Intrinsic transcript cleavage activity of RNA polymerase

MARIANNA ORLOVA*, JANET NEWLANDS†, ASIS DAS†, ALEX GOLDFARB*, AND SERGEI BORUKHOV**

*Public Health Research Institute, New York, NY 10016; and †University of Connecticut Health Center, Farmington, CT 06030

Communicated by E. Peter Geiduschek, University of California, San Diego, CA, February 10, 1995

ABSTRACT The GreA and GreB transcript cleavage factors of Escherichia coli suppress elongation arrest and may have a proofreading role in transcription. With the use of E. coli greA"greB" mutant, RNA polymerase is demonstrated to possess substantial intrinsic transcript cleavage activity. Mildly alkaline pH mimics the effect of the Gre proteins by inducing transcript cleavage in ternary complexes and antagonizing elongation arrest through a cleavage-and-restart reaction. Thus, transcript cleavage constitutes the second enzymological activity of RNA polymerase along with polymerization/pyrophosphorolysis of RNA, whereas the Gre proteins merely enhance this intrinsic property.

The hydrolytic transcript cleavage reaction was first observed in ternary elongating complexes of E. coli RNA polymerase (RNAP) (1). The cleavage is followed by the dissociation of the 3′ proximal fragment of the transcript and resumption of elongation from the newly generated 3′ terminus. Two E. coli transcription factors, GreA and GreB, facilitate transcript cleavage (2, 3). The GreA-induced cleavage removes di- and trinucleotides from the 3′ terminus. In most cases, GreB induces cleavage at the same sites as GreA; however, some ("strained") elongation complexes are directed by GreB to cleave in a longer "register", removing a 3′-terminal DNA fragment that is up to 9 nt in length (3, 4). The eukaryotic elongation factor II functionally resembles GreB, although it has no apparent sequence homology with prokaryotic Gre proteins. Elongation factor II cleaves RNA in both the long and the short registers, depending on the type of ternary complex (5–7). Similar transcript cleavage reactions have also been observed in ternary elongating complexes of vaccinia virus RNA polymerase (8) and eukaryotic RNAP III (9). The transcript cleavage factors are presumed to have two biologically important and evolutionarily conserved functions: the relief of elongation arrest, the condition of ternary complexes that can neither advance nor dissociate (3–7), and the enhancement of transcription fidelity (10, 11).

Purified GreA and GreB do not cleave free RNA, whereas even the purest preparations of recombinant, reconstituted RNAP display some residual transcript cleavage (2). We may assume that either the presence of trace amounts of Gre proteins and/or other, Gre-like factors, or RNA cleavage itself could be responsible for the residual cleavage activity. Using a greA"greB" double-mutant strain of E. coli, we demonstrate here that, in fact, RNAP itself possesses intrinsic transcript cleavage activity. Mildly alkaline pH substantially enhances this activity of RNAP and mimics the effect of the Gre proteins by antagonizing the elongation arrest through a cleavage-and-restart reaction.

MATERIALS AND METHODS

Ultrapure-grade NTPs were obtained from Boehringer Mannheim. [α-32P]CTP and [α-32P]GTP were purchased from Amersham. GreA and GreB were purified to apparent homogeneity from overproducing E. coli strains (S. Borukhov, M. Orlova, D. Koulish, S.A. Darst, and A.G., unpublished work) using a modification of the procedure described in ref. 3. Specific polyclonal antibodies directed against GreA and GreB were provided by R. Landick and H.-G. Feng (Washington University).

E. coli greA"greB" Double-Mutant Strain. A mutant strain of E. coli, AD8571, lacking functional GreA and GreB proteins was constructed by combining greA::KanR and greB alleles. The ΔompR-greB) strain, SG480 Δ76, was from T. Silhavy (12). The greA::KanR strain was constructed in several steps. The greA gene in a plasmid was first disrupted by inserting a Stu I–KanR cassette at the Dra I site, resulting in the interruption of the GreA sequence at aa 115. The truncated greA gene product fails to suppress the conditional growth defect of ts8 RNAP mutant strain (13, 14). Subsequently, a Δ69 phage carrying the greA::KanR allele was obtained by recombination between the plasmid and λΔ69 greA+ phage. A merodiploid greA+"greA::KanR strain was then constructed by lyogenic wild-type E. coli with λΔ69 greA::KanR. Phage PIG grown in the merodiploid strain was finally used to transduce the greA::KanR allele into the ΔgreB strain. The disruption of greA in the resultant strain was confirmed by linkage to a nearby Tn10 marker (15). The double-mutant strain fails to grow at high temperature (43°C), whereas neither single mutant shows this defect.

RNAP. "Recombinant" RNAP was prepared by reconstitution and assembly of individually overexpressed and purified subunits, according to ref. 16. "Natural" RNAP was isolated from greA"greB" E. coli, according to the method of Burgess and Jendrisak (17), with final purification of the RNAP holoenzyme by size exclusion and anion-exchange fast protein liquid chromatography using Superoxer 6 HR and Mono Q HR columns, as described for the preparation of the "recombinant" enzyme (16). RNAP was >99% pure as judged by Coomassie blue staining of electrophoresis gel slabs. Immunoblot analysis of 40 pmol of the material with polyclonal anti-GreA and anti-GreB antibodies did not reveal the presence of Gre proteins. The detection limit of the blot was ~1 fmol for GreA and ~30 fmol for GreB.

RNAP Ternary Complexes and RNA Chain Extension. All ternary complexes were prepared as described (2, 3) by using the isolated 202-bp DNA fragment (endpoints of −152 and +50) carrying the E. coli ribosomal rrnB P1 promoter with its initial transcribed sequence CACACUGACACGG.... (18). The "6-meric" ternary complex (i.e., bearing a 6-nt transcript) radiolabeled in the third, fourth, and sixth positions (CjAPcPcAPc) (here and elsewhere in the text boldface letters indicate radioactive phosphate) was prepared by using 0.9 μg (7.3 pmol) of the DNA fragment incubated for 10 min at 37°C with 12 μg (30 pmol) of RNAP, bovine serum albumin at 1 mg/ml, 1 mM Ca2+, 1 μM ATP, and 1 μM [α-32P]CTP (3000 Ci/mmol; 1 Ci = 37 GBq) in 35 μl of standard

Abbreviation: RNAP, RNA polymerase.

†To whom reprint requests should be addressed: Department of Microbiology and Immunology, State University of New York Health Science Center, 450 Clarkson Avenue, Box 44, Brooklyn, NY 11203-2098.
transcription buffer (40 mM Tris acetate/pH 7.9/30 mM KCl/10 mM MgCl2). Due to the presence of pyrophosphate in NTPs, the background exopyrophosphorylation resulted in significant contamination of the 6-meric complex with the 5-meric complex-bearing transcript CpApCpCpA. To counter this effect, in the second step of the reaction, unlabeled CTP (10 μM) was added, and the incubation was continued for an additional 3 min. This led to the “enrichment” of the final preparation with the 6-meric complex at the cost of “dilution” of specific radioactivity in the 3'-terminal pC. The corresponding unlabeled 6-meric complex was synthesized in one step by using 6 μM CTP throughout the reaction. The complexes were purified by gel filtration on Quick-Spin G-50 columns in 10 mM Tris-HCl buffer, pH 8.0/1 mM EDTA. The 7-meric ternary complex (CpApCpCpApCpU) was obtained by chain extension of 50 fmol of radiolabeled 6-meric complex (CpApCpCpApCp) with 10 μM UTP for 5 min at 37°C in 20 μl of standard transcription buffer. The 9-meric complex radio-labeled in the eighth position (CpApCpCpApCpUpGpA) was obtained by chain extension of 50 fmol of “cold” 6-meric complex with 10 μM UTP, 10 μM ATP, and 1 μM [α-32P]GTP (3000 Ci/mmol), for 5 min at 37°C in 35 μl of standard transcription buffer. The complex was further purified by gel filtration as described above and supplemented with 10 μM ATP to counter pyrophosphorylation.

Transcript Cleavage and Antiarrest Assays. In all transcription assays 10 μl of each reaction mixture contained 3–6 fmol of ternary complexes in specific transcription buffer. Purified GreA and GreB were used in proportion of 10 molar equivalents to the corresponding ternary complex. The crude cell lysate of greA-greB E. coli was prepared as described (2) and diluted 10-fold into the reaction samples containing yeast tRNA at 10 mg/ml.

Purified 9-meric complexes were obtained as described (3) with GreA/GreB-free RNAP. Two hundred femtomoles of radiolabeled ternary complex carrying the 6-meric transcript CpApCpCpCpApCp was incubated with 1 mM NTPs at 37°C for 30 min in 35 μl of standard transcription buffer, and the complexes were purified by G-50 Quick Spin gel-filtration.

pH-induced transcript cleavage reactions were done in three buffer systems: (i) 40 mM Pipes adjusted to pH 6.0 and 6.5 by addition of 1 M NaOH; (ii) 40 mM Tris adjusted to pH 7.0, 7.5, 8.0, 8.5, and 9.0 by addition of 1 M HCl; (iii) 40 mM CAPS adjusted to pH 9.5 and 10.0 by addition of 1 M NaOH. All buffers contained 0.1 M NaCl and 10 mM MgCl2. For pH-induced transcript cleavage and antiarrest assays, 1 μl of reaction mixture containing 3 fmol of specific ternary complexes was diluted into 10 μl of the corresponding buffer described above and incubated under conditions specified in the figure legends.

All reactions were stopped by adding EDTA to a final concentration of 50 mM. RNA products were separated by 23% denaturing PAGE as described in ref. 18 and analyzed by autoradiography; the radioactivity was then quantitated by using a scanning densitometer and a PhosphorImager.

RESULTS

Antiarrest and transcript cleavage assays were performed in a crude cell lysate of greA-greB E. coli. To exclude the possibility of RNAP contamination with trace amounts of Gre proteins and to explore the putative intrinsic cleavage activity of RNAP, a greA-greB double-mutant strain of E. coli was constructed. The entire greB gene was deleted from the chromosome, and the greA gene contained an out-of-frame insertion of the kan\* gene (see Materials and Methods for details). Fig. 1 demonstrates that a crude cell lysate of such a strain contains no detectable antiarrest (lanes 1–5) or transcript cleavage (lanes 6–9) activity. In the antiarrest assay, gel filtration-purified ternary complex prepared with “recombinant” RNAP on the rrnB P1, promoter carrying the hexameric transcript CpApCpCpApCp (lane 1) was allowed to resume elongation by adding the mixture of four elongation substrates. A substantial fraction of elongation complexes was arrested at positions +12, +13 (lane 2). In the presence of GreA (lane 3) and GreB (lane 4) the arrested complexes did not accumulate, and low-molecular-weight cleavage products appeared. However, the lysate of the greA-greB cells displayed no antiarrest effect (lane 5). The transcript-cleavage assay was done on the ternary complex carrying the nontranscript CpApCpCpApCp (lane 6). The greA-greB cell lysate had no effect (lane 7), whereas purified GreA and GreB proteins induced cleavage that removed 3'-terminal dinucleotide (lane 8) and tetranucleotide (lane 9), respectively. The lysate of the mutant cells was used in these experiments in 1:10 dilution. Under the same conditions, a lysate of the wild-type E. coli was active at various dilutions ranging from 1:10 to 1:10,000 (ref. 2 and data not shown). Thus we conclude that the double mutant has no detectable level of transcript-cleavage activity in the soluble phase of the cell.

Natural RNAP was purified from the double-mutant strain to apparent homogeneity (Coomassie blue staining of 4–20% gradient SDS slabs gels revealed no contaminating bands) and was used to form the ternary complex carrying the 9-meric transcript CpApCpCpApCpUpGpA. Fig. 2 (lanes 1–4) shows that a significant level of transcript cleavage, in both GreA and GreB modes (removing the dimer and tetramer, respectively), could be observed after prolonged incubation. The addition of 10–100–, and 1000-fold molar excess of RNAP into the reaction mixture did not enhance cleavage (lanes 5–7, 8–10, and 11–13, respectively), as judged by visual inspection of the
This conclusion was strongly supported by the serendipitous discovery that cleavage in the absence of factors can be dramatically stimulated by alkaline pH (Fig. 3). The normal enzymatic activity of RNAP varies from 100% to 13% in the pH range from 6.0 to 10.0, with an optimum at pH 7.8-8.2 (19). We found that at pH > 8.0, the nascent RNA in the 7-meric (Fig. 3A, lanes 6–10) and 9-meric (Fig. 3B, lanes 5–9) ternary complexes is cleaved at a high rate. The GreA-type cleavage is prevalent between pH 8.0 and 9.5 (Fig. 3B, lanes 5–8). The GreB-type of cleavage becomes prominent at pH 10.0 (Fig. 3B, lane 9). At the optimal conditions for polymerization (pH 7.5) and pyrophosphorolysis (pH 6.5), the endogenous transcript cleavage is negligible (Fig. 3A, lanes 2–5 and Fig. 3B, lanes 2–4). It should be noted that the apparent nonquantitative recovery of the pCpU band in Fig. 3A reflects lower specific radioactivity of $^{32}$P at the sixth position of the CpApCpCpApC substrate as compared to the third and fourth positions (see Materials and Methods).

The effect of alkaline pH on ternary transcription complexes was then studied with regard to elongation arrest at transcript positions +12, +13. Previously GreB has been shown to lift the arrest when added to the preformed arrested complexes (3). GreA did not have this effect but prevented the arrest when it was added before RNAP reached the arresting sites (3). In the experiment of Fig. 4A we studied elongation through the arresting sites at different pH ranging from 6.0 to 10.0. In each reaction, the starting ternary complex carrying 7-meric transcript CpApCpCpApCpA (lane 2) was exposed to a particular pH alone (odd-numbered lanes) or in the presence of the four NTPs (even-numbered lanes). The accumulation of the pentamer CpApCpCpA in the odd-numbered lanes and its quantitative disappearance in response to NTPs are the indication of a cleavage-and-restart reaction that progressively occurs at pH > 8.0 (lanes 7–12). The restarting material is partially chased into the runoff transcript and the arrested complexes. Due to nonspecific inhibition of elongation by alkali, the overall yield of the distinct chase products declines as the pH rises, so that at pH 10 about half of the chased material appears as a smear that can be seen at prolonged exposure of autoradiograph (data not shown). Yet, the declining ratio of the arrested complexes to the runoff transcript in the chase reactions clearly testifies to the antiarrest effect of elevated pH.

In Fig. 4B the effect of pH on the preformed arrested complexes was tested. It can be seen that at pH 9.0 and 10.0,
the transcripts in the arrested complexes were cleaved to yield the 5'-terminal pentamer CpApCpApA, which quantitatively disappears in response to the addition of NTPs (lanes 9–12). The chased radioactive material partially reappears as a high-molecular-weight smear, but no arrested complexes are formed in agreement with the result of Fig. 4A. Thus, the preformed arrested complexes undergo a spontaneous cleavage-and-restart reaction at elevated pH. From these results we conclude that alkaline conditions can mimic the antiarrest effect of both GreA and GreB.

The pH-dependent cleavage of RNA in the ternary complex can occur by two possible mechanisms. As was originally suggested (1), the trajectory of the transcript in the complex may invoke a bend associated with the strain of the RNA chain. The strained phosphodiester bond may be particularly susceptible to alkaline hydrolysis. In such a “passive” mechanism of cleavage, the reaction rate will progressively increase with pH. The alternative “base catalysis” mechanism of cleavage envisages a specific active center performing the cut in the RNA molecule in the classic enzymological sense. The latter mechanism is consistent with the hypothesis that cleavage is performed by the active site of RNA polymerization that has disengaged from the 3' terminus and snapped back to an internal position within the transcript molecule (20).

To address this question, we analyzed the pH dependence of the transcript cleavage for the 7-meric ternary complex (Fig. 5). Control experiments (data not shown) verified that each point of the titration curve reflects the initial rate of the cleavage reaction. The titration curve shows a sharp increase of the cleavage rate between pH 8.0 and 9.0, reaching a plateau above pH 9.5. This shape of the titration curve was reproduced in three independent experiments for the 7-meric substrate, as well as in three experiments for the 9-meric substrate. Such a pH dependence is likely to reflect an amino acid residue within RNAP (probably lysine, histidine, or cysteine, with an apparent pK of 8.6) for which titration is a prerequisite of the cleavage reaction, suggesting an underlying mechanism of protein-mediated catalysis (21), and argues against the notion of passive alkaline hydrolysis of a strained transcript.

**DISCUSSION**

The principal observation of this work is that internal transcript cleavage is an intrinsic function of RNAP, whereas the

**Fig. 4.** pH-induced antiarrest activity of RNAP. The starting material in all experiments was gel-filtration-purified ternary complexes formed by GreA/GreB-free RNAP carrying the radiolabeled 6-meric transcript CpApCpApCpA (4 and B, lane 1). (A) The effect of pH on RNAP reading through the elongation-arresting sites. Extension of the radiolabeled 6-meric ternary complex with UTP yielded the 7-meric complex (lane 2), which was incubated at 37°C for 15 min under the indicated conditions without (lanes 3, 5, 7, 9, 11) or with 100 μM NTPs (lanes 4, 6, 8, 10, and 12). (B) Effect of pH on preformed arrested complexes. Gel-filtration-purified arrested complexes (lane 2) were incubated at 37°C for 15 min in transcription buffers at the indicated pHs alone (lanes 3, 5, 7, 9, and 11) or in the presence of GreA and GreB (lanes 13 and 15, respectively), followed by challenge with 1 mM NTPs for an additional 15 min at 37°C (lanes 4, 6, 8, 10, 12, 14, and 15).

**Fig. 5.** pH-dependence curve of the intrinsic transcript-cleavage activity. The graph represents the accumulation of dinucleotide cleavage product pCpU after incubation of the initial radiolabeled 7-meric ternary complex carrying CpApCpApCpA in transcription buffers at pH 6.0–11.0 (experiment analogous to that shown in Fig. 3A, but with incubation time 5 min at 37°C). Each point of the titration curve corresponds to the amount of the dinucleotide cleavage product formed at the initial rate of cleavage reaction. The transcript-cleavage products were separated by denaturing gel electrophoresis and visualized by autoradiography; the radioactivity was quantitated with a PhosphorImager.
Gre factors merely enhance this function. A similar conclusion was reached recently by Rudd et al. (20) with regard to eukaryotic RNAP II, where the transcript cleavage in arrested ternary complexes can be facilitated by addition of pyrophosphate in the absence of elongation factor SII. This cleavage reaction resulted in the incorporation of the pyrophosphate to the 5' terminus of the cleaved RNA product. Because exopolyphosphorylation at the 3' terminus is the reverse reaction of RNA polymerization, the authors argued that it is the active center of RNAP that carries out the internal endopolyphosphorylolytic cleavage of the transcript (20). Our present results do not provide any evidence for or against this mechanism. It should be noted, however, that we failed to observe any pyrophosphate stimulation of transcript cleavage in the E. coli system, in both the arrested and productive ternary complexes (data not shown). Thus, some aspects of the cleavage reaction of eukaryotic and prokaryotic polymerases may differ.

Our observations together with the results of Rudd et al. (20) may explain why there is no sequence homology between the prokaryotic cleavage factors GreA and GreB and the eukaryotic elongation factor SII, even though they seem to perform the same function. Indeed, the structural constraints on the catalytic center for the sophisticated cleavage reaction can be well fulfilled at the level of polymerase, a highly conserved enzyme, whereas the external enhancing factors need not be homologous.

It has been suggested that the vital biological role of the transcript-cleavage reaction consists in the relief of the elongation arrest state, a situation that is presumed to be lethal because it would transcriptionally inactivate the gene. The demonstration that RNAP can cleave in the absence of the factors reconciles the presumed vital significance of the cleavage reaction with the fact that the GreA-GreB double mutant is viable.

We are grateful to A. Mustaev for constructive suggestions and other members of the Goldfarb laboratory for helpful discussions. This work was supported, in part, by National Institutes of Health (NIH) Grant GM49242 to A.G., NIH Postdoctoral Fellowship GM 1542102 to J.N., and NIH Grant GM 2894612 to A.D.