Bacteriophage N4 mini-virion RNA polymerase (mini-vRNAP), the 1106-amino acid transcriptionally active domain of vRNAP, recognizes single-stranded DNA template-containing promoters composed of conserved sequences and a 3-base loop–5-base pair stem hairpin structure. The major promoter recognition determinants are a purine located at the center of the hairpin loop (−11G) and a base at the hairpin stem (−8G). Mini-vRNAP is an evolutionarily highly diverged member of the T7 family of RNAPs. A two-plasmid system was developed to measure the in vivo activity of mutant mini-vRNAP enzymes. Five mini-vRNAP derivatives, each containing a pair of cysteine residues separated by ~100 amino acids and single cysteine-containing enzymes, were generated. These reagents were used to determine the smallest catalytically active polypeptide and to map promoter, substrate, and RNA–DNA hybrid contact sites to single amino acid residues in the enzyme by using end-labeled 5-iododeoxyuridine- and azidophenacyl-substituted oligonucleotides, cross-linkable derivatives of the initiating nucleotide, and RNA products with 5-iodouridine incorporated at specific positions. Localization of functionally important amino acid residues in the recently determined crystal structures of apo-mini-vRNAP and the mini-vRNAP-promoter complex and comparison with the crystal structures of the T7 RNAP initiation and elongation complexes allowed us to predict major rearrangements in mini-vRNAP in the transition from transcription initiation to elongation similar to those observed in T7 RNAP, a task otherwise precluded by the lack of sequence homology between N4 mini-vRNAP and T7 RNAP.

Bacteriophage N4 virion RNA polymerase (vRNAP) is a cysteine-less 3500-amino acid (aa) long polypeptide composed of three domains (1). The 1106-aa central domain, mini-vRNAP, possesses the same initiation, elongation, termination, and product displacement properties as full-length vRNAP (1). Mini-vRNAP shares limited sequence similarity with the T7-like RNAP family (1). T7 RNAP recognizes its cognate promoters on double-stranded templates in the absence of accessory factors. Mini-vRNAP differs in two fundamental properties from T7 RNAP: 1) N4 vRNAP requires a transcription factor, the Escherichia coli single-stranded DNA-binding protein (EcoSSB), for promoter recognition (2, 3) and RNA product displacement (4); and 2) it transcribes promoter-containing single-stranded templates with in vivo specificity (5), recognizing a 3-nucleotide loop–5-bp stem hairpin structure present four nucleotides upstream of the transcription initiation site (6, 7). Recent analysis of the mini-vRNAP template requirements identified two essential sequence-specific contacts at the promoter: the central purine of the hairpin loop (−11G) presented in the context of a loop and a major groove interaction in the stem (−8G) (7). Due to the lack of sequence similarity to T7 RNAP and to provide further insight into the mechanism of vRNAP interactions with its ligands during initiation and elongation, we have determined the sites of mini-vRNAP contacts with the initiating nucleotide, the template site of transcription initiation, and the template and transcript in elongation complexes by cross-linking to mini-vRNAP mutant enzymes and subsequent chemical cleavage at cysteines introduced at regular intervals along the polypeptide. The results identify functionally important mini-vRNAP residues; analyses of these residues based on the structure of mini-vRNAP and the structure of the T7 RNAP elongation complex predict significant structural differences between mini-vRNAP initiation and elongation complexes.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains and Plasmids—E. coli strain DH5α (supE44 ΔlacU169 (d80 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used to determine the in vivo activity of wild-type and mutant mini-vRNAP enzymes. E. coli strain BL21 (F−ompT hsdSB rB−mB−) was used for the purification of recombinant proteins. Bacteria were grown in Lennox broth base medium containing the required antibiotics.

Reporter Plasmids to Determine in Vivo Activity of Mini-vRNAP—A fragment flanked by ApaLI- and BamHI restriction sites that includes vRNAP promoter P2 (−19 to +2) followed by the lacZ′ gene from M13mp18 was inserted into ApaLI and BamHI-digested pACYC177 (New England Biolabs), disrupting the β-lactamase gene. The resulting plasmid, pIK1, was used to determine in vivo activity of N4 mini-vRNAP variants. Additional plasmids with early N4 promoters P1 (pKMK63), P2 (pKMK63), P3 (pKMK63), and P4 (pKMK63) were used to determine the in vivo activity of mutant mini-vRNAP enzymes.
(pKMk64), or P3 (pKMk65); four mutant P2 promoters (−10G to T, pKMk66; −11G to T, pKMk67; −12A to T, pKMk68; and +1C to T, pKMk69); and a control lacking the promoter (pKMk62) were constructed.

In Vivo Activity of Mini-vRNAP Variants and Mutant Promoters—E. coli strain DH5α cells carrying pK1 and a plasmid encoding a mini-vRNAP variant (see below) were grown in Lennox Broth base medium containing 100 μg/ml ampicillin and 50 μl/ml kanamycin (Sigma) until they reached a density of ~10⁶ cells/ml. Mini-vRNAP synthesis was induced by the addition of 0.2% arabinose for 20 min. β-Galactosidase activity was visualized by plating 10 μl of cells on solid medium containing appropriate antibiotics, inducers, and X-gal overnight at 37 °C, followed by incubation at 4 °C to develop the color. To quantitate levels of LacZ′ expression, cells (1 ml) were harvested and resuspended in minimal A medium supplemented with 20 μg/ml thiamine HCl, 1 mM MgSO₄, and 0.4% glycerol. Cells were disrupted with toluene, 200 μg/ml of 4 mg/ml o-nitrophenyl galactoside was added, and the appearance of o-nitrophenol was followed by measuring absorbance at 420 nm. To determine the amount of induced mini-vRNAP, cells were pelleted from a 0.5-ml aliquot of the induced culture, resuspended in loading buffer, and subjected to SDS-8% polyacrylamide gel electrophoresis and silver staining. In vivo transcription of lacZ′ from different promoters or by vRNAP variants was quantitated by dot-blot hybridization. RNA was isolated from a 1-ml culture using a Qiagen RNeasy kit and diluted such that the concentrations of total RNA were equal in the preparations. Samples were resuspended in 1 ml cold EDTA and applied to a nitrocellulose filter (Schleicher & Schuell; 0.45-μm membrane) supported in a Bio-Dot manifold (Bio-Rad). LacZ mRNA was detected by hybridization to a 25-nucleotide 32P-end-labeled oligonucleotide (5′-ATCAGTCTGCACGTTGTTAAAAC-3′) complementary to a sequence 77 nucleotides downstream from the start of the LacZ′ mRNA and quantitated by phosphorimaging using ImageQuant software.

Site-directed Mutagenesis of Mini-vRNAP and Purification of Enzymes—Mutant mini-vRNAP enzymes containing the following changes, R424H (pMKM62), D559A (pMKM64), K670R (pKMk65), Y678A (pKMk66), S280C (TAT to TGC), A843C (GCT to TGT), L954C (CTT to TGT), D900C (GAT to TGT), A918C (GCA to TGC), and W975C (TGG to TGC) were constructed in pEKD27 using the QuikChange site-directed mutagenesis procedure (Stratagene). The sequences of all polymerase expression constructs were confirmed. All enzymes were synthesized to wild-type levels after induction, and all dicysteine-substituted polymerases exhibited wild-type activity in vivo and in vitro. Mini-vRNAP enzymes were purified as described previously (8).

UV Cross-linking of Mini-vRNAP to SIdU-substituted Oligonucleotides—SIdU-substituted P2–3 DNA oligonucleotides (7) were synthesized by Integrated DNA Technologies, and UV cross-linking to RNA or DNA in stalled elongation complexes was performed as described previously (4). Cross-linked products were analyzed by SDS-PAGE and phosphorimaging.

Mapping of Cross-links in Mini-vRNAP—Cys-containing mutant mini-vRNAP (1–10 pmol), radiolabeled as a result of cross-linking, was incubated for 15 min at 37 °C in 10 μl of 8 M urea, 0.2 M Tris-HCl, pH 8.3, and 5 mM dithiothreitol. After addition of 2-nitro-5-thiocyanato benzolic acid (NTCB) to 1% final concentration, the mixture was incubated for 15 min at 37 °C. The pH was adjusted to 9.3–9.5, and the reaction mixture was incubated at 37 °C for 60 min to cleave the specific peptide bond at the modified cysteine residues (9). The reaction was terminated by addition of dithiothreitol-containing SDS loading buffer, and the products were analyzed by SDS-PAGE and phosphorimaging. Ion-metal affinity chromatography (IMAC) was used to differentiate C-terminal from N-terminal His6-tagged products (8).

UV Cross-linking of Mini-vRNAP to Azidophenacyl (AzPh)-modified Oligonucleotides—Phosphorothioate-substituted P2-3 DNA oligonucleotides were synthesized by Integrated DNA Technologies. Attachment of the AzPh group to phosphorothioate DNA was performed as described (10), with modifications. 50 μl of 0.1 mM DNA was mixed with 5 μl of 1 N potassium phosphate, pH 7.0, and 75 μl of 20 mM p-azidophenacyl bromide (in methanol) in an amber 1.5-ml microtube, incubated at room temperature for 3 h in the dark, and precipitated with ethanol. The AzPh-DNA was pelleted by centrifugation, washed two times with 70% ethanol, and resuspended in 20 μl of water. The recovery of DNA was estimated spectrophotometrically. 5′-End labeling of AzPh-modified oligonucleotides and UV cross-linking were performed as described previously (4, 7), except that irradiation was for 5 min for AzPh-derivatized oligonucleotides. The cross-linked products were analyzed by SDS-PAGE and phosphorimaging.

Catalytic Auto-Labeling and Run-off Transcription Assays—The catalytic auto-labeling assay has been described previously (1). Run-off transcription reactions were performed for 5 min at 37 °C in the presence of the specified template and mini-vRNAP, and 10 μM EcoSSB when present and analyzed as described (4). A 92-bp heat-denatured fragment, generated by ApaLI and EcoRI restriction of plasmids pKMK62 to pKM69, was used as a template to test the activity of promoters P1, P2, and P3 and P2 promoter mutants (see Fig. 1). Transcription of this fragment yields a 66-nucleotide RNA product. The template 12P2-52 has been described (4).
The 1106-aa mini-vRNAP polypeptide was defined by trypsinolysis of the 3500-aa vRNAP polypeptide, followed by catalytic autolabeling (1). To further determine the extent of the vRNAP catalytic domain, we constructed mutant enzymes with N- and C-terminal truncations. Removal of as many as 26 N-terminal residues had no effect on mini-vRNAP activity in vivo (Fig. 2A) or in vitro (data not shown). However, deletion of eight residues from the mini-vRNAP C terminus significantly reduced and deletion of 14 and 18 residues abolished activity in vivo (Fig. 2A). The effect of the C-terminal truncations was not as deleterious in vitro. The largest deletion (CΔ18) resulted in a 2-fold loss of activity in formation of the first phosphodiester bond as measured by catalytic autolabeling (Fig. 2B) and almost complete inactivation in run-off transcription assays on a single-stranded promoter-containing template (Fig. 2C). Shorter C-terminal truncations had no effect on catalytic autolabeling and resulted in only partial loss

**FIGURE 1.** *In vivo* system to detect activity of mini-vRNAP and vRNAP promoters. *A*, sequences of the bacteriophage N4 early promoters. Arrows, inverted repeats; +1, site of transcription initiation; −11, center of hairpin loop. *B*, *in vivo* X-gal-based detection of wild-type mini-vRNAP activity on different promoters determined by β-galactosidase α-peptide complementation. *C*, *in vivo* transcriptional activity of mini-vRNAP on different promoters determined by LacZ mRNA hybridization. The percentage of reporter transcripts relative to that from the P3 promoter is shown. *D*, *in vitro* run-off transcription of 100 nM mini-vRNAP on 10 nM templates containing wild-type (P1, P2, and P3) and P2 mutant promoters in the presence of 10 μM EcoSSB. The arrow indicates run-off product. The percentage of product relative to that from the P3 promoter is indicated.

**RESULTS**

Development of a vRNAP Promoter-Reporter System to Detect *in Vivo* Activity of Mini-vRNAP Mutant Enzyme—A two-plasmid system composed of the β-galactosidase α-peptide cloned under the control of N4 promoter P2 into pACYC177 and the N4 mini-vRNAP gene cloned under the PBAD control into a second compatible plasmid (1) was developed. Both plasmids were introduced into *E. coli* DH5α (lacZΔM15), yielding expression of the α-peptide and generation of enzymatically active β-galactosidase upon induction of mini-vRNAP synthesis with arabinose (Fig. 1B, P2). No α-peptide was synthesized in the absence of mini-vRNAP expression (data not shown), from a plasmid lacking a vRNAP promoter (Fig. 1B), or from a plasmid in which promoter P2 directed transcription away from the reporter gene (data not shown). As expected, the levels of β-galactosidase expression were dependent on the activity of the promoters tested (Fig. 1, compare B and D; P1, P2, and P3 sequences in A). Promoter mutations that decreased activity *in vitro* (Fig. 1D, P2 −10T, −11T, −12T, and +1T) had similar effects in the *in vivo* reporter system (Fig. 1B). To ascertain that the levels of enzymatically active β-galactosidase reflected LacZ mRNA levels, total RNA was isolated, and LacZ mRNA was detected by hybridization to a radiolabeled probe. A close correlation was observed between the levels of LacZ mRNA and β-galactosidase activity (Fig. 1, B and C).

Characterization of Mini-vRNAP Mutant Enzymes Bearing Deletions at the N or C Terminus—The 1106-aa mini-vRNAP polypeptide was defined by trypsinolysis of the 3500-aa vRNAP polypeptide, followed by catalytic autolabeling (1). To further determine the extent of the vRNAP catalytic domain, we constructed mutant enzymes with N- and C-terminal truncations. Removal of as many as 26 N-terminal residues had no effect on mini-vRNAP activity in vivo (Fig. 2A) or in vitro (data not shown). However, deletion of eight residues from the mini-vRNAP C terminus significantly reduced and deletion of 14 and 18 residues abolished activity *in vivo* (Fig. 2A). The effect of the C-terminal truncations was not as deleterious *in vitro*. The largest deletion (CΔ18) resulted in a 2-fold loss of activity in formation of the first phosphodiester bond as measured by catalytic autolabeling (Fig. 2B) and almost complete inactivation in run-off transcription assays on a single-stranded promoter-containing template (Fig. 2C). Shorter C-terminal truncations had no effect on catalytic autolabeling and resulted in only partial loss
of activity in run-off transcription assays. In vitro run-off transcription reactions were performed in the absence and presence of EcoSSB, which is required for transcript displacement and template reutilization (Fig. 2D) (4). All C-terminally truncated enzymes were activated by EcoSSB, indicating that the C-terminal 18 residues are not required for interaction with EcoSSB.

Mapping of NTP and Template Sites of Interaction at the Active Center of Mini-vRNAP—Three motifs, A, B, and C, characteristic of the T7 family of RNAPs are present in mini-vRNAP. Motifs A and C contain the catalytically essential aspartates, whereas motif B residues are involved in NTP binding (1). A fourth motif, TxGR, is characteristic of the structurally related DNAPI family, whereas RNAPs have the DxxGR motif that plays a role in stabilizing the RNA-DNA hybrid in T7 RNAP (11). Mutant mini-vRNAP enzymes possessing single amino acid substitutions within the four polymerase motifs important for activity in vitro (boldface in Fig. 3A) were stable in vivo and produced at wild-type levels (Fig. 3B, top panel). Enzymes containing R424I, D559A, K670R, or D951A substitutions displayed reductions in in vivo activity similar to those observed in vitro using purified proteins (Fig. 3B, bottom panel) (1). The Y678F polymerase was an exception because it displayed greatly reduced activity in vivo, whereas it is fully active in transcription assays containing only rNTPs in vitro (1). The Y678F substitution resulted in decreased discrimination against dNTP incorporation in vitro. We surmise that dNTP incorporation reduces the synthesis or the stability of full-length reporter gene transcripts in vivo, as indicated by the low level of the transcript in vivo (Fig. 3B, in vivo transcript levels under each lane).

UV cross-linking of mini-vRNAP to 20-mer P2–3 DNA oligonucleotides is highly efficient when 5IdU is present at the center of the hairpin tri-loop (−11), at the transcription start site (+1), or at the 5′-end of the oligonucleotide (+3) (7). Cross-linking of mutant mini-polymerases to −11 5IdU-substituted DNA was not affected by substitutions in the conserved motifs (Fig. 3C); therefore, these mutations do not perturb binding to the promoter. RNAP R424I cross-linking to +1 and +3 5IdU-substituted oligonucleotides was substantially decreased, indicating defects in interaction with the template at the active site (Fig. 3C). We have previously shown that GTP, the initiating nucleotide, inhibits mini-vRNAP cross-linking to +1 5IdU-substituted template (7). In contrast, +1 5IdU cross-linking to R424I, D559A, K670A, and D951A RNAPs was not as affected by the presence of GTP (Fig. 3D), indicating defects in GTP binding due to changes in the GTP-binding (Lys-670), catalytic (Asp-559, Asp-951), or template-binding (Arg-424) sites at the active center.

To map the targets of NTP and template interaction on the mini-vRNAP polypeptide, we took advantage of the lack of cysteines in vRNAP. We constructed five mutant (C1–C5) N-terminally His6-tagged mini-vRNAPs, each containing a pair of Cys residues ~100 residues apart introduced at different positions in the polypeptide (Fig. 4A, top panel). All enzymes were synthesized to wild-type levels after induction and were active in vivo (data not shown). Cys-specific NTCB cleavage of the C1–C5 enzymes (Fig. 4A, bottom panel) was followed by IMAC to assign cleavage products. As an example, the results for the C3 enzyme are shown in Fig. 4B, where cleavage products larger than 55 kDa are shown. Cleavage at Cys-510 is usually incomplete, yielding a noncleaved 2N product in addition to the 1N and 2C products (Fig. 4B, bottom panel). NTCB-cleaved fragments containing the N-terminal His6 tag are retained on the column and eluted with imidazole (1N and 2N) (Fig. 4, E) whereas the C-terminal fragment (2C) (Fig. 4B, FT) flows through. Because NTCB cleavage is not equally efficient at all sites and some of the fragments have anomalous migration, this procedure was performed with each of the dicysteine-containing enzymes (data not shown) to confirm the assignment of the fragments in Fig. 4A.

The mini-vRNAP site of interaction with the initiating nucleotide was identified by analysis of the NTCB cleavage patterns of C1–C5 polymerases radiolabeled by autocatalysis using the
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FIGURE 4. Mapping of derivatized initiating nucleotides to mini-vRNAP residues Lys-670 and Lys-437. A: top panel, schematic representation of position of Cys substitutions in C1–C5 mutant polymerases; bottom panel, NTCB cleavage pattern of C1–C5 (1–5) polymerases. The nomenclature of the NTCB cleavage products for each enzyme is presented for enzyme C3 in B. B: assignment of polymerase C3 cleavage products by IMAC. Top panel, IMAC analysis. L, load; FT, flow-through; E, imidazole eluate; M, molecular mass markers. Silver-stained gels are shown. Bottom, correspondence of NTCB cleavage products to the N- and C-terminal fragments of His-tagged (box) C2 and C4 enzymes. C: catalytic auto-labelling using bATP and bAMP confirmed that bATP cross-links to both Lys-670 and Lys-437 (Fig. 4 B, compare WT, K670R, and K437R). These results place Lys-437 as well as Lys-670 close to the initiating nucleotide in the mini-vRNAP initiating complex.

The NTCB cleavage patterns of C1–C5 mutant polymerases cross-linked to a +1 5IdU-substituted, radiolabeled, promoter-containing oligonucleotide mapped the site of interaction with the transcription start site (+1) to a region of the enzyme (aa 604–709) containing motif B Tyr-678 (Fig. 5 A, compare WT, K670R, and K437R), whereas bAMP exclusively cross-links to Lys-437 (Fig. 5 A, compare WT, K670R, and K437R). These results place Lys-437 as well as Lys-670 close to the initiating nucleotide in the mini-vRNAP initiating complex.

4-hydroxybenzaldehyde esters of GTP (bGTP), ATP (bATP), and AMP (bAMP), and appropriate templates (Fig. 4 C) (1). bGTP and bATP cross-linked to two mini-vRNAP regions: aa 409–510 (labeling of 1N in enzyme C3 and of 2C in enzyme C2) and aa 604–709 (labeling of 2C in enzyme C3), which contain the TaxGR and B motifs, respectively; in contrast, bAMP cross-linked exclusively to the aa 409–510 region, and no cross-linking to the 2C fragment generated by NTCB cleavage of enzyme C3 was detected. Previously, we demonstrated that motif B Lys-670 plays a role in NTP binding (1). Because the benzaldehyde nucleotide derivatives cross-link specifically to primary amines, the second target should be a lysine present between residues 409 and 510 and residing in close proximity to the initiating nucleotide bound in the active center. Two lysines, Lys-430 and Lys-437, are located close to the TxxGR motif (12). The K430A substitution did not affect mini-vRNAP activity, whereas both K437A and K437R substitutions resulted in very low polymerase activity in vivo and in vitro (data not shown). Catalytic auto-labeling using bATP and bAMP confirmed that bATP cross-links to both Lys-670 and Lys-437 (Fig. 5 D, compare WT, K670R, and K437R), whereas bAMP exclusively cross-links to Lys-437 (Fig. 5 D, compare WT, K670R, and K437R). These results place Lys-437 as well as Lys-670 close to the initiating nucleotide in the mini-vRNAP initiating complex.

The NTCB cleavage patterns of C1–C5 mutant polymerases cross-linked to a +1 5IdU-substituted, radiolabeled, promoter-containing oligonucleotide mapped the site of interaction with the transcription start site (+1) to a region of the enzyme (aa 604–709) containing motif B Tyr-678 (Fig. 5 A, compare WT, K670R, and K437R), which is responsible for the discrimination of incoming dNTPs (1). 5IdU cross-links preferentially to aromatic residues. To confirm that Tyr-678 is the target of the +1 5IdU cross-link, we compared the cross-linking efficiencies of wild-type and Y678A polymerases. Mutation at Tyr-678 did not affect promoter binding because the wild-type as well as the Y678F and Y678A enzymes cross-linked to −11 5IdU with the same efficiency (Figs. 3 C and

FIGURE 5. Mapping of the mini-vRNAP site of interaction with the +1 position of promoter DNA to Tyr-678. A, NTCB cleavage of C1–C5 polymerases cross-linked to +1 5IdU 32P-5′-end-labeled DNA. +1 5IdU cross-links to a residue located between 604 and 709. Phosphorimage of a 10% SDS-polyacrylamide gel is shown. Asterisks indicate the bands of interest. B, UV cross-linking of wild-type (WT) and Y678A polymerases to −11 and +1 5IdU 32P-5′-end-labeled DNAs. I, silver-stained gel; II, phosphorimage. Relative cross-linking of the mutant polymerase is indicated.
linking to a series of 5'-end-labeled oligonucleotides, each singly substituted with 5IdU at positions -1, +1, +2, +3 + 4, +5, +6, +7, +11, and +15 (Fig. 6A). Cross-linking to position +6 was highly efficient (Fig. 6A). The site of cross-linking was mapped to the aa 918–1020 interval using the C1–C5 mutant polymerases (Fig. 6B, left panel). Cross-linking to single Cys-containing RNAP mutants W975C and F996C (Fig. 6B, right panel) indicated that the template +6 position interacts with a residue present between amino acids 996 and 1020, which contains a single aromatic residue, Tyr-1014.

Identifying Promoter Phosphate Backbone Contacts on the Mini-vRNAP Polypeptide—To identify mini-vRNAP contacts with the promoter phosphate backbone, we used a set of AzPh-modified P2–3 oligonucleotides bearing the UV-activated cross-linkable group singly attached to each phosphate position (Fig. 7, top panel). The modification is characterized by low cross-linking selectivity and a 10-Å distance from the active group to the target site. Analysis of efficiency of cross-linking to promoters derivatized with AzPh at different positions indicated possible mini-vRNAP contacts with the −12/−13, −11/−12, −6/−7, −5/−6 phosphates as well as those around the transcription start site. No contacts close to position −8 were detected. Hairpin contacts at −11/−12 and −5/−6 were mapped further because these cross-links were strongest despite reduced mini-vRNAP binding to the modified oligonucleotides (Fig. 7, Kd values). NTCB cleavage of C1–C5 polymerases mapped cross-links to AzPh at −11/−12 and −5/−6 between residues 812 and 918 and between residues 200 and 306, respectively (Fig. 7, B and C). Using single Cys-substituted polymerases, these contacts were localized further to the aa 843–854 and aa 260–280 regions, respectively (Fig. 7, B and C). However, the low selectivity and 10-Å radius of AzPh cross-linking resulted in harder to interpret NTCB cleavage patterns. Mutational analysis within the mapped regions revealed that alanine substitutions of Trp-848, Lys-849, and Lys-850 in the aa 843–854 region and of Lys-268, Ile-272, and Tyr-273 in the aa 260–280 region significantly affected mini-vRNAP promoter-binding affinity and partially reduced the efficiency of −11/−12 AzPh and −5/−6 AzPh cross-linking, respectively (12).

Interaction of Mini-vRNAP with the DNA Template and Nascent RNA during Elongation—Interactions between mini-vRNAP and the template and product during elongation were analyzed in stalled elongation complexes (SECs) formed in the absence of CTP on P2–23 templates (Fig. 8A, top). First, 5IdU was introduced at every second or third position of the transcribed region of the P2–23 template, or 5IdU was
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A. DNA templates used in SECs for UV cross-linking with DNA (1–4) and RNA (5–8). Sequences between position +9 and +12 in the DNA templates are presented. The sequence shared by all templates is shown above. Position numbers indicate the distance from the transcription start site (+1). The arrow indicates the position of the active site in the SEC. B. mini-vRNAP UV cross-linking to 5IdU at positions +9 to +12 of 2P-S'-end-labeled P2–23 DNA templates (1–4, top) and to 5IU, incorporated into the corresponding positions of 2P-S'-end-labeled nascent RNA (5–8, bottom). The distances from the active center (AC) in the SECs are indicated. C. Mapping of mini-vRNAP contacts to positions −10 of template DNA and −10 of nascent RNA in SECs formed using DNA templates 2 and 6 (A), and the S280C and S286C and the D900C and V917C polymerase variants, respectively. Phosphorimages of UV-cross-linked polymerase NTCB cleavage products are shown. Asterisks indicate bands of interest.

incorporated in the respective RNA product positions. Results of these experiments indicated that efficient cross-linking occurred at template positions +9 to +12 (data not shown). When 5IdU or 5IU was subsequently introduced in stalled transcription elongation complexes.

Discussion

Bacteriophage N4 vRNAP, present in four copies per virion (13), is injected into the E. coli cell with the phage linear double-stranded DNA genome at the start of the N4 infection cycle. vRNAP-catalyzed transcription of the phage early genes requires the activities of host DNA gyrase and single-stranded DNA-binding protein, EcoSSB. In vitro, vRNAP is inactive on linear double-stranded templates, but using EcoSSB for template recycling, it transcribes denatured genomic N4 DNA or promoter-containing single-stranded DNA with in vivo specificity and high efficiency (5). Development of the two-plasmid vRNAP promoter-reporter system described here allowed us to assay the in vivo activity of wild-type and mutant N4 early promoters and vRNAP variants. We found that the very C-terminal region of mini-vRNAP, defined originally by trypsinolysis (1), is essential for N4 mini-vRNAP activity in vitro: deletion of 14 residues from the C terminus (mini-vRNAP CΔ14) resulted in a 10-fold decrease in transcription in vivo. However, purified mini-vRNAP CΔ14 was as active as the wild-type enzyme in run-off transcription assays. Transcription by all C-terminally truncated enzymes was activated by EcoSSB, indicating that this region is not the target for EcoSSB interaction. T7 RNAP has a C-terminal Phe-Ala-Phe-Ala sequence (“foot” motif); deletions of the last two residues lead to a decrease in processivity as well as a 30-fold weaker binding to the promoter (14), whereas substitutions or deletions of any of the side chains cause a decrease in transcription initiation (15). The foot is sensitive to proteolysis in the apoenzyme, whereas it is protected in the initiation complex (15) due to interactions with a hydrophobic pocket that cause a structural rearrangement of the active site conformation (16). Mini-vRNAP does not have any obvious sequence similar to this foot motif. The recently published crystal structure of mini-vRNAP revealed that its very C-terminal 14 residues form part of a 44-residue protruding α-helix (α-44, extended foot), with practically no contacts with the rest of the protein (12). Helix 44 has several positively charged residues on its surface that might be required for interaction with the non-template strand, which is absent in the run-off transcription assays. The actual function and interaction partners of the C-terminal helix remain to be determined.

Here, we used chemical and photocross-linking of derivatized nucleotides and oligonucleotides to map mini-vRNAP contacts with initiating nucleotides, template DNA, and product RNA (Fig. 9). T7 RNAP Lys-631, residing in motif B (RxxKx-xYG) (17), was identified as the target of polymerase autolabeling using the cross-linkable o-formylphenyl ester of GMP as an initiating substrate (18). Previously, we showed that Lys-670, which resides in the corresponding position of mini-vRNAP motif B, is involved in nucleotide binding (1). Using catalytic autolabeling, we confirmed that Lys-670 interacts directly with initiating nucleotide triphosphate hydroxybenzaldehyde ester derivatives. In addition, we identified another lysine, Lys-437, which is essential for mini-vRNAP transcription activity and cross-links to initiating nucleotide mono- and triphosphates. Lysine 437 is located within 18 Å of Asp-559 and Asp-951, the catalytically essential aspartates in motifs A and C, and of Lys-670 in the polymerase-promoter complex (20). It is worth noting that T7 RNAP Lys-441 resides in an almost identical position near the active site of T7 RNAP. Based on the crystal structure of the T7 RNAP initiation complex, it has been suggested that T7 RNAP Lys-441 is involved in the selection of
Although the RNA-DNA hybrid is only 8 bp long in the T7 RNAP elongation complex structure, there are several aromatic residues (Tyr-178, Phe-751, and Phe-755) positioned where the −10 bases of DNA and RNA would be exposed. These aromatic residues are located 14 Å away from the last RNA-DNA hybrid base pair. When a 10-bp-long extended hybrid is modeled into the T7 RNAP elongation complex structure, Tyr-178 and Phe-751/Phe-755 lie within 7 Å of −10 DNA and −10 RNA bases in the major groove of the hybrid, respectively (data not shown). Remarkably, T7 RNAP Tyr-178 and mini-vRNAP Phe-281, as well as T7 RNAP Phe-751/Phe-755 and mini-vRNAP Tyr-910, reside in loop structures on the surface of their respective proteins in the same location and orientation with respect to promoter-binding elements and active centers when the apo-structures of both polymerases are compared (12, 23). T7 RNAP residues Phe-751 and Phe-755 are present in the specificity loop, whereas mini-vRNAP Tyr-910 resides in the hairpin stem of the specificity loop (the mini-vRNAP specificity loop is devoid of aromatic residues). Whereas Tyr-178 and Tyr-751/Phe-755 are closely located in the T7 RNAP elongation complex, they are 60 Å apart in the T7 RNAP initiation complex. However, there is no biochemical evidence indicating that Tyr-178 and Phe-751/Phe-755 interact with the −10 DNA and −10 RNA bases in the T7 RNAP elongation complex. On the basis of the contacts with RNA and DNA in the mini-vRNAP stalled elongation complex, we suggest that mini-vRNAP must undergo a significant structural rearrangement on its way from transcription initiation to elongation as observed for T7 RNAP. Experiments to stabilize the mini-vRNAP elongation complex for further crystallization are in progress.

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