Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2120–2124, March 1994
Biochemistry

RNA polymerase binding using a strongly acidic hydrophobic-repeat region of σ^{54}

(σ factors/transcription)

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Communicated by Boris Magasanik, November 22, 1993

ABSTRACT **σ^{54} is a rare bacterial protein that substitutes for σ^{70} in the case of *Escherichia coli* genes transcribed by certain activators with enhancer protein-like properties. It contains a strongly acidic region of previously unknown function. Gel mobility-shift assays using σ^{54} deletion mutants show that this region is essential for σ^{54} to bind the core RNA polymerase and recruit it to the promoter. Multiple-point mutational analysis shows that the acidic amino acids and overlapping periodic hydrophobic amino acids are necessary for this binding. Related sequences are not found within the core binding determinant of σ^{70}, which binds the same core RNA polymerase. This comparison suggests that the core RNA polymerase interacts differently with the two σ factors, likely contributing to the critical differences in transcription mechanism in the two cases.**

σ^{54} is a bacterial σ factor that directs core RNA polymerase to distinctive promoters for expression of genes involved in diverse metabolic functions (1, 2). σ^{54} is the only known σ factor that is not a member of the σ^{70} family of proteins (1–3). The transcription mechanism of σ^{54}-associated RNA polymerase is unusual in that it is similar to that of eukaryotic polymerase II in several regards (1–5). By contrast with σ^{70}-associated polymerase, promoters transcribed by the σ^{54}-associated polymerase all require auxiliary activators, which have enhancer-like properties (2, 5–9). Core promoter elements can be recognized at the σ^{54} polymerase and polymerase II promoters prior to the association of the RNA polymerases (4, 10–12). The association of the polymerase does not directly trigger transcription; in both cases ATP hydrolysis is required to open the start-site (4, 13, 14). By contrast, there are not yet examples of enhancer-dependent transcription or of ATP-dependent melting among the numerous promoters transcribed by the σ^{70} family of holoenzymes (2). Because the same core RNA polymerase transcribes from both types of bacterial promoters, these differing properties appear to be conferred on the polymerase by the different σ factors.

These differences may be related to a number of motifs in σ^{54} that are unusual for bacterial transcription factors but are more common in eukaryotic transcription factors (15). These include two hydrophobic heptad repeats; one overlaps a glutamine-rich region and the other overlaps a strongly acidic region. Because neither motif is present in the σ^{70} family of proteins (16) and because similar motifs exist in factors involved in mammalian enhancer-dependent transcription (17), it was suggested that they might be important for the unique function of σ^{54} (15). Previously, we reported that deletions that disrupt either of these motifs destroy the function of σ^{54} at the *glnA* P_2 promoter (15). The deletion mutants continued to protect the *glnA* P_2 promoter against dimethyl sulfate attack *in vivo* but failed to support open promoter complex formation. On this basis it was suggested that they cooperated to form part of a distinct activation domain on σ^{54} (15). The precise function of this hypothetical domain and the roles for the various motifs within it could not be specified.

In this report we establish a gel mobility-shift assay to assess the functional defects associated with these unusual motifs. The assay detects σ^{54} binding to DNA, known to occur at special promoter sequences in the absence of RNA core polymerase (10), and σ^{54} binding to the core polymerase itself (11). The results show that the strongly acidic region of σ^{54} is essential for the binding of RNA polymerase. Construction and analysis of point mutations showed that destruction of either multiple acidic or multiple hydrophobic residues within this region was accompanied by a severe loss of ability to bind RNA polymerase. These critical residues and the hypothetical structure they form are absent from σ^{70}; this suggests that RNA polymerase is bound quite differently by the two σ factors, which can account for the different mechanisms by which the polymerase forms transcription complexes at the two types of promoters.

MATERIALS AND METHODS

Escherichia coli strain YMC109^{tk} (*thi, endA, hsr,* Δ*lacU169, rpoN*::Tn*10*/F′ *pro lacI*^{q}*ZU118*, Tn*5-102*), lacking a wild-type chromosomal copy of the σ^{54} gene, was used as a host (18). The σ^{54} gene was introduced into the host via plasmid pTH7 (19) carrying either wild-type or mutant forms of σ^{54}. Mutants were made by oligonucleotide-directed mutagenesis with the mutagenesis system kit (Version 2.1) and the protocols recommended by Amersham. Purified σ^{54} and core RNA polymerase were from Felix Claverie-Martin and Boris Magasanik (Massachusetts Institute of Technology).

The functionality of the σ^{54} mutants was determined on W-Arg minimal medium (60 mM K_2HPO_4/33 mM KH_2PO_4/0.4% glucose/2 mg of thiamine per ml/0.4 mM $MgSO_4$/0.10 mg of arginine per ml/1.5% agar) plates as described (15). Briefly, overnight cultured cells grown in Luria broth (LB) medium supplemented with ampicillin (100 μg/ml), tetracycline (5 μg/ml), and kanamycin (40 μg/ml) were streaked on plates with W-Arg medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal, 40 μg/ml). The YMC109^{tk} cells carry a chromosomal *glnA* P_2-*lacZ* fusion, which allows the assay of σ^{54}-dependent β-galactosidase expression (18). The cells carrying wild-type σ^{54} gave blue-colored colonies on W-Arg/X-Gal plates, whereas cells without the expression plasmid did not grow. The results of the growth tests are shown below.

Gel Mobility-Shift Assay. Ten milliliters of cells harboring pTH7 were grown in LB medium supplemented with antibi-

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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otics as before at 37°C until OD_{600} reached 0.5 unit, at which time isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μg/ml. The cells were grown for an additional 60 min, chilled on ice for 10 min, and pelleted at 4°C. The cells were washed once in 1 ml of buffer B (20), resuspended in 100 μl of buffer B, and then sonicated for three 10-sec bursts on ice. After the sonication, 1 μl of 100 mM dithiothreitol was added, and cell debris was pelleted at 14,000 × *g* for 30 min at 4°C. The supernatant cell extracts were used immediately or occasionally stored in 40% glycerol at −20°C.

A ^{32}P-labeled 55-base-pair fragment carrying the sequence from −45 to +10 of *Rm nifH* promoter (21) was used as a probe. Briefly, the two 37-base-long oligomers with 19 overlapping bases were hybridized. The duplex was then labeled with [γ-^{32}P]ATP, the ends were filled with Klenow DNA polymerase, and the labeled probe was purified on a 12% native polyacrylamide gel.

Purified proteins were incubated with binding buffer [40 mM Hepes, pH 8.0/10 mM $MgCl_2$/100 mM KCl/1 mM dithiothreitol/0.1 mM EDTA/0.1 mg of bovine serum albumin per ml/5% (vol/vol) glycerol] at 30°C for 10–15 min before adding ≈3 pmol of the labeled probe. After 5 min of additional incubation, the samples were loaded onto 5% native polyacrylamide gel buffered with 50 mM Tris base/50 mM boric acid/1.6 mM EDTA (TBE). Electrophoresis was at room temperature at constant 200 V until the xylene cyanol dye in an adjacent lane reached the bottom of the gel. The unbound probe was run off.

One microliter of the crude cell extracts was incubated with the binding buffer supplemented with 0.01 μg of poly(dI·dC) per ml, 1 mM ATP (not required), and 3.5% (wt/vol) PEG at 30°C for 10–15 min. Labeled probe (≈3 pmol) was then added, and the samples were loaded onto a 5% native polyacrylamide gel. The enclosure surrounding the gel apparatus (self-enclosed Bio-Rad Mini-PROTEAN II) was bathed in an ice-water bath, and the electrophoresis was at constant 400 V until the xylene cyanol dye in an adjacent lane reached the bottom of the gel.

RESULTS

Gel Mobility-Shift Assay of Purified Proteins. The positions of protein complexes with a labeled *Rm nifH* promoter probe were established by using purified proteins. Fig. 1 shows that purified *E. coli* σ^{54} alone bound to the probe (lanes 1–3, band "S"). RNA polymerase (core) alone gave a faint higher mobility shift than σ alone, as expected based on its high molecular mass and ability to bind DNA fragments nonspecifically (lane 4, "C" arrowhead). When core RNA polymerase and σ^{54} were incubated together, the holoenzyme that formed led to the appearance of a higher supershifted band (lanes 5–7, "H" arrowhead). The holoenzyme bound to the probe at a lower concentration than did σ^{54} alone (compare lanes 1 and 5), consistent with the report that core plays a role in stabilizing DNA binding by σ^{54} (11). We conclude that the assay is capable of detecting σ^{54} interactions with both DNA and RNA polymerase core. On the basis of coimmunoprecipitation assays, these interactions have been suggested to be relatively weak (22).

Gel Mobility-Shift Assays of Wild-Type σ^{54} in Crude Cell Extracts. To facilitate the study of various σ^{54} mutants, we adapted the gel mobility-shift protocol for use in crude cell extracts. In this system, overexpression of σ^{54} is induced with IPTG (19). We confirmed that wild-type σ^{54} and its mutants are present in the extracts, using procedures described previously (23). Lane 3 of Fig. 2 shows the lower wild-type σ^{54}–probe band and the upper holoenzyme–probe band observed when using extracts made from cells induced with IPTG. The source of the splitting of the upper band into a closely spaced doublet is not known. In the absence of IPTG induction, only the upper holoenzyme band was observed (lane 2); no lower band was seen presumably because there is little free σ^{54} left to bind probe. When σ^{54} was completely absent (when using extracts from cells without expression plasmid), neither holoenzyme binding nor σ^{54} binding was observed (lane 1). Other comparisons in Fig. 2 confirm the specificity of the interaction. Unlabeled σ^{54} competitor DNA containing the σ^{54}-dependent *glnA* P_2 promoter titrated holoenzyme binding away from the labeled probe (lane 5), whereas the unlabeled σ^{70} promoter, *glnA* P_1 did not (lane 6). The binding of free σ^{54} to the probe was not lessened by the unlabeled σ^{70} promoter, as expected (lane 6). Unlabeled *glnA* P_2 promoter DNA lessened binding to the protein somewhat (lane 5), consistent with its lower binding affinity (6) compared with the *Rm nifH* probe (lane 4). Unlabeled *Rm nifH* (lane 4) competed effectively for both holoenzyme and free σ, as expected. In these extracts RNA polymerase core-alone binding was less prominent because the overproduction of σ^{54} led to titration of available RNA polymerase core. We conclude that crude extracts can be used to assess the ability of σ^{54} to bind DNA and to bind RNA polymerase core.

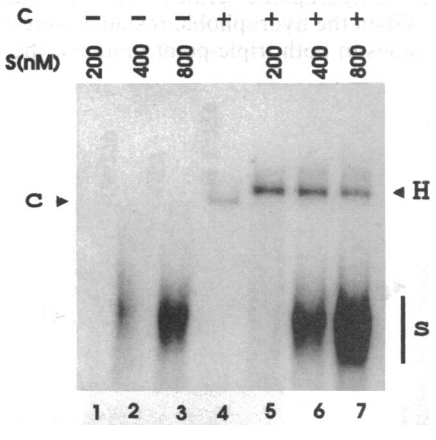

FIG. 1. Gel mobility-shift assay of the purified σ^{54} and RNA core polymerase to synthetic ^{32}P-labeled *Rm nifH* promoter. The nominal concentrations of σ^{54} ("S") are shown above the corresponding lanes. PEG was present at 28% (wt/vol) in lanes 1 and 2 and was reduced to a suboptimal 14% in lane 3 to keep the final volume constant. The concentration of core enzyme (C) in lanes 4–7 is 25 nM. H, C, and S refer to the holoenzyme, core enzyme, and σ^{54} binding positions, respectively.

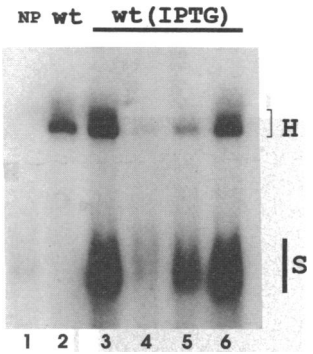

FIG. 2. Gel mobility-shift assay of wild-type σ^{54} in crude cell extracts. Lanes: 1, YMC109^{tk} cells with no plasmid (NP); 2, cells containing plasmid pTH7 carrying wild-type (wt) σ^{54} (19) before IPTG induction; 3–6, cells with plasmid pTH7 after IPTG induction. Unlabeled competitors were present in lanes 4–6—namely, σ^{54} promoter *Rm nifH* (0.35 μg/μl), σ^{54} promoter *glnA* P_2 (0.44 μg/μl), and σ^{70} promoter *glnA* P_1 (0.44 μg/μl), respectively.

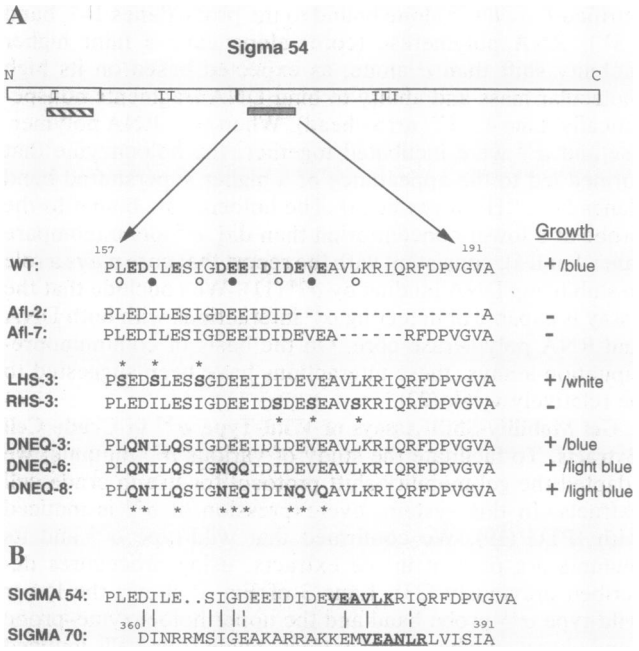

FIG. 3. (*A*) Amino acid sequence in single-letter code of acid-rich region that overlaps with hydrophobic heptad repeat. A schematic of the σ^{54} gene is shown. The hatched bar underneath the region I represents the glutamine-rich hydrophobic heptad repeat region, and the filled bar underneath the region III represents acid-rich hydrophobic heptad repeat region. The numbers above the amino acids represent the positions of the beginning and the end amino acids. Acidic amino acids of wild-type σ^{54} are shown in bold type. Amino acid residues that lie on the "a" and "d" positions of a hydrophobic heptad repeat are shown by the open and closed circles underneath. The deleted regions in the deletion mutants are indicated by the dashed lines, and the point mutations are indicated by the bold letters. Stars indicate the residues that are mutated. Viability and color of colonies resulting from σ^{54}-dependent β-galactosidase expression on W-Arg/X-Gal plates are also shown. (*B*) Sequence comparison (GAP alignment) of acid-rich hydrophobic heptad repeat region of σ^{54} and core binding determinant of σ^{70} (24). This best match is determined to be statistically not significant. The dots indicate the gap created by the GAP alignment program. The underlined residues in boldface letters indicate a possible but statistically insignificant similarity within a conserved σ^{70} sequence (16, 24).

Gel Mobility-Shift Assays of σ^{54} Deletion Mutants. These experiments were repeated with extracts containing mutants with previously reported deletions within the hydrophobic heptad repeat region overlapping either the glutamine-rich (Sal-58 mutant) or the acid-rich (Afl-2 mutant) (Fig. 3*A*) motifs of σ^{54} (15). It has been reported (15) that these deletions had the common effect of leading to a loss of recognition of the *glnA* P_2 (−12) promoter element recognition but retaining promoter binding through the (−24) element. It was recently reported that *in vitro* dimethyl sulfate footprinting of the Sal-58 mutant on the *Rm nifH* promoter showed the same protection pattern (11). The results show that σ^{54} alone binds to the probe used here (Fig. 4 *Left*, lanes 3 and 4, "S" bands), implying that the major σ–DNA interaction is through the −24 promoter region for both mutants (also true for the holoenzyme–DNA interaction; see below). The mobility of these mutant σ^{54}–DNA complexes differs from that of wild type. This may be due to altered conformations of the protein caused by the deletions.

However, the key result is that the Afl-2 mutant differed from the Sal-58 mutant in the ability to produce a supershifted band in the holoenzyme binding position. The Sal-58 mutant still produced holoenzyme capable of probe binding (Fig. 4 *Left*, lane 3, bands within H bracket), consistent with the known properties of this mutant in other assays (11, 15). However, the mobility shift of the Sal-58 holoenzyme was different from that of wild-type σ^{54} holoenzyme; this may be due to a different conformation assumed by Sal-58 holoenzyme after binding to the core (11). Mutant Afl-2 showed severe defects in the ability to produce a band in the supershifted position characteristic of holoenzyme binding (lane 4). Although this mutant σ failed to bind core RNA polymerase in this *in vitro* assay, previous *in vivo* dimethyl sulfoxide footprinting showed binding to DNA at the *glnA* P_2 promoter (15). This may indicate that σ^{54} alone can bind the promoter *in vivo* (see ref. 25). Elsewhere, we extended this experiment with a series of 16 deletion mutants covering most of the σ^{54} gene (25); only deletions in the *Afl* region show a loss of ability to bind core polymerase while retaining full ability to bind DNA as free σ.

Importance of the Acidic and Hydrophobic Residues for Core Binding. The region overlapping the *Afl* deletion is strongly acidic and contains a hydrophobic heptad repeat. To assess the contribution of these residues to the binding of the RNA polymerase core, we introduced a series of point mutations. These are shown in Fig. 3*A*.

First, we introduced multiple point mutations in hydrophobic residues. These converted residues in the two halves of this region to hydrophilic serines (see LHS-3 and RHS-3 of Fig. 3*A*). When the hydrophobic residues were converted to polar residues in both triple-point mutants, the ability of

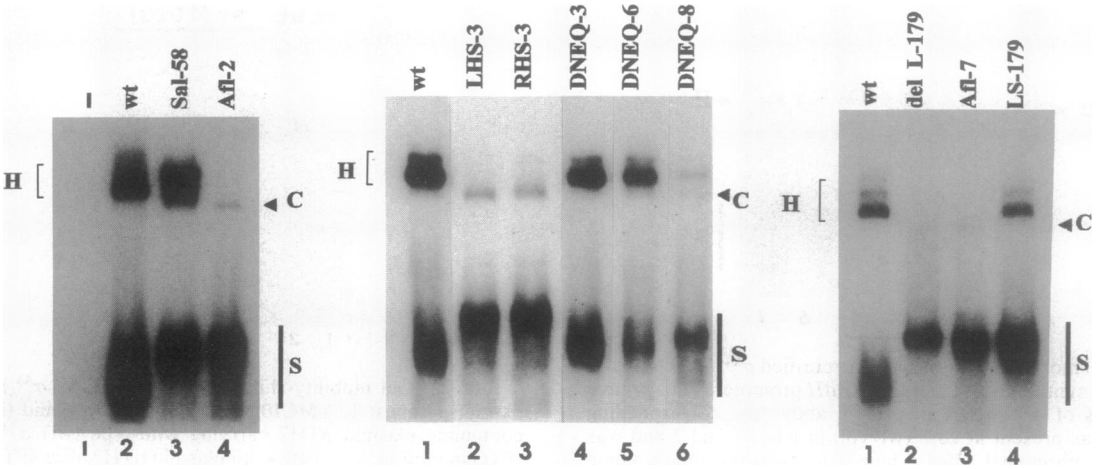

FIG. 4. Gel mobility-shift assay of σ^{54} mutants in crude cell extracts. Assays were done as indicated in Fig. 2. The type of σ^{54} is indicated above the lanes.

Table 1. Percent efficiency of complex formation relative to wild type

	σ–DNA	σ–pol–DNA
Wild type	100	100
DNEQ-3	115	65
DNEQ-6	40	40
DNEQ-8	60	10

The quantification of σ^{54} binding to DNA and core RNA polymerase (pol). The data are the average of four sets of experiments. The binding is normalized to wild-type σ^{54}.

σ^{54} to bind core polymerase was abolished (Fig. 4 *Center*, lanes 2 and 3; no bands in the H bracket). However, the binding of free mutant σ^{54} to the probe was retained (Fig. 4 *Center*, lanes 2 and 3, S bands), confirming that the hydrophobic residues are essential for binding of σ^{54} to core RNA polymerase but not necessary for binding to DNA. The prominent importance of the hydrophobic residues was confirmed by comparison with the triple acidic substitution (see below), which was far less damaging to core polymerase binding. Growth tests showed that the hydrophobic residues on the right side of the region were more critical for cell viability (Fig. 3*A*). The viability of the LHS-3 mutant was probably related to the overexpression of σ^{54} from the expression vector *in vivo*; a very small fraction of the overexpressed σ associated with core may support growth, although the colonies were small and white.

The other major feature of this region is the high concentration of acidic residues (Fig. 3*A*). We converted a number of these acidic residues to their corresponding amides and assayed the mutant proteins. Fig. 4 *Center* shows that as more acidic amino acids were mutated to the corresponding amide residues, the ability to form a supershifted holoenzyme band decreased. The loss of three or six acidic residues (mutants DNEQ-3 and DNEQ-6) led to a partial loss of holoenzyme formation (lanes 4 and 5 compared with lane 1). Eliminating the charge associated with eight residues (mutant DNEQ-8) led to a severe defect in formation of a polymerase–σ^{54}–DNA complex (H bands in lane 6 vs. lane 1). This loss by DNEQ-8 (but not necessarily by DNEQ-6) was primarily due to an inability to form holoenzyme because there was a reduction by a factor of 10 in holoenzyme binding accompanying a <2-fold loss of DNA binding (Table 1). The mobility of free σ^{54} binding to the probe varied somewhat among the mutants. The expression level of all DNEQ proteins was comparable (data not shown), indicating that the decreased holoenzyme formation by the DNEQ-8 mutant is not due to instability. The retention of viability but loss of dark-blue color by DNEQ-8 indicated that the residual holoenzyme formed from the overexpressed σ^{54} is somewhat functional.

The previously studied nonviable small deletion mutant Afl-7 (15) lies just to the C-terminal side of the acidic region (see Fig. 3). This mutant also failed to bind RNA polymerase (Fig. 4 *Right*, lane 3). Although Afl-7 covers one member of the hydrophobic repeat (L179), changing this residue to serine had no effect on polymerase binding (Fig. 4 *Right*, lane 4) or viability. However, a single deletion mutant (Leu-179) did eliminate polymerase binding (Fig. 4 *Right*, lane 2) and viability, suggesting that the register between the regions that flank Leu-179 may be important.

DISCUSSION

These results show that a region of σ^{54}, previously identified as potentially interesting based on its resemblance to mammalian activation domains (15), functions to bind RNA polymerase. This region may be represented as a helical wheel in which there are opposing hydrophobic and acidic surfaces (15). Analysis of point mutations shows that both types of residues are necessary for RNA polymerase binding to σ. Further analysis will be needed to learn the structure of this region and the role of each type of residue in interacting with the polymerase. Nonetheless, it is interesting to note that acidic motifs can play a critical role in eukaryotic pol II transcription (26–28), and discussions focus on their ability to interact with other proteins (29–32).

σ^{54} and σ^{70} participate in divergent transcription mechanisms but bind the same core RNA polymerase (4). To assess the similarity in sites that specify polymerase binding, we compared the σ^{54} site with the proposed core polymerase binding site on σ^{70} (24). The GAP alignment program of the University of Wisconsin Genetics Computer Group (UWGCG) shows no significant match between the two sequences (the best alignment is shown in Fig. 3*B*). We note also that the σ^{70} region contains neither multiple acidic residues nor a hydrophobic heptad repeat, both of which are necessary for σ^{54} binding to polymerase. The adjacent C-terminal residues may be somewhat similar to residues conserved in the σ^{70} family (16, 24) (see the underlined boldface residues in Fig. 3*B*), although the match is statistically insignificant. Because deletions within this segment destroy core polymerase binding, it is possible that this small sequence adjacent to the acidic hydrophobic region plays a role in polymerase binding; but overall, the sites appear to be remarkably different, not only in sequence but also in having large but opposing charges.

The marked dissimilarity in the binding sites suggests that σ^{54} and σ^{70} interact with RNA polymerase differently, which may account for the very different properties of the polymerase in the two types of transcription complexes. The σ^{70} element is largely conserved within the large family of σ^{70}-like proteins, suggesting that these bind polymerase similarly (16, 22). Part of this binding involves the unmasking of the DNA-binding domain of σ^{70} (33), which is not the case with σ^{54}, where DNA binding can occur in the absence of core polymerase (10, 11). The acidic–hydrophobic repeat binding region used by σ^{54} is different enough to imply that it interacts with a region of the core polymerase different from that used by σ^{70}. These different ways of binding polymerase could conceivably be related to the unique propensity of the σ^{54} polymerase to bind in a stable, transcriptionally inert, closed complex prior to activation (6, 13, 34, 35), which has been proposed as important for its enhancer-dependent transcription. σ^{54} and σ^{70} differ in many aspects; it is possible that σ^{54} uses its unique core-binding determinants to position polymerase so as to interfere with its melting and transcription function within the closed complex. In this regard it is interesting to note that the electrostatic properties of the acidic region of σ^{54} identified here mimic the acidity of known polyanionic inhibitors of polymerase, such as heparin and single-stranded DNA (36). Activators might relieve such σ^{54}-specific inhibition, contributing to the unique mechanism of σ^{54}-dependent transcription.

We thank Drs. F. Claverie-Martin and B. Magasanik for providing purified proteins, Dr. M. Buck for sharing his data before publication, and Drs. D. Eisenberg and J. Gober for their valuable comments on the manuscript. This work was supported by National Institutes of Health Grant GM35754; Y.T. and C.W. were trainees under Training Grant GM07185.

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