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A mutation in region 1.1 of σ^{70} affects promoter DNA binding by *Escherichia coli* RNA polymerase holoenzyme

Christina Wilson Bowers and Alicia J.Dombroski[1]

Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center, 6431 Fannin JFB1.765, Houston, TX 77030, USA

[1]Corresponding author
e-mail: dombros@utmmg.med.uth.tmc.edu

The sigma subunit of eubacterial RNA polymerase is essential for initiation of transcription at promoter sites. It directs recognition of DNA sequences by holoenzyme ($\alpha_2\beta\beta'\sigma$) and facilitates subsequent steps in the initiation pathway. The primary σ factor from *Escherichia coli*, σ^{70}, has four regions that are conserved among members of the σ^{70} family. Previous work has shown that region 1.1 modulates DNA binding by regions 2 and 4 when σ is separated from the core subunits, and is required for efficient progression through the later steps of initiation in the context of holoenzyme. In this report, we show that an amino acid substitution at position 53 in region 1.1, which converts isoleucine to alanine (I53A), creates a σ factor that associates with the core subunits to form holoenzyme, but the holoenzyme is severely deficient for promoter binding. The I53A phenotype can be suppressed by truncation of five amino acids from the C-terminus of σ^{70}. We propose that the behavior of σ^{70}-I53A is a consequence of impaired ability to undergo a critical conformational change upon binding to the core subunits, which is needed to expose the DNA-binding domains and confer promoter recognition capability upon holoenzyme.

Keywords: initiation/RNA polymerase/sigma/transcription

Introduction

Eubacterial RNA polymerase is composed of a core component ($\alpha_2\beta\beta'$) and the variable sigma (σ) subunit. Because σ factor is responsible for promoter recognition in the context of RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$), employment of different σ factors facilitates utilization of distinct promoter sequences (Travers and Burgess, 1969; Losick and Pero, 1981), permitting rapid response to environmental stimuli and an effective means for positive regulation of gene expression (reviewed in Gross *et al.*, 1992).

Transcription initiation by *Escherichia coli* RNA polymerase can be characterized by several steps (reviewed in Record *et al.*, 1996; deHaseth *et al.*, 1998). First, holoenzyme recognizes and binds to the promoter to form an initial closed complex upstream of the transcription start site, designated RP_{c1}. RP_{c1} isomerizes to a second closed complex, RP_{c2}, in which interaction of RNA polymerase with the template extends to contacts downstream of the transcription start site, but the two DNA strands remain base paired. Then, DNA melting accompanies formation of one or more open complexes (RP_o). In the presence of initiating nucleoside triphosphates (NTPs), initiated complexes (RP_{init}) are formed, which are competent to begin RNA synthesis. Although the σ subunit is required for specific promoter binding, increasing evidence also implicates σ factor in facilitating subsequent aspects of the process of transcription initiation (Juang and Helmann, 1994; Rong and Helmann, 1994; Roberts and Roberts, 1996; Huang *et al.*, 1997; Marr and Roberts, 1997; Wilson and Dombroski, 1997).

The amino acid sequences of the σ subunits are highly conserved, and the largest family of σ factors comprises those related to the primary σ factor from *E.coli*, σ^{70} (Lonetto *et al.*, 1992). This group of proteins is most similar in four regions (Figure 1) (Stragier *et al.*, 1985; Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988). Regions 4 and 2 are involved in recognition of the –35 and –10 promoter consensus elements, respectively (Gardella *et al.*, 1989; Siegele *et al.*, 1989; Zuber *et al.*, 1989; Daniels *et al.*, 1990; Waldburger *et al.*, 1990). However, in the absence of the core subunits of RNA polymerase, σ^{70} is unable to recognize and bind promoter DNA unless its N-terminus is truncated to remove portions of region 1.1 (Dombroski *et al.*, 1992). This autoinhibitory property of region 1.1 appears to primarily affect DNA binding by region 4, leading to the proposal that regions 1.1 and 4 are in close proximity in native σ^{70}, and that binding of σ^{70} to the core subunits induces a conformational change that unmasks the DNA-binding domains to allow promoter recognition by holoenzyme (Dombroski *et al.*, 1992, 1993).

The σ subunit is also required for events beyond DNA recognition as indicated by several observations. First, the core RNA polymerase subunits ($\alpha_2\beta\beta'$) cannot catalyze strand opening on covalently closed circular DNA in the absence of σ^{70} (Saucier and Wang, 1972). Secondly, mutations have been identified in the σ subunit that affect the transition between promoter binding and initiation of transcription (Jones and Moran, 1992), and that specifically affect DNA melting during open complex formation (Helmann and Chamberlin, 1988; Juang and Helmann, 1994; Rong and Helmann, 1994). Thirdly, both holoenzyme and σ factor interact with non-template bases in the –10 consensus of single-stranded DNA to stabilize the open complex (Roberts and Roberts, 1996; Huang *et al.*, 1997; Marr and Roberts, 1997). Finally, σ^{70} lacking the first 75 or 100 amino acids of region 1.1 results in a reduced rate of both open and initiated complex formation by holoenzyme, and σ^{70} lacking both regions 1.1 and 1.2 only allows formation of RP_{c1} (Wilson and Dombroski, 1997).

Fig. 1. Linear diagram of σ^{70}. Conserved regions 1–4 are indicated (Lonetto *et al.*, 1992). Regions 2 and 4 are involved in recognition of the –10 and –35 promoter elements. Core-binding determinants, as well as amino acids important for promoter DNA melting, have been found in region 2 (Lesley and Burgess, 1989; Juang and Helmann, 1994; Rong and Helmann, 1994). Region 1 is shown subdivided into regions 1.1 and 1.2, and has been implicated in inhibition of DNA binding by σ^{70} (Dombroski *et al.*, 1992, 1993) and in transcription initiation by holoenzyme (Wilson and Dombroski, 1997). The approximate position of amino acid 53 is shown with an asterisk (*). Each derivative was purified as a hexahistidine-tagged protein with the following sequence at the N-terminus: RGSHHHHHHGSSGLVPRGSGLGTRL.

To understand better the role of region 1.1 of σ^{70} in transcription initiation, we made substitutions at several amino acids that are invariant or highly conserved among the primary σ factors, and occur between positions 50 and 75 of σ^{70}. Position 53 was one of several sites chosen (analysis of other substitutions in this region will be published separately). Here we show that conversion of Ile53 to alanine (I53A) generates a σ^{70} derivative that binds to the core subunits, but is ineffective at making the conformational transition(s) that permit holoenzyme to bind efficiently to promoter DNA. An intragenic suppressor of the inactive I53A allele was identified and consists of a deletion of five C-terminal amino acids along with amino acid substitutions at positions 567, 609 and 610. However, simply a C-terminal truncation of five amino acids is sufficient to suppress the I53A phenotype. We suggest that σ^{70}-I53A remains in a conformation that is autoinhibited for DNA binding in the absence of the core subunits, even after association with core. The intragenic suppressor enhances the ability of σ^{70}-I53A to undergo the conformational change that normally is required to unmask the DNA-binding domains and allow promoter recognition.

Results

The identity of amino acid 53 of σ^{70} affects the function of holoenzyme in vivo

Using site-directed mutagenesis, substitutions of alanine (A), phenylalanine (F) and valine (V) were made for the highly conserved isoleucine (I) at position 53 of σ^{70} in region 1.1. Plasmids carrying the mutated *rpoD* genes were transformed into a strain carrying the *rpoD800* (*rpoD285*) allele, which encodes σ^{70} with a temperature-sensitive (*ts*) growth phenotype, and is not viable at 44°C. The *rpoD800* strain expressing σ^{70} (WT) was able to grow at the non-permissive temperature, while σ^{70}-I53A was unable to complement the *ts* growth defect of *rpoD800* (Figure 2).

Further support for the inactivity of σ^{70}-I53A *in vivo* was obtained in another strain background (CAG 20176) where the chromosomal copy of *rpoD* is under the control of a synthetic *trp* promoter. In the absence of indole-3-acrylic acid (IAA), the expression of *rpoD* from the chromosome is insufficient and σ^{70} must be provided from a plasmid to sustain viability. CAG20176 expressing σ^{70}(WT) was able to grow well in the absence of IAA, while σ^{70}-I53A was unable to substitute for σ^{70} at 28°C (Table I).

To understand better the amino acid requirement at position 53, we also tested the growth characteristics of the *rpoD800* strain expressing either σ^{70}-I53V or σ^{70}-I53F. The phenylalanine substitution resulted in a σ factor that fully complemented the mutant strain at the non-permissive temperature. The valine substitution produced a functional σ^{70}, but the strain grew more slowly and formed smaller colonies on agar plates (Figure 2). When these mutants were tested for their ability to sustain viability in CAG20176, both σ^{70}-I53F and σ^{70}-I53V resembled σ^{70} (Table I). Based upon the behavior of the I53 mutants *in vivo*, it appears that the primary requirement at that position is a large hydrophobic side chain rather than for isoleucine *per se*. However, because substitution of valine at position 53 resulted in suboptimal growth in the *rpoD800* strain, there may be an additional side chain requirement that may include parameters of size, shape and hydrophobicity. I53 normally may interact with some other hydrophobic surface on RNA polymerase.

Structural characterization of σ^{70}-I53A in vitro

To address the possibility that σ^{70}-I53A might be generally misfolded, and inactive *in vivo* for that reason, we used circular dichroism (CD) as an indication of overall conformational integrity (Callaci and Heyduk, 1998; Owens *et al.*, 1998). Figure 3A shows the spectra of both σ^{70} and σ^{70}-I53A at 25°C. The superimposability of the two curves suggests that the inactivity of σ^{70}-I53A is unlikely to be attributed to gross structural perturbation of the native polypeptide.

Another commonly used probe of protein structure is limited proteolytic digestion. σ^{70} is cleaved into a series of characteristic bands using partial trypsinolysis (Lowe *et al.*, 1979; Severinova *et al.*, 1996; Callaci and Heyduk, 1998). By performing this analysis at several different temperatures, we tested whether the structure of σ^{70}-I53A might be perturbed such that an increase in temperature would result in either a greater extent of proteolysis or an alteration in the pattern of bands. The same proteolytic products were generated by partial trypsinolysis of both σ^{70} and σ^{70}-I53A (Figure 3B). Additionally, the extent of digestion for σ^{70}-I53A was equivalent to σ^{70} at 25, 37 and 44°C. While these experiments cannot rule out the possibility that a minor local structural difference between these σ factors accounts for differences in behavior, they indicate that the overall structure of σ^{70}-I53A resembles that of σ^{70}.

Transcription by σ^{70}-I53A holoenzyme in vitro

Holoenzyme was reconstituted by adding σ^{70} or σ^{70}-I53A to purified core RNA polymerase (E). The ability of these enzymes to facilitate transcription initiation from the strong σ^{70}-dependent λP_R promoter (Hawley and McClure, 1980; Roe *et al.*, 1984) was tested using a run-off transcription assay to generate an 80 nucleotide product (Wilson and Dombroski, 1997). Eσ^{70}-I53A was severely defective in generating run-off transcripts, displaying a significantly slower rate of transcript production as compared with Eσ^{70} (Figure 4). Transcription by Eσ^{70}-I53A improved slightly when performed at 42°C (data not

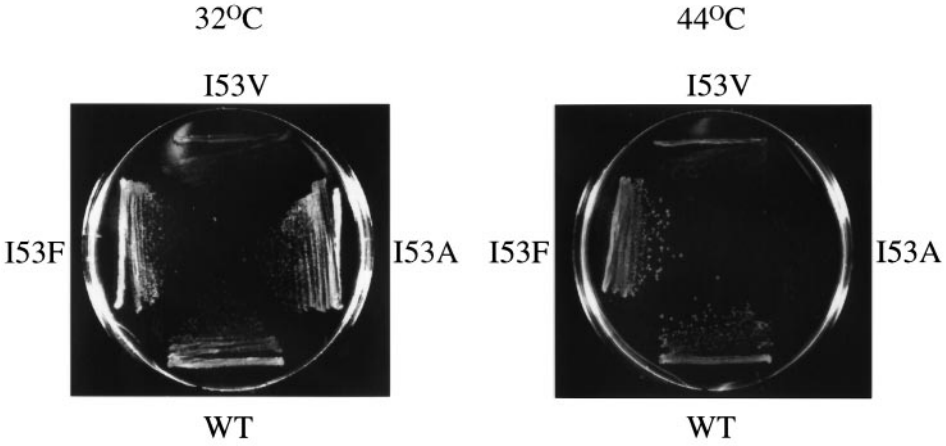

Fig. 2. Complementation of the *ts* growth phenotype of strain 19284 (*rpoD800*). LB agar plates with ampicillin (100 μg/ml), kanamycin (30 μg/ml) and 2% glucose were incubated at either the permissive (32°C) or the non-permissive (44°C) temperature, as indicated. The *rpoD* genes tested for complementation were provided on pQE30T (Wilson and Dombroski, 1997). The hexahistidine-tagged wild-type σ^{70} (WT) and mutants (I53A, I53F and I53V) are indicated next to the relevant quadrant of the plate.

Table I. Function of σ^{70} derivatives *in vivo* in strain CAG20176

σ^{70} derivative	–IAA/+IAA	WT/mutant
Wild-type	0.75	1.00
I53A	0.19	3.90
I53V	0.84	0.89
I53F	0.97	0.77
I53Asup	0.50	1.50
I53A/CΔ5	0.68	1.10

The mutated *rpoD* genes were present on pQE-30T. The chromosomal copy of *rpoD* is under the control of a synthetic *trp* promoter. Growth in LB media requires IAA unless a functional σ^{70} is provided from the plasmid copy of *rpoD*. Values for –IAA/+IAA approaching 1.0 indicate increased ability to substitute for the chromosomal copy of *rpoD*. Values normalized to the behavior of the wild-type control are shown in the WT/mutant column. These experiments were repeated at least three times with an error of not more than 20%.

shown), agreeing with the idea that σ^{70}-I53A is not thermolabile or misfolded.

DNA binding by σ^{70}-I53A holoenzyme in vitro

The first step in transcription initiation is recognition and binding of the promoter by RNA polymerase to form a closed complex. Nitrocellulose filter binding has been used to evaluate the kinetics and mechanism of DNA binding by RNA polymerase at the λP_R promoter (Hinkle and Chamberlin, 1972a,b; Strauss *et al.*, 1980). Eσ^{70} or Eσ^{70}-I53A was added to 5′-end-labeled λP_R promoter DNA 230 bp in length, and samples were removed and filtered through nitrocellulose. It was shown previously that under the conditions used in this experiment, most of the retained complexes are heparin-stable open complexes and thus represent specific binding to the promoter (Roe *et al.*, 1984). Eσ^{70}-I53A was unable to form filter-retainable complexes even after extended times (Figure 5), indicating weak DNA-binding ability for this enzyme.

We conducted DNase I and $KMnO_4$ footprinting analysis of Eσ^{70} and Eσ^{70}-I53A on the λP_R promoter as previously described (Wilson and Dombroski, 1997). The mutant holoenzyme bound to the same region of the promoter as wild-type polymerase in DNase I experiments but with lower affinity, as expected based on the nitrocellulose filter binding results (data not shown). Open complexes formed more slowly as determined by $KMnO_4$ reactivity, but at the same position on the promoter as the control (data not shown).

Core binding by σ^{70}-I53A

One simple explanation for the apparent DNA-binding deficiency of Eσ^{70}-I53A was that it might have reduced affinity for association with the core subunits. The ability of the mutant σ factor to assemble into holoenzyme was evaluated using native gel-shift experiments and immunoaffinity methods. In both cases, hexahistidine-tagged σ^{70} and σ^{70}-I53A were prepared from [^{35}S]methionine/cysteine-labeled total cell extracts. In the gel shift experiments, ^{35}S-labeled σ factor was added to unlabeled core RNA polymerase to reconstitute holoenzyme using increasing amounts of σ factor. Electrophoresis on a native polyacrylamide gel differentiated between free σ factor and σ factor bound to core by virtue of the size difference between the two species. No difference was noted between σ^{70} and σ^{70}-I53A in equilibrium binding to core (data not shown).

A similar experiment was conducted using monoclonal antibodies to the β′ subunit to extract complexes between the labeled σ subunit and core from solution by co-immunoaffinity. The amount of σ factor bound to core was similar for σ^{70} and σ^{70}-I53A (Figure 6A). As a more rigorous test of potential core-binding differences between the two σ factors, we evaluated the ability of σ^{70}-I53A to compete with σ^{70} for association with core. Holoenzyme was formed by incubating ^{35}S-labeled σ^{70} or σ^{70}-I53A with core, followed by addition of either unlabeled σ^{70} or σ^{70}-I53A. Using co-immunoaffinity to isolate holoenzyme complexes, we showed that unlabeled σ^{70}-I53A competed equally effectively with labeled ^{35}S-σ^{70}-I53A and with ^{35}S-σ^{70} for binding to core, and conversely that unlabeled σ^{70} competed with both labeled σ factors (Figure 6B). Taken together, our data are consistent with the interpretation that the I53A substitution does not compromise the ability of the mutant σ factor to bind to core.

Studies have indicated that major conformational changes accompany σ factor binding to core (Polyakov

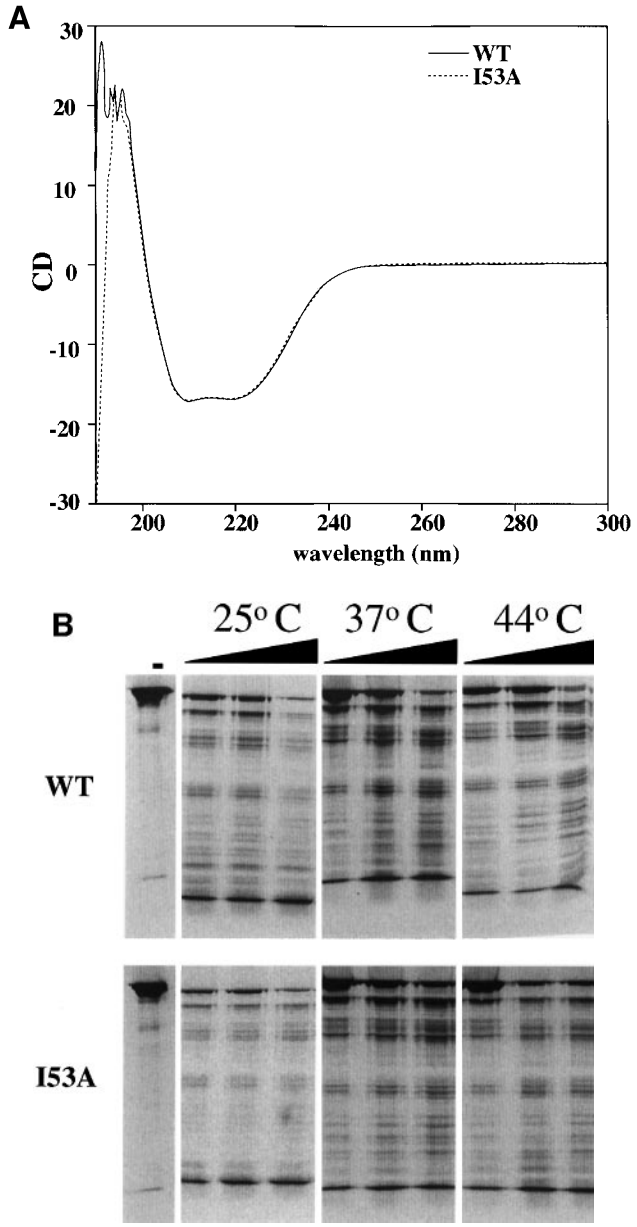

Fig. 3. Structural analysis of σ^{70}-I53A. (**A**) CD spectra of hexahistidine-tagged wild-type σ^{70} (WT) and mutant (I53A) σ^{70} performed at 25°C. (**B**) Limited trypsinolysis as a function of temperature. ^{35}S-Labeled σ factor was digested with increasing amounts of trypsin at 25, 37 and 42°C, and the bands were resolved on an 8% SDS–polyacrylamide gel. The first lane (–) shows untreated σ factor. The hexahistidine-tagged wild-type σ^{70} (WT) and mutant (I53A) σ^{70} are indicated.

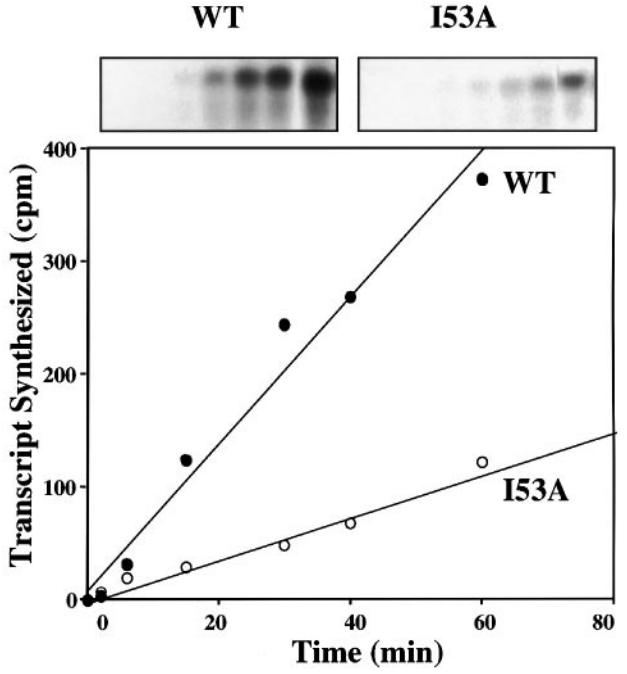

Fig. 4. Run-off transcription as a function of time *in vitro*. Reconstituted holoenzymes containing either hexahistidine-tagged wild-type σ^{70} (WT) or mutant (I53A) σ^{70} were used to transcribe a linear DNA template containing the λP_R promoter at 37°C. The 80 nucleotide transcript is shown above the plot as resolved on an 8% denaturing polyacrylamide gel. The plot shows quantification of the gel bands, where transcripts synthesized (c.p.m.) are plotted as a function of time (min).

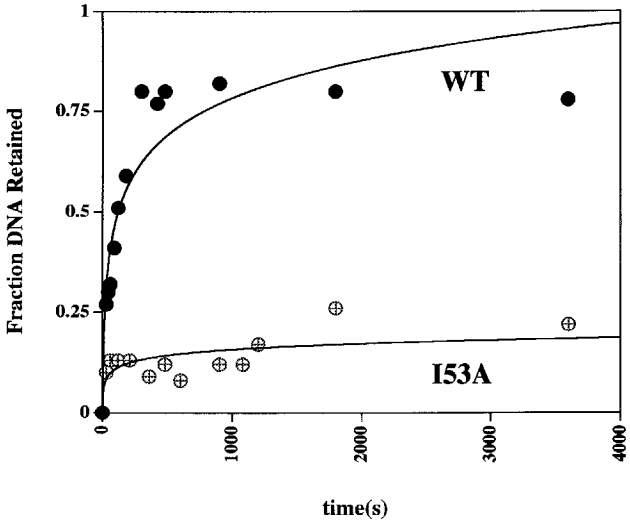

Fig. 5. Binding of holoenzyme to λP_R promoter DNA. Reconstituted holoenzymes containing either hexahistidine-tagged wild-type σ^{70} (WT) or mutant (I53A) σ^{70} were mixed with ^{32}P-labeled linear DNA of 230 bp in length containing the λP_R promoter at 37°C in binding buffer. Samples were removed at the times indicated, filtered through nitrocellulose and washed with a 10× volume of wash buffer.

et al., 1995), and it has been proposed that the conformation of σ^{70} must be altered such that the N-terminal inhibitory domain no longer prevents DNA recognition once σ^{70} associates with core (Dombroski *et al.*, 1992, 1993). One explanation for the behavior of σ^{70}-I53A is that although it interacts with core, it fails to undergo effectively the appropriate conformational change(s) that unmask the DNA-binding domains and permit promoter recognition by holoenzyme.

Isolation of a suppressor of the I53A mutation

The failure of σ^{70}-I53A to complement the *ts* growth phenotype of the *rpoD800* strain provided a means to identify suppressors that would permit growth at the non-permissive temperature. pQE30T-σ^{70}-I53A was shuttled through a strain defective in DNA mismatch repair (*mutDmutY*), and transformed into the *rpoD800* strain. Colonies that survived at 44°C were selected for further analysis. The mutated plasmids were isolated and reintroduced into the *rpoD800* strain to determine if the sup-

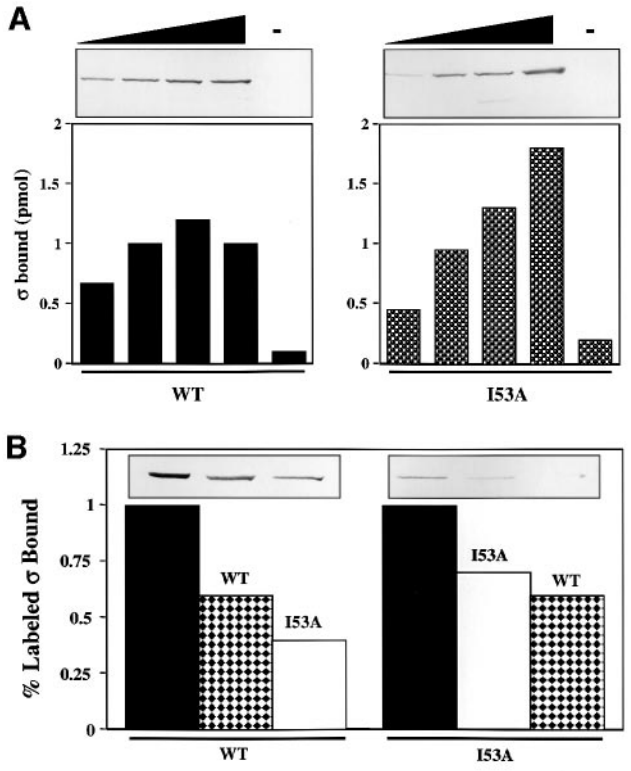

Fig. 6. Binding of σ factor to the core subunits of RNA polymerase. (**A**) Equilibrium binding. ^{35}S-labeled hexahistidine-tagged wild-type σ^{70} (WT) or mutant σ^{70} (I53A) was mixed with purified core RNA polymerase ($\alpha_2\beta\beta'$) to form holoenzyme, which was extracted from solution using monoclonal antibody to the β′ subunit (see Materials and methods). The wedges indicate increasing amounts of σ factor (4–28 pmol) added to core (3.5 pmol). The bar plots indicate the amount of σ factor recovered in pmoles, and the values have been corrected to account for the difference in specific activity of the radioactive σ factor preparations. The lane marked (–) is a control containing no core polymerase and thus indicates the background retention of labeled σ factor. (**B**) Competition binding. ^{35}S-labeled hexahistidine-tagged wild-type σ^{70} (WT) or mutant σ^{70} (I53A) was mixed with core RNA polymerase ($\alpha_2\beta\beta'$) to form holoenzyme, followed by addition of unlabeled competitor σ factor. Complexes were extracted from solution using monoclonal antibody to the β′ subunit (see Materials and methods). The labels above the bars indicate the identity of the unlabeled competitor σ factor. The labels below the bar chart indicate the identity of the ^{35}S-labeled σ factor. The plots are normalized to the amount of σ factor bound to core in the absence of competitor. Representative experiments are shown. They were repeated at least three times and we estimate the error to be no more than 14%.

pressor was associated with the plasmid. In several cases, the phenotype did not accompany the plasmid. These suppressors were not pursued for this study, and may reside in the genes for other subunits of RNA polymerase. One plasmid-associated suppressor candidate was identified and sequenced. This suppressor consisted of a five amino acid deletion from the C-terminus of σ^{70}-I53A, accompanied by a leucine (L) to proline (P) substitution at position 609, an arginine (R) to alanine (A) substitution at position 610, and a methionine (M) to isoleucine (I) substitution as position 567, between regions 4.1 and 4.2. We evaluated each of these changes more carefully to determine if one, or all, were necessary for the suppression of the I53A phenotype. Removal of just the C-terminal five amino acids (σ^{70}-I53A/CΔ5) resulted in suppression (Figure 7) of *rpoD800* at 44°C. Both the original suppressor with multiple changes and σ^{70}-I53A/CΔ5 were also able to substitute for σ^{70} *in vivo* in strain CAG20176, where the chromosomal copy of *rpoD* was turned off (Table I). Complementation of *rpoD800* required that expression of σ^{70} from the plasmid be maintained at lower levels than for complementation of CAG20176. If the I53A change causes σ^{70} to be 'locked' into a conformation that is autoinhibitory for DNA binding, then removal of the C-terminal residues in the suppressor may 'loosen' the structure of the protein enough to allow the σ^{70}-I53A to make the conformational transitions upon core association that are required for efficient promoter binding. We were unable to purify either the original suppressor or σ^{70}-I53A/CΔ5 in an active form for analysis *in vitro*; however, the genetic evidence strongly supports the fact that they must function *in vivo* and reverse the effect of the I53A substitution.

Discussion

The σ subunit of RNA polymerase must participate in a number of macromolecular interactions during the process of transcription initiation. One of the earliest interactions is between σ factor and the core subunits of polymerase to form holoenzyme. Binding of σ^{70} to core is accompanied by significant conformational changes in RNA polymerase holoenzyme (Wu *et al.*, 1976; Darst *et al.*, 1989; Polyakov *et al.*, 1995). In particular, the σ subunit is proposed to undergo conformational alterations upon interaction with core based upon several lines of evidence. First, σ^{70} does not bind to DNA with measurable affinity or specificity in the absence of the core subunits, despite the fact that the promoter recognition determinants for holoenzyme are located within the σ subunit. Previous studies have proposed that the N-terminal domain (region 1) of σ^{70} modulates DNA-binding activity, and that a conformational change in σ factor upon binding to core unmasks the DNA-binding domains to permit promoter recognition (Dombroski *et al.*, 1992, 1993). Secondly, it has been demonstrated recently that RNA polymerase interacts with the non-template strand of DNA in the –10 promoter region and this interaction is mediated by the σ subunit (Ring *et al.*, 1996; Roberts and Roberts, 1996). However, polypeptides of σ^{70} containing region 2 only bind to the non-template strand of the promoter if they are associated with core RNA polymerase, invoking a conformational change upon core binding that allows single-stranded DNA binding (Severinova *et al.*, 1996; Marr *et al.*, 1997). Finally, introduction of a fluorescent probe in region 2.3 of σ^{70} has been used to demonstrate core-induced conformational changes in σ factor that lead to increased affinity for non-template strand DNA in the open complex, and implicates core as an allosteric regulator of σ^{70} (Callaci *et al.*, 1998).

In this report, we describe a substitution of isoleucine to alanine at position 53 of σ^{70} that severely impairs the promoter-binding capability of holoenzyme. This alteration occurs in region 1.1, which has been implicated in controlling the DNA-binding ability of purified σ^{70} (Dombroski *et al.*, 1992, 1993). Previous studies postulated that region 1.1 inhibits DNA binding by interacting with the C-terminal DNA-binding region, specifically region 4 (Dombroski *et al.*, 1993). The behavior of Eσ^{70}-I53A is

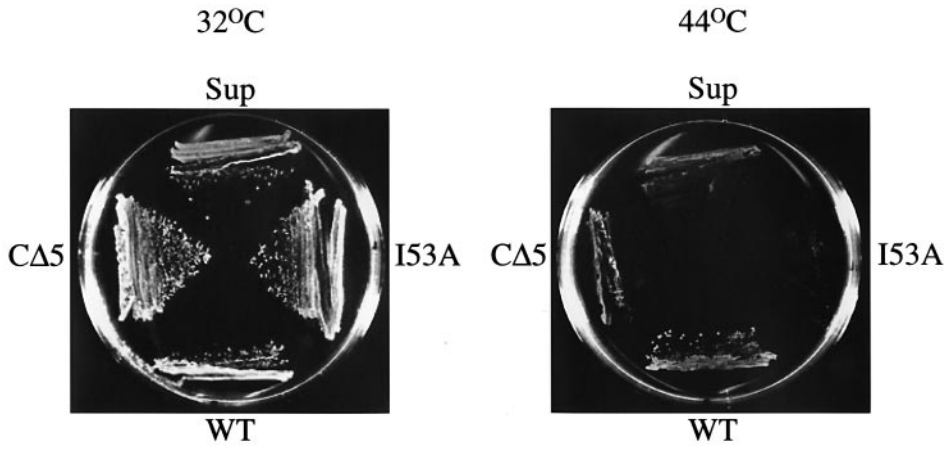

Fig. 7. Complementation of the *ts* growth phenotype of strain 19284 (*rpoD800*) by suppression of I53A. LB agar plates with ampicillin (100 μg/ml), kanamycin (30 μg/ml) and 2% glucose were incubated at either the permissive (32°C) or the non-permissive temperature (44°C), as indicated. The *rpoD* genes tested for complementation were provided from pQE30T (Wilson and Dombroski, 1997). The hexahistidine-tagged wild-type σ^{70} (WT), mutant σ^{70} (I53A) and suppressors (CΔ5, Sup) are indicated. Sup is the original suppressor and includes CΔ5 along with M567I, L609P and R610A.

consistent with σ^{70}-I53A being defective in escaping the autoinhibitory conformation although it can associate as well as σ^{70} with the core subunits of RNA polymerase. This model is supported by several observations. Eσ^{70}-I53A is significantly functionally impaired because it cannot substitute for Eσ^{70} *in vivo* and has greatly reduced transcriptional activity *in vitro*. This defect is more pronounced *in vitro* at 25°C, consistent with a conformational change problem (data not shown). We do not believe that its inactivity is due to major structural perturbations based upon the CD spectra, proteolytic susceptibility and core-binding affinity, all of which displayed behavior similar to σ^{70}. The most consequential property of this mutant is its failure to confer adequate promoter-binding ability upon holoenzyme, thus causing a major detrimental effect at the earliest stages of transcription initiation. A plausible explanation for the weakened ability of Eσ^{70}-I53A to bind stably to DNA is that although the σ factor has associated with core, the conformational change(s) necessary to expose the DNA-binding domains occur very inefficiently, or aberrantly. It is unclear whether interaction of holoenzyme with DNA is necessary for the appropriate conformational change in σ^{70} to occur, or whether it only requires association with core and thus occurs prior to DNA binding. This model is supported further by the fact that a suppressor of the σ^{70}-I53A phenotype *in vivo* consists of a truncation of five amino acids from the C-terminus (σ^{70}-I53A/CΔ5). We imagine that this suppressor functions by loosening the intramolecular contacts between the N- and C-terminal domains that act negatively to affect DNA binding by σ factor, thus relieving the autoinhibitory behavior that normally is eliminated when σ^{70} binds to core.

We are currently in the process of identifying the interacting sites within σ^{70} biochemically. Increasing evidence implicates many structural/conformational transitions in both holoenzyme and σ factor occurring during transcription initiation. The results presented here indicate that the event of core binding can be separated from the conformational change in σ factor that must occur during or following core binding to alleviate autoinhibition of DNA binding.

Materials and methods

Materials

Restriction endonucleases and T4 DNA ligase were from New England Biolabs and Promega. Vent^R DNA polymerase was from New England Biolabs. Buffer and gel components were from Sigma or Fisher Scientific. Oligonucleotides were synthesized by Genosys, Gibco-BRL Life Technologies or Integrated DNA Technologies. Sequences are available upon request.

Plasmid constructions and mutagenesis

Oligonucleotide site-directed mutagenesis (Kunkel *et al*., 1987) was used to replace the conserved isoleucine codon (ATT) with an alanine codon (GCT), a phenylalanine codon (TTT) and a valine codon (GTT), corresponding to amino acid position 53. The mutagenized *rpoD* gene sequences were amplified from M13 RF DNA by PCR using oligonucleotides designed to incorporate *Kpn*I and *Hin*dIII restriction sites at the 5′ and 3′ ends of the *rpoD* gene fragments. Following restriction digestion, the fragments were ligated into pQE-30T resulting in a hexahistidine tag at the N-terminus of σ^{70} (Wilson and Dombroski, 1997). Plasmids were next transformed, along with the wild-type (WT) *rpoD* pQE-30T derivative, into *E.coli* strain 19284 (*rpoD800*, *srl* W3110::Tn*10 recA*, *lacI*^{q}) (Wilson and Dombroski, 1997). Transformants were streaked onto Luria broth (LB) plates containing ampicillin (100 μg/ml), kanamycin (30 μg/ml) and 2% glucose, and scored for the ability to complement the *ts* growth defect of *rpoD800* at 44°C.

Overproduction and purification of σ derivatives

Hexahistidine-tagged σ^{70} and σ^{70}-I53A were overproduced and purified as previously described (Wilson and Dombroski, 1997). The following modifications were introduced for purification of ^{35}S-labeled proteins. Overnight cultures (100 ml) were grown at 37°C in minimal M9 medium supplemented with a mixture of amino acids (A, R, N, G, H, I, L, K, P, S, T and V), ampicillin (100 μg/ml), tetracycline (10 μg/ml) and 2% glucose. These cultures were diluted into 1 l each of minimal M9 medium supplemented with amino acids and 100 μg/ml ampicillin, grown at 37°C to an OD_{450} of 0.5 (±0.1) and induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. After 20 min of induction, 1 mCi of Trans ^{35}S-label™ (L-methionine, L-cysteine, 1000 Ci/mmol) (ICN) was added and incubation continued for 2.5 h. For purification of σ factor, a 0.5 g cell pellet was resuspended in 15 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9) supplemented with lysozyme (0.3 mg/ml final concentration). Lysis was accomplished by alternately freezing samples on dry ice and thawing in a 37°C water bath. Purification continued as previously described (Wilson and Dombroski, 1997).

Structural analysis of σ factor derivatives

The CD spectra of σ^{70} and σ^{70}-I53A were measured using a JASCO 710 CD Spectrophotometer. Purified protein was dialyzed against transcription buffer to remove glycerol [40 mM Tris–HCl pH 7.5, 5 mM

$MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol (DTT)] and adjusted to a final concentration of 0.33 μg/μl. Measurements were made using a 1 mm pathlength cell and data analyzed using the JASCO software.

^{35}S-labeled σ^{70} and σ^{70}-I53A proteins (2 μg) were digested with increasing amounts of trypsin (Fisher Scientific) (0.0125, 0.0625 and 0.25 μg) at 25, 37 or 44°C in trypsin buffer (0.01 mM EDTA, 0.01 mM Tris pH 7.9, 1 mM DTT, 0.05 M NaCl and 10% glycerol). Fragments were resolved on 8% SDS–polyacrylamide gels and visualized by autoradiography.

Transcription by σ^{70}-I53A holoenzyme in vitro

Multi-round run-off transcription from the 230 bp λP_R template fragment was performed as previously described (Wilson and Dombroski, 1997), except that reactions were initiated by addition of transcription buffer (see above) containing template (8.8 nM) and nucleoside triphosphates (200 μM ATP, CTP, UTP and 20 μM GTP), omitting the template pre-incubation step. Reaction aliquots (10 μl) were removed to 5 μl of formamide stop solution (0.04% xylene cyanol, 0.04% bromophenol blue), heated for 4 min at 100°C and resolved on 8% denaturing polyacrylamide gels. The transcript synthesized as a function of time was quantified using a Packard Instantimager.

Immunoaffinity core association assay

For equilibrium binding, 3.5 pmol of core RNA polymerase was incubated with 4, 8, 16 and 28 pmol of ^{35}S-labeled σ factor in protein dilution buffer [5% glycerol, 0.3 M NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5, 1 mM β-mercaptoethanol, 0.1 mM EDTA, 0.05% Tween and 100 μg/ml bovine serum albumin (BSA)] in a final reaction volume of 15 μl. Control samples contained σ factor only. The mixtures were incubated for 10 min at 37°C, followed by a 1 h incubation on ice with monoclonal antibody (NT73) to the β′ subunit of RNA polymerase (gift of R.Burgess and N.Thompson). Protein A–Sepharose beads (100 μl; purchased from either Fisher or Pierce Biochemicals) equilibrated in protein dilution buffer (50% slurry) were added and incubated for an additional 2 h on ice. The beads were washed three times with protein dilution buffer, to remove unbound σ factor, followed by the addition of 4× SDS protein loading dye. Samples were heated to 100°C for 10 min and supernatants loaded onto 8% SDS–polyacrylamide gels. Dried gels were subjected to autoradiography and the bands quantified with a Packard Instantimager.

For the competition assay, 8 pmol of core RNA polymerase was incubated with 20 pmol of ^{35}S-labeled σ factor, for 15 min at 37°C, and then 150 pmol of unlabeled competitor σ factor was added to a final volume of 30 μl and incubated for an additional 15 min at 37°C. Subsequent incubation steps and controls were performed as described above.

Promoter-binding analysis

5′ [γ-^{32}P]ATP-end-labeled λP_R promoter DNA (237 bp in length) was prepared as previously described (Wilson and Dombroski 1997). Holoenzyme–promoter complexes were formed at 37°C in binding buffer (40 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 100 μg/ml BSA), following the method of Roe *et al.* (1984). Briefly, 1 nM polymerase was incubated with 0.1 nM template to minimize the effect of non-promoter modes of binding (Roe *et al.*, 1984). Binding was performed both in the presence and absence of 50 μg/ml of heparin. DNA samples and RNA polymerase were pre-equilibrated separately at 37°C, and then mixed; sample aliquots were removed at the desired time points and filtered through nitrocellulose filter discs (GS 0.22 μm; Millipore). Discs were washed with 500 μl of wash buffer (10 mM Tris pH 7.5, 0.1 mM EDTA, 0.1 M NaCl), dried under an infrared heat source and subjected to liquid scintillation counting.

Suppressor isolation and analysis in vivo

pQE30T-σ^{70}-I53A was shuttled through the mutator strain *E.coli* TX3683 (*mutD5*, *mutY*::Tn*10*) (gift of M.Winkler). Mutagenized plasmid was purified from enriched cultures and transformed into *E.coli* 19284 (*rpoD800*ts background, see above). pQE30T-σ^{70} and unmutagenized pQE30T-σ^{70}-I53A were included as positive and negative controls. Suppression was defined as growth at 44°C. Plasmid was recovered from candidate survivors that grew at 44°C, and retransformed into *E.coli* 19284 to confirm that the suppression phenotype was plasmid dependent. The ability of the suppressor, as well as the site-directed mutants, to substitute for σ^{70} *in vivo* was also evaluated in strain CAG20176 at 28°C as previously described (Wilson and Dombroski, 1997). Intragenic suppressor candidates were sequenced using a Sequenase kit (Amersham) and by automated sequencing (Applied Biosystems, University of Texas Medical School). The multiple changes to the original suppressor were separated using oligonucleotide site-directed mutagenesis and PCR.

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