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σ factor selectivity of *Escherichia coli* RNA polymerase: role for CRP, IHF and Lrp transcription factors

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***osmY* is a stationary phase-induced and osmotically regulated gene in *Escherichia coli* that requires the stationary phase RNA polymerase ($E\sigma^S$) for *in vivo* expression. We show here that the major RNA polymerase, $E\sigma^{70}$, also transcribes *osmY in vitro* and, depending on genetic background, even *in vivo*. The cAMP receptor protein (CRP) bound to cAMP, the leucine-responsive regulatory protein (Lrp) and the integration host factor (IHF) inhibit transcription initiation at the *osmY* promoter. The binding site for CRP is centred at –12.5 from the transcription start site, whereas Lrp covers the whole promoter region. The site for IHF maps in the –90 region. By mobility shift assay, permanganate reactivity and *in vitro* transcription experiments, we show that repression is much stronger with $E\sigma^{70}$ than with $E\sigma^S$ holoenzyme. We conclude that CRP, Lrp and IHF inhibit open complex formation more efficiently with $E\sigma^{70}$ than with $E\sigma^S$. This different ability of the two holoenzymes to interact productively with promoters once assembled in complex nucleoprotein structures may be a crucial factor in generating σ^S selectivity *in vivo*.**

Keywords: repressors/RNA polymerase/*rpoS*/σ factor/stationary phase

Introduction

Entry of *Escherichia coli* cells into stationary phase results in complex morphological and physiological changes (Siegele and Kolter, 1992; Hengge-Aronis *et al*., 1993). Stationary phase *E.coli* cells become more resistant to a variety of stresses including starvation, near UV radiation, high temperature, hydrogen peroxide, acidic pH and high medium osmolarity (Loewen and Hengge-Aronis, 1994). These properties result from the induction of a set of specific genes. Many of these genes are expressed under the control of the major regulator of the general stress response, σ^S, the product of the *rpoS* gene (Lange and Hengge-Aronis, 1991). σ^S has been shown to interact with core RNA polymerase and to transcribe several promoters, thus appearing as a 'second principal' σ factor in stationary phase *E.coli* (Mulvey and Loewen, 1989; Nguyen *et al*., 1993; Tanaka *et al*., 1993).

σ^S is homologous to σ^{70}, the major σ factor, even in the domains involved in interaction with the –10 and the –35 consensus regions of a promoter (Lonetto *et al*., 1992). In agreement with these properties, σ^S and σ^{70} show overlapping promoter specificities *in vitro* (Nguyen *et al*., 1993; Tanaka *et al*., 1993; Ding *et al*., 1995; Kusano *et al*., 1996; Nguyen and Burgess, 1997; Ballesteros *et al*., 1998; Bordes *et al*., 2000). However, in contrast to the relaxed specificity observed under *in vitro* conditions, many promoters are known to be transcribed *in vivo* solely by one of the two holoenzymes. This discrepancy between the analogous recognition properties *in vitro* and the *in vivo* $E\sigma^S$ recognition specificity has been pointed out (for reviews see Hengge-Aronis, 1999; Ishihama, 1999). In this respect, higher concentrations of potassium glutamate and lower template supercoiling have been shown to contribute to $E\sigma^S$ selectivity of some stationary phase-specific promoters (Ding *et al*., 1995; Kusano *et al*., 1996; Nguyen and Burgess, 1997). These experimental conditions for increased $E\sigma^S$ selective transcription *in vitro* seem to be in agreement with the *in vivo* intracellular conditions prevailing in late stationary phase or after osmotic upshift.

osmY (also called *csi-5*) is an example of a gene that is strongly dependent on σ^S *in vivo* (Lange *et al*., 1993; Yim *et al*., 1994) but transcribed by RNA polymerase containing either σ^S or σ^{70} *in vitro* (Ding *et al*., 1995; Kusano *et al*., 1996). This promoter is induced not only during transition into stationary phase but also in response to increased medium osmolarity during exponential growth (Yim and Villarejo, 1992; Hengge-Aronis *et al*., 1993). *osmY* expression is controlled at the transcriptional level from a single promoter under both conditions and the gene encodes the periplasmic protein OsmY of unknown function (Yim and Villarejo, 1992; Lange *et al*., 1993; Yim *et al*., 1994). The *osmY* promoter sequence possesses a –10 region (TATATT) with strong similarity to the σ^{70}/σ^S consensus, but a –35 region (CCGAGC) with a poor match to the –35 consensus sequence of σ^{70}. Genetic data indicated that this promoter is repressed by three global regulators: integration host factor (IHF), cAMP–receptor protein (CRP) and leucine-responsive regulatory protein (Lrp) (Lange *et al*., 1993).

In the present study, we asked whether the presence of the regulatory factors could affect preferentially one of the two holoenzymes ($E\sigma^S$ or $E\sigma^{70}$) for *in vitro* transcription initiation, thus mimicking the σ factor selectivity observed *in vivo*. We report direct evidence that IHF, cAMP–CRP and Lrp mainly inhibit $E\sigma^{70}$-dependent expression at the *osmY* promoter.

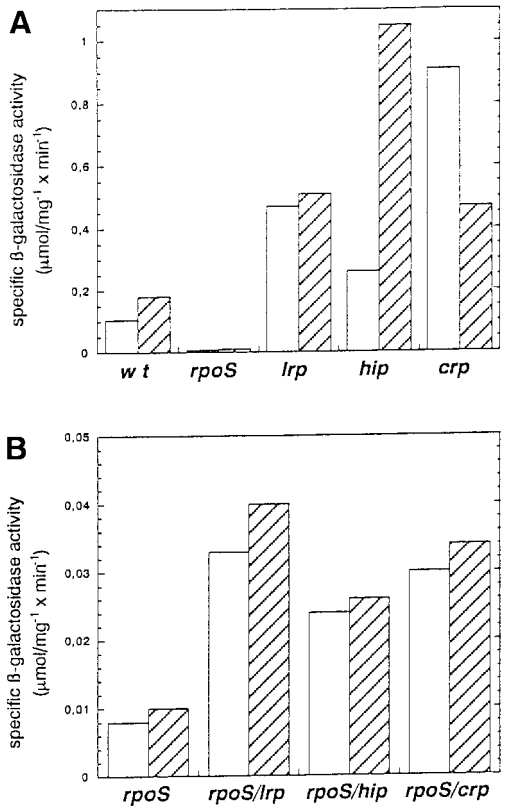

Fig. 1. Effect of single or double deficiencies in σ^S, Lrp, IHF and CRP on the expression of the *osmY* gene. Strain RO151, which carries the single copy *osmY* (*csi-5*)::*lacZ* transcriptional fusion, and its derivatives carrying a *nlpD-rpoS* deletion (which has the same effect on *osmY* expression as a mutation affecting *rpoS* alone), *lrp*::Tn*10*, *hip*::*cat* or a deletion in *crp*, were grown in minimal medium M9 supplemented with 0.1% glucose. β-galactosidase activities were determined during late exponential phase (white bars) or in stationary phase (overnight cultures; hatched bars). (**A**) Mutants defective in a single regulatory gene. (**B**) Mutants carrying lesions in *rpoS* in combination with a mutation in one of the other regulatory genes. Measurements were performed in triplicate and average values are given (±10%).

Results

Inhibition by Lrp, IHF and cAMP–CRP in the control of osmY expression in vivo in the presence or absence of σ^S

Using a single copy transcriptional *lac* fusion to the chromosomal copy of *osmY*, Lange *et al*. (1993) have shown that expression of the *osmY* promoter depends on the presence of an intact *rpoS* allele in all growth media and genetic backgrounds. However, the conditions that strongly increase σ^S levels are not always sufficient to induce the *osmY* promoter fully. In minimal medium, the depletion of glucose, ammonium or phosphate ions results in a clear σ^S induction, but only in a modest increase of the β-galactosidase activity of the *osmY::lac* fusion (Weichart *et al*., 1993). Besides σ^S availability, other transcription factors such as Lrp, IHF and CRP negatively regulate *osmY* expression. Figure 1 shows the β-galactosidase activities of cells grown in M9 medium containing 0.1% glucose until late log phase or stationary phase. A significant increase in activities can be observed in *lrp*, *hip* and *crp* strains in both conditions.

Lrp, a small homodimeric protein, acts as a transcriptional regulator by binding to DNA. Its activity is modulated by the presence of leucine or alanine (for a review see Newman and Lin, 1995). Lrp activates some genes involved in anabolism and represses others involved in catabolism. Lrp levels are constitutively high during growth and after entry into stationary phase in glucose minimal medium (Landgraf *et al*., 1996; Azam *et al*., 1999). The absence of Lrp derepresses expression of the *osmY* fusion by a 3- to 4-fold factor in late log and stationary phases. In the absence of σ^S, although the activities are ~10-fold lower, a similar derepression is observed (Figure 1B).

IHF is a small heterodimeric histone-like protein that binds and bends DNA (Friedman, 1988). It is involved in a great variety of processes including replication, site-specific recombination and transcriptional regulation. The absence of a functional IHF protein leads to a 2-fold increase in *osmY::lac* expression in late log phase but to a 6-fold increase in stationary phase (Figure 1A). This result is consistent with the dramatic increase in the amount of IHF observed in stationary phase cell cultures, partly under the dependence on σ^S (Aviv *et al*., 1994; Azam *et al*., 1999). The derepression in the *rpoS* background is still observed but with little difference between stationary phase and late log cells (Figure 1B), consistent with the lack of IHF stationary phase induction in the *rpoS* mutant (Aviv *et al*., 1994).

The presence of a mutation in the *crp* gene leads to a 9-fold increase in late log phase expression of the fusion. Much of this effect is probably indirect, i.e. due to increased σ^S levels in the *crp* mutant (Lange and Hengge-Aronis, 1994). The inhibitory effect of CRP appears to be only 2-fold in the stationary phase. Since stationary phase levels of σ^S are similar in wild-type and in the *crp* backgrounds, this inhibitory effect of CRP is probably a direct effect on *osmY* expression. In the *rpoS* mutant, CRP inhibits the *osmY::lac* activity to the same extent in stationary phase and in late log phase (i.e. 4-fold). These data suggest that, in stationary phase, the direct inhibitory effect of CRP on *osmY* expression is more pronounced with Eσ^{70} than with Eσ^S.

In conclusion, the *in vivo* data show that Lrp, IHF and CRP are able to repress *osmY*. The quantitative interpretation of these data, however, is complicated by the fact that the regulators involved also affect the cellular levels of each other (Aviv *et al*., 1994; Lange and Hengge-Aronis, 1994; Bouvier *et al*., 1998). This means that the regulatory effects observed may be the sum of direct and indirect effects. We therefore decided to investigate the *in vitro* ability of Lrp, IHF and CRP to bind to the *osmY* promoter, and the direct effects of these regulators on open complex formation and *in vitro* transcription of *osmY*.

In vitro analyses of the binding of Eσ^S, Eσ^{70}, cAMP–CRP, IHF and Lrp at the osmY promoter region

To analyse precisely binding of the two holoenzymes and the repressors in the *osmY* promoter region, DNase I footprinting experiments were performed using a radio-labelled PCR *osmY* fragment extending from positions –175 to +48 with respect to the transcriptional start site.

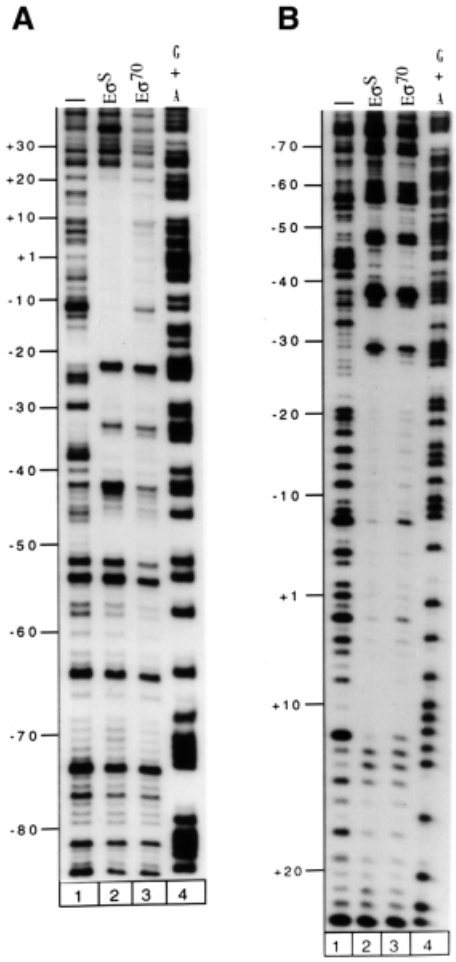

Fig. 2. DNase I footprint analysis of the complexes formed by $E\sigma^S$ or $E\sigma^{70}$ holoenzymes (150 nM) at *osmY*. After DNase I attack, the heparin-resistant complexes were purified and samples were analysed on a 7% denaturing polyacrylamide gel, which was calibrated using a sequencing reaction for G + A (lane 4). Results obtained on the non-template and template strands are shown in (**A**) and (**B**), respectively: DNA alone (lane 1), $E\sigma^S$ (lane 2) and $E\sigma^{70}$ (lane 3).

Both RNA polymerases are clearly able to bind to the *osmY* promoter. They protect DNA from position –51.5 to +19.5 on the non-template strand and from –54.5 to +12.5 on the template strand (Figure 2A and B, respectively). However, significant differences in the protection patterns were observed: (i) $E\sigma^S$ strongly protects the sequence downstream of –26.5 while protection by $E\sigma^{70}$ in this region is clearly weaker (Figure 2); (ii) $E\sigma^{70}$ strongly protects the region upstream of –35, especially on the non-template strand; and (iii) on the same strand, an $E\sigma^S$-specific hypersensitive site is observed at –42.5 (Figure 2A).

The IHF protein at 100 nM binds to the far upstream region of the promoter protecting the template strand between positions –110.5 and –82.5 from DNase I cleavage (Figure 3A). IHF recognizes a specific DNA sequence centred at position –98.5, which is only distantly related to the consensus sequence (WATCAANNNNTTR; Figure 4B; Craig and Nash, 1984; Goodrich *et al*., 1990; Rice *et al*., 1996).

In the presence of cAMP, protection by the CRP protein is observed in the –10 region and extends from at least –19.5 to +1.5 on the template strand (Figure 3B). The sequence centred at –12.5 contains between –20 and –15, the TGTGA motif, the best conserved element of the palindromic CRP-binding site, whereas only two positions match the consensus in the symmetric element between –9 and –5 (Figure 4B; Kolb *et al*., 1993). Thus, its affinity is ~4- to 5-fold lower than at *lac* (data not shown). The specific binding sites for IHF and CRP at *osmY* are indicated in Figure 4.

In contrast to CRP and IHF, Lrp does not bind to a single site, but binds co-operatively to the whole promoter region from –90 to +1 (Figure 3C). As previously reported, there is a periodic pattern of protection and enhanced cleavages separated by 10–11 bp (Wiese *et al*., 1997; Marschall *et al*., 1998). The most highly hypersensitive bands appear around positions –57.5 and –35.5 on the template strand and around –33.5 and –12.5 on the non-template strand, suggesting the existence of several Lrp-binding sites. In addition, circular permutation analysis of the mobilities of the *osmY*–Lrp complexes showed that Lrp bends the promoter region and forms a nucleoprotein complex at *osmY*. The *in vitro* methylation of the *dam* site at positions –15 and –16 did not significantly alter the Lrp footprinting pattern (data not shown).

Since the regions protected by IHF and CRP partially overlapped with those protected by Lrp, we tested the competition between these DNA-binding proteins on the non-template strand of the *osmY* promoter. After the simultaneous addition of IHF (50 nM) and CRP (50 nM), the specific protection patterns of both proteins were observed, showing that IHF and CRP binding were not mutually exclusive (Figure 3D, lane 7). In contrast, a mixture of CRP (50 nM) and Lrp (8 nM) resulted only in tight binding of CRP (Figure 3D, lane 8). Finally, when IHF (50 nM) and Lrp (8 nM) are added together, tight Lrp binding is observed whereas IHF binding is only slightly affected (Figure 3D, lane 5). Together, these results indicate that at the concentrations tested on linear template, Lrp is able to bind in the *osmY* promoter region even in the presence of IHF, while the presence of CRP leads to its exclusion from this promoter sequence.

$E\sigma^S$ forms an open complex at osmY even in the presence of repressors

A simple approach was used to monitor *in vitro* the effect of these DNA-binding proteins on open complex formation with both forms of RNA polymerase. Open complexes with $E\sigma^S$ or $E\sigma^{70}$ holoenzymes are known to be resistant to heparin challenge whereas binary complexes containing DNA and CRP or IHF are chased off quickly by this competitor (Figure 5A and B, lane 7). We first checked for the formation of heparin-resistant complexes with both holoenzymes. After a 20 min incubation of the *osmY* promoter with each reconstituted RNA polymerase (50 nM), 75% of heparin-resistant complex is observed with $E\sigma^S$ (Figure 5, lane 2) and 60% with $E\sigma^{70}$ after heparin challenge (Figure 5, lane 4). In a second set of experiments, the repressor was incubated first with the *osmY* promoter without RNA polymerase. Full occupancy of CRP, IHF and Lrp DNA-binding sites is observed, as

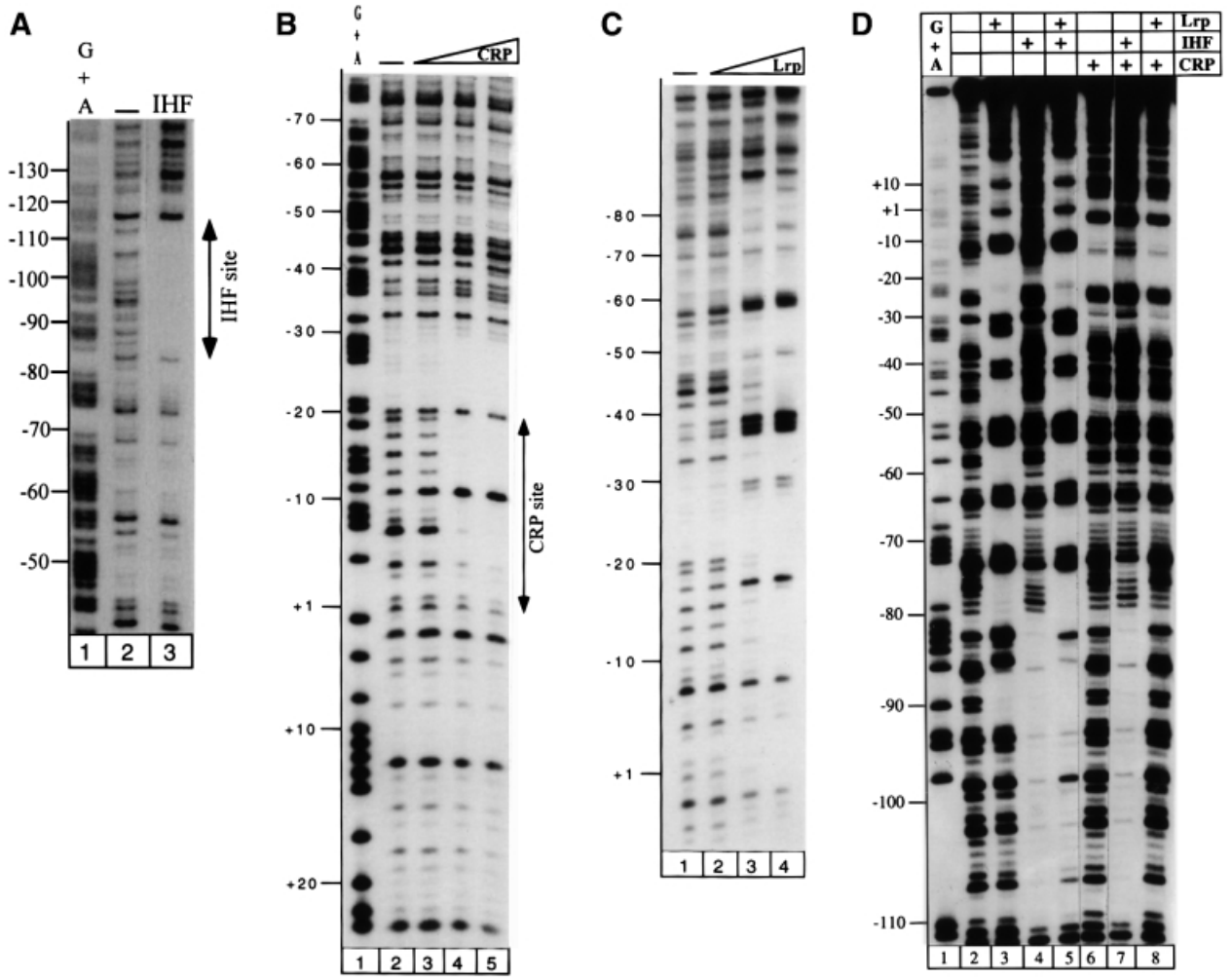

Fig. 3. DNase I footprint analysis of the complexes formed at the template strand of the *osmY* promoter with IHF (**A**), CRP (**B**), Lrp (**C**) or at the non-template strand with a mixture of repressors (**D**). The reaction mix was treated with DNase I as described in Materials and methods. Samples were analysed on a 7% denaturing polyacrylamide gel, which was calibrated using a sequencing reaction for G + A (Maxam and Gilbert, 1977). Repressor concentration: (A) IHF (100 nM, lane 3); (B) CRP (10, 50 and 150 nM, lanes 3–5); (C) Lrp (5, 25 and 125 nM, lanes 2–4); and (D) IHF (50 nM), CRP (50 nM) and Lrp (8 nM). Note that the DNA fragment has been overdigested by DNase I in (A) lane 2 with respect to lane 3. The reactive bands upstream of the IHF-binding site are located at the same positions in lanes 2 and 3 but are overexposed in lane 3.

shown in Figure 5 (lane 6). As expected, the addition of heparin totally dissociates CRP–*osmY* and IHF–*osmY* complexes whereas the Lrp–*osmY* complex appears less sensitive (Figure 5, lane 7). We then added $E\sigma^S$ or $E\sigma^{70}$ to these complexes for 20 min and followed the time course of formation of any heparin-resistant complex. A striking difference was observed: $E\sigma^S$ is able to form a heparin-resistant complex on a promoter template pre-bound to IHF or cAMP–CRP (compare lane 3 in Figure 5A, B and C). Only in the presence of Lrp was open complex formation slightly reduced (Figure 5C, lane 3). In the case of $E\sigma^{70}$ RNA polymerase, however, binding to the *osmY* DNA fragment is highly inhibited by the presence of each repressor (Figure 5, lane 5). A simple conclusion can be drawn from these results: whatever the nature of the repressor, the formation of a heparin-resistant complex is hardly affected with $E\sigma^S$ and strongly inhibited with $E\sigma^{70}$.

To confirm that $E\sigma^S$ forms an open complex even in the presence of repressor, we used potassium permanganate reactivity. $KMnO_4$ specifically reacts with thymine residues in single-stranded regions of DNA and has been used extensively to probe open complex formation (Sasse-Dwight and Gralla, 1989). When RNA polymerase forms an open complex at the *osmY* promoter, the melted region extends from –12 to –1 on the template strand for both holoenzymes (Figure 6, lanes 3 and 4). However, the reactivity pattern is different between both holoenzymes, with an enhanced reactivity of the thymines located in the downstream part of the transcription bubble observed with $E\sigma^S$. When CRP and IHF are added before RNA polymerase, open complex formation with $E\sigma^S$ remains unaffected whereas it is decreased with $E\sigma^{70}$ (Figure 6, lanes 5–12). However, previous incubation with Lrp at 10 nM diminishes permanganate reactivity with both holoenzymes but to a greater extent with $E\sigma^{70}$ (Figure 6, lanes 13 and 14). At a higher Lrp concentration (50 nM), open complex formation is inhibited completely with both holoenzymes (Figure 6, lanes 15 and 16). These observations are in agreement with the previous gel shift experiments and strongly suggest that in the presence of

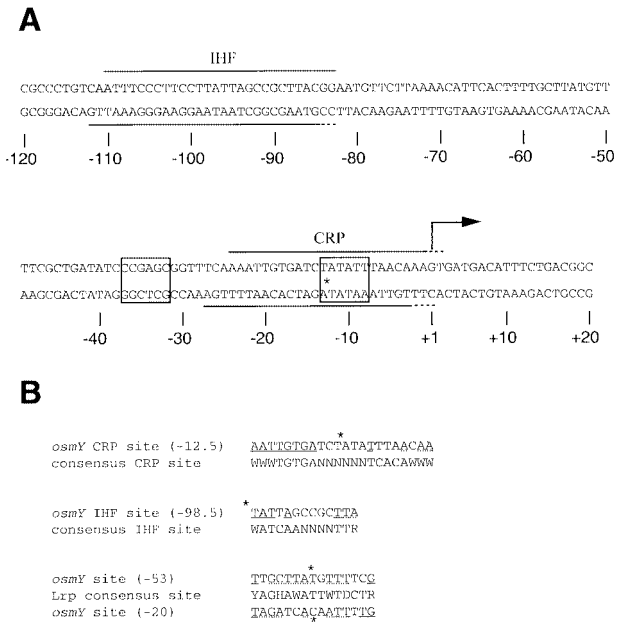

Fig. 4. (**A**) Summary of the protection patterns observed at the *osmY* promoter in the presence of IHF and CRP. The centre of the CRP-binding site is indicated by an asterisk, –10 and –35 regions are boxed. (**B**) Alignment of the *osmY*-binding sites with the respective consensus sites of CRP, IHF and Lrp where W = A/T, R = A/G, Y = C/T, H = 'not G', D = 'not C' (Craig and Nash, 1984; Kolb *et al*., 1993; Cui *et al*., 1996). The centres of the CRP- and Lrp-binding sites, indicated by asterisks, are numbered with respect to the transcription start site. For IHF, only the most conserved half of the binding site is indicated (Goodrich *et al*., 1990). Nucleotides matching the consensus sequences are underlined.

repressors, $E\sigma^S$ is more efficient at forming an open complex than $E\sigma^{70}$ at the *osmY* promoter.

The inhibitory effects of repressors on transcription activity are more drastic with $E\sigma^{70}$ than with $E\sigma^S$

In order to test whether the inhibitory effects observed on open complex formation translate into different transcriptional activities, single round transcription assays were used. When transcription was performed from the *osmY* promoter in front of two *rrnB* transcriptional terminators on a supercoiled plasmid, both RNA polymerases were able to generate similar amounts of transcripts (Figure 7A and B, lane 1), in agreement with the results obtained by Ding *et al*. (1995). Binding of IHF, CRP or Lrp to the supercoiled *osmY* plasmid, before RNA polymerase addition and subsequent incubation for 20 min at 37°C, reduces transcription activity with either $E\sigma^S$ or $E\sigma^{70}$ holoenzymes, indicating a direct inhibitory effect of each repressor on overall transcription activity (Figure 7A and B, compare lane 1 with lanes 2–4). Further, a significant differential effect is observed between the two RNA polymerases as $E\sigma^{70}$ is at least 2-fold more sensitive to repression than $E\sigma^S$ (Figure 7A and B, lanes 1–4).

An additional effect was observed when a linear plasmid cut with the unique restriction enzyme, *Afl*III, was used as a template. In the absence of repressors, $E\sigma^{70}$-mediated transcription from this template appears very weak as compared with $E\sigma^S$-mediated transcription (Figure 7A and B, lane 5). Thus, the state of DNA

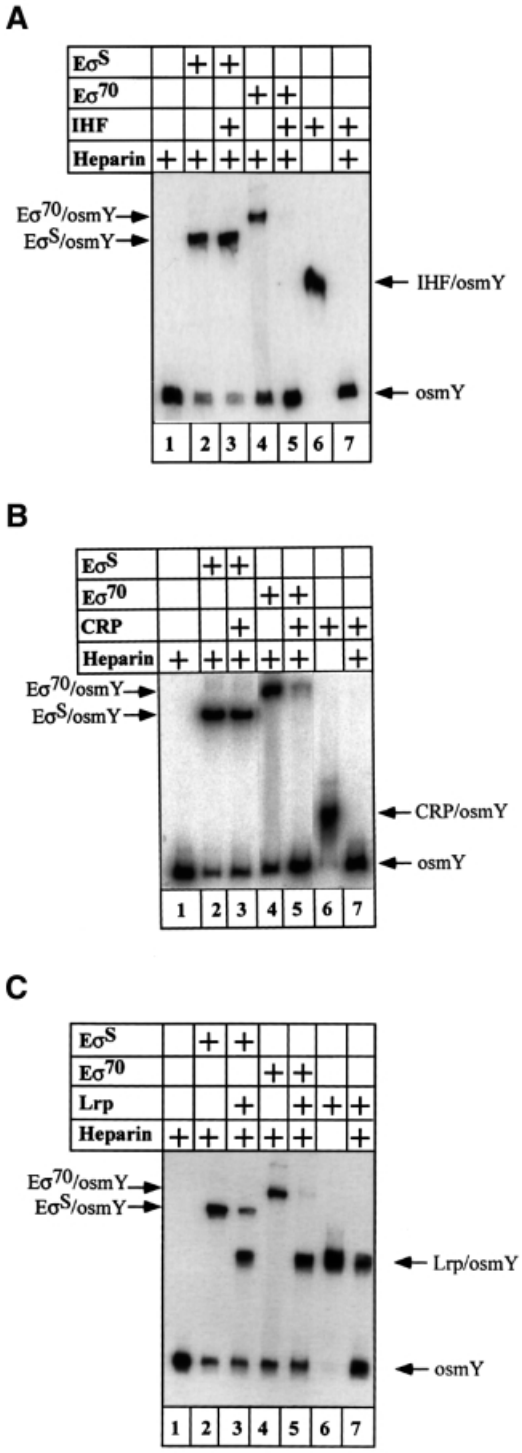

Fig. 5. Repressor effect on $E\sigma^S$ and $E\sigma^{70}$ DNA binding and open complex formation. The radioactively labelled *osmY* promoter (0.2 nM) was mixed for 20 min at room temperature with the following repressors: (**A**) IHF (100 nM), (**B**) CRP (30 nM) and (**C**) Lrp (1.5 nM). $E\sigma^S$ or $E\sigma^{70}$ holoenzymes (50 nM) were then added and incubated for 20 min at 37°C. The samples were heparin challenged before loading onto a 5% TBE polyacrylamide gel (except for lane 6). Lanes contain: DNA alone (1); DNA + $E\sigma^S$ (2); DNA + $E\sigma^{70}$ (4); DNA + repressor + $E\sigma^S$ (3); DNA + repressor + $E\sigma^{70}$ (5); DNA + repressor without heparin (6); or DNA + repressor with heparin (7).

supercoiling significantly affects the rate of *osmY* transcription by $E\sigma^{70}$. This result agrees with previous *in vitro* transcription data obtained by Kusano *et al*. (1996). Due to

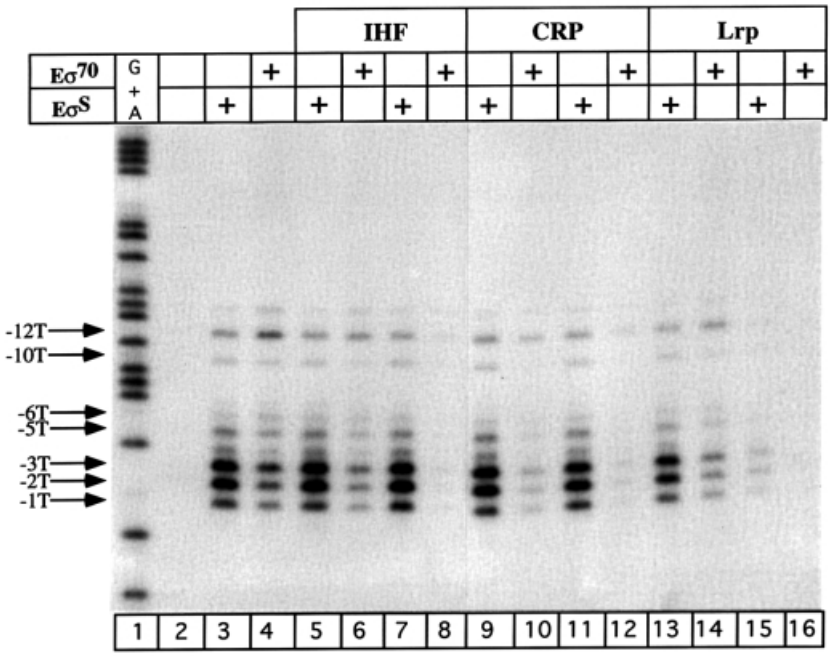

Fig. 6. $KMnO_4$ reactivity patterns of Eσ^S and Eσ^{70} at the template strand of the *osmY* promoter in the absence and presence of repressors. Lane 1 shows a sequencing reaction for G + A and lane 2 represents DNA without protein treated with $KMnO_4$. Except for these two lanes, odd and even numbers correspond to Eσ^S and Eσ^{70}, respectively. IHF (100 nM, lanes 5–6; 500 nM, lanes 7–8), CRP (5 nM, lanes 9–10; 25 nM, lanes 11–12) and Lrp (10 nM, lanes 13–14; 50 nM, lanes 15–16) were added to the *osmY* promoter before RNA polymerase. Reactive thymines, from –12 to –1, are indicated on the left of the gel.

this low actual transcriptional activity of Eσ^{70}, the inhibitory effects of CRP, Lrp and IHF when added before RNA polymerase to the linear template were less visible but seemed qualitatively similar to those observed on supercoiled DNA. Restricting the length of the template used and therefore the competing effect of other promoters makes the effects even clearer. When transcription was performed on a small *osmY* fragment carrying the transcriptional terminator generated by PCR, the inhibitory effects of the repressors appeared very different for the two RNA polymerases (Figure 7C): IHF, CRP and Lrp affected Eσ^{70} activity more dramatically than Eσ^S (Figure 7D).

Discussion

A paradox in the regulation of osmY and other σ^S-controlled genes

The *osmY* gene belongs to a class of genes that are dependent specifically on σ^S *in vivo*. However, like many other σ^S-dependent promoters, the *osmY* promoter can be transcribed efficiently by Eσ^S and Eσ^{70} holoenzymes under standard *in vitro* conditions. Thus, one puzzling question is to understand why the *osmY* promoter is not expressed during exponential phase by Eσ^{70}. Three DNA-binding proteins (IHF, CRP and Lrp) repress this promoter *in vivo* and *in vitro* (Figures 1 and 7; Lange *et al*., 1993). These proteins appear to affect *osmY* transcription by the Eσ^{70} RNA polymerase during exponential phase, since mutations that eliminate these proteins derepress *osmY* expression during this phase of the growth cycle (Lange *et al*., 1993). At the onset of the stationary phase, when σ^S is induced, the *osmY* promoter is activated even though the same set of repressors is still present (IHF and the cAMP–CRP complex even at increased concentrations; Azam *et al*., 1999).

In this study, we therefore asked whether repression exerted on Eσ^S is weaker than on Eσ^{70} and by what molecular mechanisms this different effect is maintained. First, the repressor-binding sites as well as the positioning of RNA polymerases at *osmY* had to be identified in order to understand the inhibitory effect. Since transcription initiation is a multistep process that includes RNA polymerase binding, open complex formation, initiation of RNA synthesis and promoter clearance, it was then essential to investigate whether the efficiency of any of these steps at the *osmY* promoter was (i) different for the two holoenzymes and (ii) differentially affected by IHF, cAMP–CRP or Lrp in the presence of the two holoenzymes.

Transcription initiation at the osmY promoter by Eσ^S and Eσ^{70} in the absence of the repressors

Our DNase footprint experiments show that Eσ^S and Eσ^{70} both recognized the *osmY* promoter with globally the same pattern. However, the stationary phase holoenzyme protects especially the downstream part of the *osmY* promoter (from –30) whereas the exponential phase holoenzyme protects the upstream region (from –35) more strongly (Figure 2). Our results are in good agreement with previous observations suggesting that the –35 region as well as the –10 region are recognized by Eσ^{70} whereas Eσ^S mainly recognizes the –10 region (Hiratsu *et al*., 1995; Kolb *et al*., 1995; Tanaka *et al*., 1995; Marschall *et al*., 1998; Colland *et al*., 1999).

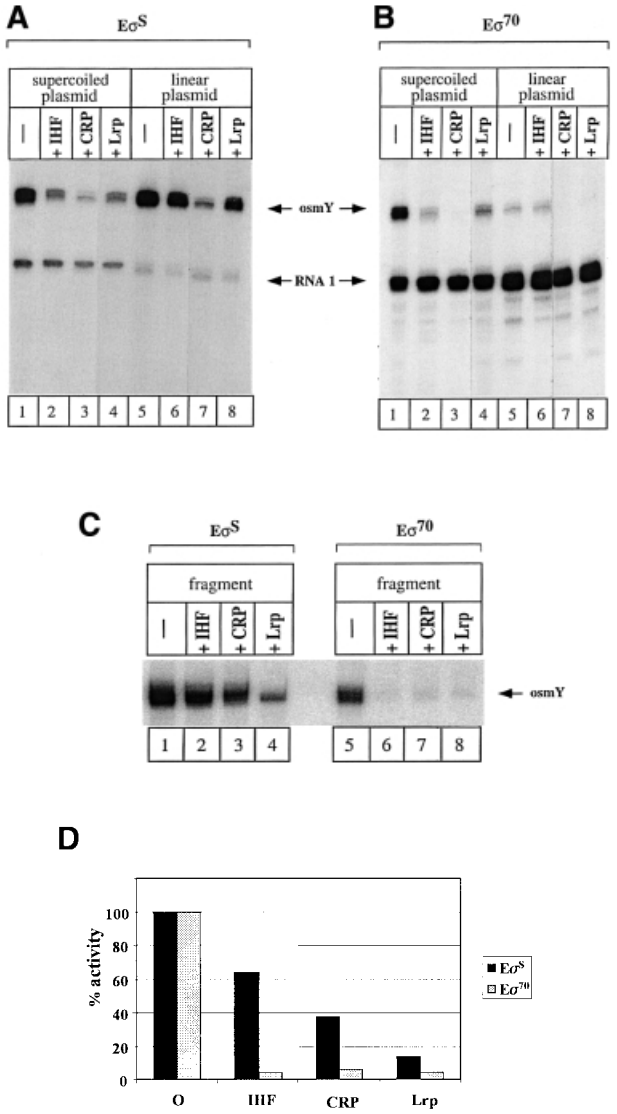

Fig. 7. Repressor effect on $E\sigma^S$- and $E\sigma^{70}$-dependent transcriptional activity. Single-round transcription experiments were performed using plasmid pJCDO2 (4 nM) (A and B) or a 343 bp *osmY* fragment (6 nM) (C) as template. (**A** and **B**) Supercoiled (lanes 1–4) or linearized (lanes 5–8) pJCD02 was incubated with holoenzyme (50 nM) containing either σ^S (A) or σ^{70} (B). (**C**) The 343 bp *osmY* fragment was incubated with holoenzyme (50 nM) containing either σ^S (lanes 1–4) or σ^{70} (lanes 5–8). In (A), (B) and (C), transcription was performed with holoenzyme alone (lanes 1 and 5) or with holoenzyme + IHF (lanes 2 and 6), cAMP–CRP (lanes 3 and 7) or Lrp (lanes 4 and 8). (**D**) Quantification of the relative intensities of the transcripts in (C) using a PhosphorImager.

Both holoenzymes are also able to form open complexes as demonstrated by heparin resistance (Figure 5) and permanganate footprinting (Figure 6). An enhanced permanganate reactivity of thymines in the downstream part was observed with $E\sigma^S$, suggesting a slightly different positioning of the transcription bubble, closer to the messenger start site in this case, as previously observed at the *bolA*P1 promoter (Nguyen and Burgess, 1997).

On supercoiled plasmid templates, $E\sigma^S$ and $E\sigma^{70}$ produce comparable amounts of *osmY* transcripts. This was observed both after a 20 min pre-incubation between promoter and RNA polymerase as in Figure 7A and B (lane 1) and after shorter incubation times, i.e. transcription initiation is fast and occurs at similar rates for both holenzymes on supercoiled templates (data not shown). In contrast, on the linearized plasmid, $E\sigma^{70}$ produced far fewer transcripts than $E\sigma^S$, even after longer incubation periods, consistent with results previously reported (Kusano *et al.*, 1996). This reduction in transcript synthesis is specific for $E\sigma^{70}$ transcription on linear *osmY* template and could also be observed, although less dramatically, on a smaller *osmY* fragment (Figure 7C, lanes 1 and 5). Again this difference in efficiency between $E\sigma^S$ and $E\sigma^{70}$ persisted even after very long times of incubation of the *osmY* fragment with RNA polymerase. Since after 20 min incubation no significant difference between the two holoenzymes was detected in the formation of the heparin-resistant complexes, reactive to permanganate (Figures 5 and 6), our data suggest that the open complexes formed by $E\sigma^S$ and $E\sigma^{70}$ have different abilities to initiate transcription. This hypothesis is supported by the reduced permanganate reactivity at positions –1, –2 and –3 of the $E\sigma^{70}$ complex, which could possibly mean that the +1 start is not as accessible to the incoming initiating ribonucleoside triphosphate as it is in the $E\sigma^S$ complex. The different transcriptional activities of $E\sigma^S$ and $E\sigma^{70}$ on linear templates might reflect a difference in their rates of promoter clearance on DNA that is not negatively supercoiled. This is certainly an attractive possibility that deserves further study.

The modulation of the $E\sigma^{70}$ activity by supercoiling may have a functional significance. While the entire chromosomal DNA is never completely relaxed, it is not excluded that, locally, certain promoter regions might become relatively relaxed by the action of topology-affecting 'histone-like' proteins such as H-NS or HU, and may therefore be transcribed less well by $E\sigma^{70}$ than by $E\sigma^S$. This also implies that changes in supercoiling on the one hand and the presence of DNA-binding proteins on the other, both of which may differentially affect transcription by $E\sigma^S$ and $E\sigma^{70}$, should not be seen as completely independent parameters.

Positioning of the DNA-binding proteins cAMP–CRP, IHF and Lrp is consistent with their inhibitory role in the control of osmY

By assaying open complex formation and transcription *in vitro*, we demonstrated a direct and independent role of each DNA-binding protein (CRP, IHF and Lrp) in repressing *osmY* expression (Figure 7). Besides being specific gene regulators, all three proteins bend DNA by >80° and appear as global organizers of the nucleoid structure (Schultz *et al.*, 1991; Wang and Calvo, 1993; Rice *et al.*, 1996).

At the *osmY* promoter, the CRP-binding site is centred at position –12.5, overlapping the –10 region of the promoter. Thus, CRP-induced repression can be understood easily as a competition between CRP and RNA polymerase for DNA occupancy. Contrary to the proximal positioning of CRP, the IHF site is located in the far upstream region of the *osmY* promoter (Figure 4). The inhibitory effect of IHF at other promoters has been shown previously when it is bound at similar positions (Huang *et al.*, 1990; Pratt *et al.*, 1997). The IHF- and CRP-binding sites are separated by 86 bp, i.e. within a distance of eight B-DNA turns assuming a DNA helix repeat of 10.8 bp per

turn, close to the *in vivo* situation. This positioning places the IHF- and CRP-induced bends in-phase and participates in the formation of a higher order curved DNA structure in conjunction with the intrinsic curvature of the *osmY* promoter sequence (Lange *et al*., 1993).

The Lrp protein binds the *osmY* promoter co-operatively and with high affinity to several sites, irrespective of the presence of leucine, as was observed previously for other promoter regions of Lrp-controlled genes (Wang and Calvo, 1993; Marschall *et al*., 1998; Zhi *et al*., 1999). Since a phasing of protected regions, extending over a region of >100 bp, was observed every 10–11 bp at *osmY* and at other promoters, it is likely that Lrp causes DNA to be wrapped around a core of Lrp molecules.

In summary, positioning of the binding sites for cAMP–CRP, IHF and Lrp is consistent with their repressing role in *osmY* control as well as with the formation of a complex nucleoprotein structure at this promoter (Lanzer and Bujard, 1988; Rojo, 1999).

The repressors differentially affect open complex formation by $E\sigma^S$ and $E\sigma^{70}$

Both $E\sigma^S$ and $E\sigma^{70}$ RNA polymerases initiate transcription from the *osmY* promoter *in vitro* (Figure 7; Ding *et al*., 1995), but a closer analysis demonstrates that the details of promoter binding and open complex formation are slightly different. IHF, CRP and Lrp not only inhibit *osmY* promoter activity, but our data indicate that these repressors differentially affect $E\sigma^S$ and $E\sigma^{70}$ holoenzymes. The formation of heparin-resistant complexes, the melting of the DNA strands and the transcriptional activity (especially on supercoiled templates) are all less affected by the repressors when $E\sigma^S$ is used rather than $E\sigma^{70}$ (Figures 5, 6 and 7). The same qualitative result was obtained irrespective of whether the repressors were added first or a mixture of the repressor and RNA polymerase was added to DNA (data not shown).

A number of mutually not exclusive mechanisms might be involved in this differential inhibitory effect of the repressors. (i) In contrast to $E\sigma^{70}$, which requires at least two anchoring regions (the –10 and –35 hexamers) for specific DNA–protein interactions (for a review see Helmann and deHaseth, 1999), the $E\sigma^S$ RNA polymerase appears to recognize a smaller part of the promoter (such as an extended –10 region) (Colland *et al*., 1999). The mechanical constraints required for the proper phasing of the two recognition domains (Buckle *et al*., 1999) are thus more stringent for $E\sigma^{70}$ and therefore more sensitive to repressor action or to the formation of complex nucleoprotein structures in this region. (ii) Using dimethylsulfate (DMS) protection experiments, a striking difference is observed between both holoenzymes on the *osmY* template strand. G-14 is weakly protected in the major groove from DMS attack by $E\sigma^S$, but not by $E\sigma^{70}$, which generates a strong hyper-reactivity of this base (F.Colland and A.Kolb, unpublished results). Taking into account the known location of CRP on one side of the DNA in close contact with the minor groove at this position (Schultz *et al*., 1991; Kolb *et al*., 1993), it is possible that the first contacts between $E\sigma^S$ and the *osmY* promoter occur on the DNA face opposite to CRP. This might ultimately lead to the removal of CRP from DNA by a mechanism similar to the displacement of nucleosomes by transcription factors (Kingston, 1997). This differential positioning of the two holoenzymes just upstream of the –10 region could explain why CRP represses $E\sigma^{70}$ more efficiently at *osmY*. (iii) Finally, specific protein–protein interactions between $E\sigma^{70}$ and the repressors might also take place and render $E\sigma^{70}$ inefficient at initiating transcription at the *osmY* promoter.

It should be noted that a differential effect on the activity of the two holoenzymes is not necessarily restricted to repressor action, nor has the favoured holoenzyme always to be $E\sigma^S$. At the *csiD* promoter, cAMP–CRP activates in concert with $E\sigma^S$, but not with $E\sigma^{70}$ (although it somewhat improves $E\sigma^{70}$ binding, it fails to stimulate open complex formation by $E\sigma^{70}$; Marschall *et al*., 1998). At the *aidB* and *osmC*p1 promoters, Lrp specifically interferes with activation by $E\sigma^S$ (Landini *et al*., 1996; Bouvier *et al*., 1998). Similarly, at the *alkA* promoter, the Ada protein specifically shuts off $E\sigma^S$-dependent transcription (Landini *et al*., 1999).

Conclusion: generation of $E\sigma^S$ selectivity at the osmY promoter

Intracellular salt concentration (Ding *et al*., 1995), negative global or local DNA supercoiling (Kusano *et al*., 1996; this study) or an anti-σ factor, such as Rsd, which may reduce the cellular $E\sigma^{70}$ concentration (Jishage and Ishihama, 1998, 1999), have been proposed to be involved in the ability of a promoter to discriminate between $E\sigma^S$ and $E\sigma^{70}$, i.e. two RNA polymerase holoenzymes that exhibit very similar if not the same basic promoter sequence recognition. In addition, a possible involvement of DNA-binding proteins such as H-NS, Lrp and CRP has been suggested (Olsen *et al*., 1993; Arnqvist *et al*., 1994; Barth *et al*., 1995; Yamashino *et al*., 1995; Bouvier *et al*., 1998; Marschall *et al*., 1998; Hengge-Aronis, 1999). In this study, we demonstrate that the three global regulators IHF, CRP and Lrp play a major role in promoting or even generating σ^S selectivity at the *osmY* promoter by interfering more strongly with transcription initiation by $E\sigma^{70}$ than that by $E\sigma^S$. It is interesting to note that the expression of many σ^S-dependent genes is affected by various combinations of these and a few more regulatory proteins, most of which are histone-like proteins (Hengge-Aronis, 1996). Further studies will be needed to determine whether the modulation of σ factor specificity by these abundant nucleoid-associated proteins is a general rule at σ^S-dependent promoters.

Materials and methods

Bacterial strains and plasmids

All strains/alleles used for the experiment shown in Figure 1 were described in Lange *et al*. (1993) and were constructed by P1 transduction. Specific mutant alleles used were: Δ(*nlpD-rpoS*)360, *lrp-201*::Tn*10*, Δ*hip-3*::*cat* and Δ*crp96* (linked to *zhd-732*::Tn*10*). The *osmY* PCR fragment from –175 to +48 has been cloned in the *Hin*cII site of pJCD0 to generate pJCD02 (Marschall *et al*., 1998). The 343 bp *osmY* fragment used for transcription was synthesized by PCR with *Pwo* polymerase using pJCD02 as template, a specific *osmY* primer 5′-TTCAGT-TCCACCAGACCC-3′ (from –175 to –158) and a plasmid primer 5′-GGATTTGTCCTACTCAGGAG-3′.

Protein purification and holoenzyme reconstitution

IHF was a gift of F.Boccard. Lrp proteins were received from J.Calvo and J.Rouvière-Yaniv. The *E.coli* CRP was prepared as described in Ghosaini

et al. (1988). The σ^{70} and σ^{S} factors were purified from the overproducing strains M5219/pMRG8 and BL21(DE3)/pLysS/pETF, respectively, according to the described purification procedures (Gribskov and Burgess, 1983; Tanaka *et al*., 1995). Log-phase core enzyme was prepared according to Lederer *et al*. (1991). Reconstitution of active holoenzymes was achieved by incubating 1 vol. of 5 µM core enzyme with 2 vols of each σ factor at 10 µM for 20 min at 37°C (σ:core = 4). The reconstituted holoenzyme was then diluted at room temperature in buffer A [40 mM HEPES pH 8.0, 10 mM magnesium chloride, 100 mM potassium glutamate, 4 mM dithiothreitol (DTT) and 500 µg/ml bovine serum albumin (BSA)].

$KMnO_4$ and DNase I footprinting

The labelled *osmY* fragment was generated by PCR with the primers 5′-TTCAGTTCCACCAGACCC-3′ and 5′-GATATCTACGCATTGA-ACG-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). This fragment was purified on a glass fibre column (High pure PCR product purification kit, used according to the recommendations of the manufacturer, Boerhinger Mannheim). Complexes with the labelled promoter region (at 2 nM final concentration) were formed for 20 min at 37°C in 15 µl of buffer A (for $KMnO_4$, DTT was omitted) using each repressor and/or reconstituted RNA polymerase (200 nM final concentration). In one set of experiments, 2.5 µl of DNase I solution (1 µg/ml in 10 mM Tris–HCl, 10 mM magnesium chloride, 10 mM calcium chloride, 125 mM potassium chloride) were added and incubated at 37°C for 20 s, or for 30 s when RNA polymerase was present in the mixture. The reaction was stopped by the addition of 200 µl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA, 50 µg/ml calf thymus DNA, and put on ice. In the other set, 1.5 µl of $KMnO_4$ solution (50 mM) was added to the complexes for 30 s at 37°C. The reaction was stopped by adding 2.5 µl of 2-mercaptoethanol (2 M). Then, all the samples were phenol extracted and precipitated with ethanol. With the $KMnO_4$ samples, the ethanol precipitates were resuspended in 100 µl of piperidine (1 M), heated at 90°C for 30 min and evaporated until dryness. Then, 20 µl of water were added and evaporated (twice). $KMnO_4$ and DNase I samples were resuspended in 5 µl of 20 mM EDTA in formamide containing xylene cyanol and bromophenol blue and loaded on a 7% denaturing polyacrylamide gel.

Gel retardation assays

Repressors [CRP (30 nM), IHF (100 nM) and Lrp (1.5 nM)] were complexed to the radioactively labelled *osmY* promoter (0.2 nM) for 20 min at room temperature in buffer A. Eσ^{70} or Eσ^{S} reconstituted holoenzymes (50 nM) were then added and incubated for 20 min at 37°C in a final volume of 10 µl. After addition of heparin (55 µg/ml), the mixture was loaded onto a 5% native polyacrylamide gel. The gel was fixed, dried before being autoradiographed and quantified using a PhosphorImager (Molecular Dynamics).

Run-off transcription assays

Single-round transcription by reconstituted RNA polymerase was carried out under the standard conditions described previously, using supercoiled plasmid (pJCD02 prepared from an overnight culture of a *recA1* strain), linear plasmid (pJCD02 digested with *Afl*III) or a 343 bp *osmY* fragment generated by PCR using *Pwo* polymerase. DNA plasmid (8 nM), previously incubated with IHF (250 nM), cAMP–CRP (100 nM), Lrp (200 nM) or buffer at room temperature for 20 min, was next incubated with each reconstituted holoenzyme (50 nM) in buffer A at 37°C for 20 min in 10 µl final volume. Alternatively, the 343 bp *osmY* fragment (12 nM), previously incubated with IHF (250 nM), cAMP–CRP (50 nM), Lrp (50 nM) or buffer at room temperature for 20 min was used as template for transcription under the same conditions. Elongation was started by the addition of 5 µl of a pre-warmed mixture containing 600 µM ATP, GTP and CTP, 30 µM UTP, 0.5 µCi of [α-^{32}P]UTP and 600 µg/ml 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).

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