Sequences within the DNA Cross-linking Patch of $\sigma^{54}$ Involved in Promoter Recognition, $\sigma$ Isomerization, and Open Complex Formation

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The bacterial RNA polymerase holoenzyme containing the $\sigma^{54}$ subunit functions in enhancer-dependent transcription. Mutagenesis has been used to probe the function of a sequence in the $\sigma^{54}$ DNA binding domain that includes residues that cross-link to promoter DNA. Several activities of the $\sigma$ and holoenzyme are shown to depend on the cross-linking patch. The patch contributes to promoter binding by $\sigma^{54}$ and holoenzyme and is involved in activator-dependent $\sigma$ isomerization. As part of the $\sigma^{54}$-holoenzyme, some residues in the patch limit basal transcription. Other cross-linking patch sequences appear to limit activator-dependent open complex formation. Deletion of 19 residues adjacent to the cross-linking patch resulted in a holoenzyme unable to respond to activator but capable of activator-independent (bypass) transcription in vitro. Overall results are consistent with the cross-linking patch directing interactions to the −12 promoter region to set basal and activated levels of transcription.

$\sigma$ factors play a pivotal role in bacterial transcription. Through their association with core RNA polymerase they function in promoter-specific initiation. DNA contact by $\sigma$ is required for promoter recognition and can contribute to the DNA opening event in open promoter complex formation. Two classes of $\sigma$ factor exist, apparently unrelated by sequence (1, 2). Members of the class related to the $\sigma^{30}$ of Escherichia coli form holoenzymes that are active for transcription in the absence of accessory proteins (3, 4). In contrast, the $\sigma^{54}$-type factor forms a holoenzyme silent for transcription until acted upon by an enhancer binding activator protein. In a reaction requiring nucleoside triphosphate hydrolysis by the activator protein, the $\sigma^{54}$-holoenzyme bound in a closed complex isomerizes, and start site proximal DNA is melted out to form a stable open promoter complex (5–9). Thus, one core RNA polymerase can participate in two distinct mechanisms of transcription initiation.

Key functional activities of $\sigma^{54}$ are arranged in discrete sequences across three regions identified by sequence alignment (Fig. 1). The amino-terminal Region I includes sequences that are essential for enhancer responsiveness, whereas Region II sequences assist in promoter recognition (9–12). Region III contains determinants for core polymerase binding at its amino end and a domain containing motifs for DNA binding toward the carboxyl-terminal end (13, 14). The $\sigma^{54}$-holoenzyme recognizes promoters with conserved sequences at −24 and −12 relative to the transcription start site, and $\sigma$ contacts these sequences (15). Determining the amino acids in $\sigma$ that direct promoter binding is important for understanding how the initial specific closed complex is formed. Additionally, whether $\sigma$-DNA contacts contribute to post binding steps such as DNA melting can be evaluated. Biochemical and genetic experiments with Klebsiella pneumoniae and E. coli $\sigma^{54}$ have shown that the major DNA-contacting surfaces of $\sigma^{54}$ are located in a carboxyl-terminal domain of approximately 150 amino acids (14–17). Within the holoenzyme-promoter closed complex, DNA sequences that must be melted to form open complex are contacted by $\sigma^{54}$ (14, 15, 17, 18). The DNA binding domain is likely to be functionally involved in open complex formation.

Cross-linking methods are useful for identifying $\sigma$-DNA interacting domains. Native promoter DNA can be specifically cross-linked to $\sigma^{54}$ and its holoenzyme by a “zero length” UV light-catalyzed reaction (19). The DNA cross-linking patch of $\sigma^{54}$, composed of residues 329–346 (17), sits at the amino terminus of the DNA binding domain and is linked by a predicted surface-exposed element to an adjacent domain that enhances DNA binding activity (20, 21). We have shown that the invariant residue Arg-336, within the cross-linking patch, is important for maintaining the holoenzyme as a closed complex, silent for transcription before activation (22). Deletion of Region I ($\Delta$I) or mutation of Arg-336, each, alter the interaction of holoenzyme with −12 promoter sequences and allow the holoenzyme to isomerize independent of activator (9, 22, 23). Region I binds core polymerase, and deletion of Region I alters the conformation of the carboxyl-terminal DNA binding domain of $\sigma^{54}$ as part of the holoenzyme, suggesting a linked interaction between amino and carboxyl parts of $\sigma^{54}$ (13, 24).

Identification of $\sigma^{54}$ sequences involved in interaction with promoter DNA provides a basis for understanding the mechanism of transcription initiation by enhancer binding activators. To further explore the involvement of the DNA cross-linking patch in $\sigma^{54}$ function, conserved residues within the patch were mutated. Single amino acid substitutions were obtained that led to defects in $\sigma$ promoter binding, $\sigma$ isomerization, and activator-dependent transcription. We also deleted the adjacent surface-exposed element, amino acids 310–328, which links the cross-linking patch with a domain that enhances DNA binding (20, 21). Deletion of the linking element resulted in...
FIG. 1. K. pneumoniae σ^{24} is divided into three regions (I-III) based on sequence alignments (2). The amino-terminal Region I is required for response to activator and masks single-stranded DNA binding activity in the σ^{24}-holoenzyme (9, 39, 40). Region II is acidic and variable among members of the σ^{24} protein family. Region III includes the sequence binding determinants: the sequence that cross-links to promoter DNA (X.L.), a helix-turn-helix motif (HTH), and the conserved eight-residue sequence termed the RpoN box and sequences that modulate DNA binding (MOD) (17, 21, 41, 42). The amino acid sequence of the cross-linking patch is shown. Conserved residues that have been mutated are underlined.

helenzyme unable to form open complex but able to initiate transcription in the absence of activator from supercoiled DNA.

MATERIALS AND METHODS

DNA Manipulations—The K. pneumoniae rpoN gene codons 328 (Trp), 338 (Asp), and 342 (Arg) were changed to CGA (Ala); codon 340 (Leu), in line with other substitutions of Leu in σ^{24} (10), was changed to AGC (Ser); and codons 310–328 were deleted using the Sculptor (Trp), 338 (Asp), and 342 (Arg) were changed to CGA (Ala); codon 340 (Leu), 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.9; 100 m M glycine buffer, pH 8.6, for 2 h. [32P]DNA was detected by a phosphorimaging device. Reactions were replicated to enhance reliability of the data.

Transcription Assays—S. meliloti nifH promoter DNA as plasmid pMKC28 was used as the template in transcription experiments (22). Assays (at 30 °C) contained holoenzyme assembled from 100 nM E. coli core RNA polymerase and a 6-fold excess of σ^{24}. Pre-incubation of promoter DNA (20 nM) and holoenzyme was for 10 min in STA buffer with ATP (4 mM) and activator protein PopFΔHTH (4 μM) unless otherwise stated in the figure legend. A mixture of the remaining 3 NTPs (GTP and CTP 0.2 mM, UTP 0.05 mM) and 1 μCi of [α-32P]UTP (plus 100 μg/ml heparin) was then added for a further 10 min. Reactions were stopped by mixing in formamide-sequencing loading buffer and heated to 90 °C, and half the sample was directly run on 6% denaturing sequencing gels. Reactions were replicated to enhance reliability of the data. Transcripts were detected by phosphorimaging device. Transcription from nifH on pMKC28 yields a 469-base product.

Gel-shift Assays—These were conducted using linear end-labeled S. meliloti nifH promoter fragments as described previously (9, 30). Reactions were replicated to enhance reliability of the data. Either an 88-mer homoduplex or heteroduplex molecule from 60 to 28, the latter with the −10 to −1 or −12 to −11 non-template strand sequence mismatched, was used. One strand was [32P]DNA, and the other unlabeled strand was annealed at a 2-fold excess. Binding reactions with unlabeled competitor DNA were in STA buffer at 30 °C, and bound and unbound DNA was resolved on 4–4.5% native polyacrylamide Bio-Rad mini-protein II gels run at room temperature at 60 V in 25 mM Tris, 200 mM glycine buffer. Phosphorimaging gels were detected by a phosphorimaging device.

RESULTS

Mutations in σ^{24}—Enzyme and chemical cleavage of σ^{24} suggested that the patch of σ^{24} that can be UV-cross-linked to promoter DNA resided between or included residues Trp-328 to Thr-340 (14, 17). To further study the importance of this patch for σ^{24} function, single amino acid substitutions of conserved residues W328A, R336A, D340A, L340F, R342A, and deletion of an adjacent surface-exposed region Δ310–328 were created by site-directed mutagenesis. Preparation of R336A has been

1 The abbreviations used are: o-Cup, ortho-copper phosphonohydrazine; ΔI, σ^{24} lacking Region I.
described previously (22). In each case the integrity of the mutated rpoN was established by DNA sequencing. Immuno-blotting using polyclonal anti-σ54 antibody confirmed that all mutants were expressed as intact protein (data not shown).

**In Vivo Function and Transcription Activities of the σ54 Mutants—**Initially the ability of each mutant σ54 to complement an rpoN K. pneumoniae mutant for growth on arginine as a nitrogen source was tested (Table I). The Δ310–328 mutant did not allow growth, whereas L340S allowed approximately 25% growth compared with wild type. The mutants W328A, D338A, and R342A were largely unimpaired for growth. Using the rpoN K. pneumoniae strain, each σ54 mutant was tested for transcription activity using σ54-dependent reporter promoters fused to lacZ. Five test promoters were used to evaluate any promoter-specific effects: the wild type K. pneumoniae nifH and mutants nifH049, nifH050, nifH053, and nifH054, all of which respond to activator NifA. The nifH049, -050, -053, and -054 promoters are mutants of the K. pneumoniae nifH promoter (see Table II) with changed affinities for σ54 (18). Bacteria were grown under anaerobic nitrogen-limiting conditions to induce synthesis and activity of chromosomal nifA. Overall, the in vivo transcription results mirror the initial growth test. Δ310–328 gave no detectable transcription activity, whereas L340S produced significantly lower transcription activity compared with wild type σ54-holoenzyme at all promoters (Table II, data in parentheses). At the K. pneumoniae nifH promoter, all mutants gave reduced levels of activity compared with wild type (Table II, column 2, data in parentheses). At the K. pneumoniae nifH049 promoter, all point mutant transcription activity increased with D338A and R342A, producing close to wild type level (Table II, column 3, data in parentheses). At the nifH049 promoter, the activity of L340S, although low, was 4-fold that seen at the K. pneumoniae nifH promoter. As discussed previously (22), the “T-tract” in the nifH049 promoter may be compensating for a lost or reduced contact made by σ54 as part of the holoenzyme (18). Greater affinity for the promoter could increase occupancy and potentially favor initiation by holoenzymes, which form open complexes slowly.

To further explore the stimulatory effect of the T-tract we used a K. pneumoniae nifH mutant promoter series in which each C from −17 to −15 has been deleted by T (27) (Table II). These data show that for wild type and all mutant σ54-holoenzymes, a C to T change at −17 or −15 produces at least a 2-fold increase in transcription activity and is at least as stimulatory for transcription as the −17 to −15 CCG to TTT change (Table II). Except for L340S, which showed a 3-fold increase in activity, the C to T change at −16 was not stimulatory for transcription. The L340S σ54-holoenzyme, which has the lowest activity compared with wild type σ54-holoenzyme (Table II, data in parentheses), was highly stimulated by the C to T change at −15, producing a 10-fold increase above its activity at the wild type nifH promoter. In vivo experiments using a consensus promoter sequence where the consensus −12 core promoter sequence (TTGC) was randomly mutated suggested that the −15 consensus T was important for maintaining promoter function (31). The results suggest that the L340S mutation may cause a disruption in correct recognition and/or binding to the K. pneumoniae nifH-12 promoter region, which is to some degree rescued by the C to T change at −15.

Overall the in vivo analysis revealed that substitutions in the cross-linking patch and deletion of adjacent residues leads to three classes of mutant: class I, no transcription activity (Δ310–328); class II, reduced transcription activity (L340S); and class III, significant levels of activity stimulated further by a high affinity promoter. To facilitate further analysis of any defects in the mutant σ54 proteins, we performed SDS-polyacrylamide gel electrophoresis. Core Binding—An essential early step in transcription is the binding of σ to core to form the holoenzyme. To determine whether any mutation grossly altered the interaction of σ with core, a native gel holoenzyme assembly assay was conducted. With the exception of L340S, each mutant shifted all core at a molar ratio of 1:1 (σ54:core), indicating that core binding was intact and could not readily account for the defects in transcription seen in vivo (data not shown). In contrast to the other mutants and wild type σ54, L340S and its holoenzyme did not enter the native gel, indicating this protein has altered physical properties. All other mutant holoenzymes appeared to have a mobility indistinguishable from wild type holoenzyme. This suggests that, with the exception of L340S, the conformation of the holoenzyme is not grossly changed by the cross-link patch substitution or deletion of residues 310–328. The native gel also showed that free-running W328A σ54 (not associated with core) has increased mobility compared with wild type σ54, suggesting that the W328A substitution has altered the conformation of the σ54 protein (data not shown).

**Table I**

| Complementation for growth on arginine in an rpoN K. pneumoniae mutant | Growth on (NH4)2SO4 | Growth on arginine |
|---|---|---|
| WT | ++ + | ++ + |
| Δ310–328 | ++ + | + + |
| W328A | ++ + | + + |
| D338A | ++ + | + + |
| ΔI340S | ++ + | + + |
| ΔR342A | ++ + | + + |

**Table II**

| β-Galactosidase activity from the K. pneumoniae nifH mutant promoter series | nifH promoter | nifH049 | nifH050 | nifH053 | nifH054 |
|---|---|---|---|---|---|
| WT | 1 (100) | 2 (100) | 2 (100) | 1 (100) | 2 (100) |
| W328A | 1 (73) | 2 (65) | 2 (52) | 1 (71) | 2 (70) |
| ΔD338A | 1 (93) | 2 (105) | 2 (93) | 1 (105) | 2 (94) |
| ΔΔI340S | 1 (3) | 4 (6) | 6 (9) | 3 (8) | 10 (10) |
| ΔΔR342A | 1 (70) | 2 (90) | 2 (72) | 1 (77) | 2 (85) |
structural defects (as indicated by native gel mobility) rather than loss of an essential DNA contact.

The reduced DNA binding of W328A and R342A indicates that they are functionally important residues within the DNA cross-linking patch of $\sigma^{54}$ that contribute to DNA binding. We directly confirmed this by conducting a DNA cross-linking assay (Fig. 2B). The formation of two products in the cross-linking assay probably reflects stable cross-linking of $\sigma^{54}$ to either both strands or one strand of the DNA (17). Interestingly R342A, which showed reduced DNA binding in the gel-shift assay, gave wild type levels of cross-link product. All $\sigma^{54}$ proteins were present at a concentration judged to bind 50% of the DNA in the gel mobility shift assay (A). Lane 1, DNA alone. Lane 2, wild type $\sigma^{54}$. Lane 3, W328A

footprinting. In the DNase I and S1 assays, all mutant $\sigma^{54}$ gave footprints indistinguishable from wild type $\sigma^{54}$ (data not shown). In the o-Cup footprinting assay, D358A and R342A $\sigma^{54}$ gave similar footprints to wild type $\sigma^{54}$ (data not shown). o-Cup footprints of W328A and $\Delta 310-328$ did not show the typical cut at $-5$ on the top strand, suggesting that Trp-328 is involved in a start site proximal promoter interaction (Fig. 2C).

Taken together, the gel-shift, DNA cross-linking, and o-Cup footprinting assays establish that residues W328A and R342A both contribute to the DNA binding activity of $\sigma^{54}$ and that each mutant has a different qualitative and quantitative effect on the $\sigma^{54}$ DNA interaction.

$\sigma^{54}$ Interactions with Melted DNA—Artificially melted DNA at $-12/-11$ simulates the nucleation of promoter DNA melting seen within the natural holoenzyme closed complex (23). The $-12/-11$ position was identified as a nucleation site by o-Cup footprinting and by gel mobility assays with DNA fork junction probes (23, 32). Heteroduplex S. meliloti nifH DNA, where the top strand bases at $-12$ and $-11$ are mismatched, was used to investigate the interactions of the mutant $\sigma^{54}$ with the $-12$ promoter region early melted DNA. As with fork junction DNA, where the bottom strand thymine residue is unpaired compared with double-stranded DNA, the $-12/-11$ early melted DNA was preferred for wild type $\sigma^{54}$ binding (32). Like wild type $\sigma^{54}$, mutants W328A, D358A, and R342A all showed a preference for binding the early melted DNA (Fig. 3). In contrast, the $\Delta 310-328$ $\sigma^{54}$ showed no preference for binding the early melted DNA and bound it less well than native double-stranded DNA (Fig. 3).

A second assay to examine whether the $\sigma^{54}$ interaction with melted DNA changed was performed by measuring the ability of $\sigma$ to bind heteroduplex DNA that is mismatched from $-1$ to $-10$, DNA which is melted in the later stages of open complex formation (9, 22). Wild type and mutant $\sigma^{54}$ including $\Delta 310-328$ $\sigma^{54}$ showed a preference for binding the $-10/-1$ late-melted DNA (Fig. 3). It seems that $\sigma^{54}$ has a modest preference for binding to melted DNA structures that are normally only created when holoenzyme is bound to the promoter. This suggests that, normally as part of the holoenzyme, $\sigma^{54}$ is likely to be intimately involved in the initiation and maintenance of the DNA strand separation needed for open complex formation (9, 22, 32). Apparently the cross-linking patch mutants assayed here, unlike $\Delta 310-328$ $\sigma^{54}$, do not change the binding preference of $\sigma^{54}$ but do contribute to overall binding activity.

$\sigma^{54}$ Isomerization—When activator and hydrolyzable nucleoside triphosphate are added to $\sigma$ bound to early melted DNA,
a new $\sigma^{54}$-DNA complex results (the supershifted band) with an extended $\sigma$ DNase I footprint indicating that activator interacts with $\sigma$ and causes it to isomerize and increase its interaction with DNA. All cross-linking patch mutant $\sigma$s were tested for isomerization. For activation we used a deletion mutant form of the activator PspF that lacks its DNA binding domain, PspFAB (29), to simplify the gel-shift assay. In the isomerization assay, mutants D338A and R342A $\sigma^{54}$ behaved as wild type (data not shown). The $\Delta310-328$ $\sigma^{54}$ mutant did not respond to activator to produce a supershift (data not shown), whereas W328A produced only a small amount of supershift (Fig. 4). The lack of any supershifted band for $\Delta310-328$ is found also with $\sigma^{54}$ lacking Region I and correlates with an inability for transcription in vivo. The defective response of W328A to activator in the supershift assay was investigated further. Titration of the W328A protein did not increase the amount of supershifted complex (Fig. 4), nor was the amount of supershifting DNA increased by longer incubation times (data not shown). Replacing PspF with the activator NiFa, increasing the amount of PspF, or increasing the amount of DNA probe in the reaction did not result in an increased conversion of DNA-bound $\sigma^{54}$ to the isomerized form (data not shown). Exposing the W328A protein to PspF and nucleotide before adding the DNA did not detectably change the percentage of supershifted product (data not shown). Overall, these results show that the W328A protein is unable to be rescued for $\sigma^{54}$ isomerization. Whether its altered conformation or its changed DNA interaction per se, detected by $\sigma$-Cup footprint (Fig. 2C), reduces $\sigma$ isomerization is not clear. Nonetheless, Trp-328 contributes to $\sigma^{54}$ isomerization.

**Holoenzyme Interaction with Native DNA—**Gel-shift assays using the *S. meliloti nifH* promoter 88-mer were conducted to study the effect of the $\sigma^{54}$ mutants on holoenzyme DNA binding. The mutant W328A that showed decreased $\sigma^{54}$ DNA binding activity formed holoenzyme that bound DNA with approximately the same affinity as wild type holoenzyme, suggesting that core was still able to stabilize the $\sigma$ on DNA (Fig. 5). In contrast, the R342A holoenzyme showed a decrease in DNA binding compared with wild type. At 80 nM, the wild type holoenzyme bound 39% of the DNA, whereas the R342A holoenzyme bound 17% (Fig. 5). This result suggests that residue Arg-342 makes an important contribution to the DNA binding energy of the holoenzyme in the closed complex, consistent with the reduced R342A $\sigma^{54}$ DNA binding (Fig. 2A). The $\Delta310-328$ and L340S holoenzymes bound approximately 2-fold more of the DNA probe than wild type holoenzyme, approximately equivalent to the DNA binding activities observed for deleted Region I $\sigma^{54}$ and deleted Regions I and II $\sigma^{54}$ (11). This suggests that these mutants may have allowed the holoenzyme to form a conformation equivalent to that of the deleted Region I $\sigma^{54}$ holoenzyme, which has an extended DNA contact (9). Because L340S $\sigma^{54}$ alone does not bind DNA and has altered physical properties (aberrant mobility on native gel), the above result suggests that on binding to core to form the holoenzyme, L340S is reconfigured and revealed to have much of its DNA binding activity intact. The interaction with promoter DNA apparently stabilizes a more native conformation of the L340S holoenzyme. It seems L340S alters $\sigma^{54}$ conformation but in a way readily rescued in the holoenzyme DNA complex. On association with core RNA polymerase to form the holoenzyme, the conformation of $\sigma^{54}$ is altered (20, 24). The mutants W328A, L340S, and $\Delta310-328$ may be changed for core-dependent DNA binding.

**Holoenzyme Interaction with Melted DNA—**Closed promoter complexes formed with the $\sigma^{54}$-holoenzyme are very heparin-sensitive, but holoenzyme binding to early melted DNA results in an increased stability toward heparin (30). Stability may reflect altered $\sigma^{54}$-core interactions, which are normally disrupted by heparin (13). Heparin stability of holoenzymes on the early melted DNA was tested to evaluate the contribution of the cross-linking patch to stability. All mutant holoenzymes except $\Delta310-328$ bound the DNA with an affinity similar to wild type $\sigma^{54}$-holoenzyme with between 70 and 85% of the DNA bound with 100 nM holoenzyme (Fig. 5, top). The $\Delta310-328$ holoenzyme bound about 56% of the probe (Fig. 6). The holoenzyme-DNA complex was then challenged for 5 min with the polyanion heparin. Of the initial complex formed for wild type, W328A, D338A, and R342A holoenzymes, 37–50% of the complex was heparin-stable after 5 min (Fig. 6). For the $\Delta310-328$ and L340S holoenzymes, only 7 and 23% of the complexes were heparin-stable after 5 min. This suggests that the $\Delta310-328$ and L340S $\sigma^{54}$ disrupt holoenzyme interactions in the −12/−11 promoter region, which leads to heparin stability. For $\Delta310-328$, the defect may be in DNA binding to early melted DNA, for L340S in core interactions (see above). In both cases, defects in stability on the early melted DNA correlate with an activator-independent transcription activity (Ref. 32 and see below).
Holoenzyme binding to late melted (−10/−1) DNA was assayed. Wild type holoenzyme is able to form a heparin-stable interaction with this premelted DNA template when activated. The Δ310–328, as shown for deleted Region I σ^54 and the R336A holoenzyme (10, 22), was able to form a heparin-stable complex on premelted DNA in the absence of activation (data not shown). This result suggests the Δ310–328, like deleted Region I σ^54 and R336A σ^54, may result in activator-independent holoenzyme isomerization (9, 22). The unactivated L340S holoenzyme on the late melted DNA gave slightly increased stability toward heparin compared with wild type holoenzyme (data not shown), suggesting an improved interaction with single strand DNA and a low level of holoenzyme isomerization. The mutants W328A, D338A, and R342A holoenzymes behaved like wild type holoenzyme and did not form heparin-stable complexes on the late melted DNA in the absence of activator and NTP (data not shown).

Holoenzyme Footprinting—As previously reported for holoenzyme with σ^54 lacking its Region I sequence and the R336A holoenzyme, there is a correlation between activator-independent heparin-stable binding of holoenzyme to late melted DNA (−10/−1 bubble), activator bypass transcription, and altered holoenzyme-DNA interactions at start site and −12 proximal promoter sequences as detected by S1 and o-Cup footprints (9, 22). In the S1 footprint assay, only Δ310–328 holoenzyme gave the extended footprint to +20 (Fig. 7A, lane 4), equivalent to that made by activated wild type σ^54-holoenzyme or the deregulated deleted Region I σ^54 and R336A σ^54, may result in activator-independent holoenzyme isomerization (9, 22). The unactivated L340S holoenzyme on the late melted DNA gave slightly increased stability toward heparin compared with wild type holoenzyme (data not shown), suggesting an improved interaction with single strand DNA and a low level of holoenzyme isomerization. The mutants W328A, D338A, and R342A holoenzymes behaved like wild type holoenzyme and did not form heparin-stable complexes on the late melted DNA in the absence of activator and NTP (data not shown).

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When the holoenzymes were footprinted with o-Cup, both Δ310–328 and L340S holoenzymes gave footprints in which the
In Vitro Transcription—For the φ54-holoenzyme complex to be converted into an open complex, the closed complex must interact with an activator of the φ54-holoenzyme in a reaction that requires nucleotide hydrolysis. Single round in vitro transcription assays were conducted to study the effect of the mutants on activator-dependent and -independent transcription. Using supercoiled DNA, the level of φ54-dependent transcription from the S. meliloti nifH promoter was measured with PspFΔHTH as activator (Fig. 8A). We also measured any potential of the mutant φ54 to support transcription without activator by preincubating holoenzyme and template DNA with GTP before heparin challenge (Fig. 8B). The initiating sequence of the S. meliloti nifH promoter is GGG (+1 to +3); thus, the addition of GTP before heparin could allow open complexes to initiate and form heparin-stable complexes. Transcripts are then extended from the heparin-stable activator-independent initiated complex that has formed. In activator-dependent assays, ATP and PspFΔHTH were added before the heparin challenge. Hence transcripts in this assay arise from heparin-stable uninitiated open complexes, since ATP is not an effective initiating nucleotide at the S. meliloti nifH promoter.

Using saturating levels of PspFΔHTH, the activator-dependent assay revealed a trend for transcription activity levels closely following the pattern seen in the in vivo assays (see Table I). The Δ310–328 holoenzyme essentially produced no activated transcript, whereas W328A, D338A, and R342A gave levels closest to wild type activity. The L340S mutant produced approximately 18% of the transcripts made by wild type holoenzyme. With holoenzyme present at 100 nM, the amount of PspFΔHTH required for half-maximal transcription was measured as an indicator of the apparent affinity of each closed complex for activator. Mutants W328A, D338A, and R342A showed similar half-maximal PspFΔHTH requirements as the wild type holoenzyme. The L340S holoenzyme required approximately 2-fold more activator, similar to results previously reported for R336A (22). The apparent reduced affinity for activator of the L340S mutant may be indirect and due to its aberrant structure rather than loss of any specific activator contact. As previously reported for R336A holoenzyme (22), a time course of exposure to heparin before the addition of the NTP extension mix showed no significant difference in stability of the transcription-competent open complexes between L340S and wild type φ54-holoenzyme (data not shown). This together with the closed complex (Fig. 5) and open complex gel mobility assay data (Fig. 9) suggests that L340S holoenzyme is defective at some step where the closed complex is converted to the open complex but that Leu-340 is unimportant for the stability of the resultant open complex. Activator-dependent transcription was also tested on a linearized DNA template. All mutants were active for transcription on the linear DNA, suggesting no obvious defect in DNA melting (data not shown). However, mutants D338A and R342A were more active than the wild type φ54-holoenzyme for activated transcription from the linear DNA template. With PspFΔHTH at 800 nM, the D338A and R342A φ54-holoenzymes were 42% and 24%, respectively, more active for transcription than wild type φ54-holoenzyme (data not shown). This suggests that the D338A and R342A φ54-holoenzymes could be enhanced for a DNA-melting activity that is not detected when transiently melting supercoiled DNA is used as template.
In the activator-independent assay (GTP addition before heparin), among the mutants investigated here only Δ310–328 and, to a much lower extent, L340S holoenzyme (both of which showed altered interaction in the heteroduplex and footprinting assays) produced discernable levels of transcripts (Fig. 8D, lanes 5 and 9). The native gel mobility data indicate that L340S is structurally disrupted. Δ310–328 has lost a potential surface-exposed loop connecting the carboxyl-terminal DNA binding domain with the rest of σ54. In both cases the activator bypass activity and correlating holoenzyme activities (instability on early melted DNA, stability on late melted DNA, and altered footprints) might be due to local or more extensive structural disruptions in σ54, interfering with correct functioning of Arg-336 or the Region I of σ54, both of which are known to function to restrict the holoenzyme to the transcriptionally silent closed complex (9, 22, 32).

**Stable Initiated Complex Formation**—In the single round transcription assay, abortive initiation and promoter escape activities can influence the amount of transcript produced. Therefore, to investigate the ability of holoenzyme to form heparin-stable initiated complexes on linear DNA, we used a gel mobility shift assay. To ensure that the polymerase was saturated with σ54, increasing amounts of σ were added to a constant concentration of core (33). For activation, we used PspFΔHTH with GTP as the hydrolyzable nucleotide to allow open complexes to initiate. The Δ310–328 holoenzyme was unable to efficiently form heparin-resistant complexes and, as suggested by the in vivo transcription assays and DNA binding assays, appears to form a closed complex unable to respond to activator. Although the L340S holoenzyme was very active for closed complex formation, it appears that little of the closed activator. The L340S holoenzyme was shown to be defective in class II L340S holoenzyme was shown to be defective in transcription initiation (Fig. 7A, lane 5). The W328A holoenzyme was approximately 60% as active as wild type for stable initiated complex formation, whereas D338A and R342A σ54-holoenzymes, as suggested by transcription on linear DNA, were in repeated experiments consistently improved compared with wild type for stable complex formation (Fig. 9). The increase in initiated complex formation as the ratio of σ to core polymerase is increased must reflect that D338A and R342A σ54 have an altered affinity for core within the assay. It is notable that R342A holoenzyme, which formed low levels of closed complexes, was not obviously defective for stable complex formation. Thus, the mutation R342A appears important for σ54 and holoenzyme DNA binding but unimportant for the post DNA binding step of stable initiated complex formation. Closed complexes that form with R342A may convert more efficiently to initiated complexes than wild type.

The reduced activity of the W328A holoenzyme for initiated complex formation might be due to a defect in DNA strand separation. A repeat of the initiated complex assay at 15 °C showed that stable initiated complex formation by W328A holoenzyme was not especially temperature-dependent (data not shown). Cold-sensitive DNA opening is evident for mutants in σ70 that are believed to lack single-stranded DNA binding activity (34). The insensitivity of the W328A holoenzyme activity to lower temperature suggests residue Trp-328 is not involved in DNA opening in the way that aromatic residues in σ70 are.

**DISCUSSION**

The σ54-holoenzyme closed complex is converted to an open complex via a reaction employing activator nucleoside triphosphate hydrolysis (5–9). In vivo and in vitro transcription assays identified three classes of mutant in the patch of σ54 that can be cross-linked to DNA. The Δ310–328 σ54 was inactive for transcription; L340S, like R336A (22), gave reduced transcription activity; and the remaining mutants gave close to wild type transcription levels. The cross-linking patch contains residues both important for promoter binding (Trp-328 and Arg-342) and σ isomerization (Trp-328) as well as restricting basal transcription (amino acids 310–328 and Leu-340) and setting the level of open complex formation (Trp-328, Asp-338, Leu-340, and Arg-342). DNA footprints suggest that the cross-linking patch directs interactions with promoter DNA near –12. The –12 promoter sequence has a crucial role in σ54-dependent transcription, providing interactions that set basal transcription and which start DNA melting (23, 31, 32). Mutations in the –12 region produced promoters that increased basal transcription or diminished activation (31). Some sequences in the cross-linking patch apparently contribute to interactions with the –12 promoter region important for control of σ54-holoenzyme transcription activity. Interactions of the three classes of mutant σ and their holoenzymes with both native and heteroduplex promoter DNA templates are discussed in the context of the σ54 transcription initiation mechanism.

The class I mutant Δ310–328 holoenzyme was inactive for activator-dependent transcription in vivo and in vitro due to the inability of its closed complex to respond to activator and form an open complex. The Δ310–328 holoenzyme did allow activator bypass transcription in vitro (Fig. 8B, lane 5) and formed a heparin-stable complex on late melted DNA (data not shown), showing the conformation of the holoenzyme had changed. The Δ310–328 and wild type σ54 had similar affinities for native promoter DNA (Fig. 2A). Like the deleted Region I σ54-holoenzyme (11) the Δ310–328 holoenzyme was enhanced for DNA binding (Fig. 5). Also, as for deleted Region I and R336A holoenzymes (9, 22), S1 footprinting showed the Δ310–328 holoenzyme has isomerized to give an extended footprint (Fig. 7A, lane 4), and CUP footprinting revealed an altered interaction at –12 (Fig. 7B, lane 4). The Δ310–328 σ54 was defective for binding to early melted DNA (Fig. 3), and its holoenzyme was unstable on this template (Fig. 6). Possibly, this critically relates to the inability to be activated because the initial nucleation of DNA melting may not have occurred. Furthermore, residues 310–328 are likely surface-exposed (20) and could be a site for activator contact (35). Removal of these residues could change the holoenzyme conformation such that it is unable to respond to activator and isomerize properly. Interestingly, single-stranded DNA binding activity has been implicated for a region of σ54 between amino acids 329 and 364 (21). Deletion of residues 310–328 may cause a local change in conformation, which reveals this single strand DNA binding activity. Thus Δ310–328 appears to behave like deleted Region I σ54. Both are unable to respond to activator and form an unstable holoenzyme interaction with early melted DNA. Instead, a stable interaction with late melted DNA is favored. In contrast, wild type holoenzyme can form an activator-independent heparin-stable complex on early melted DNA but cannot on late melted DNA (9, 30). Mutant σs such as Δ310–328 and deleted Region I σ54, which exhibit a reverse activity with heparin-stable holoenzyme complex forming on late melted DNA are also capable of activator bypass transcription (see Fig. 8B, lanes 3 and 5). The bypass activity of the Δ310–328 σ54-holoenzyme supports the idea that disrupting σ54 recognition of early melted DNA near the –12 promoter region directs the holoenzyme to form a more stable complex with melted downstream promoter sequences and which, under specialized conditions, can allow activator-independent transcription (9, 22, 32).

The class II L340S holoenzyme was shown to be defective in
conversion from closed to open complex (compare Fig. 5 with Figs. 8A and 9). The purified L340S σ54 protein was physically disrupted and could not be directly assayed for DNA or core binding. However, when the L340S σ54 was mixed with core and promoter DNA, a closed complex with normal gel mobility was observed. Interaction between σ, core, and DNA apparently caused the σ to become restructured and to form a closed complex capable of transcription. The L340S holoenzyme showed a 10-fold increase in transcription activity at the K. pneumoniae nifH promoter when the C at −15 was changed to T (Table II). Replacing the K. pneumoniae nifH −15 C with a T is stimulatory for binding of σ54 and its holoenzyme (19). In the context of a consensus promoter, the −15 consensus T has been shown to be important for maintaining promoter function (31). Correct location of the −12 promoter sequence where open complex formation is initiated is necessary for regulated transcription initiation (32). Possibly a T at −15 helps correct a defective interaction between L340S holoenzyme and −12 promoter sequences. Like Δ310–328 holoenzyme, the L340S holoenzyme was enhanced for closed complex formation (Fig. 5) and gave weak activator bypass transcription (Fig. 8B, lane 9). However, unlike Δ310–328 holoenzyme, enhanced DNA binding by L340S holoenzyme did not correlate with an extended S1 footprint (Fig. 7A, lane 8), suggestive of extensive holoenzyme isomerization. The L340S holoenzyme showed reduced stability on the early melted DNA (Fig. 6) and slightly increased stability on the late melted DNA (data not shown). The o-Cup footprint shows that the −12 interaction of L340S holoenzyme has changed (Fig. 7B, lane 8). However, the S1 footprint was not extended (Fig. 7A, lane 8), suggesting that DNA interactions at −12 do not always function to strongly restrict polymerase isomerization. In the pathway from closed to open complex a change in the interaction between σ54 and the −12 promoter site may precede extension of DNA contact and stable binding of the open promoter DNA (23).

The class III mutants W328A, D338A, and R342A gave close to wild type levels of transcription activity in vivo and in vitro and did not exhibit any activator bypass transcription activity. W328A and R342A σ54 were shown to be defective for DNA binding (Fig. 2A), but apparently not to a level that greatly restricted transcription formation (Fig. 8A). W328A is part of a small hydrophobic patch flanked by positively charged residues that is highly conserved in σ54 proteins. Proteolysis with chymotrypsin has demonstrated that the hydrophobic patch containing Trp-328 is probably surface-exposed and therefore a candidate for ligand interaction (14, 20). The 3-fold decrease in DNA binding by W328A σ54 suggests that this tryptophan residue contributes to binding energy, possibly via a direct DNA contact (36). The increased native gel mobility of the W328A protein together with its altered DNA cross-link product (Fig. 2B) suggests that the W328A σ54 makes a qualitatively different interaction with the promoter DNA. The lack of the typical −5 sensitivity in the W328A σ54 o-Cup footprint (Fig. 2C) suggests that some change in DNA interaction could be at the downstream edge of the σ54 promoter DNA interaction. The W328A holoenzyme showed decreased activity for open complex formation (Fig. 9), which was apparently unrelated to any easily detected defect in DNA melting or transcript formation. The W328A holoenzyme exhibited reduced stability on the early melted DNA (Fig. 6) but behaved as wild type holoenzyme on late melted DNA (data not shown). The W328A σ54 was the only mutant examined to have a defect in σ isomerization (Fig. 4). The results suggested that the W328A-DNA complex was inhibitory to σ isomerization. It appears that Trp-328 is likely a surface-exposed residue involved in a promoter start site proximal interaction and is important for σ isomerization and open complex formation on linear DNA. However, mutation of Trp-328 apparently has little overall effect on normal function in the context of the holoenzyme. Presumably other steps in the pathway to transcript formation are more limiting than those that depend on Trp-328. Some reaction equilibria might be changed by inclusion of core enzyme, depending upon the assay substrate and type of defect in the σ.

The R342A holoenzyme gave reduced levels of closed complex (Fig. 5), but apparently at a level unimportant for open complex formation (Fig. 9). Both R342A and D338A gave enhanced levels of open complex formation (Fig. 9), suggesting that some sequences in the cross-linking patch may contribute negatively to open complex formation. Alteration of −12 promoter sequences has been shown to increase transcription activity (31, 37, 38). Possibly the R342A and D338A mutations have disrupted σ54 promoter interactions that function to restrict open complex formation in some contexts. In addition, the increase in open complex formation as the ratio of the mutant σ (D338A and R342A) to core polymerase was increased suggests these mutations may have an effect on the σ interface with core polymerase, an effect not detected as an altered affinity for core in the core polymerase binding assay.

Taken together these results are consistent with amino acids within the DNA-cross-linking patch of σ54 contributing to several functions of the σ54 polymerase (22), including establishing activator responsiveness for σ isomerization (Trp-328, this paper). Altered interactions in the −12 region, as shown by o-Cup and S1 footprinting together with reduced stability of mutant holoenzymes on the early melted DNA, and an ability to transcribe in the absence of activator are all consistent with an interaction with the −12 promoter region being changed (23, 31, 32). Independent assays of σ54-DNA proximity relationships are consistent with this conclusion.3 We cannot yet say if the cross-linking patch directly contacts the −12 proximal sequences and some changes in core interaction may also contribute.

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