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Recognition of a Human Arrest Site Is Conserved between RNA Polymerase II and Prokaryotic RNA Polymerases*

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Abstract

DNA sequences that arrest transcription by either eukaryotic RNA polymerase II or *Escherichia coli* RNA polymerase have been identified previously. Elongation factors SII and GreB are RNA polymerase-binding proteins that enable readthrough of arrest sites by these enzymes, respectively. This functional similarity has led to general models of elongation applicable to both eukaryotic and prokaryotic enzymes. Here we have transcribed with phage and bacterial RNA polymerases, a human DNA sequence previously defined as an arrest site for RNA polymerase II. The phage and bacterial enzymes both respond efficiently to the arrest signal *in vitro* at limiting levels of nucleoside triphosphates. The *E. coli* polymerase remains in a template-engaged complex for many hours, can be isolated, and is potentially active. The enzyme displays a relatively slow first-order loss of elongation competence as it dwells at the arrest site. Bacterial RNA polymerase arrested at the human site is reactivated by GreB in the same way that RNA polymerase II arrested at this site is stimulated by SII. Very efficient readthrough can be achieved by phage, bacterial, and eukaryotic RNA polymerases in the absence of elongation factors if 5-Br-UTP is substituted for UTP. These findings provide additional and direct evidence for functional similarity between prokaryotic and eukaryotic transcription elongation and readthrough mechanisms.

The *in vitro* rate of transcript elongation by RNA polymerases is complex. There is a wide variation in the time RNA polymerase spends inserting a nucleotide into the different positions of a growing RNA chain. The dwell time at a specific site can vary as a function of the template and transcript sequence and structure. Certain sites at which the dwell time is exceptionally long, but through which transcription by RNA polymerase II can be stimulated by elongation factor SII, are referred to as arrest sites. These have been operationally defined through *in vitro* experiments on defined DNA sequences (1– 6). Similarly, DNA sequences have been identified at which elongation by *Escherichia coli* RNA polymerase ceases but can be reactivated by GreB, a factor that is functionally related to SII (7–11). Arrest sites are often, but not always, associated with tracts of A-T base pairs. Relatively few such sites have been described in either eukaryotic or prokaryotic gene sequences and no consensus sequence has emerged that enables their prediction *a priori* from raw DNA sequence. A regulatory role of such sites has been suggested, but their specific biological function is unknown.

Studies of the mechanism of chain polymerization by DNA-dependent RNA polymerases and factor-dependent transcript elongation in particular have exploited arrested elongation complexes as an experimental tool. We and others have used an arrest site from a human

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histone gene (called site Ia) as a model (2, 12–15). At this site, RNA polymerase II is converted into an elongation-incompetent enzyme that is unable to extend RNA chains despite the presence of a high concentration of all four nucleoside triphosphate substrates (2). The rate at which RNA polymerase II, which has reached the arrest site, acquires the arrested configuration appears to be a first-order process (16). A similar time-dependent decay of elongation potential at specific bacterial DNA sequences has been observed for bacterial RNA polymerase (7, 17). The Ia arrest site is coincident with a bend in the DNA helix and the extent of bending is correlated with the efficiency of arrest (18). The unusual structure of this region of DNA duplex, or the transcription bubble formed from it, may account for the difficulty RNA polymerase II has in transcribing through it (18).

SII is thought to stimulate arrested transcription by allosterically activating an intrinsic ribonuclease activity in RNA polymerase II that shortens the nascent RNA at its 3′-end. This chain can be re-extended, thus providing another opportunity for RNA synthesis through the arrest site (reviewed in Refs. 19 and 20). SII-activated nascent RNA cleavage has been observed *in vitro* for RNA polymerases II from evolutionarily diverged species including yeast and humans (4, 21–24). A similar activity has also been described for bacterial RNA polymerases (8, 25). Evidence that RNA cleavage is causally involved in transcription elongation has been well documented for RNA polymerase II and bacterial RNA polymerase. Eukaryotic RNA polymerases I and III, vaccinia virus, and bacteriophage T7 RNA polymerases also display nascent RNA nuclease activities (26 –32). A role for the nuclease activity in the stimulation of elongation is less clear for these enzymes.

The striking evolutionary conservation observed in the two largest subunits of the multisubunit DNA-dependent RNA polymerases suggests that the fundamental mechanisms of RNA chain synthesis and extension are conserved (33). In fact, models of transcriptional arrest have been constructed from studies on prokaryotic and eukaryotic RNA polymerases and are thought to be applicable to both classes of enzyme (34). Most models describe a pattern of translocation of RNA polymerase relative to DNA that is dynamic; *i.e.* at certain DNA sequences movement of the protein along DNA is not necessarily commensurate with RNA chain elongation (10, 11, 34 –36). Evidence for sequence-dependent variations in the structure of *E. coli* and RNA polymerase II elongation complexes that accompany the state of arrest has been presented (9 –11, 15, 17, 37, 38). In one model, RNA polymerase at an arrest site can undergo limited RNA chain extension and at least some portion of the enzyme (perhaps its leading edge) does not translocate relative to DNA during this interval of chain polymerization (9 –11, 15, 19, 35). In a second model, chain extension is initially accompanied by translocation of the enzyme on the template. The arrest process is postulated to involve the subsequent sliding back of RNA polymerase on the template to an upstream position (14, 17, 34, 39). In both models, arrest is accompanied by an unusual upstream location of the DNA-protein contacts after the synthesis of arrested RNA and the displacement of the 3′-end of RNA from the active site. Both models suggest that the activation of cleavage of the nascent RNA by elongation factors results in the correct repositioning of the active site relative to the 3′-hydroxyl group of the RNA chain and this re-establishes elongation competence.

Here we have extended the comparison of functional similarities between bacterial and eukaryotic RNA polymerases by studying elongation through a well characterized RNA polymerase II arrest site by these enzymes as well as by bacteriophage SP6 RNA polymerase. All can specifically recognize the human arrest site, albeit with varying efficiencies. Each enzyme can also evade arrest at this site *in vitro* by incorporating the base analog 5-Br-UMP into RNA.

EXPERIMENTAL PROCEDURES

Proteins and Reagents

E. coli RNA polymerase was isolated as described from *E. coli* bearing inactivated *gre*A and *gre*B genes (AD8751; gift of A. Das, University of Connecticut; see Ref. 40) and transformed with a plasmid encoding the largest subunit of RNA polymerase bearing a hexahistidine tag (courtesy of R. Landick, University of Wisconsin). Nickel-purified enzyme was then subjected to phosphocellulose chromatography as described (41). Recombinant GreB was purified as described (11) from an overexpressing *E. coli* strain (RL 747; courtesy of R. Landick, University of Wisconsin). 5-Bromouridine 5′-triphosphate was purchased from Sigma. Nucleotides used in transcription were fast protein liquid chromatography-purified (Amersham Pharmacia Biotech). [α-^{32}P]CTP was purchased from Amersham Pharmacia Biotech or NEN Life Science Products. Pansorbin® (formalin-fixed *Staphylococcus aureus*) was purchased from Calbiochem. Partially purified general transcription factors and RNA polymerase II were isolated from rat liver as described (42) or were purified from *E. coli* (TFIIB; Ref. 43). Recombinant human SII was expressed in *E. coli* containing the plasmid pT7–7/Met (courtesy of C. Kane, University of California, Berkeley) and purified by phosphocellulose chromatography as described (44). Labeled marker RNAs were synthesized *in vitro* by *E. coli* RNA polymerase from the plasmid pKK34 –121 (45), which bears two tandem promoters and two tandem terminators.

Templates

The plasmid pGEMTerm was constructed by inserting a 285-base pair *Taq*I fragment from the human histone gene H3.3 (2) into *Acc*I-cut pGEM2 (Promega Corp., Madison, WI). To construct p*rrn*Ia, a polymerase chain reaction was performed using pKK34 –121 (45) and the primers 5′-GTTACGGCTTCGAAACGCTC-3′ and 5′-GGCGAAGCGAAAAAGAGTCG-3′. The resulting 270-base pair polymerase chain reaction product was digested with *Bst*UI and the resulting 185-base pair fragment was inserted into the *Sma*I site of pGEMTerm. pAd-Term-2 has been described previously (2).

In Vitro Transcription with E. coli RNA Polymerase

Each reaction contained 42 mM Tris, pH 8.0, 14 mM $MgCl_2$, 300 mM β-mercaptoethanol, 20 mM NaCl, 0.4 mM EDTA, 2% glycerol, 50 μg/ml bovine serum albumin, 0.12 mM each ATP, UTP, and GTP (unless otherwise indicated), 200 ng *Pst*I-cut p*rrn*Ia, 5 μCi [α-^{32}P]CTP (400 Ci/mmol, Amersham Pharmacia Biotech), and 0.5 μg *E. coli* RNA polymerase. Initiated complexes were formed by incubation of this mixture at 22 °C for 2–5 min for a labeling pulse. Complexes were then precipitated with 0.5 μg anti-RNA IgG per reaction and Pansorbin® (46, 47) and washed twice with equal volumes of reaction buffer to generate “washed” complexes. Transcription was completed with the addition of 50 μg/ml heparin, 7 mM $MgCl_2$, 0.12 mM each ATP, UTP, and GTP, and varying concentrations of CTP for 10 min at 30 °C. In some experiments, arrested Ia complexes were washed with 3 rounds of immunoprecipitation, treated with 2 μg GreB and 7 mM $MgCl_2$. Reactions were stopped by the addition of 0.3 M sodium acetate, 40 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.25 μg yeast RNA. RNA was precipitated with ethanol and dissolved in 80% (v/v) formamide, 0.05% (w/v) bromphenol, and 0.05% (w/v) xylene cyanol in 89 mM Tris, pH 8, 89 mM boric acid, 1 mM EDTA (TBE). Samples were loaded on 5% (w/v) polyacrylamide, 0.25% (w/v) bisacrylamide gels cast with 50% (w/v) urea in TBE, exposed to autoradiographic film (Eastman Kodak Co.), and imaged with a Fuji (Stamford, CT) BAS1000 phosphorimager.

Transcription with SP6 RNA Polymerase

One reaction contained 10 units of SP6 RNA polymerase (New England Biolabs, Beverly, MA or Life Technologies, Inc.), 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 28 units of RNasin™ (Promega, Madison, WI), 0.5 μg of linearized pGEMTerm, 10 μCi [α-^{32}P]CTP (400 Ci/mmol), 2.4 mM each of ATP, UTP, GTP, and varying concentrations of CTP. Reactions were incubated at 37 °C as indicated, stopped with 0.375 M sodium acetate and 25 mM EDTA, extracted with phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and loaded onto a 7% (w/v) acrylamide, 0.35% (w/v) bisacrylamide gel in 50% (w/v) urea and TBE.

Rat Liver RNA Polymerase II Transcription

A reaction equivalent contained 2 μg of RNA polymerase II, 2 μg of fraction D, 8.4 units RNasin™, 2.2% (w/v) polyvinyl alcohol, 200 ng of pAdTerm2 cut with *Pst*I or *Nde*I, 0.5 mg/ml acetylated bovine serum albumin (New England Biolabs), 20 mM Tris-HCl, pH 7.9, 2 mM dithiothreitol, 150 mM KCl, and 20 mM HEPES, pH 7.9, in a total volume of 20 μl, and incubated at 30 °C for 30 min. An additional 30 μl of the same buffer lacking KCl and containing recombinant TFIIB and 1.5 μg fraction TFIIE/TFIIF, was added and incubated 20 min at 30 °C. 20 μCi [α-^{32}P]CTP (400 Ci/mmol), 7 mM $MgCl_2$, and 20 μM each ATP and UTP were added and incubated at 30 °C for 15 min. Heparin (10 μg/ml) and all four nucleotides (800 μM unless otherwise indicated) were added. Reactions were stopped with 0.2 M Tris-HCl, pH 7.5, 25 mM EDTA, 0.3 M NaCl, and 2% (w/v) SDS. RNA was isolated and separated on a 5% polyacrylamide, 0.25% bisacrylamide gel in 50% urea, TBE.

RESULTS

Our experimental system employs a template containing the adenovirus major late promoter located upstream of a well characterized arrest site from a human histone gene (Ia; Fig. 1*A*). Due to the template sequence, elongation complexes bearing a 14-base ^{32}P-labeled transcript can be assembled *in vitro* with partially purified general transcription factors and rat liver RNA polymerase II in the absence of GTP (2, 42). Upon addition of heparin to prevent reinitiation and all four nucleoside triphosphates, synchronous elongation can be monitored for a single round of transcription.

At site Ia, approximately 50% of the RNA polymerase II molecules cease elongation in the presence of a high level (800 μM) of each nucleoside triphosphate (2). There is a direct correlation between the concentration of the next nucleotide (CTP) needed for chain elongation at site Ia, and the fraction of RNA polymerase II that reads through the site (16). Here we have examined the elongation kinetics at different concentrations of CTP (Fig. 2*A*). At all concentrations, RNA polymerase II preferentially stops at site Ia. At 380 μM CTP the maximal level of readthrough attainable (≈50%) in the absence of SII was observed. At lower concentrations, average elongation rates were correspondingly slowed and transcript Ia represented an increased fraction of the total RNA synthesized (Fig. 2*A*, *lanes 6–20*). This is not simply because the enzymes are starved for CTP, since an early burst of synthesis of the cytidine containing, ≈200-base Ia RNA is evident in each case (by 10, 20, and 60 min for 60, 20, and 10 μM CTP, respectively), yet read-through is largely unchanged over the ensuing period. When elongation complexes assembled in reactions containing 6 μM CTP or 380 μM CTP were challenged with an additional 380 μM CTP, only a small amount of additional run-off transcript was observed (Fig. 2*B, lane 1 versus 2*, *4 versus 5*). Hence, the enzymes at site Ia were no longer elongation competent even when the CTP level was increased to an amount sufficient for high levels of readthrough when it was provided early in the reaction. SII enabled quantitative readthrough of site Ia confirming that these RNAs are associated with potentially active elongation complexes (Fig. 2*B*, *lanes 3* and *6*).

Substitution of 5-bromo-UTP for UTP reduces the extent of termination by *E. coli* RNA polymerase at terminators and improves readthrough at bacterial arrest sites, presumably because of stronger base pairing by the brominated derivative in RNA with the cognate template bases (39, 48 –50). Similarly, we observed very efficient readthrough by RNA polymerase II with 5-Br-UTP in the absence of SII (Fig. 2*C*). Indeed, there was virtually complete readthrough after 10 min of elongation. This is despite the fact that Br-UTP is used less well as a substrate than UTP, as is apparent from the slower average elongation rate in its presence (Fig. 2*C*, compare 2 min samples between Br-UTP and UTP). The particularly strong effect seen here may be due to the sequence of site Ia, which encodes seven consecutive U residues at the 3′-end of the transcript (Fig. 1*A*). Substitution of 5-iodo-UTP for UTP had a similar effect (data not shown).

To analyze the ability of *E. coli* RNA polymerase to read through the human arrest site, we constructed a plasmid (Fig. 1*B*) containing the P_2 promoter from the *E. coli rrnB* operon upstream of the same H3.3 arrest sequences transcribed by RNA polymerase II in Fig. 2. *E. coli* RNA polymerase was purified *via* a hexahistidine-tagged β' subunit from a strain in which the *greA* and *greB* genes, which encode transcript cleavage/elongation factors, were inactivated (40). Initiated transcription complexes bearing short ^{32}P-labeled RNAs (Fig. 3*A*, *lane 1*) were immunoprecipitated with an anti-RNA monoclonal antibody as described previously for RNA polymerase II complexes (47). New initiation events were suppressed by the addition of heparin. At 1 mM each of all 4 NTPs, elongation of the radioactive RNA chains yielded 80 –90% readthrough in 10 min (data not shown). In contrast, when the level of CTP was reduced, *E. coli* RNA polymerase preferentially accumulated at site Ia (Fig. 3*A*, *lanes 3–6*). At 1 μM CTP, a concentration that supports long transcript synthesis from another promoter on the plasmid (transcript "*p*"; Fig. 3*A*, presumably the ampicillin resistance gene), 90% of the enzymes transcribing from *rrn*P_2 stopped at site Ia (*lane 3*). Arrest efficiencies dropped with increasing CTP levels (*lanes 4–6*). To demonstrate that these were arrested polymerases, we incubated elongation complexes assembled at each CTP concentration for 10 more minutes with an additional 1 mM CTP (Fig. 3, *A*, *lanes 7–11,* and *B*). Complexes at site Ia were refractory to this high CTP chase, except at the very low CTP concentration of 100 nM, at which many of the enzymes had not yet reached site Ia during the initial incubation (Fig. 3*A*, *lane 2 versus 7*). At 1 μM CTP, 80% of the RNA polymerases were unable to elongate past site Ia after incubations of up to 2 h (Fig. 4, *lanes 1–4* and *6*). Chasing these complexes with 1 mM CTP yielded little additional extension of the Ia transcript (70% arrest, *lanes 5* and *7*). When GreB was included in the chase with 1 mM CTP, the Ia transcript was quantitatively extended to run-off (data not shown), demonstrating the high degree of stability of this complex since neither the RNA nor the DNA dissociated from RNA polymerase. At relatively low levels of CTP, *E. coli* RNA polymerase paused but did not become arrested at site II (Fig. 4), a previously described tract of six T residues followed by a C residue (Fig. 1*A* and Ref. 2).

In the presence of elongation factor GreB, *E. coli* RNA polymerase becomes released from arrest-like sites in bacterial DNA, such as those found immediately downstream of the *rrnB*-P_1 and phage T7 A1 promoters (7–11, 17, 39). Template sequences associated with bacterial and phage transcription terminators may represent similar functional signals for RNA polymerase (7, 10, 11). To test if GreB could act upon *E. coli* RNA polymerase arrested at human site Ia, we challenged isolated Ia complexes with all four NTPs and GreB (Fig. 5, *lane 5*). This treatment resulted in the efficient readthrough of site Ia.

When RNA polymerase II elongation complexes arrested at site Ia are depleted of NTPs, SII-activated nascent transcript cleavage can be observed (21). This represents an intermediate that precedes factor-dependent readthrough (37). Similar GreB-stimulated cleavage events have been observed for *E. coli* RNA polymerase blocked by bacterial DNA

sequences (8, 25). Here, GreB-activated cleavage was observed at site Ia when the complexes were depleted of NTPs by immunoprecipitation using an anti-RNA antibody (Fig. 5, *lane 2*). As seen for the RNA polymerase II-SII interaction, GreB-activated nascent RNA cleavage by *E. coli* RNA polymerase was sensitive to the anionic detergent sarkosyl and resistant to the polyanion heparin (Fig. 5, *lanes 3* and *4*, respectively). The cleavage product could be shown to be an elongation intermediate since it was detected when GreB was added to Ia complexes with ATP, UTP, GTP, and the chain-terminating CTP-analog, 3′-dCTP (Fig. 5, *lane 6*). A similar RNA cleavage pattern has been observed for SII-activated cleavage of transcript Ia by RNA polymerase II (51).

We have previously measured the first-order rate at which RNA polymerase II residing at site Ia becomes arrested (16). Here, we carried out a similar set of experiments to test the propensity of *E. coli* RNA polymerase to become arrested as a function of time at site Ia. Ia complexes (Fig. 6*A*, *lane 1*) were washed free of NTPs by immunoprecipitation, treated with $MgCl_2$ and GreB to generate a truncated transcript (*lane 2*), and washed again by immunoprecipitation to remove GreB. Sodium chloride (0.5 M) was added with nucleotides to suppress the activity of any residual GreB that might remain. (Similar results were obtained when sarkosyl was employed for this purpose.) These complexes were then provided with a high concentration (1 mM) of all four NTPs to permit readthrough (*lane 3*), or only ATP, UTP, and GTP to support limited chain re-extension back to site Ia (*lane 4*). After increasing intervals of time with only ATP, UTP, and GTP, samples were challenged with 1 mM CTP for 10 min to assess their ability to extend RNA chains in a factor-independent manner (*lanes 5–10*). As CTP was withheld for increasing amounts of time, the fraction of RNA polymerase that became arrested, *i.e.* refractory to a chase with 1 mM CTP, increased correspondingly. In contrast to RNA polymerase II, which almost completely loses its elongation potential while dwelling at site Ia for only a few seconds at 28 °C (Ref. 16 and Fig. 6*B*), *E. coli* RNA polymerase acquired the arrested state very slowly at 30 °C with a half-life of ≈700 s and an apparent first-order rate constant of 0.001 s^{-1}. These complexes remained active since they could be elongated when GreB was added with NTPs and magnesium (data not shown). This value is 14-fold slower than RNA polymerase II's acquisition of the arrested state at site Ia (50 s; Ref. 16) and almost 2-fold slower than a previous estimate of *E. coli* RNA polymerase loss of elongation competence at a site in phage T7 DNA that has the properties of an arrest site (7).

Incorporation into RNA of 5-Br-UMP in place of UMP, results in reduced arrest efficiency of *E. coli* RNA polymerase at a site in the phage T7 A1 transcription unit (39). This, combined with the finding that 5-Br-UMP incorporation had a profound influence on arrest by RNA polymerase II at site Ia (Fig. 2*C*), led us to expect a similar effect on *E. coli* RNA polymerase at site Ia. Indeed, the arrest efficiency was strongly reduced when 5-Br-UTP was used as a substrate (Fig. 7, *lanes 1–6 versus 7–12*). After 1 h of elongation, 82% of the enzymes became arrested at site Ia in the presence of UTP *versus* 42% when Br-UTP was employed. Again this was despite a significant slowing of the average elongation rate in the presence of the brominated base, as was evident from the slower accumulation of the large plasmid-derived RNA in the Br-UTP experiment (transcript "*p*"; Fig. 7, compare 5 min samples).

Relatively little is known about transcriptional arrest for the single subunit RNA polymerases encoded by bacteriophages. Recently, however, an RNA cleavage activity intrinsic to the enzyme has been described for T7 phage RNA polymerase (32). No cleavage/elongation stimulating factors comparable with SII or GreB have yet been identified for phage enzymes. The human DNA fragment containing site Ia was inserted downstream of a bacteriophage SP6 RNA polymerase promoter (Fig. 1*C*). As observed for *E. coli* RNA polymerase (data not shown), relatively little enzyme stopped at site Ia at high

NTP levels; 90% reached the end of the linear template (Fig. 8*A*, *lane 5*). Upon reduction of the CTP concentration, transcription became halted specifically at sites Ia and II, both of which are followed by a C residue, such that run-off levels of only 77 and 23% were attained at 100 and 10 μM CTP, respectively (*lanes 3* and *4*). A similar result was obtained at 10 μM CTP when the plasmid was linearized at a downstream site (*lane 2*). Transcription did not become blocked at a large number of other template positions at which CTP was required, nor at a previously characterized site called Ib, which is located between site Ia and II and is a stretch of five T residues followed by an A residue (2). Hence, when CTP was limiting, phage SP6 RNA polymerase specifically recognized these human sites with high efficiency. The short transcripts were not extended to run-off length when a high level of CTP was added, nor when heparin was added to prevent new initiation events (Fig. 8*A*, *lanes 6–8*). Substitution of 5-Br-UTP for UTP again resulted in a large increase in readthrough efficiency at levels of CTP that would otherwise restrict readthrough (Fig. 8*A*, *lane 9*). The previously described retardation of electrophoretic mobility of bromouridine-containing RNA relative to natural RNA, is evident in this experiment (*lane 6–8 versus lane 9*). When UTP and 5-Br-UTP were mixed in varying ratios but the total uridine nucleotide concentration was kept constant at 1 mM, there was a direct correlation between the level of the brominated base and extent of readthrough of sites Ia and II (Fig. 8*B*). In contrast to RNA polymerase II and *E. coli* RNA polymerase, the SP6 enzyme appeared to terminate at these sites since most Ia and II transcripts were released from the template as assessed by immobilized template experiments (data not shown).

DISCUSSION

Three phylogenetically distinct RNA polymerases recognized and stopped transcription at a human arrest site *in vitro,* albeit to differing extents. We conclude that at least some of the molecular events that characterize the arrest process are conserved between RNA polymerases and that the arrest site described here can be a general impediment to elongation.

RNA polymerase II has the highest propensity for arrest. It does so efficiently, even when NTP levels are not limiting (2). *E. coli* RNA polymerase only becomes arrested at limiting NTP concentrations, presumably because the elongation rate of the complex is slowed. Nevertheless, transcription stops specifically at the arrest site providing additional evidence that this site has unique features that serve to impede transcription elongation. Like RNA polymerase II, *E. coli* RNA polymerase remains template-engaged for long periods of time, is stable to isolation *via* its RNA moiety, and can resume elongation by the action of a dissociable factor that activates nascent RNA cleavage. Elongation factor GreB stimulates transcription through site Ia in a manner similar to, if not indistinguishable from, that described for SII and RNA polymerase II. This functional conservation is remarkable since the elongation factors are significantly different both in primary sequence and three-dimensional structure (52–54). Phage SP6 RNA polymerase also stops elongation efficiently at site Ia only when NTP-restricted. It had a distinct response to the arresting DNA sequence in that it appeared to release the RNA at the arrest site thereby terminating transcription. Since there is currently no known factor that can influence elongation by phage RNA polymerase, it is difficult to assess whether under some conditions restarting of enzyme that stopped at the arrest site is possible. A recently described nuclease activity of T7 RNA polymerase, an enzyme related to that of phage SP6, has not yet been linked to the stimulation of elongation as described for nascent RNA cleavage by bacterial and eukaryotic RNA polymerases (32).

Two obvious intrinsic differences between these enzymes are their elongation rates and their size and subunit structure. *In vitro* elongation rates of ≈230, 12–18, and 7–10 bases/sec have

been measured for purified T7 phage RNA polymerase, bacterial RNA polymerases, and calf thymus RNA polymerase II, respectively (55–57). Mutants of all three classes of enzyme have been found that reduce their elongation rates and increase termination or arrest efficiencies (44, 58 – 62). An increase in dwell time at a termination or arrest site by mutations in polymerase or due to substrate unavailability, may provide an opportunity for the enzyme to acquire a specific conformation favoring the termination or arrest pathway (63). Reaction conditions or factors that accelerate average elongation rates can increase the readthrough efficiency of RNA polymerase II (16, 64). Presumably, reduced dwell time diminishes the probability that polymerase will succumb to the effect of the arrest or terminator sequence. Termination and arrest efficiencies may also be affected by alteration of specific protein-protein or protein-nucleic acid interactions that may be independent of effects upon elongation rate or dwell time (61, 65).

E. coli RNA polymerase and rat liver RNA polymerase II both interact with proteins that stimulate nascent RNA cleavage and reactivate arrested enzymes. The general means by which this occurs appears conserved, *i.e.* elongation factor binds arrested polymerase and allosterically activates an intrinsic ribonuclease activity. However, this similarity does not appear to extend to cross-species function of GreB with rat RNA polymerase II or SII with bacterial RNA polymerase (data not shown). This may have been anticipated since GreB and SII lack significant homology in either primary sequence or three dimensional structure in their RNA polymerase binding domains (8, 53, 54, 66).

In a comparison of the rates at which the bacterial and eukaryotic RNA polymerases acquire factor dependence, we found that *E. coli* RNA polymerase becomes arrested much less readily than RNA polymerase II. Differences in the efficiency of arrest could be due to a higher rate of nascent RNA cleavage by *E. coli* RNA polymerase relative to RNA polymerase II in the absence of cleavage factor, the so-called “intrinsic” cleavage rate (40). In principle, a polymerase that is more efficient at carrying out the intrinsic reaction would be less dependent upon an elongation factor for readthrough. Alternatively, distinct readthrough efficiencies could be related to species-specific rates of a kinetic step that contributes to a conformational change. One potential step may be the tightness of binding of the RNA and/or DNA to their cognate binding sites on the elongating enzyme (7, 14, 17, 35, 39, 67–70). Physical evidence that a conformational change is associated with arrest has been presented in investigations of prokaryotic and eukaryotic enzymes.

SP6 RNA polymerase is a single large polypeptide chain of ≈98 kDa. The elongating form of *E. coli* RNA polymerase is a 4 subunit enzyme of ≈380 kDa; the largest two, β and β', contribute most or all of the determinants of catalytic function. RNA polymerase II consists of 10 –12 subunits, the two largest of which have regions of homology to the prokaryotic β and β' subunits (33). Structural analyses of representatives from these three classes of RNA polymerase, suggest that the elongating molecules encircle the DNA duplex template almost completely, akin to the sliding clamp subunit of DNA polymerases that impart processivity to those enzymes (71–74). The arrest site described here is coincident with a region of non-B DNA and this structural feature may be an important determinant in provoking arrest (18). Differences between enzymes could also result from differences in contacts between the enzyme and duplex DNA, melted DNA strands, or nascent RNA any of which may be a determinant of the extent to which arrest (or termination) takes place.

Incorporation of 5-Br-UMP into RNA reduces the *in vitro* termination efficiency of *E. coli* RNA polymerase (49) and vaccinia virus RNA polymerase (75). More recent work has shown that arrest by this enzyme is also suppressed by 5-Br-UTP or 5-Br-CTP (39). We show that all three enzymes examined here have significantly increased levels of readthrough of site Ia when 5-Br-UTP is used as a substrate. This extends the finding to

phage and eukaryotic RNA polymerases and represents another feature of elongation held in common by them. The relative stability of double helical nucleic acid is increased when the 5-position of the uridine ring is brominated (48). This property has been exploited to probe the role of an RNA-DNA hybrid in transcription complex stability and function and has led to suggestions of a role for a nascent RNA-DNA helix in arrest and termination by *E. coli* RNA polymerase (34, 39, 49). In one report, a single substitution of 5-Br-UMP for UMP at a position 7 bases upstream from the 3′-end, reduced arrest by almost 2-fold (39). One model applied to both bacterial and eukaryotic enzymes suggests that arrest results from sliding of RNA polymerase upstream from the template site occupied after the polymerization of an arrested RNA chain. There would be a commensurate upstream transposition of the region of melted DNA duplex. The region of RNA base paired to DNA is also postulated to shift upstream leaving the 3′ terminus of the transcript unpaired to template bases. This event may be suppressed if a tighter RNA-DNA hybrid could be generated at the end of the transcript. Application of this “sliding-clamp” model of arrest to RNA polymerase II predicts that incorporation of 5-Br-UMP into RNA would reduce arrest as seen for *E. coli* RNA polymerase (14, 17, 39). Evidence presented here is consistent with this idea. The effect of Br-UTP on elongation by SP6 RNA polymerase (Fig. 8) is also consistent with prior observations that an RNA-DNA hybrid is important for lateral stability of phage polymerase on DNA and that bacteriophage T7 RNA polymerase terminates at sites at which the RNA is U-rich but which lacks a discernible stem-loop secondary structure (70, 76 –79).

Alternatively, a direct RNA polymerase-RNA interaction may be perturbed by the incorporation of Br-UMP into RNA. 5-Br-uracil bears an additional 80 Da of mass per base than uracil. The van der Waals radius of Br is 1.95 Å, similar to the size of a methyl group (2.0 Å). Thus, this modification represents a significant increase over natural RNA in the occupied mass and volume that must be accommodated in the active site of the enzyme. If all seven RNA bases were paired to template A residues, these new features of bromine-derivatized RNA would be found in the major groove of the hybrid helix. Conceivably, the rate of an important conformational change within the protein, or between the protein and RNA, could be altered by brominating the RNA. In fact, alteration of protein-RNA contacts has been suggested as the basis for reduced termination efficiency of vaccinia virus RNA polymerase after incorporation of 5-Br-UMP (75). The termination signal for this polymerase is a tract of U near, but not at, the RNA 3′ terminus. This termination sequence in single-stranded RNA is recognized by a termination factor as the RNA emerges from the vaccinia virus elongation complex (80, 81). In this case, the effect of bromine in RNA is not likely to act at the level of RNA-DNA base pairing, but instead by perturbing productive protein-RNA contacts. A similar effect was observed when phosphorothioate nucleotides were incorporated into the vaccinia virus RNA termination sequence (82). In our system however, we do not find that incorporation of α-thio-UMP into transcript Ia by RNA polymerase II increases readthrough; in fact it dramatically reduces readthrough (data not shown). Our experiments, therefore, do not allow us to distinguish between steric effects on the protein structure and an alteration in hybrid stability due to Br-UMP in RNA.

The specific features of an arrest site that impede elongation by RNA polymerases are not known. Nonetheless, the sites are a useful tool for *in vitro* analysis of polymerase and elongation factor function. Their biological role and genomic distribution in prokaryotes and eukaryotes are also poorly understood and will likely be the focus of future work.

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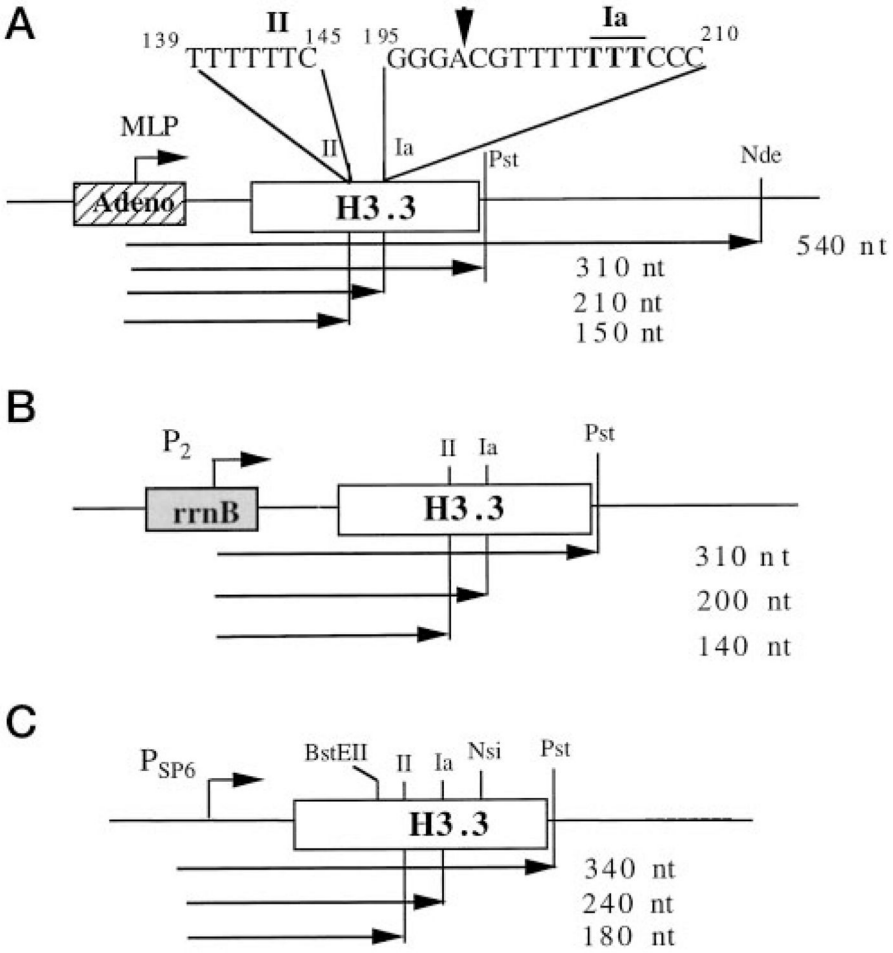

Fig. 1. Templates used for *in vitro* transcription

A, pAdTerm-2 contains the core adenovirus major late promoter (−50 to +10) and a 285-base pair segment of the human histone H3.3 gene. The sequence around arrest site Ia (*overlined*) and a related site (site II; Ref. 2) is shown. Numbering refers to base positions in the transcript relative to the transcription start (+1). The phosphodiester bond cleaved by RNA polymerase II in arrested Ia complexes is indicated (*downward arrowhead*). Approximate sizes of the expected transcripts are shown. *Bold* T represents the positions of the RNA 3′ termini determined previously (37). *B,* p*rrn*-Ia contains DNA sequence from the P_2 promoter of the *E. coli rrn*B operon inserted upstream of the human H3.3 sequences in pGEMTerm (see below). Approximate lengths of transcripts initiating from P_2 are indicated on the *right. C,* pGEMTerm contains the human H3.3 sequences inserted into pGEM2 downstream of an SP6 RNA polymerase promoter. Approximate transcript lengths are indicated on the right.

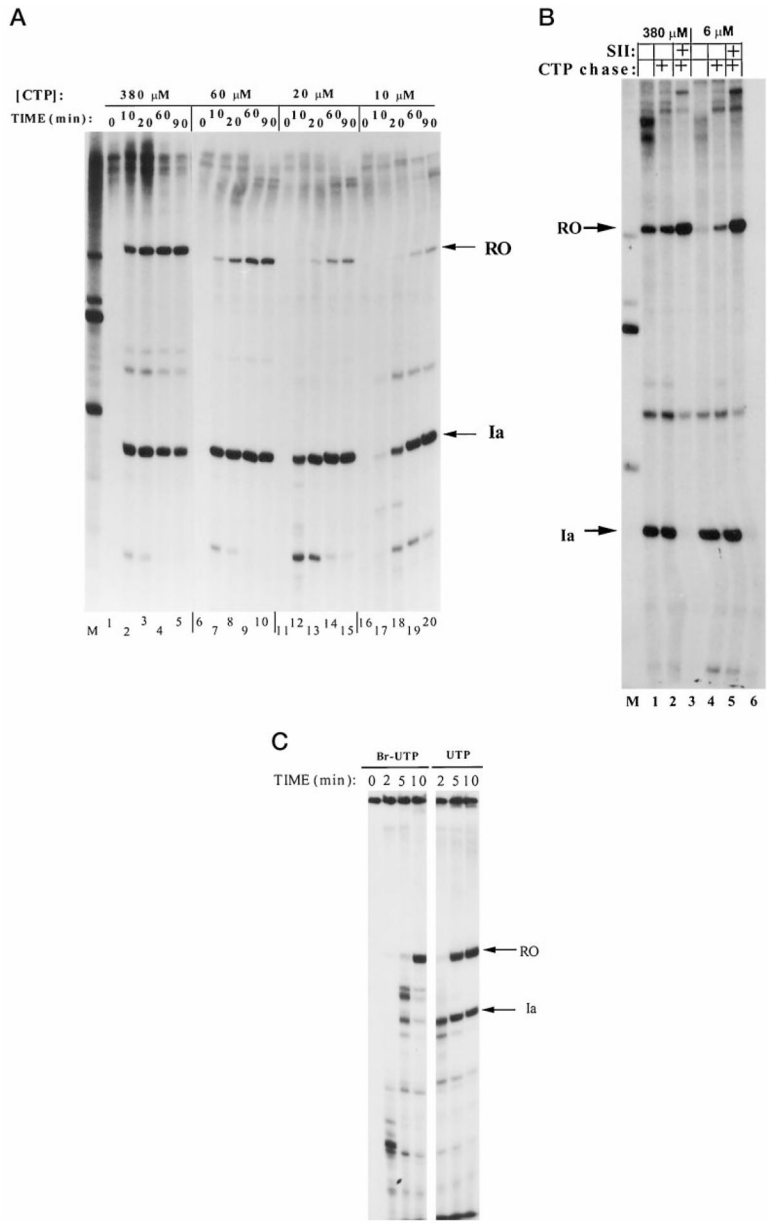

Fig. 2. *In vitro* transcription by RNA polymerase II on pAdTerm-2 cut with *Nde*I (*A* and *B*) or *Pst*I (*C*)

A, time course of elongation at varying CTP concentrations. Transcription was carried out in the presence of ATP, UTP, GTP, and the indicated concentrations of CTP. Samples were stopped at the indicated times and separated by gel electrophoresis. Run-off (*RO*) and Ia (*Ia*) RNAs are indicated with *arrows. Lane M,* reference RNAs of 260, 380, 420, and 540 bases. *B,* CTP "chase" of arrested complexes. Arrested complexes were assembled by incubation for 1 h at 28 °C with 380 μM (*lanes 1–3*) or 6 μM (*lanes 4–6*) CTP and 800 μM each ATP, UTP, and GTP. Samples were stopped (*lanes 1* and *4*) or incubated for 15 min with an additional 380 μM CTP without (*lanes 2* and *5*) or with recombinant human SII (0.5 μg; *lanes 3* and *6*). *Lane M,* reference RNAs of 260, 380, 420, and 540 bases. *C,* readthrough in the presence of 5-Br-UTP. Initiated complexes bearing a 14-base RNA were assembled and

prepared for electrophoresis (*lane 0*) or were incubated for the indicated times with 700 μM each ATP, GTP, CTP, and either 700 μM 5-Br-UTP or UTP.

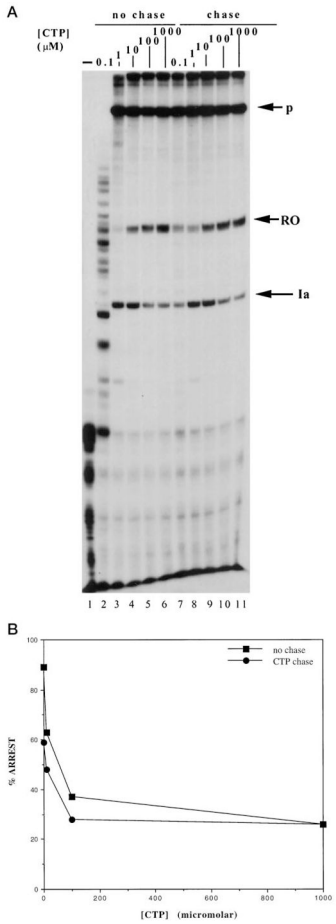

Fig. 3. Effect of CTP concentration on elongation by *E. coli* RNA polymerase

A, pulse-labeled elongation complexes were assembled on *Pst*I-cut p*rrn*-Ia, washed by immunoprecipitation (*lane 1*), and incubated for 10 min at 30 °C with ATP, GTP, and UTP (100 μM each), $MgCl_2$ (7 mM), heparin (50 μg/ml), and the indicated amounts of unlabeled CTP. Samples were challenged with water (*lanes 2–6*) or an additional 1 mM CTP (*lanes 7–11*) and incubated for 10 min at 30 °C. A long transcript derived from a promoter located elsewhere on the plas-mid is indicated (*p*). *B,* the radioactivity in the run-off and Ia RNAs from *lanes 2–6* and *8–11* of *panel A* were quantitated and percent arrest was calculated (= 100 × (phosphorimager units for Ia RNA divided by the sum of the units in Ia RNA and run-off RNA)).

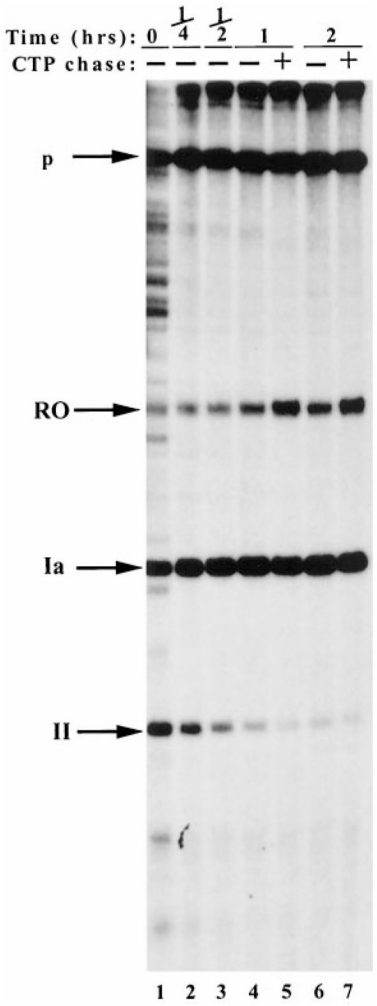

Fig. 4. Time course of elongation by *E. coli* RNA polymerase at 1μM CTP.

Arrested elongation complexes were assembled with 1 μM CTP (*lane 1*) as shown in Fig. 3, *lane 3*, and incubated for the indicated additional times at 30 °C. After 1 (*lane 5*) or 2 h (*lane 7*) a portion of the sample was adjusted to 1 mM each ATP, UTP, GTP, and CTP, and incubation continued for 10 min. RNA identities including pause site II are indicated on the *left*.

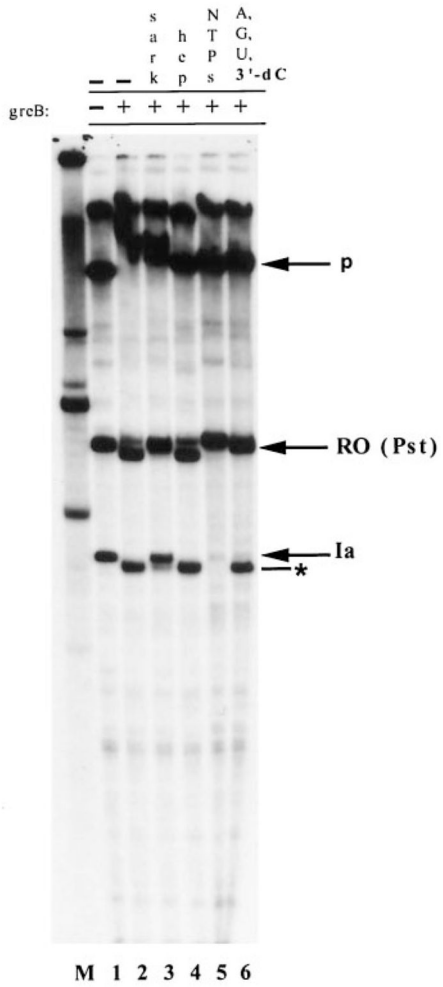

Fig. 5. Effect of GreB on arrested elongation complexes

Initiated complexes were assembled with *E. coli* RNA polymerase as described under "Experimental Procedures." Arrested elongation complexes were formed by incubation with 100 μM CTP and 800 μM each ATP, GTP, and UTP and washed by immunoprecipitation. Complexes were incubated with 7 mM $MgCl_2$, and either buffer (*lane 1*) or 2 μg GreB (*lanes 2–6*). Sarkosyl (0.25%; *lane 3*), heparin (10 μg/ml; *lane 4*), 1 mM each of all four nucleotides (*lane 5*), or 1 mM each ATP, UTP, GTP, and 3′-deoxy-CTP (*lane 6*) were also added, and incubation was continued for 5 min at 30 °C. The major GreB cleavage product is indicated (*). *Lane M,* reference RNAs of 260, 380, 420, and 540 bases

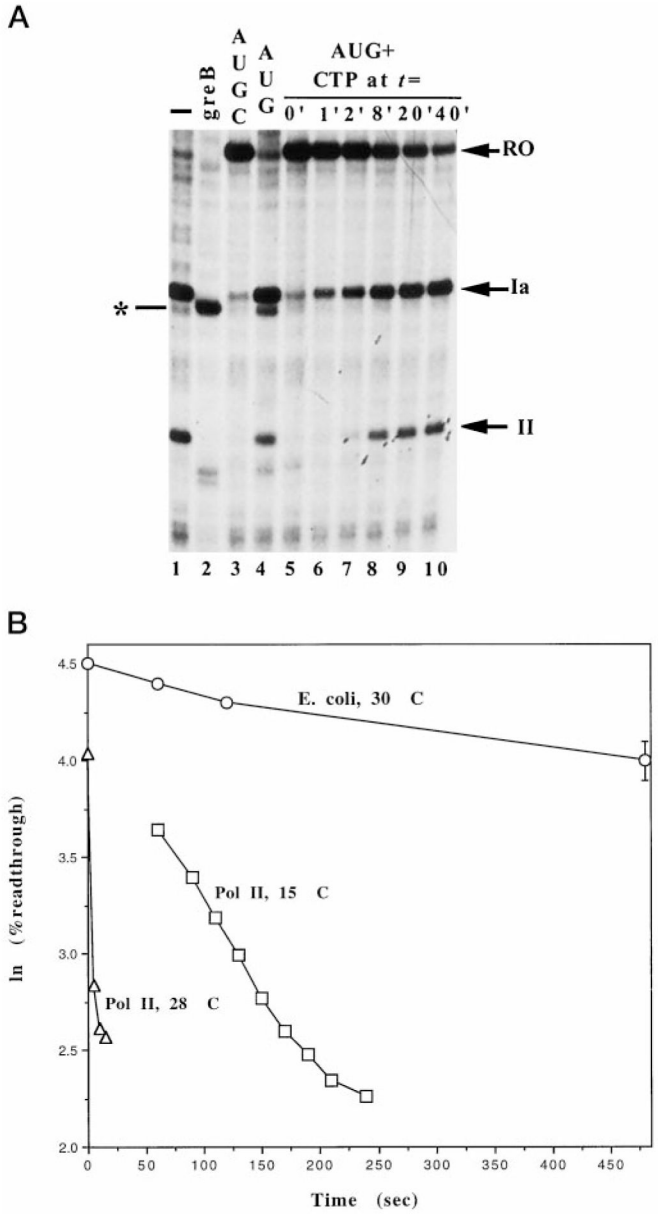

Fig. 6. Rate of arrest by *E. coli* RNA polymerase at site Ia

A, arrested complexes were assembled (*lane 1*) as shown in Fig. 3, *lane 3*, washed by immunoprecipitation, and treated with 7 mM $MgCl_2$ and GreB (1.3 μg) for 5 min at 30 °C (*lane 2*). These cleaved complexes were washed again and incubated with 0.5 M NaCl, 7 mM $MgCl_2$ and 1 mM each ATP, UTP, and GTP (*lane 4*) or all four NTPs (*lane 3*) for 10 min at 30 °C. An aliquot of washed, cleaved complexes were adjusted to 0.5 M NaCl, 7 mM $MgCl_2$ and 1 mM each ATP, UTP, and GTP. At the indicated times (*lanes 5–10*), samples were made of 1 mM CTP at 30 °C and incubated for 10 min. Run-off (*RO*), transcripts Ia and II, and the major cleavage product (*) are indicated. *B,* comparative first-order plot showing loss of factor-independent elongation ability for *E. coli* RNA polymerase and rat liver RNA polymerase II at site Ia at the indicated temperatures. The natural logarithm of percent readthrough (=100 × (phosphorimager units in run-off RNA divided by sum of units in run-off RNA and Ia RNA)) was plotted *versus* time before CTP was added. The average

of three independent trials (including the experiment shown in Fig. 6*A*) were plotted for *E. coli* RNA polymerase. Standard deviation was calculated for each time point and is represented by error bars. Points for which no error bars are visible had standard deviations that were too small to permit error bars to be drawn. Measurements made in similar experiments on RNA polymerase II at 15 or 28 °C were replotted from Ref. 16 without error bars.

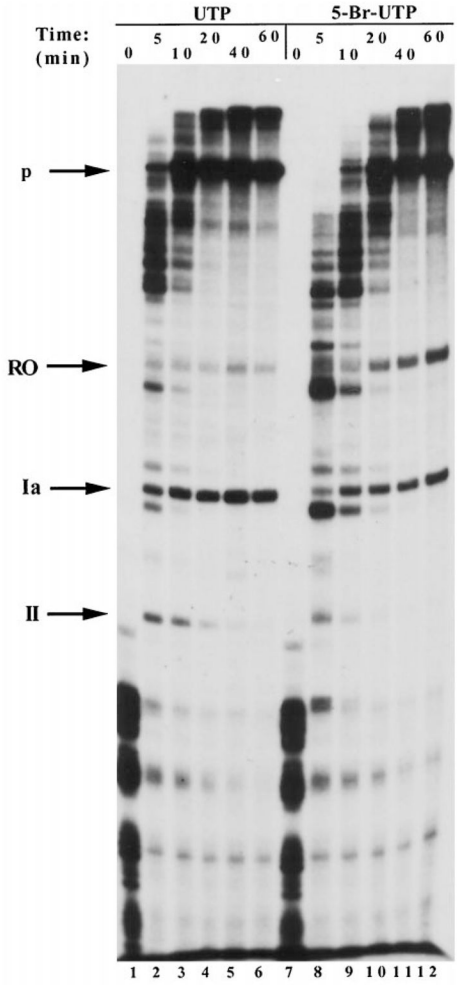

Fig. 7. Readthrough of site Ia by *E. coli* RNA polymerase in the presence of 5-Br-UTP

Pulse-labeled elongation complexes were assembled on *Pst*I cut p*rrn*-Ia, washed by immunoprecipitation (0 min), and provided with 7 mM $MgCl_2$, 50 μg/ml heparin, 1 μM CTP, 120 μM each ATP and GTP and either 120 μM UTP or 5-Br-UTP as shown, and incubated for the indicated times at 30 °C.

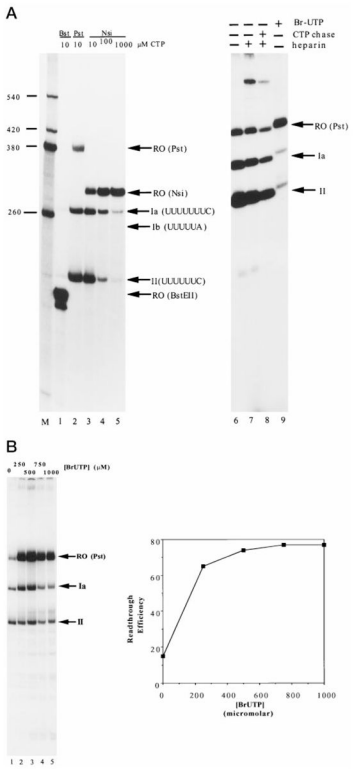

Fig. 8. Transcription through site Ia by phage SP6 RNA polymerase

A, pGEMTerm DNA cut with *Bst*EII (*lane 1*), *Pst*I (*lane 2*), or *Nsi*I (*lanes 3–5*) were transcribed *in vitro* with SP6 RNA polymerase in the presence of 2.4 mM each ATP, UTP, and GTP, and the indicated concentration of CTP for 2 h (*lanes 1–5*) or 30 min (*lanes 6–9*). In *lane 9*, UTP was replaced with 2 mM 5-Br-UTP. After incubation, samples were made of 100 μg/ml in heparin (*lanes 7* and *8*) and 1 mM in CTP (*lane 8*) and incubated for an additional 15 min. The addition of heparin results in a new slower migrating band, the identity of which is unknown. *Lane M,* reference RNAs of 260, 380, 420, and 540 bases. *B,* reactions containing 2.4 mM each ATP and GTP, 10 μM CTP, and either 1 mM (*lane 1*), 750 μM (*lane 2*), 500 μM (*lane 3*), 250 μM (*lane 4*), or no UTP (*lane 5*) were incubated for 30 min at 37 °C. The indicated concentrations of 5-Br-UTP were added to each to bring the total uridine nucleotide concentration to 1 mM. Percent readthrough (100 × (phosphorimager units in the run-off RNA)/(units in run-off RNA + transcript Ia + transcript II)), was plotted *versus* Br-UTP concentration.