The elongation factor RfaH and the initiation factor $\sigma$ bind to the same site on the transcription elongation complex

Anastasiya Sevostyanova*, Vladimir Svetlov*, Dmitry G. Vassylyev†, and Irina Artsimovitch*‡

*Department of Microbiology, Ohio State University, 484 West Twelfth Avenue, Columbus, OH 43210; and †Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, 402B Kaul Genetics Building, 720 Twentieth Street South, Birmingham, AL 35294

Edited by Jeffrey W. Roberts, Cornell University, Ithaca, NY, and approved December 6, 2007 (received for review September 5, 2007)

RNA polymerase is a target for numerous regulatory events in all living cells. Recent studies identified a few “hot spots” on the surface of bacterial RNA polymerase that mediate its interactions with diverse accessory proteins. Prominent among these hot spots, the $\beta'$ subunit clamp helices serve as a major binding site for the initiation factor $\sigma$ and for the elongation factor RfaH. Furthermore, the two proteins interact with the nontemplate DNA strand in transcription complexes and thus may interfere with each other’s activity. We show that RfaH does not inhibit transcription initiation but, once recruited to RNA polymerase, abolishes $\sigma$-dependent pausing. We argue that this apparent competition is due to a steric exclusion of $\sigma$ by RfaH that is stably bound to the nontemplate DNA and clamp helices, both of which are necessary for the $\sigma$ recruitment to the transcription complex. Our findings highlight the key regulatory role played by the clamp helices during both initiation and elongation stages of transcription.

clamp helices | RNA polymerase | transcription factor | nontemplate DNA

Bacterial RNA polymerase (RNAP) is a principal target for numerous accessory proteins and small ligands that fine-tune gene expression profiles to match the cell needs. Competition (or cooperation) among these regulators for the finite number of targets on the RNAP surface determines the patterns of gene expression. The classical paradigm for the partitioning of the regulatory space is $\sigma$ competition (1) with different initiation $\sigma$ factors competing for binding to the core enzyme (subunit composition $\alpha_2\beta'\beta\omega$) and, when successful, directing it to a subset of $\sigma$-specific promoters. The $\sigma$-subunit makes many contacts to the core RNAP among which the $\beta'$ subunit clamp helices ($\beta'$ CH, a coiled-coil motif comprising residues 260–309 in the Escherichia coli enzyme) are thought to constitute the major $\sigma$ binding site in the free RNAP (2, 3) as well as in the transcription elongation complex (4). Our recent finding that the $\beta'$ CH is also required for recruitment of the elongation factor RfaH (5) suggested that competition for this site may regulate gene expression far beyond $\sigma$-specific promoter recognition.

RfaH reduces pausing and termination thereby suppressing transcriptional polarity in long operons encoding virulence and fertility determinants (6, 7). RfaH action depends on the $\text{ops}$ DNA sequence (GGCGGTAAGT) located in the transcribed regions of RfaH-controlled operons (7). 

**Results**

RfaH Abrogates $\sigma$-Induced Pausing. To test whether RfaH can prevent $\sigma$-dependent pausing, we first constructed templates (Fig. 2) with the extended $-10$ (TGCATATAAT) sequence positioned downstream from the $\text{ops}$ element that has been shown to mediate efficient RfaH recruitment in vitro (6, 7). We prepared halted radiolabeled G37 TECs and monitored RNA chain extension upon addition of the NTP substrates in the presence of RfaH, $\sigma$, or both. Addition of the wild type (WT) $\sigma^{70}$ to $1 \mu$M [at or below its physiological concentration (13)] induced pausing at position 118, at the same distance from the $-10$ element as observed in earlier studies of $\sigma$-induced pauses (9, 10, 12); 14.5% of TECs remained paused at this site (called $\sigma^P$ thereafter) after a 16-min incubation. The full-length RfaH (at 40 nM) increased the rate of elongation (as seen from the accumulation of the runoff transcript) and, when present with

Several lines of evidence support this view. First, the isolated N-domain no longer requires the $\text{ops}$ element for function. Second, RfaH reduces pausing at all sites in vitro (7) yet increases pausing at the $\text{ops}$ element located downstream from the identical $\text{ops}$ site that mediates RfaH recruitment to the TEC (our unpublished observations), indicating that the N-domain retains the ability to interact with $\sigma$. Third, the RNAP variant missing the CH tip fails to respond to either full-length RfaH or the N-domain (5), arguing that the contacts to the CH are required regardless of the recruitment mechanism. Last, RfaH does not dissociate from the RNAP after its recruitment to $\text{ops}$ (our unpublished observations). Although RfaH and $\sigma$ lack any sequence or structure similarity and recognize very different DNA elements, their targets on the TEC are topologically similar (Fig. 1). $\sigma^{70}$ binding to the TATA-like element, which is also located in the NT strand, mediates promoter recognition during initiation (8) and RNAP pausing at promoter-proximal (9–11) and downstream sequences (12). Moreover, RfaH (5) and $\sigma$ (3) likely bind to the adjacent sites on the $\beta'$ CH (Fig. 1B). Thus, RfaH would be expected to “insulate” the TEC from $\sigma$ rebinding because the $\beta'$ CH is thought to be the only part of core that contacts $\sigma$ during elongation (4). In contrast, RfaH is not likely to interfere with $\sigma$ action during initiation when $\sigma$ makes numerous interactions that engage $\sim$10,000 $\text{Å}^2$ of the core surface.

**Author contributions:** A.S. and I.A. designed research; A.S., D.G.V., and I.A. performed research; V.S. contributed new reagents/analytic tools; A.S. analyzed data; and V.S., D.G.V., and I.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

†To whom correspondence should be addressed. E-mail: artsimovitch.1@osu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0708432105DC1.

© 2008 by The National Academy of Sciences of the USA
apparent competition with $\sigma^{70}$ requires stable RfaH recruitment to the TEC.

RfaH Likely Prevents $\sigma$ Recruitment Directly. RfaH could eliminate $\sigma$-induced pause directly, by making stable contacts to TEC and sterically excluding $\sigma$, or indirectly, through its general antipaus- ing activity (7). We favor the first possibility because such a dramatic effect of RfaH on pausing has not yet been seen at any other pause/arrest sites; on the template used in Fig. 2 and many others the isolated N-domain reduces pausing (e.g., at the hisP site) on average by 3-fold. To exclude the indirect model, we used a “fast” RNP variant ($\beta^{\Delta\gamma 43-1130}$) that is defective in both pausing and response to RfaH yet retains the ability to bind to RfaH (14). The mutant enzyme paused at $\sigma P$ as efficiently as the WT RNAP (Fig. 2), and this pause was abolished by the N-domain. These observations argue against the antipaus- ing mechanism of RfaH action at $\sigma$-induced pauses and suggest competition for the same binding site. In support of this model, we found that $\sigma^{70}$ apparently interfered with RfaH recruitment to the ops site positioned immediately downstream from the extended −10 element [supporting information (SI) Fig. 6].

Contacts to CH and NT DNA Are Essential for the RfaH and $\sigma$ Action During Elongation. Molecular modeling suggests that RfaH makes only a few contacts with the short exposed NT DNA segment and the adjacent regions on RNAP (5). Likewise, the $\alpha P$TEC interface is also limited, as most of the $\sigma$ contacts to core are lost upon the transition from initiation to elongation (4). Thus, the recruitment of RfaH and $\sigma$ and their apparent competition during elongation should be very sensitive to changes in interactions with the NT strand and the $\beta'$ CH.

We probed the role of the NT DNA interactions using substitutions in ops, the −10 hexamer, and RfaH and $\sigma^{70}$ residues that are thought to make sequence-specific contacts to the DNA.
As expected, full-length RfaH did not affect elongation or interfere with the $\sigma$ action on the template with the scrambled $\text{ops}$ (7) whereas the N-domain abrogated the $\sigma^P$ (Fig. 3A); the same result was obtained on templates with single substitutions in the $\text{ops}$ (data not shown). A single base substitution in the extended $-10$ element, $-12T$ to C, eliminated pausing by the WT $\sigma^{30}$; the pause was partially restored ($1.0 \pm 0.3\%$ vs. $3.0 \pm 0.4\%$) by the addition of $\sigma$ Q437H variant, which acts as an allele-specific suppressor of the $-12T$ to C mutation in vivo (15). Consistent with the in vivo data, the Q437H variant was less effective in recognizing the WT $-10$ element ($14.5 \pm 0.7$ vs. $8.3 \pm 1.9\%$ of $\sigma^P$-paused TECs). Last, we showed that R73A, a substitution in the putative DNA-binding site that greatly reduces RfaH binding to the $\text{ops}$ and its postrecruitment effects on elongation (our unpublished observations), also abolished RfaH's effect on $\sigma$-dependent pausing (Fig. 3A). Taken together, these data highlight the importance of RfaH and $\sigma$ contacts to the NT strand.

Next, we turned to the analysis of the CH contacts with $\sigma$ and RfaH. We reasoned that $\sigma$ Glu-407 should be required for $\sigma$-dependent pausing: this residue is located near $\sigma$ Leu-402, which is essential for promoter-proximal $\sigma$-dependent pausing (16) and interacts with the CH directly, and E407K substitution has been shown to destabilize $\sigma$ interactions with the CH (17, 18). Indeed, $\sigma^{70}$ E407K failed to induce pausing at the $\sigma^P$ site ($1.2 \pm 0.1\%$) (Fig. 3B). We next tested whether $\sigma^{70}$, which shares the $-10$ recognition determinants (19) with $\sigma^{70}$, will recognize the $-10$ element as a pause signal. In agreement with ref. 20, we found that $\sigma^{70}$ did not induce RNAP pausing significantly above background ($2.4 \pm 0.6\%$) (Fig. 3B). This observation is consistent with the report that $\sigma^{30}$ binds less tightly to the core enzyme and dissociates more rapidly after initiation (21), as well as with the sequence differences between the two $\sigma$ factors both in the CH recognition region and in the $\sigma^P$ region, reported to affect $-10$ recognition allosterically (22). Importantly, $\sigma^{70}$ has a Glu residue at the position that corresponds to Glu-406 in $\sigma^{30}$; Q406A substitution confers a defect in binding to core RNAP comparable to that of E407K (17). To probe the importance of the RfaH/CH interface, we used Y8A, a substitution at the proposed RfaH/RNAP interface (Fig. 1B) that is expected to weaken hydrophobic contacts between the N-domain and RNAP. We found that Y8A substitution greatly reduced RfaH effects on $\sigma$-dependent pausing ($11.5 \pm 2.7\%$) (Fig. 3B) and elongation (data not shown). Thus, weakening RfaH interactions with the CH (by RfaH Y8A or $\beta'$ I290R substitution; see below) compromises its anti-$\sigma$ activity. In turn, weakening of $\sigma$ contacts with the NT DNA or the $\beta'$ CH reduces pausing, making RfaH competition irrelevant.

RfaH and $\sigma^{70}$ Recognize Distinct Subsets of Determinants on the $\beta'$ CH.

The modes of $\sigma^{30}$ ($\sigma^{70}$ homolog in Thermus thermophilus) and RfaH binding to the CH are quite different (Fig. 1B): $\sigma$ forms a network of polar interactions with the CH covering nearly all their surface exposed in the core enzyme (3), whereas the N-domain of RfaH is predicted to make predominantly van der Waals contacts (5) with two hydrophobic residues at the very tip of the $\beta'$ CH (Ile-290 and Ile-291 in E. coli). This suggests that it may be possible to selectively destabilize the CH contacts with RfaH or $\sigma^{70}$.

Indeed, substitutions of Ile-290 and Ile-291 for Arg abolish regulation by RfaH but do not prevent transcription initiation (5). We found that $\beta'$ I290R RNAP paused at the $\sigma^P$ site whether or not RfaH was present (Fig. 3B), albeit with a lower efficiency than the WT enzyme; thus, it appears that this substitution did not obliterate the $\sigma/\text{CH}$ interaction while completely abolishing RfaH action (and presumably binding to the TEC).

In search for a substitution in the CH that would severely destabilize $\sigma^{70}$ contacts during elongation, we constructed two $\beta'$ variants that were reported to have a reduced affinity to $\sigma^{70}$, R293Q and R275Q (2). The purified $\beta'$ R293Q core RNAP did not behave any differently from the WT enzyme in any assay used (SI Fig. 7 and data not shown); in fact, in the T. thermophilus holoenzyme (3) the corresponding $\beta'$ residue (Arg-568) makes no contacts to $\sigma$. In contrast, the $\beta'$ R275Q enzyme was defective in $\sigma$-pausing (SI Fig. 8) but displayed no other defects in transcript elongation, including response to RfaH (SI Fig. 7 and data not shown). Given the dramatic effect of $\sigma$ E407K on pausing (Fig. 2), we also designed a core variant with the substitution of $\beta'$ Arg-278, a residue that interacts with $\sigma$ Glu-407 in the holoenzyme, for Glu. As expected, this substitution had an effect comparable to the $\sigma$ E407K change: $\beta'$ R278E RNAP failed to pause at the $\sigma^P$ site yet was similar to the WT enzyme in its elongation pattern and response to RfaH (Fig. 3B and SI Fig. 7). These data lead us to conclude that the $\beta'$ CH determinants that mediate its binding to $\sigma$ and RfaH are not identical.
RfaH and σ70 Do Not Compete During Initiation. During elongation, both σ and RfaH are thought to make only a few interactions with the TEC (4, 5). In contrast, in the initiation complex σ makes multiple contacts to both the core enzyme and the promoter DNA elements; thus, a loss of just one of these contacts may still allow for σ function. Indeed, E407K variant unable to pause at the perfect −10 element (Fig. 3B) supported transcription initiation. We therefore reasoned that RfaH would not be able to interfere with σ function at promoters, given that it requires a specific sequence for recruitment and binds to core RNAP weakly, and only when its diffusion is limited by immobilization on a matrix (7). Indeed, RfaH (either full-length or the N-domain alone) did not inhibit transcription initiation from the PR promoter (Fig. 4) under conditions that are expected to favor RfaH over σ; a large molar excess of RfaH was preincubated with core RNAP before the addition of σ70, template encoding Apk promoter, NTP substrates, and a 32P-labeled DNA oligonucleotide used as a loading control. Samples were analyzed on a 12% denaturing gel; a representative gel is shown. The fraction of halted A26 complex (corrected by using the 45-mer as standard) formed in a single-round assay was quantified relative to that in the absence of RfaH. The assay was repeated five times; the fraction of A26 RNA was between 94% and 102% and independent of RfaH concentration.

Discussion

In this work we demonstrate that the elongation factor RfaH antagonizes σ function during elongation but not initiation of transcription. This anti-σ activity of RfaH requires its stable association with the TEC and is mediated by the CH domain of the RNAP β' subunit and by the NT DNA strand. Interestingly, the β' CH utilizes a different set of interactions to bind to σ and RfaH, extensive polar contacts in the first case and a hydrophobic patch in the second case (Fig. 1B). We show that these two sets of interactions can be selectively disrupted by substitutions in the β' CH—we argue that RfaH sterically occludes the σ target site and thus prevents its reloading onto the TEC at sites that resemble the −10 consensus. On the other hand, although their recognition DNA sequences are very different (Fig. 1A), RfaH and σ could compete for binding to the NT DNA in the TEC directly, using essentially the same target, the fork junction between the upstream DNA duplex and the surface-exposed NT strand.

We used the recently determined structure of a bacterial TEC (25) to visualize the contacts of σ and RfaH within the TEC (Fig. 5). We assumed that the protein structure remains largely unaltered and modeled the NT strand in a conformation resembling that in the DNA duplex, with most bases exposed and stacking on each other while avoiding close contacts of its phosphate backbone with the protein. The modeling was aided by the restraints imposed on the positions of the first annealed

![Fig. 4](image_url). RfaH does not compete with σ70 during initiation. Core RNAP was preincubated with increasing concentrations of RfaH (full-length or the N-domain alone) before addition of σ70, template encoding Apk promoter, NTP substrates, and a 32P-labeled DNA oligonucleotide used as a loading control. Samples were analyzed on a 12% denaturing gel; a representative gel is shown. The fraction of halted A26 complex (corrected by using the 45-mer as standard) formed in a single-round assay was quantified relative to that in the absence of RfaH. The assay was repeated five times; the fraction of A26 RNA was between 94% and 102% and independent of RfaH concentration.

![Fig. 5](image_url). Contacts to the NT DNA strand. Shown are structural models of RfaH (A) and σ (B) bound to the TEC. The RNAP core is shown in gray with the CH highlighted in cyan. The template and non-template strands are shown in red and blue, respectively. The registers (relative to the active site) from −9 to −6 represent the single-stranded NT DNA, whereas those of −10, −11, etc., correspond to the upstream DNA duplex; the numbering does not correspond to positions in the nascent RNA transcript because RfaH- and σ-paused TECs are backtracked, placing the 3′ end of the RNA ahead of the active site. This figure was prepared with Molscript (39).
DNA base pair and the first unpaired NT strand nucleotide in the transcription bubble: the TEC structure and a recent biochemical study (26) indicate the 9-bp RNA/DNA hybrid, with only one DNA base pair melted in the active site. The model of the RfaH N-domain was fitted to the tip of the CH as described previously (5). The TEC/σ model was generated through superposition of the β’ CH domain in the holoenzyme (3) with that in the TEC (25); the CH appears substantially displaced (by ~7 Å) toward the main channel in the latter structure. The TEC/σ modeling reveals that only σ region 2 is likely to maintain stable contacts with the core enzyme. As noted above, contacts of the σ3-4 linker and σ9 observed in the holoenzyme are incompatible with the TEC structure. Moreover, the σ domain that encompasses σ9-3-1 likely also loses its contacts with RNAP because its binding site in the TEC appears stably blocked by the upstream DNA duplex. This view agrees with the previous model (4) and the experimental data implicating the β’ CH as the major σ binding site on the TEC.

These models are hypothetical and cannot be used for the detailed structural analysis—we cannot rule out the alterations in the RNAP structure upon binding of σ or RfaH (e.g., additional displacement of the mobile CH) or a somewhat distinct conformation of the ab initio modeled NT DNA strand (e.g., some bases may be trapped in protein pockets rather than exposed). Assuming, however, that these putative changes are not very dramatic, the models suggest three implications. First, consistent with our data (Fig. 2), RfaH and σ are expected to compete for the binding site on the TEC during elongation. Second, both proteins may establish base-specific contacts with two to three exposed bases of the NT strand at the upstream edge of the transcription bubble. Last, although the upstream NT bases are most proximal to σ/RfaH, they are not directly accessible to σ Gln-437 and RfaH Arg-73, the residues that are thought to form sequence-specific contacts with the DNA. This observation suggests that the -10 and ops sequence elements may favor formation of a DNA loop within the melted NT strand to allow for the base-specific contacts with σ and RfaH, respectively. After this initial recruitment, further compaction of the DNA may be induced by protein–DNA interactions in both types of paused complexes; these structural transitions may represent structural changes occurring during elongation (27) rather than abortive initiation (28).

The β’ CH has been long known to play an important regulatory role by recruiting the σ initiation factors to core RNAP. We show that, despite its rather limited size (~50 residues), the β’ CH exhibits the potential for unexpected mechanistic and functional diversity that allows it to recruit regulators that act during elongation and have no apparent similarity to σ. We found that RfaH prevents σ-induced pausing at a ~10-like element in vitro (Fig. 2); other regulatory proteins that target the β’ CH during elongation would be expected to compete with σ as well. Obvious candidates include various RfaH paralogs, such as the elongation factor NusG and ActX in E. coli, and yet unknown proteins that bind to the NT DNA, either specifically or nonspecifically [e.g., during transcription-dependent AID-mediated cytidine deamination (29)].

RfaH increases expression of distal genes in long operons by facilitating bypass of many consecutive roadblocks as RNAP traverses the entire length of the operon. Both nucleic acid signals and DNA-bound proteins can delay RNAP progression along the template, prompting emergence of regulators that enable transcription through both types of obstacles. Our results argue that RfaH plays a dual antipausin role: it prevents the TEC isomerization into off-pathway states at factor-independent pause signals (14) and insulates the TEC from spurious rebinding of σ to ~10-like sequences, which triggers pausing. Consistent with the proposed RfaH/σ competition, the second mechanism is highly specific toward σ in the context of the TEC, because RfaH is unable to facilitate transcription through an EcoRIQ1011 roadblock preformed on double-stranded DNA (I.A., unpublished observations). Notably, σ-induced pauses hinder transcript elongation yet are unavoidable because they depend on the same set of contacts that mediate the indispensable function of σ at promoters. Interference with σ-dependent pausing (but not initiation) may thus constitute an essential part of the RfaH regulatory function. This “competition” could work both ways: whereas RfaH would inhibit σ loading during elongation, σ rebinding to the termination complex triggered by conformational changes in RNAP (30) may induce RfaH release.

Our findings also have implications for σ release and rebinding to the transcription complex. The release of σ was proposed to occur deterministically, after the nascent RNA reaches a defined length of at least 8 nt and actively decrypts σ, or stochastically, after entering the elongation phase when most of the σ contacts to core are lost (reviewed in ref. 4). Even though association constant of σ70 drops from 2 × 10^15 to 5 × 10^12 M⁻¹ upon transition from initiation to elongation (24), the estimated in vivo concentration of σ70 is 15 μM (13), and its effective concentration may be higher because of the macromolecular crowding. Thus, σ could stay bound to the TEC, a scenario supported by some studies (31, 32). Although the vast majority of σ70 appears to be released from the elongating RNAP rapidly in vivo (21, 33), in some operons σ hangs along (34), suggesting that the TEC/σ association can be regulated by environmental conditions, specific sequences, or accessory factors. For example, the elongation factor NusA was proposed to bind to the CH (35) and thus could trigger σ displacement. We note, however, that although NusA and σ may compete for binding to the β flap (17, 36), their competition for the CH appears unlikely: NusA does not eliminate σ-dependent pausing (12), does not compete with RfaH (I.A., data not shown) or NusG (37), and may coexist with σ within the TEC (31, 38). Instead, our data suggest that NusG and its paralogs play the role originally assigned to NusA: RfaH would exclude σ from RNAP molecules transcribing the ops-containing operons whereas NusG is expected to act on the rest of the transcriptome.

The enthusiastic but short-lived demise of the σ-release paradigm painted a new picture of transcription, overloaded with “memories” of past regulatory decisions in form of idle σ-subunits forever bound to their core RNAPs. The ChIP-on-chip data (21, 33), together with our findings that elongation factors can insulate the TEC from rebinding of σ, restore the σ release to its place in the transcription cycle. Perhaps even more important than preventing σ-induced pausing, this insulatory effect may reset the memory of past initiation events and increase responsiveness of σ competition as a regulatory developmental switch.

Materials and Methods
Proteins and Reagents. All general reagents were obtained from Sigma and Fisher; NTPs from GE Health; PCR reagents were from Fermentas and Roche; restriction and modification enzymes were from NEB; and [32P]NTPs were from GE Health. Oligonucleotides were obtained from Integrated DNA Technologies. DNA purification kits were from Promega. Substitutions in the E. coli rpoC gene (encoding the β’ subunit) were constructed by site-directed mutagenesis; sequences of all plasmid constructs were verified at the Ohio State University Plant Microbe Genomics Facility. All plasmid constructs used in this work are listed in Table 1.

RfaH variants, σ70, and altered RNAPs were purified as described previously (5). σ28 was a gift of Jay Gralla (University of California, Los Angeles, CA).

Pause Assays. Linear DNA template generated by PCR amplification (40 nM), holo RNAP (30 nM), ApU (100 μM), and starting NTP subsets (indicated in figure legends; the NTP used for labeling at 1 μM and two others at 5 μM) were mixed on ice in GBB buffer (20 mM Tris/HCl/20 mM NaCl/14 mM MgCl₂/5% glycerol/14 mM 2-mercaptoethanol/0.1 mM EDTA, pH 7.9). Halted radiolabeled TECs were formed at 37°C for 15 min and incubated with RfaH variants.
and \( \nu^{30} \) (at 40 nM and 1 \( \mu \)M, respectively, where indicated) for 3 min at 37° C. Elongation was restarted by the addition of the NTPs (150 \( \mu \)M ATP, CTP, and UTP, and 10 \( \mu \)M GTP) and rifampicin (25 \( \mu \)g/ml). Aliquots were withdrawn at 15, 30, 60, 120, 480, and 960 sec.

**Halted A26 Complex Formation.** Core RNAP (40 nM) was preincubated with different concentrations of RifA in 25 \( \mu l \) of GBB buffer at 37° C for 25 min. An equal volume of the prewarmed mix of the linear pIA253 DNA template (200 nM), \( \nu^{30} \) (40 nM), ApU (200 \( \mu \)M), ATP and UTP (10 \( \mu \)M), GTP (2 \( \mu \)M), 10 \( \mu \)Ci of \( [\alpha-\text{32P}] \text{GTP} (3,000 \text{ Ci/mmol}) \), and 2 nM of the ssDNA standard in the GBB buffer was added, followed by the 25-min incubation at 37° C.

### ACKNOWLEDGMENTS
We thank Georgiy Belogurov, Rachel Mooney, and Bob Landick for discussions. This work was supported by National Institutes of Health Grants GM67153 (to I.A.) and GM74252 (to D.G.V.).

---