Roles for the C-terminal Region of Sigma 54 in Transcriptional Silencing and DNA Binding*

Lei Wang and Jay D. Gralla‡
From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90095-1569

Twenty-one conserved positively charged and aromatic amino acids between residues 331 and 462 of sigma 54 were changed to alanine, and the mutant proteins were studied by transcription, band shift analysis, and footprinting in vitro. A small segment corresponding to the rpoN box was found to be most important for binding duplex DNA. Two amino acids, 52 residues apart, were found to be critical for maintaining transcriptional silencing in the absence of activator. These two activator bypass mutants and several other mutants failed to bind the type of fork junction DNA thought to be required to maintain silencing. The two bypass mutants showed a binding pattern to DNA probes that was unique, both in comparison to other C-terminal mutants and to previously known N-terminal bypass mutants. On this basis, a model is proposed for the role of the C terminus and the N terminus of sigma 54 in enhancer-dependent transcription.

Sigma 54 is unique among bacterial sigma factors with regard to both amino acid sequence and transcription mechanism. It is not a member of the sigma 70 family of proteins and is uniquely required to transcribe from enhancer-dependent promoters. As with most sigmas, promoter recognition involves two DNA sequence elements separated by a defined number of base pairs. Initially the holoenzyme binds the two sigma 54-specific promoter elements, termed −24 and −12 in reference to locations that include conserved nucleotides (1). The holoenzyme typically remains tightly bound until signal transduction leads to activation by a protein bound to a remote enhancer element (2−5). The holoenzyme is initially inactive, because it cannot open the DNA and engage the transcription start site; the enhancer protein overcomes this block, thus allowing the DNA to open and transcription to begin (reviewed in Ref. 6). The use of enhancers and the common regulation at the DNA melting step differentiates this class of holoenzymes from all others in bacteria (reviewed in Ref. 7). All sigma factors use the common core enzyme so these differences are solely attributed to the nature of the sigma factor.

In addition to binding duplex DNA, sigma factors bind single-stranded DNA and structures with single strand and duplex DNA juxtaposed (fork junction structures, Ref. 8). In the case of sigma 54, these latter interactions are central to control by the holoenzyme (6, 9−11). Within the inactive closed complex containing sigma 54 holoenzyme, a single base pair adjacent to the −12 recognition element is transiently melted (12). This provides a transient double-strand/single-strand fork junction structure. Interaction at this fork is repressive in the sense that the conformation of sigma bound to it helps keep the holoenzyme silent by blocking its ability to melt DNA. Activators can overcome this silencing by triggering conformational changes in both sigma and holoenzyme (6, 10, 11). Both the silent state and the active state rely on a complex network of interactions that centrally involve the promoter −12 element (9, 13−15). Interactions with the −24 elements are simpler and are the dominant factor in directing general DNA binding (16−18).

The motifs on sigma 54 that direct these various DNA interactions are not well established but appear to involve primarily N-terminal and C-terminal sequences. A short N-terminal region is required for regulation; numerous deregulated bypass forms of sigma have been identified with changes in the first 50 amino acids (19−22). Holoenzymes containing these mutant forms of sigma can transcribe in vitro in the absence of activator. These holoenzymes have also lost the capacity to bind the fork junction structure associated with the silent state of the closed complex. They have also gained an ability to interact with downstream single-stranded DNA (9).

By contrast, mutations in the C-terminal region can destroy general DNA binding and this property is not shared by N-terminal mutants (16, 18). Because the −24 interaction is dominant for DNA binding, it is presumed that the C terminus recognizes this element. The C-terminal region also contains determinants that are needed for activation of the holoenzyme (21, 23) and both ends of the protein have been proposed to participate in recognition of the −12 promoter element (17, 24). Recently a deregulated, bypass point mutation has been identified within the C-terminal region (23). It is obvious that the C-terminal region of sigma 54 has a particularly complex array of functions.

Subregions of potential importance within the C terminus have been identified. Among these are the following: a block of 10 almost completely conserved amino acids termed the rpoN box (25), a segment originally suggested to have the potential to form a helix-turn-helix (HTH, Ref. 26), and a segment that can be cross-linked to DNA (27). Collectively, these segments and others that have proposed functions (28) cover a region of 150 amino acids. This extensive region may contain activities that contribute to recognition of duplex DNA, fork junction DNA, and single strand DNA.

In studies of sigma 54 and sigma 70, the most prominent residues involved in DNA recognition are positively charged and aromatic amino acids (24, 28−32). The C terminus of sigma 54 contains many such residues, and these have a tendency to cluster within the motifs suggested to be important. To learn the role of these residues and to identify the array of functions

* This work was supported by National Institutes of Health Grant GM35754. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ To whom correspondence should be addressed. Tel.: 310-825-1620; Fax: 310-267-2302; E-mail: gralla@chem.ucla.edu.

This paper is available on line at http://www.jbc.org
within the C terminus we have mutated each of these residues individually, purified the mutant proteins, and characterized them using biochemical assays. The collection displays a rich array of properties, which allows the role of the C terminus in both regulation and DNA binding to be understood to a much greater extent.

**MATERIALS AND METHODS**

**Strains, Plasmids, and Site-directed Mutagenesis**—The plasmid pASS54, derived from expression plasmid pJF5401, carries the Escherichia coli sigma 54 gene (33). This plasmid was subjected to site-directed mutagenesis at the desired positions with QuikChange site-directed mutagenesis kit (Stratagene). The presence of the correct mutation was confirmed by sequencing. The strain E. coli YMC109 lacking a wild-type chromosomal copy of the sigma 54 gene was used as the host.

**Protein Purification**—Sigma 54 and its derivatives were partially purified by modified methods based on those described (13). In brief, 10 ml of Luria-Bertani medium with 100 μg/ml ampicillin was inoculated with YMC109 cells transformed with expression vector that carries the appropriate sigma 54 mutant. The cell culture was grown to OD of 1 OD at 30 °C with vigorous aeration. The culture was shifted to 43 °C and grown for another 3–4 h to induce expression. Cells were collected and suspended in 300 μl of buffer S (10 mM Tris-HCl, pH 8.0, 200 mM KCl, 0.1 mM dithiothreitol and 5% glycerol) and disrupted by sonication. After centrifugation of the cell lysate, the pellet containing the inclusion body was dissolved in 150 μl of buffer S plus 4 mM guanidine-HCl and 0.1% Nonidet P-40 (nonionic detergent). Sonication was used to help dissolve the pellet, which was then dialyzed, first against buffer S with 1 mM guanidine-HCl, then against buffer S, and finally against buffer S with 40% glycerol. After each dialysis, the undissolved material was discarded. The protein concentration was estimated on a SDS-polyacrylamide gel against known protein markers.

**In Vitro Transcription**—Standard one-round in vitro transcription was used as described previously (13). The activated transcription reaction mixture contained 100 nM NtrC, 45 nM sigma 54 holoenzyme (core RNA polymerase is from Epicentre Technology), 5 mM supercoiled DNA template pTH8, 10 mM carbamyl phosphate, 0.05 mM GTP, 0.05 mM CTP, 4 μCi of [32P]UTP, 50 μM unlabeled UTP, and 3 mM ATP in transcription buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM of bovine serum albumin, and 3.5% polyethylene glycol), in a total reaction volume of 10 μl. When NtrC is present, the reaction mixture was preincubated without GTP, UTP, and CTP for 20 min at 37 °C. Then, heparin was added to destroy any residual closed complex. [32P]UTP was added to initiate a round of transcription. No activator was included, and in control experiments one round of activated transcription with wild-type holoenzyme was used for comparison. The template contains a downstream terminator, permitting a discrete RNA to be assayed.

In this assay, wild-type and 19 of the 21 mutant proteins showed very low levels of activator-independent transcription, less than 5% of the amount produced by activated wild-type holoenzyme (Fig. 1). Two of the mutant sigmas give high transcription levels. R336A gives a 90% signal in this assay, consistent with expectations from the prior report (23). In addition K388A gives a high signal, at approximately the 50% level. We note that these residues are not close to each other. One is within the region that can cross-link to DNA, and the other is within or directly adjacent to the putative ITH motif. We also note that residues that are very close to each of these bypass mutants, such as Lys-331 and Arg-342 that surround Arg-336, and Arg-383, Tyr-389, and Arg-394 that surround Lys-388, do not show this property with any mutated. These properties and others to be assayed below are collected in Table I. We infer that silencing in the absence of activator is conferred by widely separated amino acids within the C terminus. In contrast to bypass mutants in the N terminus, the silencing determinants do not involve extensive adjacent stretches of amino acids.

**RESULTS**

We identified 21 highly conserved positively charged and aromatic amino acids within the 150 amino acid region from residue 319 to 469. Each of these was conserved in at least 18 of the 21 sigma 54 sequences available at the start of this study. They were changed individually to alanine by site-directed mutagenesis. The plasmids carrying mutated sigma 54 genes were transferred individually to a strain lacking endogenous sigma 54. The 21 mutants and the wild-type sigma protein were partially purified in 2 batches of 11 proteins each. In general the proteins were judged to be 50–80% pure. The single exception was R383A, which was poorly induced, possibly because of a folding defect, and thus was much less pure. As a comparison for this form of sigma a mock purification was done from a strain lacking plasmid and thus containing only contaminating proteins. The 22 forms of sigma were used in equal amounts for biochemical experiments, as judged from staining of the protein gels. The holoenzymes were formed using a 4:1 ratio of sigma to core.

**Activator-independent Bypass Transcription in Vitro**—Although the N terminus is the primary locus of in vitro deregulated bypass mutants, a recent in vitro study found this property to be associated with a C-terminal change (23). This mutant, R336A, is within the collection just described. To determine whether R336 is unique or belongs to a family that determines the requirement for activator, all altered sigma 54 holoenzymes were tested with a protocol for activator-independent transcription in vitro.

The activator-independent bypass transcription assay was done using plasmid pHTh8 that carries the gnaP2 promoter as a template (19). Holoenzyme, template DNA and ATP, GTP, and CTP were preincubated to allow the potential for initiation without activator. Then, heparin was added to destroy any residual closed complex. [32P]UTP was added to initiate a round of transcription. No activator was included, and in control experiments one round of activated transcription with wild-type holoenzyme was used for comparison. The template contains a downstream terminator, permitting a discrete RNA to be assayed.

In this assay, wild-type and 19 of the 21 mutant proteins showed very low levels of activator-independent transcription, less than 5% of the amount produced by activated wild-type holoenzyme (Fig. 1).
teins were assayed for transcription using a protocol that strictly depends on activator. In this protocol heparin is added after activator but prior to addition of initiating nucleotides. The experiment is done under optimal conditions for in vitro transcription, including 37 °C and high concentrations of all proteins. Under these conditions, transcription depends strictly on activator with very little bypass transcription. This stringency was shown in prior experiments using strong N-terminal bypass mutants (19) and is also true for the C-terminal bypass mutants just described (not shown). Fig. 2 shows the results of this activated transcription assay using the mutant holoenzymes. The data from several experiments is compiled in Fig. 2B, where the transcription level is normalized to that of the wild-type holoenzyme.

Under these conditions, 15 of the 21 mutant holoenzymes behave normally, giving a level of activated transcription not significantly different from wild-type. Of the six defective mutants, four are in the rpoN box region, K455A, R456A, Y461A, and R462A. A fifth mutant is R383A, which because of its low purity cannot be firmly assigned as being defective in activated transcription. The remaining mutant, F355A, is the least defective of the group (see also Ref. 30). We infer that residues within the rpoN box region are particularly important for obtaining a signal in the activated transcription experiment. Other segments of the C terminus seem to be less important in this assay. We note that the two mutants that gave bypass transcription in Fig. 1 are not in the rpoN box, suggesting that the determinants for silencing and those required to lead to activated transcription are at least partially separable.

In the next experiment, the efficiency of the activated transcription assay was lowered to see if secondary defects would turn up in residues outside the rpoN box region. This simply involved lowering the temperature from 37 to 15 °C. Under these conditions, several other mutants showed partial defects (Fig. 3). Now only 8 of the 21 mutants retained a transcription level of greater than 80% of the wild-type protein (Fig. 3B). The four mutants in the rpoN box that showed defects under optimal conditions were now even more defective. R383A and F355A were not much affected by the change in temperature. Seven mutants showed defects only in this assay, and they were not clustered in any region, being scattered between amino acids 336 and 460. It appears that residues throughout the C-terminal region play some role in activated transcription. These include Arg-336 and Lys-388, which play a primary role in silencing and now are seen to have their activated transcription levels reduced to half of the wild-type level under these suboptimal conditions. We note that the data makes no clear distinction between positively charged and aromatic residues; both types are among the six residues that are most defective and among the eight residues that show no defect in either assay.

DNA Binding—These data indicate that the rpoN box residues play a primary role in transcription. A secondary role is played by residues scattered throughout the C-terminal 150 amino acids. As closed complex formation is a prerequisite for transcription we next assayed duplex DNA binding using the 21 mutant holoenzymes. The stability of closed complexes is generally less than that of open complexes and so two different assays were used, DNase footprinting and a band shift analysis. A fragment of the nifH promoter, which contains the central consensus sequence of the −24 and −12 elements, was used in both assays.

Closed complex footprints are known to cover these two elements but yield incomplete protection; the interaction does not extend very far beyond the elements, in particular leaving the transcription start site unprotected (34, 35). Under the conditions of our experiment, the overall promoter occupancy with wild-type holoenzyme in closed complexes was 50–70% for the best protected positions in optimal experiments. As seen in Fig. 4, the footprints gave partial protection over the −12 and −24 elements (compare the two leftmost lanes in Fig. 4A) and no protection further downstream. We used three of the strongest bands to analyze the quantitative extent of protection using phosphorimager technology. Fig. 4A shows that position −1 is of equal intensity in the two leftmost lanes, without and with wild-type holoenzyme. This band is used to normalize the signal to ensure that binding by the 21 mutants is not improperly analyzed because of altered loading or extent of digestion. The degree of protection was judged by comparing the signals of mutants with wild type at the −21 and −10 band positions. That is, after normalizing the signal from each lane to equalize the −1 band intensity, the protection using bands at −21 or −10 was taken as the extent of binding in the closed complex. The data were not significantly different using the two bands, and the results using −10 are quantified in Fig. 4B. In this display, wild type is taken as 1.0 so the extent of protection by each mutant can be compared directly to wild type.

The most dramatic result is that each of four mutations in the rpoN box lead to by far the lowest extent of protection (R455A, R456A, Y461A, and R462A in Fig. 4B). R455A and R456A have been shown elsewhere to bind core polymerase and form holoenzymes, so this is not the source of the defect in DNA binding (31). These four mutants are the same ones that showed the greatest defect in transcription so the cause of this is very likely to be an inability to bind duplex DNA to form a closed complex. The fifth change in the rpoN box, K460A, is partially down in DNA binding, mimicking its partial loss of transcription under suboptimal conditions (compare Figs. 4B and 3B).

Other mutations, scattered about the C-terminal region, show partial reductions in closed complex formation using this assay. These partially defective mutants range over a nearly 150-amino acid region. This is roughly the same collection of mutants that showed partial defects in transcription under suboptimal conditions. Moreover, approximately one-third of the mutants show little evidence of defects, being at or near the levels of wild-type holoenzyme in both this assay and the sub-
optimal transcription assay. It appears that both binding and transcription have similar amino acid requirements.

Because protection within closed complexes is fairly weak, we sought to confirm these results using a band shift assay. At very high concentrations of holoenzyme most mutants bound DNA well (not shown), and so the concentration was lowered to 15 nM to reveal significant differences (Fig. 5). Two shifted bands could be seen, a lower one that was not very sensitive to protein concentration or to mutation and an upper one with an intensity that varied with the protein type and its concentration (Fig. 5A). The upper band was used in the quantitative analysis (see Fig. 5B).

For the most part, the result of the band shift analysis was consistent with that of the footprint analysis. There were only three exceptions; F355A, F402A, and R383A, which bound better in the band shift analysis than in the footprint analysis. These three mutants were partially defective in both assays, and the higher extent of binding seen in band shifts was better correlated with the extent of transcription under suboptimal conditions. To learn if the level of binding by R383A was specific, we compared it to a mock-purified protein preparation obtained from nontransformed cells (Fig. 5A, host). No upper band was present in the mock control, indicating that the R383A signal likely comes from the cloned sigma 54. However, because of the low purity of this protein and the uncertainty that misfolding is the cause of its low induction, we cannot be certain of the cause of the lowering of its binding to DNA.

These experiments were repeated at lower temperature (15 °C, not shown), but the results were not different. Four of five rpoN box mutants showed very little binding in both footprint and band shift assays. Several other mutants (K331A, K400A, F403A, R421A, and K460A), showed reduced binding in both assays. It appears that the rpoN box region is the most critical for DNA binding, but other residues throughout the C terminus make an important quantitative contribution to affinity. In many cases, the lowering of occupancy within closed complexes likely accounts for the lowering of activated transcription under suboptimal conditions.

| Sigma 54 | bypass trxn* | Activated trxn | DNA binding | T12 binding | T9 binding | supershift |
|----------|--------------|---------------|-------------|-------------|-----------|-----------|
|          | 37 °C        | 15 °C         |             |             |           |           |
| WT       | -            | +             | +           | +           | +         | +         |
| K331A    | +            | +             | +           | ++          | +/++      | +         |
| R336A    | +            | +             | +           | +           | +         | +         |
| R342A    | +            | +             | +           | ++          | +/++      | +         |
| F355A    | -            | +             | +           | +           | +/++      | +         |
| K363A    | -            | +             | +           | ++          | +/++      | +         |
| R383A    | -            | +             | +           | ++          | +/++      | +         |
| R388A    | +            | +             | +           | ++          | +/++      | +         |
| Y389A    | +            | +             | +           | ++          | +/++      | +         |
| R394A    | +            | +             | +           | ++          | +/++      | +         |
| F397A    | -            | +             | +           | +           | +/++      | +         |
| K400A    | -            | +             | +           | +           | +/++      | +         |
| Y401A    | -            | +             | +           | +           | +/++      | +         |
| R402A    | -            | +             | +           | +           | +/++      | +         |
| F403A    | -            | +             | +           | +           | +/++      | +         |
| R421A    | -            | +             | +           | +           | +/++      | +         |
| K425A    | -            | +             | +           | +           | +/++      | +         |
| R455A    | -            | +             | +           | +           | +/++      | +         |
| R456A    | -            | -             | -           | -           | -/++      | -         |
| K460A    | -            | +             | +           | +           | +/++      | +         |
| Y461A    | -            | +             | +           | +           | +/++      | +         |
| R482A    | -            | +             | +           | +           | +/++      | +         |

* trxn, transcription.
C-terminal mutants to this probe to learn if they retained the ability to recognize such junctions. The results of binding to the T12 fork probe are shown in Fig. 6B, lower panel. The data show that there is a very wide variation in the extent to which this probe is bound by the different mutants. 10 of 21 mutants showed nearly undetectable levels of binding. Eight of these are in a segment from amino acids 400 to 462 that contains the rpoN box but extends well beyond it. It is not surprising that rpoN box mutants would fail to bind probe T12 as they are defective in general DNA recognition. The extension of the fork junction binding defect into the region adjacent to the rpoN box is interesting. However, because these two mutants F403A and R421A have partial defects in DNA binding, we cannot assess how much of their defect in T12 recognition is specifically related to fork junction recognition.

The two other mutants that do not recognize the fork junction probe T12 are R336A and K388A. These are the two sigma mutants identified as bypass mutants in the activator-independent transcription assays described above. The failure of these C-terminal bypass mutants to bind this probe mimics the behavior of the N-terminal bypass mutants assayed previously (9, 36). The results indicate that failure to bind this probe is a common property of bypass mutants.

Sigma needs to retain the ability to recognize templates with single-stranded DNA near the start site to transcribe efficiently. We assessed this property by assaying binding to probe T9 (see Fig. 6A, Ref. 9). This probe contains the full −12 and −24 duplex elements but does not contain the tight binding fork junction. Most important, it contains single-stranded DNA from position −29 to +1. Fig. 6A, upper panel shows that many mutants have lowered levels of T9 binding compared with wild type (Fig. 6, left). The worst binders here are primarily the same mutants that failed to bind probe T12, probably because they are largely defective in recognition of the duplex part of the probe (discussed above).

However, the two striking exceptions are mutants R336A and K388A. The contrast is remarkable in that if one compares the upper and lower panels of Fig. 6B, these two mutants uniquely display strong T9 binding and a complete absence of T12 binding. Recall that these are the only two mutants that show the in vitro transcription bypass phenotype. That is, the bypass phenotype in this group of 21 mutants strictly correlates with a lost ability to bind the optimal fork junction while retaining the ability to recognize downstream single-stranded DNA.

Additionally, the mutant Y401A appears to have unique properties. It shows significantly reduced T9 binding but binds
reasonably well to T12 and very well to duplex DNA. T9 binding should rely critically on recognition of downstream single-stranded DNA. This suggests that the tyrosine might be a determinant that assists in recognition of the single-stranded DNA in the open complex.

These two C-terminal mutants share one property that is quite different from all the N-terminal bypass mutants that have been subjected to the same assay. N-terminal bypass mutants bind probe T9 much better than does wild-type protein (Ref. 9 and unpublished data). Fig. 6B shows that the C-terminal bypass mutants R336A and K388A do not share this property as their ability to bind T9 is essentially unchanged. Thus their defect is associated much more selectively with the loss of the ability to bind the T12 fork junction, as will be discussed below.

**Effect of Mutation on Response to Activator in a Band Shift Assay**—Recently, we developed a band shift assay in which sigma 54 holoenzyme-DNA complexes change their mobility in response to added activator (10). The altered complex appears not to contain activator but rather to represent an activator-induced conformational change (6, 10, 11). Because the change does not require ATP hydrolysis, it appears to represent an early step in response to the interaction with activator. Regardless of the details, this assay measures the capacity of the sigma 54 holoenzyme to make a response to activator. We used a high concentration of NtrC to allow it to act from solution (37) as the probe shown to be responsive lacks the activator NtrC binding site. The probe used, based on prior results, is shown in Fig. 7A. It contains two determinants of interest. One is the optimal fork junction, which terminates at base pair −12 and contains an unpaired bottom strand nucleotide (as discussed above). The other is the non-template (top) single strand, which is required for response to activator. Thus the probe could potentially be bound in at least two types of complexes, one involving the optimal fork junction and one resulting from a response to activator. We will assay each of the mutant holoenzymes to determine whether they have retained the ability to sense the presence of activator.

As shown in Fig. 7C, the wild-type holoenzyme binds this probe, and a new weak upper band is formed in response to activator (compare the two leftmost lanes; band indicated by the arrow). In the absence of activator, this upper band is absent for the wild-type and all mutant holoenzymes. Each mutant holoenzyme binds to this probe similar to the prior experiment using the closely related probe T12 containing only the optimal fork junction. In the presence of activator, most of the mutant holoenzymes give an upper band (Fig. 7B, upper panel). The exceptions are simply the rpoN box mutants; this is not surprising as they were found not to bind DNA in the prior assays. The other mutants that show partial defects in forming
this band are also partially defective in general DNA binding, so the data do not show specific defects in response to activator. Indeed the intensity of this new band correlates well with activated transcription levels under suboptimal conditions (compare Figs. 7B and 3). The amount of this band is always fairly small, probably because the activation system (activation from solution and probe mimics), is weak.

One aspect of the results is new and revealing. Several of these mutants (K460A, R421A, F403A, K400A, K388A, and R336A) bind the optimal fork junction very poorly (see the absence of a strong lower band in both B and C in Fig. 7). Nonetheless, each of these gives a normal amount of upper band in response to activator. This indicates that there is an uncoupling of events associated with two important processes, binding to the optimal fork junction and the response to activator, implying that the two have different determinants, which was not known previously. Below we put these results in context and attempt to ascertain the functions directed by the C-terminal domain of sigma 54.

**DISCUSSION**

A variety of biochemical phenotypes were observed when conserved positively charged and aromatic amino acids in the C terminus of sigma 54 were mutated. 16 lysines and arginines and 5 phenylalanines and tyrosines were changed individually to alanines. The strongest defects were associated with seven of these; three arginines, three lysines, and one tyrosine. Five failed to transcribe and four of these were located within the highly conserved rpoN box between amino acids 455 and 462. Footprinting and band shift assays indicated that the defect was at the level of DNA binding. With regard to regulation, no mutants were identified that bound DNA normally but failed to transcribe. However, two widely separated mutants, R336A and K388A, were found to be deregulated in that they lost the ability to keep the holoenzyme silent in the absence of activator. Band shift assays identified unique properties associated with these mutants that can account for their in vitro bypass transcription phenotype. Mutations in other residues within this 150 amino acid region showed detectable but less severe defects. Below we discuss the implications of these results for the mechanism of action of the sigma 54.

**Primary DNA-binding Determinants**—Data from both footprint and band shift assays indicate that mutations in the rpoN box are far more damaging to DNA binding than mutations elsewhere. Prior systematic studies of mutants defective in DNA binding have either excluded the rpoN box (28) or focused elsewhere in the C-terminal region. These other mutants also include several with regulatory defects. The construct suggests that the rpoN box may contact the −24 region with the rest of the C terminus contributing to the complex network of interactions that use the −12 region for both binding and regulation.

**Transcription Regulation Mutants**—Two clear cut regulatory mutants were found within the collection, R336A (found previously in Ref. 23) and K388A. These mutants transcribed at the 50–90% level in the absence of activator and fully activated transcription. Both showed minor defects in DNA binding and in transcription under suboptimal conditions. None of the other mutants showed any detectable tendency toward this activator bypass transcription. These 19 nonbypass proteins included those with a similar type of amino acid changed at locations near to bypass mutants. For example, R336A was a bypass mutant, whereas R342A and R331A were not, and K388A was a bypass mutant, whereas R394A and R383A were not. The data indicate that there is remarkable selectivity for these two well separated individual residues with regard to the ability to silence transcription in the absence of activator. This selectivity is in marked contrast to the bulk of bypass mutants, which reside in the N terminus; numerous changes throughout the N-terminal 50 amino acids can lead to the bypass phenotype (19, 20, 22, 33). The contrast indicates that the N terminus is a discrete regulatory motif, whereas the C terminus contains scattered residues that contribute to regulation.

No positive control or pure isomerization mutants were found that bound DNA normally but failed to melt it and transcribe. This supports the view that transcriptional regulation and aspects of DNA recognition may be inextricably linked. The linkage was proposed to occur through the −12 recognition element because certain promoter mutations there lead to bypass transcription (14, 15). Mutants with greater effects on isomerization than on binding have been reported (9, 19, 21, 38, 39), and some of the mutants identified here may fall in that category. Some of these are within the N terminus where there is at least one cluster of residues with a role in mediating activation (33, 36). Overall, it appears that both silencing and activation likely rely on a complex network of interactions involving the N-terminal regulatory module, residues scattered about the C terminus, and DNA sequences overlapping the −12 promoter element.

**Implications for the Mechanism of Silencing and Activation**—The data revealed unique binding properties of the two bypass mutants, and this has implications for the mechanism by which transcription is silenced prior to activation. Approximately one-half of the mutants showed very significant impairment in binding a probe (T12) that contained a double-strand/single-strand junction at position −12 and the bottom single-stranded fork. We suggested previously that binding to structures of this type is required to fully maintain the silenced state (6, 9). However, the data make it apparent that loss of binding to this structure is not sufficient to impose the bypass phenotype. Most of these mutants also show reduced ability to bind a probe (T9) that presents single-stranded bottom strand DNA but has the fork junction placed in a physiologically inappropriate location. There are only two exceptions, R336A and K388A, that fully retain T9 binding; these are the same two mutants that show the bypass phenotype. We note that other mutants such as those at positions 400, 403, 421, and 460, bind duplex DNA no differently from K388A but do not show bypass transcription. Thus it appears that selective loss of fork junction T12 binding by K388A and R336A is a prerequisite for bypass transcription. But in addition, a bypass...
simple determinants of the response to activator. Perhaps using the top strand of the fork. Thus the determinants for required for activation from those required for silencing (33). It has not been allow it to change as activation occurs.

The numerous other mutated residues, largely N-terminal, that fail to bind the T12 probe may be part of the large network of interactions that indirectly support this binding and allow it to change as activation occurs.

The changes that occur upon activation have been proposed to involve a switching of sigma binding preference from the bottom strand of the fork to the top strand (6). It has not been possible to cleanly separate the determinants on sigma that are required for activation from those required for silencing (33). The current data show that several mutants that have lost binding to the bottom strand fork structure still retain the ability to initiate a response to activator. This involves binding using the top strand of the fork. Thus the determinants for silencing and activation are not the same within the C terminus, and indeed it is still not clear if the C terminus contains simple determinants of the response to activator. Perhaps these determinants are primarily within the N terminus (19, 22, 33, 36), and changes within this regulatory module are transmitted to the C terminus, which appears to be in close physical proximity (40). This transmission would alter a complex structural network and switch the interaction with the –12 junction from the bottom to the top strand of the fork, which may be required to initiate activation.

Acknowledgments—We thank Yuli Guo and other members of the group for advice.

REFERENCES
1. Merrick, M. J. (1993) Mol. Microbiol. 10, 903–909
2. Reitzer, L. J., and Magasanik, B. (1986) Cell 45, 785–792
3. Morett, E., and Buck, M. (1989) J. Mol. Biol. 210, 65–77
4. Sasse-Dwight, S., and Gralla, J. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8938–8941
5. Popham, D. L., Sztco, D., Keener, J., and Kustu, S. (1989) Science 243, 629–635
6. Gralla, J. D. (2000) Nature Struct. Biol. 7, 530–532
7. Buck, M., Gallegos, M., Studholme, D., Guo, Y., and Gralla, J. (2000) J. Bacteriol. 182, 4129–4136
8. Guo, Y., and Gralla, J. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11655–11660
9. Guo, Y., Wang, L., and Gralla, J. D. (1999) EMBO J. 18, 3746–3756
10. Guo, Y., Lew, C., and Gralla, J. (2000) Genes Dev. 14, 2242–2255
11. Cannon, W. V., Gallegos, M.-T., and Buck, M. (2000) Nature Struct. Biol. 7, 594–601
12. Morris, L., Cannon, W., Claverie-Martin, F., Austin, S., and Buck, M. (1994) J. Biol. Chem. 269, 11563–11571
13. Wang, J. T., Syed, A., and Gralla, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9538–9543
14. Wang, L., and Gralla, J. D. (1998) J. Bacteriol. 180, 5626–5631
15. Wang, L., Guo, Y., and Gralla, J. D. (1999) J. Bacteriol. 181, 7558–7565
16. Sasse-Dwight, S., and Gralla, J. D. (1996) Cell 84, 945–954
17. Hsieh, M., and Gralla, J. D. (1994) J. Mol. Biol. 239, 15–24
18. Hsiieh, M., and Gralla, J. D. (1995) Science 270, 992–994
19. Syed, A., and Gralla, J. D. (1997) Mol. Microbiol. 23, 987–995
20. Kelly, M. T., and Hover, T. R. (1999) J. Bacteriol. 181, 3351–3357
21. Casaz, P., Gallegos, M. T., and Buck, M. (1999) J. Mol. Biol. 292, 229–239
22. Chaney, M., and Buck, M. (1999) Mol. Microbiol. 33, 1260–1269
23. van Slotten, J. C., Broughton, W. J., Hwang, W. C., and Stanley, J. (1990) J. Mol. Biol. 217, 5073–5074
24. Coppeed, J. R., and Merrick, M. J. (1991) Mol. Microbiol. 5, 1309–1317
25. Cannon, W., Claverie-Martin, F., Austin, S., and Buck, M. (1994) Mol. Microbiol. 11, 227–236
26. Guo, Y., and Gralla, J. D. (1997) J. Bacteriol. 179, 1239–1245
27. Fenton, M., Lee, S. J., and Gralla, J. D. (2000) EMBO J. 19, 1130–1137
28. Oguzia, J. A., Gallegos, M.-T., Chaney, M. K., Cannon, W. V., and Buck, M. (1999) Mol. Microbiol. 33, 873–885
29. Oguzia, J. A., Gallegos, M.-T., Chaney, M. K., Cannon, W. V., and Buck, M. (1999) Mol. Microbiol. 33, 1045–1054
30. Helmann, J. D., and deHaseth, P. L. (1999) Biochemistry 38, 5959–5967
31. Syed, A., and Gralla, J. D. (1999) J. Bacteriol. 180, 5619–5625
32. Cannon, W., Claverie-Martin, F., Austin, S., and Buck, M. (1999) Mol. Microbiol. 33, 287–298
33. Cannon, W., Missalildis, S., Smith, C., Cottier, A., Austin, S., Moore, M., and Buck, M. (1995) J. Mol. Biol. 248, 781–803
34. Kelly, M. T., and Hover, T. R. (2000) J. Bacteriol. 182, 513–517
35. North, A. K., and Kustu, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 65–77
36. Kelly, M. T., and Hoover, T. R. (2000) J. Bacteriol. 182, 513–517
37. North, A. K., and Kustu, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 65–77
38. Cannon, W., Gallegos, M. T., Casaz, P., and Buck, M. (1999) Genes Dev. 13, 357–370
39. Gallegos, M. T., and Buck, M. (2000) J. Mol. Biol. 297, 849–859
40. Casaz, P., and Buck, M. (1999) J. Mol. Biol. 285, 507–514

2 Y. Guo and J. Gralla, unpublished data.