Conservation of sigma–core RNA polymerase proximity relationships between the enhancer-independent and enhancer-dependent sigma classes

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Two distinct classes of RNA polymerase sigma factors (σ) exist in bacteria and are largely unrelated in primary amino acid sequence and their modes of transcription activation. Using tethered iron chelate (Fe-BABE) derivatives of the enhancer-dependent σ^{54} , we mapped several sites of proximity to the β and β' subunits of the core RNA polymerase. Remarkably, most sites localized to those previously identified as close to the enhancer-independent σ^{70} and σ^{38} . This indicates a common use of sets of sequences in core for interacting with the two σ classes. Some sites chosen in σ^{54} for modification with Fe-BABE were positions, which when mutated, deregulate the σ^{54} –holoenzyme and allow activator-independent initiation and holoenzyme isomerization. We infer that these sites in σ^{54} may be involved in interactions with the core that contribute to maintenance of alternative states of the holoenzyme needed for either the stable closed promoter complex conformation or the isomerized holoenzyme conformation associated with the open promoter complex. One site of σ^{54} proximity to the core is apparently not evident with σ^{70} , and may represent a specialized interaction.

Keywords: enhancers/ σ factors/protein footprinting/RNA polymerase

Introduction

The *Escherichia coli* RNA polymerase is a multisubunit enzyme that exists in two major forms with respect to its subunit composition. The core enzyme ($\alpha_2\beta\beta'$; E) is capable of processive transcription elongation followed by termination, but is unable to initiate transcription specifically unless it is in its σ -bound holoenzyme form ($E\sigma$). The σ subunit confers promoter-specific DNA binding and transcription initiation capabilities on the RNA polymerase. Based on structural and functional criteria, the seven different σ factors (σ^{70} , σ^{54} , σ^{38} , σ^{32} , σ^{28} , σ^{24} and σ^{18}) identified in *E. coli* are grouped into two classes. Most σ factors, with the exception of σ^{54} , belong to the σ^{70} class, the major σ factor in *E. coli* that is involved in expression of most genes during exponential growth. σ^{54} (σ^N) represents a unique class that differs functionally from the σ^{70} class. The presence of σ^{54} has been well

documented in several proteobacteria as well as in the Gram-positive *Bacillus subtilis* (Debarbouille *et al.*, 1991) and in *Planctomyces limnophilus* (Leary *et al.*, 1998). σ^{54} directs its holoenzyme to recognize and bind promoters with conserved –12/–24 regions located between positions –11 and –26 relative to the transcription start. These can be considered the functional analogue of the –10/–35 core promoter elements recognized by σ factors belonging to the σ^{70} class. In *E. coli*, both σ^{70} , the major σ factor for growth-related genes, and σ^{54} are always present, but other minor σ^{70} class members are induced under certain stress conditions (Jishage *et al.*, 1997).

Although σ^{54} binds the same core RNA polymerase as the σ^{70} class members, each σ class confers different properties on the holoenzymes formed. $E\sigma^{54}$ causes transcription to assume several features reminiscent of higher eukaryotic mechanisms (Sasse-Dwight *et al.*, 1990). In marked contrast to the σ^{70} –holoenzyme, which often initiates transcription in the absence of transcription activators, the σ^{54} –holoenzyme remains in a closed promoter complex in the absence of activator protein. Isomerization of this closed complex to a transcriptionally competent open complex depends upon the activity of an activator protein bound to a DNA sequence with enhancer-like properties coupled to γ – β bond nucleoside triphosphate hydrolysis by the activator protein (Weiss *et al.*, 1991).

The functional domain organization of σ^{54} is complex and appears to differ significantly from that of the members of the σ^{70} class. In particular, overall DNA binding by σ^{70} is modulated by N-terminal sequences, whereas N-terminal sequences in σ^{54} are required for activation. Extensive deletion and mutational analyses of σ^{54} have allowed functions to be assigned to different regions of the protein (Merrick *et al.*, 1993; Wong *et al.*, 1994; Cannon *et al.*, 1995; Gallegos and Buck, 1999). The N-terminal region I of σ^{54} is regulatory and closely implicated in polymerase isomerization and DNA melting (Cannon *et al.*, 1999; Gallegos *et al.*, 1999; Guo *et al.*, 1999). It plays a central role in mediating the response to activator proteins and in binding early melted DNA structures (Gallegos and Buck, 2000; W.Cannon, M.T.Gallegos and M.Buck, submitted), but is dispensable for core and overall DNA binding (Cannon *et al.*, 1999). Region II is acidic and not conserved. Region III includes a major core RNA polymerase-binding determinant and sequences that directly contact DNA and that enhance σ^{54} –DNA binding (Merrick and Chambers, 1992; Cannon *et al.*, 1997; Gallegos and Buck, 1999) (Figure 1A). Emerging roles for region III appear to be in maintaining the closed promoter complex in a transcriptionally silent state and in generating polymerase isomerization upon activation (Chaney and Buck, 1999).

Fig. 1. (A) Schematic representation of *Klebsiella pneumoniae* σ^{54} domain organization. The locations of the native cysteine residues are indicated. (B) Sites where a single cysteine was introduced and conjugated with Fe-BABE. The arrow indicates that conjugation was performed under denaturing conditions.

The core RNA polymerase-binding interface of σ^{54} comprises at least two functionally distinct sequences: a 95 amino acid residue sequence (120–215) within region III, which binds the core strongly, and a second sequence within region I that binds the core more weakly (Gallegos and Buck, 1999). Comparison of hydroxyl radical-mediated cleavage profiles of wild-type and region I-deleted ($\Delta R1\sigma^{54}$) proteins indicated that the overall solvent-exposed surface of σ^{54} is not greatly altered by deletion of region I (Casaz and Buck, 1999). Residues protected from hydroxyl radical-mediated cleavage in the holoenzyme made with $\Delta R1\sigma^{54}$ largely coincided with residues protected specifically by DNA in the wild-type holoenzyme, implying a role for region I in establishing an appropriate conformation of the DNA-binding domain in the wild-type holoenzyme (Casaz and Buck, 1999). Thus, it is possible that the interaction of region I with core RNA polymerase is important for controlling DNA-binding domain conformation.

To date, little is known about the location of σ^{54} -binding sites on the core RNA polymerase. Recently, we showed by small-angle X-ray scattering studies that core RNA polymerase-binding fragments of σ^{54} (amino acids 70–324) and the crystal structure of core RNA polymerase-binding σ^{70} fragment (amino acids 114–448) have a similar envelope shape, leading to the suggestion that both σ classes might be located in similar places on the core (Svergun *et al.*, 2000). However, the sequence differences that exist between σ^{54} and σ^{70} must also account for the markedly different properties of the two holoenzymes. The affinities of σ^{54} and σ^{70} for the core RNA polymerase measured *in vitro* are similar (Gallegos and Buck, 1999). Both σ factors could use the same binding site on core RNA polymerase, have overlapping sites or utilize additional independent binding sites. Recent protein–protein interaction and footprinting studies have revealed that the β' subunit of core RNA polymerase provides the major binding interaction for σ^{70} in the holoenzyme, while the β subunit contributes a further binding interaction (Arthur and Burgess, 1998; Owens *et al.*, 1998; Katayama *et al.*, 2000). In the present work, we have used tethered

nucleophile-mediated footprinting to extend our understanding of the σ^{54} -core RNA polymerase interface. Using (*p*-bromoacetamidobenzyl)-EDTA Fe (Fe-BABE) derivatives of σ^{54} , we mapped several sites of proximity to the β and β' subunits of the core RNA polymerase. Remarkably, most sites localized to those previously identified as close to enhancer-independent σ^{70} (Owens *et al.*, 1998) and σ^{38} (A.Kolb, personal communication). This indicates use of the same or overlapping sequences in core RNA polymerase for interaction with the two σ classes. One site of σ^{54} proximity to the β' subunit apparently is not evident with σ^{70} , and may represent a specialized interaction of σ^{54} with core RNA polymerase.

Results

Location of Fe-BABE conjugation targets on σ^{54}

The bifunctional chelating agent Fe-BABE covalently conjugates to the free sulfhydryl groups of cysteine residues. After the addition of ascorbate and peroxide, the Fe-BABE generates nucleophiles, which cleave polypeptide chains in proximity (~ 12 Å) to the chelate, independently of the amino acid sequence involved (Rana and Meares, 1991; Ishihama, 2000). Using information from extensive mutational analysis of σ^{54} , we selected five sites (E36, C198, R336, C346 and R383, described below) of potential interest for tethering Fe-BABE. Figure 1B shows the schematic representation of the primary structure of σ^{54} , in which the positions of introduced cysteine residues are indicated.

Cys198 and Cys346. The σ^{54} from *Klebsiella pneumoniae* contains two highly conserved cysteine residues at amino acid positions 198 and 346. Cys198 is located in a 95 amino acid sequence (120–215), which binds core RNA polymerase strongly and is likely to include most of the fold critical for forming stable holoenzyme (Gallegos and Buck, 1999). Cys346 borders a DNA-interacting patch in σ^{54} (Cannon *et al.*, 1994) and is strongly protected by the core in hydroxyl radical cleavage experiments when region I is absent (Casaz and Buck, 1999). By changing Cys198 and Cys346 to alanine, we constructed two mutant σ^{54} proteins with single cysteines at positions 198 (C346A) and 346 (C198A). The Cys(–)*rpoN* construct was used to purify the cysteine-free double mutant (C198A and C346A) protein, which was used as the negative control protein in the cleavage assays. The Cys(–) σ^{54} protein was found to maintain 90% of its transcriptional activity (see later), implying that substitution of the conserved Cys198 and Cys346 with alanine does not significantly affect the conformation of the mutant protein.

Cys36. In the next step, single cysteine residues were introduced into Cys(–) σ^{54} . Region I of σ^{54} has multiple roles in regulating σ^{54} function: (i) inhibiting polymerase isomerization in the absence of activation and directing fork junction DNA binding (Wang *et al.*, 1995; Syed and Gralla, 1998; Cannon *et al.*, 1999; Guo *et al.*, 1999); and (ii) stimulating initiation of open complex formation in response to activation (Sasse-Dwight and Gralla, 1990; Syed and Gralla, 1998; Casaz *et al.*, 1999; Gallegos and Buck, 2000). The low affinity of region I sequences for

core RNA polymerase and its protection from proteolysis by core (Casaz and Buck, 1997, 1999; Gallegos and Buck, 1999) prompted us to introduce a single cysteine residue into region I in the Cys(-) σ^{54} protein. The increased protease sensitivity of residue E36 detected upon open complex formation provided us with a potential target for introducing a cysteine that could be in close proximity to the core RNA polymerase subunits in closed complexes (Casaz and Buck, 1997).

Cys336. Recently, we showed that a single amino acid substitution, R336A, in the region III DNA-binding domain of σ^{54} allows holoenzyme to isomerize and transcribe without activator (Chaney and Buck, 1999), a phenotype previously only associated with region I mutations (Hsieh and Gralla, 1994; Hsieh *et al.*, 1994; Syed and Gralla, 1998; Casaz *et al.*, 1999). We justify our choice of R336 for introducing a single cysteine residue by suggesting that R336 is a part of a network of interactions necessary for maintaining the transcriptionally silent state of the holoenzyme and thus might interact directly or indirectly with core RNA polymerase subunits.

Cys383. The highly conserved arginine at position 383 is located within the second helix of the proposed helix–turn–helix motif in region III of σ^{54} , suggested to make direct contact with the conserved –12 promoter sequences (Coppard and Merrick, 1991; Merrick and Chambers, 1992). Recent evidence shows conserved arginine residues in the region III DNA-binding domain of σ^{54} prevent polymerase isomerization (Chaney and Buck, 1999). Furthermore, the –12 promoter sequence is part of a molecular switch that has to be thrown by the action of the activator for transcription to proceed (Guo *et al.*, 1999) through a network of communication possibly involving R383, other σ^{54} parts and/or the core subunits. We therefore targeted R383 to introduce a single cysteine residue.

Single cysteine σ^{54} mutants are active for transcription

Next, the activity of each single cysteine mutant and the Cys(-) σ^{54} protein was compared with the activity of wild-type σ^{54} in an *in vitro* activator-dependent single round transcription assay using the *E. coli glnHp2-m12* promoter as the template (Claverie-Martin and Magasanik, 1992). Assays were conducted with subsaturating amounts of holoenzyme to allow quantitative detection of holoenzyme activities. The mutant proteins were found to exhibit >50% of the activity of wild-type σ^{54} (Figure 2A). We therefore conclude that a replacement of all endogenous cysteines with alanine residues and the introduction of cysteine residues at alternative positions (see above) had no gross negative effect on the function of the protein. Also, because transcriptional activity of σ^{54} involves numerous unique stereospecific interactions, the preservation of transcriptional activity in the single cysteine mutants and the Cys(-) σ^{54} protein suggests that the conformation of the mutant proteins important for function is not altered significantly.

Region I sequences and R336 are involved in preventing unregulated polymerase isomerization (Syed *et al.*, 1997; Syed and Gralla, 1998; Casaz *et al.*, 1999; Chaney and

Fig. 2. *In vitro* transcription activity of σ^{54} single cysteine mutants on supercoiled *E. coli glnHp2-m12* promoter. (A) Activator-dependent (using PspFΔHTH) and (B) activator-independent transcription activity of σ^{54} single cysteine mutants and Cys(-) σ^{54} relative to the wild type. $\Delta(21-27)\sigma^{54}$ (Gallegos and Buck, 2000) was used as the positive control (Positive) in the activator-independent assays.

Buck, 1999). We therefore tested whether the single cysteine σ^{54} mutants were active for unregulated transcription (also called ‘activator bypass’ transcription), i.e. whether they allowed polymerase isomerization in the absence of activator proteins. The protocol for these assays begins by incubating the holoenzyme with GTP, CTP and UTP. The first six bases transcribed from the *E. coli glnHp2-m12* template are UGUCAC (+1 to +6), so transcripts can be initiated under these conditions if an activator-independent open complex forms. ATP and [α - 32 P]UTP are then added, together with heparin, to destroy residual closed complexes and unstable open complexes, and allow elongation of any initiated transcripts. E36C and R336C showed activator-independent transcription activity (Figure 2B). The lack of unregulated transcription activity from the Cys(-) σ^{54} protein suggests that the bypass phenotype of E36C and R336C is attributable to the substitutions at amino acid residues at positions E36 and R336, and not because of the replacement of the two endogenous cysteines at positions C198 and C346. Consistent with previous observations by us (Casaz *et al.*, 1999; Chaney and Buck, 1999) and others (Hsieh and Gralla, 1994; Hsieh *et al.*, 1994; Syed and Gralla, 1998), E36 and R336 in σ^{54} have a function in inhibiting polymerase isomerization in the absence of activation.

Derivatization of single cysteine σ^{54} mutants with the chemical protease Fe-BABE

Each single cysteine σ^{54} mutant and Cys(-) σ^{54} were derivatized with the chemical cleavage reagent Fe-BABE under native conditions and the conjugation yield was determined fluorometrically by the CPM test (*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide) (Greiner *et al.*, 1997) from the fluorescence difference between the conjugated and unconjugated proteins. The conjugation yield for the single cysteine mutants E36C, R336C, C198A and R383C was estimated to be >40% (Table I). The mutant σ^{54} harbouring the native single cysteine at position C198 (the C346A mutation) proved difficult to conjugate under native conditions (conjugation yield of 20%), implying that C198 is probably slightly buried or located in a hydrophobic region in the folded state because the hydrophobic reagent CPM reacted with high yield (data not shown) while the hydrophilic molecule Fe-BABE did not. When conjugation with C198 was performed under denaturing conditions, the yield increased to 50%. The Cys(-) σ^{54} protein failed to react detectably with CPM and Fe-BABE under native and denaturing conditions, directly implying that under the conditions used, Fe-BABE conjugation has only occurred at the free sulfhydryl side groups of cysteine residues.

The Fe-BABE-derivatized single cysteine mutants of σ^{54} are active for holoenzyme formation and transcription

We investigated the properties of the derivatized single cysteine mutants of σ^{54} proteins *in vitro* to determine whether core RNA polymerase binding and transcription were affected. Native gel holoenzyme assembly assays were used to detect complex formation between core RNA polymerase and σ^{54} . The holoenzyme migrates more slowly than the core enzyme. With the exception of the conjugated R336C mutant, all derivatized σ^{54} mutant proteins bound core RNA polymerase with the same apparent affinity as the non-conjugated wild-type σ^{54} (Figure 3A). Residue 336 lies outside the minimal core-binding region defined by deletion mutagenesis (Gallegos and Buck, 1999), but adjacent to a region in σ^{54} (amino acids 290–310) that is protected by core RNA polymerase from hydroxyl radical-mediated cleavage (Casaz and Buck, 1999) and within a sequence (amino acids 325–440) strongly protected by core RNA polymerase when region I is removed (Casaz and Buck, 1999). Because the unconjugated R336C binds core RNA polymerase with an affinity similar to that of wild-type σ^{54} , we conclude that the presence of the 490 Da Fe-BABE molecule at position R336 has caused the R336C mutant to undergo a conformational change that diminishes binding to the core RNA polymerase. We also note that conjugated Cys198 binding to core RNA polymerase results in two different holoenzyme conformers, one of which has a slower native gel mobility (Figure 3A, lane 10, marked with an arrow).

To determine the effect of Fe-BABE conjugation on the transcriptional activity of each single cysteine σ^{54} mutant, single-round transcription assays were also performed after conjugation with the Fe-BABE probe. Assays were conducted with subsaturating amounts of holoenzyme to allow detection of activity differences. After correction for

Table I. Fe-BABE conjugation efficiency

Residue	Mutation	Conjugation efficiency (%)
36	E36C	53
198	C346A	46 ^a
336	R336C	51
346	C198A	47
383	R383C	76
Cys(-)	C198A/C346A	3/5 ^a

^aDenotes conjugation under denaturing conditions.

the presence of unconjugated σ^{54} proteins, the conjugates showed >40% transcription activity relative to wild-type σ^{54} , with the exception of the R336C mutant, which has a markedly reduced transcription activity (10%) following conjugation (Figure 3B), consistent with our previous observation that the conjugated R336C mutant has reduced affinity for the core. Nevertheless, the conjugated R336C σ^{54} forms a holoenzyme that is active for a significant level (10%) of activated transcription.

We used the activator bypass transcription assay on the conjugated E36C and R336C mutants as a further indicator of conformational preservation of these proteins following conjugation. We reasoned that if the E36C and R336C mutants retained bypass activity following Fe-BABE conjugation, then the presence of the Fe-BABE molecule at these positions did not greatly affect the conformation of the conjugated proteins. Both mutants E36C and R336C retained their bypass activity following conjugation with Fe-BABE (Figure 3C). In conclusion, the core RNA polymerase-binding assays, the activator-dependent and the bypass transcription assays altogether suggest that Fe-BABE conjugation did not grossly affect the overall conformation and structural integrity of the mutant σ^{54} proteins.

Enhancer-dependent σ^{54} - and enhancer-independent σ^{70} -interacting sites on β and β' core subunits are conserved

Holoenzymes were formed with conjugated and unconjugated single cysteine mutants of σ^{54} and cleavage reactions were carried out essentially as previously described (Owens *et al.*, 1998). In our approach, we assigned the cleavage sites on the β and β' subunits by comparing the relative migration of the Fe-BABE cleavage products with sequence-specific markers generated by cleaving β and β' subunits at either cysteine or methionine residues [using 2-nitro-5-thiocyanobenzoate (NTCB) and cyanogen bromide (CNBr), respectively]. The cleavage sites for each β and β' marker fragment were assigned by calculating the molecular weight of each fragment with respect to the locations of cysteine or methionine residues in the amino acid sequence of β and β' subunits (Figure 4A) using a plot of log molecular weight versus the relative migration distance of a known set of marker proteins (data not shown).

Initially, we showed that self-cleavage of σ^{54} did not occur (data not shown), indicating that cutting of β and β' was attributed to binding of intact σ^{54} proteins. Ascorbate-

Fig. 3. (A) Formation of holoenzyme by the unconjugated (–) and Fe-BABE-conjugated (+) σ^{54} single cysteine mutants. Migration positions of free core RNA polymerase, holoenzyme and free σ protein are indicated. (B) *In vitro* transcription activity as in Figure 2. The activities of the σ^{54} single cysteine mutants before (black bars) and after (white bars) Fe-BABE conjugation are compared. The values for conjugated proteins are corrected for the presence of unconjugated proteins. (C) Activator bypass activity of the E36C and R336C σ^{54} proteins after Fe-BABE conjugation (C); the activator bypass activity of unconjugated proteins (UC) is shown for comparison.

and hydrogen peroxide-treated and untreated reactions of each single cysteine mutant σ^{54} –holoenzyme were separated, blotted and visualized by immunostaining with affinity-purified β and β' N-terminus-specific antibodies (Figure 4B and C). The lack of cleavage of free core RNA polymerase and the holoenzyme formed with Cys(–) σ^{54} (Figure 4B and C, controls) protein serves as an essential control experiment, demonstrating that under the conditions used there is no artefactual cutting of the core RNA polymerase subunits. Hence, we believe that each cut (Figure 4B and C) is attributed to a specific Fe-BABE form of σ^{54} created by derivatizing a single cysteine residue.

Cys198 and Cys346. Initially, we looked for the β and β' interactions made by the σ^{54} single cysteine mutants with Fe-BABE conjugated to the endogenous cysteines

at positions Cys198 (C346A) and Cys346 (C198A), respectively. Cys198 cleaves β and β' at multiple sites. On the β subunit, strong cleavage by Cys198 is seen within the Rif1 cluster (between conserved β regions C and D) and in conserved region G (Figure 4B, Cys198, lane 4). A few weak cleavages are also seen within the DR1 region. Moderate cleavage by Cys198 of the β' subunit occurs in conserved regions B and C and within region G (Figure 4C, Cys198, lane 2). In contrast, Cys346 only moderately cleaves β and β' at single sites; these map proximal to the conserved region D on the β subunit and within conserved regions C and D on the β' subunit (Cys346, lane 6, Figure 4B and C, respectively). These results are consistent with previous observations: Cys198 is located within a sequence

Fig. 4. (A) Immunoblot of markers generated by treating core RNA polymerase with NTCB (N) and CNBr (C), respectively, using anti- β or anti- β' N-terminus-specific antibodies. Pre-stained molecular weight markers (M; Bio-Rad, Low Range SDS-PAGE Standards) were used as reference molecular weight markers. Holoenzyme cutting by σ^{54} single cysteine-Fe-BABE conjugates. Immunostained blots of SDS-PAGE detecting either (B) β or (C) β' subunit fragments by using affinity-purified N-terminal antibodies. All reactions were either treated (+) or untreated (-) with ascorbate and hydrogen peroxide.

(amino acids 120–215) in region III of σ^{54} that has a strong affinity for the core RNA polymerase (Gallegos and Buck, 1999), while Cys346 is located within a region (amino acids 325–340) that is weakly protected

by core RNA polymerase during hydroxyl radical cleavage (Casaz and Buck, 1999), consistent with the differences in the β and β' cleavage patterns generated by the Cys198- and Cys346-conjugated σ^{54} proteins.

Fig. 5. (A) Summary of the cleavage data in Figure 4 showing the proximities of σ^{54} residues to surfaces on the β and β' subunits. The thickness of the lines indicates the cleavage efficiency observed on immunoblots. (B) Surfaces (shaded boxes) proximal to σ^{70} as determined by Fe-BABE cleavage studies (Owens *et al.*, 1998). Conserved regions in β and β' are indicated along the horizontal axes.

Cys 36 and Cys336. Next, we used the activator-independent single cysteine mutants of σ^{54} with Fe-BABE conjugated to Cys36 (E36C) and Cys336 (R336C) in the Cys(-) σ^{54} background to map their interaction sites on the β and β' subunits of core RNA polymerase. Cys36 is in region I of σ^{54} , which functions to keep the closed complexes in a transcriptionally silent state in the absence of activation (Gallegos *et al.*, 1999) and has been shown to have weak affinity for core RNA polymerase (Gallegos and Buck, 1999). The core RNA polymerase cleavage by Cys36 shows that region I interacts strongly with β and β' subunits. Cleavage sites map to within the Rif1 cluster and the conserved region D on β and within conserved region C on β' (Cys36, lane 2, Figure 4B and C). Cys336 is located within a sequence in σ^{54} that lies outside the minimal DNA-binding domain, but nevertheless cross-links to DNA (Chaney and Buck, 1999; M.K.Chaney, M.S.Pitt and M.Buck, submitted). Remarkably, the cleavage data indicate that Cys336 interaction sites on both β and β' subunits unambiguously overlap with those of Cys36. Given the shared activator-independent transcription phenotypes of the E36C and R336C σ^{54} mutants, we rationalize this observation by suggesting that E36 and R336 belong to an interface between σ^{54} and core RNA polymerase that acts to prevent polymerase isomerization in the absence of activators, and propose that the activator probably functions in changing the interface.

Cys383. Attempts to cleave core RNA polymerase by Cys383 (R383C)-conjugated σ^{54} revealed no discernible cleavage of either the β or β' subunit (Cys383, lane 10, Figure 4B and C, respectively). In σ^{54} , Cys383 is proposed to fall within the recognition helix (helix 2) of the putative helix–turn–helix motif and to contribute to the recognition of the -12 promoter element by the σ^{54} -holoenzyme (Coppard and Merrick, 1991; Merrick and Chambers, 1992). We can conclude that Cys383 is not located proximal (at least not within a radius of 12 Å) to the core RNA polymerase subunits β and β' and may be facing promoter DNA rather than core.

A comparison with σ^{70} -core RNA polymerase proximity. Because binding of σ^{54} to core RNA polymerase results in

the formation of a holoenzyme that has very distinct functional properties compared with the σ^{70} -holoenzyme, we compared our cleavage data with those obtained for σ^{70} -holoenzyme cleavage by Fe-BABE-modified σ^{70} (Owens *et al.*, 1998; Figure 5). Notably, cleavage by Cys198 is within the β' subunit conserved region G, whereas an interaction of σ^{70} with this region has not been observed. Several lines of evidence show that the N-terminal portion of the β' subunit is involved in the specific binding of the σ^{70} subunit (Luo *et al.*, 1996; Arthur *et al.*, 1998; Owens *et al.*, 1998; Katayama *et al.*, 2000). The interaction made by Cys198 within conserved β' region G may well be σ^{54} specific. Interestingly, a part of the sequence in β' involved in chelating Mg^{2+} to the active centre of RNA polymerase is in conserved region G (Zaychikov *et al.*, 1996). We note that Cys198 is also proximal to the β' rudder, a region distant from the active centre of the RNA polymerase (Zhang *et al.*, 1999; Figure 5A), suggesting significant conformational changes occurring in β' following σ binding. Remarkably, other surfaces of the core RNA polymerase proximal to the two classes of σ factors appear to overlap greatly (residues 480–600 and around residue 900 ± 10 in the β subunit, and between residues 200 and 350 ± 20 in the β' subunit). Some are proximal to the parts of the RNA polymerase catalytic centre, contributed by β conserved regions D and H and β' conserved regions D and H (Mustaev *et al.*, 1997). Locating the proximity sites of σ^{54} and σ^{70} within the recently resolved crystal structure of *Thermus aquaticus* core RNA polymerase (Zhang *et al.*, 1999) serves to illustrate further the degree of overlap between the core surfaces that are proximal to the two classes of σ factors (Figure 6).

Discussion

The tethered hydroxyl radical footprinting methodology using Fe-BABE is a powerful tool to study protein–protein interactions that occur during transcription, and has been applied successfully previously (Miyake *et al.*, 1998; Owens *et al.*, 1998). It is particularly well suited to the study of proximity relationships of a single site on one

Fig. 6. RIBBONS diagrams of the three-dimensional structure of core RNA polymerase from *T. aquaticus* (Zhang *et al.*, 1999). The β subunit is shown in pink and the β' subunit in blue. The two α subunits are shown in green and yellow. (A) σ^{70} - and (B) σ^{54} -interacting surfaces on the β and β' subunits are indicated in blue and red, respectively.

subunit to one or more other sites. The results can add considerably to our understanding of the overall architecture of large macromolecular complexes. We have used the Fe-BABE cleavage methodology to look at proximity relationships between functional domains of σ^{54} and the core RNA polymerase subunits β and β' , and to widen our understanding of this specialized form of bacterial enhancer-dependent transcription.

Residue 36 in region I

Our cleavage data for Cys36 are consistent with the demonstrated interaction of region I sequences with core RNA polymerase (Casaz and Buck, 1997, 1999; Gallegos and Buck, 1999). Partial proteolysis studies on the σ^{54} -holoenzyme have shown that an N-terminal region of σ^{54} spanning residues 36–100 is protected from proteolytic cleavage (Casaz and Buck, 1997) and free hydroxyl radical footprinting experiments have shown that core RNA polymerase protects a region of σ^{54} comprising residues 36–140 from hydroxyl radical cleavage (Casaz and Buck, 1999). Even though region I of σ^{54} is dispensable for the binding of both the core RNA polymerase and DNA, it is essential in mediating the response to activator proteins (Sasse-Dwight and Gralla, 1990; Hsieh and Gralla, 1994; Hsieh *et al.*, 1994; Wong *et al.*, 1994; Cannon *et al.*, 1995; Casaz *et al.*, 1999). The proximity of region I E36 to core RNA polymerase provides further physical evidence consistent with the view that region I interacts with core RNA polymerase to exert some of its effects, including the inhibition of polymerase isomerization and the stabilization of holoenzyme on melted DNA (Cannon *et al.*, 1999; Gallegos and Buck, 1999, 2000). It seems that each of these functions is achieved in part through interacting with core RNA polymerase, but we cannot say precisely whether proximity relationships revealed by the Fe-BABE data correlate with one or both roles of region I. The interface

of σ factors with core RNA polymerase is now recognized as being functionally specialized, contributing to more than just anchoring core and σ together, and is likely to contribute to communicating information about promoter conformation to the core RNA polymerase (Gross *et al.*, 1998; Sharp *et al.*, 1999).

Residue 336 in region III

We previously concluded that an interaction between the N- and C-terminal sequences of σ^{54} in the holoenzyme is closely associated with inhibiting polymerase isomerization (Chaney and Buck, 1999), consistent with the view that the σ^{54} region I sequences may be in communication with other sequences in σ^{54} (Cannon *et al.*, 1995; Casaz and Buck, 1999), probably including the major C-terminal DNA-binding domain (Wong *et al.*, 1994; Taylor *et al.*, 1996; Guo and Gralla, 1997; Guo *et al.*, 1999). The recently discovered role of residue 336 in region III of σ^{54} in maintaining closed complexes in a transcriptionally silent state in the absence of activation (Chaney and Buck, 1999), and the unambiguous similarity of Cys336 cleavage data to those of Cys36, together argue that residues around 336 contribute to the same interface with core RNA polymerase. Our Fe-BABE cleavage data provide indirect structural evidence for an interaction between regions I and III occurring via the core RNA polymerase. However, the similarity in the cleavage patterns seen with Cys36 and Cys336 must also be viewed in the context that both mutants (E36C and R336C) form the deregulated holoenzyme conformation, in which σ^{54} regions I and III may be proximal to each other, hence providing similar cleavage patterns. Thus, we do not discount the possibility that regions I and III may normally only interact with each other following polymerase isomerization after activation, or at some earlier intermediate step en route to the open complex.

Residue 198 in region III

Residue 198 seems to be able to establish two proximities to the β subunit. According to the *T.aquaticus* core RNA polymerase structure, these are well separated in space (Zhang *et al.*, 1999). Possibly, binding of σ^{54} results in two different holoenzyme conformers, or movement in core RNA polymerase upon the binding of σ^{54} brings the two sites closer together. Inspection of the native gel showing core RNA polymerase binding of the Fe-BABE-modified Cys198 σ^{54} supports the former suggestion. We also note that core RNA polymerase binding of a small fragment of σ^{54} (amino acids 120–215) resulted in two discrete complexes (Gallegos and Buck, 1999), consistent with the two binding modes suggested by the Cys198 Fe-BABE cleavage data. The 120–215 amino acid fragment seems to contain the major high affinity determinant for core RNA polymerase binding. A short σ^{70} similarity sequence of σ^{54} important for core RNA polymerase binding is found between residues 175 and 189 (Tintut and Gralla, 1995). Kinetic studies have shown that initial binding of σ^{54} to core RNA polymerase is followed by a slower rearrangement of the holoenzyme (D.J.Scott, A.L.Ferguson, M.T.Gallegos, M.S.Pitt and J.G.Hoggett, unpublished data). The relationship of this conformational change to interactions made by sequences around Cys198 remains to be determined. Mutational analysis around position Cys198 has shown that the integrity of this σ^{54} sequence is important for a holoenzyme stability that is conditional upon particular pre-melted DNA sequences (M.S.Pitt, M.T.Gallegos and M.Buck, in preparation). Communication of promoter structure with the core RNA polymerase through a patch in σ^{54} within the 120–215 fragment involving Cys198 seems likely. Intermolecular interactions within region III that enhance DNA binding of σ^{54} are evident (Cannon *et al.*, 1997).

Residue 346 in region III

C346 shows a similar proximity relationship to core RNA polymerase as does residue 336; however, substitutions in C346 do not result in deregulation of RNA polymerase isomerization. The cutting by Fe-BABE at 346 was weaker than that at 336, indicating a greater distance to core RNA polymerase and/or geometric constraint on positioning the Fe-BABE on core RNA polymerase.

Residue 383 in region III

The failure of the Fe-BABE derivative at position 383 to cleave core RNA polymerase is consistent with a role in DNA binding *per se* rather than in interacting with core RNA polymerase. DNA interaction studies with Fe-BABE at position 383 might reveal such a role.

Overview

In conclusion, our Fe-BABE data provide a framework for interpretation of some of the genetic and biochemical data on σ^{54} . The most remarkable observation is the unambiguous similarity in the core RNA polymerase surfaces proximal to the activator-independent and activator-dependent σ classes. This observation is supported further by small-angle X-ray scattering data on σ^{54} , which suggest that although σ^{54} and σ^{70} are unrelated by primary amino acid sequence, they both share a significant overall structural similarity (Svergun *et al.*, 2000), and by protein

footprinting studies (Travaglia *et al.*, 1999). Possibly, the two different σ classes occupy similar positions in the core RNA polymerase and use some overlapping points of interaction to contribute to the shared functionalities of the holoenzymes. For transcription by σ^{54} , the progression from the closed to the open promoter complex must occur along one or more pathways in which changing interactions between σ and DNA and σ and core RNA polymerase occur to establish the alternative functional states of the holoenzyme. It seems doubtless that some of the proximity relationships we have detected will change upon activation of the σ^{54} -holoenzyme. Those associated with σ^{54} residues 36 and 336 seem most likely to change, given that these are associated with σ^{54} sequences needed to maintain the stable closed promoter complex and that these, when mutated, lead to activator-independent isomerization and binding to pre-melted DNA. It seems reasonable to assume that one or more of the σ -core and σ -DNA interactions associated with maintaining the stable closed complex conformation must be changed by activator to initiate the holoenzyme isomerization. How activator and its nucleotide hydrolysis trigger these changes remains to be determined.

The proximity relationships we have determined with Fe-BABE-modified σ^{54} proteins fall into two different classes with respect to the phenotypes of the σ^{54} single cysteine mutants. The σ^{54} Fe-BABE derivatives at Cys36 and Cys336 show deregulated transcription, whereas the Fe-BABE derivatives at Cys198 and Cys346 holoenzymes do not. Therefore, it is formally possible that the proximity relationships deduced apply to two different conformers and therefore to two functional states of the holoenzyme. In contrast to the Cys198 and Cys346 holoenzymes, Cys36 and Cys336 holoenzymes may have a conformation that could resemble more the conformation of the activated σ^{54} -holoenzyme in open promoter complexes. The extent to which the conformations of regulated and deregulated σ^{54} holoenzymes differ and how conformations change upon interacting with DNA remains to be determined. It is interesting that Cys198 is proximal to sequences in the β' region G that are involved in maintenance of the active site of the RNA polymerase.

Materials and methods**Site-directed mutagenesis**

The pET28b(+) (Novagen)-based plasmid pMTH σ^N (Gallegos and Buck, 1999), which directs the synthesis of the N-terminal His₆-tagged σ^{54} from *K.pneumoniae*, was used as the template for construction of single cysteine mutants of σ^{54} using the Quickchange mutagenesis kit (Stratagene). Briefly, mutated DNA was synthesized by *Pfu* DNA polymerase in a reaction that contains pMTH σ^N and a large molar excess of complementary mutagenic oligonucleotides. Following 30 cycles of heating at 95°C for 1 min, 50°C for 1 min and 72°C for 14 min, the reactions were treated with *DpnI* to remove the parental DNA. The resulting reaction mix was used to transform *E.coli* JM109 cells. Mutant clones were verified by DNA sequencing. Initially, the two single cysteine σ^{54} mutants, C198A (pSRW198) and C346A (pSRW346), were constructed by replacing cysteine residues at positions Cys198 and Cys346 with alanine. An *NdeI*–*Bam*HI fragment from pSRW198 carrying the C198A mutation and a *Bam*HI–*Hind*III fragment from pSRW346 carrying C346A were cloned into pET28b(+) (Novagen) to create pSRWCys(–). For the introduction of new cysteine residues at positions 36, 336 and 383 in σ^{54} , pSRWCys(–) was used as the template plasmid in the site-directed mutagenesis reactions to create pSRW36, pSRW336 and

pSRW383. The DNA sequences of each single cysteine σ^{54} mutant construct and that of *cys(-)rpoN* were confirmed by DNA sequencing.

Protein overproduction and purification

The plasmids directing the overproduction of Cys(-) σ^{54} and the single cysteine mutants were overexpressed in *E. coli* B834(DE3) cells. Proteins were purified by nickel affinity chromatography and eluted with an imidazole gradient essentially as described previously for wild-type σ^{54} (Gallegos and Buck, 1999). All purified proteins were dialysed against TGED buffer [10 mM Tris-HCl pH 8.0, 5% (v/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol (DTT)] to which 50% (v/v) glycerol and 50 mM NaCl were added, and stored at -70°C (long-term storage) or -20°C (short-term storage). The activator protein PspF Δ H Δ TH used in the *in vitro* transcription assays was purified and stored essentially as described (Jovanovic *et al.*, 1996). Core RNA polymerase was purified from *E. coli* W3350 cells (Fujita *et al.*, 1987; Kusano *et al.*, 1996).

Conjugation of σ^{54} single cysteine mutants with Fe-BABE

With the exception of C346A, each purified σ^{54} protein was dialysed overnight at 4°C against conjugation buffer [10 mM MOPS pH 8.0, 0.2 M NaCl, 5% (v/v) glycerol, 2 mM EDTA]. Conjugation reactions were initiated by adding a 10-fold molar excess of Fe-BABE (300 μM) (Dojindo Chemicals, Japan) to each σ^{54} mutant (20 μM) at pH 8.0. After 1 h incubation at 37°C , excess Fe-BABE was removed by overnight dialysis against storage buffer (without DTT) at 4°C . The concentration of free cysteine sulfhydryl groups on the conjugated and unconjugated σ^{54} mutants and Cys(-) σ^{54} was determined fluorometrically by the CPM test (Parvari *et al.*, 1983) and the conjugation yield was calculated as described previously (Greiner *et al.*, 1997). The C346A preparation in storage buffer was dialysed overnight at 4°C against conjugation buffer containing 6 M deionized urea and treated essentially as described above.

Core RNA polymerase-binding assays

Escherichia coli core RNA polymerase (250 nM) and different amounts of unconjugated and conjugated mutant σ^{54} proteins were mixed together in Tris-NaCl buffer [40 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT, 100 mM NaCl] and incubated at 30°C for 10 min, followed by the addition of glycerol bromophenol blue loading dye. Samples were loaded onto native 4.5% polyacrylamide Bio-Rad Mini-Protein II gels and run at 50 V for 2 h at room temperature in Tris-glycine buffer (25 mM Tris, 200 mM glycine). Proteins were visualized by Coomassie Blue staining.

In vitro transcription assays

Supercoiled pFC50-m12 (Claverie-Martin and Magasanik, 1992) containing the *E. coli* *glnHp2*-m12 promoter was used as the template for the *in vitro* transcription assays. Activator-dependent transcription was performed essentially as described previously (Chaney and Buck, 1999), except that 30 nM σ^{54} holoenzyme, 10 nM template DNA (30 nM core RNA polymerase:120 nM σ^{54}) were used. For activation, 4 μM PspF Δ H Δ TH was added with 4 mM ATP to the final reaction volume of 10 μl . The reactions were incubated for 20 min to allow open complexes to form. The remaining rNTPs (CTP and GTP at 0.1 mM and UTP at 0.05 mM), 1.5 μCi of [α - ^{32}P]UTP and heparin (100 $\mu\text{g}/\text{ml}$) were added and incubated for a further 20 min at 30°C . The reactions were stopped with 4 μl of formamide loading buffer and, after heating for 5 min at 95°C , 7 μl of the samples were loaded on a 6% denaturing sequencing gel. The dried gel was analysed on a phosphorimager. The activator-independent transcription assay was performed essentially as described above, but in the absence of the PspF Δ H Δ TH activator protein. The *E. coli* *glnHp2*-m12 start sequence is TGTCAC (+1 to +6). To allow activator-independent initiated complex formation, 4 mM GTP, 0.1 mM UTP and 0.1 mM CTP were incubated with the holoenzyme prior to challenge with heparin and the addition of 0.1 mM ATP and 1.5 μCi of [α - ^{32}P]UTP.

Core RNA polymerase cleavage by Fe-BABE-conjugated σ^{54}

The *E. coli* RNA polymerase holoenzymes were prepared by incubating core RNA polymerase with Fe-BABE-conjugated σ^{54} mutants and Cys(-) σ^{54} (1:1 molar ratio) at 30°C for 10 min in cleavage buffer [10 mM MOPS pH 8.0, 10 mM MgCl_2 , 10% (v/v) glycerol, 200 mM NaCl, 2 mM EDTA]. Cleavage reactions were initiated by the rapid sequential addition of freshly prepared ascorbate (5 mM final) and hydrogen peroxide (5 mM final) and were allowed to proceed for 2 min at 30°C . Reactions were stopped with $5\times$ SDS sample buffer [62.5 mM Tris-HCl pH 8.2, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 25 mM EDTA, 0.02% (w/v) bromophenol blue] and immediately frozen in liquid nitrogen and stored at -70°C . The cleaved fragments were separated,

blotted, and visualized by immunostaining with affinity-purified subunit β and β' N-terminus-specific antibodies essentially as described previously (Greiner *et al.*, 1996).

Assignment of cleavage sites on β or β' subunits

The β and β' subunits were cleaved at either cysteine or methionine residues using the sequence-specific chemical reagents NTCTB and CNBr, respectively. NTCTB cleavage was performed as described previously (Jacobson *et al.*, 1973). For the NTCTB cleavage, core RNA polymerase was buffer exchanged into unfolding buffer (0.1 M MOPS pH 8.5, 8 M urea) and incubated for 10 min at 37°C . Cleavage was initiated by adding a 5-fold molar excess of NTCTB (in 0.1 M MOPS buffer) over total sulfhydryl groups. After overnight incubation at 37°C , the cleavage reactions were stopped with $5\times$ SDS sample buffer [125 mM Tris-HCl pH 6.8, 4% (w/w) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue] and analysed by SDS-PAGE and immunostaining with β and β' N-terminus-specific antibodies. CNBr cleavage was performed essentially as in Grachev *et al.* (1989). Initially, core RNA polymerase was treated with 10% SDS and incubated at 37°C for 30 min. For denaturation, 1 M HCl and 1 M CNBr in acetonitrile were then added and incubated at room temperature for 6 h. The reaction was stopped with $5\times$ SDS sample buffer and analysed as described above. The cleavage sites for each NTCTB- and CNBr-generated fragment of β and β' were assigned by using a third-order polynomial fit of log molecular weight versus the migration distance on SDS-PAGE of a known set of marker fragments and by β and β' amino acid sequence analysis.

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