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Escherichia coli transcript cleavage factors GreA and GreB stimulate promoter escape and gene expression *in vivo* and *in vitro*

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ABSTRACT **The process of RNA chain initiation by RNA polymerases plays a central role in the regulation of transcription. In this complex phase of transcription, short oligomers are synthesized and released from the enzyme–promoter complex in a reaction termed abortive initiation. The polymerase undergoes many cycles of abortive initiation prior to completion of the initiation process, which is signaled by the translocation of the enzyme away from the promoter, release of σ factor, and formation of an elongation complex in which the RNA is stably bound. We have studied the parameters that affect escape from the promoter by *Escherichia coli* RNA polymerase for the phage T7 A1 promoter, the phage T5 N25 promoter, and the chimeric promoter T5 $N25_{antiDSR}$. The latter site contains a synthetic initial transcribed region that reduces its ability to synthesize RNA both *in vivo* and *in vitro*. Clearance from T5 $N25_{antiDSR}$ can be stimulated up to 10-fold *in vitro* by addition of the *E. coli* transcript cleavage factor GreA or GreB, but these factors have little effect on transcription from the normal T7 A1 or T5 N25 promoters. Using an *E. coli* strain lacking GreA and GreB, we were also able to show stimulation of transcription by the Gre factors from the T5 $N25_{antiDSR}$ promoter *in vivo*. The stimulation of RNA chain initiation by Gre factors, together with their known biochemical properties in the transcription elongation reaction, suggests some specific models for steps in the transcription initiation reaction.**

Transcription of a single RNA chain by DNA-dependent RNA polymerases proceeds in a series of phases commonly designated as promoter binding and activation, RNA chain initiation, RNA chain elongation, and termination–release (1). Each step is mechanistically complex, and regulation of gene expression *in vivo* can occur at any step (for reviews, see refs. 2–5). Although primary attention has focused on the promoter-binding phase of transcription initiation, the RNA chain initiation phase is biochemically distinct and plays a central role in clearance of the promoter and in determining promoter strength. Recent studies have revealed regulatory mechanisms that target the RNA chain initiation process (6–10).

The mechanism of RNA chain initiation is now known to be complex. Initially, it was supposed that initiation was completed by synthesis of the first phosphodiester bond, yielding a dinucleoside tetraphosphate (1). Subsequently, it has been found that initiation is actually a multistep process in which the RNA polymerase in the open promoter complex repeatedly synthesizes short oligonucleotides ranging from 2 nt up to 9 nt, or larger, and releases these in a process called abortive initiation (11–15). Abortive initiation is well documented for bacterial, bacteriophage, and eukaryotic RNA polymerases, at least for RNA synthesis *in vitro*.

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The escape of the RNA polymerase from the initiation phase is best documented for *Escherichia coli* RNA polymerase and is marked by three distinct events: (*i*) the ternary complex of polymerase, DNA, and nascent transcript becomes highly stable to dissociation (16–18); (*ii*) the polymerase moves along the DNA away from the promoter (14, 18); (*iii*) the σ subunit is released from the polymerase (14). We will refer to the overall transition from initiation to elongation as promoter escape.

Changes in the initial transcribed sequence (ITS), in the promoter recognition region, or even upstream of the promoter recognition region can affect the size and yield of abortive transcripts and markedly alter the ability of the polymerase to escape from the promoter (6, 7, 10, 13, 16, 19–22). It follows that the different steps in initiation can play a major role in limiting promoter strength, measured as the ability to produce full-length transcripts (6, 10). However, at this time there is no simple explanation for how these different sequences act to alter promoter clearance and/or the yield and size of abortive products.

MATERIALS AND METHODS

E. coli strains MC4100 (23), AD8571 (24), and DG156 (25) are as referenced. Plasmid DNAs used in this study included pAR1707 (T7 A1 promoter: ref. 14); pDS3/PN25 (T5 N25 promoter), and pDS3/$PN25_{antiDSR}$ (T5 $N25_{antiDSR}$ promoter; ref. 20); pDNL278 and pGF296, the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible plasmids that overproduce GreA and GreB, respectively; and vector plasmids pKK232-8 (26) (Pharmacia) and pACYC184 (27) (New England Biolabs).

E. coli RNA polymerase holoenzyme PC-20 was prepared from DG156 (28); about 30% of the enzyme was active. RNA polymerase ΔAB was prepared from strain AD8571 (29) by using a Mono Q column (Pharmacia) to recover the holoenzyme. GreA and GreB proteins were purified from *E. coli* JM109 cells harboring the pDNL278 and pGF296, respectively (30).

Promoter fragment templates were prepared by PCR amplification (L.M.H. and M.J.C., unpublished work). The 200-bp fragments usually span the region from −150 to +50 around the transcription start site, so that the productive runoff RNA obtained can be displayed in the same gel with abortive transcripts. The ITSs are as follows: T7 A1, 5′-AUCGAGAGGGACACGGCGAA-3′; N25, 5′-AUAA-AUUUGAGAGAGGAGUU-3′; and T5, $N25_{antiDSR}$, 5′-AUCCCGGAAUCCUCUUCCCG-3′.

Single-promoter fragment templates were transcribed under extensive synthesis conditions (31, 32). Under these conditions the RNA polymerase synthesizes many transcripts from a single DNA template. This requires that the enzyme recycle

Abbreviations: CAT, chloramphenicol acetyltransferase; IPTG, isopropyl β-D-thiogalactopyranoside; ITS, initial transcribed sequence.

quantitatively, and this was tested carefully for all reactions (31). Reaction mixtures (10–100 μl) were incubated at 37°C and contained 20 nM template DNA, 50 nM RNA polymerase, 40 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, acetylated bovine serum albumin at 10 μg/ml, and [γ-^{32}P]ATP (10–20 cpm/fmol) in the presence of all four NTPs, each at 20 μM. Optimal KCl concentrations were 190 mM for T7 A1, 250 mM for N25, and 150 mM for T5 $N25_{antiDSR}$ promoter. RNA products were recovered by glycogen/ethanol precipitation (33) and resolved by 20% PAGE carried out in 7 M urea with a salt-gradient buffer (34).

GreA cleavage products were obtained by *in vitro* transcription of the T7 A1 or T5 $N25_{antiDSR}$ promoter template in standard reaction mixtures with each of the four [α-^{32}P]NTPs. The reaction was catalyzed by a GreA/polymerase mixture of 2:1 for 30 min at 37°C. The abundant cleavage products were recovered from gel pieces by passive diffusion and were characterized by paper electrophoresis and nearest-neighbor analysis (L.M.H. and M.J.C., unpublished work).

A 224-bp T5 $N25_{antiDSR}$ promoter fragment was inserted at the blunted *Bam*HI cloning site upstream of the chloramphenicol acetyltransferase (CAT) structural gene in vector pKK232-8, to yield plasmid pKKN25anti. Next, a 1.8-kb *Xmn* I fragment containing the T5 $N25_{antiDSR}$ promoter–CAT fusion was isolated from pKKN25anti and ligated into the 3.0-kb *Sca* I–*Xmn* I fragment of pACYC184 to create pAC4N25anti-CAT. The corresponding plasmid pAC4N25-CAT was prepared by replacing the 241-bp *Xmn* I–*Pst* I T5 $N25_{antiDSR}$ promoter fragment in pAC4N25anti-CAT with a 210-bp N25 promoter fragment. The integrity of the promoter region in both constructions was verified by DNA sequencing. Double-plasmid transformants were grown at 30°C in Luria–Bertani (LB) medium with tetracycline and ampicillin each at 20 μg/ml; kanamycin at 25 μg/ml was also included in the cultures of AD8571 to select for the Km^r trait associated with the *greA* disruption. Cell lysates were prepared from 1-ml samples (35) and assayed for protein and CAT activity (36).

RESULTS

To understand the role of promoter escape in transcription initiation, we have analyzed the extent of productive and abortive RNA synthesis from three promoters: T7 A1, T5 N25, and T5 $N25_{antiDSR}$ (L.M.H. and M.J.C., unpublished work). Both T7 A1 and T5 N25 are strong bacteriophage promoters, whereas T5 $N25_{antiDSR}$ has a synthetic DNA sequence from +3 to +20 that reduces promoter strength *in vivo* and *in vitro* by a factor of ≈10 (6). We found that the T5 $N25_{antiDSR}$ promoter, with a synthetic ITS, failed to escape from the promoter under most transcription conditions (up to millimolar NTP concentrations), whereas T7 A1 and T5 N25 failed to escape only at low NTP concentrations (1–3 μM). However, abortive synthesis was seen at all NTP concentrations tested. This suggests that all promoters may be limited in the promoter escape reaction at very low NTP concentrations, but that the T5 $N25_{antiDSR}$ promoter may be an extreme case and is limited even at high NTP concentrations.

With the finding that many standard RNA polymerase preparations are contaminated by GreA and/or GreB transcript cleavage factors (37), we wondered whether, at low NTP concentrations, the slow rate of chain initiation and elongation coupled with cleavage by the Gre factors might act to keep the polymerase at the promoter region. We tested this possibility by comparing the transcription of the three promoters as a function of NTP concentration, using a conventional RNA polymerase preparation and an RNA polymerase prepared from a *greA*$^-$*greB*$^-$ strain, ΔAB. Both enzyme fractions gave rise to identical patterns of abortive and productive transcription from each promoter at NTP concentrations ranging from 1 to 100 μM (results not shown). These results rule out the notion that the submolar amount of contaminating Gre factors found in some RNA polymerase preparations play any role in the restriction of promoter escape.

In the course of these studies, we obtained a quite surprising result. When purified GreA and/or GreB was added at high molar ratio (10:1) to the ΔAB polymerase, dramatic differences in both the pattern and extent of transcription of the T5 $N25_{antiDSR}$ promoter were observed (Fig. 1). The most striking effect of GreA or GreB was on productive RNA synthesis to form the 65-nt runoff product, which was dramatically stimulated. For comparison, this RNA was hardly detectable in the absence of Gre factors even after an hour of synthesis with the ΔAB enzyme (Fig. 1, lanes 1–4).

Quantitation of the stimulation of clearance from the T5 $N25_{antiDSR}$ promoter by GreA and/or GreB is shown in Fig. 2*A*. At a 10:1 molar ratio, GreA stimulated clearance about 10-fold, whereas GreB produced a 14-fold effect, after 1 hr of

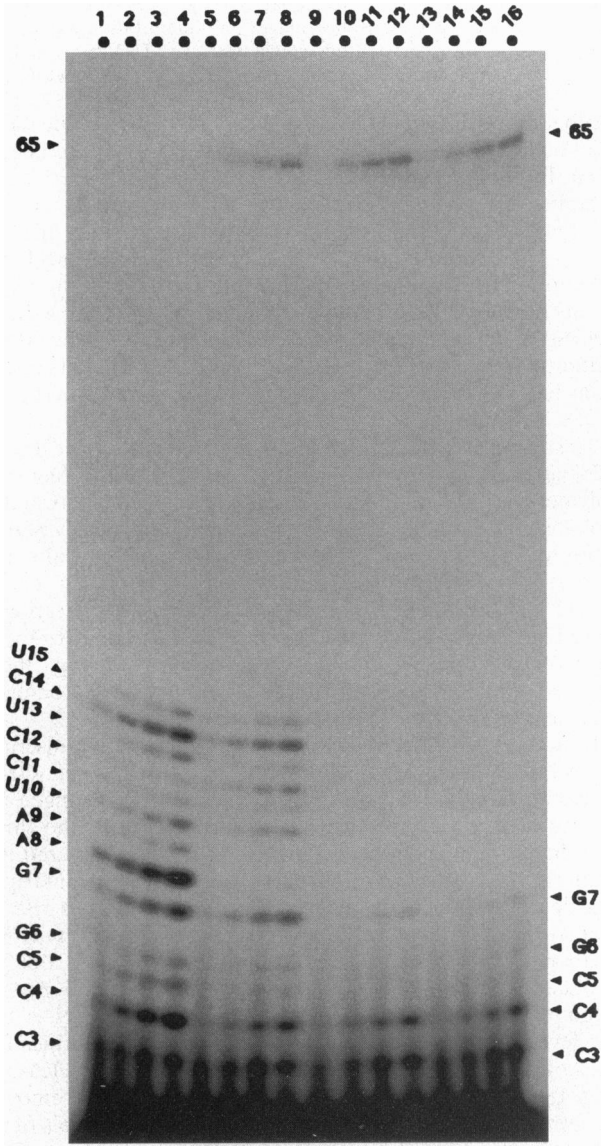

FIG. 1. Transcription from the T5 $N25_{antiDSR}$ promoter with GreA and/or GreB supplementation. Four sets of transcription reactions were run with RNA polymerase ΔAB alone (lanes 1–4), ΔAB plus GreA (1:10) (lanes 5–8), ΔAB plus GreB (1:10) (lanes 9–12), or ΔAB plus GreA and GreB (1:10:10) (lanes 13–16). Final concentrations of RNA polymerase and Gre factors were 50 nM and 500 nM, respectively. Samples (10 μl) were withdrawn at 6, 15, 30, and 62 min and resolved by PAGE. Numbers at left and right indicate the size of the transcripts.

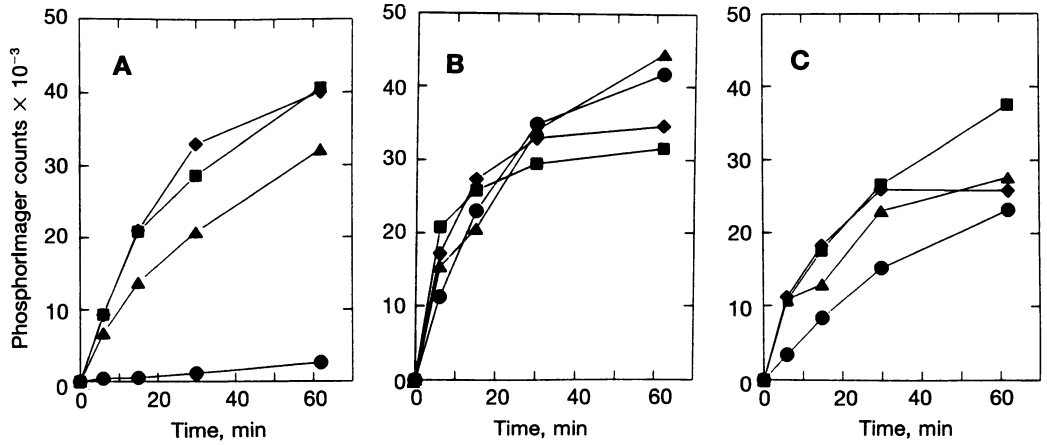

FIG. 2. Quantitation of productive RNA synthesis in the presence and absence of Gre factors. The gel shown was scanned in a Molecular Dynamics model 425E PhosphorImager and quantitation was performed with the IMAGE QUANT software. PhosphorImager counts, the arbitrary unit of quantitation, was plotted against time. The T5 $N25_{antiDSR}$ (*A*), T7 (*B*), and N25 (*C*) promoter templates were used in transcription reactions with ΔAB enzyme alone (●) or in the presence of GreA (▲), GreB (■, o)r GreA and GreB (◆).

incubation. GreA and GreB together gave rise to the same level of stimulation as GreB alone, suggesting that the factors act at the same point.

Similar transcription time courses were performed with T7 A1 and T5 N25 promoters. As was found with the T5 $N25_{antiDSR}$ promoter, GreA and GreB cleavage produced different patterns of abortive products at both promoters (gel results not shown). Surprisingly, however, GreA and/or GreB cleavage failed to stimulate the rate of further productive RNA synthesis from the T7 A1 promoter (Fig. 2*B*), and clearance from the T5 N25 promoter was only weakly stimulated (Fig. 2*C*).

The stimulatory effect of GreA on clearance from the T5 $N25_{antiDSR}$ promoter was dependent on the ratio of factor to polymerase; a direct proportionality could be demonstrated at molar ratios from 1 to 10; increasing the GreA/polymerase ratio to 20 gave no additional enhancement (results not shown). GreA/polymerase titrations performed with T7 A1 and T5 N25 promoters also showed no changes above ratios of 10:1. Thus, contrary to our initial expectation that GreA and GreB might impede the promoter escape reaction, these factors stimulated that reaction *in vitro*, and in a promoter-specific manner.

In addition to affecting the yield of productive transcripts from the T5 $N25_{antiDSR}$ promoter, both GreA and GreB cleaved within the ITS region of the transcript and changed the pattern of abortive products formed from each of the three promoters. That the reaction involved was cleavage, and not simply suppression of the formation of abortive transcripts, was demonstrated by using [α-^{32}P]NTP, which gives rise to characteristic 3′-terminal fragments in the cleavage reaction (data not shown; see *Materials and Methods*). For the T5 $N25_{antiDSR}$ promoter, GreA cleavage (Fig. 1, lanes 5–8) targeted predominantly the 8-mer and 9-mer abortive RNAs, but the level of other abortive products was also significantly reduced; e.g., 5-mer and 13-mer. In contrast, GreB cleavage (Fig. 1, lanes 9–12) appeared to have quantitatively removed all abortive RNAs longer than the 7-mer, and the levels of 7-, 6-, and 5-mer were reduced substantially as well. Despite the sharp differences in pattern, both GreA and GreB cleaved to yield mostly dinucleotide and trinucleotide products (L.M.H. and M.J.C., unpublished work). With both factors present, the pattern obtained was essentially identical to that obtained with GreB alone (Fig. 1, lanes 13–16). As was found with the T5 $N25_{antiDSR}$ promoter, GreA and GreB cleavage produced different patterns of abortive products at both T7 A1 and T5 N25 promoters as well (gel results not shown).

To probe the *in vivo* role of Gre factors in promoter clearance and gene expression, dual plasmid experiments with the promoter–CAT fusion on a pACYC184-derived vector and the inducible *greA* or *greB* gene on the compatible pBR322-derived vector were performed in the wild-type (MC4100) and *greA*$^-$ *greB*$^-$ (AD8571) hosts. CAT activity in logarithmic-stage lysates was measured as a function of time. In making this comparison, we assumed that a difference in CAT activity measured was directly due to a difference in the amount of CAT protein in the cell, which in turn was directly attributable to the difference in the level of CAT mRNA. In support of this premise, the stability of CAT mRNA made by the two promoter–CAT fusion constructs was equivalent (results not shown).

In such strains, N25 is a 3-fold more active promoter than T5 $N25_{antiDSR}$ *in vivo* in the wild-type cells and 8-fold more active in the *greA*$^-$ *greB*$^-$ cells (data not shown). The comparison between MC4100 and AD8571 suggests that the absence of GreA and GreB depressed the rate of expression of both promoters in AD8571, but the T5 $N25_{antiDSR}$ promoter was affected more severely than N25 (≈4-fold drop for T5 $N25_{antiDSR}$ and <2-fold drop for N25).

The effect of plasmid-expressed GreA or GreB on promoter expression *in vivo* was directly assessed in the same host strain (AD8571) by either supplying or withholding these factors (Fig. 3). In the presence of the *greA* plasmid, expression from T5 $N25_{antiDSR}$ promoter was elevated about 5-fold in the absence of IPTG induction; with IPTG induction, an 8-fold effect was obtained (Fig. 3*A*). By contrast, N25 was only weakly affected by GreA overproduction with or without IPTG induction (Fig. 3*B*). GreB supplementation produced essentially the same outcome, an ≈5-fold stimulation of T5 $N25_{antiDSR}$ promoter expression (Fig. 3*C*) and no stimulation of N25 expression (Fig. 3*D*). We surmised that the Gre proteins were probably expressed well from the *trc* promoter, even in the absence of IPTG. This was confirmed by protein gel analysis of the cellular lysates. The leaky nature of the *trc* promoter used does not alter our conclusions regarding the stimulatory effects of Gre factors, since the presence of the parent plasmid did not lead to any stimulation.

DISCUSSION

We have shown that the transcription elongation factors GreA and GreB are able to facilitate productive initiation of RNA chains *in vitro* and *in vivo* from a particular promoter. The factors appear to act to facilitate promoter escape. Although

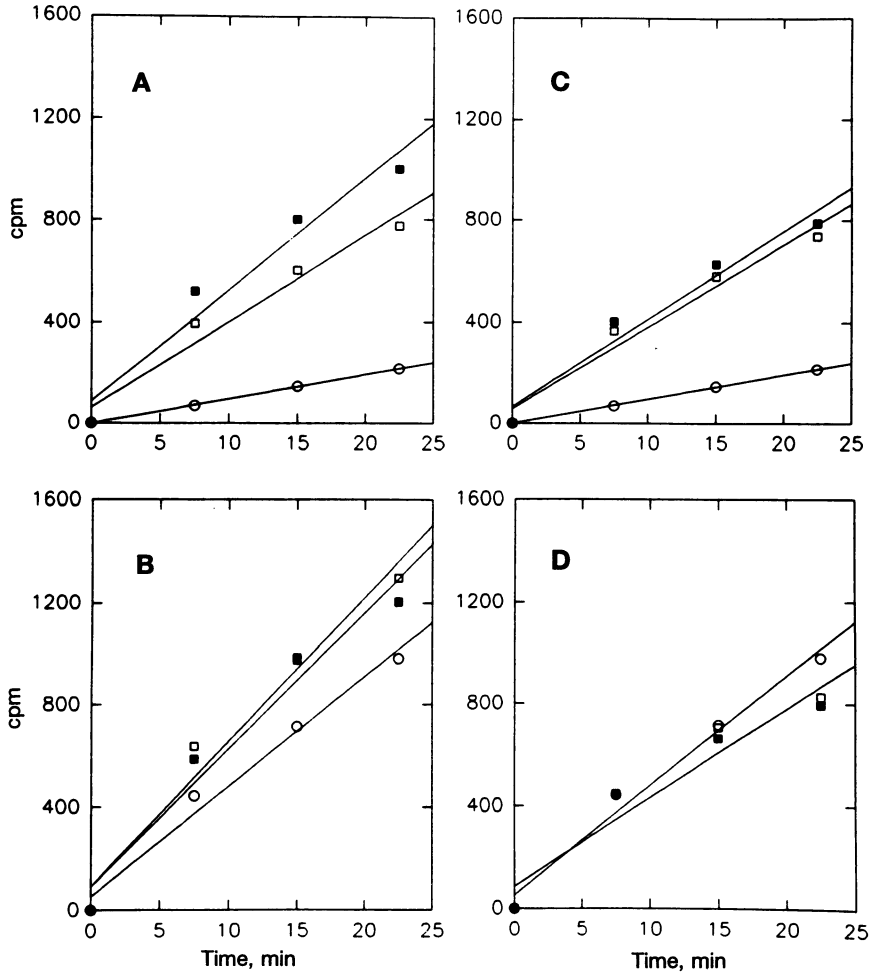

FIG. 3. Effect of Gre plasmid supplementation on promoter–CAT gene expression in AD8571. Lysates were prepared from logarithmic-stage cultures of AD8571 harboring pAC4N25anti-CAT with pDNL278 (GreA) (*A*), pAC4N25-CAT with pDNL278 (*B*), pAC4N25anti-CAT with pGF296 (GreB) (*C*), or pAC4N25-CAT with pGF296 (*D*). ○, Promoter–CAT fusion plasmid alone or with *gre*⁻ vector plasmid; □, promoter–CAT fusion with Gre plasmid, without IPTG induction; ■, promoter–CAT fusion with Gre plasmid, with IPTG induction. CAT activity normalized to the amount of lysate protein (cpm of [^{14}C]chloramphenicol acetylated per picogram of protein) was measured as a function of time.

we have not yet identified a normal *E. coli* promoter that is affected, it seems quite likely that there are such promoters. Further, since the initiation reaction is a complex and regulated process in eukaryotic cells as well as bacteria, it seems likely that similar factors and effects will be seen for RNA polymerase II and other eukaryotic RNA polymerases.

What is the mechanism by which the Gre factors enhance productive chain initiation? To discuss this problem we must first consider several questions: By what mechanism does the RNA polymerase carry out the various steps involved in the chain initiation and promoter escape reaction? Why does the polymerase form and release abortive transcripts, and what determines the size and yield of these transcripts in relation to productively initiated chains? What is the normal role of the Gre factors in transcription elongation, and do they affect productive initiation in a similar manner?

In point of fact, we know almost nothing about these questions. The biochemical activities of the purified Gre factors have been studied by several groups. It is clear that these factors can facilitate specific cleavage of the nascent RNA transcript in ternary complexes of RNA polymerase (30, 37, 38) and that in some cases this can relieve a state called transcription arrest, in which the RNA polymerase complex is unable to normally resume transcription. Weak effects on the rate of transcription initiation, comparable to those we see with the T5 N25 promoter, have been reported (30).

However, cells in which both known Gre factor genes have been disrupted are still viable (39); hence, neither GreA nor GreB appears to play an essential role in the cell. In fact, it is quite likely that the RNA polymerase catalytic site itself catalyzes the factor-dependent cleavage reactions and can catalyze factor-independent cleavage as well (39).

Similarly, we have only an elementary understanding of the initiation process. It is clear that the efficiency of promoter clearance can be affected by changes in both the promoter recognition regions and the ITS, and these same DNA regions can alter the distribution and yield of abortive products (6, 10, 16, 20). A simple model for initiation (40) has led to the suggestion that the formation of abortive products competes with the addition of the next nucleotide in a kinetic competition mode. However, we will show elsewhere that this mode is not correct for sites that give the majority of abortive products in the three promoters studied here (L.M.H. and M.J.C., unpublished work). That is, increasing the concentration of the nucleotide immediately downstream of an abortive product does not necessarily decrease abortive release of that product, and may actually increase abortion. In addition, no one has come up with a satisfactory explanation as to why abortive release of short RNAs should be characteristic of all transcription initiation.

A simple explanation for the action of the Gre factors in promoter clearance might be that for the T5 $N25_{antiDSR}$ promoter, the RNA polymerase pauses or becomes arrested at one or more positions prior to undergoing promoter clearance (22, 26). In fact, this promoter is unusual in forming much longer aborted transcripts (up to 16 nt) than are formed by other promoters that have been studied (L.M.H. and M.J.C., unpublished work). In this event, since the Gre factors are able to cleave these nascent RNAs prior to their release (41), this would allow the RNA polymerase to resynthesize the transcript and possibly have an increased opportunity to read through the pause. There is often a fraction of RNA polymerase that reads directly through a pause site, and hence repeated trials might well facilitate readthrough. This model has been proposed by

Feng *et al.* (30) to account for the weak stimulation that they see with Gre factors.

This is a plausible model, and as it predicts, we do see significant cleavage of longer abortive transcripts from the T5 $N25_{antiDSR}$ promoter. However, we have not been able to show that the initial transcribing complexes (ITCs) formed with T5 $N25_{antiDSR}$ have detectably long lifetimes. Gel exclusion chromatography can detect ITCs with lifetimes of a minute or more (14), and this does not detect any stable ITCs from the T5 $N25_{antiDSR}$ promoter (data not shown). In addition, there is evidence, at least for the RNA polymerase II system, that elongation through a transcription arrest site does not simply involve multiple tries at the site but directly requires some action of the protein factor involved in readthrough (42).

For these reasons we favor an alternative explanation, based on recent speculations about the mechanism of the transcript cleavage reaction and consistent with a model suggested by Feng *et al.* (30). One reason to favor this model is that it provides a different view of the abortive initiation process and explains the lack of kinetic coupling. This alternative starts with the observations of Rudd *et al.* (43), who showed that during transcript cleavage by RNA polymerase II, pyrophosphate could be incorporated at the 5′ terminus of the released RNA. This strongly suggests that the catalytic site for the cleavage reaction is the same site on RNA polymerase that catalyzes the nucleotide addition reaction. This view is also supported by the finding that the *E. coli* RNA polymerase purified from *greA–greB* disruption mutants is still able to carry out transcript cleavage, with a specificity different from the factor-dependent reaction (24). If this is correct, then the cleavage reaction must involve the movement of the catalytic site on RNA polymerase backwards along the RNA chain for a distance of up to 17 nt! Such a movement was suggested by Chamberlin (44) in the discussion of a model for the mechanism of RNA chain elongation, as an explanation for the formation of transcription-arrested RNA complexes, and is consistent with footprinting studies of arrested complexes by Krummel and Chamberlin (17).

If this idea is correct, then one role of the Gre factors is to facilitate cleavage and hence reactivation of elongation complexes where the catalytic site has moved away from the 3′-hydroxyl end of the RNA. However, it is equally plausible that the presence of the Gre factor may favor binding of the catalytic site at the 3′ end and hence prevent transcription arrest in the first place. In fact, Goldfarb and coworkers (38) described such an example where GreA can prevent formation of arrested complexes but cannot reactivate them once they have formed. Let us assume that during the formation of oligomers in the initiation reaction, the catalytic site can slip back in a sequence-dependent manner. Because of the instability of the ITCs, this will lead to release in a reaction that will be unaffected by the presence of the next NTP. If the Gre factors interact with this complex prior to the movement of the site away from the 3′ hydroxyl, they can facilitate passage through this site and enhance productive initiation. By this view the Gre factors play a role, along with nucleotide sequence, in maintaining the position of the catalytic site at the end of the RNA and suppressing abortion of the transcript (30) or, should it slip back along the RNA, they facilitate cleavage and hence restoration of the normal elongation state.

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