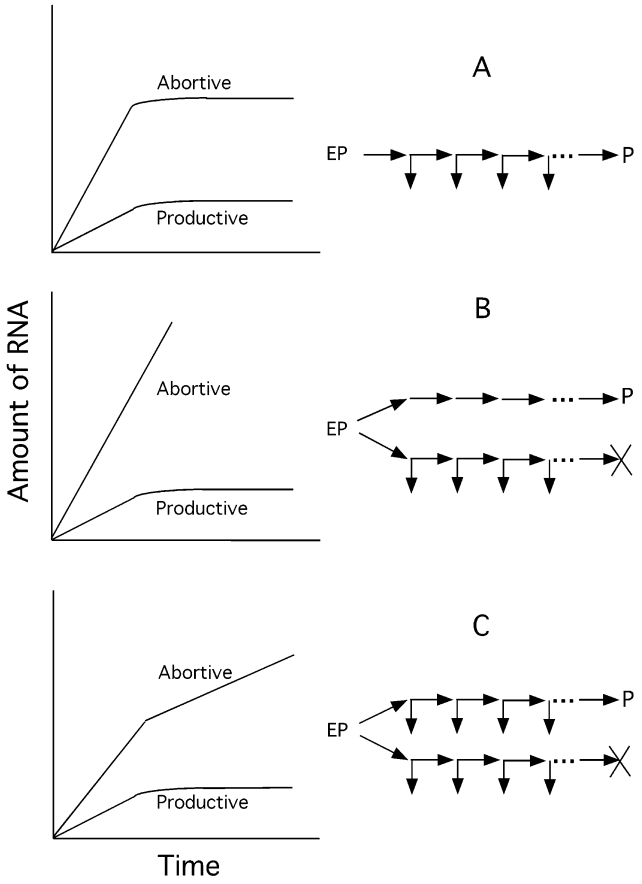


FIGURE 1: Three models of the transcript initiation process. See the text for a complete explanation. (A) Sequential model. (B) Branched pathway model. (C) Composite model. Each model is represented schematically on the right, with its attendant productive and abortive transcription kinetics plotted on the left. EP is the enzyme−promoter complex and P the productive transcript.

Single-Round Transcription Assay by Formation of Halted Elongation Ternary Complexes. Halted elongation ternary complexes (ECs) can be made during transcription by withholding one or more of the NTP substrates, depending on the transcribed sequence. To make G29 ternary complexes on the T5 N25 promoter, *E. coli* RNA polymerase (10 nM) was incubated with 30 nM PCR promoter template in buffer III at 37 °C for 10 min to form open promoter complexes. Transcription was initiated by the addition of ATP, UTP, GTP, and [α-^{32}P]ATP (100 μM each) to a specific activity of ~5 cpm/fmol. The reaction mixture was incubated at 37 °C, and 10 μL aliquots were removed at various time points and added to an equal volume of FLB. To make the A21 EC on *PrmP20* or *PrmT5A20* promoter, the nucleotide substrates consisted of ApU, ATP, CTP, and GTP (100 μM each).

Single-Round Transcription Assay in the Presence of the Competitor Promoter Prm11+1C. The *E. coli* RNA polymerase holoenzyme (10 nM) was incubated with the test promoter template (30 nM) in buffer III at 37 °C for 15 min to form the open complexes. Transcription was initiated by 100 μM NTP with [α-^{32}P]ATP at 5 cpm/fmol and 300 nM Prm11+1C competitor promoter. Aliquots (10 μL) were withdrawn at the desired times, mixed with an equal volume of FLB, and subsequently analyzed by high-voltage gel electrophoresis.

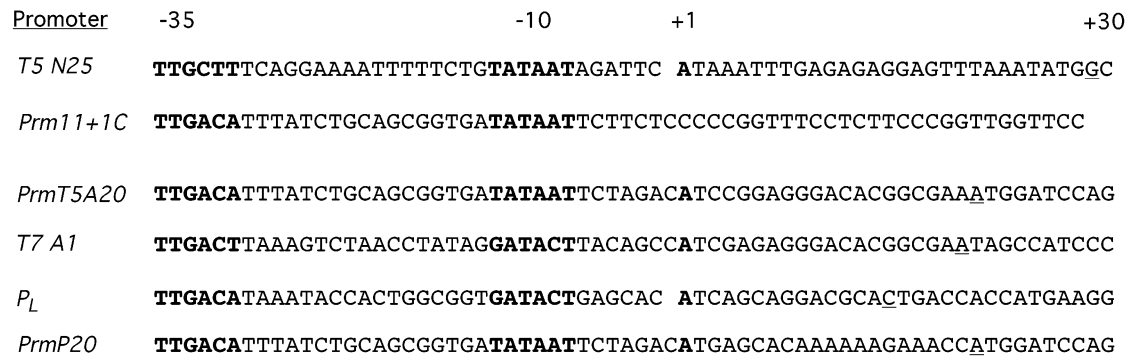


FIGURE 2: Promoters used in this study. Promoter fragments prepared by PCR usually spanned nucleotides −150 to 60, but only sequences from nucleotide −35 to 30 are shown. The −35 and −10 consensus elements and the +1 transcriptional start site are highlighted in bold. The underlined nucleotide on each template represents the position where RNA polymerase can be halted during elongation when using a select mix of NTP substrates and primer. Prm11+1C is a competitor promoter used in the single-round transcription assays.

Pulse Labeling of the Transcription Reaction Prestarted with Unlabeled Nucleotides. A 120 μL transcription reaction mixture containing 30 nM T5 N25 promoter template, 10 nM *E. coli* RNA polymerase, and unlabeled ATP, GTP, and UTP (100 μM each) in buffer III was incubated at 37 °C. At 0, 5, 10, and 30 min, 30 μL samples were removed and placed in reaction tubes containing [γ-^{32}P]ATP to achieve a specific activity of ~10 cpm/fmol. These radioactive reaction mixtures were further incubated at 37 °C; 0, 5, 10, 20, and 30 min after the addition of label, 5 μL aliquots were withdrawn and mixed with an equal volume of FLB for gel electrophoresis.

pppApU Dinucleotide Assay for Estimating the Fraction of Unproductive ITC. Ni^{2+}−NTA agarose beads were washed three times in buffer W before use. To measure the rate of synthesis of pppApU from the unproductive ITC, a 100 μL reaction mixture containing 30 nM PCR promoter template, 10 nM *E. coli* His_6-tagged RNA polymerase, and a subset of nucleotides (ATP, GTP, and UTP for the T5 N25 promoter and ApU, ATP, GTP, and CTP for the PrmP20, PrmT5A20, T7 A1, and λ P_L promoters) in buffer III was mixed with the washed Ni^{2+}−NTA agarose beads and incubated at 37 °C for 30 min to partition the polymerase into halted ECs and unproductive ITCs. Free nucleotides were removed by five washes (1 mL each). The pellet containing the halted ECs and the unproductive ITCs was resuspended in buffer III along with 100 μM ATP and UTP with [γ-^{32}P]ATP at 5 cpm/fmol. The reaction mixture was incubated at 37 °C for pppApU synthesis, presumably only from the unproductive ITCs. Samples of 5 μL were withdrawn at 0, 2, 5, 10, 20, and 30 min, and mixed with an equal volume of FLB for analysis by gel electrophoresis. To assay the rate of pppApU synthesis from the total ITCs in the reaction, the same procedure that was described above was followed but without NTP during the first incubation. The percentage of unproductive ITC was obtained by taking the ratio of the rate of pppApU synthesis of the unproductive ITCs versus the total ITCs and multiplying this ratio by 100.

Gel Mobility Shift Assay for Estimating the Percentage of Unproductive ITC. A gel mobility shift assay was performed according to the method of Levin et al. (*10*). Open complexes were formed at PrmP20 and PrmT5A20 promoters by incubating the 5′-^{32}P-end-labeled promoter (30 nM) with 10 nM RNA polymerase in buffer III at 37 °C for 10 min. To the reaction mixtures were added ApU, ATP, GTP, and CTP (100 μM each) to form the A21-halted EC. To resolve the unproductive ITC and the halted EC, reaction mixtures were mixed with $^1/_{10}$ volume of a sucrose dye buffer (60% sucrose and 0.25% each bromophenol blue and xylene cyanol) and loaded directly onto a 4% native polyacrylamide gel (37.5:1). Gel electrophoresis was carried out at 200 V and 4 °C until the XC dye has migrated 16 cm from the well.

Recovery of RNA Polymerase from the Unproductive ITCs and Halted ECs. The biotinylated T7 A1 promoter template (30 nM) and *E. coli* His_6-tagged RNA polymerase (10 nM) were mixed with 100 μM ApU, ATP, GTP, and CTP to form the halted A20 EC and unproductive ITCs. These complexes were pelleted with streptavidin-conjugated magnetic beads. The pellet was washed twice with buffer W (1 mL each) to remove the released σ factor, free RNA polymerase, nucleotides, and abortive RNA transcripts, then resuspended in 1 M KCl, and incubated for 10 min at 25 °C to disrupt the unproductive ITC. Previously, A20 EC on the T7 A1 promoter template was shown to be resistant to disruption by 1 M KCl (*32*). His_6-tagged RNA polymerase released into the supernatant from the unproductive ITC was pelleted with Ni^{2+}−NTA agarose beads and washed three times with buffer W to remove the residual KCl. RNA polymerase molecules recovered from the affinity beads were then used in a single-round transcription reaction to form the halted G29 complex on the T5 N25 promoter template.

To recover the RNA polymerase associated with the A20 complex, streptavidin magnetic beads from above containing these complexes on the biotinylated T7 A1 promoter were washed three times with buffer W to remove free RNA polymerase and residual KCl. The A20 complexes were then incubated in 100 μM NTP at 37 °C for 10 min to chase the elongation complexes off the template DNA. The released His_6-tagged core RNA polymerase was recovered using Ni^{2+}−NTA agarose, supplemented with σ^{70}, and then used in a single-round transcription by forming the G29 complex on the T5 N25 promoter.

RESULTS

Rationale and Experimental Approach. Investigation into the relationship of abortive initiation and promoter escape has led to several models that account for the high level of abortive initiation at *E. coli* promoters. The "sequential" model, represented in Figure 1A, postulates a uniform population of initial complexes all going through several rounds of abortive initiation before leaving the promoter to

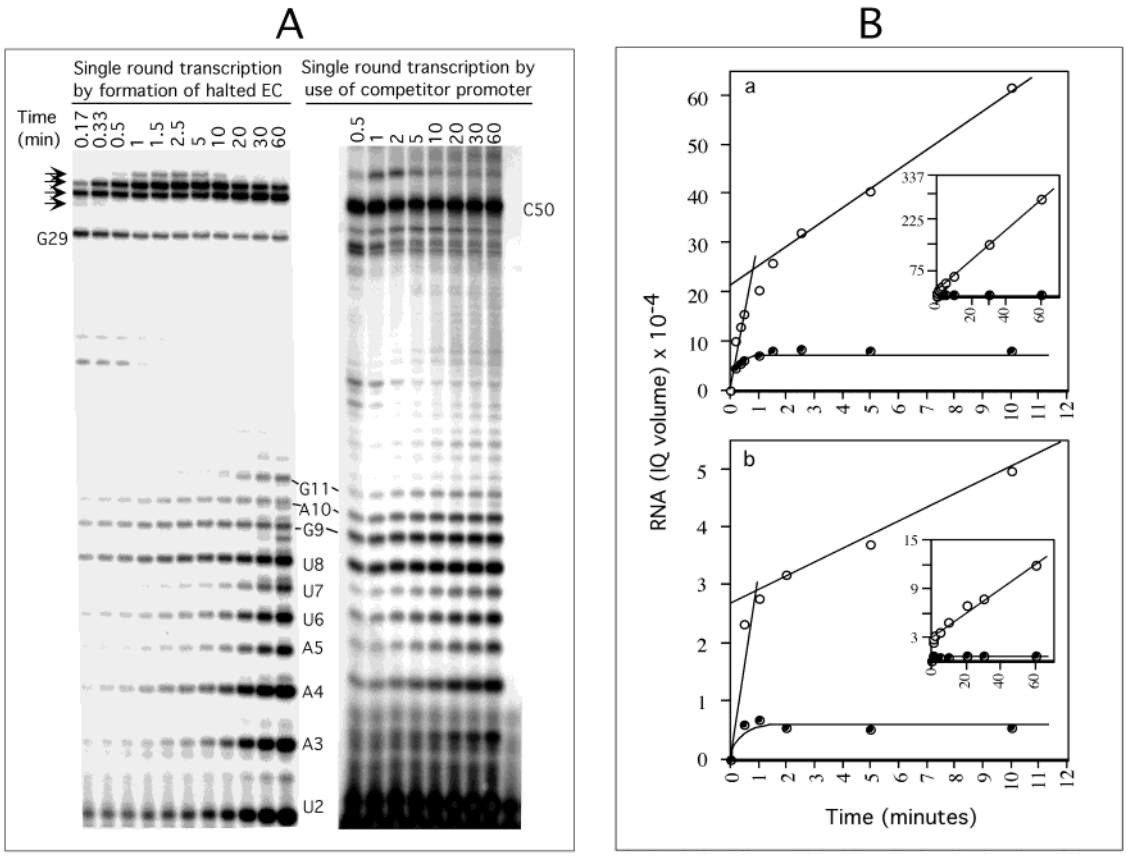


FIGURE 3: Single-cycle transcription time course of T5 N25. (A) Polyacrylamide gel profiles: (left) single-round condition imposed by halting elongation at G29 and (right) single-round condition achieved with the addition of excess competitor DNA. Assay conditions are as described in Materials and Methods. Abortive and productive transcripts are designated by a letter and number combination; the letter represents the identity of the 3′-most nucleotide of the RNA, and the number corresponds to the length of the RNA. Arrows point to read-through products from the G29 complex at U35, U37, and G39, all preceding a C residue in the sequence. C50 is the runoff RNA. (B) Kinetics of abortive and productive synthesis from single-round transcription. Gels shown in panel A were quantitated using ImageQuant (IQ): the left panel gave rise to graph a, and the right panel yielded graph b. The inset in each graph shows the full time course up to 1 h. (○) Total abortive RNA and (●) productive transcripts. The *X*-axis is the reaction time in minutes and the *y*-axis the amount of RNA in arbitrary units of IQ volume.

enter elongation (*33*). According to this model, all initial complexes eventually undergo promoter escape. Contrary to this view, the results of Kubori and Shimamoto (*24*) suggest the existence of two classes of the ITC during initiation; one class produces abortive RNA exclusively, and the other is responsible for productive transcription exclusively. Their "branched pathway" model is represented in Figure 1B. Formally, a third model is possible; of the two classes of the ITC, one class yields abortive RNA exclusively while the other undergoes promoter escape after an obligate sequence of abortive synthesis. We call this the "composite" model, and it is illustrated in Figure 1C. The first goal of this study is to determine which of these three models most accurately represents the actual mechanism of transcript initiation.

As shown in Figure 1, these three models can be distinguished by the kinetics of single-round transcription by monitoring the rates of abortive and productive synthesis. For the "sequential" model, the rates of both productive and abortive RNA synthesis should increase linearly at first, but level off simultaneously after all of the RNA polymerase molecules have escaped the promoter and completed full-length synthesis. For the branched pathway model, the kinetics of single-round transcription predict a single rate of abortive synthesis by the moribund complexes. There is also a single rate of productive synthesis that increases with time initially but reaches a plateau after all of the productive complexes escape the promoter. In the composite model, there are two classes of ITCs: productive and unproductive. Unlike the branched pathway model, however, the productive ITCs also undergo abortive initiation, prior to promoter escape. Hence, abortive RNA synthesis in this model is predicted to show biphasic kinetics in which an initial phase represents the combined rates of abortive synthesis from both classes of ITCs and a second phase showing a reduced rate of abortive synthesis due solely to the unproductive ITCs. The rates of both abortive and productive synthesis should undergo the biphasic switch at the same instance, corresponding to the time required for the productive ITCs to complete the initiation−elongation transition and full-length RNA synthesis.

Kinetics of RNA Synthesis from Single-Round Transcription Are Consistent with the Composite Model. Single-round transcription kinetics of abortive and productive synthesis were determined for the T5 N25 promoter using two different approaches. In the first approach, RNA polymerase was blocked from multiple rounds of initiation by the formation of halted EC. In the presence of a subset of NTPs (ATP, GTP, and UTP), ECs are halted at the 29 position (G29 complex) on the T5 N25 promoter (Figure 3A, left panel).

The formation of halted ECs, however, is not 100% effective since transcripts longer than G29 are also observed. These longer RNAs correspond to halted complexes at U35, U37, G39, and A49 all followed by a C residue, and represent read-through products from trace contamination of the missing C nucleotide (*5*, *10*). To calculate the amount of productive transcripts, we summed all of the read-through RNA species as well as the G29 product.

In the second approach, a competitor promoter was added in large molar excess to the test promoter to block reinitiation by free RNA polymerase (Figure 3A, right panel). We constructed the competitor promoter Prm11+1C lacking A in the nontemplate strand (Figure 2). Thus, when [γ-^{32}P]-ATP is used as the radioactive substrate, only RNA products from the test promoter would be labeled. Moreover, Prm11+1C has a run of five C residues near the transcriptional start site that induces reiterative synthesis to trap the RNA polymerase at the promoter (data not shown). When added in a 10−20-fold molar excess with respect to a test promoter, Prm11+1C was shown to outcompete the test promoter for free RNA polymerase without disrupting the open complexes at the test promoter, effectively limiting transcription to a single cycle (*34*).

Single-round transcription kinetics of abortive and productive RNA synthesis from the T5 N25 promoter are graphed in Figure 3B. Whether by formation of the halted ECs or by the use of a competitor promoter, the kinetics are distinctly biphasic (Figure 3B, graphs a and b). The extent of productive synthesis increased linearly for ~1 min and then leveled off (●). Abortive products also accumulated sharply for ~1 min (○), after which the rate of abortive synthesis somewhat decreased and remained constant for more than 1 h (see the insets in Figure 3B). More importantly, the time at which productive synthesis leveled off coincides with the time at which the switch between the two phases of abortive synthesis occurred. Thus, the biphasic nature of the abortive rate, the coincidence of the biphasic transition of abortive and productive synthesis, and the continued abortive synthesis after the cessation of productive synthesis are consistent with the kinetics predicted by the composite model. Furthermore, single-round transcription of four other promoter templates (PrmP20, PrmT5A20, T7 A1, and λ P_L) all exhibited similar kinetics of abortive and productive RNA synthesis as described above (*34*; data not shown). These results support the existence of two distinct classes of ITC; one class is able to escape the promoter after some rounds of abortive synthesis, and the other class remains at the promoter to carry out abortive initiation continuously.

Pulse Labeling of the Transcription Reaction Corroborates the Existence of Unproductive ITCs. To independently confirm the occurrence of ITCs that remain at the promoter, a pulse labeling experiment was performed. In this experiment, single-cycle transcription was started at the T5 N25 promoter with unlabeled ATP, GTP, and UTP to form the G29 complex. At various times during the reaction, [γ-^{32}P]-ATP was added to label the newly initiated transcripts. The result is shown in Figure 4. As expected, the zero-minute control yields the transcript pattern seen earlier where the productive RNA level rapidly plateaus but the abortive RNA level increases linearly with time (see Figure 3A, left panel). When [γ-^{32}P]ATP was added 5 and 10 min into the reaction, the extent of labeling of productive transcripts decreased

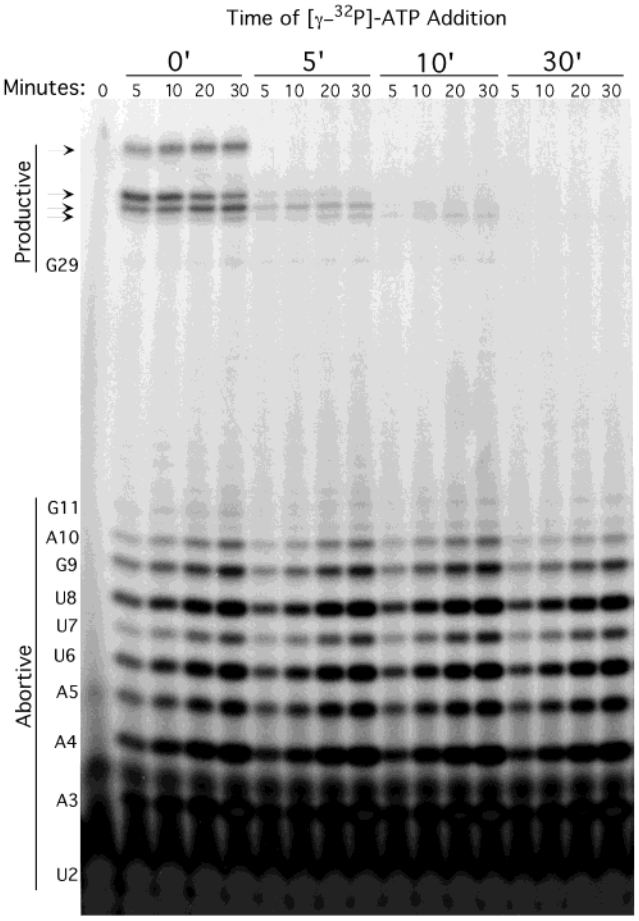


FIGURE 4: Pulse labeling of transcription reactions prestarted with unlabeled nucleotides demonstrates the existence of unproductive ITCs. T5 N25 open promoter complexes were incubated with unlabeled ATP, UTP, and GTP (100 μM each) at 37 °C, and [γ-^{32}P]-ATP was added to the reaction mixture at 0, 5, 10, and 30 min. The radioactive reaction mixtures were further incubated and aliquots sampled at 5, 10, 20, and 30 min.

drastically and almost disappeared completely after 30 min. (The small amount of productive RNA that is labeled could be due to the recycling of RNA polymerase molecules that have run off the productive complexes.) In contrast, the synthesis of labeled abortive transcripts persisted; the addition of [γ-^{32}P]ATP, even after a 30 min preincubation with unlabeled NTP, gave rise to an undiminished rate of abortive synthesis. These observations confirm the existence of a fraction of RNA polymerase molecules that remain at the promoter and carry out abortive initiation continuously without escape (*24*).

Estimation of the Fraction of Unproductive ITC at a Given Promoter. Given the existence of two classes of ITCs, we were interested in knowing what fraction of the total ITCs was of the unproductive class. We performed two independent assays to answer this question. The first assay involves measuring the rate of pppApU dinucleotide synthesis from the unproductive ITCs and the total ITCs in the transcription reaction. The rate of dinucleotide synthesis from the total ITCs is readily measured when the transcription reaction is provided with just [γ-^{32}P]ATP and UTP; that of the unproductive ITCs can be probed specifically after first partitioning the RNA polymerase molecules into the productive and unproductive classes. If rates of dinucleotide synthesis for the unproductive ITCs and the total ITCs are

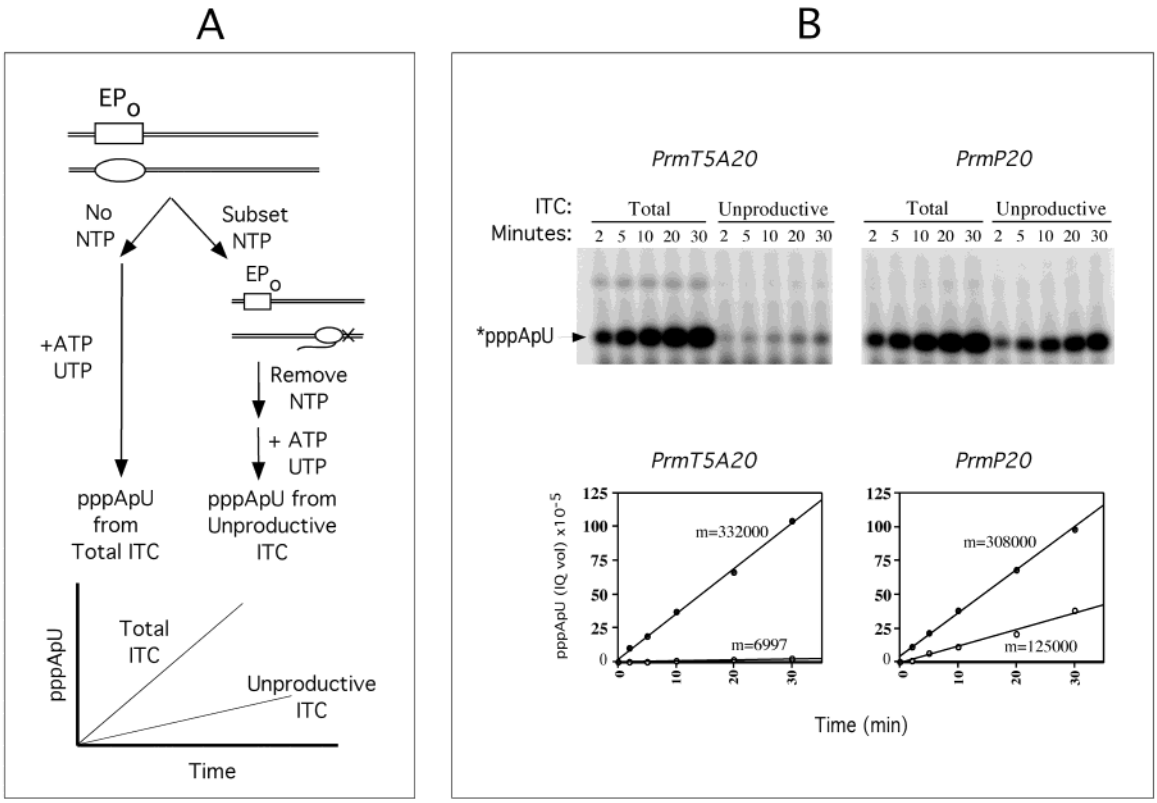


FIGURE 5: Determination of the fraction of unproductive ITC at a promoter using the dinucleotide assay. (A) Experimental outline of the dinucleotide assay. The rationale and details of this procedure are described in the text. A transcription reaction was first performed with 100 μM ApU, ATP, CTP, and GTP at 37 °C for 30 min to partition RNA polymerase into the productive EC (A21 for both promoters) and the unproductive ITC: (rectangle) RNA polymerase associated with the unproductive ITC and (oval) enzyme associated with the productive ITC and EC. (B) Dinucleotide assay performed on PrmT5A20 and PrmP20 promoters: (left panels) PrmT5A20 promoter and (right panels) PrmP20 promoter; (top) gel profiles and (bottom) quantitation of pppApU synthesis; and (●) pppApU synthesis by the total ITCs and (○) pppApU synthesis by the unproductive ITCs. The slope (*m*), the rate of pppApU synthesis, is indicated for each line. Dividing the rate from the unproductive ITCs into that of the total ITCs gives an estimate of the fraction of unproductive ITC at each promoter.

assumed to be equivalent, their relative rate gives an estimate of the unproductive fraction.

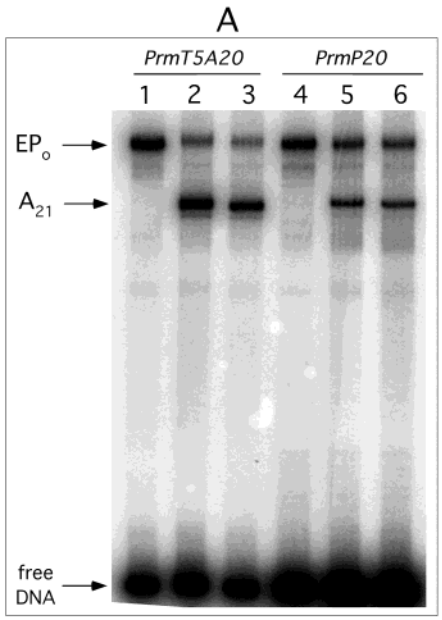
A diagram illustrating the partitioning of ITCs for the "dinucleotide" assay is shown in Figure 5A. Using this assay, we found the percentage of unproductive ITC at two promoters differed substantially: 2% at PrmT5A20 and 40% at PrmP20 (Figure 5B). Given the caveat mentioned above, we were unable to discern whether the large disparity in these percentages reflects the difference either in the unproductive fraction at these promoters or in the enzymatic rates of dinucleotide synthesis by different populations of ITCs.

To gain an independent assessment, we employed the gel shift assay. In this gel analysis, the unproductive ITCs are unstable and readily release their RNA, thus exhibiting an electrophoretic mobility indistinguishable from that of the open complex (*5*, *10*, *35*). The percentage of unproductive ITCs can be calculated as the fraction of EP_o to the sum of EP_o and EC in the gel. A caveat exists in this analysis as well, in that the value derived from this assessment assumes the absence of catalytically inactive EP_o (*10*, *36*).

Using the electrophoretic mobility assay, the unproductive fraction determined was 18% for PrmT5A20 and 49% for PrmP20 (Figure 6). These values are higher than those obtained from the dinucleotide assay probably because of the presence of catalytically inactive EP_o complexes that comigrated with the unproductive ITCs. Even with this discrepancy, the large disparity in the unproductive fraction for PrmT5A20 and PrmP20 suggests that the promoter sequence itself exerts a significant influence on the formation of productive and unproductive ITCs. To examine the generality of this observation, we performed the dinucleotide assay on three additional promoters, λ P_L, T7 A1, and T5 N25. As summarized in Figure 6C, the percentage of unproductive ITC varies widely among different promoters: 2% for PrmT5A20, 7% for T7 A1, 9% for T5 N25, 22% for λ P_L, and 40% for PrmP20. This result provides evidence that strongly supports the role of promoter sequence, especially the initial transcribed region (compare the sequences of PrmP20 and PrmT5A20 in Figure 2), in the formation of unproductive ITCs.

Inactive RNA Polymerase Molecules Are Not the Cause of Unproductive ITCs. To test whether the unproductive ITCs are formed from inactive RNA polymerase, the dinucleotide assay was performed using several RNA polymerase preparations containing different percentages of active molecules at the time of use (*27*). Our results show a lack of correlation; that is, an RNA polymerase preparation with 65% active molecules gave a percentage of unproductive ITC similar to that of another preparation with 18% active molecules (data not shown).

To completely rule out the possibility that unproductive ITCs are formed from inactive enzyme molecules, we devised a procedure for recovering the RNA polymerase molecules associated with either class so that their transcriptional properties can be compared. The procedure for isolating these two pools of RNA polymerase is outlined in



B

	PrmP20	PrmT5A20
Dinucleotide assay	40 ± 2	2 ± 1
Gel shift assay	49 ± 2	18 ± 1

C

Promoter	% Unproductive ITC
PrmT5A20	2 ± 1
T7 A1	7 ± 1
T5 N25	9 ± 2
P_L	22 ± 2
PrmP20	40 ± 2

FIGURE 6: Estimation of the percentage of unproductive ITC by the gel shift assay. (A) Profile of transcription complexes on a native 4% polyacrylamide gel: lanes 1−3, transcription at the PrmT5A20 promoter; lanes 4−6, transcription at the PrmP20 promoter; lanes 1 and 4, EP_o formation reactions; and lanes 2, 3, 5, and 6, duplicate reactions in which the EP_o complexes were incubated with 100 μM ApU, ATP, CTP, and GTP at 37 °C for 30 min to form the A21 ternary elongation complexes. The fraction of unproductive ITC was estimated by dividing the radioactivity associated with the EP_o band into that of all complexes (EP_o and A21 bands). (B) Percentages (with one standard deviation) of the unproductive ITC from the PrmT5A20 and PrmP20 promoters as determined by the two assays. (C) Percentages of unproductive ITCs determined for a number of promoters using the dinucleotide assay.

Figure 7A. His_6-tagged RNA polymerase was first partitioned into unproductive ITCs and A20-halted EC on a 5′-biotinylated T7 A1 promoter template, and the mixture was treated with 1 M KCl to disrupt the unproductive ITCs without affecting the stability or activity of the A20 complexes (*32*). His_6-tagged RNA polymerase molecules released from the unproductive ITCs were recovered subsequently using Ni^{2+}−NTA agarose beads. To isolate RNA polymerase from the productive A20 complexes, NTP was added to chase the core enzyme off the promoter template. The His_6-tagged core enzyme thus released was reconstituted with σ^{70} and recovered with Ni^{2+}−NTA agarose beads. Both fractions of RNA polymerase were then examined for their ability to carry out productive versus abortive synthesis at the T5 N25 promoter under single-round conditions.

As shown in Figure 7B, the kinetics of abortive and productive RNA synthesis by the two RNA polymerase populations was essentially indistinguishable, from each other, as well as from that of the original enzyme (compare Figures 7C and 3B). The synthesis of productive transcripts by RNA polymerase recovered from the unproductive ITCs rules out the possibility that these complexes are formed from inactive enzymes incapable of escaping the promoter. Indeed, our results suggest that active RNA polymerase molecules are involved in the formation of both productive and unproductive complexes. Furthermore, with each round of promoter binding and activation, RNA polymerase is partitioned anew into the productive and unproductive fractions, the ratio being a function of the promoter sequence.

Properties of the Productive ITC. With the demonstration that unproductive ITCs can account for the bulk of abortive RNA synthesis, it is reasonable to ask whether the productive ITCs undergo abortive initiation prior to escape from the promoter region. Previously, Kubori and Shimamoto (*24*) showed that RNA polymerase recovered from a productive complex is subsequently capable of abortive and productive synthesis. However, the data presented, in the form of a single reaction time point, preclude a determination of the source of abortive transcripts, whether from the productive ITCs, the unproductive ITCs, or both. We show in this report that it is possible to deduce the source of abortive transcripts by the kinetics of abortive and productive RNA synthesis from the same single-cycle transcription reaction (Figures 3 and 7). From the biphasic nature of abortive synthesis and the temporal coincidence of the phase transition with the cessation of productive synthesis, we conclude that the productive ITCs also carry out abortive synthesis as a prelude to promoter escape.

The Nature and Concentration of Initiating Nucleotide Substrates Affect the Formation of Unproductive ITCs. The NTP concentration has been shown to play an important role in the process of promoter escape, although the concentration effect is inconsistent with a kinetic competition for productive elongation (*37*, *58*). This observation led us to examine the effect of varying NTP concentrations on the fraction of unproductive ITCs formed at various promoters, as estimated by the dinucleotide assay. We examined two nucleotide concentrations: all four NTPs at either 100 or 500 μM. At 100 μM, the unproductive fractions at T5 N25, PrmT5A20, and PrmP20 were 9, 7, and 12%, respectively; at 500 μM, the percentages were reduced to 4, 5, and 5%, respectively (Table 1). A high NTP concentration, therefore, appears to reduce the percentage of unproductive ITC during the transcription initiation process.

We further noticed that the percentage of unproductive ITC estimated by the dinucleotide assay varies dramatically depending on the nucleotide mixture used in the transcription reaction. For example, at PrmP20, the unproductive ITC

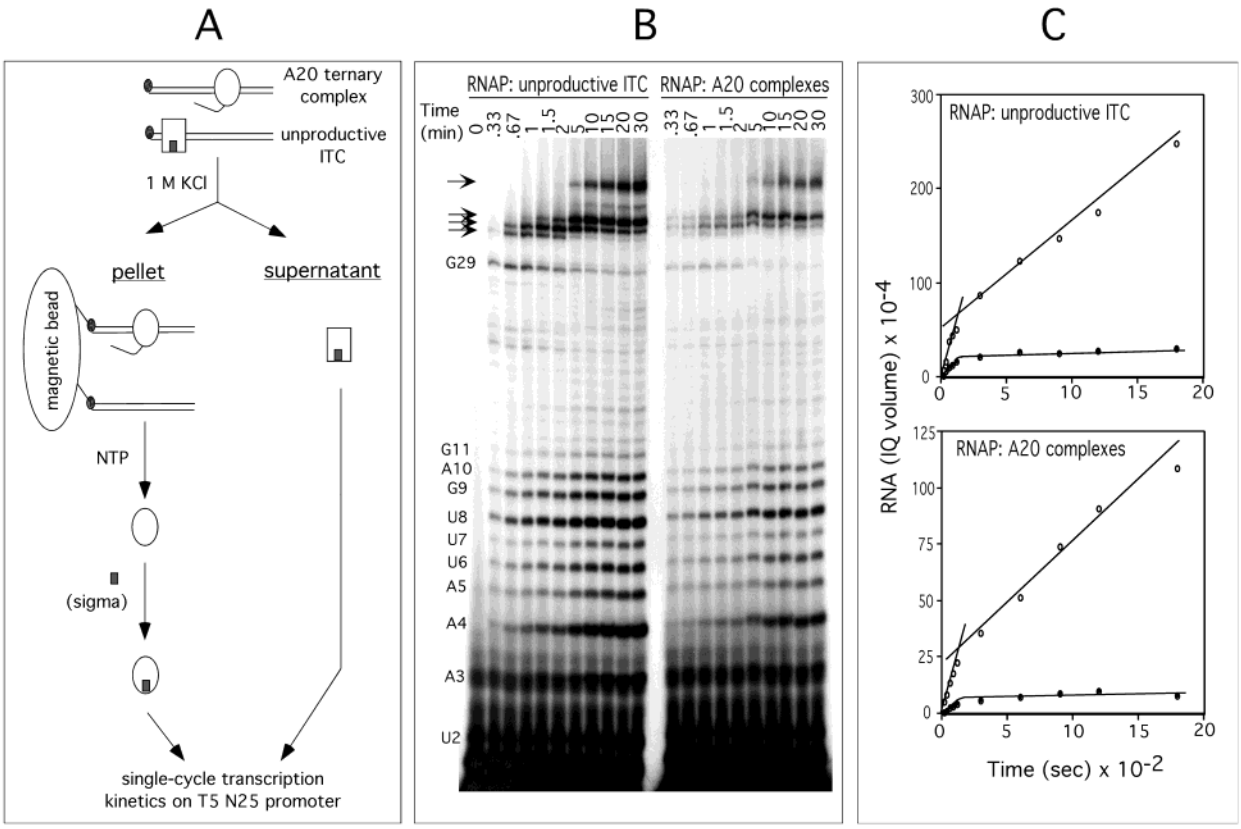


FIGURE 7: Recovery of RNA polymerase from the productive EC and the unproductive ITC and comparison of their transcriptional properties. (A) Outline of the procedure for recovering *E. coli* RNA polymerase from the unproductive ITCs and the halted ECs. Details of this experiment using the T7 A1 promoter are described in the text. RNA polymerases isolated from these fractions were then tested in single-round transcription at the T5 N25 promoter. (B) Single-round transcription of T5 N25 by RNA polymerase recovered either from the unproductive ITCs (left) or from the halted ECs (right). (C) Kinetics of abortive and productive synthesis by the two pools of RNA polymerase. The *X*-axis is the time of reaction in seconds and the *y*-axis the amount of RNA in arbitrary units of IQ volume: (●) total productive RNA and (○) total abortive products.

Table 1: Effect of Initiating Nucleotide Concentration on the Percentage of Unproductive ITC[a]

promoter	25 μM ApU	100 μM ApU	500 μM ApU	100 μM NTP	500 μM NTP
T5 N25	ND	ND	ND	9	4
PrmT5A20	ND	ND	ND	7	5
PrmP20	68	40	16	12	5

[a] A concentration of 100 μM elongating NTP was used. The percentage of unproductive ITC was estimated by the dinucleotide assay, after a prior partitioning of RNA polymerase into the productive and unproductive fractions. In the case of ATP as the initiating nucleotide, partitioning was achieved with the use of competitor promoter Prm11+1C. When ApU served as the initiating nucleotide, partitioning was achieved by the formation of halted ECs.

formed in the presence of a subset of nucleotides [ApU, ATP, GTP, and CTP (100 μM each)] was 40%, but only 12% if all four natural NTP (100 μM) substrates (along with the competitor promoter Prm11+1C) were used to limit transcription to a single cycle. A key difference between these two determinations is the nature of the initiating nucleotide, ApU dinucleotide in one and ATP in the other. This prompted further examination of the effect of varying the type and concentration of the initiating nucleotide on the formation of unproductive ITC.

As shown in Table 1, the percentage of unproductive ITC was found to vary inversely with the ApU concentration. At PrmP20, the percentage of unproductive ITC was 16, 40, and 68% at 500, 100, and 25 μM ApU, respectively, when the other three nucleotides (ATP, GTP, and CTP) were provided at a concentration of 100 μM to form the A21 complex. Thus, a high concentration of the initiating nucleotide lowers the fraction of unproductive ITC; this holds true also with ATP as the initiating nucleotide (data not shown). However, a much higher concentration of ApU than of ATP was necessary to achieve the same lower percentage of unproductive ITC, suggesting that ApU is not as effective as an initiating substrate as ATP. This result confirms an earlier observation with the T7 A1 promoter, in which a high concentration of the ApU dinucleotide was required to convert EP_o into the A20 complex (*10*).

Further tests revealed a similar dependence on the non-initiating nucleotides such that low concentrations led to high percentages of the unproductive ITCs (data not shown). Thus, the nature and concentration of both the initiating and elongating nucleotides are important in controlling the partitioning reaction.

Comparison of Abortive Patterns between the Unproductive and the Total ITC. Which step(s) in the transcription initiation process present a barrier to promoter escape by the unproductive ITCs? The answer to this question can be gained ideally by comparing the abortive patterns between the productive and unproductive ITCs. Since it is not possible to determine the abortive pattern from the productive ITCs alone, we instead compared the abortive patterns of the

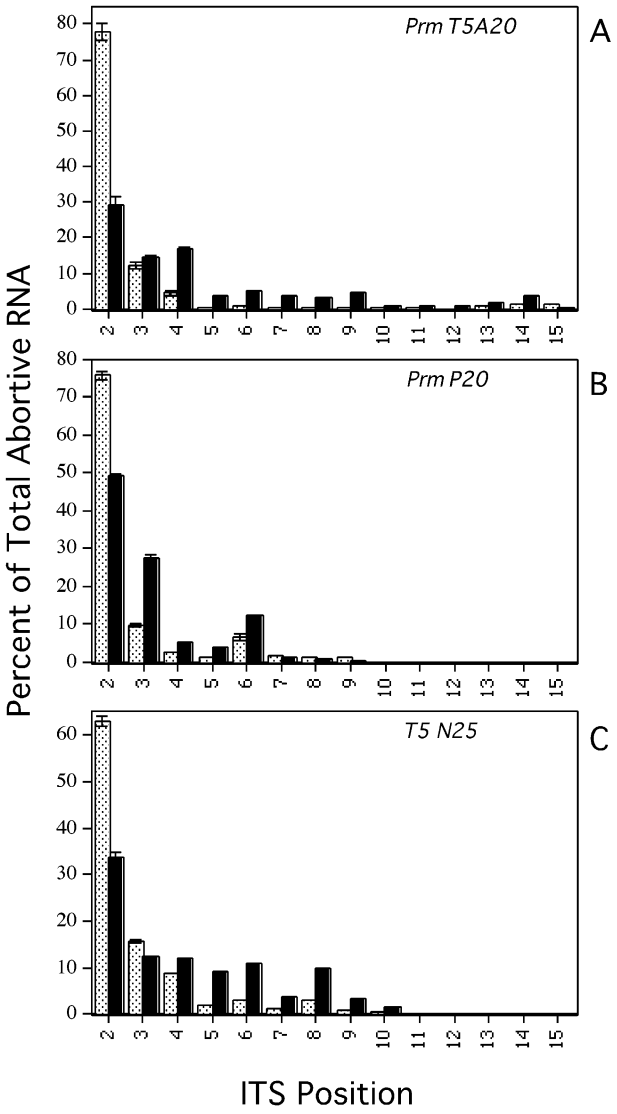


FIGURE 8: Comparison of abortive patterns generated by the unproductive ITCs and the total ITCs at three promoters. The abortive patterns were determined by calculating the yield of each abortive transcript as a percent of total abortive RNAs. Abortive RNA levels from the total ITCs are derived from steady-state transcription reactions, and those of the unproductive ITCs were from single-round transcription assays, after first allowing the productive ITCs to escape the promoter region: (A) PrmT5A20 promoter, (B) PrmP20 promoter, and (C) T5 N25 promoter. These promoters were shown previously to form 2, 40, and 9% of unproductive ITCs, respectively (see Figure 6C). The *X*-axis shows ITS positions and the *y*-axis the percentage of total abortive RNA.

unproductive ITCs and the total ITCs at three promoters: PrmT5A20, PrmP20, and T5 N25 (Figure 8). At all three promoters that were examined, the unproductive ITCs consistently abort to a higher level at position 2 (65−80%) than the total ITCs (30−50%), indicating the likelihood that most unproductive ITCs become arrested at position 2.

DISCUSSION

In this study, we provide strong evidence supporting the existence of two major classes of complexes, the productive ITC and the unproductive ITC, during transcript initiation. Similar complexes were observed earlier (*24*, *38*), but extensive characterization documented here allows us to describe the properties of these complexes more definitively. Our results show that the productive ITCs are competent at promoter escape in synthesizing the full-length RNA; however, promoter escape follows an obligate series of abortive synthesis. This finding dispels the notion that abortive initiation is simply an artifact of *in vitro* transcription, a reaction belonging only to the so-called moribund complex (*24*). The unproductive ITCs, on the other hand, are trapped at the promoter and carry out abortive synthesis exclusively, mostly the 2−3 nt products. The unproductive ITCs characterized in this study differ from the moribund complexes in that they are not derived from inactive RNA polymerase molecules, nor are they inactivated during the transcription reaction, on the way to forming the dead-end complexes (*24*, *25*). Most importantly, we show that both fractions of RNA polymerase recovered from the productive and unproductive ITCs, when set to re-bind another promoter and perform single-cycle transcription, were catalytically active and carry out productive and abortive synthesis with a pattern nearly identical to that of the original enzyme (Figures 3 and 7).

Our results further show that the level of partitioning of RNA polymerase into productive and unproductive complexes is determined intrinsically by sequence information embedded in individual promoters (Figure 9). Additionally, the partitioning ratio is subject to modulation by extrinsic factors, such as NTP concentration and the nature of the initiating nucleotide. Table 1 shows that the formation of productive ITC is favored by higher nucleotide substrate concentrations. We do not know the exact mechanism by which NTP exerts this stimulatory effect, but can offer two speculative ones. One is that NTP at a high concentration can act as an allosteric activator, effecting a conformational change that converts the unproductive ITCs into the productive ITCs (*39*). Alternatively, there may not be a conformational difference between these two types of complexes. Rather, the unproductive ITCs as a class may have higher apparent K_S values for the nucleotide substrates. A substantial fraction of the unproductive ITC is then able to advance through the initial transcribed sequence region only at an elevated NTP concentration to escape the promoter. For the promoters we examined, the evidence at hand precludes a distinction between these two models.

Conformational change, however, lies at the heart of moribund complex formation on λ P_R. These complexes, by exonuclease III footprinting, show a backtracked conformation, suggesting that they gradually undergo arrest as initial transcription continues (*25*). Consistent with transcriptional arrest, the moribund complexes on λ P_R can be reactivated by transcript cleavage factors GreA and GreB (*25*). With the partitioning reaction yielding a mixture of productive and unproductive ITCs, it is reasonable to ask whether Gre factors stimulate productive synthesis by altering the distribution in favor of the productive ITC. Experiments with the promoters we characterized here showed that addition of the transcript cleavage factors GreA and GreB to the transcription reaction mixture, whether before or after the formation of unproductive ITC, has no effect on the percentage of unproductive ITC formed (data not shown). Thus, Gre factors neither prevent the formation of the unproductive ITC nor convert the unproductive ITC into the productive class. This result is consistent with the observation that, on the promoters we examined, a large fraction of the unproductive ITC is blocked

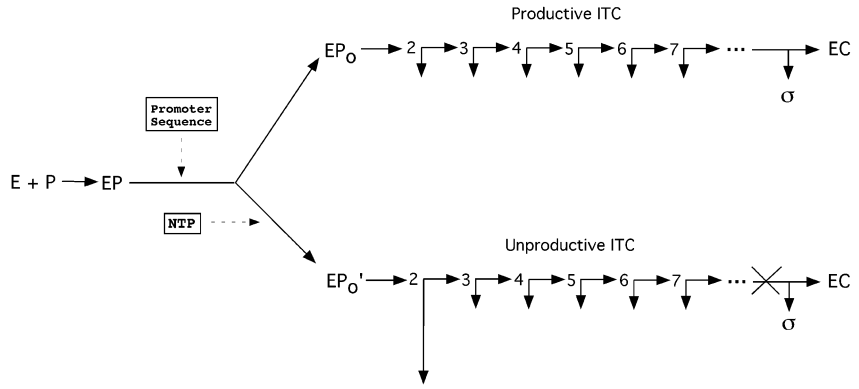


FIGURE 9: Proposed model illustrating the various steps involved during transcription initiation. After promoter recognition and binding, RNA polymerase−promoter complexes are postulated to partition into two different classes of open promoter complexes, EP_o and EP_o'. The EP_o complexes subsequently give rise to the productive ITCs, which then go through the steps of abortive initiation prior to escaping from the promoter to enter the elongation phase. In contrast, the EP_o' complexes are postulated to give rise to the unproductive ITCs, most of which become arrested at position 2 and some of which become blocked at the step(s) just before or during the promoter escape transition. The unproductive ITCs carry out abortive initiation but do not escape the promoter.

at the 2 position and that Gre factors have no effect on the synthesis and abortive release of 2 nt RNA transcripts (*7*, *40*).

In light of major differences in the properties of moribund complexes versus the unproductive ITCs, that is, moribund complexes undergo inactivation to form dead-end complexes and are susceptible to Gre factor-mediated reactivation, while the unproductive ITCs we characterized do not become inactivated and are not rescued by the action of Gre, we may have documented the existence of different classes of unproductive ITCs. These complexes all share the property of carrying out mostly abortive initiation without the facile ability to escape the promoter. Their differences may explain why Gre factors stimulate productive synthesis in a promoter-dependent manner (*7*).

Our findings join a long line of studies that with increasing frequency document the existence of many classes of transcription complexes, especially during the elongation phase when RNA polymerase encounters various DNA sequence signals that can cause it to pause, arrest, or terminate (*32*, *41*−*44*). Here we show the existence of multiple biochemical classes of transcription complexes at the initiation stage, an observation reported in earlier studies (*24*, *35*, *45*, *46*). Indeed, the formation of ITCs that fail to escape the promoter had been reported much earlier (*10*, *36*), although the RNA polymerase molecules in such complexes were thought to be catalytically inactive. In this context, promoters that specify reiterative slippage at the initiation stage constitute yet another class of unproductive ITCs (*47*); although the "slipped" transcripts do not re-enter templated transcription in general, this class of complexes at the pyrimidine operon promoters can be reversibly activated by varying the concentration of UTP *in vitro* and *in vivo* (*48*−*52*). Recently, Pol II complexes that undergo reiterative slippage during the initiation stage have also been documented (*53*). On these complexes, the efficiency of slippage is also found to depend on the concentration and choice of initiation nucleotide or dinucleotide. Most interestingly, the Pol II slippage transcripts appear to re-enter templated transcription.

In all the examples presented here, the promoter sequence itself is shown to exert the primary influence on the formation of unproductive ITCs, the extent of which can be modulated by reaction parameters such as the nature and concentration of the nucleotide substrates. Our results confirm the hypothesis that formation of multiple types of complexes, whether during initiation or elongation, is driven by DNA sequence.

The Position of Partitioning Can Be Inferred from Single-Cycle Transcription Kinetics and the Effect of NTP Concentration. At which step during transcription initiation is the unproductive ITC formed? Our finding that the unproductive ITCs synthesize mostly 2−3 nt abortive RNA points to its formation before the catalysis that forms the first phosphodiester bond. The kinetics of abortive synthesis during single-cycle transcription, revealing that abortive synthesis continues for more than 2 h after productive synthesis has abruptly ceased, also support this conclusion. Were partitioning to take place later in the abortive cycling process, the unproductive ITC fraction would gradually convert into the productive ITCs by repartitioning after each round of abortive release and reinitiation. The observation that the NTP concentration and the nature of the initiating nucleotide exert an influence on partitioning further focuses the partitioning event to the *de novo* RNA chain initiation reaction or before. For λ P_R, branching to form the moribund complexes was also localized to the binary complex stage (*25*).

Potential Barriers to Escape from the Promoter. To escape from the promoter, RNA polymerase must successfully negotiate two distinct types of reactions. First, it has to synthesize the nascent RNA transcripts without aborting them. Second, it must be able to translocate downstream from the promoter by displacing the σ−DNA contacts, and binding tightly to its transcript (*4*−*6*, *21*). We shall refer to the first phase as the nucleotide addition−abortive initiation phase and to the second as the transitional or promoter escape phase. Our results show that promoter escape barriers can occur at either phase.

An example of a promoter escape barrier at the nucleotide addition−abortive initiation phase is illustrated by our finding that the molar yield of the 2 nt abortive transcript is exceptionally high in the unproductive ITC fraction. Thus, a substantial fraction of unproductive ITCs appears to be blocked after catalysis of the formation of a single phosphodiester bond. Is this blockage related to the observation that the nature and concentration of the initiating nucleotide

affect the extent of unproductive ITC formation? This is likely if the architecture of the catalytic center in this class of unproductive ITCs is altered such that the binding of the initiating nucleotide is impaired. Such a blockage might then be alleviated by a high concentration of the initiating nucleotide.

Among the unproductive ITCs, there exists a fraction that is blocked at the transitional step. These complexes are not blocked at the nucleotide addition phase as evidenced by the synthesis of abortive transcripts up to 9−15 nt in length, but are unable to bring about the promoter escape transition to produce the full-length RNA. Consistent with this notion, a number of RNA polymerase mutants that are able to perform abortive initiation but unable to achieve promoter escape have previously been characterized (*54*−*56*). Alternatively, the promoter escape transition can be brought about sooner by RNA polymerase mutations in a specific region of σ^{70} (*57*). Our investigation into the role of the initial transcribed sequence shows that, on a number of promoters, sequence variation in the region of nucleotides 16−20 can greatly influence the promoter escape transition with little effect on the abortive initiation process (*34*). The current available evidence suggests that the actual promoter escape transition is dependent on the specifics of RNA polymerase−promoter DNA interaction and may vary from promoter to promoter.

REFERENCES

1. McClure, W. R., Cech, C. L., and Johnston, D. E. (1978) *J. Biol. Chem. 253*, 8941.
2. Carpousis, A. J., and Gralla, J. D. (1980) *Biochemistry 19*, 3245.
3. Munson, L. M., and Reznikoff, W. S. (1981) *Biochemistry 20*, 2081.
4. Carpousis, A. J., and Gralla, J. D. (1985) *J. Mol. Biol. 183*, 165.
5. Krummel, B., and Chamberlin, M. J. (1989) *Biochemistry 28*, 7829.
6. Metzger, W., Schickor, P., Meier, T., Werel, W., and Heumann, H. (1993) *J. Mol. Biol. 232*, 789.
7. Hsu, L. M., Vo, N. V., and Chamberlin, M. J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11588.
8. Marr, M. T., and Roberts, J. W. (2000) *Mol. Cell 6*, 1275.
9. Luse, D. S., Kochel, T., Kuempel, E. D., Coppola, J. A., and Cai, H. (1987) *J. Biol. Chem. 262*, 289.
10. Levin, J. R., Krummel, B., and Chamberlin, M. J. (1987) *J. Mol. Biol. 196*, 85.
11. Martin, C. T., Muller, D. K., and Coleman, J. E. (1988) *Biochemistry 27*, 2690.
12. Whipple, F. W., and Sonenshein, A. L. (1992) *J. Mol. Biol. 223*, 399.
13. Chamberlin, M. J., and Hsu, L. M. (1996) in *Regulation of Gene Expression in Escherichia coli* (Lin, E. C. C., and Lynch, A. S., Eds.) pp 7−25, R. G. Landes Co., Austin, TX.
14. Dvir, A. (2002) *Biochim. Biophys. Acta 1577*, 208.
15. Hsu, L. M. (2002) *Biochim. Biophys. Acta 1577*, 191.
16. Cheetham, G. M. T., and Steitz, T. A. (1999) *Science 286*, 2305.
17. Korzheva, N., Mustaev, A., Kozlov, M., Malhotra, A., Nikiforov, V., Goldfarb, A., and Darst, S. A. (2000) *Science 289*, 619.
18. Murakami, K. S., Masuda, S., Campbell, E. A., Muzzin, O., and Darst, S. A. (2002) *Science 296*, 1285.
19. Hansen, U. M., and McClure, W. R. (1980) *J. Biol. Chem. 255*, 9564.
20. Bernhard, S. L., and Meares, C. F. (1986) *Biochemistry 25*, 5914.
21. Straney, D. C., and Crothers, D. M. (1987) *J. Mol. Biol. 193*, 267.
22. Bar-Nahum, G., and Nudler, E. (2001) *Cell 106*, 443.
23. Mukhopadhyay, J., Kapanidis, A. N., Mekler, V., Kortkhonjia, E., Ebright, Y. W., and Ebright, R. H. (2001) *Cell 106*, 453.
24. Kubori, T., and Shimamoto, N. (1996) *J. Mol. Biol. 256*, 449.
25. Sen, R., Nagai, H., and Shimamoto, N. (2000) *J. Biol. Chem. 275*, 10899.
26. Gonzalez, N., Wiggs, J., and Chamberlin, M. J. (1977) *Arch. Biochem. Biophys. 182*, 404.
27. Chamberlin, M., Nierman, W., Wiggs, J., and Neff, N. (1979) *J. Biol. Chem. 254*, 10061.
28. Uptain, S. M. (1997) Ph.D. Thesis, University of California, Berkeley, CA.
29. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res. 16*, 7351.
30. Hsu, L. M. (1996) *Methods Enzymol. 273*, 59.
31. Sheen, J. Y., and Seed, B. (1988) *BioTechniques 6*, 942.
32. Arndt, K. M., and Chamberlin, M. J. (1990) *J. Mol. Biol. 213*, 79.
33. McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171.
34. Vo, N. V. (1998) Ph.D. Thesis, University of California, Berkeley, CA.
35. Straney, D. C., and Crothers, D. M. (1985) *Cell 43*, 449.
36. Hinkel, D. C., Ring, J., and Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 197.
37. Nierman, W. C., and Chamberlin, M. J. (1980) *J. Biol. Chem. 255*, 4495.
38. Susa, M., Sen, R., and Shimamoto, N. (2002) *J. Biol. Chem. 277*, 15407.
39. Foster, J. E., Holmes, S. F., and Erie, D. A. (2001) *Cell 106*, 243.
40. Feng, G. H., Lee, D. N., Wang, D., Chan, C. L., and Landick, R. (1994) *J. Biol. Chem. 269*, 22282.
41. Reynold, R., Bermudez-Cruz, R. M., and Chamberlin, M. J. (1992) *J. Mol. Biol. 224*, 31.
42. Levin, J. R., and Chamberlin, M. J. (1987) *J. Mol. Biol. 196*, 61.
43. Kainz, M., and Roberts, J. W. (1992) *Science 255*, 838.
44. Chan, C. L., and Landick, R. (1994) in *Transcription: Mechanisms and Regulation* (Conaway, R. C., and Conaway, J. W., Eds.) pp 297−321, Raven Press, New York.
45. Suh, W. C., Ross, W., and Record, M. T., Jr. (1993) *Science 259*, 358.
46. Zaychikov, E., Denissova, L., Meier, T., Gotte, M., and Heumann, H. (1997) *J. Biol. Chem. 272*, 2259.
47. Jacques, J.-P., and Kolakofsky, D. (1991) *Genes Dev. 5*, 707.
48. Liu, C., Heath, L. S., and Turnbough, C. L., Jr. (1994) *Genes Dev. 8*, 2904.
49. Tu, A. H., and Turnbough, C. L., Jr. (1997) *J. Bacteriol. 179*, 6665.
50. Han, X., and Turnbough, C. L., Jr. (1998) *J. Bacteriol. 180*, 705.
51. Jin, D. J. (1994) *J. Biol. Chem. 269*, 17221.
52. Qi, F., and Turnbough, C. L., Jr. (1995) *J. Mol. Biol. 254*, 552.
53. Pal, M., and Luse, D. S. (2002) *Mol. Cell. Biol. 22*, 30.
54. Kashlev, M., Lee, J., Zalenskaya, K., Nikiforov V., and Goldfarb, A. (1990) *Science 248*, 1006.
55. Lee, J., Kashlev, M., Borukhov, S., and Goldfarb, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6018.
56. Sagitov, V., Nikiforov, V., and Goldfarb, A. (1993) *J. Biol. Chem. 268*, 2195.
57. Chan, C. L., and Gross, C. A. (2001) *J. Biol. Chem. 276*, 38201.
58. Hsu, L. M., Vo, N. V., Kane, C. M., and Chamberlin, M. J. (2003) *Biochemistry 42*, 3777−3786.
59. Vo, N. V., Hsu, L. M., Kane, C. M., and Chamberlin, M. J. (2003) *Biochemistry 42*, 3798−3811.

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