**The Transcription Initiation Pathway of Sigma 54 Mutants That Bypass the Enhancer Protein Requirement**

**IMPLICATIONS FOR THE MECHANISM OF ACTIVATION**

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In vitro transcription, DNase I footprinting, and abortive initiation assays were used to characterize transcription using mutant forms of sigma 54 shown previously to bypass certain enhancer requirements in vitro. The holoenzymes containing these sigma mutants produce low levels of open complexes at both the glnAp2 and glnHp2 promoters. The open complexes are unusual in that they are destroyed by heparin. Enhancer protein and ATP convert them into a stable heparin-resistant state. The enhancer response occurs over a similar range of NtrC concentration as occurs with the wild-type holoenzyme, indicating that the activation determinants have been largely preserved within these mutants. One-round transcription assays show that the mutant holoenzymes can be driven to transcribe both promoters without NtrC. The unstable opening induced by these mutations apparently serves as a conduit that can shuttle templates into transcriptionally competent complexes. The results lead to a model in which activation occurs in two steps. First, the enhancer complex overcomes an inhibitory effect of the sigma 54 leucine patch and unlocks the melting activity of the holoenzyme. Second, different sigma 54 determinants are used to drive stabilization of the open complexes, allowing the full transcription potential to be realized.

Sigma 54 is necessary for transcribing a class of bacterial genes that depend on activators (such as NtrC/NRI) that bind and function from remote DNA sites (for reviews, see Refs. 1 and 2). These activator-binding sites can be moved to various locations and retain function, allowing them to be termed bacterial enhancers (see Ref. 3). This class of promoters is distinct from the promoters used by the sigma 70 family of proteins, which generally include at least one nearby regulatory DNA site (4, 5). Although the promoter classes are clearly distinct, sigma 54 and sigma 70 use the same RNA polymerase core for transcription. Thus, sigma 54 is thought to alter the holoenzyme so as to confer on the polymerase the property of enhancer responsiveness (6). This is facilitated by the unique amino acid sequence of sigma 54, which is unrelated to that of any other sigma factor (7). By contrast, all other sigma factors constitute a family of sequence-related proteins that form holoenzymes that do not respond to distant enhancers.

The mechanism by which the sigma 54 holoenzyme responds to enhancer-binding proteins has been studied in detail. One major aspect of the response that differs strongly from transcription by the sigma 70 family of proteins is an energy requirement. Thus, the activator must hydrolyze ATP and transfer this energy to the remainder of the transcription complex in order for the polymerase to function (8, 9). Prior to physiological activation, the inactive form of the holoenzyme can be bound to DNA, but cannot begin RNA synthesis (10) because the transcription start site is not melted (11, 12). In the activation event, activator-dependent ATP hydrolysis drives DNA melting and thus converts the inactive closed complex to an active open complex. As the enhancer sites are not directly adjacent to the promoter, the intervening DNA is looped out to enable the activator protein to contact the polymerase (13).

Because the RNA core polymerase that participates in this mechanism is the same one used to transcribe sigma 70-dependent promoters, it appears that this unique mechanism is imposed by association with sigma 54, i.e. the sigma 70 form of polymerase neither responds to enhancers nor needs ATP hydrolysis to melt promoter DNA (reviewed in Refs. 5 and 14). Recently, mutant forms of sigma 54 were isolated that can still associate with polymerase and direct its binding to DNA but that cause the polymerase to behave more like the sigma 70 holoenzyme in transcription (6). The mutations allow the polymerase to transcribe the glnAp2 promoter in vitro without the need for enhancer protein. The mutant holoenzymes can also transcribe without the need for hydrolysis of the β-γ bond of ATP.

Our prior study (6) indicated that changes within a short leucine-rich region of sigma 54 were important for allowing these energy and enhancer requirements to be bypassed in vitro. Two mutant forms of sigma 54 were studied, the double leucine mutant LS2633 and the triple leucine mutant HRS456 (6). Neither mutant requires ATP or the activator NtrC for transcription in vitro. Both mutant proteins allow the polymerase to catalyze detectable levels of glnAp2 promoter melting in the absence of enhancer protein and ATP. As assayed by permanganate probing, these levels are fairly low, 15% or less of what is attainable in a fully activated (presence of NtrC and ATP) wild-type complex. The combination of the enhancer-binding protein NtrC and ATP stimulates the low permanganate signal of the mutant holoenzymes, bringing it closer to the fully activated level.

The properties of these mutants should provide a substantial basis for learning how sigma 54 confers on polymerase the need for and ability to use enhancers and ATP. One complication relates to prior studies showing that some leucines in the region play a positive role in allowing sigma 54 to direct formation of closed complexes that fully use the –12 element of the glnAp2 promoter (15). Another complication is that the mutations also lie within a region that has been proposed to
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allow the positive response to enhancers, and it is not known if the changes alter the ability to respond properly. It is also necessary to further understand the relationship between the observed low levels of melting and the higher levels of in vitro transcription.

In this report, we investigate these and other issues by expanding the range of assays used to study these two mutant forms of sigma 54. These include direct assays for the formation of certain critical intermediate complexes along the transcription pathway: DNase footprinting assays for closed and open complexes and an abortive initiation assay for the extent to which the mutant holoenzymes can make the first mRNA bond. In addition, the ability of the mutant holoenzymes to transcribe the glnHp2 promoter was assayed to assess whether the mutants could function on the large class of sigma 54 promoters that depend on the DNA-bending protein integration host factor (IHF) (16–19). The results suggest an expanded model for the action of activators and for the role of the sigma 54 leucine patch in this process.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pYS1 (pGEM-3 vector, Promega) contains the glnA regulatory region and downstream coding region derived from pL1 (3) and was used for DNase I footprinting by primer extension procedures (20). Plasmid pTHS (21) was used for transcription and diffusion from gel only in the downstream sequence. Plasmid pFC50 (16) contains the glnH regulatory and promoter regions. All were obtained from B. Magasanik (Massachusetts Institute of Technology).

NtrC was purified as described (22), as was wild-type sigma 54 (23). The HRS456 and LS2633 forms of sigma 54 were found in inclusion complexes and an abortive initiation assay for the extent to which the mutations alter the ability to respond properly. It is also not known if these might be unstable and in equilibrium with closed complexes. In this experiment, we use a quantitative abortive initiation assay to further understand the relationship between the changes alter the ability to respond properly.

Abortive Initiation—RNA polymerase holoenzymes containing sigma 54 mutants HRS456 and LS2633 were studied previously under conditions where activator was absent (6). Per-manganate probing indicated that the mutations could trigger detectable but low levels of open DNA at the glnHp2 promoter start site. The amount of melting was thought to be <15% of the activated wild type, but the accuracy of this value was limited by the nature of the technique. The mutations allowed higher levels of heparin-resistant transcription to be achieved, but only using a preincubation with nucleotides to allow a short RNA chain to form. The results suggested that the mutations allowed the holoenzyme to form open complexes with DNA, but that these might be unstable and in equilibrium with closed complexes. In this experiment, we use a quantitative abortive initiation assay to test and expand these ideas.

Abortive initiation has been used to quantify the extent of open complex formation at promoters (27–29) and has been applied previously using holoheme containing wild-type sigma 54 and the activator NtrC (23, 25). The glnHp2 promoter start site sequence is UACC (positions −1 to +4). When the UpA dinucleotide and radioactive CTP are the only sources of ribonucleotide (dATP is present to support ATPase activity), the wild-type holoenzyme reiteratively synthesizes the trinucleotide UAC. As a control, Fig. 1 confirms that large amounts of the first RNA chain to form. The results suggested that the mutations allowed the trinucleotide to be inactivated at 37°C possibly due to aggregation, and so all procedures avoided lengthy preincubations. The concentrations of all proteins are nominal in that they are calculated from the absorbance.

Abortive Initiation—The reactions were similar to those described by Tintut et al. (25). 20-μl reactions contained a 5 m concentration of either a linearized DNA template pTHS (glnAp2) or linear template pFC50 (glnHp2). When pFC50 was used, 100 μJ IHF was included in the reaction and GpU was used in place of UpA as dinucleotide primer. Other components included in the reaction were 100 μM NtrC (when stated otherwise), 10 mM carboxyl phosphate (Resh; 26, Sigma), 100 mM glnA 54, 38 mM Escherichia coli RNA core polymerase (1 unit; Epicenter Technologies Corp., Madison, WI), 10 mM dATP, 0.5 mM unlabeled CTP, and 50 μM unlabeled CTP in buffer containing 50 mM HEPES, pH 7.9, 10 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 1 mM diithiothreitol, 50 ng of bovine serum albumin, and 5% (w/v) polyvinyl alcohol. As assembled on ice, and after the addition of labeled CTP, the reactions were incubated at 37°C for 20 min. For the heparin challenge reactions, the unlabeled CTP (25 μM) was present for the first 10 min of incubation, followed by heparin (25 μg/ml for 2 min). Labeled CTP (4 μCi + 25 μM unlabeled CTP) was added for the last 10 min. The reaction was stopped by the addition of an equal volume of urea-saturated formamide mixture (95% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromphenol blue). The products were separated on a 20% urea-polycrylamide gel (19:1 monomethacrylamide:bisacrylamide) and then visualized and quantitated by direct phosphorimaging.

DNase I Footprinting—20-μl reactions contained 200 ng of supercoiled DNA template pYS1 bearing the glnAp2 promoter, 200 nM NtrC, 10 mM carboxyl phosphate, 200 mM NaCl, 5 units of E. coli RNA core polymerase, and 4 mM ATP in the same buffer as used in the abortive initiation assay. All components were assembled on ice and then incubated at 37°C for 20 min. The remaining procedure was as described by Tintut et al. (25). Briefly, 2 μl of 0.5 μg/ml DNase I (Sigma) in 25 mM CaCl2, 50 mM MgCl2, and 1.2 X 10⁻⁴ M HCl was added for 30–40 s. The reaction was quenched with 40 μl of Tris-HCl, pH 8.0, + 2 μl of 0.5 M EDTA; extracted with 100 μl of phenol + 100 μl of chloroform; and heated to 90°C for 3 min. The aqueous phase was removed and passed through a Sephadex G-50 packed 1 cc syringe spin column equilibrated in water. 32P-End-labeled oligonucleotide primer Gdn4 (5′-TCAGTACGTTTGACCGG-3′) was added and extended with Tdq polymerase enzyme (Promega) in a thermocycler (MJ Research, Inc.) for 35 cycles. The entire extension product was separated by urea-polycrylamide gel electrophoresis. The dried gel was analyzed on a PhosphoImager (Molecular Dynamics, Inc.).

One-round Run-off Transcription—Supercoiled pTHS was used for glnAp2 transcription, and pFC50 linearized with EcoRI was used for glnHp2 transcription. The reaction conditions were as described for abortive initiation, except that 0.5 mM each ATP and GTP and 4 μCi of [α-32P]CTP (75 μCi/ml) were added (note UTP was omitted). The sequence of the glnAp2 template will enable the polymerase to elongate to position +20, where the first U is encountered. For the glnHp2 promoter, the dinucleotide GpU was added (position +2 is U), allowing ATP, GTP, CTP, and GpU to direct elongation to position +13. The reactions were incubated at 37°C for 10 min, and then 0.5 mM UTP and 25 μg/ml heparin were added to the reaction. The reaction was incubated 10 min further and was then quenched by the addition of urea-saturated formamide dye. The mixtures were electrophoresed on a 6% urea gel. The run-off transcript was then analyzed by phosphorimaging.

RESULTS

Abortive Initiation—RNA polymerase holoenzymes containing sigma 54 mutants HRS456 and LS2633 were studied previously under conditions where activator was absent (6). Per-manganate probing indicated that the mutations could trigger detectable but low levels of open DNA at the glnHp2 promoter start site. The amount of melting was thought to be <15% of the activated wild type, but the accuracy of this value was limited by the nature of the technique. The mutations allowed higher levels of heparin-resistant transcription to be achieved, but only using a preincubation with nucleotides to allow a short RNA chain to form. The results suggested that the mutations allowed the holoenzyme to form open complexes with DNA, but that these might be unstable and in equilibrium with closed complexes. In this experiment, we use a quantitative abortive initiation assay to test and expand these ideas.

Abortive initiation has been used to quantify the extent of open complex formation at promoters (27–29) and has been applied previously using holoheme containing wild-type sigma 54 and the activator NtrC (23, 25). The glnHp2 promoter start site sequence is UACC (positions −1 to +4). When the UpA dinucleotide and radioactive CTP are the only sources of ribonucleotide (dATP is present to support ATPase activity), the wild-type holoenzyme reiteratively synthesizes the trinucleotide UAC. As a control, Fig. 1 confirms that large amounts of this product form (see Ref. 25) when the activator NtrC is included in the reaction (lane 1). When NtrC is not included,
only a very low signal is seen (lane 4). This low signal is primarily due to nonspecific activity of the core polymerase (data not shown).

When the holoenzymes containing sigma mutants are assayed without NtrC, they show detectable levels of the same abortive initiation product (Fig. 1, lane 5 for HRS456 and lane 6 for LS2633). These levels are significantly higher than the low background signal seen for wild-type sigma without NtrC (lane 4). The data were analyzed using a PhosphorImager and normalized, taking the signal from the wild type with NtrC as 100% (lane 1). After subtracting the background signal of lane 4, the results show 11–12% signals for the two mutants (lanes 5 and 6). The 11–12% signal seen in these experiments is in good agreement with levels of promoter opening estimated from prior permanganate footprinting studies of these same mutant polymerases (6). Those prior studies also showed that the mutant holoenzymes could still respond to NtrC to yield greater levels of start site opening. This is also reflected in the abortive initiation assay, where strongly enhanced signals are observed in the presence of NtrC: HRS456 shows a signal comparable to that of the wild type (lane 2), and the LS2633 signal is increased to 65% (lane 3).

We suggested previously that the open complexes formed by these mutants might be unstable, i.e. in dynamic equilibrium with closed complexes (6). To test this directly, the abortive initiation reactions were allowed to proceed as described above for half the reaction time (10 min). At this time, heparin (25 μg/ml) was added for 2 min, followed by [α-32P]CTP for another 10 min. Any heparin-resistant complexes can then produce radioactive abortive initiation product during the final 10 min of reaction.

Fig. 2 shows that the open complexes formed by the HRS456 holoenzyme in the absence of the enhancer complex are largely heparin-sensitive (compare lanes 4 and 6 with heparin with lanes 3 and 5 without heparin). Heparin reduces the signal to close to background levels (lanes 7 and 8 without sigma), and quantitation indicates that at least 80% of the complexes are destroyed by heparin. Neither ATP alone nor NtrC alone can increase the signal significantly or induce resistance to heparin (lanes 4 and 6).

By contrast, when NtrC and ATP are added together, the complexes that form are largely resistant to heparin (Fig. 2, compare lanes 1 and 2). Quantitation shows that now only 25% of the complexes are destroyed by heparin. Even this 25% is likely an overestimate of the actual heparin sensitivity because the initial 10-min incubation is probably not long enough to allow all the complexes to reach the open complex stage. Overall, the heparin experiments indicate that open complexes formed by the HRS456 holoenzyme alone are likely in rapid equilibrium with heparin-sensitive closed complexes; NtrC and ATP enhance the stability of the open complexes, leading to heparin resistance.

Next, we use this protocol and an NtrC titration (10) to determine whether the mutant holoenzymes respond to the presence of activator in the same manner as does the wild type. The amount of abortive product observed for the wild type increases up to 100 nM NtrC (Fig. 3, filled circles). For the mutants, the curves originate at slightly elevated levels, reflecting their ability to catalyze some amount of abortive initiation in the absence of NtrC. Both curves rise roughly over the same range of NtrC concentrations as does the wild-type curve. This indicates that there are no strong defects in the ability of the mutant holoenzymes to respond to activator. However, the maximum level attainable by mutant LS2633 is only about two-thirds that of the wild type. This suggests that there is a defect in the ability of LS2633 to form open complexes that cannot be fully overcome by the addition of activator.

DNase I Footprinting—The HRS456 and LS2633 sigma mutants have been studied previously in vivo (15). The experimental context was in a background containing activator and over-expressed levels of sigma. In vivo dimethyl sulfate footprinting indicated that mutant LS2633 (and to a lesser extent, mutant HRS456) had certain defects compared with the wild type. Transcription levels were lower, and especially for LS2633, promoter recognition was impaired. We now use DNase I footprinting to assess promoter binding by the mutant holoenzymes in vitro.

At the glpAp2 promoter, DNase I footprinting can distinguish closed and open complexes by the extent of downstream protection (12, 30). Closed complexes can form without activator and ATP and protect an ~35-base pair segment upstream of the start site. This is confirmed for the wild type in Fig. 4A (lane 5, where the upstream protection is evident, compared with lane 8, which lacks polymerase). When NtrC and ATP are added, the protection extends farther downstream, as confirmed in Fig. 4A (lane 2).

When HRS456 and LS2633 polymerases were footprinted...
without activator, both mutants showed partial protection patterns. In both cases, the partial protection was restricted to the upstream region of the promoter (Fig. 4A, lane 6 for HRS456 and lane 7 for LS2633; both compared with template alone in lane 8). Thus, the predominant type of complex formed by the two mutant holoenzymes is similar to the complex that forms when wild-type polymerase forms a closed complex. The experiment also shows that the mutants form weaker closed complexes than does the wild type as the degree of protection in either of lanes 6 and 7 is less than that shown by the wild type in lane 5. Although not fully evident in the exposure shown in Fig. 4A, the amount of protection by LS2633 (lane 7) is significantly less than that seen using HRS456 polymerase (lane 6).

In the presence of NtrC and ATP, the HRS456 polymerase protection strengthens in the upstream region and extends into the downstream region (Fig. 4A, lane 3). The result confirms that open complexes have formed and that they have protection patterns similar to those formed by activated wild-type polymerase. The protection is somewhat weaker than that of wild-type polymerase (compare lanes 2 and 3). For LS2633, the footprinting pattern seen in the presence of NtrC and ATP (lane 4) is essentially that of an open complex, but with clearly reduced occupancy. The partial protection is fairly uniform over both the upstream and downstream regions (compare lane 4 with lanes 1 and 8 (controls)). By comparison with lane 7, the effect of NtrC and ATP is to extend the partial protection into the downstream region and to slightly increase the overall extent of protection.

These footprinting results lead to several conclusions. First, the mutant holoenzymes form closed and open complexes that cover the same regions as the wild type. Second, the mutants form mostly closed complexes without activator and ATP and mostly open complexes with these components. Third, the mutants show modest defects in promoter occupancy, especially LS2633, consistent with results of in vivo footprinting (15).

Another observation from the DNase I footprinting is that NtrC and ATP not only increase the downstream protection of HRS456 polymerase, but also increase the overall protection of the promoter. There have been prior suggestions that activators might have a secondary function independent of triggering the transition between closed and open complexes by the sigma 54 holoenzyme. In these "recruitment" models, activators can assist the holoenzyme to form closed complexes more readily (see Ref. 31). The model cannot be tested easily using typical footprinting conditions because they are such that closed complexes already form fairly efficiently in the absence of NtrC on the glnAP2 promoter. Thus, any recruitment effect of NtrC will be difficult to detect. To avoid this problem and to allow assessment of the recruitment model in this system, we established conditions that decrease the efficiency of HRS456 closed complex formation. This involves increased ionic strength (100 mM KCl instead of 10 mM KCl).

Under these conditions, closed complex protection by the HRS456 holoenzyme (Fig. 4B, lane 3) is weak. By contrast, the wild-type holoenzyme still forms closed complexes fairly well (lane 4). Both of these results pertain in the absence of activator. When NtrC, but not ATP, is included during formation of HRS456 closed complexes, there is no increase in protection (compare lanes 1 and 3; inclusion of ATP yields a normal open complex protection pattern in both case (data not shown)). We

Fig. 4. A, DNase I footprints of the wild-type, HRS456, and LS2633 holoenzymes at the glnAp2 promoter. Where NtrC phosphate is indicated, it was present at 200 nM and included 4 mM ATP. The open complex footprint is from approximately positions –34 to +23, and the closed complex is from approximately positions –34 to –2. The presence of various components is indicated. B, DNase I footprints using higher ionic strength to reduce occupancy. Conditions were as described for A, except with 100 mM KCl instead of 10 mM KCl in the reaction buffer.
infer that NtrC alone cannot significantly recruit RNA polymerase into closed complexes at the glnAP2 promoter.

In Vitro Transcription—The above results indicate that when either mutant holoenzyme is reacted with the glnAP2 promoter, an equilibrium mixture of mostly closed and some open complexes forms, along with some unoccupied DNA. We adapted a one-round transcription protocol (10) to assay the potential of the mutant holoenzymes to participate in a process that leads to transcription elongation. Several potentially complicating factors in prior conditions (6) are removed from consideration. Because only a single round of transcription is allowed, effects of sigma and NtrC on reinitiation will be eliminated (30). The protocol also minimizes kinetic differences as it simply collects those transcribing complexes that are heparin-resistant and then subsequently counts them by allowing each to elongate to a single transcript.

The transcription protocol starts with formation an equilibrium mixture of complexes. ATP, GTP, and CTP are added, allowing the polymerase to elongate to position +20 (Ref. 25; details are given under “Experimental Procedures”). A mixture of UTP and heparin is then added; heparin inactivates any polymerases that are not engaged (6), and UTP allows the engaged polymerase to finish its round of transcription. Fig. 5 shows that the mutant holoenzymes produce significant amounts of transcript (lanes 5 and 6 versus lane 4 (wild type)). Repeated experiments do not indicate any reproducible differences in the signal associated with LS2633 compared with HRS456. This is somewhat in contrast to modestly lower signals for LS2633 in both abortive initiation and footprinting. In the presence of 100 nM NtrC (the optimal amount from above) and ATP, all three polymerases produce roughly equivalent signals in this transcription assay (compare lanes 1–3). Repeated experiments indicate that there is no significant stimulation of signal by NtrC for either mutant in this one-round transcription assay. The detection of a low level of transcript using unactivated wild-type sigma (lane 4) is unexpected, and preliminary results suggest that it is a consequence of the use of supercoiled DNA and lower salt solutions.2

We infer from these results that, under the conditions of the one-round transcription assay, all complexes that are present may be driven by elongation substrates into complexes that produce transcript. As speculated previously, it appears that as open complexes are depleted by elongation, the predominant closed complexes are driven to open to restore the perturbed equilibrium. These too then elongate, eventually driving DNA into the elongation complex state. Thus, whether one starts with the highest numbers of open complexes and the least free DNA (wild-type holoenzyme with NtrC) or the lowest number of open complexes and the most free DNA (LS2633 holoenzyme without NtrC), the result is the same in this assay; DNA can be driven efficiently into productive transcription complexes in the presence of all four nucleoside triphosphates.

IHF-dependent glnHp2 Promoter—The above experiments have been done at the glnAP2 promoter, which is representative of one of the two classes of sigma 54-dependent promoters (see Ref. 4). The second class of promoters differs in that maximal transcription requires the function of the DNA-bending protein IHF. The IHF-binding sites of such promoters lie between the binding sites for polymerase and the enhancer. The mechanism of transcription initiation is believed to be similar in the two classes of promoters; IHF is thought to assist in looping DNA and to increase activator-enzyme contact (16). We wished to determine whether the mutant forms of sigma 54 could also direct activator-independent transcription at the glnHp2 promoter, which is a representative of this second major class of promoters (16).

A one-round transcription assay was adapted, for the initial transcribed sequence of GUCACCGU. Linear DNA, IHF, core polymerase, sigma 54, dinucleotide GpU, CTP, ATP, and GTP were present prior to the addition of heparin and UTP. Fig. 6 shows the transcripts observed from all three holoenzymes when NtrC is included in the reaction (lanes 1–3). When NtrC is not included, only mutants LS2633 and HRS456 produce transcript (lanes 5 and 6); the wild type does not (lane 4). The modest differences among the signal intensities seen in Fig. 5 were not seen reproducibly in several repetitions. Basically, the transcription results reflect those obtained using the non-IHF promoter glnAP2.

Fig. 7 shows that the mutant holoenzymes produce very low levels of abortive initiation product in the absence of activator. The signal for both LS2633 (lane 6) and HRS456 (lane 5) is greater than the background signal (lane 4), but is quite weak. This is not due to an inability of the system to produce abortive initiation products because high amounts can be produced when NtrC is added to the reaction (lanes 1–3). Quantitative analysis showed that, in the absence of NtrC, both mutant holoenzymes produced only ~3% of the abortive initiation product compared with the fully activated wild type. Apparently, the equilibrium mixture of complexes that form at the glnHp2 promoter includes fewer open complexes (3%) than glnAP2 (10–15%). However, even this exceptionally low level of open complex formation is sufficient to lead to substantial amounts of productive transcription in this one-round assay, as shown in Fig. 6.

DISCUSSION

The results presented here lead us to propose an expanded model for sigma 54 enhancer-dependent transcription. We will use the data obtained to propose a two-step model for activation, shown in Fig. 8 and developed below. In this model, when either mutation or the enhancer complex disrupts the leucine patch, only incomplete open complexes form. These must be activated again by the enhancer, in a step that involves different sigma 54 determinants, to become fully functional.

Two Steps in Activation—Several experiments show that the glnAP2 open complexes formed by leucine patch disruption are unstable in that they are in dynamic equilibrium with closed complexes. DNase footprinting and abortive initiation show

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that only approximately one-tenth of the complexes in the mixture are open. Even these open complexes can be destroyed by heparin treatment, in contrast to known properties of typical open complexes (10, 12). This confirms that they are in rapid equilibrium with the closed complexes present, which are known to be destroyed by heparin. Thus, we suggest that the leucine mutations overcome the barrier to opening the DNA, but fail to stabilize the open complexes once they form (Fig. 8, step 1).

The data indicate that the stabilization can be done by NtrC and ATP. Experiments show that activator and ATP increase the number of mutant open complexes. These open complexes are now largely heparin-resistant. Thus, although leucine patch disruption allows some opening to occur (Fig. 8, step 1), the enhancer function is still needed for optimal complex formation (step 2). Data in the literature show that open complexes formed by the wild-type holoenzyme in the presence of NtrC and ATP are long-lived (8, 30). Activator and ATP have an important role both in overcoming the initial energy barrier to melting (step 1) and in stabilizing complexes once the DNA is opened (step 2).

The data show that leucine patch disruptions lead to less opening at the HIF-dependent glnHp2 promoter. Because both glnHp2 and glnAp2 can be driven to transcribe well in vitro, it appears that the leucine disruption mutants work in vitro on both classes of sigma 54 promoters. The differences in the extent of opening at the two promoters probably simply reflect the number of mutant open complexes. These open complexes to elongate, templates are driven inevitably toward elongation as open complexes are progressively de-

This result emphasizes the importance of keeping the unactivated promoter tightly closed because, in this case of only 3% leakage into open complexes, there is a significant level of transcription in vitro. This is likely a major reason that a high energy requirement is built into the opening reaction (8).

Role of the N-terminal Region in the Two-step Model—The current data show that the leucine disruption mutants respond to NtrC over approximately the same concentration range as the wild type, implying that the leucines are not absolutely essential recognition determinants for NtrC. In vivo studies suggests that full induction may depend on other residues that are near the leucine patch (15). Thus, step 2 may depend on determinants that include the N-terminal glutamine-rich region.

The leucine mutants studied here had been previously characterized by mRNA analysis and in vitro footprinting in cells containing NtrC (15, 33). Both mutants were found to yield reduced mRNA amounts, with LS2633 being lower. The leucines changed in this mutant lie on a heptad repeat, the integrity of which is required for optimal protection of the −12 promoter element. This recognition defect, seen in vivo, probably accounts for the reduced levels of complex formation seen in the above in vitro experiments. Thus, the same residues appear to be involved in two properties of the closed complex: protecting the −12 promoter element and keeping the holoenzyme melting function in check. Support for this dual role comes from studies showing that nucleation of melting occurs within the −12 element (34).

How do these features work within the two-step model of Fig. 8 to allow the unique enhancer responsiveness of sigma 54-dependent promoters? We believe that a critical difference associated with sigma 54 transcription is a high barrier to accomplish step 1. The leucine patch appears to lock the holoenzyme complex in a form that is tightly closed. This keeps transcription to a minimum, even when the polymerase is fully bound to DNA. The bound holoenzyme provides an easy activation target for a looping enhancer protein. The absence of such a feature in sigma 70 means that it is difficult to construct a promoter in which polymerase can bind, but be prevented from transcribing until an activation signal is received.

NtrC and ATP unlock this inhibitory effect of the leucine patch, but the model suggests that this is insufficient for high level activation. Thus, NtrC and ATP are also needed for step 2, in which the unlocked open complex is stabilized. It is this step, which is not facilitated by leucine disruptions, that probably allows the highest transcription levels to be obtained in vivo. One unexpected aspect of the model is that it suggests that NtrC and ATP are required in two separate steps involving two separate determinants within the sigma 54 holoenzyme. We are currently attempting to validate this model and to determine what the determinants are and how they work.

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