The $\sigma^{54}$ subunit of the bacterial RNA polymerase requires the action of specialized enhancer-binding activators to initiate transcription. Here we show that $\sigma^{54}$ is able to melt promoter DNA when it is bound to a DNA structure representing the initial nucleation of DNA opening found in closed complexes. Melting occurs in response to activator in a nucleotide-hydrolyzing reaction and appears to spread downstream from the nucleation point toward the transcription start site. We show that $\sigma^{54}$ contains some weak determinants for DNA melting that are masked by the Region I sequences and some strong ones that require Region I. It seems that $\sigma^{54}$ binds to DNA in a self-inhibited state, and one function of the activator is therefore to promote a conformational change in $\sigma^{54}$ to reveal its DNA-melting activity. Results with the holoenzyme bound to early melted DNA suggest an ordered series of events in which changes in core to $\sigma^{54}$ interactions and $\sigma^{54}$-DNA interactions occur in response to activator to allow $\sigma^{54}$ isomerization and the holoenzyme to progress from the closed complex to the open complex.

Accessing the information in DNA often relies upon the action of DNA-binding proteins that are able to generate non-canonical B-DNA structures. Recombination, replication, methylation, repair, and transcription are processes that proceed through intermediates in which DNA is distorted. The process of RNA transcript formation by all RNA polymerases must involve a DNA-melting event to reveal the template DNA strand (1–3). Distortion of the DNA leading to the nucleation of strand separation occurs within the closed complex formed between RNA polymerases and the promoter. Following isomerization of the polymerase, full stable DNA opening is evident, which is thought to have spread from the initial nucleation site. Single-stranded DNA-binding activities in the RNA polymerase are required for DNA opening, and in the case of the bacterial RNA polymerase, the $\sigma$ subunit plays an important role (1, 3–7). For the $\sigma^{70}$-type factor, binding to the core enzyme induces conformational changes in a single-stranded DNA-binding region of the protein. As a consequence of these conformational changes, $\sigma^{70}$ gains specificity for the non-template strand of the melted region in the open complex (8, 9).

The $\sigma^{54}$-type factor, unrelated by sequence to $\sigma^{70}$, the determinants of single-stranded DNA binding are less well described. Single-stranded DNA binding by $\sigma^{54}$ is evident, however (4, 10, 11). The sequences that $\sigma^{54}$ recognizes as single-stranded DNA are between the −12 promoter element and the start site (4). Importantly, the activity of the $\sigma^{54}$ holoenzyme is tightly regulated at the DNA-melting step, but promoter binding to form the initial $\sigma^{54}$ holoenzyme closed complex is not highly regulated (12).

The closed complexes formed with the $\sigma^{54}$ holoenzyme are silent for transcription unless acted upon by an enhancer-binding activator protein (13–15). A network of protein and DNA interactions involving $\sigma^{54}$ function to maintain a stable holoenzyme conformation that rarely changes spontaneously to allow DNA melting and transcript initiation (11, 16–22). The conformationally restricted closed promoter complex isomerizes to an open promoter complex (in which the DNA strands are melted out) in a reaction in which the activator consumes ATP or another nucleoside triphosphate (14). As a part of this reaction pathway, $\sigma^{54}$ contributes to the creation of a local structural distortion within the closed complex (23). $\sigma^{54}$ binds tightly to the distorted promoter DNA and can be shown to isomerize independently of the core RNA polymerase in a reaction that has all the remaining requirements for open complex formation (4, 24). Isomerization is associated with an increased DNase I footprint of $\sigma^{54}$ on DNA, extending toward the transcription start site (24).

Here we use DNA footprinting to show that the DNA within the isomerized $\sigma^{54}$-DNA complex has melted and that some melting is negatively regulated by Region I of $\sigma^{54}$. However, extensive melting requires Region I. Additionally, changed interactions between $\sigma^{54}$ and the nucleated DNA are evident in complexes in which melting has occurred. We show that the presence of core RNA polymerase inhibits those changes in $\sigma^{54}$-DNA interactions that occur in response to activator, consistent with the view that tight binding to the early melted DNA limits DNA opening (4). The results provide clear evidence in favor of an activation mechanism in which conformational changes in a basal $\sigma^{54}$-DNA complex are brought about by the enhancer-binding activator. Activator-independent melting suggests that the activator does not function exclusively as a site-specific DNA helicase for DNA opening (11, 18, 22).

**EXPERIMENTAL PROCEDURES**

**DNA and Proteins**—The promoter fragments used in this work were *Escherichia coli* glnHp2 from −60 to +28 in which T at −13 was replaced by G and the −11 to −10 sequence was replaced with a CATG mismatch to create a short unpaired DNA element next to a consensus GC (−13−12) promoter element or a mismatched sequence between −11 and −6 (see Fig. 1). *Sinosrhizobium melloti* nifH promoter fragments from −60 to +28 either containing a CATG mismatch immediately adjacent to the consensus GC element or with the A-12 top strand base missing (gapped duplex) were also used (see Fig. 1) (24). Synthetic
DNA strands from −60 to +28 were annealed to create the duplex, with either strand 5'-32P-end-labeled. The unlabeled strand was at 2-fold molar excess.

The Klebsiella pneumoniae σ54 protein, its Region I-deleted derivative lacking the first 56 amino acids (ΔIσ54), and Region I (amino acids 1–56) were prepared as described previously (11, 25). The activator was E. coli PspF lacking a functional C-terminal DNA-binding domain (PspFΔHTH) (26).

DNA Binding Assays—End-labeled DNA (16–100 nM) and 1 mM σ54 or ΔIσ54 in a 10-μl reaction in buffer containing 25 mM Tris acetate (pH 8.0), 8 mM magnesium acetate, 10 mM KCl, 10 mM dithiothreitol, and 3.5% (w/v) polyethylene glycol 8000 were incubated for 5 min at 30 °C. Activator PspFΔHTH (0.5–4 mM) and dGTP, GTP, or GTPγS (4 mM each) were added for a further 10 min. Region I was at 0.5 μM. Where indicated, heparin (100 μg/ml) was added for 5 min prior to gel loading. Free DNA was separated from σ54-bound DNA on 4.5% native polyacrylamide gels run in 25 mM Tris and 200 mM glycine at room temperature.

DNA Footprints—Binding reactions were conducted as described above; footprinting reagents were added; reactions were terminated; and bound and unbound DNAs were separated on native gels as described above. DNA was then excised, processed, and analyzed on a denaturing 10% polyacrylamide gel. Free DNA was separated from σ54-bound DNA on 4.5% native polyacrylamide gels run in 25 mM Tris and 200 mM glycine at room temperature.

DNA Footprints—Binding reactions were conducted as described above; footprinting reagents were added; reactions were terminated; and bound and unbound DNAs were separated on native gels as described above. DNA was then excised, processed, and analyzed on a denaturing 10% polyacrylamide gel. Free DNA was separated from σ54-bound DNA on 4.5% native polyacrylamide gels run in 25 mM Tris and 200 mM glycine at room temperature.

Gel-isolated DNA was eluted into 0.1 mM EDTA (pH 8.0) (DNase I) or H2O (KMnO4) overnight at 37 °C. KMnO4-oxidized DNA was cleaved with 10% (v/v) piperidine at 90 °C for 20 min. Recoveries of isolated DNA were determined by dry Cerenkov counting, and equal numbers of counts were loaded onto gels.

RESULTS

Previously, we showed that purified σ54 bound to the S. meliloti nifH promoter was able to isomerize if the DNA template had an unpaired sequence downstream of the GC element of the promoter (24). The isomerization also required activator and nucleoside triphosphate hydrolysis and was characterized as an extended σ54-DNA interaction toward the transcription start site. The unpaired DNA downstream of the GC promoter element was suggested to mimic the nucleation of DNA melting (early melted DNA) seen in σ54 closed complexes, which normally requires σ54 and core RNA polymerase (23). Here we have used variants of the σ54-dependent E. coli glnH2 promoter (27) (Fig. 1) to explore DNA melting by σ54. We chose to generate a nucleated glnH2 promoter because the high AT content of the sequence should facilitate the detection of unstacked T residues using KMnO4 as a footprinting reagent (28). Base unstacking occurs when DNA melts, and the associated increased reactivity to KMnO4 is readily detected.

Isomerization of the σ54-glnH2 Complex—Initially, we used a gel shift assay to show that σ54 bound to the modified glnH2 promoter with unpaired DNA at −11 to −10 and, in a reaction

FIG. 1. Sequence of the E. coli glnH2 and S. meliloti nifH derivatives used in this work. Compared with the original glnH2 m12 promoter sequence (27), heteroduplex promoter fragments contained either unpaired DNA from −11 to −10 with A-10 (bottom strand) replaced with G to form a pre-melted structure comparable to that for the early melted S. meliloti nifH DNA (24) or from −11 to −1 (highlighted). In glnH2 m12, the wild-type TA base pair at −13 is replaced by GC to increase σ binding. S. meliloti nifH promoter fragments are as described previously (24).
σ\textsuperscript{54}.DNA Melting

requiring hydrolyzable nucleoside triphosphate (dGTP) and activator, produced a supershifted heparin-resistant complex (ssso-DNA, Fig. 2A, lane 4). As seen before, the same mobility-supershifted complex formed with activators of different molecular weights, providing evidence that the activator was not stably associated with the isomerized complex (Ref. 24 and data not shown). Formation of this complex required σ\textsuperscript{54} Region I (Fig. 2A, compare lanes 4 and 8). Addition of Region I in trans to Δσ\textsuperscript{54} resulted in a new species with similar mobility to that of the supershifted complex, independent of activator and nucleotide (Fig. 2A, lanes 9 and 10). Using Dnase I footprinting, we showed that the DNA within the isomerized σ\textsuperscript{54} complex was protected more than in the complex formed with σ\textsuperscript{54} in the absence of activator and nucleotide (Fig. 2B). The downstream edge of the σ\textsuperscript{54} footprint extended to about −5 (Fig. 2B, lane 3). In the isomerized complex, the footprint extended clearly to +2, but a partial footprint to +5 was also detected (Fig. 2B, lane 4). The upstream edge of the footprint was not easily discernible due to background DNA fragments in the undigested sample (Fig. 2B, lanes 1 and 5). Binding of Δσ\textsuperscript{54} did not lead to the extended DNase I footprint of σ\textsuperscript{54} seen with the activator-dependent isomerized complex, but weakly footprinted to about −7 (Fig. 2B, lanes 9 and 10). Addition of Region I in trans to Δσ\textsuperscript{54} resulted in a DNase I footprint indistinguishable from that of the non-isomerized σ\textsuperscript{54}-DNA complex (Fig. 2B, compare lanes 7 and 8 with the lane 3).

The overall results showed that σ\textsuperscript{54}-DNA interactions at glnH2 are changed by the action of activator in a nucleotide-dependent manner. Non-hydrolyzable nucleotide GTP\textsubscript{S} did not substitute for dGTP to produce an extended footprint (data not shown). Qualitatively, the results are similar to those obtained with the S. meliloti nif\textsubscript{H} promoter (24) and clearly indicate that activator-dependent σ\textsuperscript{54} isomerization may be readily demonstrated with a number of different promoters.

Activator-dependent DNA Melting by σ\textsuperscript{54}—We used KMnO\textsubscript{4} to probe for DNA melting within isomerized complexes. KMnO\textsubscript{4} footprints at 30 °C using the S. meliloti nif\textsubscript{H} early-melted DNA (−12/−11) in the isomerized complex did not convincingly show extra DNA melting, but the unpaired T residue at −12 of the heteroduplex region was much less reactive in both σ\textsuperscript{54}-DNA complexes (data not shown). These KMnO\textsubscript{4} footprints were repeated at 37 °C and with a promoter derivative having a single base pair of heteroduplex at −12 (top strand A replaced with C) (24). Results with the −12/−11 heteroduplex showed no extra DNA melting at the elevated temperature, but the unpaired T residue at −12 in the isomerized complex was more reactive to KMnO\textsubscript{4} (data not shown). However, footprints using the C-12 heteroduplex DNA in the isomerized complex showed that, at 37 °C, the template strand T at −9 had a 2-fold increase in KMnO\textsubscript{4} reactivity, indicating some extra DNA melting (data not shown). This contrasts with results obtained with glnH2 derivatives (see below and Fig. 3), where considerable extra DNA melting in the isomerized complex was seen at 30 °C. It seems that melting of the nif\textsubscript{H} promoter within the isomerized complex occurs less frequently than with glnH2 and may relate to differences in the ease with which the DNA strands of the two promoters can separate (see “Discussion”).

Footprints of the glnH2 −11/−10 promoter DNA show convincingly that the isomerized complex has extra DNA melting. As shown in Fig. 3A, σ\textsuperscript{54} strongly protected the template strand unpaired T residue at −11 from KMnO\textsubscript{4} attack (compare lanes 2 and 3). In the isomerized complex, this protection was lost, and KMnO\textsubscript{4} reactivity was evident at the unpaired T residue at −11 as well as at the new positions −9 and −7 (Fig. 3A, compare lanes 3 and 4). The bases at −11, −9, and −7 must be within an altered DNA structure compared with the free DNA.
and the non-isomerized $\sigma^{54}$-DNA complex. It seems that activator brings about some extra DNA melting as well as changing the interaction of $\sigma^{54}$ with the T residue at -11. The KMnO$_4$ reactivity of the bands was quantified to enhance the reliability of the interpretation (Table I). The same patterns of $\sigma^{54}$-DNA binding assays did not show a constant increase in the reactivity of T at -9 when $\sigma^{54}$ was bound. The increase was seen in three independent experiments. Controls in which either activator or hydrolyzable nucleotide (GTPyS) was used showed that the extra DNA melting at -9 and -7 and the changed footprint at -11 required activator plus hydrolyzable nucleotide (data not shown). In the absence of these components, the $\sigma^{54}$-DNA footprint remained unchanged, and the only complex evident in the gel shift assay was the fast running $\sigma^{54}$-DNA complex (data not shown).

$\sigma^{54}$-DNA was used to probe the non-template strand in $\sigma^{54}$-DNA and isomerized $\sigma^{54}$-DNA complexes forming with the modified glnHp2 promoter. As shown in Fig. 3B (lane 3 and lane 2) and Table I, no sequences were significantly more KMnO$_4$-reactive than in the unbound DNA (Fig. 3B, lane 2). For the $\sigma^{54}$-DNA complex, no further changes in KMnO$_4$ reactivity were detected in response to activator and hydrolyzable nucleotide (Fig. 3B, lane 7). Region I supplied in trans did not result in a significant change in KMnO$_4$ reactivity to suggest a shift in the footprint toward that of the non-isomerized $\sigma^{54}$-DNA complex (Fig. 3A, compare lanes 3 and 5; and Table I). It seems that, although Region I binds to the -12/-11-$\sigma^{54}$ complex, this does not lead to large changes in KMnO$_4$ reactivity (24).

Interactions of $\sigma^{54}$ with DNA Pre-opened from -11 to -6 and a Gapped Structure—The extra DNA opening from -9 to -6 seen in the activator-dependent isomerized complex (Fig. 3) could arise from activator functioning as a DNA helicase. To explore this issue, we wished to learn if pre-opening the DNA from -11 to -6 would allow the $\sigma^{54}$ to bind promoter DNA in an isomerized state without activator. The interaction of $\sigma^{54}$ with glnHp2 promoter DNA mismatched from -11 to -6 (see Fig. 1) to mimic the DNA opening seen in the activator-dependent isomerized complexes was examined in the presence and absence of activator. Gel shift assays showed that $\sigma^{54}$ bound to the -11/-6 opened DNA to give an initial complex (ss$\sigma^{54}$-DNA) with reduced mobility compared with the -11/-10 opened DNA complex (Fig. 4, compare lanes 2 and 5). The reduced mobility was similar to that of the activator-dependent isomerized complex (ssRNA-DNA) forming on the -11/-10 opened DNA (Fig. 4, compare lanes 3 and 5). The mobility of the $\sigma^{54}$ complex with DNA opened from -11 to -6 was unchanged by activator and hydrolyzable nucleotide (Fig. 4, compare lanes 4 and 5). To more fully understand the properties of these slow running complexes, we probed them by DNase I and KMnO$_4$ footprinting. Using glnHp2 promoter DNA mismatched from -11 to -6, $\sigma^{54}$ gave a short DNase I footprint to -5 and no extra KMnO$_4$ reactivity, and footprints were insensitive to activator and nucleotide (data not shown). We conclude that the reduced mobility of the $\sigma^{54}$ complex with DNA opened from -11 to -6 is due to activator-induced DNA melting and that the $\sigma^{54}$-DNA complex with DNA opened from -11 to -6 is due to activator-induced DNA melting.

Activities of Region I include contributions to the DNA-binding function of $\sigma^{54}$, particularly the recognition and creation of the nucleated DNA near -12, and an interaction with core RNA polymerase (4, 16, 23, 31, 34). Removal of Region I results in activator-independent transcription if the DNA is transiently opened and allows the holoenzyme to engage with pre-melted DNA (11, 18, 20, 25). Using KMnO$_4$ to probe the template strand of the glnHp2-$\Delta\sigma^{54}$ complex, we found some evidence for weak activator-independent melting. As shown in Fig. 3A (lane 8), the T residue at -9 showed some increased KMnO$_4$ reactivity in the $\Delta\sigma^{54}$ complex compared with the zero protein control (lane 2). This region of extra DNA melting appears to be a subset of that found within the activator-dependent complex forming with full-length $\sigma^{54}$ (Fig. 3A, compare lanes 4 and 8). The restricted pattern of melting at -9 seen with $\Delta\sigma^{54}$ was independent of nucleotide and activator (Fig. 3A, compare lanes 7 and 8). Quantitative treatment of the KMnO$_4$ reactivity (Table I) showed a constant increase in the reactivity of T at -9 when $\Delta\sigma^{54}$ was bound. The increase was seen in three independent experiments. The T residue at -11 in the $\Delta\sigma^{54}$ complex was slightly more protected compared with the isomerized $\sigma^{54}$ complex (Fig. 3A), but significantly more reactive than $\sigma^{54}$ alone to KMnO$_4$ attack (compare lanes 3, 4, and 8; also see Table I). It seems that removal of Region I partially deregulates $\sigma^{54}$ melting activity. Region I supplied in trans did not result in a significant change in KMnO$_4$ reactivity to suggest a shift in the footprint toward that of the non-isomerized $\sigma^{54}$-DNA complex (Fig. 3A, compare lanes 3 and 5; and Table I). It seems that, although Region I binds to the -12/-11-$\sigma^{54}$ complex, this does not lead to large changes in KMnO$_4$ reactivity (24).

$\sigma^{54}$-DNA was used to probe the non-template strand in the $\Delta\sigma^{54}$-DNA complexes forming with the modified glnHp2 promoter. As shown in Fig. 3B (lanes 5–8) and Table I, no sequences were significantly more KMnO$_4$-reactive than in the unbound DNA (Fig. 3B, lane 2). For the $\Delta\sigma^{54}$-DNA complex, no further changes in KMnO$_4$ reactivity were detected in response to activator and hydrolyzable nucleotide (Fig. 3B, lane 7). Region I supplied in trans to $\Delta\sigma^{54}$-DNA binding assays did not change non-template strand KMnO$_4$ reactivity (Fig. 3B, lanes 5 and 6).
to the altered DNA conformation and that pre-opening the DNA does not drive the change in $s^{54}$ needed for the extended downstream DNase I footprint to at least 12 seen in activator-dependent isomerized complexes (Fig. 2B, lane 4) (24). Instead, a change in $s^{54}$ conformation driven by the activator seems to be necessary for the extended footprint to 12. The insensitivity of the $s^{54}$ complex on the $2^{11}/2^{16}$ opened DNA to activator suggests that the DNA from $2^{9}$ to $2^{6}$ should be in a double-stranded form for activator to act on $s^{54}$, consistent with prior work with the S. meliloti nifH promoter (24).

We previously observed with the S. meliloti nifH promoter that removal of the top strand A-12 residue resulted in a $s^{54}$-DNA complex that did not form a new slow running species when incubated with activator and hydrolyzable or non-hydrolyzable nucleoside triphosphate (Fig. 5A, compare lanes 2, 4, and 5 with lane 6) (24). $\Delta l^{54}$ formed a slower running complex when Region I was in trans (Fig. 5A, compare lanes 2, 6, and 7). To characterize these complexes and to determine their relationship to the isomerized complex, we used DNase I and KMnO$_4$ footprinting. The S. meliloti nifH promoter −12 gap (top strand) probe (see Fig. 1) gave a DNase I footprint with a distinct region that was cut poorly compared with the intact probe (data not shown). This region centered over the −12 gap.
and extended ~4 bases on either side, suggesting a locally altered DNA structure refractory to DNase I cutting. When $\sigma^{54}$ bound, extra cutting from -10 to -1 also was evident, suggesting that $\sigma^{54}$ stabilized a double-stranded DNA structure otherwise absent from the unbound gapped DNA (data not shown). There was no difference in the DNase I footprint under activating conditions (data not shown). The effects of activating conditions were then gauged by KMnO$_4$ footprinting. When $\sigma^{54}$ bound the gapped duplex, the single-stranded T residue at -12 (template strand) was protected from attack by KMnO$_4$, and a modestly increased reactivity to KMnO$_4$ was seen at T-9 indicative of some activator- and nucleotide-independent DNA melting (Fig. 5B, compare lanes 2 and 3). The same pattern was seen in the presence of activator and nucleotide (Fig. 5B, compare lane 3 with lanes 5–7) without evidence for extra activation-dependent melting. This suggests that a mismatch at -12 is needed for activator response. The slow running $\Delta$$\sigma^{54}$ complex with Region I in trans footprinted like wild-type $\sigma^{54}$ (Fig. 5B, compare lanes 3 and 8), whereas the $\Delta$$\sigma^{54}$ complex showed less KMnO$_4$ reactivity at T-9 (compare lanes 8 and 9). This suggests that Region I stabilizes the melted DNA at -9. The DNA melting seen with the gapped DNA when bound by $\sigma^{54}$ (Fig. 5B) is consistent with the proposed role of the -12 nucleotide in restricting melting prior to activation (4). The gapped DNA allows melting within the $\sigma^{54}$-DNA complex that is not evident with homoduplex DNA (Fig. 5B and data not shown).

**Interactions of the $\sigma^{54}$ Holoenzyme with Early Melted DNA—**

The early melted DNA structure just downstream of the GC promoter that enables $\sigma^{54}$ isomerization is believed to exist in closed promoter complexes, but is apparently absent in the activator-dependent open complex (23, 24). The chemical activities at -12/-11 seen in closed complexes are not evident in open complexes; rather, new melting is evident nearer the transcription start site (23). To explore the activator responsiveness of the $\sigma^{54}$ holoenzyme on the S. meliloti nifH and E. coli glnH2 early melted DNAs, we conducted gel shift and footprinting assays. As noted above, the $\sigma^{54}$ holoenzyme bound to the early melted DNA assumes a complex with greater resistance to heparin than does the holoenzyme complex on homoduplex DNA (31). This has been suggested to involve a changed interaction between $\sigma^{54}$ and core RNA polymerase since the binding of $\sigma^{54}$ to core RNA polymerase in the absence of early melted DNA is heparin-sensitive (31, 34). Gel shift assays with either the S. meliloti nifH (Fig. 6A) or the E. coli glnH2 (data not shown) early melted DNA in the presence of the $\sigma^{54}$ holoenzyme showed, that under activating conditions, no new slow running holoenzyme-DNA complex was detected (compare lanes 6 and 7). A reduction in $\sigma^{54}$ holoenzyme concentration led to increased formation of the supershifted $\sigma^{54}$-DNA complex (ss-$\sigma$-DNA, Fig. 6A, compare lanes 2–6) in the presence of activator and nucleotide, reflecting free $\sigma^{54}$.

We next used DNase I and KMnO$_4$ footprinting to characterize the $\sigma^{54}$ holoenzyme-DNA complexes on the early melted DNA and to learn if they had isomerized. Results showed that, under non-activating conditions, the isolated $\sigma^{54}$ holoenzyme complex gave a short DNase I footprint to -5 and no extra KMnO$_4$ reactivity (data not shown). The footprints were essentially as for $\sigma^{54}$, except that some extra protection from DNase I by the $\sigma^{54}$ holoenzyme upstream of -34 was observed with the E. coli glnH2 promoter (data not shown). We next footprinted the $\sigma^{54}$ holoenzyme-DNA complexes under activating conditions. The isolated holoenzyme complexes gave footprints indistinguishable from those obtained under non-activating conditions, suggesting that core-bound $\sigma^{54}$ was not able to isomerize efficiently (data not shown). Further experiments with the use of initiating nucleotide (GTP) to potentially sta-

![Fig. 6. Holoenzyme binding to S. meliloti nifH early melted DNA.](image)

**A**. Gel mobility shift assay. Reactions contained S. meliloti nifH -12/-11 DNA (16 nM). Holoenzyme (E-$\sigma^{54}$) was formed from 200 nM $\sigma^{54}$ plus increasing amounts of core RNA polymerase (E) at 10, 25, 50, or 100 nM (indicated by the triangle above lanes 3–6). dGTP (1 mM), PspF$\Delta$HTH (4 nM), and $\sigma^{54}$ (200 nM) (lanes 1 and 2) were added where indicated (+). Unactivated holoenzyme (100 nM core RNA polymerase and 200 nM $\sigma^{54}$)-DNA complex (E-$\sigma$-DNA) is shown in lane 7. After binding, heparin (100 nM) was added for 5 min. No new $\sigma^{54}$ holoenzyme-DNA species was detected under activation conditions. **B**, core binding to the $\sigma^{54}$-DNA complex (graphed in C). A -35 to +6 base pair S. meliloti nifH -12/-11 DNA fragment (500 nM) (24) was used to form a supershifted complex (ss-$\sigma$-DNA) with 300 nM $\sigma^{54}$, 1 mM GTP, and 4 nM PspF$\Delta$HTH (lane 2). GDP (10 mM) was added for 1 min to inhibit activator function, and then increasing amounts of core (E) were added (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 nM) for 5 min before gel loading (lanes 3–8). Lane 9 shows core (1.0 nM) binding to DNA. C, quantification of complexes from B (lanes 2–8). ○, $\sigma^{54}$ holoenzyme-DNA; □, supershifted $\sigma^{54}$-DNA complex; ■, $\sigma^{54}$-DNA.
in which activator-dependent changes were evident (data not shown). We conclude that $\sigma^{54}$ does not isomerize efficiently when bound to core RNA polymerase in assays using early melted DNA probes.

We confirmed (data not shown), using $S$. meliloti nifH – 60 to +28 homoduplex DNA, that the heparin-resistant open promoter complexes formed by the action of activator and GTP had extended DNase I footprints to at least +13, definition of an exact end point being limited by the resolution of the gel and fragment size to +28 (14, 20, 35). However, the efficiency of activator-dependent stable complex formation was low in this assay. To address the issue that the $\sigma^{54}$ holoenzyme bound to the early melted DNA might form new activator-dependent complexes (not distinguished from the activator-independent complexes because of the common property of heparin resistance) but with low efficiency, we also used KMrO$_4$ as a probe of isomerization events. Here isomerization would be evident as increased reactivity to KMrO$_4$ rather than protection from DNaseI (see Fig. 3A). Unlike the activator- and nucleotide-dependent complexes forming with $\sigma^{54}$ and the early melted $glnHp2$ promoter DNA (Fig. 3A), the $\sigma^{54}$ holoenzyme did not respond to activator to yield detectable extra melting or any associated loss of KMrO$_4$ reactivity of the $glnHp2$ heteroduplex sequence (data not shown). The overall results show that the holoenzyme complex, in contrast to $\sigma^{54}$, is poorly (if at all) responsive to activator conditions when bound to the early melted DNA. As discussed below, this may relate to unusually stable complex formation between the early melted DNA and the $\sigma^{54}$ holoenzyme.

Core RNA Polymerase Binding to Isomerized $\sigma^{54}$-DNA.—Having shown that $\sigma^{54}$ bound to early melted DNA forms an isomerized complex (Ref. 24 and this work), but that $\sigma^{54}$ holoenzyme does so inefficiently if at all (see above), we conducted an experiment to determine whether the conformation of the isomerized $\sigma^{54}$-DNA complex allowed core RNA polymerase binding. In this assay, the isomerized complex was formed using an end-labeled $-35$ to +6 $S$. meliloti nifH – 12/–11 DNA fragment (24) in excess of $\sigma^{54}$ to diminish the amount of free $\sigma^{54}$ and to ensure that core RNA polymerase interactions were potentially largely with DNA-bound $\sigma^{54}$. Isomerization reactions were carried out and stopped by adding GDP (Fig. 6B, lane 2) (24). Increasing amounts of core RNA polymerase (E) were then added to bind $\sigma^{54}$-DNA complexes. As shown in Fig. 6B, addition of increasing amounts of core RNA polymerase (lanes 3–8) depleted the $\sigma^{54}$-DNA complex; and in parallel, an increasing amount of the $\sigma^{54}$ holoenzyme bound to DNA (E-$\sigma^{54}$-DNA) was detected. The amount of isomerized $\sigma^{54}$-DNA complex (ssr-DNA) remained relatively constant throughout the titration with core RNA polymerase. It seems that core RNA polymerase preferentially binds the non-isomerized $\sigma^{54}$-DNA complex. At high core RNA polymerase concentrations (in excess of 0.6 $\mu$m) (Fig. 6B, lanes 7 and 8), some core bound to DNA was detected, and this contributed to the apparent increased amount of $\sigma^{54}$ holoenzyme bound (graphed in Fig. 6C) since core-DNA and holoenzyme-DNA complexes were not fully resolved. We infer that weak binding of the isomerized $\sigma^{54}$-DNA complex by core RNA polymerase is because the interface between core and $\sigma^{54}$ and DNA has changed upon isomerization. This leads to the suggestion that movements in $\sigma^{54}$ and DNA are concerted with some in core RNA polymerase for forming the natural open promoter complex and is discussed below.

DISCUSSION

DNA Melting by $\sigma^{54}$—Activator-dependent isomerization of $\sigma^{54}$ results in the spreading of DNA melting away from the nucleated DNA structure and toward the transcription start site (Fig. 7). A structure melted over at least 6 base pairs is generated. Interactions with the nucleated DNA located at –11/–10 are lessened in the isomerized complex compared with the initial $\sigma^{54}$-DNA complex. Although removal of Region I sequences allows some weak DNA melting by $\sigma$, this is not as extensive as activator-driven isomerization. A comparison of results obtained with $\Delta\sigma^{54}$ and intact $\sigma^{54}$ shows that the full DNA-melting activity of $\sigma^{54}$ has some determinants outside of regulatory Region I (for weak melting at –9 and some that depend upon Region I (for extra melting from –8 to –6).

Isomerized $\sigma^{54}$-nifH promoter complexes failed to show KMrO$_4$ reactivity as extensively as $glnHp2$ (Fig. 3 and data not shown). If this reflects less melting (as opposed to shielding from KMrO$_4$), differences in intrinsic DNA opening rates between the two promoters in combination with the sequence-independent single-stranded DNA-binding activity in $\sigma^{54}$ (36)
could contribute. DNA opening at −9 seen in natural nifH open complexes but weakly detected in isomerized complexes with σ54 might therefore reflect a stabilizing contribution from the core enzyme (37). Clearly, changes in the σ54-DNA relationship seen in DNase I footprints of isomerized complexes in which melting may not have occurred is consistent with the idea that activator changes σ54 structure and that pre-opening the DNA does not drive this change (22, 24).

Comparisons of the closed and activator-dependent open σ54 holoenzyme-promoter complexes using KMnO₄ and ortho-copper phenanthroline footprinting suggest that the structure of the DNA immediately downstream of the GC element differs between these complexes and that contact with G-13 is also the DNA immediately downstream of the GC element differs per phenanthroline footprinting suggest that the structure of DNA through heteroduplex formation does not bypass activation (14). Changes in promoter DNA and σ54 holoenzyme conformation are probably coupled through σ54 Region I to maintain either the closed or open state of the promoter. Open promoter complexes that form in deregulated transcription by the σ54 holoenzyme are unstable compared with activator-dependent open complexes (18, 19, 25). Their instability may be related to incomplete DNA melting beyond −9 as a consequence of the mutations in Region I of σ54 and a need for Region I to stabilize melted DNA (Fig. 5B).

Role of Activator—Results with DNA mismatched from −11 to −6 across the sequences melted in the isomerized σ54-DNA complex support the view that activator drives a conformational change in σ54 rather than generating isomerization by solely creating an opened DNA structure for σ54 binding. For both transcription and σ54 isomerization, pre-opening of the DNA through heteroduplex formation does not by-pass activator requirements (22), unless the structure recognized by σ54 at −11 is destroyed (36). Rather, activator-dependent conformational changes in σ54 and the holoenzyme seem necessary for open complex formation and σ54 isomerization (24, 36). DNase I footprinting shows that σ54 clearly binds to double-stranded DNA ahead of the locally melted DNA or the fork junction that forms next to the GC promoter element in closed complexes (this work and Refs. 4 and 24). This suggests that the further melting of the double-stranded DNA within the σ54-DNA complex is an active process in the sense that it is not a primary result of a domain of σ54 translocating along the duplex and trapping DNA strands at a fraying fork junction. Energy for duplex destabilization may come from the tight binding of σ54 to the initially locally melted DNA. An activator-driven change in σ54-DNA interaction might release σ54 from binding to the double-stranded DNA downstream of the −12 GC and switch σ54 to a conformation that then allows it to bind single-stranded DNA. Combined with the single-stranded DNA-binding activities in σ54 (4, 36) and the core RNA polymerase (39), σ54 isomerization would lead to formation of the stable open promoter complex.

Stable Holoenzyme Binding—When bound to core RNA polymerase, activator-dependent isomerization of σ54 was not evident, in marked contrast to its efficient isomerization without core. It seems that the short sequence of heteroduplex downstream of GC inhibits isomerization of σ54 within the holoenzyme since, on linear DNA, activator-dependent isomerization of the holoenzyme is evident (11, 23, 39). The activator-dependent movement of σ54 across the sequence opened downstream of GC implied by the results of our KMnO₄ footprints may be inhibited when σ54 is bound by core RNA polymerase. This suggests that isomerization of σ54 within the holoenzyme may normally require that the local DNA opening next to GC does not strongly persist. In the homoduplex, the DNA can base pair again, but not in the heteroduplex. Consistent with this view is the observation that the local DNA distortions downstream of GC and present in the closed complex are apparently changed in the open complex, as judged by the reduced sensitivity of the DNA to two chemical probes of DNA structure (23). The spread of melting observed in the σ isomerization assays would then normally be associated with a changing of the DNA structure believed to be locally melted in the closed complex, achieved through a breaking of DNA contacts and a rebinding of σ54 to DNA. These considerations suggest an ordered series of events in which changes in core RNA polymerase to σ54 interactions and σ54-DNA interactions occur in response to activator to allow σ54 isomerization and the holoenzyme to progress from the closed complex to the open complex. This view is consistent with activator interacting with both core RNA polymerase and σ54 (24, 40) to achieve isomerization of the holoenzyme and with the view that the promoter sequences around GC contribute to preventing the holoenzyme from isomerizing prior to activation and to set the target of the activator (4, 24).

Core-σ Interactions and DNA Melting—Results of core RNA polymerase binding with the isomerized and non-isomerized σ54-DNA complexes strongly suggest that some points of interaction between σ54 and core are changed upon isomerization of the σ54-DNA complex. The poorer core binding of the isomerized complex likely correlates with the changes in protease sensitivity of σ54 in the isomerized complex (24). Changed DNA structure within the isomerized σ54-DNA complex may also contribute to poor binding by core RNA polymerase. The strong reduction in isomerization of σ54 resulting from core RNA polymerase binding prior to exposure to activating conditions (Fig. 6A) and the weak binding of isomerized σ54 to core RNA polymerase (Fig. 6, B and C) is striking. It seems that normally for efficient σ54 holoenzyme isomerization and open complex formation, activator-dependent changes in σ54 structure would occur in concert with a changed binding of parts of σ54 to core RNA polymerase; but on the early melted DNA, this is not occurring properly. As indicated above, when using the early melted DNA as template, the failure to reconfigure interactions with DNA next to the GC promoter may simply strongly stabilize a σ54 conformation that is unfavorable for core RNA polymerase binding. We therefore suggest that, in closed complexes, activator drives a conformational change in σ54 that results in altered contacts with the early melted DNA (as detected in our KMnO₄ footprinting; see above), allowing binding of σ54 and core RNA polymerase to permit full σ54 isomerization and the associated isomerization of the closed complex to the open complex. It seems that Region I of σ54 greatly contributes to these events through (i) its requirement for creating the early melted DNA when the holoenzyme binds homoduplex DNA, (ii) directing σ54 binding to the early melted DNA in heteroduplexes and associated fork junction structures, (iii) a binding interaction with core RNA polymerase, and (iv) the changing Region I structure in isomerized σ54 and the activated σ54 holoenzyme (reviewed in Ref. 12). The recent demonstration that Region I sequences localize over the −12 promoter region is fully consistent with these observations and points to a central role of the protein and DNA elements that localize there in establishing new interactions that allow DNA melting and a changing binding relationship between core subunits and σ54 (41). We also note that the refractory behavior of
the $\sigma^{54}$ holoenzyme bound to the early melted DNA and the poor core binding of isomerized $\sigma^{54}$ are fully consistent with the view that interactions $\sigma^{54}$ makes with the $\sigma^{54}$ promoter region, in particular sequences just downstream of GC, are key in limiting spontaneous activator-independent open complex formation (4).

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