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Transcript Cleavage by *Thermus thermophilus* RNA Polymerase

EFFECTS OF GreA AND ANTI-GreA FACTORS*

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All known multisubunit RNA polymerases possess the ability to endonucleolytically degrade the nascent RNA transcript. To gain further insight into the conformational changes that govern transcript cleavage, we have examined the effects of certain anions on the intrinsic transcript cleavage activity of *Thermus thermophilus* RNA polymerase. Our results indicate that the conformational transitions involved in transcript cleavage, and therefore backtracking, are anion-dependent. In addition to characterizing the intrinsic cleavage activity of *T. thermophilus* RNA polymerase, we have identified, cloned, and expressed a homolog of the prokaryotic transcript cleavage factor GreA from the extreme thermophiles, *T. thermophilus* and *Thermus aquaticus*. The thermostable GreA factors contact the 3′-end of RNA, stimulate the intrinsic cleavage activity of *T. thermophilus* RNA polymerase, and increase the k_{app} of the cleavage reaction 25-fold. In addition, we have identified a novel transcription factor in *T. thermophilus* and *T. aquaticus* that shares a high degree of sequence similarity with GreA, but has several residues that are not conserved with the N-terminal "basic patch" region of GreA. This protein, Gfh1, functions as an anti-GreA factor *in vitro* by reducing intrinsic cleavage and competing with GreA for a binding site on the polymerase.

RNA polymerase has been shown to possess a surprising activity that serves to release complexes from an arrested state. Specifically, RNA polymerase (RNAP)[1] appears to be able to catalyze the endo- and exonucleolytic cleavage of the RNA transcript, rapidly releasing the 3′-terminal fragment, which can be as large as 17 nucleotides in length, and resuming synthesis from the 5′-terminal fragment (1, 2). Two accessory proteins, GreA and GreB, have been found to stimulate this cleavage activity in *Escherichia coli* (1, 3), and an ortholog of these factors (SII) has been found in eukaryotes (4, 5). Transcript cleavage induced by GreA and GreB reduces abortive initiation (6) and misincorporation (7) and regulates pausing and arrest during elongation (3, 7–9). Taken together, these results suggest that transcript cleavage plays an important role in maintaining processive and accurate synthesis of the RNA transcript *in vivo*. There is strong evidence that this cleavage activity resides on RNA polymerase itself (10, 11). In addition, it has been suggested that the same amino acids that are responsible for nucleotide incorporation may be responsible for this cleavage activity (10, 11). If this hypothesis is true, it would indicate large scale movements of the catalytic site relative to the RNA transcript and the DNA template (12–14). Such conformational changes have been investigated *in vitro* using stalled complexes formed by NTP deprivation or physical obstruction (15–17). Studies of these complexes suggest that the formation of a reverse-translocated, or backtracked state, is a necessary step in the transcript cleavage reaction (13, 14). Backtracking repositions the catalytic site residues of RNAP upstream on the RNA and DNA (13, 14, 16, 18) while displacing the 3′-end of the RNA transcript from the catalytic site. It has been suggested that the 3′-end of the RNA is extruded through the secondary channel of RNAP in a backtracked complex (8, 19), which presumably positions the internal phosphodiester bonds of the transcript for hydrolysis by RNAP.

For backtracking and transcript cleavage to occur, interactions between RNAP and upstream and downstream DNA need to be altered, as well as single-stranded DNA-RNAP, RNA-RNAP, and DNA-RNA hybrid interactions. Given the number of protein-nucleic acid interactions within the ternary elongation complex, it is likely that the conformational changes associated with backtracking and transcript cleavage may be sensitive to salt concentration (20). Different components of the transcription cycle that involve large conformational changes in RNAP and nucleic acid are indeed sensitive to certain electrolytes. For example, RNAP can form an open complex at the λP_R promoter in 200 mM potassium glutamate (Kglu), but not in buffer containing 200 mM potassium chloride (KCl) (20). Similarly, individual anions within the Hofmeister series can either increase or decrease the rate of elongation and have quantitative effects on pause half-lives (21).

In this report, we present the characterization of the intrinsic cleavage activity of *Thermus thermophilus* RNAP. We find that the intrinsic cleavage activity of the polymerase is significantly influenced by the anion concentration of the transcription buffer. In addition to examining factor-independent transcript cleavage, we have also characterized the effects of two GreA homologs on the cleavage activity of *T. thermophilus* RNAP. One of the two Gre-like factors is a true homolog of GreA, containing the highly conserved "basic patch" region within its N-terminal region (22), and possessing transcript cleavage properties similar to *E. coli* GreA. Interestingly, the

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[1] The abbreviations used are: RNAP, RNA polymerase; Kglu, potassium glutamate; DTT, dithiothreitol; TEC, ternary elongation complex.

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second Gre-like factor shares a high degree of sequence identity with GreA in its C-terminal region, but several residues within its N-terminal region differ from those in the N-terminal basic patch region of GreA. This factor is only found in a limited set of organisms and appears to function as an anti-GreA factor *in vitro*. Because of its high degree of sequence similarity but lack of functional homology to GreA, we have named this new factor Gfh1 for Gre Factor Homolog 1.

MATERIALS AND METHODS

Cells and Reagents—T. thermophilus HB8 cells were purchased from the University of Wisconsin Biotechnology center. *Thermus aquaticus* cells, from which genomic DNA was obtained, were kindly provided by S. Darst (The Rockefeller University). All chemicals, reagents, and radioactive nucleotides were the same as those described in a previous study (17). *T. thermophilus* RNAP was purified as described previously (17) or purchased from Epicentre (Madison, WI, purified as described previously (23)).

Identification of GreA Homologs in T. thermophilus—The sequence of the *E. coli* GreA was used in a BLAST search of all non-redundant protein data bases to identify GreA homologs in organisms closely related to *T. thermophilus* and *T. aquaticus*. A putative GreA-like transcription factor was identified in the thermophile, *Thermatoga maritima*, and the primary sequence of this protein was used to search the unfinished genome of *T. thermophilus*. Two large contigs (28.7 and 36.7 kb) containing DNA sequence with homology to *T. maritima* GreA were identified. Each contig was subdivided into 8-kb units and translated in six reading frames. The protein sequence derived from each reading frame was further subdivided into 200-amino acid units, and each was used as a query sequence in a BLAST search. Two putative transcription factors bearing strong sequence similarity to *T. maritima* GreA were identified using this procedure.

Cloning and Expression of GreA Homologs from T. thermophilus and T. aquaticus—Using sequence information derived from the BLAST search, oligonucleotide primers were designed to amplify these two GreA homologs from *T. thermophilus* and *T. aquaticus* genomic DNA. The following primers were designed to PCR-amplify Gfh1 and GreA from *T. thermophilus* and *T. aquaticus*: Gfh1 forward, 5′-ATG GCG CGC GAG GTG AAG CTC-3′; Gfh1 reverse, 5′-GCC GTG GAT GGC CAC CAC CCG-3′; GreA forward, 5′-ATG AAG AAG CCC GTC TAC CTG-3′; GreA reverse, 5′-CTA TAG GGG TTT GAT CTC CAG-3′. Genomic DNA was obtained from *T. thermophilus* and *T. aquaticus* according to previously described methods (24). *T. thermophilus* and *T. aquaticus* genomic DNA (200 ng) were used as template DNA for PCR. Gfh1 PCR products were blunt-end-cloned into the vector pPCR-Script (Stratagene). These clones were then subjected to a second round of amplification with the following primers bearing unique *Nde*I and *Eco*RI restriction sites: *Nde*I-Gfh1, 5′-AGG CAA TTC CAT ATG GCG CGC GAG GTG AAG-3′; *Eco*RI-Gfh1, 5′-GTG GTG CCT CGA GCC GTG GAT GGC CAC CAC-3′. Gfh1-*Nde*I/*Eco*RI PCR products were then cloned into pET 21c (Novagen) creating the expression plasmids, pBPHTthGfh1 and pBPHTaqGfh1. GreA PCR products were directly TA cloned into the expression vector pCT/TOPO (Invitrogen) creating the expression plasmids pBPHTthGreA and pBPHTaqGreA.

Protein Expression in E. coli and Purification of Thermostable Transcription Factors—The expression plasmids harboring the genes for *T. thermophilus* and *T. aquaticus* GreA and Gfh1 were transformed into BL21(DE3) cells. Large cultures were grown to mid-log and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. After induction, cells were grown for an additional 3 h, spun at 3000 rpm for 20 min, and resuspended in extraction buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA). Resuspended cells were lysed by sonication and incubated at 65 °C for 30 min, followed by centrifugation at 13,000 rpm for 30 min at 4 °C. Proteins were precipitated from the 65 °C supernatant by adding solid ammonium sulfate to a final saturation of 90% at 4 °C, followed by centrifugation at 13,000 rpm for 30 min at 4 °C. Nucleic acids were removed from the ammonium sulfate pellet by sequentially washing the pellet three times with extraction buffer + 3 M ammonium sulfate. After washing, the pellet was resuspended in 25 ml of extraction buffer and dialyzed for 16 h against 2 liters of extraction buffer at 4 °C. The dialyzed sample was loaded onto an HQ column (Applied Biosystems) and a NaCl gradient from 150 mM to 1 M was used to elute the proteins. GreA was further purified using a Sephadex G-25 column, and Gfh1 was further purified to electrophoretic homogeneity using a Superdex 200 column (Amersham Biosciences, Inc.). Fractions were analyzed by 15% SDS-PAGE, and those containing the ~18-kDa proteins were pooled and concentrated to 0.4 mg/ml and stored in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 20% glycerol at −20 °C.

Formation of Stalled Elongation Complexes—The following transcription buffers were used to assay the effect of different anions on the intrinsic cleavage activity of RNAP: chloride-low ionic strength buffer (40 mM Tris-HCl, pH 7.9, 40 mM potassium chloride, 10 mM magnesium chloride), glutamate-low ionic strength buffer (40 mM Tris-HCl, pH 7.9, 40 mM potassium glutamate, 10 mM magnesium glutamate), and high ionic strength buffer (30 mM Hepes, pH 7.8, 200 mM potassium glutamate, 25 μg/ml acylated bovine serum albumin, 1 mM DTT, 10 mM magnesium glutamate). Each transcription buffer was supplemented with a micronutrient solution containing following mineral salts: 240 μM $CuSO_4 \cdot 5H_2O$, 830 μM $MgSO_4 \cdot 7H_2O$, 136 μM NaCl, 1 mM KNO_3, 8.1 mM $NaNO_3$, 414 μM Na_2HPO_4, 1.74 μM $ZnSO_4 \cdot 7H_2O$, 1.2 μM Na_2MoO_4, 189 nM $CoCl_2 \cdot 6H_2O$.

Open promoter complexes were formed by incubating 100 nM *T. thermophilus* RNAP and 60 nM DE13 template DNA at 55 °C for 15 min. DE13 template DNA was 5′-biotinylated and bound to streptavidin-coated magnetic beads prior to use. The DE13 DNA template contains the λP_R promoter and encodes a transcript in which first 30 nucleotides of the sequence are as follows: pppAUGUAGUAAGGAGGUUGUAUG GAA^{+24}CAACGC.

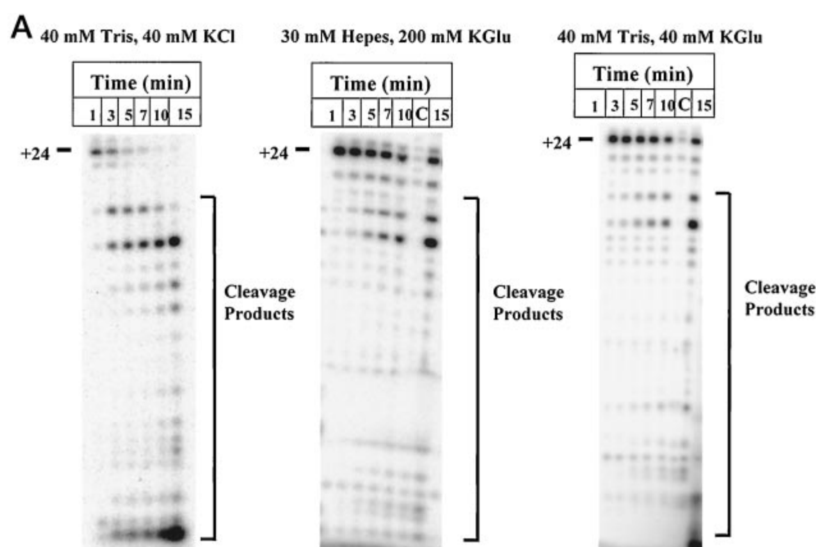
Following open complex formation, 50 μM ATP, 20 μM UTP, 10 μM GTP [α-^{32}P]GTP (200 Ci/mmol) were added to the reaction. Reactions were performed in the absence of CTP, thereby generating stalled elongation complexes containing a +24 transcript. Stalled +24 elongation complexes were purified by placing the reaction tube next to a strong magnet, and washing three times with 200 μl of 1× transcription buffer (25) without micronutrients (either chloride-low ionic strength, high ionic strength, or glutamate-low ionic strength). Stalled +24 complexes were resuspended in 30 μl of 1× transcription buffer without micronutrients and incubated at 55 °C. Aliquots were removed over a 15-min time period and quenched with 95% formamide. Transcription products were analyzed by electrophoresis on an 8 M urea/20% acrylamide gel.

Transcript Cleavage Assays—Stalled +24 TECs were generated and purified as described above and resuspended in the indicated transcription buffer. To each purified TEC containing the +24 transcript, *T. thermophilus* or *T. aquaticus* GreA or Gfh1 was added to a final concentration of 3.5 μM and incubated at 55 °C. Aliquots were removed at 1, 3, 5, 7, 10, and 15 min and quenched with 95% formamide. A 10-min aliquot was chased by adding each of 330 μM ATP, UTP, CTP, and GTP. Transcription products were analyzed by electrophoresis on an 8 M urea/20% acrylamide gel.

UV Cross-linking—Stalled ternary elongation complexes bearing the +24 transcript were prepared in high ionic strength buffer and purified as described for the transcript cleavage assays. After purification, complexes were walked to position +25 with the addition of 10 μM CTP and purified again. The +26/+27 RNA transcript bearing the photoreactive adenosine analog, 8-azido-adenosine, at its 3′-end was generated with the addition of 50 μM 8-N_3ATP (ICN Biomedicals, Inc.). 3.5 μM *T. thermophilus* GreA, *T. aquaticus* GreA, or *T. thermophilus* Gfh1 were added to 13.5-μl aliquots of the +26/+27 containing TEC in a 96-well micro titer plate. The reactions were exposed to long wavelength UV light (254 nm) for 10 min at a distance of 1 cm at 55 °C. Reactions were quenched with 15 μl of 2× J buffer (100 mM Tris acetate, pH 6.8, 4% SDS, 100 mM DTT) and analyzed by 15% SDS-PAGE followed by autoradiography.

Competitive Binding Assay—Stalled elongation complexes bearing the +24 transcript were generated as described above and distributed in reaction tubes in 13.5-μl aliquots. *T. thermophilus* GreA was added to an aliquot of stalled complexes to a final concentration of 3.5 μM, and *T. thermophilus* Gfh1 was added separately in increasing amounts to each reaction (0.875, 1.75, 3.5, and 7 μM, respectively). Each reaction was incubated at 55 °C for 3 min and quenched with 95% formamide. Transcription products were analyzed by electrophoresis on an 8 M urea/20% acrylamide gel. The gels were imaged on a Molecular Dynamics PhosphorImager and analyzed using ImageQuaNT software.

Kinetic Analysis of the Cleavage Reaction—The total amount of radioactivity in each lane was measured on a Molecular Dynamics PhosphorImager and analyzed with ImageQuaNT software. The percentage of complexes at position +24 was determined for each reaction, and the disappearance of the +24 complexes was plotted as a function of time. Each data set was fit to a single exponential ($y = A\exp(-kt)$), and the pseudo-first-order rate constant for each reaction was determined.



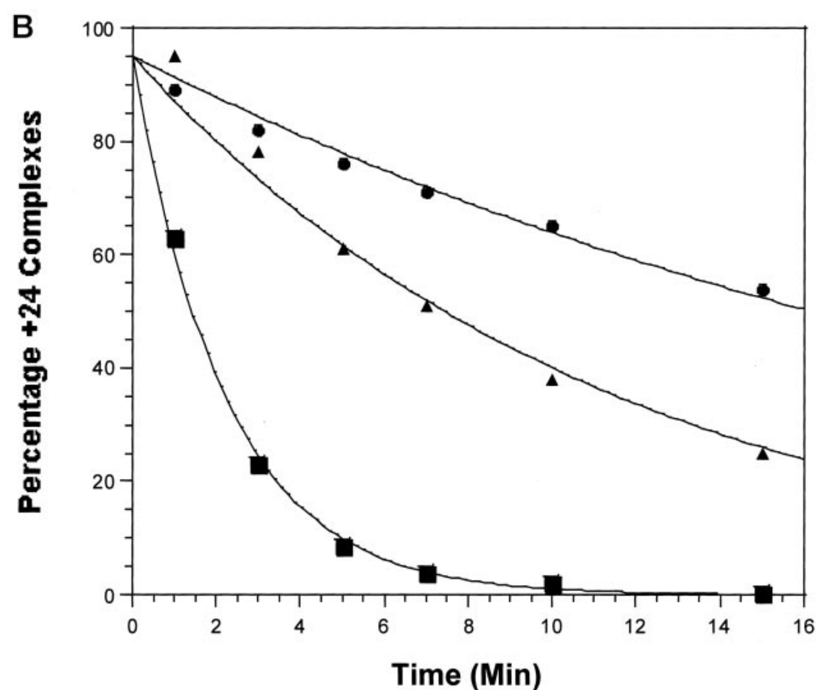


FIG. 1. **The intrinsic cleavage activity of *T. thermophilus* stalled elongation complexes is anion dependent.** *A*, stalled elongation complexes bearing the +24 transcript were generated with *T. thermophilus* RNAP in the following transcription buffers: *Left*, chloride-low ionic strength buffer (40 mM Tris-HCl, pH 7.9, 40 mM potassium chloride, 10 mM magnesium chloride); *middle*, high ionic strength buffer (30 mM Hepes, pH 7.8, 200 mM potassium glutamate, 25 μg/ml acylated bovine serum albumin, 1 mM DTT, 10 mM magnesium glutamate); and *right*, glutamate-low ionic strength buffer (40 mM Tris-HCl, pH 7.9, 40 mM potassium glutamate, 10 mM magnesium glutamate). Complexes were incubated at 55 °C, and aliquots were removed and quenched over a 15-min time period. *B*, the percentage of complexes at position +24 on the DE13 template was quantified from the gel in *A* and plotted as a function of time. *Closed circles*, high ionic strength buffer; *closed triangles*, glutamate-low ionic strength buffer; *closed squares*, chloride-low ionic strength buffer. The *curves* through the data are the best fits to single exponentials. The apparent rate constants, k_{app}, of cleavage are 0.04, 0.08, and 0.45 min^{-1} in high ionic strength buffer, glutamate-low ionic strength buffer, and chloride-low ionic strength buffer, respectively.

RESULTS

The Intrinsic Cleavage Activity of T. thermophilus Stalled Elongation Complexes Is Anion-dependent—Before examining the biochemical effects of thermostable Gre factors on transcription, it is first necessary to characterize the intrinsic transcript cleavage properties of RNAP. To investigate the intrinsic cleavage properties, stalled elongation complexes containing a transcript of defined length were generated with *T. thermophilus* RNAP. The data in Fig. 1*A* indicate that in chloride-low ionic strength buffer *T. thermophilus* RNAP possesses an intrinsic cleavage activity similar to that reported for recombinant *T. aquaticus* RNAP (26). Under these conditions, nearly 100% of the internally labeled transcripts are hydrolyzed into smaller cleavage products after 15 min of incubation at 55 °C. These data suggest that the observed transcript cleavage activity in Fig. 1*A* is the result of endogenous RNAP cleavage and not due to contamination by an exogenous transcript cleavage factor. To further ensure that no transacting transcript cleavage factor copurified with *T. thermophilus* RNAP, 10 μg of RNAP were run on SDS-PAGE and visualized by silver staining. Only bands with molecular weights corresponding to the α, β, β′, ω, and σ subunits of *T. thermophilus* RNAP are observed (data not shown). Given the rapid rate of cleavage observed in chloride-low ionic strength buffer, it is highly unlikely that the observed cleavage is due to contaminating Gre factor. Finally, the pattern of cleavage products produced in the absence of any transcription factor is significantly different from that produced in the presence of a transacting transcript cleavage factor (compare Figs. 1*A* and 3*A*).

In our previous studies examining the elongation properties of *T. thermophilus*, we did not observe a significant amount of transcript cleavage using our standard high ionic strength transcription buffer (17). To elucidate whether an individual component of the high ionic strength buffer was responsible for reducing the rate of intrinsic cleavage in *T. thermophilus* RNAP, we assayed the cleavage activity of RNAP in high ionic strength buffer, low ionic strength buffer with chloride as the anion, and glutamate-low ionic strength buffer containing glutamate as the anion (Fig. 1*A*). For each of the three different buffer conditions, the percentage of complexes at position +24

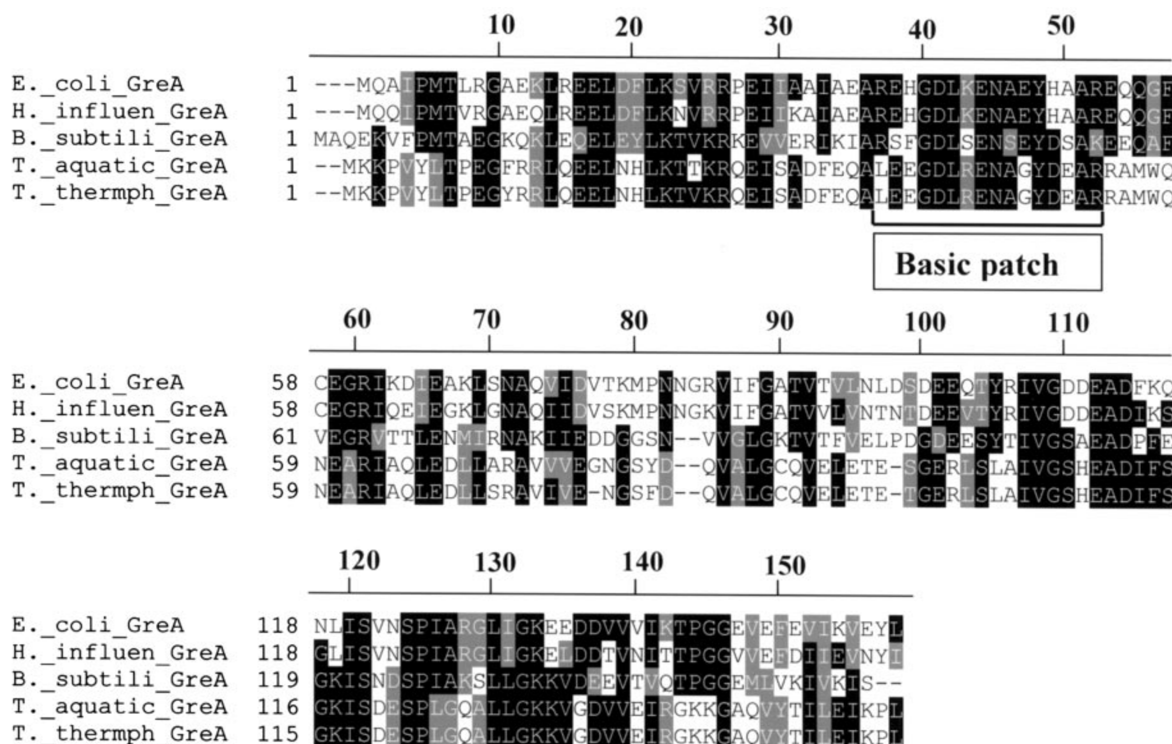


FIG. 2. **Sequence alignment of GreA from mesophilic and thermophilic bacteria.** The *residue numbers above the figure* are with respect to the *E. coli* GreA sequence. Sequences in *black* represent conserved residues, and sequences in *gray* represent conservative substitutions. GreA sequences were obtained using the BLAST search engine from NCBI. Residues 37–52 represent the basic patch element, and residues 41–57 have been shown to contact the 3′-end of RNA.

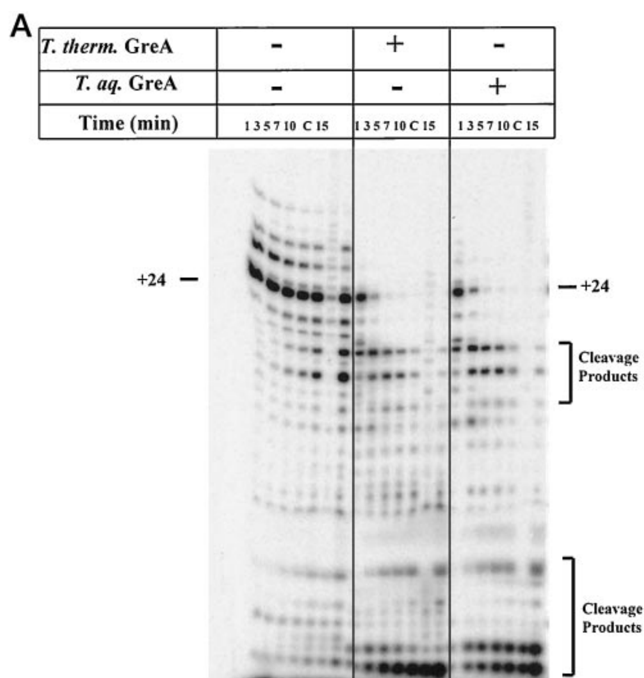
was quantified and plotted as a function of time (Fig. 1*B*). The data were fit to single exponentials ($y = A\exp(-kt)$), and the pseudo-first-order rate constants for the reactions were determined. Inspection of Fig. 1*B* reveals a significant difference in the rate of intrinsic cleavage in transcription buffer containing glutamate rather than chloride. Specifically, replacing 40 mM potassium chloride and 10 mM magnesium chloride in low ionic strength buffer with 40 mM potassium glutamate and 10 mM magnesium glutamate results in a 5-fold decrease in the rate of intrinsic cleavage (Fig. 1*B*; compare *closed squares* and *closed triangles*). Furthermore, increasing the ionic strength of the buffer by raising the potassium glutamate concentration from 40 to 200 mM results in an additional 2-fold decrease in the rate of transcript cleavage (Fig. 1*B*; compare *closed triangles* and *closed circles*). Overall, the rate of *T. thermophilus* RNAP intrinsic cleavage is 10-fold lower in high ionic strength buffer than in low ionic-strength chloride buffer. These data suggest that the presence of glutamate significantly affects the rate of RNAP intrinsic cleavage and are consistent with the limited amount of transcript cleavage we observed in our previous elongation studies (17).

Homologs of GreA Are Found in T. thermophilus and T. aquaticus—The *E. coli* transcript cleavage factor, GreA, has been shown to play important roles in transcription both *in vitro* and *in vivo* (7, 8, 27, 28). Presently, homologs of GreA have been identified in 33 prokaryotes, yet *E. coli* GreA is the only one of the identified factors that has been characterized biochemically. To gain further insight into the mechanism of factor-induced transcript cleavage in all organisms and to interpret our results in the direct context of an existing structural model of RNAP (19), we have identified, cloned, and expressed homologs of GreA from the closely related thermophiles, *T. thermophilus* and *T. aquaticus*.

The *T. thermophilus* and *T. aquaticus* homologs of GreA are 36% identical to *E. coli* GreA, ~95% identical to each other, and contain the conserved basic patch region common to all GreA factors (Fig. 2) (22). Additionally, based on the *E. coli* GreA crystal structure, a number of N-terminal structural residues shown to participate in interdomain and interhelical salt bridging are conserved in both *T. thermophilus* and *T. aquaticus* GreA (27, 29), suggesting that the overall domain organization and three-dimensional structure are conserved.

GreA-induced Transcript Cleavage by T. thermophilus RNA Polymerase Is Genus-specific—*E. coli* GreA has been shown to stimulate the intrinsic cleavage activity of *E. coli* RNAP in a stalled ternary elongation complex (3, 27, 30). Similarly, we find that *T. thermophilus* and *T. aquaticus* GreA can stimulate transcript cleavage in *T. thermophilus* RNAP complexes stalled at position +24 on template DE13 (Fig. 3*A*). In addition, the cleavage products produced in the presence of GreA are different from those that result from intrinsic cleavage (Fig. 3*A*). The pseudo-first-order rate constants for the factor-induced transcript cleavage reactions were determined and compared with the rate of intrinsic cleavage (Fig. 3*B*). The addition of 3.5 μM *T. thermophilus* or *T. aquaticus* GreA to stalled complexes increases the rate of intrinsic cleavage 25-fold, from $k = 0.04$ min^{-1} in the absence of GreA to $k = 1.0$ min^{-1} in the presence of GreA in buffer containing glutamate. Interestingly, the rate of GreA-induced transcript cleavage in high ionic strength buffer is only 2.5-fold faster than factor-independent intrinsic cleavage in the presence of chloride. Furthermore, adding *T. thermophilus* GreA to complexes in low ionic strength buffer containing chloride had no observable effect on the rate of cleavage, and no GreA-specific cleavage products were observed (data not shown).

The ability of *T. aquaticus* GreA to cross-react with *T. thermophilus* RNAP and stimulate transcript cleavage is not surprising given the close evolutionary relationship between the two species. The core RNAPs of *T. thermophilus* and *T. aquati-*



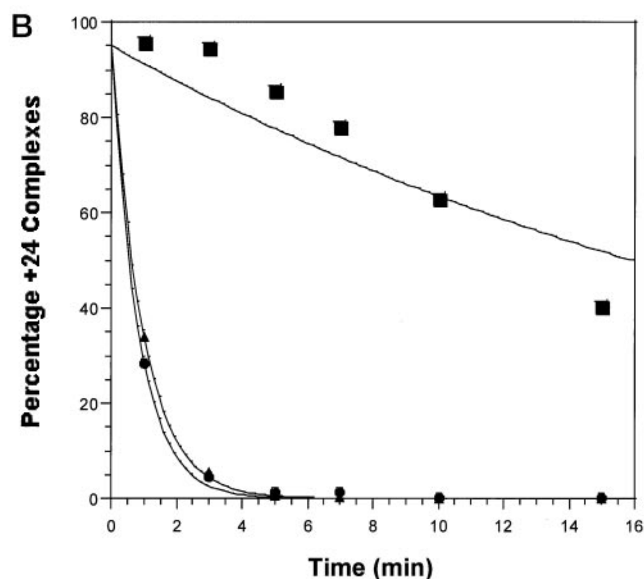


FIG. 3. ***T. thermophilus* and *T. aquaticus* GreA stimulate the cleavage of the +24 transcript.** *A*, stalled elongation complexes at +24 were generated with *T. thermophilus* RNAP, purified, and incubated at 55 °C. Aliquots were removed at 1, 3, 5, 7, 10, 15 min and quenched with 2 volumes of 95% formamide. Complexes were chased (indicated by a *C*) after 10 min in the presence of 330 μM ATP, UTP, CTP, and GTP. *Left*, *T. thermophilus* RNAP only; *middle*, *T. thermophilus* RNAP + 3.5 μM *T. thermophilus* GreA; *right*, *T. thermophilus* RNAP + 3.5 μM *T. aquaticus* GreA. *B*, the percentage of complexes at position +24 on the DE13 template was quantified from the gel in *A* and plotted as a function of time. *Closed squares*, *T. thermophilus* RNAP; *closed circle*, *T. thermophilus* RNAP + *T. thermophilus* GreA; *closed triangles*, *T. thermophilus* RNAP + *T. aquaticus* GreA. The *curves* through the data are the best fits to single exponentials. The apparent rate constants, k_{app}, of cleavage are 0.04, 1.0, and 1.0 min^{-1} for intrinsic, *T. thermophilus* GreA-induced and *T. aquaticus* GreA-induced cleavage, respectively.

cus are ~90% identical,[2] suggesting that the GreA binding site on the polymerase is well conserved between the two species. Interestingly, the cleavage pattern produced by *T. thermophilus* GreA is slightly different from that produced by *T. aquaticus* GreA (Fig. 3*A*). This species-specific difference in transcript cleavage suggests that, although the two factors are capable of stimulating the cleavage activity of RNAP, a differential interaction between the two factors and the polymerase may lead to the subtle differences in the transcript cleavage products. In contrast, *T. thermophilus* and *T. aquaticus* GreA do not stimulate the transcript cleavage activity of *E. coli* RNAP-stalled complexes at 37 °C or 55 °C even at high concentrations of GreA (140 μM), suggesting no cross-genus reactivity between the thermostable cleavage factors and the mesophilic RNAP (data not shown). These data are consistent with previous studies showing that *E. coli* GreA is incapable of stimulating the cleavage activity of *T. aquaticus* RNAP (26). Furthermore, overexpression of endogenous GreA in *E. coli* has been shown to have deleterious effects on growth (22); whereas, overexpression of *T. thermophilus* and *T. aquaticus* GreA in *E. coli* has no observable effects on growth (data not shown).

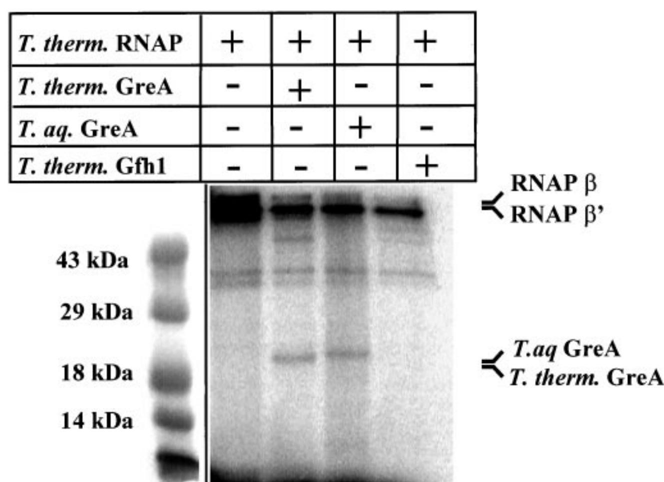


FIG. 4. ***T. thermophilus* and *T. aquaticus* GreA contact the 3′-end of nascent RNA.** Complexes stalled at +24 were walked to positions +26 and +27 with CTP and 8-N_3-ATP, purified, and subsequently exposed to UV light. Cross-links between the RNA 3′-end were visualized by autoradiography after 15% SDS-PAGE. *Lane 1*, Coomassie Blue-stained low molecular weight marker; *lane 2*, UV-irradiated complexes containing *T. thermophilus* RNAP; *lane 3*, *T. thermophilus* RNAP + *T. thermophilus* GreA, *T. thermophilus* RNAP + *T. aquaticus* GreA; *lane 4*, *T. thermophilus* RNAP + *T. thermophilus* Gfh1.

The 3′ Terminus of Nascent RNA Contacts Both T. thermophilus and T. aquaticus GreA—Both *T. thermophilus* and *T. aquaticus* GreA share a high degree of sequence identity with known GreA factors in the region of the N terminus previously shown to cross-link to the 3′-end of nascent RNA (27) (Fig. 2). To examine whether *T. thermophilus* and *T. aquaticus* GreA contact the 3′-end of RNA, stalled elongation complexes were generated with *T. thermophilus* RNAP bearing a transcript with the photoreactive adenosine analog, 8-azido-adenosine, at their 3′-end. These complexes were incubated with *T. thermophilus* or *T. aquaticus* GreA, exposed to UV light for 10 min, and analyzed by SDS-PAGE and autoradiography. Fig. 4 shows that the 3′-end of the RNA transcript cross-links to both *T. thermophilus* and *T. aquaticus* GreA as well as to the β and β' subunits of *T. thermophilus* RNAP. The ability of *T. thermophilus* and *T. aquaticus* GreA to bind *T. thermophilus* RNAP and cross-link to the 3′-end of a nascent RNA is not surprising given the sequence and structural identity of the *Thermus* GreA factors and their ability to stimulate transcript cleavage. However, these results represent the first biochemical characterization of a prokaryotic transcript cleavage factor other than *E. coli* GreA. Of greater significance is the fact that a high resolution crystal structure for *T. aquaticus* core RNAP now

[2] T. Hartsch, unpublished data.

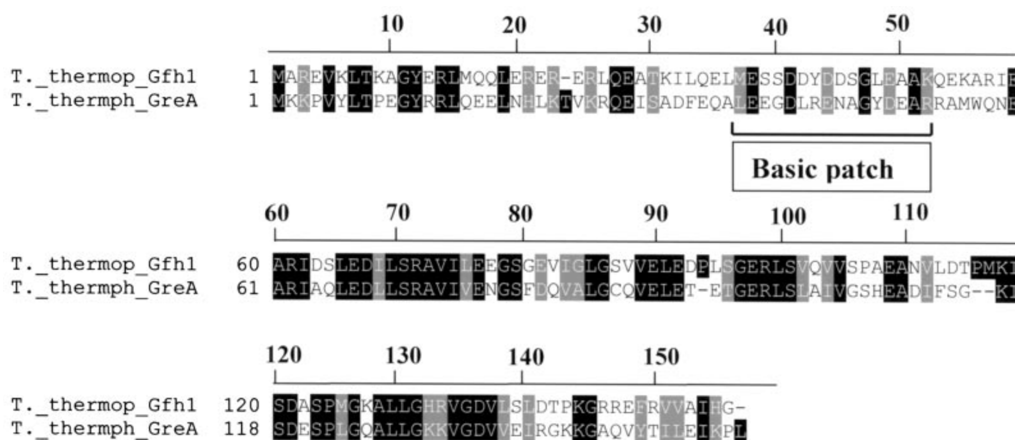


FIG. 5. **Sequence alignment of *T. thermophilus* GreA and Gfh1.** Sequences in *black* represent conserved residues, and sequences in *gray* represent conservative substitutions. The region representing the basic patch of GreA is indicated below the sequence. The *numbering above the sequence alignment* is with respect to Gfh1.

exists (19), and the characterization of the *Thermus* transcription factors can be directly applied to the only existing structural model of a prokaryotic RNAP.

T. thermophilus and T. aquaticus Possess a Novel Gre-like Factor—During the course of our genome search, we identified an uncharacterized transcription factor that shares strong sequence identity with *T. thermophilus* GreA, as well as with a putative Gre-like transcription factor in *Deinococcus radiodurans* (31). We have cloned this transcription factor from *T. thermophilus* and *T. aquaticus* and named it Gfh1 (Gre Factor Homolog 1). *T. thermophilus* and *T. aquaticus* Gfh1 are ~26% identical to *E. coli* GreA and 40% identical to *T. thermophilus* GreA and do not share significant sequence similarity with GreB. The C-terminal regions of *T. thermophilus* and *T. aquaticus* GreA and Gfh1 are highly conserved, sharing greater than 90% sequence identity in their C termini (residues 60–158). In contrast, the N-terminal domains only share ~28% sequence identity (residues 1–60). Several residues within the basic patch region (residues 37–52) of GreA are not conserved in Gfh1; however, Gfh1 does possess a number of conserved residues within its N-terminal region that have been shown to participate in interhelical and interdomain salt-bridging based on the *E. coli* GreA crystal structure (29) (Fig. 5). These results suggest that Gfh1 may adopt a similar tertiary structure to GreA but perhaps has a different function.

Gfh1 Is an Anti-cleavage and Anti-GreA Factor—Studies with the N-terminal and C-terminal domains of *E. coli* GreA indicate that the C-terminal globular domain of the protein is primarily responsible for mediating its interaction with RNAP (32). Given this information, the high level of conservation between the C termini of *T. thermophilus* and *T. aquaticus* Gfh1 and GreA suggests that Gfh1 may interact with RNAP in a manner similar to GreA. To test this hypothesis, we assayed the ability of Gfh1 to promote factor-induced transcript cleavage by *T. thermophilus* RNAP. Instead of stimulating the intrinsic cleavage activity of RNAP, Gfh1 reduces the amount of transcript cleavage in high ionic strength buffer (Fig. 6). After 15 min at 55 °C, stalled elongation complexes incubated in the presence of Gfh1 show a reduced amount of the cleavage products 1 and 2 compared with complexes incubated in the absence of Gfh1 or in the presence of GreA (Fig. 6). To ensure that the Gfh1-mediated reduction in transcript cleavage was an intrinsic property of the transcription factor and to rule out the possibility that the lack of transcript cleavage was due to buffer conditions, the activity of Gfh1 was assayed in three different buffer conditions. Gfh1 was assayed under conditions that produced a high degree of intrinsic transcript cleavage (chloride-low ionic strength buffer), conditions that produced a moderate level of transcript cleavage (glutamate-low ionic strength buffer), and conditions that may better mimic the intracellular environment of *T. thermophilus* (glutamate-low ionic strength buffer + 100 mM trehalose). Presently, little is known about the intracellular environment of *T. thermophilus*, but trehalose, a disaccharide, and glutamate are both compatible organic solutes that accumulate intracellularly in *T. thermophilus* under conditions of osmotic stress (33). In addition, chloride was excluded from this transcription buffer, because NaCl concentrations greater than ~150 mM have been shown to have deleterious effects on *T. thermophilus* growth (33). Gfh1 did not

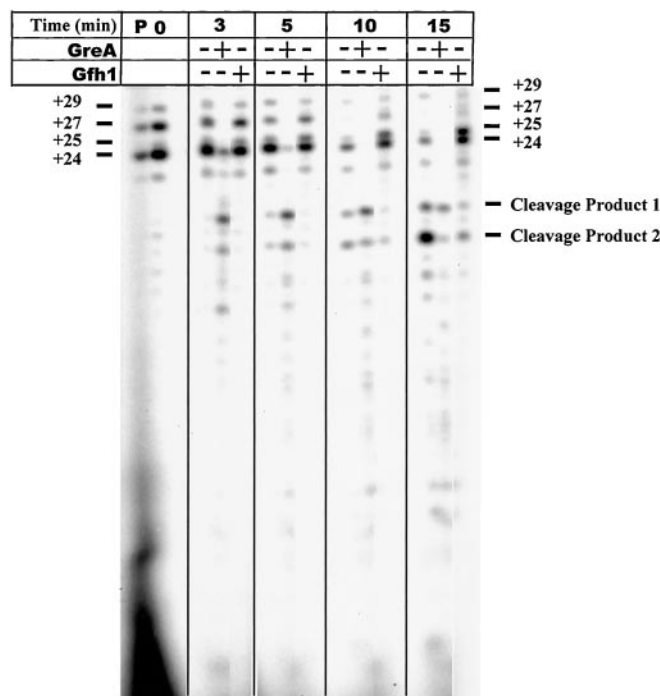


FIG. 6. ***T. thermophilus* Gfh1 does not stimulate the intrinsic cleavage activity of RNAP.** Stalled elongation complexes containing the +24 transcript were generated in high ionic strength buffer with *T. thermophilus* RNAP and purified. Stalled complexes were incubated at 55 °C in the presence or absence of 3.5 μM Gfh1. Aliquots were removed at 3, 5, 10, and 15 min and quenched with 2 volumes of 95% formamide. *Lane P* represents stalled elongation complexes prior to purification. *Lane 0* is a zero time point immediately following purification of the complexes. The data in the figure are from the same experiment and the same gel.

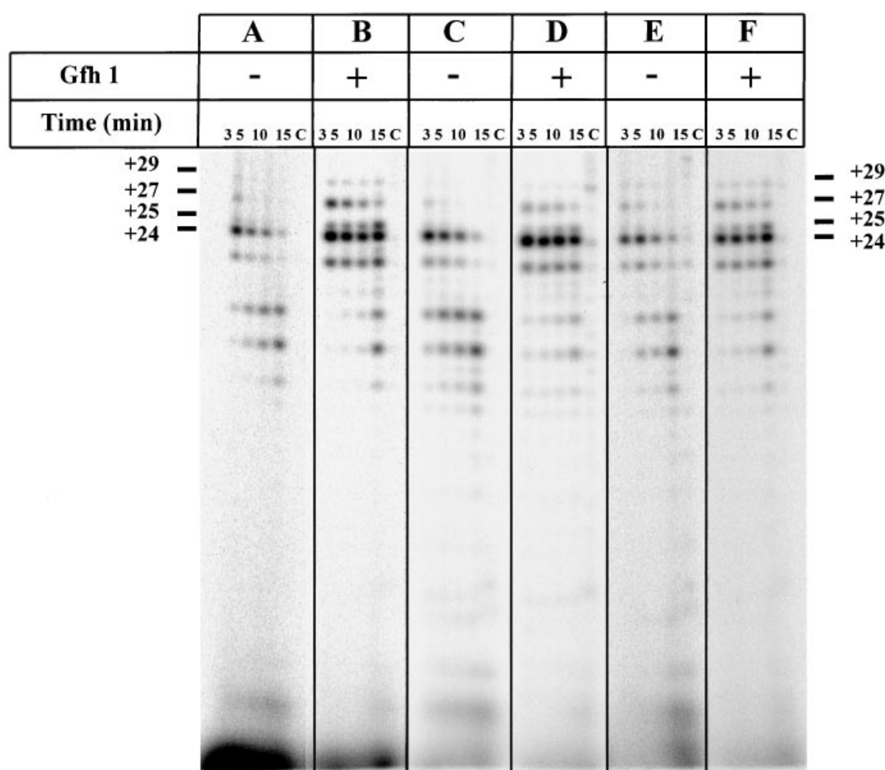


FIG. 7. ***T. thermophilus* Gfh1 reduces transcript cleavage under different buffer conditions.** Stalled elongation complexes containing the +24 transcript were generated in one of three different transcription buffers with *T. thermophilus* RNAP and purified. Stalled complexes were incubated at 55 °C in the presence or absence of 3.5 μM Gfh1. Aliquots were removed at 3, 5, 10, and 15 min and quenched with 2 volumes of 95% formamide. Complexes were chased (indicated by a *C*) after 10 min in the presence of 330 μM ATP, UTP, CTP, and GTP. *A*, chloride-low ionic strength buffer; *B*, chloride-low ionic strength buffer + Gfh1; *C*, glutamate-low ionic strength buffer; *D*, glutamate-low ionic strength buffer + Gfh1; *E*, glutamate-low ionic strength buffer + 100 mM trehalose; *F*, glutamate-low ionic strength buffer + 100 mM trehalose + Gfh1.

stimulate transcript cleavage in any of the transcription buffers that were assayed; rather, the presence of Gfh1 reduced the rate of transcript cleavage in each case (Fig. 7). Interestingly, in the presence of Gfh1, transcripts that result from misincorporation (*i.e.* transcripts that have become misincorporated at position +25 and have been elongated to +27 and +29) appear to be cleaved before the +24 transcript. In the presence of Gfh1 the percentage of +27 and +29 transcripts decreases with time as the intensity of the +25 transcript increases (Fig. 6). Additionally, +25 transcripts can be chased in the presence of NTPs, indicating that these complexes remain active. Taken together, these results suggest that Gfh1 is an anti-cleavage factor that functions by decreasing transcript cleavage and ensuring the processive elongation of nascent RNA.

In addition to lacking the ability to stimulate transcript cleavage, no cross-link between the 3′-end of transcript RNA bearing the photoreactive adenosine analog and Gfh1 was observed (Fig. 4). These results suggest that, under these reaction conditions, Gfh1 does not interact the 3′-end of RNA. However, Gfh1 may contact the 3′-end of the transcript to a lesser extent than GreA, but the weak interaction may be undetectable by cross-linking.

Based on the fact that GreA and Gfh1 share a high degree of sequence similarity, and Gfh1 reduces intrinsic cleavage, we conducted a competitive binding assay to determine if Gfh1 and GreA compete for an overlapping binding site on RNAP. Specifically, stalled elongation complexes bearing the +24 transcript were formed, and *T. thermophilus* GreA was added to stimulate transcript cleavage. Different concentrations of *T. thermophilus* Gfh1 were added simultaneously to the reactions in increasing amounts and incubated at 55 °C for 3 min. The data in Fig. 8*A* show that Gfh1 competitively inhibits GreA-induced transcript cleavage. At low concentrations of Gfh1, a large percentage of transcripts are cleaved generating fragments less than 24 nucleotides in length. In contrast, as the concentration of Gfh1 increases, the percentage of complexes $\leq$ 24 nucleotides decreases (*i.e.* there is a reduction in cleavage activity) (Fig. 8*B*). These data indicate that Gfh1 competitively inhibits GreA activity, suggesting that GreA and Gfh1 bind to overlapping sites on RNAP. Given that GreA and Gfh1 share a high degree of sequence similarity in their C termini, and that the two factors compete for RNAP binding, it is possible that Gfh1 acts as an anti-GreA factor as well as an anti-cleavage factor. Gfh1 may exist to modulate the amount of intrinsic and GreA-induced transcript cleavage *in vivo* and, therefore, may play an integral role in maintaining the processive synthesis of the RNA transcript.

DISCUSSION

Anions Modulate the Intrinsic Cleavage Activity of T. thermophilus RNAP—The catalytic activities of several *E. coli* enzymes, including RNAP, are influenced by the presence of the glutamate anion (20). Specifically, replacing chloride with glutamate expands the range of salt concentrations over which certain restriction enzymes are active and significantly increases the extent and rate of *in vitro* open complex formation by RNAP at the λP_R promoter (20). In addition to its effects on open complex formation, glutamate reduces the overall rate of elongation and dramatically increases pause half-lives relative to chloride (21). It was concluded from the work of Chan and Landick (21) that anion-protein interactions modulated the stability of certain conformations of RNAP elongation complexes that control the half-life of a pause. Similarly, we find that anions significantly affect the rate of *T. thermophilus* RNAP intrinsic cleavage. This result suggests that protein-protein or protein-nucleic acid interactions control, in part, the intrinsic cleavage activity of the enzyme.

The backward translocation of RNAP along the template DNA is believed to be a necessary precursor to transcript cleavage (13, 14, 18). It has been suggested that this backtracking extrudes the 3′-end of the transcript through the secondary channel of RNAP and positions the phosphate backbone of RNA for hydrolysis (14). Recent studies with yeast RNAP II suggest that the stability of the RNA-DNA hybrid is primarily responsible for the conformational changes leading to backtracking (34). Specifically, the more stable the hybrid, the less back-

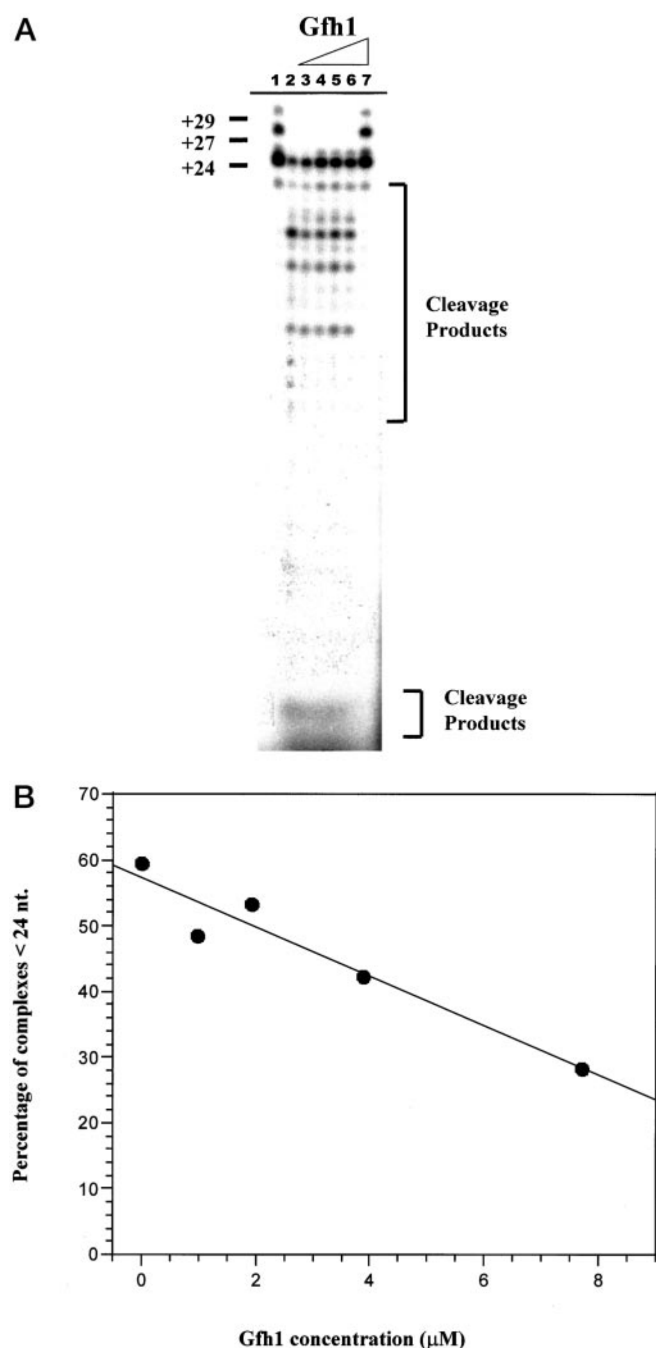


FIG. 8. ***T. thermophilus* Gfh1 competitively inhibits GreA activity.** *A*, stalled elongation complexes containing the +24 transcript were generated in high ionic strength transcription buffer and purified. *T. thermophilus* GreA was added to the stalled complexes to a final concentration of 3.5 μM (*lanes 2–6*) to promote transcript cleavage. *Lane 1* is an intrinsic cleavage control and contains only *T. thermophilus* RNAP. *T. thermophilus* Gfh1 was added in increasing amounts to *lanes 3–6* (0.875, 1.75, 3.5, and 7 μM, respectively). *Lane 7* contains *T. thermophilus* RNAP 7 μM *T. thermophilus* Gfh1 and no GreA. *B*, the data in the gel in *A* were quantified. The percentage of complexes less than 24 nucleotides in length are plotted as a function of Gfh1 concentration after incubation at 55 °C for 3 min.

tracking will occur. However, in the case of RNAP II, the upstream translocation of the RNA-DNA hybrid that leads to the backtracked state seems to be dependent on a conformational change within the polymerase (35). This result suggests that stability of the RNA-DNA hybrid and the propensity to backtrack can be influenced by exogenous agents that affect discrete conformational states of the polymerase.

Because the stability of the RNA-DNA hybrid is influenced primarily by cations, increasing the concentration of K^+ should stabilize the hybrid thereby decreasing the conformational transitions to backtracked states and, therefore, reducing the rate of transcript cleavage. Consistent with this suggestion, increasing the Kglu concentration from 40 to 200 mM causes a 2-fold reduction in the rate of cleavage. Interestingly, replacing 40 mM Kglu with 40 mM KCl results in a 5-fold increase in rate of transcript cleavage. This anion effect on intrinsic cleavage is particularly notable for two reasons. First, the chloride anion lies in the middle of the Hofmeister series and is generally thought to be inert in terms of its overall contribution to the folded tertiary structure of a protein (21), which is the reason it is commonly used in transcription buffers. Second, the fact that the rate of intrinsic cleavage activity can be decreased 5-fold by relatively low concentrations of glutamate is significant. Previous studies have shown that Kglu increases the rate of open complex formation at the λP_R promoter, but these effects were seen at concentrations of glutamate ranging from 0.3–0.5 M (20). The reduction in intrinsic cleavage at 40 mM Kglu suggests that the anion-induced change in cleavage activity is specific to the glutamate anion. This result also implies that the decreased rate of intrinsic cleavage in glutamate buffer cannot be explained entirely by changes in RNA-DNA hybrid stability. Furthermore, these data indicate that protein-protein and/or protein-nucleic acid interactions play a major role in controlling transcript cleavage.

From an experimental standpoint, the ability to modulate the intrinsic cleavage activity of *T. thermophilus* RNAP with buffer conditions is of major importance. In the past, the high rate of intrinsic cleavage demonstrated by thermostable RNAPs has limited the formation and purification of stalled complexes of specific lengths, and thus, limited the research into the mechanisms of transcription elongation. By modifying buffer conditions we were able to generate, purify, and sequentially walk stalled complexes to specific lengths. This technique was essential for several of the experiments discussed in this report.

Evolutionary Conservation of GreA—Homologs of GreA have been identified in many prokaryotes due to genome sequencing projects; however, with the exception of *E. coli* GreA, no biochemical characterization of these factors exists. The cloning, expression, and *in vitro* characterization of *T. thermophilus* and *T. aquaticus* GreA represents the first characterization of a prokaryotic transcript cleavage factor since the initial discovery of GreA and GreB in *E. coli* (1, 3). As we enter the age of genomics and sequence the genomes of ever increasing numbers of bacteria, it is prudent to confirm that the homologs of known/existing factors behave biochemically as homology searches define them.

Our characterization of *T. thermophilus* and *T. aquaticus* GreA reaffirms much of what we have already learned from the study of *E. coli* GreA; however, the subtle differences in the primary sequence of these factors, specifically in the N-terminal basic patch region, have led to new and intriguing ideas into the roles of particular residues within GreA and transcript cleavage itself. Neither *T. thermophilus* nor *T. aquaticus* GreA possess the highly conserved arginine residue at position 37 within the basic patch; instead, the thermostable GreA factors both possess a leucine at this position (Fig. 2). Based on genome information, this particular residue is conserved in ~95% of GreA homologs (22). In *E. coli* GreA, mutation of arginine 37 to alanine results in a dramatic reduction of cross-linking between the 3′-end of nascent RNA and GreA (22). Based on these data, it has been suggested that the large, positively charged side chain of arginine plays a critical role in positioning the

3′-end of RNA extruded from the secondary channel for hydrolysis by RNAP. Instead of arginine, *T. thermophilus* and *T. aquaticus* GreA have a leucine at position 37, which is sterically larger than the alanine substitution in the *E. coli* Arg-37 → Ala mutant. This characteristic of the thermostable GreA factors leads to the speculation that *T. thermophilus* and *T. aquaticus* GreA may direct the translocation of the 3′-end of RNA to the RNAP cleavage center through a steric rather than an electrostatic interaction. Although we present no direct mutational analysis of *T. thermophilus* and *T. aquaticus* GreA to confirm this hypothesis, three other organisms are known to naturally possess a residue other than arginine at position 37 of GreA. *D. radiodurans*, *Treponema pallidum*, and *Clostridium acetobutylicum* each possess either an isoleucine or a leucine at position 37 of GreA (31, 36, 37). This analysis suggests, from an evolutionary standpoint, that a positive charge at this position in the protein is not critical to the function of GreA.

GreA Dramatically Increases the Rate of RNAP Intrinsic Cleavage—The prokaryotic transcription factors GreA and GreB, as well as their functional eukaryotic homolog, SII, have been shown to stimulate the cleavage activity of RNAP (1, 27, 38). Consistent with these earlier data, we find *T. thermophilus* and *T. aquaticus* GreA increase the rate of *T. thermophilus* RNAP intrinsic cleavage ~25-fold in buffer containing glutamate. However, in transcription buffer containing chloride, the rapid rate of the intrinsic cleavage reaction rendered Gre-induced transcript cleavage indistinguishable from RNAP intrinsic cleavage. Moreover, the rate of GreA-independent cleavage in buffer containing chloride was approximately 10-fold greater than buffer containing 200 mM glutamate. This rate is nearly half the rate of GreA-induced transcript cleavage, lending further support for the idea that the interaction between the polymerase and the glutamate anion has a significant effect on the conformational transitions that lead to a cleavage-competent state.

Gfh1 Is an Anti-cleavage and an Anti-GreA Factor—Based on genome searches, Gfh1 appears to be specific only to organisms that can be classified as extremophiles. Among the 33 mesophilic organisms in which the transcript cleavage factor GreA and/or GreB have been identified, none possess this particular transcription factor. Given that, *in vitro*, Gfh1 inhibits cleavage while GreA induces cleavage and that Gfh1 competes with GreA for a binding site on RNAP, one possible role of Gfh1 may be to modulate the amount of both intrinsic and factor-induced transcript cleavage. Gfh1 may bind to RNAP under conditions that favor RNA synthesis and prevent RNAP from undergoing the conformational changes that result in backtracking and transcript cleavage. By occupying a site on RNAP that overlaps with the GreA binding site, Gfh1 would effectively exclude GreA and may function to keep RNAP in an activated state (7). Similarly, it is possible that Gfh1 may be displaced from its overlapping binding site with GreA upon a conformational change in the ternary elongation complex that shifts RNAP from an actively elongating state to stalled and, ultimately, backtracked state.

We have previously shown that the initial steps governing transcription are well conserved between *T. thermophilus* and *E. coli* (17). Similarly, we find the existence of a GreA factor in the extreme thermophile, suggesting the mechanisms that govern the fidelity of transcription are also well conserved throughout evolution. However, our genome search revealed a novel Gre-like transcription factor in both *T. thermophilus* and *T. aquaticus* that does not function as a transcript cleavage factor, but rather functions to suppress the intrinsic cleavage activity of RNAP. Homologs of Gfh1 appear to exist only in bacteria that can be classified as extremophiles, suggesting that under extreme environmental conditions more than one mechanism for modulating transcript cleavage is required to maintain the integrity of transcription.

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REFERENCES

1. Borukhov, S., Polyakov, A., Nikiforov, V., and Goldfarb, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8899–8902
2. Surratt, C. K., Milan, S. C., and Chamberlin, M. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7983–7987
3. Borukhov, S., Sagitov, V., and Goldfarb, A. (1993) *Cell* **72,** 459–466
4. Reines, D. (1992) *J. Biol. Chem.* **267,** 3795–3800
5. Sluder, A. E., Greenleaf, A. L., and Price, D. H. (1989) *J. Biol. Chem.* **264,** 8963–8969
6. Hsu, L. M., Vo, N. V., and Chamberlin, M. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11588–11592
7. Erie, D. A., Hajiseyedjavadi, O., Young, M. C., and von Hippel, P. H. (1993) *Science* **262,** 867–873
8. Artsimovitch, I., and Landick, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7090–7095
9. Marr, M. T., and Roberts, J. W. (2000) *Mol. Cell* **6,** 1275–1285
10. Rudd, M. D., Izban, M. G., and Luse, D. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8057–8061
11. Wang, D., and Hawley, D. K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 843–847
12. Mustaev, A., Kashlev, M., Zaychikov, E., Grachev, M., and Goldfarb, A. (1993) *J. Biol. Chem.* **268,** 19185–19187
13. Komissarova, N., and Kashlev, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1755–1760
14. Nudler, E., Mustaev, A., Lukhtanov, E., and Goldfarb, A. (1997) *Cell* **89,** 33–41
15. Arndt, K. M., and Chamberlin, M. J. (1990) *J. Mol. Biol.* **213,** 79–108
16. Krummel, B., and Chamberlin, M. J. (1992) *J. Mol. Biol.* **225,** 221–237
17. Xue, Y., Hogan, B. P., and Erie, D. A. (2000) *Biochemistry* **39,** 14356–14362
18. Reeder, T. C., and Hawley, D. K. (1996) *Cell* **87,** 767–777
19. Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K., and Darst, S. A. (1999) *Cell* **98,** 811–824
20. Leirmo, S., Harrison, C., Cayley, D. S., Burgess, R. R., and Record, M. T., Jr. (1987) *Biochemistry* **26,** 2095–2101
21. Chan, C. L., and Landick, R. (1997) *J. Mol. Biol.* **268,** 37–53
22. Kulish, D., Lee, J., Lomakin, I., Nowicka, B., Das, A., Darst, S., Normet, K., and Borukhov, S. (2000) *J. Biol. Chem.* **275,** 12789–12798
23. Wnendt, S., Hartmann, R. K., Ulbrich, N., and Erdmann, V. A. (1990) *Eur. J. Biochem.* **191,** 467–472
24. Kato, R., and Kuramitsu, S. (1993) *J. Biochem.* (*Tokyo*) **114,** 926–929
25. Foster, J. E., Holmes, S. F., and Erie, D. A. (2001) *Cell* **106,** 243–252
26. Minakhin, L., Nechaev, S., Campbell, E. A., and Severinov, K. (2001) *J. Bacteriol.* **183,** 71–76
27. Koulich, D., Orlova, M., Malhotra, A., Sali, A., Darst, S. A., and Borukhov, S. (1997) *J. Biol. Chem.* **272,** 7201–7210
28. Toulme, F., Mosrin-Huaman, C., Sparkowski, J., Das, A., Leng, M., and Rahmouni, A. R. (2000) *EMBO J.* **19,** 6853–6859
29. Stebbins, C. E., Borukhov, S., Orlova, M., Polyakov, A., Goldfarb, A., and Darst, S. A. (1995) *Nature* **373,** 636–640
30. Loizos, N., and Darst, S. A. (1999) *J. Biol. Chem.* **274,** 23378–23386
31. White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., Haft, D. H., Gwinn, M. L., Nelson, W. C., Richardson, D. L., Moffat, K. S., Qin, H., Jiang, L., Pamphile, W., Crosby, M., Shen, M., Vamathevan, J. J., Lam, P., McDonald, L., Utterback, T., Zalewski, C., Makarova, K. S., Aravind, L., Daly, M. J., and Fraser, C. M. (1999) *Science* **286,** 1571–1577
32. Koulich, D., Nikiforov, V., and Borukhov, S. (1998) *J. Mol. Biol.* **276,** 379–389
33. Santos, H., and da Costa, M. S. (2001) *Methods Enzymol.* **334,** 302–315
34. Kireeva, M. L., Komissarova, N., Waugh, D. S., and Kashlev, M. (2000) *J. Biol. Chem.* **275,** 6530–6536
35. Palangat, M., and Landick, R. (2001) *J. Mol. Biol.* **311,** 265–282
36. Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M. D., and Venter, J. C. (1998) *Science* **281,** 375–388
37. Nolling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., Lee, H. M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y. I., Tatusov, R. L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M. J., Bennett, G. N., Koonin, E. V., and Smith, D. R. (2001) *J. Bacteriol.* **183,** 4823–4838
38. Gu, W., and Reines, D. (1995) *J. Biol. Chem.* **270,** 30441–30447