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Positioning of σ^S, the stationary phase σ factor, in *Escherichia coli* RNA polymerase–promoter open complexes

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The σ^S subunit of RNA polymerase is the master regulator of the general stress response in *Escherichia coli* and is required for promoter recognition of many stationary phase genes. We have analysed open complexes of $E\sigma^S$ RNA polymerase, using σ^S derivatives carrying single cysteine residues at nine different positions to which the reagent FeBABE has been tethered. All holoenzymes but one formed transcriptionally active open complexes at three different promoters (*osmY, gal*P1 and *lac*UV5). The chemical nuclease FeBABE can cleave DNA in proximity to the chelate. The overall cutting pattern of $E\sigma^S$ open complexes does not depend on the nature of the promoter and is similar to that obtained with $E\sigma^{70}$, but extends towards the downstream part of the promoter. The strongest cleavages are observed with FeBABE positioned on cysteines in regions 2.2 to 3.1. In contrast to σ^{70}, region 2.1 of σ^S appears to be far from DNA. Region 4.2 of σ^S appears less accessible than its counterpart in σ^{70} and FeBABE positioned in the turn of the helix–turn–helix (HTH) motif in region 4.2 reacts only weakly with the –35 promoter element. This provides a structural basis for the minor role of the –35 sequence in σ^S-dependent promoter recognition.

Keywords: DNA cleavage/FeBABE/RNA polymerase/ stationary phase

Introduction

The RNA polymerase of *Escherichia coli* is composed of a core enzyme, containing the $\alpha_2\beta\beta'$ subunits, associated with a σ subunit (Burgess *et al*., 1969). The core enzyme carries the catalytic machinery and binds DNA non-specifically whereas the σ subunit provides the promoter recognition activity (Helmann and Chamberlin, 1988). Until now, seven different sigma factors, programmed to express distinct classes of genes, have been identified in *E.coli* (for a review see Ishihama, 1997). The σ^{70} subunit is the major σ factor and is involved in expression of most genes during exponential phase. All alternative σ factors, except σ^{54}, are related in amino acid sequence to σ^{70} and exhibit the same modular organization as σ^{70} (Lonetto *et al*., 1992). Regions 2.1 and 2.2 are involved in binding to the core of RNA polymerase. Regions 2.3 and 2.4 are implicated in melting of the promoter and in the recognition of the –10 hexamer, whereas region 4.2 binds to the –35 hexamer (Figure 1).

When cells reach the end of the exponential phase or are exposed to various stresses such as hyperosmolarity, low or high temperature, or acidic pH, a novel species of sigma factor σ^S, the product of the *rpoS* gene, comes into play (for a review see Hengge-Aronis, 1996). Its level is tightly controlled by transcriptional and translational mechanisms and by proteolysis, but in stationary phase it can reach up to 30% of the level of σ^{70} (Jishage and Ishihama, 1995). σ^S is considered the second principal sigma factor in *E.coli* (Nguyen *et al*., 1993; Tanaka *et al*., 1993). Associated with core enzyme, the σ^S subunit regulates the expression of >50 genes involved in the survival of the cells in the stationary phase and in response to different stresses (Hengge-Aronis *et al*., 1993; Loewen and Hengge-Aronis, 1994).

The σ^S protein is smaller than σ^{70} (Figure 1). It does not possess the non-conserved domain of 245 residues located between regions 1.2 and 2.1 of σ^{70} (Lonetto *et al*., 1992; Malhotra *et al*., 1996) and contains a smaller region 1, which has been implicated in modulating the DNA binding activity of free σ^{70} (Dombroski *et al*., 1992, 1993). Other domains are more conserved between σ^S and σ^{70}, especially the domains involved in the interaction with the –10 and the –35 regions of a promoter (Lonetto *et al*., 1992). A compilation of σ^S-dependent promoters deduced a –10 consensus sequence CTATACT, slightly different from the typical TATAAT hexamer, recognized by region 2.4 of σ^{70} (Hawley and McClure, 1983; Espinosa-Urgel *et al*., 1996). In contrast to $E\sigma^{70}$, the stationary phase RNA polymerase does not strictly require the presence of the –35 consensus sequence TTGACA for its activity (Hiratsu *et al*., 1995; Kolb *et al*., 1995; Tanaka *et al*., 1995). However, some σ^S-dependent promoters contain a curved upstream sequence which possibly binds the α subunit of RNA polymerase (Espinosa-Urgel *et al*., 1996; Aiyar *et al*., 1998). In addition, regions 2.5 of σ^S and σ^{70} have only a few identical residues in common. In σ^{70}, this region has been shown to be involved in the recognition of the TG motif of extended –10 sequences (Barne *et al*., 1997; Bown *et al*., 1999), whereas a similar role has yet to be demonstrated for σ^S-dependent promoters. In σ^S, region 2.5 is required for the rapid degradation of the protein by the ClpXP protease (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Schweder *et al*., 1996; Zhou and Gottesman, 1998). Despite all these differences, a number of stationary phase promoters can

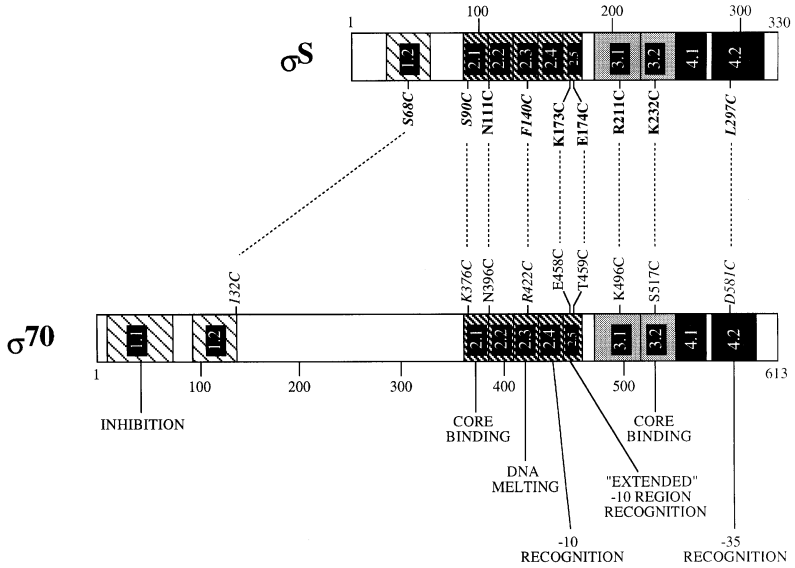


Fig. 1. Comparison of primary sequence of σ^S (330 aa) and σ^{70} (613 aa) factors. Conserved regions are represented as defined by Lonetto *et al.* (1992), with the addition of region 2.5 involved in extended –10 element recognition (Barne *et al*., 1997). Known functions of several σ^{70} regions are indicated. Positions of single cys-σ^S mutants are shown by analogy with the σ^{70} mutants (dashed line). Mutations indicated in italics correspond to non-equivalent residues in the sequence alignment between σ^S and σ^{70} (Mulvey and Loewen, 1989; Lonetto *et al*., 1992). Note that the present σ^S amino acid numbering is 12 residues shorter than previously described (KatF) and that the sequence contains some modifications due to strain heterogeneity, which do not affect the overall similarity (Tanaka *et al*., 1993).

be recognized *in vitro* by both RNA polymerases, although with different affinities (Nguyen *et al*., 1993; Tanaka *et al*., 1993; Ding *et al*., 1995; Kusano *et al*., 1996; Nguyen and Burgess, 1997; Ballesteros *et al*., 1998).

Until now, in contrast to $E\sigma^{70}$, only a few structural and genetic studies of the stationary phase RNA polymerase have been performed. As a consequence, it remains to be determined whether (i) the homologous DNA binding domains of σ^S and σ^{70} contact the same promoter regions, (ii) the corresponding but non-homologous domains of both σ factors are positioned similarly with respect to the promoter and to each other, and (iii) the same structural intermediates are involved in formation of the open complex for both holoenzymes. A powerful method using the FeBABE chemical nuclease [(p-bromoacetamidobenzyl)-EDTA Fe] tethered to a single cysteine of the protein has been applied recently to the α and σ^{70} subunits of RNA polymerase (Greiner *et al*., 1997; Murakami *et al*., 1997a,b; Miyake *et al*., 1998; Owens *et al*., 1998a,b; Bown *et al*., 1999). This approach has the unique ability to detect deoxyribose or peptide bonds in close proximity to the reactant (<12 Å). In the case of σ^{70}, DNA cleavage occurs on both DNA strands in the open complex at the *lac*UV5 promoter and extends from position –48 to +12 with cleavages generated from FeBABE tethered to regions 2.1 and 3.2 centered in the melted region around +1 (Owens *et al*., 1998a). As expected from genetic data, regions 2.3 and 4.2 map in close proximity to the –10 and –35 regions of the promoter, respectively.

Using the same method as for σ^{70}, we have introduced single cysteine residues into σ^S and explored the spatial relationships of σ^S in the open complex along the DNA at three different promoters (Figure 2A). The *lac*UV5 promoter was used to allow a direct comparison to be made with σ^{70}; however, this promoter does not depend on σ^S for expression *in vivo* (Yim *et al*., 1994). We therefore chose a well-characterized stationary phase promoter, the *osmY* promoter, which is known to operate only under the control of σ^S *in vivo* (Yim and Villarejo, 1992; Hengge-Aronis *et al*., 1993; Yim *et al*., 1994; Wise *et al*., 1996). *In vitro*, this specificity can be achieved at a high concentration of potassium glutamate (Ding *et al*., 1995; Kusano *et al*., 1996). In contrast to *lac*UV5, *osmY* does not contain any obvious consensus –35 hexamer. To investigate the role of the –35 region in $E\sigma^S$ recognition, we also probed the σ^S-DNA positioning at another promoter which has no obvious –35 consensus region, the *gal*P1 promoter (Burns *et al*., 1996). The DNA binding pattern of σ^S, as characterized by the FeBABE nuclease, appears similar at the three promoters, but distinct from σ^{70}.

Results

The FeBABE reagent covalently conjugates to the free sulfhydryl side groups of cysteine residues (Greiner *et al*., 1997). It can be introduced at single positions along the polypeptide chain by designing variants of the protein containing a unique accessible cysteine. After addition of H_2O_2 and ascorbic acid, the FeBABE nuclease generates hydroxyl radicals which cleave DNA and polypeptide chains in its close proximity. The σ^S subunit of RNA polymerase does not contain any cysteine residue. We took advantage of data obtained from previous cysteine substitutions introduced in the major σ subunit of RNA polymerase to determine which substitutions to create in σ^S.

Choice of the cysteine substitutions in σ^S

The cysteine substitutions were located at or very close to the positions previously used for σ^{70} (Figure 1). The σ^S mutants N111C, K173C, E174C, R211C and K232C correspond precisely to single cysteine mutants generated at the equivalent positions in σ^{70}. They are located at the end of region 2.1, in the middle of region 2.5, and in

Table I. FeBABE conjugation yield

Mutants	Conjugation yield (%)[a]
S68C (1.2)	65
S90C (2.1)	43
N111C (2.1)	43
F140C (2.3)	75
K173C (2.5)	40
E174C (2.5)	68
R211C (3.1)	83
K232C (3.2)	67
L297C (4.2)	10
L297C (4.2) [UF][b]	60

[a]Conjugation yield (%) was determined by measuring the concentration of free cysteine side chains with the fluorescent reagent CPM using both unconjugated and conjugated proteins (Greiner *et al*., 1997)
[b]With L297C mutant, conjugation yield was also determined with mutant conjugated under denaturing conditions [UF].

regions 3.1 and 3.2, respectively. We synthesized S90C at the beginning of region 2.1 of σ^S, corresponding to the residue adjacent to K376C in σ^{70}, which had low reactivity. Mutant F140C, located in region 2.3 of σ^S, is shifted three residues closer to the C terminus compared with the position mutated in σ^{70} because of its weak transcriptional activity. In addition, we preferred to change residue L297 into C in the helix–turn–helix (HTH) motif of region 4.2 in σ^S rather than the adjacent glycine residue whose integrity could be crucial for the proper folding of the motif. In the non-conserved region 1 of σ^S, mutant S68C in the middle of region 1.2 was constructed.

Each single cys-σ^S mutant was conjugated with the chemical cleavage reagent FeBABE. The conjugation yield was determined using the fluorescent CPM (*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide) reagent on both unconjugated and conjugated proteins as described previously (Greiner *et al*., 1997). This yield was always >40% for all conjugated mutants except for L297C (Table I). However, when FeBABE conjugation of this mutant was performed under denaturing conditions, the yield increased from 10 to 60%. This residue, localized in the HTH motif of region 4.2, is therefore not easily accessible to the reactant under the native conditions, probably being buried within the protein. The L297C mutant, however, retained almost full activity after one cycle of denaturation–renaturation treatment (see below).

The FeBABE derivatives in regions 1.2 to 4.2 of σ^S are transcriptionally active with the exception of K173C in region 2.5

The transcriptional activity of each σ^S mutant associated with core enzyme was determined to check that our mutants were active before and after conjugation. Single-round transcription assays were performed at the *osmY*, *gal*P1 or *lac*UV5 promoters (Figure 2B). Before conjugation, all mutants possessed between 60 and 100% of wild-type transcriptional activity with the exception of two mutants. R211C is fully active at *osmY* and *gal*P1 but shows reduced activity at *lac*UV5 (40%). Moreover, the K173C mutant is strongly deficient in *osmY* (16%) and *lac*UV5 (12%) transcription, but still partially active at *gal*P1 (49%) (Figure 2B). Thus, K173, localized in region

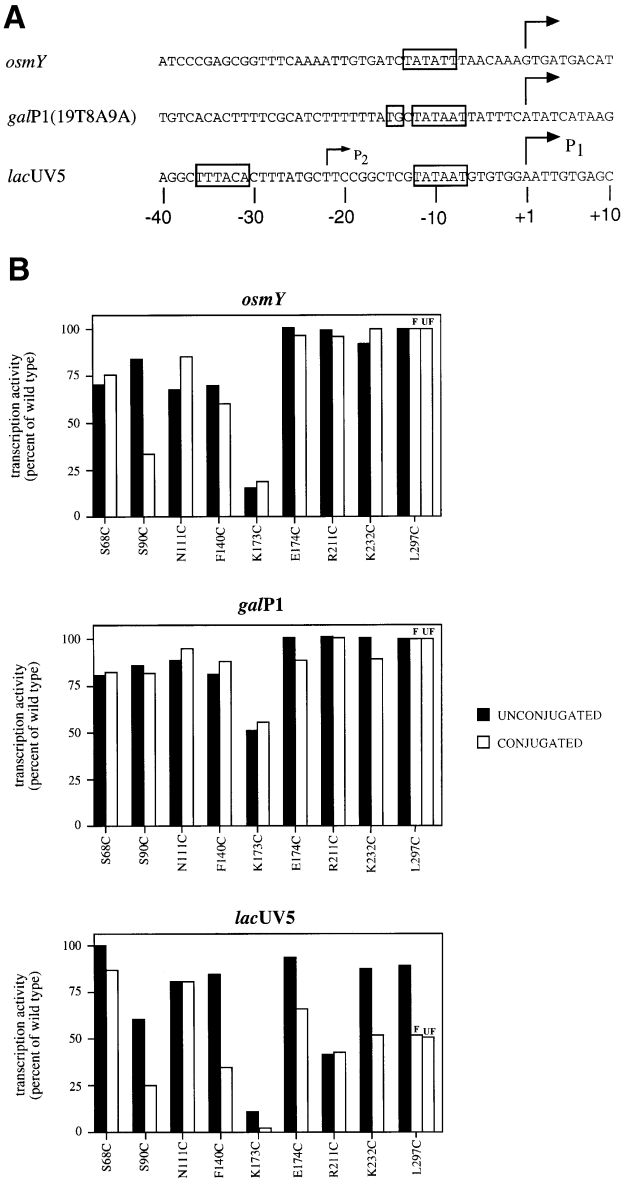


Fig. 2. (**A**) Nucleotide sequence of the *osmY*, *gal*P1 and *lac*UV5 promoters. The –10, the TG motif and the –35 sequence elements are boxed. Arrows represent the start site of transcription. At *lac*UV5, the P1 start at +1 and the P2 start at –22 are indicated.
(**B**) Transcriptional activity of Eσ^S mutants. Single-round transcription experiments were performed with each Eσ^S derivatives at the *osmY*, *gal*P1 and *lac*UV5 promoters. Transcriptional activity of unconjugated (black bars) or conjugated (white bars) σ^S mutants, reconstituted with core enzyme, is given and is accurate to ±20%. With L297C mutant, transcription was performed with mutant conjugated under native (F) or denaturing (UF) conditions. The activity of FeBABE-conjugated σ^S proteins is a corrected value for the presence of unconjugated σ^S (a_{con}; see Owens *et al*., 1998a).

2.5, is required for Eσ^S activity at *osmY* and *lac*UV5 promoters but appears to be not so crucial at the *gal*P1 promoter. After conjugation, all other FeBABE-tethered mutants exhibited at least 80% of wild-type activity at *gal*P1 (Figure 2B). At the *osmY* promoter, transcriptional activity is only reduced with two mutants: S90C (32%) and K173C (19%). Finally, at the *lac*UV5 promoter, the Eσ^S activity is especially reduced for the following mutants: S90C (25%), F140C (35%), K173C (4%) and

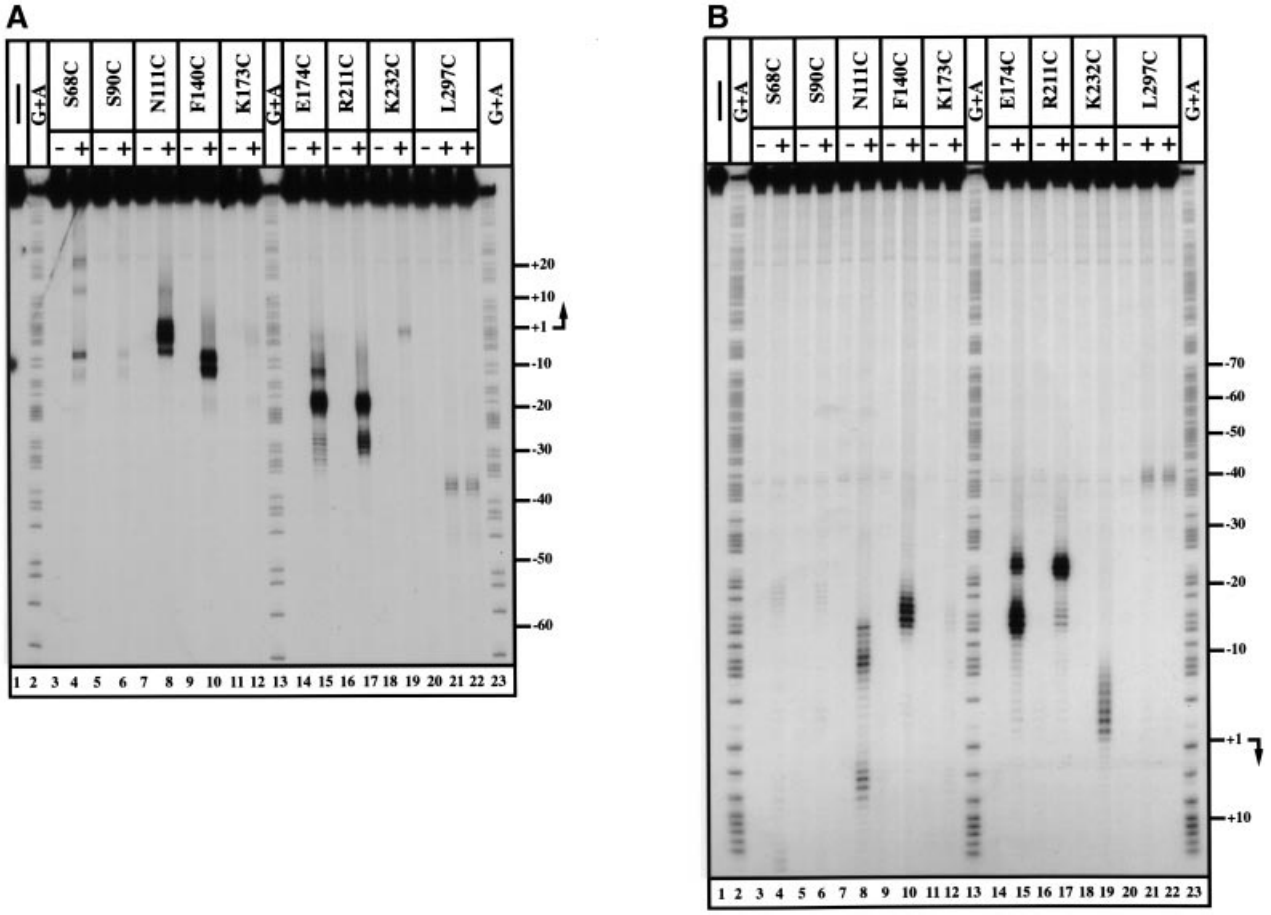


Fig. 3. *OsmY* promoter DNA cleavage pattern with the stationary phase RNA polymerase carrying FeBABE-modified σ^S. Non-template (**A**) or template (**B**) strand *osmY* promoter cleavage was performed with the Eσ^S holoenzymes modified from region 1.2 to region 4.2 of σ^S, as indicated at the top of the figures. Cleavage reactions containing FeBABE-modified σ^S proteins are indicated by '+', and those without FeBABE are indicated by '–'. The L297C mutant conjugated under native (F) or denaturing (UF) conditions is represented in lanes 21 and 22, respectively. All samples were treated equally with ascorbate and hydrogen peroxide for 10 min at 37°C. A marker ladder, constructed according to Maxam and Gilbert (1977), is shown in lanes 2, 13 and 23. The arrow at the +1 represents the start and direction of transcription.

R211C (43%). In conclusion, it is clear from Figure 2B that each conjugate retained 50% or more of its transcriptional activity at, at least, one of the three promoters used. Hence, for each of the derivatives studied, the cleavage pattern by hydroxyl radicals should be a faithful marker of the distance between the tethered residue and the bases at which the cleavages occurred.

Open complexes at osmY with σ^S derivatives conjugated with FeBABE in regions 2.2, 2.3, 2.5 and 3.1 produce high intensity promoter cleavage between positions –25 and +2

Cleavages were performed, by addition of H_2O_2 and ascorbate, on open complexes preformed for 20 min at 37°C. Control reactions showed that no cleavage occurred under these conditions with the wild-type enzyme or unconjugated cysteine derivatives, irrespective of the nature of the promoter. DNA cleavages in the promoter region of *osmY,* a typical stationary phase-dependent promoter, by holoenzymes carrying FeBABE-modified σ^S vary both in position and intensity with the different σ^S derivatives. However, the strongest DNA cleavages are produced with FeBABE positioned at N111C, F140C, E174C and R211C (Figure 3A and B). These four mutations are located between regions 2.2 and 3.1 of σ^S. The N111C mutation is located at the junction between regions 2.1 and 2.2, and the N111C FeBABE derivative cuts DNA preferentially in the transcription bubble from –9 to +2 (strong cleavage) on the non-template strand and from –14 to +7 (medium) on the template strand (Figure 3A and B, lanes 8). FeBABE positioned on F140C in the middle of region 2.3, which is rich in aromatic residues surrounded by basic residues, cuts the non-template strand from –14 to –8 (strong) and the template strand from –19 to –13 (medium) (Figure 3A and B, lanes 10). The E174C mutation is located in the middle of region 2.5 and the E174C FeBABE derivative cuts the non-template strand from –23 to –19 (strong) and with a weaker intensity around –14/–13 and from –33 to –28. On the template strand, the E174C cutting site is localized to two sequences: from –17 to –12 (strong) and –26 to –22 (medium) (Figure 3A and B, lanes 15). Within conserved region 3.1, FeBABE located at R211C cleaves at sequences –23 to –19 (strong), –30 to –27 (medium) on the non-template strand and at sequences –25 to –21 (strong), –16 to –13 (weak) on the template strand (Figure 3A and B, lanes 17). Therefore, regions 2.2 and 2.3 of σ^S are in close proximity to the melted region, and

regions 2.5 and 3.1 are localized just upstream in the –15/–20 DNA sequence.

A σ^S derivative in region 4.2 reacts weakly with the –35 region in the open complex at osmY

The other single cys-σ^S mutants result in weaker intensity cleavages. This is the case for K232C, which is located in the middle of region 3.2. FeBABE positioned at this residue specifically cleaves the template strand from –6 to +1 (Figure 3B, lane 19). A very weak cleavage is also observed with the L297C mutant, located in the turn of the HTH motif of region 4.2 (Figure 3A and B, lanes 21 and 22). The L297C cutting site is localized at –39 to –36 on the non-template strand and in sequences –42 to –39 on the template strand. This is the farthest upstream cutting site observed. In contrast, the farthest downstream cutting site is observed with residue S68C, located in the middle part of region 1.2. FeBABE-modified S68C cuts the non-template strand specifically at several dispersed positions: –15/–14, –11/–10 (more intense), +10/+12 and +19/+22 (Figure 3A, lane 4). No significant cleavage is observed when the reagent is positioned at S90C, at the border between regions 1.2 and 2.1, suggesting that this region is remote from the DNA (Figure 3A and B, lanes 6). The FeBABE mutant K173C, localized in region 2.5, does not cut the *osmY* promoter, an observation which is consistent with its weak RNA polymerase transcription activity (Figure 3A and B, lanes 12).

Cleavages at the galP1 and lacUV5 promoters confirm the overall positioning of $E\sigma^S$

The FeBABE proximity cleavages performed at open complexes of a variety of *gal* promoters with σ^{70} conjugates tethered to the DNA binding regions 2.3, 2.5 and 4.2 have shown an almost identical pattern, irrespective of the promoter sequences (Bown *et al*., 1999). It was therefore of interest to perform the σ^S-dependent cleavage experiments with the same promoters used previously for σ^{70} (Figure 2A): (i) a derivative of the *gal*P1 promoter, which has been mutated to introduce a consensus –10 element (Burns *et al*., 1996) (this sequence has an extended –10 promoter and no homology with the –35 consensus) and (ii) the *lac*UV5 promoter whose –10 and –35 hexamers are closely related to the consensus sequences. All these promoters are recognized *in vitro* by $E\sigma^{70}$ as well as by $E\sigma^S$ holoenzymes, albeit with different affinities (Tanaka *et al*., 1993, 1995; Kolb *et al*., 1995).

At *gal*P1, we observed the same cleavage profiles as at the *osmY* DNA sequence (Figure 4A and B). The only difference concerns the K173C mutation, located in region 2.5. The K173C mutant now cleaves the *gal*P1 promoter from –22 to –18 on the non-template strand and –16 to –12 on the template strand (Figure 4A and B, lanes 12). In contrast to *gal*P1 and *osmY*, the overall cleavage pattern generated by the various $E\sigma^S$ conjugates at *lac*UV5 appears more scattered and extends along the promoter sequence from positions –64 to +22 (Figure 5). For example, the predominant positions of cleavage resulting from FeBABE tethered at E174C map around position –15, and with decreased intensity around position –25 on the template strand of the *osmY* and *gal*P1 promoters (Figures 3B and 4B, lanes 15). At *lac*UV5, however, a second set of cleavages of weaker intensity is generated around positions –40 and –50 as if a fraction of RNA polymerase containing the conjugated 174C residue was now also able to recognize, at least to some extent, the *lac*P2 promoter which initiates transcription ~22 bp upstream of the *lac*P1 promoter (Malan and McClure, 1984). This was confirmed by $KMnO_4$ reactivity assays that indicated, besides the well characterized melted region of *lac*P1 around +1, a second melted region of lower intensity around –22 with the $E\sigma^S$ holoenzymes. It is well known that the cAMP–CRP complex prevents the $E\sigma^{70}$ holoenzyme from binding at *lac*P2 and activates transcription at *lac*P1 (Malan and McClure, 1984; Spassky *et al*., 1984). Similarly, the preincubation of the *lac*UV5 template with CRP and cAMP before addition of the $E\sigma^S$ holoenzyme completely turns off the *lac*P2 promoter. Under these conditions, we showed that the cleavage pattern resulting from FeBABE tethered at E174C was restricted to the most downstream sites and fully consistent with the sole occupation of the *lac*P1 promoter (data not shown). In conclusion, if we take into account the cleavages generated by the occupancy of RNA polymerase at *lac*P2, it appears that the proximity cleavage patterns induced by FeBABE-σ^S do not show major differences amongst all the promoters under study, as also shown for some FeBABE-σ^{70} derivatives at *lac* and *gal*P1.

Direct comparison between $E\sigma^S$ and $E\sigma^{70}$ DNA cleavages

A detailed analysis of some σ^{70} mutants previously used by others (Owens *et al*., 1998a; Bown *et al*., 1999) was performed to compare the positioning of $E\sigma^S$ and $E\sigma^{70}$ at *osmY, lac*UV5 and *gal*P1 directly. However, this comparison is dependent on promoter occupancy by both RNA polymerases. Since the *osmY* and *lac*UV5 promoters are not occupied with the same efficiency by $E\sigma^S$ and $E\sigma^{70}$ (Ding *et al*., 1995; Kusano *et al*., 1996), DNA cleavage intensities indicate not only proximity between σ and DNA but also promoter occupancy. In contrast, as shown by transcription assays, a similar occupancy is achieved by both wild-type holoenzymes at the *gal*P1 promoter (data not shown). Moreover, all the FeBABE conjugates of mutant σ^S and σ^{70} under study exhibited at least 80% of wild-type activity at *gal*P1, with the exception of the σ^S mutant K173C (Figures 2B and 6). Therefore, this promoter is the most suitable to compare the differences in FeBABE cleavage patterns between the two holoenzymes.

We performed DNA cleavage using σ^S and σ^{70} mutants with FeBABE located in regions 1.2, 2.2, 2.5, 3.1 and 4.2. The overall cleavage patterns by FeBABE-tethered mutant σ^S were essentially the same as those by σ^{70} (Owens *et al*., 1998a). Within region 1.2, FeBABE positioned on σ^{70} (132C) yielded no cleavage on the non-template strand of *gal*P1, in contrast to σ^S (S68C) (Figure 6, lanes 3 and 4). For conjugates located in regions 2.2 (N396C) and 3.1 (K496C) of σ^{70}, the same cleavage pattern was observed as for the corresponding σ^S mutants (Figure 6, lanes 5, 6, 12 and 13). For region 2.5, the two conjugates K173C and E458C exhibited the same cleavage pattern despite the fact that the σ^S derivative had only half the transcriptional activity of wild-type σ^S (Figure 6, lanes 7 and 8). In addition, residue T459C in region 2.5 of σ^{70} was much less reactive compared with the σ^S equivalent (E174C) (Figure 6, lanes 10 and 11). Finally, with the conjugate

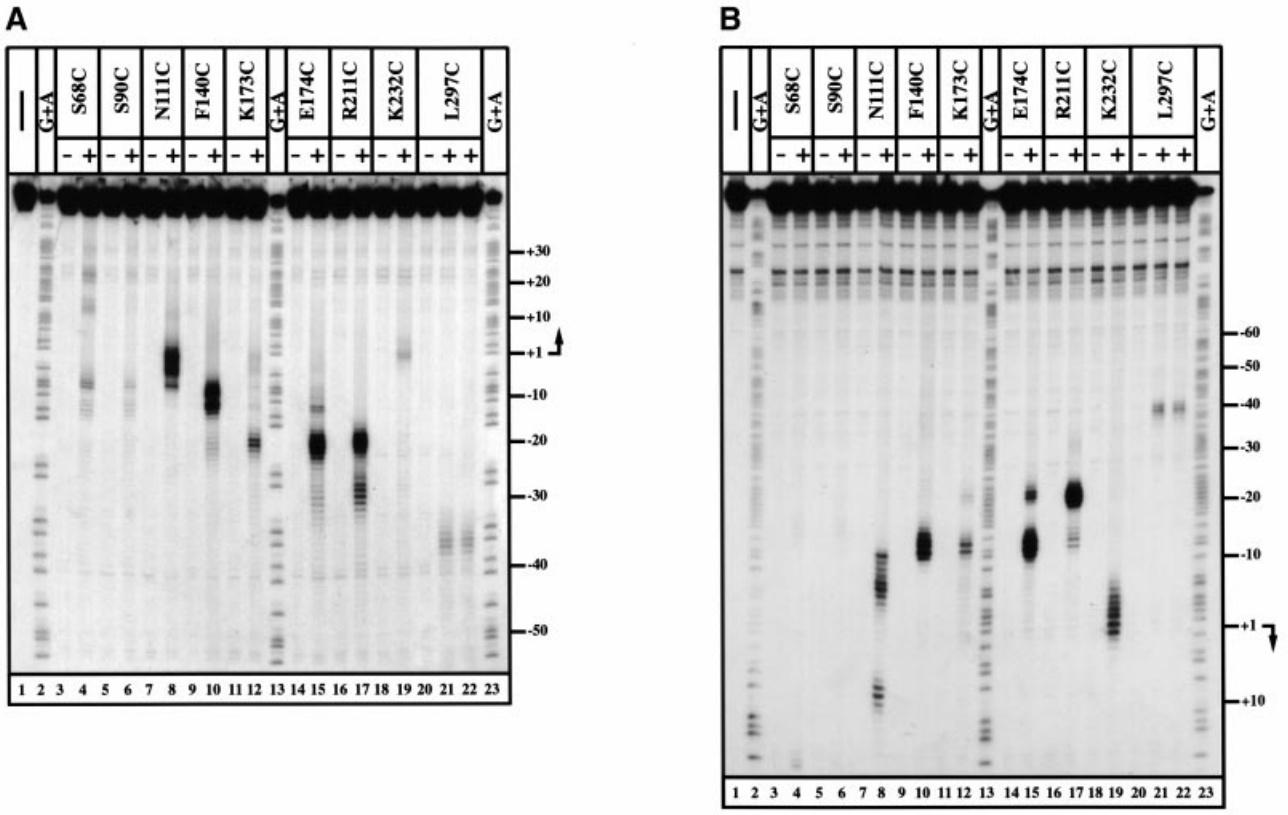


Fig. 4. *Gal*P1 promoter DNA cleavage pattern with the stationary phase RNA polymerase carrying FeBABE-modified σ^S (conditions as in legend to Figure 3).

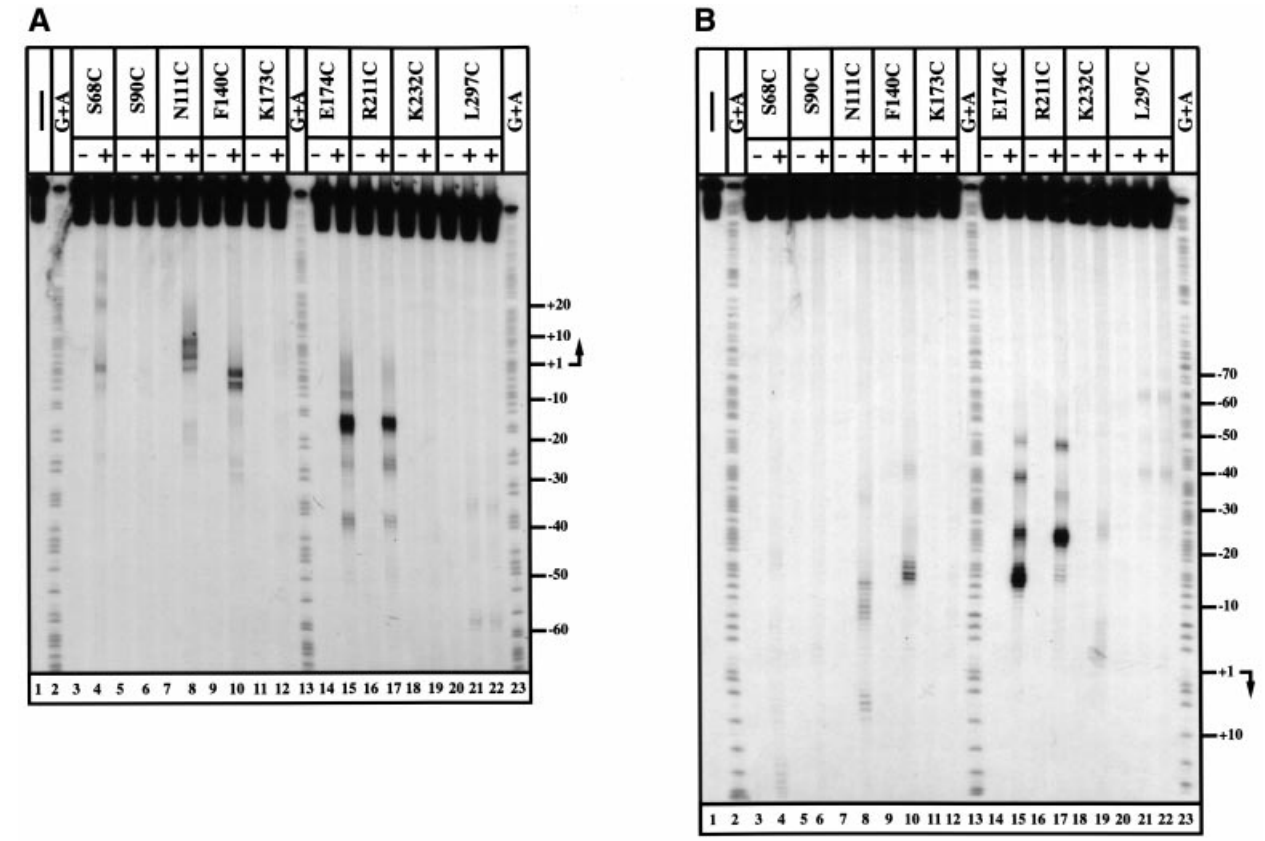


Fig. 5. *Lac*UV5 promoter DNA cleavage pattern with the stationary phase RNA polymerase carrying FeBABE-modified σ^S (conditions as in legend to Figure 3).

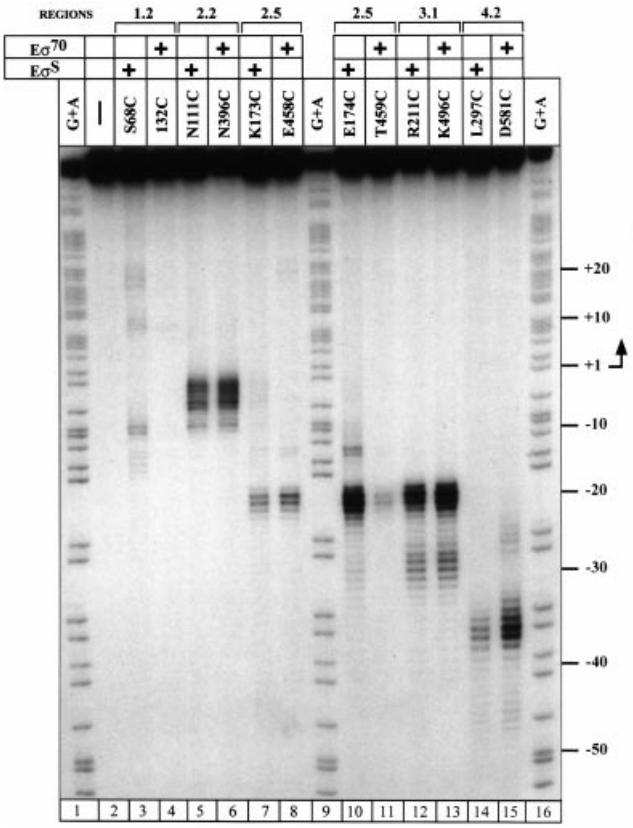


Fig. 6. Cleavage pattern with Eσ^S and Eσ^{70} RNA polymerases carrying FeBABE-modified σ at the non-template strand of the *gal*P1 promoter. Conjugation yield of the σ^{70} FeBABE derivatives was determined: 132C, 33%; N396C, 78%; E458C, 59%; T459C, 60%; K496C, 90% and D581C, 80%. After binding to core enzyme, all these σ^{70} derivatives retained >90% of the wild-type transcriptional activity at the *gal*P1 promoter.

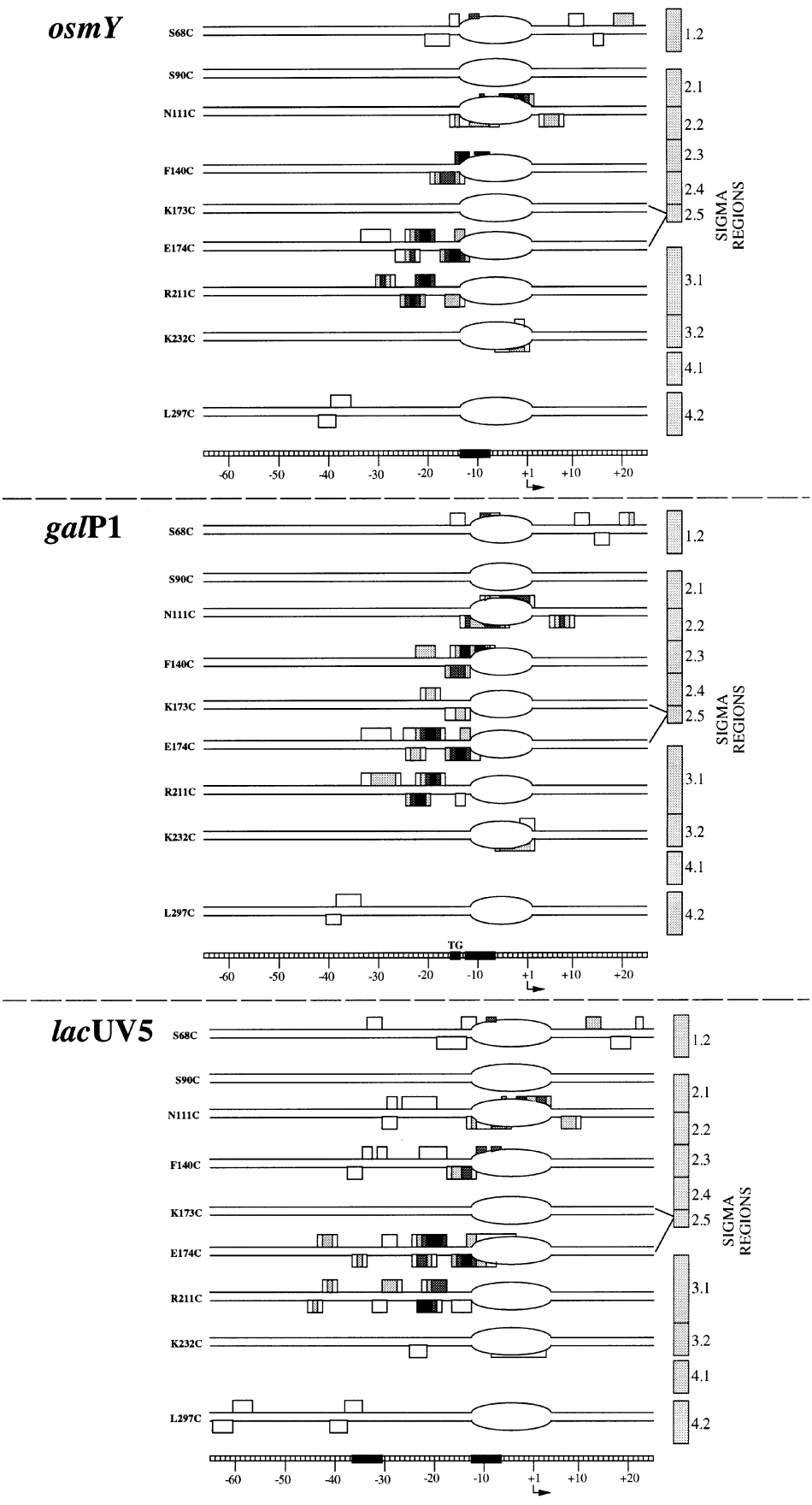
located in region 4.2 of σ^{70} (D581C), a strong cleavage was observed from –37 to –34, with additional weaker cleavages near –26 to –24 and –45/–44 on the non-template strand. In contrast, a weak cleavage was observed at position –38 to –35 with the L297C derivative of σ^S (Figure 6, lanes 14 and 15). This result was observed with all the promoters used (*osmY*, *lac*UV5 and *gal*P1), suggesting a differential positioning between both σ factors in the –35 region.

Discussion

The FeBABE reagent, covalently conjugated to the free sulfhydryl group of a cysteine residue in a protein, can cleave polypeptide chains and nucleic acid backbones located in proximity to the chelate. This methodology has been applied previously to study protein topography and proximity sites between protein and DNA (Greiner *et al*., 1997; Murakami *et al*., 1997a,b; Miyake *et al*., 1998; Owens *et al*., 1998a,b; Bown *et al*., 1999). In these studies, the DNA cleavage patterns were interpreted in terms of proximity between the conjugated residue and the cleaved deoxyribose. Proximity sites may interact, but do not necessarily have to. In the case of σ^{70}, Owens and co-workers selected the sites for cysteine substitution from the crystal structure data (Malhotra *et al*., 1996) as well as from many mutation and deletion studies. Using several Eσ^{70} FeBABE derivatives, they subsequently showed that every proximity score was in full agreement with the genetic and biochemical data (Owens *et al*., 1998a), thus assessing the validity of the approach. In the absence of any structural data for the stationary phase σ^S factor, we have modified the equivalent or neighbouring positions as in σ^{70} because this strategy should give a better comparison between the σ factors, even though it may preclude the identification of new proximities between Eσ^S and its templates. Using the FeBABE nuclease, we have mapped the positioning of σ^S, associated with the core enzyme, at three different promoter sequences. The resulting cleavage patterns resemble the σ^{70} patterns despite some significant differences in cleavage intensities between the two holoenzymes.

The absence of a given cleavage could originate from a lack in proximity of the tagged residue from DNA or from a defect of the modified RNA polymerase in promoter binding. Therefore, we have characterized the transcriptional activity of all the derivatives to check their ability to occupy the relevant promoter. Among the nine mutations introduced in σ^S, K173C is the only one resulting in a significant decrease of the activity of the protein at all promoters. The lysine in position 173, located in the putative α-helix of region 2.5, is crucial for Eσ^S activity, at least at the *osmY* and *lac*UV5 promoters. The partial restoration of activity at the *gal*P1 promoter suggests that the TG motif upstream of the –10 hexamer provides additional contacts which, at this promoter, compensate for the crucial interaction between K173 of σ^S and the –10/–20 DNA sequence (Figure 4A and B, lanes 12). Conjugation of single-cys σ^S derivatives with FeBABE does not affect their specific functions since it does not reduce their transcriptional competence at least at the *osmY* and *gal*P1 promoters. *Lac*UV5, which is not dependent upon σ^S for *in vivo* expression (Yim *et al*., 1994), appears to be the most sensitive to the various modifications introduced in the σ^S RNA polymerase. This is not unexpected since Eσ^S shows the greatest susceptibility to heparin challenge when bound at this promoter (F.Colland, unpublished data). At this promoter, as well as at *osmY*, the FeBABE cleavage intensities generated by the Eσ^S or Eσ^{70} holoenzymes are correlated with their respective promoter occupancies. In most cases, the ratio of open complex formed at *osmY* with each Eσ^S derivative appears ~2-fold higher than the one obtained with the homologous σ^{70} derivative, whereas the opposite occurs at *lac*UV5. In addition, Eσ^S partitions between the overlapping promoters *lac*P1 and *lac*P2. Despite these complexities, one can compare the positioning of the two holoenzymes on DNA both from previous data on *lac* and *gal*, and from a direct comparison performed here (Owens *et al*., 1998b; Bown *et al*., 1999; Figure 6).

The cutting pattern observed with FeBABE derivatives of Eσ^S is almost identical for all promoters studied irrespective of their sequence determinants in the –10, –15 and –35 regions, demonstrating a similar architecture of the open complex (Figure 7). This conservation has also been noticed recently for the DNA binding regions of Eσ^{70} at three variants of the *gal*P1 promoter, which differ either by the presence of the TG motif at –14/–15 or by the nature of the –35 consensus hexamer (Bown *et al*., 1999). For both holoenzymes, the cleavage patterns


osmY
S68C
S90C
N111C
F140C
K173C
E174C
R211C
K232C
L297C
1.2
2.1
2.2
2.3
2.4
2.5
3.1
3.2
4.1
4.2
SIGMA REGIONS
-60 -50 -40 -30 -20 -10 +1 +10 +20
galP1
TG
lacUV5

span at least 60 bases: Eσ^S cleaves between –42 and +22, and Eσ^{70} between –48 and +12. Within these limits, the two holoenzymes present the same general organization in the open complexes. This similar positioning of σ^S and σ^{70} relative to promoters could have been expected in view of their similar functional organization and their high degree of homology. In region 2, which is the most conserved portion of all the σ polypeptides, region 2.2 is very close to the +1 transcriptional start for both σ factors. Region 2.3, rich in aromatic residues, is implicated in DNA melting and maps in both cases very close to the unpaired segment of the non-template strand. But for σ^S only, a new cleavage is detected at the template strand around –10/–15. Region 3.1 and 3.2 show strong similarity in this comparison, while interesting differences can be noticed in regions 1.2, 2.1, 2.5 and 4.2.

Region 1.2 of σ^{70} is required for open complex formation (Wilson and Dombroski, 1997) and the corresponding FeBABE cleavage maps in the –10 region (Owens *et al*., 1998a). A similar cut is observed within the σ^S pattern but additional cleavages are also present in the far downstream region of the non-template strand around +10 and +20, leading to a shift in the downstream border of the reactivity for Eσ^S. The presence of this downstream reactivity is probably correlated with another function attributed to region 1, of facilitating RNA polymerase escape from the initiating complex (Wilson and Dombroski, 1997). Such an extended cleavage pattern, brought about by the holoenzyme carrying σ^S tagged in this region, suggests also that a strong bend is induced here upon DNA melting. None of the other σ^S derivatives associated with core enzyme display such an extended reactivity. Region 2.1 of σ^{70} is involved in core binding (Lesley and Burgess, 1989). In region 2.1 of σ^S, residue 90 must be located far from the DNA in contrast to the equivalent position in σ^{70} (Owens *et al*., 1998a). Residue 90 of σ^S might also be very close or inside the core binding site; preliminary results show indeed that its FeBABE derivative specifically cleaves the β′ subunit of core RNA polymerase (data not shown). As observed with region 2.1, the reactivity of the σ^S derivative in region 4.2 is very weak in the –35 region compared with σ^{70}. In fact, in contrast to the σ^{70} mutant, the L297C mutant of σ^S is located in a poorly accessible region (Table I). This lack of accessibility suggests an explanation for the poor efficiency of region 4.2 of σ^S in the recognition of –35 consensus sequences, in agreement with previous biochemical and genetic studies (Hiratsu *et al*., 1995; Kolb *et al*., 1995; Tanaka *et al*., 1995).

Regarding region 2.5, Bown and co-workers proposed a model for the docking of the putative α-helix of σ^{70} (residues 454–461) in the major groove of DNA between positions –14 and –20 with residue E458 in hydrogen bonding contact with bases at –14 and –15 opposite to the TG motif. In the present work, a cleavage pattern similar to that observed with the E458C FeBABE conjugate of σ^{70} at *gal* was obtained with the E174C σ^S mutant at all promoters, but with a much higher cleavage efficiency (Figures 6 and 7). E174 is adjacent to K173, the residue corresponding to E458 in σ^S sequence, whose integrity is required for Eσ^S activity at *osmY* and *lac*UV5. In contrast, at these promoters, E458 can be substituted by a cysteine without any effect on transcription activity. Thus, region 2.5 appears crucial for Eσ^S activity and comes into close proximity to the DNA just upstream of the –10 hexamer. From the analysis of the cleavage patterns and from the higher intensities observed, we also suggest that there is a much tighter anchoring of the putative α-helix in the major groove of DNA with σ^S than with σ^{70}. These observations could have functional implications since region 2.5 of σ^S seems to be especially crucial for the proper regulation and for the activity of σ^S-dependent promoters (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Schweder *et al*., 1996; Zhou and Gottesman, 1998; Becker *et al*., 1999).

In conclusion, the positioning of σ^S along the DNA in open complexes formed at three different promoters is essentially independent of the promoter sequence. The central regions of both σ factors including regions 2.2 to 3.2 all react at the same positions on the DNA. The cleavage intensities are especially high for σ^S tagged in regions 2.2, 2.3, 2.5 and 3.1 on both strands of DNA between positions –25 to +2. From the intensities of the cuts and their similar distribution on both strands, we suggest that the central part of σ^S makes the closest interactions with the core promoter. This is at variance with Eσ^{70} where the strongest cleavages are scattered from region 2.1 to region 4.2 with a clear preference for one strand at some positions. The Eσ^{70} holoenzyme recognizes an extended promoter sequence and requires at least two anchoring regions, the –10 and –35 hexamers, for specific DNA–protein interactions. The Eσ^S holoenzyme appears to interact with a smaller part of the promoter and might need only a single specific anchoring region such as an extended –10 region, in agreement with the fact that a specific –35 region is dispensable at most σ^S-dependent promoters. This strategy might be especially advantageous in the stationary phase where the DNA is protected by multiple DNA-binding proteins (such as IHF and Dps, the levels of which generally increase at the onset of the stationary phase) (for a review see Ishihama, 1999).

Materials and methods

Site-directed mutagenesis

pETF plasmid encodes wild-type σ^S subunit under the control of Ø10 promoter of bacteriophage T7 (Tanaka *et al*., 1993). A unique *Mlu*I site was introduced at residue 162 into pETF to yield the plasmid, pFC0. The wild-type σ^S protein has no cysteine residues. Plasmids overexpressing mutant σ^S subunits, which contain a single cysteine residue at positions 68 (pFC1), 90 (pFC2), 111 (pFC3), 140 (pFC4), 173 (pFC6), 174 (pFC7), 211 (pFC8), 232 (pFC9) or 297 (pFC10), were constructed from pFC0 using the Quiaquick site-directed mutagenesis kit (Stratagene). Depending on the position of the mutation, *Nde*I–*Hpa*I, *Hpa*I–*Mlu*I, *Mlu*I–*Rsr*II or *Rsr*II–*Bam*HI fragments were isolated and inserted into pFC0 between the corresponding sites. DNA sequencing of each cys-

Fig. 7. Summary of *osmY, gal*P1 and *lac*UV5 DNA cleavage by FeBABE-conjugated, stationary phase RNA polymerase. Each σ^S mutant is indicated on the left of the figure and their respective position in the primary sequence is indicated on the right. Horizontal lines correspond to DNA; boxes above these lines correspond to non-template strand cleavage and boxes below these lines correspond to template strand cleavage. The cleavage efficiency is indicated by the intensity of the shading of the box. Regions unpaired in open complexes are shown by the separation of DNA strands. Positions along the DNA template, with the +1 start site of transcription, are represented at the bottom of the figure. The –10, extended –10 and –35 consensus elements are shown by black boxes.

mutant gene was performed by the dideoxy chain termination (T7 sequencing kit from Pharmacia Biotech).

Protein purification

Mutant σ^S subunits were purified from the over-producing strain BL21(DE3)/pLysS/pFC as described for wild-type σ^S (Tanaka *et al*., 1995). Log-phase core enzyme was prepared according to Lederer *et al*. (1991). Mutant σ^{70} subunits (132C, N396C, E458C, T459C, K496C and D581C) were purified as described (Owens *et al*., 1998a; Bown *et al*., 1999).

Conjugation of σ^S and σ^{70} mutants with FeBABE

Each purified σ preparation in storage buffer [10 mM Tris pH 7.6, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 M KCl and 50% (v/v) glycerol] was dialysed overnight at 4°C against the conjugation buffer [10 mM MOPS pH 8.0, 2 mM EDTA, 0.2 M NaCl and 5% (v/v) glycerol]. Conjugation was performed by mixing 300 µM of FeBABE with 20 µM of each σ mutant for 4 h at room temperature. This mixture was then dialysed at 4°C against conjugation buffer to remove free FeBABE. As a control, the wild-type σ^S protein, devoid of cysteine residue, was incubated with FeBABE: no DNA cleavage was observed after association of this protein to the core enzyme, showing that the non-covalently bound FeBABE was totally removed by dialysis (data not shown). The L297C mutant of σ^S (20 µM final) was also unfolded by dialysis at 4°C into the conjugation buffer containing 6 M urea, then mixed with FeBABE (300 µM final) for 4 h at room temperature. The mixture was last dialysed at 4°C against the conjugation buffer to refold the L297C mutant and to remove free FeBABE. The concentration of free cysteine side chains was determined with the fluorescent CPM reagent on both unconjugated and conjugated proteins as described previously (Greiner *et al*., 1997).

Promoter DNA labelling

The *osmY* PCR fragment from –175 to +48 has been cloned in the *Eco*RI site, filled in with the Klenow fragment, of pJCD0 to generate pJCD02 (Marschall *et al*., 1998). The labelled *osmY* fragment was generated by PCR with the primers 5′-TTCAGTTCCACCAGACCC-3′ and 5′-GATATCTACGCATTGAACG-3′ using a combination of one unlabelled primer and the second primer end-labelled with phage T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol). The 164 bp *gal*P1 and 229 bp *lac*UV5 fragments were generated by PCR from pSR*gal*P1 (Burns *et al*., 1996) and pBR*lac*UV5 (Schaeffer *et al*., 1982), respectively, as template using the pBR primer 5′-GGCGTATCAC-GAGGCCCTTTCG-3′ and the *gal* primer 5′-CTTGGCGGTAACC-AGAACTC-3′ or the *lac* primer 5′-CACGACAGGTTTCCCGACTG-3′. These fragments were then purified on a glass fibre column (High Pure PCR Product Purification Kit from Boehringer Mannheim) and used for subsequent experiments.

Transcriptional activity assay before and after conjugation

Single-round transcription by reconstituted RNA polymerase was carried out under the standard conditions both before and after FeBABE conjugation, from the *osmY* linear template (pJCDO2 digested with *Afl*III), the *gal*P1 linear template (pSR*gal*P1 digested with *Afl*III) or the *lac*UV5 linear template [pOM70*lac*UV5 digested with *Afl*III (Adelman *et al*., 1997)]. Briefly, reconstitution of active holoenzymes was achieved by incubating 1 vol 5 µM core-enzyme with 2 vol each σ factor at 10 µM for 20 min at 37°C (σ:core ratio of 4:1). DNA template (4 nM) was incubated with each reconstituted holoenzyme (50 nM) in glutamate buffer (100 mM potassium glutamate, 40 mM HEPES pH 8.0, 10 mM magnesium chloride, 100 µg/ml bovine serum albumin) at 37°C for 20 min in 10 µl final volume. Elongation was started by the addition of 5 µl of a prewarmed mixture containing 600 µM ATP, GTP and CTP, 30 µM UTP, 0.5 µCi [α-^{32}P]UTP and 600 µg/ml of heparin to the template/polymerase mix, and allowed to proceed for 5 min at 37°C. Reactions were stopped by the addition of 20 mM EDTA in formamide containing xylene cyanol and Bromophenol Blue. After heating to 65°C, samples were subjected to electrophoresis on 7% sequencing gels. Run-off products were quantified using a PhosphorImager (Molecular Dynamics).

DNA cleavage by FeBABE

Each σ mutant (2 vol, 10 µM) was reconstituted with 1 vol of core enzyme at 5 µM for 20 min at 37°C. Reconstituted holoenzymes (200 nM) were incubated with non-template- or template-labelled *osmY*, *gal*P1 or *lacUV5* promoter fragments (1 nM) in glutamate buffer (10 µl final volume) for 20 min at 37°C. To remove non-specifically-bound RNA polymerase, heparin (50 µg/ml) was added 3 min before the cleavage reaction. DNA cleavage was generated by addition of ascorbate (2 mM) and H_2O_2 (1 mM), in the presence of each $E\sigma^S$ or $E\sigma^{70}$ derivative, and was then performed exactly as described by Owens *et al*. (1998a).

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