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The Helix-Turn-Helix Motif of σ^{54} Is Involved in Recognition of the −13 Promoter Region

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Residue Arg-383 in the proposed helix-turn-helix motif of the novel RNA polymerase sigma factor σ^{54} has been changed by site-directed mutagenesis to all possible alternative amino acids. Only two mutants, RK383 and RH383, are active in promoting transcription from either the *glnAp*$_2$ promoter or the *nifL* promoter. We constructed a set of mutant derivatives of *glnAp*$_2$ such that each base in the conserved GG and GC doublets at −24 and −12 was changed to all possible alternatives. All 12 mutant *glnAp*$_2$ promoters showed a marked promoter-down phenotype with wild-type σ^{54}, but RK383 suppressed changes of both G to C and G to T at −13. This result suggests that the σ^{54} helix-turn-helix is involved in recognition of the −13 region of σ^{54}-dependent promoters.

The promoter specificity of bacterial RNA polymerases is determined by the sigma subunit present in the holoenzyme. Sigma factors can be divided into two families, a major family related to the primary vegetative sigma factor, i.e., σ^{70} in *Escherichia coli* and σ^{A} in *Bacillus subtilis* (13, 16), and a minor family of which σ^{54} is presently the only member (26, 29, 30).

RNA polymerase-containing σ^{54} ($E\sigma^{54}$) is required for transcription of genes whose products have diverse physiological functions. σ^{54} was originally recognized in the enteric bacteria (17, 18), but homologs of σ^{54} have since been described in at least 12 bacterial genera, including both gram-negative and gram-positive species. Many features of σ^{54} indicate that it is neither structurally nor functionally closely related to the σ^{70} family, and studies of the regulation of a number of σ^{54}-dependent genes have suggested an underlying common mechanism of transcription initiation (23). $E\sigma^{54}$ bound to promoter DNA can isomerize from the closed to the open complex only in the presence of an activator protein that binds to specific sites upstream of $E\sigma^{54}$ and has an ATPase activity necessary for open complex formation (4, 35, 46).

All promoters recognized by holoenzymes containing σ^{70}-related sigma factors have two blocks of conserved sequences located at about 10 and 35 bp upstream of the transcription start, and these sequences are unique to the particular sigma factor (13, 38). By contrast, $E\sigma^{54}$ recognizes a promoter sequence with highly conserved GG and GC doublets 24 and 12 bp, respectively, upstream of the transcription initiation point (5, 32); furthermore, unlike σ^{70}, σ^{54} is able, in certain cases, to bind specifically to promoter DNA in the absence of core RNA polymerase (7).

Comparisons of the amino acid sequences of sigma factors in the σ^{70} family have identified four major regions of homology, two of which have been implicated in recognition of the −35 and −10 promoter regions. In each case, genetic experiments have demonstrated that modifications of the amino acid sequence in one of these regions allow the sigma factor to direct RNA polymerase to an altered promoter sequence. These studies suggest that a helix-turn-helix (HTH) motif in conserved region 4 is involved in recognizing the −35 region of the promoter (12, 42) and that a putative α helix located in region 2.4 is involved in recognizing the −10 region of the promoter (10, 42, 44, 48).

σ^{54} shows no significant homology to the σ^{70} family in its primary amino acid sequence; however, alignment of the sequences of the 14 σ^{54} homologs available to date indicates a high degree of sequence conservation and is consistent with the three-domain structure proposed by Merrick et al. (29). The polypeptide has a highly conserved glutamine-rich N-terminal domain of some 50 amino acids followed by a poorly conserved but notably acidic domain which varies in length from about 25 to 100 amino acids. The C-terminal domain is approximately 380 amino acids in length, with significant sequence conservation throughout. The domain contains two particularly notable motifs: a potential HTH motif near the carboxy terminus of the protein and an even more C-terminal block of eight almost totally conserved amino acids (9, 29). The HTH motif has been proposed both on statistical grounds (i.e., when compared with known HTH structures in other DNA-binding proteins, this region shows a very significant likelihood of conforming to such a motif [9, 11]) and on genetic grounds (i.e., the phenotypes of mutants altered in this region are consistent with such a structure [9]). Sasse-Dwight and Gralla (40) proposed a structure-function model for σ^{54} in which they suggested that the N-terminal region of the protein forms a novel intramolecular leucine zipper structure involved in recognition of the −12 region of the promoter and that an HTH motif at the C terminus facilitates interaction with the −24 promoter region.

Cassette mutagenesis of the HTH motif has shown that single amino acid changes in the predicted recognition helix (helix 2) can both impair and modify promoter recognition (9). We have therefore focused on this region in an attempt to identify which promoter element is recognized by the HTH. We have shown that a specific alteration (RK383) of a residue predicted to be surface exposed in the recognition helix of the HTH suppresses the promoter-down phenotype of two mutations at the −13 position in the *glnAp*$_2$ promoter. We therefore conclude, in contrast to Sasse-Dwight and Gralla (40), that the HTH plays a role in recognition of the −13 promoter region.

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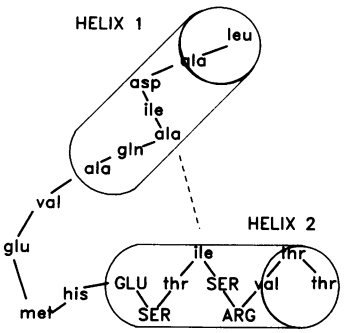

FIG. 1. Proposed HTH motif of *K. pneumoniae* σ^{54}. The predicted exposed residues in helix 2 are shown in capital letters.

MATERIALS AND METHODS

Site-directed mutagenesis. Cassette mutagenesis of *Klebsiella pneumoniae rpoN* was carried out as described by Coppard and Merrick (9). All mutants were sequenced across the region of the introduced cassette, i.e., the region encoding Met-376 to Ser-386 (9), to identify the amino acid substituted for Arg-383. As cassette mutagenesis ensures that the rest of the *rpoN* gene is not modified in any way, we can be sure that each of the mutant sigma factors contains only the stated single amino acid substitution. Oligonucleotide-directed mutagenesis of $glnAp_2$ was carried out by using either the two-primer method of Zoller and Smith (47) or a polymerase chain reaction-based method described by Landt et al. (25). The DNA template was pMM135, which carries $glnAp_2$ and three weak upstream binding sites for NtrC (9). pMM135 was constructed by cloning the 242-bp *Eco*RI-*Bam*HI fragment from pMM132 into pTZ19.

Measurement of the activity of mutant σ^{54} proteins. Protein activity was measured exactly as described previously (9). In all cases, the strain used was *K. pneumoniae* UNF2792 (*hisD2* Δ*rpoN71*::*kan recA56 sbl300*::Tn*10*) carrying *rpoN* on a derivative of pMM83 and with either pMM132 for $glnAp_2$-*lacZ* or pRD554 for *nifLp-lacZ*.

In vivo dimethyl sulfate (DMS) footprinting. Footprinting was carried out as described previously (9) except that NFDM was supplemented with 200 μg of glutamine per ml rather than 1 mg of glutamine per ml. Densitometry was carried out on a Molecular Dynamics computing densitometer.

RESULTS

Mutation of Arg-383 in the σ^{54} HTH motif. The proposed recognition helix of the σ^{54} HTH motif has four potentially surface-exposed residues which could make specific contacts with base pairs in the promoter. These residues are Glu-378, Ser-379, Ser-382, and Arg-383 (Fig. 1). Among the mutants isolated in a previous mutagenesis of this motif, we obtained alanine substitutions at each of these positions, and all except RA383 were significantly active. In the cases of Glu-378 and Ser-382, a variety of different amino acids could be introduced at these positions without dramatically impairing function. In contrast, among the mutants studied, Ser-379 could be substituted only by Thr or Ala to give an active σ^{54}, and Arg-383 was not replaceable with any of seven other residues tested. The inactivity of RA383 led us to consider the possibility that the arginine residue at this position is absolutely required to allow σ^{54} to interact with the promoter, and we therefore made all possible substitutions at this position.

TABLE 1. Effects of amino acid substitutions at Arg-383 of σ^{54} on expression from $glnAp_2$ and *nifLp*

Promoter	Activity (%) with amino acid substitution[a]:																		
	A	G	S	T	I	L	V	M	F	Y	E	D	N	Q	H	K	W	C	P
$glnAp_2$	4	5	5	5	9	5	5	8	5	6	4	8	8	8	**16**	**97**	5	7	7
nifLp	2	2	3	2	2	2	2	2	2	2	2	3	3	2	**5**	**28**	2	3	3

[a] Data are means of at least three experiments, expressed as percentage of the β-galactosidase activity from the relevant fusion in the presence of wild-type σ^{54}. The background level of expression observed in the absence of σ^{54} is 4 to 7% with $glnAp_2$ and 2 to 3% with *nifLp*.

All 19 R383 mutants were assayed for the ability to promote transcription from two σ^{54}-dependent promoters, $glnAp_2$ and *nifLp*, using translational *lacZ* fusions. Only two mutants, RK383 and RH383, showed levels of activity which were significantly above background, RK383 having 97 and 28% of wild-type activity on $glnAp_2$ and *nifLp*, respectively, and RH383 having 16 and 5% activity (Table 1).

As judged by DMS footprinting, the two promoters used differ significantly in the ability to form a closed complex with $E\sigma^{54}$. Hence, whereas a complex can easily be detected at $glnAp_2$ (39), a comparable footprint is not detectable at *nifLp* (31). Despite the fact that RK383 supports significant activity at $glnAp_2$, in vivo DMS footprinting did not detect an interaction between $E\sigma^{54}$ RK383 and this promoter under conditions in which wild-type $E\sigma^{54}$ shows clear protection of the −24 and −12 regions (Fig. 2). Hence, the mutation apparently increases either the dissociation constant for $E\sigma^{54}$ and $glnAp_2$ or the facility with which $E\sigma^{54}$ responds to the activator.

Mutations in conserved residues of $glnAp_2$. To investigate possible interactions between helix 2 of the σ^{54} HTH motif and conserved bases in the −24 or −12 region of the promoter, we constructed a set of mutant derivatives of $glnAp_2$. The conserved residues at −25, −24, −13, and −12 were altered sequentially to give 12 mutant promoters in which each of these four bases was changed to all possible alternatives. These mutations were introduced into the $glnAp_2$-*lacZ* fusion vector pMM132, and the effects of the mutations were analyzed in the presence of wild-type $E\sigma^{54}$. All of the mutations produced a marked promoter-down phenotype (Table 2). All changes to −25G, −24G, and −12C

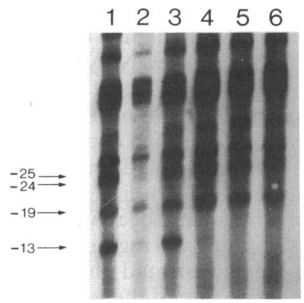

FIG. 2. DMS footprinting of $glnAp_2$ in vivo. Lanes: 1 and 2, *K. pneumoniae* UNF2972(pMM132) Δ*rpoN71*::*kan*($glnAp_2$-*lacZ*) carrying no *rpoN* plasmid (lane 1) and wild-type *rpoN* (lane 2); 3 to 6, UNF2972(pMM83-RK383) Δ*rpoN71*::*kan*(*rpoN*-RK383) carrying wild-type $glnAp_2$ (lane 3), $glnAp_2$ −13GA (lane 4), $glnAp_2$ −13GC (lane 5), and $glnAp_2$ −13GT (lane 6).

TABLE 2. Activities of mutant $glnAp_2$ promoters with wild-type σ^{54}

Substituted base	Activity (%) with wild-type residue[a]:			
	−25G	−24G	−13G	−12C
A	12	10	6	12
C	12	8	14	100
G	100	100	100	12
T	11	9	26	14

[a] Expressed as percentage of the wild-type $glnAp_2$ promoter activity (β-galactosidase units) in *K. pneumoniae* UNF2792(pMM83)(pMM132). The background level in the absence of σ^{54} is equivalent to 4 to 7%. Data are based on four or more determinations in each case.

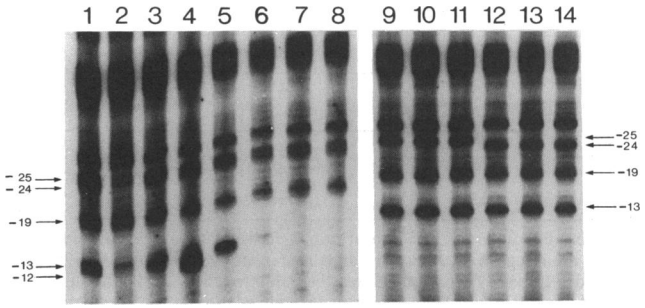

FIG. 3. DMS footprinting of the $glnAp_2$ promoter in vivo. Lanes: 1 and 2, *K. pneumoniae* UNF2972(pMM132) Δ*rpoN71*::*kan*($glnAp_2$-*lacZ*) carrying no *rpoN* plasmid (lane 1) and wild-type *rpoN* (lane 2); 3 to 14, UNF2972(pMM83) Δ*rpoN71*::*kan*($rpoN^+$) carrying the following mutant derivatives of pMM132: $glnAp_2$ with A, G, and T at −12; A, C, and T at −13; A, C, and T at −24; and A, C, and T at −25, respectively.

were equally deleterious and reduced expression to background levels comparable with those obtained in the absence of σ^{54}. By comparison, mutations at −13G had more varied phenotypes; the activities of −13GA and −13GC were near the background level (with −13GA reproducibly less active than −13GC), whereas −13GT was noticeably more active (Table 2).

Each of the mutant promoters was examined by in vivo DMS footprinting in the presence of wild-type Eσ^{54}. Whenever present, the G residues at −25, −24 and −13 showed no detectable protection (Fig. 3).

Interaction of σ^{54} R383 mutants with mutant $glnAp_2$ promoters. Previous studies using mutant derivatives of σ^{70} or σ^{A} have identified interactions between the HTH motif of the sigma factor and the −35 region of the promoter by examining the ability of sigma factor mutants to suppress specifically the promoter-down phenotype of mutations in conserved bases (12, 21, 42). Adopting a similar strategy, we constructed strains with all combinations of the 12 mutant $glnAp_2$ promoters and the 19 σ^{54} Arg-383 mutants, which were then assayed for expression of β-galactosidase from the $glnAp_2$-*lacZ* fusion (Table 3). Only RK383 produced a significant elevation of expression from any of the mutant promoters, namely, −13GC and −13GT (Table 3). DMS protection studies on these active combinations, i.e., RK383 with −13GC and −13GT, together with −13GA for control purposes, showed no evidence of protection, as judged by the intensity of bands representing −24G and −25G (Fig. 2).

The low level of activity observed with the −13GT mutation and wild-type σ^{54} was not observed with Arg-383 mutants, as expected if the activity is σ^{54} dependent, but by comparison, it was notable that the −12CG mutant promoter showed a small but significantly elevated level of activity in all of the σ^{54} mutant backgrounds (Table 3).

The phenotype of σ^{54} SA379. Previous studies had shown that Ser-379 could be substituted by both threonine and alanine to retain σ^{54} activity (9). This result could indicate either a structural requirement for a small residue at this position in the helix or a specific interaction of the hydroxyl side chain group of either serine or threonine with a base pair in the promoter, in which case the active nature of SA379 would suggest that σ^{54} can function in the absence of this interaction. In the latter case, SA379 might be expected to act as a loss-of-specificity mutant, and we therefore examined the activities of all 12 $glnAp_2$ mutants in the presence of σ^{54} SA379. All of the promoters showed no increase in activity above the basal level (data not shown).

The interaction of SA379 with wild-type $glnAp_2$ was also examined by DMS footprinting and subsequent analysis of the autoradiographs by densitometry. When protection of the promoter by SA379 was compared with that in the presence or absence of wild-type σ^{54}, SA379 showed intensities at −25, −24G, and −13G reproducibly lower than those seen in the absence of σ^{54}, indicative of a weak interaction of Eσ^{54} SA379 with the promoter (Table 4).

TABLE 3. Activities of mutant $glnAp_2$ promoters in the presence of σ^{54} mutants with substitutions at Arg-383

$glnAp_2$ mutation	Activity (%) with amino acid substitution[a]:																			
	None	A	G	S	T	I	L	V	M	F	Y	D	E	N	Q	H	K	W	C	P
None	100	4	5	5	5	9	5	5	8	5	6	4	8	8	8	**16**	**97**	5	7	7
−12CA	16	9	8	9	8	11	8	7	12	11	15	14	8	9	13	11	25	9	10	13
−12CG	16	19	17	21	19	17	15	19	22	24	14	25	19	17	16	20	23	20	18	26
−12CT	17	8	8	9	10	11	8	8	14	10	11	12	11	17	11	10	19	9	9	12
−13GA	8	7	8	9	6	10	10	8	9	8	11	10	7	9	10	7	16	10	8	10
−13GC	14	9	10	8	8	10	9	9	8	7	8	9	6	8	10	8	**78**	8	8	10
−13GT	26	6	8	8	6	14	6	7	10	9	7	11	9	12	13	13	**88**	7	11	10
−24GA	11	14	10	11	9	8	11	10	11	11	11	13	11	12	7	9	10	11	10	13
−24GC	9	9	9	7	8	8	9	8	8	9	8	8	12	8	7	7	8	7	6	8
−24GT	10	10	9	8	12	8	9	8	10	8	9	10	11	10	7	10	10	10	8	10
−25GA	13	11	7	9	11	7	8	7	11	7	8	9	12	8	7	9	9	8	8	8
−25GC	11	11	10	11	12	7	9	10	10	10	13	11	11	11	7	11	10	10	10	10
−25GT	11	11	10	9	11	8	10	8	10	13	15	14	10	10	7	8	9	11	8	10

[a] Expressed as percentage of the wild-type $glnAp_2$ promoter activity (β-galactosidase units) in *K. pneumoniae* UNF2792(pMM83)(pMM132). All data are means of at least two determinations and, in the case of RK383, of at least five determinations. The background level in the absence of σ^{54} is equivalent to 4 to 7%.

TABLE 4. Interaction of σ^{54} SA379 with $glnAp_2$

G residue	Densitometry value[a]		
	No σ^{54}	Wild-type σ^{54}	σ^{54} SA379
−25,−24	1.33 ± 0.20	0.31 ± 0.03	0.91 ± 0.17
−19	0.78 ± 0.11	0.69 ± 0.13	0.89 ± 0.24
−13	0.55 ± 0.08	0.22 ± 1.10	0.45 ± 0.09

[a] Autoradiographs of DMS footprinting gels were subjected to densitometry, and the integrated values for bands representing G residues at −25,−24, −19, and −13 were corrected against the value for the −28 band, which was assigned a value of 1.0. Data shown are means of three to seven independent experiments.

DISCUSSION

There is now a considerable body of genetic evidence to support the proposal of Losick and Pero (27) that members of the major family of RNA polymerase sigma factors confer promoter specificity on core RNA polymerase by acting as sequence-specific DNA-binding proteins that physically interact with the major conserved elements in the promoter (13).

In attempting to identify those regions of σ^{54} responsible for promoter recognition, we focused initially on the C-terminal HTH motif which constitutes one of the most highly conserved regions in the σ^{54} protein family. Among 14 published σ^{54} sequences, the region from His-377 to Arg-383, which comprises helix 2, is almost completely conserved. The residue at position 4 in the helix (Ile-381 in *K. pneumoniae* σ^{54}) is either isoleucine or valine, and the glutamate at position 1 (Glu-378) is replaced by other residues in just two cases, namely, by alanine in *Rhodobacter capsulatus* (3, 19) and by proline in *Rhodobacter sphaeroides* (28). Both of these substitutions have been made in *K. pneumoniae* σ^{54}, and they do not inactivate the protein (9).

Previous mutagenesis of residues in helix 2 identified Arg-383 as a residue which might make specific promoter contacts (9). By examining all possible substitutions at Arg-383, we have now shown that the presence of arginine at this position is not absolutely required but that other basic amino acids, most notably lysine, can be efficient substitutions. The fact that only lysine or histidine is an active substitution indicates that the nature of the charge on this residue is an important criterion in determining the facility with which $E\sigma^{54}$ forms a closed promoter complex. The greater activity of RK383 compared with that of RH383 may reflect the bulkier nature of the histidine side chain compared with those of arginine and lysine side chains or the fact that the pK_as of arginine and lysine are significantly higher than that of histidine. We interpret the suppression of the promoter-down mutations, −13GC and −13GT, by RK383 as evidence for a role of the σ^{54} HTH motif in recognition of the −13 promoter region. The loss of specificity for the −13 position observed with RK383 may be attributable to the less bulky nature of the lysine side chain compared with the arginine side chain, accommodating alternative base pairs. However, all other substitutions at Arg-383 inactivate σ^{54} rather than producing a loss of specificity, emphasizing the requirement for a basic residue in this position of the HTH motif. Of course, we cannot completely exclude the possibility that the RK383 mutation merely facilitates interaction with the −13 promoter region by another part of σ^{54}. However, we believe that the specificity of the effect that we observe makes this interpretation rather unlikely.

The importance of electrostatic interactions in the binding of $E\sigma^{54}$ is emphasized by the observations of Buck and Cannon (6), who noted that closed complex formation in vitro is favored both by lowering the salt concentration of the buffer and by the presence of a T tract between −17 and −15 and that the methyl groups in the DNA major groove contributed by the T tract enhance σ^{54}-promoter interaction (7). Furthermore, studies of protein-DNA cocrystals of both prokaryotic (lambda repressor and 434 repressor) and eukaryotic (homeodomain) proteins (22, 34) have demonstrated that registration of the HTH motif on the DNA is frequently established by contacts between the protein and nonesterified phosphate oxygens of the sugar-phosphate backbone (14, 15).

As σ^{54} is not an essential protein, the mutant derivatives can be studied in vivo in the absence of the wild-type protein. By contrast, in vivo studies with σ^{70} must be carried out in a diploid condition, and consequently change-of-specificity and loss-of-specificity phenotypes cannot be distinguished. The σ^{54} RK383 mutant is clearly a loss-of-specificity mutant, as it is active with both wild-type and mutant derivatives of $glnAp_2$. Recent studies have indicated that the σ^{70} RH588 mutant which modifies the recognition helix of the HTH motif in σ^{70} also causes loss of specificity. This mutant σ^{70} is as active as the wild type with the TTGACA consensus but recognizes −35GC and −35GA much better than does the wild-type (W. R. McClure [unpublished data], cited in reference 13). Hence, in both σ^{54} and σ^{70}, the role of the arginine residue at position 6 in the recognition helix may be not to make a specific hydrogen bond to the relevant G · C base pair but rather to contribute positive charge necessary for stabilization of the polymerase-promoter complex by facilitating interaction with the sugar-phosphate backbone.

Although Ser-379 is also predicted to be an exposed helix 2 residue with potential for making a specific contact with the promoter, we presently have no evidence for such an interaction. The conservation of Ser-379 may alternatively reflect the fact that this residue has primarily a structural role; indeed, it is notable that in a wide range of proposed HTH motifs, serine is by far the most commonly occurring amino acid at this position in helix 2 (11).

In the course of this work, we have determined the phenotypes of all possible mutations in the conserved GG and GC doublets of the $glnAp_2$ sequence. As expected, all 12 mutations caused a promoter-down phenotype, but −13GT was reproducibly less severe than all of the others. In all known naturally occurring σ^{54}-dependent promoters, the GG doublet at −25,−24 is absolutely conserved, whereas the GC doublet at −13,−12 is not (32). The C at −12 can be replaced by either A in *Rhizobium meliloti nifN* (2), *Rhizobium phaseoli nifH* (36), and *Rhizobium trifolii nifH* (41) or T in *E. coli psp* (45), and the G at −13 is replaced by T in *E. coli glnH* (33).

The low σ^{54}-independent level of expression observed in −12CG could reflect the production by this mutation of a weak σ^{70}-dependent promoter. The sequence of $glnAp_2$ between −9 and −4 is TATATT, which is an excellent match to the consensus −10 for σ^{70}. Hence, when the −12CG mutation is introduced, this sequence could behave as a promoter of the extended −10 type in which sequences upstream of −10 facilitate interaction of $E\sigma^{70}$ with the promoter in the absence of a −35 sequence (8, 20). Mutations downstream of $glnAp_2$ which produce new σ^{70}-dependent promoters have been described previously in *E. coli* (37).

The naturally occurring variation in the −12 region of σ^{54}-dependent promoters is consistent with the notion that the interaction of $E\sigma^{54}$ with the promoter sequence is absolutely dependent on contacts between σ^{54} and the GG at −25,−24 but that the sequence at −13,−12 may tolerate some deviation from GC in certain contexts. It is noticeable that four of the five natural promoters with GA at −13,−12 have a run of four T residues in the preceding −17 to −14 positions. This may compensate for the absence of C at −12, as the presence of the T tract has been shown to enhance the interaction of $E\sigma^{54}$ with the promoter (7, 32).

We studied the interaction of $E\sigma^{54}$ with all 12 mutant promoters by DMS footprinting in vivo and found that all of the mutations failed to show a detectable interaction with $E\sigma^{54}$. Variants of σ^{54} which relax the interaction of $E\sigma^{54}$ with the −13,−12 region while maintaining strong protection at −25,−24 have been described (40). We find that this phenotype cannot be mimicked by mutations in the −13 or −12 region of the promoter; i.e., wild-type $E\sigma^{54}$ requires sequence conservation in both the −24 and −12 regions to allow formation of a strong closed complex.

Structure-function relationships in σ^{54}. On the basis of alignments of certain σ^{54} primary amino acid sequences and the analysis of the phenotypes of a number of mutant σ^{54} proteins encoded by in-frame deletions in *E. coli rpoN*, Sasse-Dwight and Gralla (40) proposed a structure-function model for σ^{54}. This model made two proposals with respect to the potential interactions of σ^{54} with the promoter elements: first, that the N-terminal region of σ^{54} contains two leucine zipper motifs which can cooperate to form a DNA-binding domain responsible for contacting the −12 region of the promoter and, second, that an HTH motif in *E. coli* σ^{54} helps to bring $E\sigma^{54}$ to the promoter by making contacts in the −24 region. The results described in this report and other considerations discussed below suggest that this model should be reevaluated.

The suggestion that the N-terminal region of σ^{54} forms an intramolecular leucine zipper motif is based on a heptad repeat of hydrophobic residues flanking the central domain (region II) of σ^{54}. The primary amino acid sequence of the N-terminal domain of σ^{54} certainly has characteristics of an extended α helix. However, the heptad repeats show less conservation than is found in classical leucine zippers such as those of Fos, Jun, and GCN4 both with respect to the fact that the most N-terminal zipper contains only three conserved leucines rather than the four or five characteristically observed (1, 24) and with respect to the fact that the second proposed zipper has a high content of hydrophobic residues other than leucine at the fourth position in the heptad. Furthermore, it is now well established that the DNA-binding properties of leucine zippers arise from the juxtaposition of basic amino acid residues adjacent to each zipper which is achieved through the dimerization of the zippers (14, 43). Candidate basic regions were not identified by Sasse-Dwight and Gralla (40) and are indeed not present in the appropriate locations in σ^{54}. Finally, the proposition that the leucine zipper motif is an intramolecular structure in σ^{54} sets a new precedent, as those zippers recognized to date act in an intermolecular fashion to facilitate protein dimerization.

The role of the N terminus of σ^{54} in making contact with the −12 region of the promoter was proposed by Sasse-Dwight and Gralla (40) on the basis of the phenotype of deletions in the two heptad repeat regions which result in σ^{54} derivatives that show an interaction of $E\sigma^{54}$ with the −24 region but no protection of the −12 region. This mutant phenotype was likewise the basis for their proposal that interaction with the −24 region was determined by an HTH that lies in the C-terminal region of σ^{54}. It should also be noted that the HTH referred to by Sasse-Dwight and Gralla (40) extends from Thr-386 to Ser-405. It is not the HTH (Val-366 to Thr-386) mutagenized in this study; rather, it is located immediately downstream in a region originally considered as having the potential to form an HTH (30) but since discounted because statistical analysis suggests that it is very unlikely to form such a structure (9, 11, 29) and comparison of this region in σ^{54} amino acid sequences from other organisms does not show the degree of conservation expected for such a motif.

We are now proposing that the HTH plays a role in interaction with the −12 rather than the −24 promoter region. Structural considerations preclude the HTH being involved in interactions with both the −12 and −24 regions, and we therefore suggest that the phenotype of the N-terminal deletion mutants described by Sasse-Dwight and Gralla (40) could alternatively be explained if these deletions restrain the mutant protein when complexed with core RNA polymerase in a conformation which does not allow $E\sigma^{54}$ to make normal contacts with the −12 region. The region of σ^{54} responsible for interaction of $E\sigma^{54}$ with the −24 promoter region remains to be identified.

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REFERENCES

1. **Abel, T., and T. Maniatis.** 1989. Action of leucine zippers. Nature (London) **341**:24–25.
2. **Aguilar, O. M., H. Reilander, W. Arnold, and A. Puhler.** 1987. *Rhizobium meliloti nifN* (*fixF*) gene is part of an operon regulated by a *nifA*-dependent promoter and codes for a polypeptide homologous to the *nifK* gene product. J. Bacteriol. **169**:5393–5400.
3. **Alias, A., F. J. Cejudo, J. Chabert, J. C. Willison, and P. M. Vignais.** 1989. Nucleotide sequence of wild-type and mutant *nifR4* (*ntrA*) genes of *Rhodobacter capsulatus*: identification of an essential glycine residue. Nucleic Acids Res. **17**:5377.
4. **Austin, S., and R. Dixon.** 1992. The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation and DNA dependent. EMBO J. **11**:2219–2228.
5. **Beynon, J., M. Cannon, V. Buchanan-Wollaston, and F. Cannon.** 1983. The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. Cell **34**:665–671.
6. **Buck, M., and W. Cannon.** 1992. Activator-independent formation of a closed complex between σ^{54}-holoenzyme and the *nifH* and *nifU* promoters of *Klebsiella pneumoniae*. Mol. Microbiol. **6**:1625–1630.
7. **Buck, M., and W. Cannon.** 1992. Specific binding of the transcription factor σ^{54} to promoter DNA. Nature (London) **358**: 422–424.
8. **Chan, B., and S. Busby.** 1989. Recognition of nucleotide sequences at the *Escherichia coli* galactose operon *P1* promoter by RNA polymerase. Gene **84**:227–236.
9. **Coppard, J. R., and M. J. Merrick.** 1991. Cassette mutagenesis implicates a helix-turn-helix motif in promoter recognition by the novel RNA polymerase sigma factor σ^{54}. Mol. Microbiol. **5**:1309–1317.
10. **Daniels, D., P. Zuber, and R. Losick.** 1990. Two amino acids in an RNA polymerase σ factor involved in the recognition of adjacent base pairs in the −10 region of a cognate promoter. Proc. Natl. Acad. Sci. USA **87**:8075–8079.
11. **Dodd, I. B., and J. B. Egan.** 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nu-

cleic Acids Res. **18**:5019–5026.
12. **Gardella, T., H. Moyle, and M. M. Susskind.** 1989. A mutant *Escherichia coli* σ^{70} subunit of RNA polymerase with altered promoter specificity. J. Mol. Biol. **206**:579–590.
13. **Gross, C. A., M. Lonetto, and R. Losick.** Sigma factors. *In* K. Yamamoto and S. McKnight (ed.), Transcriptional regulation, in press. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Harrison, S. C.** 1991. A structural taxonomy of DNA-binding domains. Nature (London) **353**:715–719.
15. **Harrison, S. C., and A. K. Aggarwal.** 1990. DNA recognition by proteins with the helix-turn-helix motif. Annu. Rev. Biochem. **59**:933–969.
16. **Helmann, J. D., and M. Chamberlin.** 1988. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. **57**: 839–872.
17. **Hirschman, J., P. K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the *ntrA* product is a sigma factor. Proc. Natl. Acad. Sci. USA **82**:7525–7529.
18. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA **82**:8453–8457.
19. **Jones, R., and R. Haselkorn.** 1989. The DNA sequence of the *Rhodobacter capsulatus ntrA*, *ntrB* and *ntrC* gene analogues required for nitrogen fixation. Mol. Gen. Genet. **215**:507–516.
20. **Keilty, S., and M. Rosenberg.** 1987. Constitutive function of a positively regulated promoter reveals sequences essential for activity. J. Biol. Chem. **262**:6389–6395.
21. **Kenney, T. J., and C. P. Moran.** 1991. Genetic evidence for interaction of σ^A with two promoters in *Bacillus subtilis*. J. Bacteriol. **173**:3282–3290.
22. **Kissinger, C. R., B. Liu, E. Martin-Blanco, T. B. Kornberg, and C. O. Pabo.** 1990. Crystal structure of an engrailed homeodomain-DNA complex at 2.8Å resolution: a framework for understanding homeodomain-DNA interactions. Cell **63**:579–590.
23. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53**:367–376.
24. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**:1759–1764.
25. **Landt, O., H.-P. Grunert, and U. Hahn.** 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene **96**:125–128.
26. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. **174**:3843–3849.
27. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **25**:582–584.
28. **Meijer, W. G., and F. R. Tabita.** 1992. Isolation and characterisation of the *nifUSVW-rpoN* gene cluster from *Rhodobacter sphaeroides*. J. Bacteriol. **174**:3855–3866.
29. **Merrick, M., J. Gibbins, and A. Toukdarian.** 1987. The nucleotide sequence of the sigma factor gene *ntrA* (*rpoN*) of *Azotobacter vinelandii*: analysis of conserved sequences in NtrA proteins. Mol. Gen. Genet. **210**:323–330.
30. **Merrick, M. J., and J. R. Gibbins.** 1985. The nucleotide sequence of the nitrogen-regulation gene *ntrA* of *Klebsiella pneumoniae* and comparison with conserved features in bacterial RNA polymerase sigma factors. Nucleic Acids Res. **13**:7607–7620.
31. **Minchin, S. D., S. Austin, and R. A. Dixon.** 1989. Transcriptional activation of the *Klebsiella pneumoniae nifLA* promoter by NTRC is face-of-the-helix dependent and the activator stabilizes the interaction of sigma 54-RNA polymerase with the promoter. EMBO J. **8**:3491–3499.
32. **Morett, E., and M. Buck.** 1989. *In vivo* studies on the interaction of RNA polymerase-σ^{54} with the *Klebsiella pneumoniae* and *Rhizobium meliloti nifH* promoters: the role of NifA in the formation of an open promoter complex. J. Mol. Biol. **210**:65–77.
33. **Nohno, T., and T. Saito.** 1987. Two transcriptional start sites found in the promoter region of *Escherichia coli* glutamine permease operon, *glnHPQ*. Nucleic Acids Res. **15**:2777.
34. **Pabo, C. O., A. K. Aggarwal, S. R. Jordan, L. J. Beamer, U. R. Obeysekare, and S. C. Harrison.** 1990. Conserved residues make similar contacts in two repressor-operator complexes. Science **247**:1210–1213.
35. **Popham, D. L., D. Szeto, J. Keener, and S. Kustu.** 1989. Function of a bacterial activator protein that binds to transcriptional enhancers. Science **243**:629–635.
36. **Quinto, C., H. de la Vega, M. Flores, J. Leemans, M. A. Cevallos, M. A. Pardo, R. Azpiroz, M. L. Girard, E. Calva, and R. Palacios.** 1985. Nitrogenase reductase: a functional multigene family in *Rhizobium phaseoli*. Proc. Natl. Acad. Sci. USA **82**:1170–1174.
37. **Reitzer, L. J., R. Bueno, W. D. Cheng, S. A. Abrams, D. M. Rothstein, T. P. Hunt, B. Tyler, and B. Magasanik.** 1987. Mutations that create new promoters suppress the σ^{54} dependence of *glnA* transcription in *Escherichia coli*. J. Bacteriol. **169**:4279–4284.
38. **Reznikoff, W. S., D. A. Siegele, D. W. Cowing, and C. A. Gross.** 1985. The regulation of transcription initiation in bacteria. Annu. Rev. Genet. **19**:355–387.
39. **Sasse-Dwight, S., and J. D. Gralla.** 1988. Probing the *Escherichia coli glnALG* upstream activation mechanism *in vivo*. Proc. Natl. Acad. Sci. USA **85**:8934–8938.
40. **Sasse-Dwight, S., and J. D. Gralla.** 1990. Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor σ^{54}. Cell **62**:945–954.
41. **Scott, K. F., B. G. Rolfe, and J. Shine.** 1983. Biological nitrogen fixation: primary structure of the *Rhizobium trifolii* iron protein gene. DNA **2**:149–155.
42. **Siegle, D. A., J. C. Hu, W. A. Walter, and C. A. Gross.** 1989. Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. J. Mol. Biol. **206**:591–603.
43. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science **246**:911–916.
44. **Waldburger, C., T. Gardella, R. Wong, and M. M. Susskind.** 1990. Changes in conserved region 2 of *Escherichia coli* σ^{70} affecting promoter recognition. J. Mol. Biol. **215**:267–276.
45. **Weiner, L., J. L. Brisette, and P. Model.** 1991. Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on σ^{54} and modulated by positive and negative feedback mechanisms. Genes Dev. **5**:1912–1923.
46. **Weiss, D. S., J. Batut, K. E. Klose, J. Keener, and S. Kustu.** 1991. The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. Cell **67**:155–167.
47. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded template. DNA **3**:479–488.
48. **Zuber, P., J. Healy, H. Luke Carter III, S. Cutting, C. P. Moran, Jr., and R. Losick.** 1989. Mutations changing the specificity of an RNA polymerase sigma factor. J. Mol. Biol. **206**:605–614.