Roles for Inhibitory Interactions in the Use of the −10 Promoter Element by σ70 Holoenzyme*

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A panel of seven −10 region DNA mutants was tested for holoenzyme binding against a panel of 13 region 2 mutants of σ70. No patterns were noticed that would indicate unique interactions between individual amino acids and individual −10 region bases. Instead, certain amino acid substitutions led to increased holoenzyme binding to DNA, implying that the wild type interactions are associated with an inhibitory component. These inhibitory interactions were stronger on DNA containing non-consensus sequences, like those of typical promoters. In addition, the DNA segment downstream from the −10 element was also inhibitory to binding when in duplex form but stimulated binding when in single strand form. Overall, the data suggest that −10 region duplex recognition and melting have a large component of unfavorable protein:DNA interactions, particularly when the bases are non-consensus, and that this contributes to setting physiologically appropriate variations in transcription rate.

Escherichia coli gene transcription is accomplished by multisubunit RNA polymerase holoenzymes, with the σ70 form being most common. σ70 promoters typically contain two moderately conserved sequence elements separated by an optimal spacer length of 17 bp (1, 2). Naturally occurring promoters diverge from the consensus sequences to varying degrees, and these variations are required to obtain physiologically appropriate transcription rates. Other nearby DNA sequences also contribute to promoter activity (3, 4).

The role of the −10 element is complex as it is recognized initially as duplex DNA and continues to function after the DNA is opened. The dominant function is associated with the non-template strand (5) and particularly with the −11 to −7 fork junction nucleotides at the boundary between duplex and non-template single strand DNA (6). The structure of holoenzyme bound to fork junction DNA (a duplex with a single strand tail from −11 to −7) is available (7). Protein:DNA contacts are not visible, but it is clear that certain amino acids in region 2 of σ70 are close enough to the non-template single strand to make contact. There is no structure with −10 duplex DNA, so information about −10 duplex recognition comes solely from biochemical studies.

In addition to marking promoters for recognition, the −10 element contributes to the rate of open complex formation, and this is known to proceed through binding and isomerization steps (8, 9). The open complex pathway includes several intermediates in which the conformational state of the DNA and polymerase varies (10–12). The final functional state is characterized by the resistance of the isomerized holoenzyme to the polyanionic inhibitor heparin (13). This isomerization of the enzyme can be measured independently of the opening of the DNA by using fork junction probes in which the DNA is premelted (14, 15). Such studies have indicated that the −10 region consensus bases have a critical role in enzyme isomerization (15). The bases also play a role in duplex recognition but have a lesser influence on binding single strand DNA, where interactions with the backbone appear to be more important (16).

There have been systematic binding studies changing either the bases in the −10 region or the amino acids in the σ region 2 that may recognize them (17, 18). In general, these and prior genetic experiments suggested the importance of a collection of clustered amino acids, many of which are aromatic (19) and basic residues. The various studies are in substantial, but not full, agreement. This may be due to using different promoter contexts (17, 18). A detailed nucleotide:amino acid recognition pattern has not yet emerged from these studies, although individual interactions have been proposed both in terms of structure and in terms of function.

To extend our knowledge of these interactions, we have combined the study of mutant proteins with the study of mutant −10 region DNA. Duplex and single strand DNA binding and enzyme isomerization were each assayed using combinations of mutant proteins and mutant promoters. The results do not support a model in which extensive unique favorable interactions are made by pairs of amino acids and nucleotide bases. They reveal an unexpected phenomenon; certain amino acids and DNA downstream from the −10 element are designed to contribute to inhibition of binding or isomerization. These unfavorable interactions can be stronger with non-consensus −10 elements, and this may contribute to setting physiologically appropriate transcription rates.

EXPERIMENTAL PROCEDURES

Proteins and DNA—The plasmid pQE30-rpoD was overexpressed, and σ70 and mutants were purified as described (31). Numerous clones had bands that migrated to lower positions on protein gels and were discarded. The DNA from candidates that passed this mobility screen was sequenced, and if the DNA contained the correct mutation, the protein was expressed and purified, and the mobility was rechecked. The E. coli RNA polymerase core is a commercial product from Epicenter Technologies. Oligonucleotides and probes were gel-purified and prepared as described (6). Briefly, the bottom strand of each probe was labeled with γ-32P/ATP. The 40-μl mixture, containing 4 pmol of kinased DNA and 6 pmol of complementary strand in 20 μM Tris-HCl, pH 7.5/50 mM NaCl, was annealed by rapid heating to 95 °C and slow cooling to room temperature in a PCR thermocycler (MJ Research). The resulting annealed probes were diluted in Tris-EDTA buffer containing

Received for publication, July 10, 2003, and in revised form, August 4, 2003
Published, JBC Papers in Press, August 4, 2003, DOI 10.1074/jbc.M307412200

This paper is available online at http://www.jbc.org

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Printed in U.S.A.
50 mM NaCl to the desired concentration. Proper annealing was monitored by electrophoresis. Plasmid pTH5-UV5 was constructed by inserting a 300-bp fragment containing the lac UV5 promoter into pTH5 (32) through unique restriction sites of BamHI and HindIII. Changing pTH5-UV5 by the Strategene site-directed mutagenesis kit created the point mutation −11C in the UV5 promoter.

**Electrophoresis Mobility Shift Assay**—Mobility shift assays with and without heparin were as described (15). Briefly, 20 nM core was mixed with 50 mM NaCl to the desired concentration. Proper annealing was monitored by electrophoresis. Plasmid pTH5-UV5 was constructed by inserting a 300-bp fragment containing the lac UV5 promoter into pTH5 (32) through unique restriction sites of BamHI and HindIII. Changing pTH5-UV5 by the Strategene site-directed mutagenesis kit created the point mutation −11C in the UV5 promoter.

**In Vitro Transcription**—Transcription was as follows: 20 nM core and 50 mM α70 were incubated with buffer A (described above) and 2.5 mM pTH5-UV5 for 10 min at 37 °C. Reactions were further incubated with an NTP mix consisting of 0.5 mM ATP, GTP, and UTP for 5 min at 37 °C. 1 μl of a CTP mix (1 mM CTP, 0.2 μCi/μl α-[32P]CTP, and 100 μg/ml heparin) was added for 20 min at 37 °C. Sample volumes were doubled with urea dye mix, heated 90 °C for 5 min, and run on a 6% PAGE with urea in 1× TBE.

**RESULTS**

**Effects of DNA Downstream from −10 on Holoenzyme Binding**—Prior studies using a variety of promoter sequences and probes have indicated that the DNA downstream from −10 has a role in open complex formation (14, 17). This region has the potential to interact with subunits within the polymerase core (20). To consolidate and extend the information on this region, we conducted binding studies using the homologous series of probes shown in Fig. 1. These contain the downstream −6 to +1 region in either duplex or single strand form; control probes lack this region.

The primary observation from these data is that the probe containing the duplex region from −6 to +1 (lane 1) is bound less well than all other probes. The inhibition by this region only occurs when it is in the duplex form (lane 2 versus lane 1); when it is single-stranded, the downstream sequence stimulates (lane 2 versus lane 4), as seen previously (15, 17). Thus the opening of the downstream DNA should be accompanied by two favorable changes, a relief from duplex inhibition and a stabilization due to the presence of the single strand. To bypass these differences and focus solely on the −10 region, the −10 duplex probe (7/11 probe in lane 2) and the −10 fork junction probe (7/12 probe in lane 5) were used for binding studies.

The amino acid changes made in region 2 are listed in Fig. 2, first column. The 5 aromatic between residues 425 and 434 were changed individually to alanines; for 2 of these whose aromatic character was suggested to be important (17), substitutions were also made that retain aromaticity. Other nearby residues suggested to be of importance were changed to serine to maintain the hydrophilicity of the side chains (17). The 13 mutant αs were purified in parallel with wild type and assembled into holoenzymes. Some of these were studied previously and were found to function in intact holoenzymes on wild type promoter probes (17, 18, 21). The other mutant αs were confirmed to bind core polymerase using surface plasma resonance (17), and holoenzyme binding is confirmed by the mobility in the band shift assays shown below.

**Binding of Mutant Holoenzymes to G-substituted Duplex DNA**—First, the mutant holoenzymes were tested for binding to wild type duplex DNA by EMSA.1 As shown above, the probe is duplex with the lac UV5 sequences −11 to −7, and the incubation and electrophoresis were done on ice to preserve duplex character. Nine of these mutants were studied previously on either lac UV5 duplex probes extending to +1 or APr, duplex probes extending to −12 (17); each was either partially or severely defective in binding in one or both of these contexts. The -fold reduction in binding by each holoenzyme in the current study is shown in Fig. 2 in the WT (wild type) column and also includes the data for the 4 new aromatic substitution mutants. Changes of 1.5-fold or less are not indicated in the table. Examples of the EMSA assays are shown in Fig. 3, top.

Of the 9 mutants studied previously in different duplex contexts, only 3 do not show loss of binding in the current experiments using consensus UV5 duplex probes extended to −7 (Y430A, W433A, and Q437S). These also show no significant loss of binding in prior studies on different probes (17). The 4 new mutants, at Tyr-430 and Trp-43, were designed to preserve the aromatic character of these 2 amino acids. The data show that aromatic substitutions at Trp-433 yield slight reductions, whereas alanine has no effect. Aromatic substitutions at Tyr-430 are severely defective in binding, whereas alanine has no effect. It appears that the aromatic character of these residues does not have an important role in directing duplex binding.

These experiments were repeated using a series of six probes in which guanine substitutions were made individually at each position in the consensus. Each probe contains a G-C pair at the indicated position. The EMSA data for these additional 84 combinations are also shown in Fig. 2. The results are arranged as a table in which guanine-substituted DNA (in columns) is paired with mutated amino acids (in rows).

Collectively, the data do not reveal any clear patterns that suggest unique interactions between single amino acids and nucleotides. This would be suggested if, for example, the binding reduction caused by a nucleotide change was not further reduced by an amino acid substitution; if there was unique pairing of nucleotide with amino acids, then changing either or both would lead to the same reduction in binding. The data do not show any such unique pairings. For example, Y425A reduces binding to wild type DNA and all mutant DNAs. Although there are some differences in the quantitative effects of amino acid substitutions at various promoter sequences, we do not consider these to be significant enough to merit interpretation.

The one potential exception concerns mutant W434A. This

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1 The abbreviations used are: EMSA, electrophoretic mobility shift assay; WT, wild type.
substitution reduces binding on wild type DNA but not on DNA substituted at the fork junction positions −12 and −11. That is, the lower binding caused by these mutant DNAs is not further reduced when Trp-434 is changed to alanine. The substitution does further reduce the lowered binding associated with other mutant promoter sequences. The comparison suggests that Trp-434 could interact with the pair of fork junction nucleotides on duplex DNA.

A striking aspect of the data involves mutant Q437S, and to a lesser extent, Y430A. The Q437S substitution actually increases binding on all mutant promoter sequences (the effect is shown for three promoters in Fig. 3). The effect is strongest when position −12 is altered, where wild type holoenzyme binding is reduced 5-fold (Fig. 2, top row). When Q437S holoenzyme is used on −12-substituted DNA, the defect is almost fully rescued (Fig. 3). Gln-437 was found previously to be a site for the suppression of a −12 mutant promoter (22–25), consistent with these in vitro data. However, the in vitro data show effects on other promoters, suggesting that the in vivo suppression is not likely to be allele-specific. A much smaller effect is seen with Y430A, which enhances binding to a −11-substituted promoter (Fig. 3), but the low signal makes this difficult to quantify. From these data, we infer that Gln-437 and probably Tyr-430 interfere with duplex binding, and this inhibition can be relieved by alanine substitution.

Binding to Fork Junction Probes Containing Single-stranded DNA—Prior studies indicated that binding to fork junction DNA containing the melted non-template strand is very tight and involves a large contribution from the DNA backbone (16). Bases at position −11 and to a lesser extent −7 make a contribution (confirmed in Fig. 4, top row). Most of the protein mutants qualitatively reduce binding to this probe as they did on duplex DNA (compare Fig. 4, first column, with Fig. 2). The exception is T440S, whose effect on duplex recognition is not seen in the context of melted fork junction DNA. Of the mutants studied, it appears that only T440S fails to retain an influence after duplex DNA is melted.

The experiments were repeated, pairing each of the 14 mutants with each of the 6 guanine-substituted probes (Fig. 4). For the most part, the mutants lead to modest decreases in binding or have little effect; there is no indication of specific pairing for stabilizing interactions. However, a suppression effect of Y430A was seen again in the data. Y430A partially rescues the defect caused by substitution of guanine at position −11 (Fig. 5), but no rescue is seen of the −7 position substitu-
tation (not shown). The effect is comparable with that seen at the same position on duplex DNA (see above). Q437A does not rescue either defect (Fig. 5), in contrast to its general suppression on duplex DNA. It appears that the inhibitory effect of Tyr-430 on the non-consensus \( \text{H}11002 \text{H} \) promoter is retained after DNA melting, but the general inhibition by Gln-437 is relieved in this context.

**Binding to Abasic Probes**—Recently, we reported experiments using abasic probes in which each consensus base was removed individually, leaving the backbone intact (16). We repeated aspects of these experiments as comparisons for the extent of changes in binding shown here. The abasic duplex probes were all bound at levels higher than the intact parent (Fig. 6 top); this is likely because the abasic probes facilitate DNA melting, and the melted probes are bound more tightly (as shown above). The removal of any base had little effect using fork probes with single strand tails (Fig. 6, middle); as discussed previously, the affinity for these probes appears to rely significantly on the DNA backbone, and a non-consensus base can be inhibitory. When the bases at \(-11\) and \(-7\) are removed together, the binding is decreased more than 10-fold (Fig. 6, compare middle and top WT (wild type) lanes).

The new experiment is shown in Fig. 6 (bottom) where the double abasic probe is tested for binding against the panel of \( \sigma \) mutants. Most substitutions reduce the low level of binding even further (the bands for the mutants co-migrate with bound core polymerase). However, the Y430A holoenzyme not only binds but largely rescues the defect caused by the 2 abasic residues. This is a further indication that the wild type Tyr-430 has the potential to participate in inhibitory interactions with \( \text{H}11002 \text{H} \) region DNA. The fact that the effect is most strongly revealed when bases are removed and \( \text{H}9268 \) is mutated indicates that the inhibitory interactions rely on both \( \sigma \) and the DNA bases.

**Binding to Isomerized Holoenzyme**—The above experiments reflect how the melted \(-10\) region is bound rather than how it becomes melted. They also reflect interactions that could include both isomerized and non-isomerized forms of the holoenzyme. To evaluate binding contributions made by the isomerized polymerase, the experiments were repeated after a heparin challenge to dissociate unisomerized forms of holoenzyme. Only one context revealed an inhibitory interaction (data not shown). This was an increase in binding by Q437S holoenzyme on the wild type promoter. Other guanine-substituted

|        | wt | 12G | 11G | 10G | 9G | 8G | 7G |
|--------|----|-----|-----|-----|----|----|----|
| *wt sigma* |    | 70% | 70% | 18% | 70% | 70% | 35% |
| Y425A  | 2  | 3   | 3   | x   | x  | x  | 2  |
| K426S  | 3  | 6   | 4.5 | x   | 2  | 2  | 2  |
| F427A  | 6  | 2.5 | 6   | 2.5 | 3  | 3  | 2  |
| Y430A  |    |     |     |     |    |    |    |
| Y430F  |    |     |     |     |    |    |    |
|        |    | no bind |       | no bind | 2 up | no bind | no bind | x | x | x | 2 |
| W433A  |    |     |     |     |    |    |    |
| W433F  |    |     |     |     |    |    |    |
| W433Y  |    |     |     |     |    |    |    |
| W434A  |    |     |     |     |    |    |    |
| R436S  |    |     |     |     |    |    |    |
| Q437S  |    |     |     |     |    |    |    |
| T440S  |    |     |     |     |    |    |    |

*Fig. 4.* Binding changes associated with mutant fork junction T7/B12 probes and mutant holoenzymes. See the legend for Fig. 2 for details. Results are from 2-5 individual experiments. The error for the 11G and 10G probes is \( \pm 10\% \). For the WT, 12G, 9G, and 7G probes, the error is \( \pm 20\% \), except for K426S and F427A on the 7G probe at \( \pm 50\% \). Error using the 8G probe is \( \pm 35\% \).

*Fig. 5.* EMSA with mutant \( \sigma \)s on the T7/B12 fork probe. 11G refers to the non-consensus guanine base change at position \(-11\). Only WT and two mutant \( \sigma \)s discussed in the text are labeled. Other changes are as in Fig. 3.
promoters could not be reliably assessed for these σ mutant effects because binding in the heparin challenge assay was essentially undetectable. We conclude that the inhibitory structure still has the potential to affect the isomerized enzyme, but it is difficult to assess the range of this effect.

Transcription by Y430A Holoenzyme—These experiments suggest that mutations at Gln-437 and Tyr-430 could enhance transcription, depending on −10 promoter sequence. This is in a sense already known in vivo for Gln-437; Q437R and Q437H were isolated as suppressors of a −12 region mutant promoter (23, 25). In the current context, this can simply be seen as a relief from unfavorable interactions, not restricted to position −12, that involve Gln-437. As no suppressors at Tyr-430 are known, we tested Y430A against a −11-substituted promoter, at which it relieved inhibition of both duplex and fork + single strand binding (see above).

Fig. 7 shows that Y430A holoenzyme is competent to transcribe a consensus promoter on plasmid DNA in vitro and does so at essentially wild type levels (Fig. 7, lanes 1–4). It appears that Tyr-430 is not needed for optimal transcription in this context. When −11 is substituted, transcription is not detectable using wild type holoenzyme under these conditions (Fig. 7, lanes 5 and 6). When Y430A holoenzyme is used, a weak but clearly detectable transcript appears (Fig. 7, lanes 7 and 8). We infer that the inhibitory structure involving Tyr-430 is felt both in binding and in transcription of a non-consensus promoter and can be partially relieved by alanine substitution.

DISCUSSION

There are two main observations in this work, neither of which was expected when it was begun. First, certain amino acid substitutions in region 2 of σ70 are found to make σ bind better rather than worse. This leads to the idea that the wild type amino acids can participate in unfavorable interactions and that these are part of the natural design of σ70 function. Second, no amino acid substitution leads to defects that are selectively associated with individual −10 region nucleotides. Taken together, the data suggest the existence of a complex network of contacts involving region 2 of σ70 and the −10 promoter region that include both favorable and unfavorable interactions.

Inhibitory Interactions along the Open Complex Pathway—We identified 2 amino acids that have the potential to inhibit holoenzyme in various assays. Gln-437 inhibited duplex DNA binding and did so on both consensus and non-consensus promoters. That is, when Gln-437 was changed to alanine, binding improved. Tyr-430 alanine substitution also could improve binding, but the Y430A holoenzyme differed in two respects. First, the improvement was seen clearly only in the context of promoters where the −11 position had been altered. Second, Y430A could lower binding in the context of other DNA probes. Gln-437 was previously the site of suppression in vivo (23, 25) of a −12 promoter change, but the current data suggest that this is not truly allele-specific. The current data also showed that Y430A could weakly suppress a −11 promoter change for transcription in vitro. Because we have not tested every amino acid change in region 2 nor every nucleotide change in the DNA, it is possible that other inhibitory interactions exist.

Several other features of the data also point to the importance of inhibitory interactions in controlling the pathway to the formation of open complexes. Nucleotide substitution in the −10 region in single-stranded form can inhibit binding. This defect is not caused simply by the loss of a contact to the consensus base because removal of a single base has little effect (Ref. 16 and see above). Moreover, the overall pattern of binding losses shows no obvious one-to-one pairing of nucleotides and amino acids (discussed above). Taken together, these independent observations suggest that a large component of sequence-specific recognition involves unfavorable interactions dictated by non-consensus DNA bases. That is, inclusion of a non-consensus base should lead to active interference with the progress of open complex formation. The process should be slowed further by the inhibitory influence of duplex DNA between −6 and the start site (Fig. 1).

A very considerable body of data, both structural and biochemical, exists on the interaction between σ region 2 and the promoter −10 element DNA (see the Introduction). The current data are for the most part consistent with those studies but cannot distinguish among the models proposed. The inconsistencies can largely be attributed to context as the magnitude of defects clearly depends on which promoter probe is used and which promoter elements are associated with it (see also Ref. 16). Overall, it appears that the most important amino acids are between positions 414 and 440 of σ (17, 18), and the most important nucleotides for σ interaction are those on the non-template strand (5) at the fork junction positions −12 and −11 (14, 26). High resolution structures of the holoenzyme exist (7, 27), but resolution is lacking in the σ component bound with fork junction DNA, in which the overall holoenzyme structure is clearly changed. The manner in which these determinants combine to contribute to positive and negative effects will likely require both further structural analysis and biochemical kinetic studies. The key new point here is that the kinetic pathway is likely to be strongly influenced by unfavorable interac-
tions with −10 region bases that become increasingly important as the bases deviate from the consensus.

**Implications for σ and Promoter Function**—The interaction between σ and fork junction DNA elements appears to have widespread significance. In the case of σ54 holoenzyme, these interactions have a very strong inhibitory component (28); indeed, they prevent prebound polymerase from melting the DNA until signaled by an activator (26). In the case of σ38 holoenzyme, no inhibitory interactions were detected in experiments similar to those conducted here (29, 30). That case is the polar opposite of σ54 as binding is very weak but DNA melting is facile. The weak inhibitory structure associated with the fork junction at σ70 promoters represents an intermediate case. This would be appropriate as the σ70 holoenzyme has the most diversity in terms of promoter use, and therefore, physiology could benefit from being regulated at both the binding and the open complex steps. The very large majority of σ70 promoters have non-consensus −10 regions, and these should influence the degree of active inhibition applied to holoenzyme interactions. In this context, amino acids such as Gln-437 and Tyr-430 help holoenzyme to function appropriately by moderating DNA binding and complex isomerization so as to make σ70 promoters subject to widespread regulation.

**Acknowledgment**—We thank Randy Yang for assistance in the initial stages of this work.

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