Control of transcription frequently involves the direct interaction of activators with RNA polymerase. In bacteria, the formation of stable open promoter complexes by the $\sigma^{54}$ RNA polymerase is critically dependent on $\sigma^{54}$ amino Region I sequences. Their presence correlates with activator dependence, and removal allows the holoenzyme to engage productively with melted DNA independently of the activator. Using purified Region I sequences and holoenzymes containing full-length or Region I-deleted $\sigma^{54}$, we have explored the involvement of Region I in transcription activation. Results show that Region I in trans inhibits a reversible conformational change in the holoenzyme believed to be polymerase isomerization. Evidence is presented indicating that the holoenzyme (and not the promoter DNA per se) is one interacting target used by Region I in preventing polymerase isomerization. Activator overcomes this inhibition in a reaction requiring nucleotide hydrolysis. Region I in trans is able to inhibit activated transcription by the holoenzyme containing full-length $\sigma^{54}$. Inhibition appeared to be noncompetitive with respect to the activator, suggesting that a direct activator interaction occurs with parts of the holoenzyme outside Region I. Stabilization of isomerized holoenzyme bound to melted DNA by Region I in trans occurs largely independently of the initiating nucleotide, suggesting a role for Region I in maintaining the open complex.

$\sigma^{54}$-Holoenzyme binds promoters in a transcriptionally inactive state, forming stable closed complexes (5–7). Isomerization of the closed complex to a transcriptionally competent open complex requires an activator protein bound to a DNA sequence with enhancer-like properties (5, 8) and nucleotide hydrolysis by the activator protein (6, 9, 10). Activation appears to involve direct contact between the activator and the holoenzyme (11–13). Isomerization from the closed complex to the open complex is believed to involve a major conformational change in the holoenzyme to reveal single-strand DNA binding determinants as one step and DNA melting to reveal the template strand (14, 15) as a second step. The pathway to stable DNA melting within the holoenzyme appears to involve at least one unstable intermediate and to be driven by nucleotide hydrolysis by the activator (15).

The functional domain organization of $\sigma^{54}$ is complex, but different activities reside in different sequences: DNA binding motifs are localized in the carboxyl-terminal region, the central domain is needed to bind polymerase, and amino-terminal sequences are required for proper regulation of activation (16–20). When the amino-terminal domain (Region I) is deleted, $\sigma^{54}$ can still bind RNA polymerase and direct it to DNA (14, 21, 22). However, the bound holoenzyme fails to respond to activator and form a stable open complex that can initiate transcription. If stably or transiently melted DNA is used, the holoenzyme with Region I deleted can produce transcripts, showing that its catalytic activity is intact (14, 22). This transcript is unusual in that it results from heparin-sensitive transcription, and its production is not enhanced by the addition of activator, implying that the amino terminus of $\sigma^{54}$ contains essential activator response determinants.

Region I of $\sigma^{54}$ is closely implicated in polymerase isomerization and DNA melting and is a major determinant of enhancer responsiveness. Current models suggest that Region I contains determinants for nucleating DNA melting, for inhibiting polymerase isomerization, and for the interaction of holoenzyme with melted DNA (14, 15, 22–24). Some properties of the holoenzyme that depend upon Region I sequences likely rely upon the interaction that Region I makes with the core RNAP subunits (17); others may depend upon more direct domain communication between Region I sequences and other parts of $\sigma^{54}$ and possibly DNA. The conformation of the carboxyl-terminal $\sigma^{54}$ DNA-binding domain is changed in the holoenzyme when Region I is deleted (25, 26), implying that Region I contributes to the physical properties of the holoenzyme, some of which involve sequences that are closely associated with the DNA binding function of $\sigma^{54}$. In initial complexes that form between holoenzyme and promoter DNA, the $\sigma^{54}$ contacts DNA that is melted in open complexes (27–29), suggesting that the $\sigma^{54}$ DNA-binding domain may influence open complex formation.

To explore the involvement of Region I sequences in enhancer- and nucleotide-dependent activation of transcription, we have taken advantage of the domain structure of $\sigma^{54}$ and worked with purified partial sigma sequences. We now show
that the polymerase isomerization that occurs when Region I sequences are deleted is reversed by Region I added in trans and that inhibition of isomerization requires a Region I-protein interaction. We also show that Region I sequences in trans stabilize holoenzyme bound to melted DNA. Activator overcomes the inhibition caused by Region I in trans in a reaction requiring NTP hydrolysis, providing a formal demonstration that the anti-inhibition function of the activator occurs via its NTP hydrolysis activity. Transcription by the wild type holoenzyme was inhibited in a noncompetitive manner when the holoenzyme was challenged with Region I in trans, indicating that the activator may interact with parts of the holoenzyme outside of Region I for regulated stable open complex formation. The positive and negative activities of Region I appear to be crucial for the ordered progression from the closed complex to the open complex.

EXPERIMENTAL PROCEDURES

Proteins—These were prepared as described previously (16, 20, 25). The Klebsiella pneumoniae $\sigma^{54}$ protein (amino acids 1–477) and $\Delta\sigma^{54}$ (amino acids 57–477) were used to form holoenzymes (Fig. 1A). Purified amino-terminal sequences of $\sigma^{54}$ (amino acids 1–56) were obtained by overproducing an amino-terminal histidine-tagged fragment as a soluble sequence in E. coli (Ref. 17; Fig. 1A). Purified E. coli core RNA polymerase was from Epicentre Technologies. A purified carboxyl-terminal-deleted form of activator, PspF, polymerase was from Epicentre Technologies. A purified carboxyl-terminal-deleted form of activator, PspF, was from activation assay. Working protein solutions and stocks were stored at $-20^\circ$C and $-70^\circ$C, respectively, in 10 mM Tris-HCl, pH 8.0, 50% (v/v) glycerol, 0.1 mM EDTA and 1 mM diithiothreitol (TGED) containing 50–250 mM NaCl. Protein concentrations were determined using the Bio-Rad Protein Assay kit.

DNA Footprinting Assays—S1 nuclease footprinting was performed with linear homoduplex DNA prepared by primer extension of a single-strand M13mp19 clone of the Sinorhizobium meliloti $nifH$ promoter (14). Reactions (25 $\mu$L) were conducted in STA buffer at 30 °C and contained 1.6 nM DNA together with 100 nM holoenzyme (core: $\sigma$ at a 1:2 ratio) and activator PspF$\Delta$HTH (4 $\mu$L) and GTP (4 $\mu$L) where needed. When used, Region I (2 $\mu$L) was added to the reaction before holoenzyme assembly. Holoenzymes and DNA were incubated for 10 min, and for activation assays, PspF$\Delta$HTH and GTP were added for an additional 10 min. S1 nuclease (700 units; Amersham Pharmacia Biotech) was added for 5 min before reactions were terminated by the addition of 10 mM EDTA followed by rapid phenol extraction. DNA was recovered by ethanol precipitation and run on 6% denaturing polyacrylamide gels. Markers were generated by chemical cleavage of the DNA with piperidine after partial methylation with dimethyl sulfate.

Native Gel Complex Formation Assays—A gel shift assay (25, 32) was used to detect holoenzymes bound to a radioactively labeled S. meliloti $nifH$ heteroduplex 1 promoter DNA fragment in which top strand sequences 10 to 1 are mismatched (14). Typical holoenzyme interactions were carried out in STA buffer. Holoenzyme core: $\Delta\sigma^{54}$ ratio, 1:2) was assembled at 30 °C for 5 min, and then heteroduplex DNA (16 nM) was added for another 5 min, followed by glycerol bromphenol blue loading dye (final concentration, 10% glycerol) and, if required, heparin (final concentration, 100 $\mu$g/ml). Region I was added either before holoenzyme assembly or for 5 min after formation of the $\Delta\sigma^{54}$-holoenzyme-DNA complex. Samples were then loaded onto 4.5% native polyacrylamide gels to separate free DNA and bound DNA that were detected by autoradiography. Quantitative data were derived from phosphorimager analyses.

In Vitro Transcription Assays—For transcription from supercoiled DNA, template DNA (10 $\mu$L) was plasmid pMKC28 (21). Holoenzyme was assembled at 30 °C for 5 min, and then DNA was added for another 5 min. Region I (0.5 $\mu$L) was added either before holoenzyme assembly or for 5 min after formation of the holoenzyme-DNA complex. Then, if necessary, nucleotide and activator were added for 10 min, followed by heparin (100 $\mu$g/ml) plus the remaining ribonucleotides (0.1 mM of each) and 3 $\mu$Ci of $[\alpha-32P]UTP$. After 10 min, RNA was precipitated and analyzed on 6% sequencing gels. Detection was by autoradiography and phosphorimaging.

RESULTS

Inhibition of RNAP Isomerization by Region I—We previously showed that deletion of amino acids 1–56 (removing all of Region I) from K. pneumoniae $\sigma^{54}$ allowed the holoenzyme to isomerize independently of the activator (14). The assay used employed S1 nuclease footprinting of holoenzyme on linear DNA templates containing the S. meliloti $nifH$ promoter as the binding site for the holoenzyme. The $\sigma^{54}$-holoenzyme showed a short footprint from $-33$ to $-5$, whereas the $\Delta\sigma^{54}$-holoenzyme footprinted over and beyond the transcription start site to $+20$. Activated $\sigma^{54}$-holoenzyme showed a similar extended footprint, and differences between $+8$ and $+20$ could be related to the absence of Region I in the $\Delta\sigma^{54}$-holoenzyme. To determine whether the conformational change in the holoenzyme resulting in the extended footprint was reversible, we added Region I (amino acids 1–56) back in trans to S1 footprint assays. Results in Fig. 1B show that the extended footprint of $\Delta\sigma^{54}$-holoenzyme is lost when Region I is added in trans, demonstrating that polymerase isomerization does occur as a reversible manner. $\sigma^{54}$-holoenzyme footprinting were essentially insensitive to Region I in trans (lane 3 versus lane 4), but the $\Delta\sigma^{54}$-holoenzyme footprinted (lane 5 versus lane 6) upon addition of Region I in trans (loss of $a$ reactivity). Footprints of $\sigma^{54}$- and $\Delta\sigma^{54}$-holoenzymes in the presence of Region I were essentially the same (lanes 4 and 6). Controls showed that Region I alone did not footprint the linear DNA (compare lane 2 and lane 7). Activated $\sigma^{54}$-holoenzyme is shown as a control (lane 9 versus...
Inhibition of Stable Complex Formation on Melted DNA by Region I—Removal of Region I sequences reveals a single-strand DNA binding activity in the holoenzyme and allows engagement with melted DNA sequences (14). We have used a heteroduplex DNA molecule with a region of unpaired DNA between −10 and −1 presenting stably melted DNA sequences. Experimentally, the stability of holoenzyme complexes with the melted DNA can be measured in a challenge assay in which pre-bound complexes are formed and then heparin is added. Bound and unbound labeled DNA are separated by native gel electrophoresis. ΔIR54-holoenzyme can bind stably to heteroduplex DNA in a reaction inhibited by the addition of Region I in trans to the holoenzyme before engagement with DNA (14). As suggested by the results shown in Fig. 1B, inhibition by Region I could occur by preventing polymerase isomerization and masking holoenzyme single-strand DNA binding activity.

To probe interactions involved in Region I inhibition, we titrated ΔIR54-holoenzyme with purified Region I sequences and with heteroduplex DNA. The number of ΔIR54-holoenzyme/heteroduplex DNA complexes surviving a 5-min heparin challenge was measured. We initially determined the minimum amount of Region I (residues 1–56) needed to cause significant inhibition of stable complex formation with 100 nM holoenzyme and 16 nM DNA (Fig. 2A). We observed that 0.3 μM Region I caused a 40% reduction of heparin-resistant complex formation. Keeping Region I (0.3 μM) and DNA constant but increasing the amount of holoenzyme (0.1–0.5 μM), we observed that more stable complexes formed (Fig. 2B, lanes 2, 4, 7, 10, and 13). The addition of extra Region I reversed this effect (lanes 5, 8, 11, and 14). This result suggests that the holoenzyme is one target of Region I. Using 32P-labeled ΔIR54, we observed that increasing amounts of heteroduplex DNA did not overcome the Region I-dependent inhibition (data not shown), consistent with Region I interacting with holoenzyme rather than the free heteroduplex DNA to achieve inhibition. The 5-fold excess of Region I required for in trans inhibition (compared with its normal in cis functioning) may reflect the weak binding to the ΔIR54-holoenzyme or the ΔIR54-holoenzyme/DNA complex. Our experiments do not establish which holoenzyme form (free or bound to DNA) is the target for Region I-dependent inhibition of the activity required for interaction with melted DNA.

Stabilization of Holoenzyme Complexes on Melted DNA by Region I—We previously established that initiating nucleotide increased the stability of the ΔIR54-holoenzyme on heteroduplex DNA (14). We now find that stabilization is also afforded by Region I sequences in trans when added once the ΔIR54-holoenzyme is bound to the DNA and can occur in a nucleotide-independent manner. First, we determined the effect of Region I concentrations on stable complex formation with 100 nM holoenzyme and 16 nM DNA (Fig. 3A) and observed that 2 μM Region I caused a 30% increment of heparin-resistant complex formation. We also analyzed the decay with time of complexes between heteroduplex DNA and ΔIR54-holoenzyme in a heparin challenge experiment (Fig. 3B). Clearly, Region I sequences added after DNA binding afford a stabilization (which is only modestly increased by the initiating nucleotide GTP; data not shown). Therefore, Region I sequences have a role in stabilizing the holoenzyme at the promoter when the DNA is stably melted out, and this is of potential significance for maintaining the open complex.

Effect of Region I on Bypass Transcription—ΔIR54-holoenzyme transcribes independently of activator from stably or transiently melting DNA templates containing σ54 promoter sequences (14, 21, 22). Using the S. meliloti nifH promoter on a supercoiled plasmid, we showed that transcription by the ΔIR54-holoenzyme was inhibited by the addition of Region I to the holoenzyme before the template and initiating nucleotide GTP (Fig. 4, lanes 3 and 4). The presence of GTP is required to stabilize the polymerase as an initiated complex before the heparin challenge, and the addition of remaining nucleotides is required for transcript elongation (compare lanes 1 and 3). Thus, the effects of Region I on transcript formation are consistent with the inhibition of polymerase isomerization and the binding of melted DNA demonstrated above (Figs. 1–3).

The addition of Region I to template-bound ΔIR54-holoen-
enzymatic inhibition overcomes Region I Inhibition—Having established that Region I in trans inhibited bypass transcription by the ΔIσ54-holoenzyme (Fig. 4), we investigated whether activator protein PepFΔIHTH could overcome the inhibition and whether this required NTP hydrolysis. We also investigated whether activator and Region I in trans would allow the ΔIσ54-holoenzyme to transcribe via a heparin stable intermediate that would form independently of transcript initiation, as is the case with the wild type holoenzyme (Fig. 5). Footprints (Fig. 1) indicated that σ54-holoenzyme and ΔIσ54-holoenzyme plus Region I in trans were similar, inferring that ΔIσ54-holoenzyme could form an activable closed complex in the presence of Region I in trans. Bypass transcription is characterized as requiring the polymerase to form one or more phosphodiester bonds in the nascent transcript to establish heparin-resistant initiated complexes (24, 33). However, activated transcription results in the formation of a heparin stable open promoter complex without phosphodiester bond formation (6). Because of the complicating factor of activator hydrolyzing a nucleoside triphosphate to drive open complex formation, we used different GTP analogues to allow either activator function and RNA phosphodiester bond formation (GTP), activator function (dGTP) only, or phosphodiester bond formation (GTPγS or GMP-PNP) only. We used GTPγS (or GMP-PNP, data not shown) elongation substrates with a nonhydrolyzable γ-β bond to explore activator NTP hydrolysis requirements. We chose dGTP as a hydrolyzable nucleotide unable to support transcription elongation rather than ATP or deoxy GTP to stop nonspecific initiation or inhibition of the subsequent transcript elongation from the S. meliloti nifH promoter (data not shown).
Experiments with GTP—Fig. 5 shows transcript levels resulting from bypass transcription using the $\Delta\sigma^{54}$-holoenzyme and its inhibition by Region I added before DNA binding (lanes 1 and 2). The activator did not stimulate transcription by the $\Delta\sigma^{54}$-holoenzyme, but it did reproducibly overcome a large fraction of the inhibition of bypass transcription seen when Region I was added in trans (lanes 7 and 8). Controls with $\sigma^{54}$-holoenzyme showed that GTP supported activator-dependent transcription (lane 13), and that Region I slightly inhibited activation (lane 14).

Experiments with GTP$\gamma$S—Activators of the $\sigma^{54}$ RNA polymerase must hydrolyze the $\gamma$-P bond of a NTP to stimulate the formation of open complexes (6). This is true for PspF$\Delta$HTH and the wild type holoenzyme at the nifH promoter because no transcripts are detected when GTP$\gamma$S replaces GTP (Fig. 5, lanes 15 and 13). However, bypass transcription occurs with GTP$\gamma$S and is unchanged by the presence of PspF$\Delta$HTH (lanes 1, 3, and 9), confirming that it is the activator involvement in open complex formation that correlates with $\gamma$-P bond hydrolysis. To determine whether $\gamma$-P bond hydrolysis was needed for the activator-dependent relief of inhibition caused by Region I, we conducted transcript assays using GTP$\gamma$S. Results showed that $\gamma$-P bond hydrolysis was not needed for the inhibitory effect of Region I upon bypass transcription (lanes 3 and 4), but that it was clearly needed for relief of inhibition by the activator (lanes 9 and 10 versus lanes 7 and 8). We conclude that the anti-inhibition of Region I activity by the activator requires NTP hydrolysis (lanes 9 and 10 versus lanes 7 and 8), as does formation of the open complex (lanes 13 and 15).

Experiments with dGTP—Transcripts are synthesized with the wild type holoenzyme when dGTP is used as the hydrolyzable nucleotide by PspF$\Delta$HTH to form heparin stable open complexes (Fig. 5, lane 17), but dGTP does not support bypass transcription, as expected from the selectivity of RNA polymerase (lanes 5, 6, 11, and 12). In these assays, heparin resistance must be established before the addition of elongation substrates. Results confirm that heparin resistance in bypass transcription requires phosphodiester bond formation. The PspF$\Delta$HTH and dGTP combination did not stimulate stable complex formation by the $\Delta\sigma^{54}$-holoenzyme (lanes 5 and 11) or when Region I was in trans (lanes 6 and 12). We attempted to form stable activator-dependent noninitiated open complexes with the $\Delta\sigma^{54}$-holoenzyme plus Region I in trans using different combinations of activators and promoters, but none yielded any (data not shown). Region I in cis appears to be necessary for the formation of a heparin stable noninitiated open complex via the action of the activator.

Inhibition of Activated Transcription by Region I—We attempted to determine whether Region I interacted directly with the activator. Using PspF$\Delta$HTH and dGTP to drive stable open complex formation by the wild type holoenzyme, we sought an inhibitory effect of Region I supplied in trans to measure the extent to which providing extra activator would overcome the inhibition. Transcript assays were conducted in which $\sigma^{54}$-holoenzyme was pre-mixed with Region I and then added to template, after which activator and dGTP were added. Results (Fig. 6) showed that Region I reduced the activated transcript levels (see also Fig. 5, lanes 14 and 18). Plots of the levels of transcription in the presence and absence of Region I as a function of increasing activator concentration are shown in Fig. 6. The activator partially overcomes the inhibitory effect of Region I but does not restore transcription to the level obtained without Region I. Such behavior is consistent with a noncompetitive inhibition by Region I. The inhibition of transcription seen at a high activator concentration and in the presence of 0.5 or 1 $\mu$M Region I could mask relief of Region I-dependent inhibition; however, the 2 $\mu$M Region I data argue against this. Results suggest that Region I in trans is not interacting directly with the activator to cause inhibition, but that Region I is responsible for a conformational change in the holoenzyme that stabilizes the inhibited state. Lineweaver-Burk plots of PspF$\Delta$HTH concentrations versus transcripts levels were consistent with the inhibition being noncompetitive (data not shown).

**DISCUSSION**

The amino-terminal Region I of $\sigma^{54}$ is pivotal in establishing enhancer-dependent transcription in bacteria. It has multiple roles and is strongly implicated in influencing the activities of the sigma and its holoenzyme that likely work together for the ordered progression from a closed complex to an open complex. These activities include DNA interactions in the closed complex that involve the $\sim 12$ promoter element (18–20, 22, 34, 35), the nucleation of DNA melting and its associated fork junction binding by sigma (23, 36), the inhibition of polymerase isomerization (14), and an interaction with core polymerase (17). Our results now show that stabilization of $\Delta\sigma^{54}$-holoenzyme binding to melted DNA is also assisted by Region I sequences. Although not yet directly demonstrated, a stabilizing effect of Region I upon holoenzyme binding to a transition state of the DNA along the melting pathway would lower the activation energy for the strand separation process and increase the rates of open complex formation. The inability to detect stable open complex formation with holoenzymes lacking Region I may be attributable in part to the absence of stabilizing effects of Region I that help form and maintain the opened DNA.

It seems that Region I inhibits polymerase isomerization and engagement with melted DNA through a protein contact, consistent with the demonstration that Region I sequences bind to the core polymerase (17). Region I is also responsible for some interactions between the holoenzyme and the $\sim 12$ promoter element (18–20, 22, 34, 35), and because Region I stabilizes the holoenzyme on melted DNA, a Region I-melted DNA interaction is possible. It is likely that the protein and/or DNA interactions made by Region I are changed by the activator to allow polymerase isomerization and DNA melting to occur. Overcoming the inhibitory effects of Region I to allow polymerase isomerization appears to be one role played by the activator and its associated NTP hydrolysis. Because polymerase isomerization can be driven simply by the removal of Region I and reversed by its addition in trans, the energy costs appear to be modest for this step and can be accounted for in terms of interactions that Region I makes within the holoenzyme. For the $\sigma^{54}$-holoenzyme, the energy for isomerization is suggested to be equivalent to burying a few hydrophobic residues (37).
Region I of $\sigma^{54}$ has many leucine residues, some of which appear to be critical for keeping the holoenzyme in the inhibited state and could similarly be involved in isomerization (15, 24).

We were unable to form activator-dependent heparin stable open promoter complexes using Region I in trans to complement the defect in the holoenzyme assembled with $\Delta\sigma^{54}$. It is possible that the normal cis configuration of Region I restricts some interactions and favors others so that a sequential pathway for stable DNA melting can be followed. Supplied in trans Region I may have additional freedom to interact outside the normal reaction ordinal and thus fail to make stable activator-dependent complexes with the $\Delta\sigma^{54}$-holoenzyme. It is possible that Region I in trans cannot maintain a strained or stressed intermediate required for DNA strand opening. Stress generated in $\sigma^{54}$ by Region I may be transferred to DNA in a reaction that is part of the sequence of events that leads to stable DNA opening, as appears to be the case for the $\sigma^{70}$-holoenzyme (38).

An anti-inhibition activity of activator upon Region I supplied in trans was observed and suggests that a simpler set of Region I-dependent interactions is involved in this partial reactivation of the activation pathway. The ability of the activator to overcome the in trans inhibition was clearly dependent upon NTP hydrolysis, providing a formal demonstration that one function of the activator is to overcome the inhibition exerted by Region I and hence to allow polymerase isomerization. The noncompetitive inhibitory effect of Region I suggests that sequences in the holoenzyme outside of Region I contain determinants for a direct interaction with the activator.

Results suggest that Region I can be viewed as a protein domain that functions to control an equilibrium between alternate states of the holoenzyme by favoring either the closed complex (blocking polymerase isomerization) or the open complex (stabilizing holoenzyme on melted DNA). To explain the opposing effects of Region I, we suggest that Region I is involved in several different networks of protein-protein and protein-DNA interactions that maintain different conformations of the holoenzyme. In this model, the activator functions to change interactions so as to relieve inhibition and then allow other interactions to be established that more directly favor DNA melting by the holoenzyme.

Subunits of the bacterial core RNA polymerase have homologous counterparts in eukaryotic polymerases, and several subunits can represent different functional domains of a single bacterial protein (39). Separation of functions in this manner is commonplace among many different protein families. Although database searches indicate that Region I of $\sigma^{54}$ has no obvious homologue among other transcription factors, except for the fact that some others are also glutamine-rich, it is clear that Region I can function as an isolated domain to control RNAP activity. It is possible that the Region I domain originated as a separate factor to control RNA polymerase activity (it has positive and negative activities) and was recruited to form part of $\sigma^{54}$, enabling tightly regulated enhancer-dependent transcription.

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