Mapping Interactions of Escherichia coli GreB with RNA Polymerase and Ternary Elongation Complexes*

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Escherichia coli GreA and GreB modulate transcription elongation by interacting with the ternary elongation complex (containing RNA polymerase, DNA template, and RNA transcript) to induce hydrolytic cleavage of the transcript and release of the 3′-terminal fragment. Hydroxyl radical protein footprinting and alanine-scanning mutagenesis were used to investigate the interactions of GreB with RNA polymerase alone and in a ternary elongation complex. A major determinant for binding GreB to both RNA polymerase and the ternary elongation complex was identified. In addition, the hydroxyl radical footprinting indicated major conformational changes of GreB, in terms of reorientations of the N- and C-terminal domains with respect to each other, particularly upon interactions with the ternary elongation complex.

In vitro studies have shown that a number of prokaryotic and eukaryotic RNA polymerases can cleave and release fragments of 1–18 nucleotides from the 3′-end of the RNA transcript (1–6) within a ternary elongation complex (TEC). 1 The 5′-terminal fragment of the transcript, bearing a free 3′-OH, remains bound in the complex and can then be extended by RNA polymerase (RNAP) upon the addition of nucleotides.

One group of transcription elongation factors, which includes prokaryotic GreA and GreB and eukaryotic SII (TFIIS), acts by stimulating the endonucleolytic cleavage reaction within the elongation complex (7). Although the in vivo role for this group of elongation factors remains unknown, studies suggest their cleavage stimulating activity may (i) maintain transcription fidelity by facilitating the removal of misincorporated nucleotides (8, 9), (ii) enhance the productive initiation of RNA chains (10, 11), and (iii) serve to rescue arrested complexes (12, 13).

In an arrested state, the RNAP cannot continue RNA synthesis but also does not dissociate from the DNA template and RNA transcript (14). According to one model, arrest is associated with backward translocation of the RNAP, which disengages the 3′-end of the transcript from the catalytic center of the enzyme. Factor-stimulated cleavage of an internal phosphodiester bond (wherever the RNAP catalytic center is positioned) results in the generation of a new 3′-OH in register with the catalytic center, which allows for renewed RNA synthesis (15). Evidence for this model comes from experiments indicating the RNAP catalytic site also performs the cleavage reaction (3) and that backtracking occurs for both eukaryotic and prokaryotic RNAPs (16–19).

The 2.2-Å resolution crystal structure of GreA (20) comprises an N-terminal domain (NTD) consisting of an antiparallel α-helical coiled-coil, which extends into solution, and a globular C-terminal domain (CTD). Based on the high sequence similarity, the functional similarities, and the fact that GreA and GreB have identical CD spectra, homology modeling was used to derive a structural model of GreB (21). The structures led to a model of how the Gre factors interact with RNAP (20, 21). One face of each factor is strongly acidic, whereas the opposite face, in the case of GreA, is neutral with a small basic patch and, in the case of GreB, is highly basic. It was posited that upon interaction with TECs, the acidic face of a Gre factor would be oriented away from the acidic surface of RNAP (the pI of Escherichia coli core RNAP is 5.34). Consequently, the opposite face would be positioned to interact with the TEC (20, 21). The structural differences between the Gre factors could be related to their functional differences. GreA generally induces the cleavage of short fragments (2–3 nucleotides) from the 3′ terminus of the RNA, whereas GreB can induce cleavage of longer (2–18 nucleotides) fragments (13). Functional studies of Gre factor domains have led to the conclusion that the CTD is primarily involved in binding RNAP, whereas the NTD participates directly to induce the transcript cleavage reaction (22, 23), possibly by interacting with the 3′-end of the transcript (20, 21).

Earlier, we reported the low resolution, three-dimensional structure of E. coli core RNAP complexed with GreB (23), which localized the binding site for GreB on the RNAP structure. The present work investigates sites on GreB that are involved in interactions with RNAP and a TEC. GreB was chosen for this work because it binds to RNAP with a considerably higher affinity than GreA (24). Hydroxyl radical protein footprinting (25) was used to identify areas within GreB involved in interactions with core RNAP and a TEC, either through direct binding or indirectly through conformational changes. Alanine-scanning mutagenesis (26) was used to further delineate a putative RNAP-binding region detected from both TEC and core RNAP footprints on GreB. Upon binding core and TECs, conformational changes within GreB were observed and suggest how the domains communicate with each other for proper orientation during interactions with a ligand.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of GreB Derivatives—A modified pET-16b (Novagen) plasmid containing coding sequences for a heart muscle kinase (HMK) site directly upstream of the hexahistidine (H16) tag was used (27). The GreB open reading frame was inserted between the NdeI and BamHI restriction sites of this plasmid, yielding plasmid PK-pET16Gb. E. coli BL21(DE3) cells transformed with PK-
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pET16Gb expressed GreB with an N-terminal HMk site (LRRASV) and His tag (HMk-His6-GreB) in soluble form.

HMk-His6-GreB was purified as described for overexpressed wild type GreB (28) with modifications. Specifically, cells were grown in 2 liters of LB medium with ampicillin (100 μg/ml) to an A600 of 0.35. Expression of the recombinant protein was induced by adding 0.12 μmol of 3-β-thiogalactoside to 1 mM, and cells were grown for an additional 3 h at 37 °C. Cells harvested by centrifugation were resuspended in lysis buffer (40 mM Tris-HCl, pH 8.0, 1 mM NaCl, 5% glycerol, 10 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride) and lysed using a French press. After removal of cellular debris by centrifugation (10,000 × g, 20 min), Poly-P was added to a final concentration of 0.3% v/v. The precipitate formed was pelleted by centrifugation (10,000 × g, 25 min) and discarded. Powdered (NH4)2SO4 was then added to the supernatant to a final concentration of 0.8 M, and the formed precipitate was pelleted by centrifugation (10,000 × g, 25 min) and discarded. The supernatant was then dialyzed against binding buffer (40 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 0.8 M (NH4)2SO4, and 5 mM imidazole) to remove EDTA and DTT prior to the elution step. The recombinant HMk-His6-GreB was pooled and dialyzed into storage buffer (40 mM glycerol, 1 M NaCl, 10 mM EDTA, and 1 mM DTT). Fractions containing pure HMk-His6-GreB were pooled and dialyzed into storage buffer (40 mM Tris-HCl, pH 8.0, 1 mM NaCl, 10 mM EDTA, 40% glycerol, and 1 mM DTT) and kept at -20 °C.

Alanine substitution mutants of HMk-His6-GreB were generated using the QuikChange site-directed mutagenesis system (Stratagene). All mutant proteins were purified as described above except the Poly-P and (NH4)2SO4 precipitation steps were omitted, and E. coli lysates were applied directly onto the Poros MC20 column in lysis buffer B (40 mM Tris-HCl, pH 8.0, and 1 mM NaCl). The column was washed with lysis buffer B containing 45 mM imidazole prior to the elution step. Instead of being loaded onto the butyl-Toyopearl column, the MC20 column eluates were concentrated using a Millipore centrifugal filter and loaded onto a 16/60 Superdex 75 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 40 mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM NaCl, 10 mM EDTA, and 1 mM DTT. Fractions containing pure mutant protein were pooled, dialyzed into storage buffer, and stored at -20 °C.

To prepare Elongation Complex Formation—Picoquant quantities of pure TEC23 (T7A1 promoter-containing DNA template, E. coli RNAp, and 23-mer transcript) were generated in a scaled up and modified version of that described previously (29). The DNA template was a polynucleoside chain-reaction derived 375-base pair fragment where one of the oligonucleotides used in the polynucleoside chain reaction was biotinylated (for immobilization during TEC formation). A HindIII restriction enzyme was added to the 23-mer transcript. The complex was incubated with 25 pmol of HMk-His6-GreB at 25 °C for 5 min prior to the addition of cleavage reagents. The data were analyzed as described previously (30, 32). Briefly, the nucleotide sequence difference profile of GreB complex (I complex + Iresolv) versus residue number, were calculated (where I complex is the corrected intensity for the complex, and Iresolv is the corrected intensity for GreB alone). Ten difference plots were generated from four independent experiments for the Core/GreB interaction and 12 difference plots were generated from two independent experiments for the TEC/GreB interaction. Core/GreB complex, GreB and

Hydroxyl Radical Protein Footprinting—Purified HMk-His6-GreB was "P" (or "P")-end-labeled in a 400-μl reaction containing 40 μg (5 μM) of HMk-His6-GreB, 100 units of recombinant HMk-Sigma (Sigma), 0.05 μM [γ-32P]ATP (2000 Ci/mmol; NEN Life Science Products), 20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, and 12 mM MgCl2. The kinase was reconstituted in 40 mM DTT, and 0.02 μM [γ-32P]ATP (6000 Ci/mmol; NEN Life Science Products) was substituted for [γ-32P]ATP when "P"-end-labeled protein was desired. Following an incubation time of 15–20 min at 37 °C, 20 μl of N6-NTA agarose beads, prepared as described (30), were added to the reaction (10 μl of buffer A, 100 μl of buffer C, and 10 mM MgCl2) were added. The sample was gently mixed and incubated on ice for 15 min. The beads were then pelleted and washed (performed as described above for TEC formation) twice with 1 ml of buffer A to remove free label. The beads were resuspended in 200 μl of buffer A plus 250 mM imidazole (1 mM imidazole stock solution pH 7.5 with concentrated HCl), incubated on ice for 10 min, then pelleted by centrifugation and discarded. The 200-μl supernatant, now containing the eluted, labeled protein, was diluted with 300 μl of buffer A and concentrated in a Microcon 10 microconcentrator (Amicon) to approximately 100 μl. The sample was then diluted again with 400 μl of buffer A and re-concentrated in the same microconcentrator. This dilution and concentration steps lowered the imidazole level to ~20 mM. The total yield of labeled protein was 6 mg (285 pmol) in a 100-μl volume. Prior to each footprinting experiment, labeled GreB was subjected to SDS-polyacrylamide gel electrophoresis and phosphorimagining to confirm that no other contaminating proteins were labeled (data not shown). In addition, control experiments showed no incorporation of label into native GreB under the phosphorylation conditions described (data not shown).

Footprinting reactions were performed at 25 °C for 20 min in a mixture containing labeled HMk-His6-GreB free or with a ligand, 10 mM Mops/NaOH (pH 7.2), 200 mM NaCl, 10 mM MgCl2, 1 mM (NH4)2SO4, 1 mM ATP, 2 mM EDTA, 200 μM ascorbate, 1 mM H2O2. Reactions were initiated by simultaneously adding Fe-EDTA (freshly prepared complex of 10 mM (NH4)2Fe(SO4)2 and 20 mM EDTA), ascorbate, and H2O2. For core RNAP footprints on HMk-His6-GreB, 25 (or 50) pmol of core RNAP was added to 23-mer transcript containing 25 pmol of HMk-His6-GreB at 25 °C for 5 min prior to the addition of cleavage reagents. The final concentrations of core and HMk-His6-GreB in these footprinting reactions were 1.2 (or 2.4) and 1.25 μM, respectively. For TEC23 footprints on HMk-His6-GreB, 2–4 pmol of TEC23 were incubated with 1 pmol of HMk-His6-GreB at 25 °C for 5 min prior to the addition of cleavage reagents, and MgCl2 was omitted from the buffer to preclude cleavage of the transcript. The final concentrations of TEC23 and HMk-His6-GreB in these footprinting reactions were 0.08 and 0.03 μM, respectively. Reactions were terminated as described (30), products loaded onto Tricine/SDS 16.5% T, 3% C polyacrylamide gels (31), and analyzed by PhosphorImager analysis (Molecular Dynamics model Storm).

The data were analyzed as described previously (30, 32). Briefly, PhosphorImager intensities were integrated across full lane widths and plotted versus electrophoretic mobilities using IMAGEQUANT (Molecular Dynamics). To correct for lane-to-lane distortions, intensity plots were aligned using the program ALIGN (30). Subsequent data analysis was performed using Microsoft EXCEL. Intensity plots were normalized to correct for gel loading and cleavage efficiencies, and electrophoretic mobilities were converted into the position of the cleavage site in the sequence by subtracting the band migration distance from the sequence (I complex + Iresolv) versus residue number, were calculated (where I complex is the corrected intensity for the complex, and Iresolv is the corrected intensity for GreB alone). Ten difference plots were generated from four independent experiments for the Core/GreB interaction and three difference plots were generated from two independent experiments for the TEC/GreB interaction. Core/GreB complex, GreB and...
Purified TECs containing radiolabeled RNA 6-mers (ApUpCpApUpC), generated from the A promoter, were incubated with either the GreB derivative or wild type GreB (WT-Greb). A 23% polyacrylamide (20:3 acrylamide:bisacrylamide by weight) gel was used to separate reaction products, which were then visualized by autoradiography. Lane 1 contains RNA 6-mer incubated under the same conditions but without added GreB. Lanes 2–4 and 5–7 show products of cleavage by increasing amounts of HMK-His6-Greb and WT-Greb, respectively. RNA 4-mer cleavage product (ApUpCpA) is marked on right side of autoradiogram.

**RESULTS**

**Activity and Hydroxy Radical Cleavage of a GreB Derivative**—For end-labeling GreB, a derivative containing an N-terminal heart muscle kinase (HMK) recognition site, hexahistidine tag (His6), and 9-amino acid linker was generated. This GreB derivative (HMK-His6-Greb), containing 22 non-native amino acids in total, behaved as wild type GreB by several criteria. First, HMK-His6-Greb was soluble when over-expressed in E. coli. Second, it was equally active in in vitro transcript cleavage assays (Fig. 1). Third, it bound core RNAAP in a direct assay for binding to RNAAP in tubular crystals (23).

Finally, it bound core RNAAP in a native gel-shift assay (see Fig. 4) with an affinity essentially the same as that observed for WT GreB. The dissociation constant ($K_d$) for HMK-His6-Greb was approximately $0.87 \times 10^{-7}$ M, compared with a $K_d$ of $1-2 \times 10^{-7}$ M in the case of WT GreB (24).

FIG. 1. Cleavage assay showing activity of GreB derivative. Purified TECs containing radiolabeled RNA 6-mers (ApUpCpApUpC), generated from the A promoter, were incubated with either the GreB derivative or wild type GreB (WT-Greb). A 23% polyacrylamide (20:3 acrylamide:bisacrylamide by weight) gel was used to separate reaction products, which were then visualized by autoradiography. Lane 1 contains RNA 6-mer incubated under the same conditions but without added GreB. Lanes 2–4 and 5–7 show products of cleavage by increasing amounts of HMK-His6-Greb and WT-Greb, respectively. RNA 4-mer cleavage product (ApUpCpA) is marked on right side of autoradiogram.

**Molecular weight standards** were generated by residue-specific cleavage (30) of HMK-His6-Greb in reactions containing CNBr (methionine-specific), endopeptidase Lys-C (lysine-specific), endopeptidase Glu-C (glutamic acid-specific), or endopeptidase AspN (aspartic acid-specific). Knowledge of the residue specificity for each endopeptidase, as well as the primary amino acid sequence for GreB, allowed us to determine how many bands (i.e., molecular weight standards) and their mobilities relative to each other would be generated under limited cleavage conditions.

Native Polyacrylamide Gel Electrophoresis Binding Assay—Reaction mixtures (6 ml) containing 10 mM Mops/NaOH, pH 7.2, 230 mM NaCl, 10 mM MgCl2, 7% glycerol, the indicated amounts of core RNAAP, and $^{32}$P-labeled GreB derivatives (166 nM for experiments shown in Fig. 4, A and B, 125 nM for experiments shown in Fig. 4, C and D) were incubated for 5 min at 25 °C. Following the incubation, 1 µl of each reaction was loaded onto a 4–15% gradient PhastGel (Amersham Pharmacia Biotech) and run for 160 V·h at 15 °C using native buffer strips. Gels were visualized by phosphorimaging (Molecular Dynamics Storm), and intensities of core-Greb derivative complexes were quantitated using Imagequant (Molecular Dynamics). The amount of bound derivative was determined by multiplying the intensity value for the complex by the amount of derivative in 1 µl of a reaction (see below). The total intensity value was determined by spotting 1 µl of a reaction into the gel after an electrophoresis run and quantitating the spot intensity.

**Hydroxy Radical Footprinting of Core RNAAP on HMK-His6-Greb**—To determine the sites of GreB that interact with core RNAAP, cleavage profiles of radical-cleaved HMK-His6-Greb alone and in the presence of saturating amounts of core RNAAP were quantitatively compared (Fig. 3A). Cleavage products near the full-length protein (ranging from positions 140 to 158) were not visible when $^{32}$P-labeled GreB was used due to masking by the intensity of the uncleaved peptide. Consequently, experiments using $^{32}$P-labeled GreB were conducted to allow these products to be resolved more clearly on gels (Fig. 3B). These and other images were subjected to quantitative analysis (see “Experimental Procedures”), and the results are presented in the form of a difference plot (Fig. 3C).

Regions of statistically significant protection (negative values) and enhancement (positive values) of radical cleavage by the binding of core RNAAP were observed (Fig. 3C). In general, protection of a site on GreB from radical cleavage in the presence of core RNAAP can result from a direct association of RNAAP with that site or from a conformational change in GreB that altered the local environment, making cleavage less likely. Enhancement of GreB cleavage in the presence of core RNAAP...
most likely results from a conformational change in GreB that makes cleavage more likely. Statistically significant protection of GreB from radical cleavage by binding core RNAP occurred in two regions of the NTD, centered at residues 27 (just after the proline kink in the middle of α1) and 69 (at the end of α2), and two in the CTD, centered at residues 122 (near the beginning of α3) and 143 (in the loop between β6 and β7). An area of enhanced radical cleavage was observed in the loop L2 connecting the NTD with the CTD (centered at residue 78). Two areas of enhanced radical cleavage were observed in the CTD, cen-
tered at residues 95 (most of β3 and the loop between β3 and β4) and 110 (in the loop L3).

The complicated pattern of enhancements and protections from radical cleavage is difficult to interpret, even when mapped onto the modeled GreB structure (Fig. 6B). However, functional studies of the isolated Gre factor domains revealed that the main determinants for binding to RNAP lie within the CTD. For instance, the CTD alone, but not the NTD, can competitively inhibit the activity of full-length GreB when added in trans (22). Furthermore, CTD, but not NTD, was found to associate with core RNAP in a direct binding assay (23). Therefore, in order to locate the main determinants within GreB for binding to RNAP, we focused on the GreB-CTD. Direct contact of regions of GreB with RNAP would be expected to result in protection from radical cleavage. There were two regions of protection from radical cleavage in the CTD (Fig. 6B). Protection from radical cleavage is not diagnostic of direct protein-protein contacts since protection could also result from conformational changes. Therefore, we used alanine-scanning mutagenesis (26) to assess directly the role of side chains within the GreB-CTD/RNAP interaction areas identified by hydroxyl radical protein footprinting.

Alanine-scanning Mutagenesis within the CTD RNAP-binding Area—Surface-exposed amino acids within the GreB-CTD/RNAP interaction areas (from residues 116–127 and 134–148) were identified using the program GRASP (33) and then were individually substituted with alanine. The measured CD spectra for each purified mutant GreB was identical to that obtained for GreB (data not shown); for GreB spectra and measurement conditions, see Ref. 21, indicating that each mutant was properly folded. Each mutant was also active in the transcript cleavage assay (data not shown).

To assess binding to core for each mutant, a native gel-shift assay was used. 32P-Labeled HMK-His6-GreB was shown in a gel-shift experiment to form complexes with core RNAP (Fig. 4A). Incubating increasing amounts of core RNAP with a constant amount of 32P-labeled HMK-His6-GreB allowed for a K_d measurement of 87 nM (Fig. 4A and B), essentially the same as that obtained by other methods (24). Competition experiments, which tested the specificity of binding, indicated that core-32P-labeled HMK-His6-GreB complexes did not form in the presence of a 20-fold molar excess of unlabeled GreB but did form in the presence of a 20-fold molar excess of an unlabeled and unrelated 30-kDa protein, FKBP12–30 (Fig. 4C). In addition, HMK-His6-GreB did not form a complex with the 204-kDa γ-protein complex (γ2βδσεϕ) of E. coli DNA polymerase III in this assay (data not shown).

Alanine substitution for residues 121 (Asp) and 123 (Pro) resulted in an approximately 2-fold decrease in core binding (Fig. 4D). These residues are both highly solvent-exposed and form a small patch on the face of the GreB-CTD (Fig. 6C). These results indicate that the protection from radical cleavage within this area of GreB most likely results from a direct interaction with core RNAP.

Hydroxyl Radical Footprint of TECs on HMK-His6-GreB—Next, we investigated the interactions between GreB and a TEC, which is the true substrate for the activity of GreB. To achieve a hydroxyl radical footprint of a TEC on GreB, the TEC must be stable over the time course and conditions of the footprinting reaction but also sensitive to GreB-induced transcript cleavage. A TEC that met these requirements contained the 23-mer RNA (TEC23) initiated from the bacteriophage T7.
A1 promoter. In the absence of Mg\(^{2+}\), GreB binds the TEC but transcript cleavage cannot proceed (20). TEC23 was found to be resistant to induced cleavage by a 50-fold molar excess of HMK-His\(_6\)-GreB in a reaction lacking Mg\(^{2+}\) (Fig. 5A, lane 5). In the presence of Mg\(^{2+}\), TEC23 was sensitive to induced cleavage by a 5-fold molar equivalent of HMK-His\(_6\)-GreB (Fig. 5A, lane 1). Importantly, in the absence of Mg\(^{2+}\) but in the presence of hydroxyl radical cleavage reagents, transcript cleavage also did not occur over the time of the experiment (Fig. 5A, lanes 7–9). This indicates that Fe\(^{2+}\) did not adventitiously substitute for Mg\(^{2+}\) and induce transcript cleavage. It also indicates that the backbone of the RNA transcript was completely protected by the RNAP from radical cleavage, which is consistent with recent RNase protection studies showing that a 23–24-nucleotide transcript was protected from low concentrations of RNase T1 within the elongating complex (34).

TECs were generated by a “walking” procedure that involved immobilizing the TEC to a support matrix (35, 36). It was found that HMK-His\(_{23}\)-GreB nonspecifically bound the support matrix used in the production of TEC23 (data not shown), so it was therefore necessary to release TEC23 from the matrix prior to footprinting (see “Experimental Procedures”). The elution step resulted in a large loss of TEC (85%), and consequently, the amounts used in footprinting reactions were less than the amount of core RNAP used in core footprinting reactions (2–4 pmol of TEC23 as opposed to 25–50 pmol of core). To compensate for less ligand, the amount of HMK-His\(_{23}\)-GreB was lowered (1 pmol as opposed to 25 pmol) yet allowed for clearly detecting certain enhancements and protections (Fig. 5B). However, as can be seen from comparing gels in Figs. 3A and 5B, the lower amounts of labeled HMK-His\(_{23}\)-GreB led to a lower signal-to-noise ratio for the TEC23 footprinting reactions that also precluded the use of \(^{32}\)P-labeled protein. Quantitative analysis was therefore crucial in detecting all interactions, but cleavage products near the uncut full-length protein (beyond residue 140) could not be defined as in core footprinting (Fig. 3B).

In the absence of Mg\(^{2+}\), HMK-His\(_{23}\)-GreB was mixed with saturating amounts of TEC23 and hydroxyl radical footprinting performed to probe the interaction between GreB and the ternary complex (Fig. 5C). Within the CTD, protection from

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**Fig. 4. GreB binds RNAP core in a gel-shift assay.** A, end-labeled GreB (166 nt) was incubated with the indicated amounts of core, and GreB-core complexes were visualized by phosphorimaging. Due to the pI of GreB derivatives being very near the pH of the gel (pH 8.8), much of the free fraction did not migrate into the gel but remained in the well area (also see C). B, binding curve of labeled GreB/Core interaction. Data for curve come from three independent experiments similar to that shown in A. Nonlinear regression analysis of the binding data revealed a \(K_d\) of 87 nt with a standard error of 17.45 (Prism, GraphPad, San Diego). C, specificity of binding. Labeled GreB (125 nt) incubated with the indicated amounts of core and competitors; a, specific competitor (unlabeled HMK-His\(_{23}\)-GreB); b, nonspecific competitor (FKBP12–30). Competitors at 20-fold molar excess to labeled GreB. D, bar graph showing core binding affinity for alanine substitution mutants and labeled WT GreB. A 125 nt concentration of core was used in these binding studies.

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\(^2\) E. Nudler, personal communication.
radical cleavage was still observed in the RNAP interaction region defined above (centered at residue 122), but the strong enhanced cleavage in the loop connecting β3 and β4 was not. Striking changes in the protection pattern within loop L2 and the NTD were observed with GreB bound to TEC23 compared with core RNAP. In the loop L2, linking the NTD and CTD, the significant enhancement of cleavage seen upon binding to core RNAP was not observed upon binding to TEC23. The region of protection by core RNAP centered at residue 69, at the C-terminal end of α2, was absent with TEC23, but a new region of protection emerged, centered at residue 54, toward the N-terminal end of α2. In two separate regions of the NTD, very

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**Fig. 5.** Hydroxyl radical footprint of TEC23 on GreB. A, transcript cleavage assay of purified TEC23 (0.01 pmol/lane) incubated with HMK-His₆-GreB in footprinting reaction buffer with indicated modifications. A 12% polyacrylamide (19:1 acrylamide to bisacrylamide), 7 M urea gel was used to resolve the reaction products, which were then visualized by autoradiography. Bar graph shows normalized cleavage of 23-mer RNA with numbers on x axis corresponding to lanes in autoradiogram. B, phosphorimaged gel of hydroxyl radical cleaved GreB and GreB in complex with TEC23. Enhanced and protected areas are marked with arrows and brackets, respectively. C, difference plot showing normalized intensity difference (I_complex − I_config) plotted against residue number (I_complex and I_config are the corrected intensities for the GreB-TEC23 complex and GreB, respectively). Statistically significant differences according to a Student’s t test (confidence level of 0.9) are denoted by black bars.
strong enhancements of radical cleavage with TEC23 were observed. These were clearly visible on the autoradiogram even without quantitative analysis (Fig. 5B). One site of enhanced cleavage comprised the N terminus of GreB, from residues 1–13. The second area extended from residues 24 to 41, essentially helix α1b.

DISCUSSION

Although the detailed molecular mechanism by which the Gre factors induce transcript cleavage is unknown, previous biochemical and structural results have led to working hypotheses regarding the interactions between the Gre factors, the RNAP, and the RNA transcript. Functional studies of the isolated Gre factor domains revealed that the main determinants for binding RNAP lie within the CTD. For instance, the CTD alone, but not the NTD, competitively inhibits the activity of full-length GreB when added in trans (22). Furthermore, CTD, but not NTD, associates with core RNAP in a direct binding assay (23). On the other hand, the NTD appears to be involved directly in inducing the transcript cleavage reaction, a function likely aided by being directed to the RNAP via CTD binding. The amino acid sequence of the NTD determines the type of transcript cleavage (GreA or GreB-like), irrespective of the CTD sequence (21). Moreover, NTD alone, when added in sufficient amounts, can induce transcript cleavage (22). Cross-linking studies suggest that the NTD may function by interacting directly with the RNA transcript at a region near the distal end of the coiled-coil finger (20, 21). Finally, the unusual charge distribution around GreA and GreB suggests how they may interact with the elongation complex (20, 21). One face of the Gre factors is very acidic, and the opposite face is slightly basic (GreA) to strongly basic (GreB). Since the RNAP itself is very acidic, it was proposed that GreA or GreB interacts in such a way that the acidic face is oriented away from the RNAP (20, 21). This would presumably position the basic regions appropriately to contact the negatively charged transcript. It is interesting to interpret the results of this hydroxyl radical protein footprinting study in the context of these models for Gre factor function.

Upon interacting with both core RNAP or TEC, the same region of the GreB-CTD, near the beginning of α3 (centered at residue 122), becomes significantly protected from radical cleavage. Furthermore, alanine substitutions of two side chains...
in the heart of the protected region (Asp-121 and Pro-123) result in mild but significant defects in RNAP binding. These results, taken together, indicate that this region comprises a major determinant for binding GreB to RNAP (Fig. 6C). Interestingly, Pro-123 is absolutely conserved in all known Gre factor sequences. Asp-121 is conserved in GreB sequences but not GreA, which may suggest one reason for the lower affinity of GreA for RNAP (24). This binding determinant is located opposite the acidic face of the Gre factors, in a neutral region of GreA and a basic region of GreB, supporting the earlier proposals that GreA and GreB interact with the RNAP with their acidic faces oriented away from the acidic RNAP (20, 21).

Finally, the changes in radical cleavage of the GreB-CTD on binding to either RNAP or TEC are qualitatively very similar (compare Fig. 6, B and C), consistent with CTD playing the role of an anchor, holding the NTD in the vicinity of the RNAP during the course of the transcript cleavage reaction.

In contrast to the CTD, striking differences in the radical cleavage patterns of the L2 loop (linking the NTD to the CTD) and the NTD, when binding RNAP versus TEC, were observed. In the L2 loop, the significant enhancement of radical cleavage seen with core RNAP was missing with TEC. This is suggestive of possible conformational changes in the form of a reorientation of NTD with respect to CTD upon binding core RNAP and again upon interacting with TEC. Reorientations of the NTD with respect to the CTD would be expected to alter the conformation of the L2 loop linking them.

In the NTD, major changes of the cleavage pattern were observed between the complex with core RNAP and TEC, consistent with a direct role of the NTD in the transcript cleavage reaction. In the complex with core RNAP, a region at the C-terminal end of α2 (centered at residue 69) was protected from radical cleavage. This protection could be due to either direct interactions with RNAP or to a conformational change. The protected region is on the same face of GreB as the RNAP binding determinant in the CTD, but if the protection around residue 69 does arise from contacts with RNAP, they cannot be essential contacts since CTD alone specifically binds RNAP (22, 23). In the complex with TEC, the protection around residue 69 was no longer present (further arguing against this region playing an important role in binding RNAP), but a new region of protection was observed closer to the N-terminal end of α2 (centered around residue 54). Interestingly, specific cross-links between both GreA and GreB and cross-linkable nucleotide analogs incorporated into the 3’-end of the RNA transcript were mapped in this region. The RNA cross-links were mapped to peptide fragments between residues 41 and 57 of GreA (20) and residues 47 and 63 of GreB (21). It is interesting to speculate that the protection from radical cleavage in this region upon GreB binding to TEC is due to an interaction with the RNA 3’-end. It is not possible, however, to firmly make this conclusion from the current footprinting data.

Finally, two regions of the NTD exhibit very strong enhancements of radical cleavage when bound to TEC. One region (residues 24–41), comprising essentially helix α1b, includes a region that was protected from radical cleavage when bound to core RNAP. Thus, this region went from being protected from radical cleavage in the presence of core RNAP to very strongly enhanced cleavage with TEC. Even stronger enhancement of radical cleavage with TEC was observed near the GreB N terminus (from about residues 1–13). In free GreB, only background levels of cleavage were observed in this region, and the susceptibility to cleavage was not altered upon binding to core RNAP. It is striking to note that in the model of the GreB structure (which was based on the crystal structure of GreA), within this region, residues 4–7 are completely buried within

The protected region is on the same face of GreB as the RNAP binding site, but it should be noted that in the GreA crystal structure (which was based on the crystal structure of GreA), the exposure of the previously buried N-terminal arm.

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