Region 2.5 of the *Escherichia coli* RNA polymerase σ^{70} subunit is responsible for the recognition of the 'extended -10' motif at promoters

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At some bacterial promoters, a 5'-TG-3' sequence element, located one base upstream of the -10 hexamer element, provides an essential motif necessary for transcription initiation. We have identified a mutant of the *Escherichia coli* RNA polymerase σ^{70} subunit that has an altered preference for base sequences in this 'extended -10' region. We show that this mutant σ^{70} subunit substantially increases transcription from promoters bearing 5'-TC-3' or 5'-TT-3' instead of a 5'-TG-3' motif, located one base upstream of the -10 hexamer. The mutant results from a single base pair substitution in the *rpoD* gene that causes a Glu to Gly change at position 458 of σ^{70} . This substitution identifies a functional region in σ^{70} that is immediately adjacent to the well-characterized region 2.4 (positions 434–453, previously shown to contact the -10 hexamer). From these results, we conclude that this region (which we name region 2.5) is involved in contacting the 5'-TG-3' motif found at some bacterial promoters: thus, extended -10 regions are recognized by an extended region 2 of the RNA polymerase σ^{70} subunit.

Keywords: promoter recognition/RNA polymerase/sigma subunits/transcription initiation

Introduction

Transcription initiation is a complex process which involves rallying many diverse molecular interactions to facilitate promoter recognition by RNA polymerase (RNAP) and DNA unwinding around the transcription start-point. In bacteria, one RNAP with subunit composition $\alpha_2\beta\beta'$ (E) is responsible for all transcription. However, although this core enzyme is capable of transcript elongation, it cannot initiate transcription without enlisting a specific transcription factor, σ . It is σ that directs RNAP to specific promoters, and σ is also involved in promoter melting. *Escherichia coli* contains many classes of promoter, all of which are recognized by separate σ factors. The major σ factor, σ^{70} , makes sequence-specific contacts with two hexamer elements at promoters, the -10 and -35 sequences, that are separated by an optimal spacing of 17 bp (Hawley and McClure, 1983; Harley and Reynolds, 1987): the consensus sequences for -10 and -35 hexamers are 5'-TATAAT-3' and 5'-TTGACA-3', respectively (Figure 1).

Sequence analysis of bacterial σ factors has revealed four conserved important regions, regions 1, 2, 3 and 4 (reviewed by Helmann and Chamberlin, 1988). The analysis argues that region 2 is subdivided into four segments (2.1–2.4) and region 4 is divided into two (4.1 and 4.2). A series of studies, based mainly on suppression genetics, has shown that sub-region 2.4 (located at the C-terminal end of region 2) interacts directly with promoter -10 hexamer elements, whilst sub-region 4.2 (located at the C-terminal end of region 4) interacts directly with promoter -35 hexamer elements. For example, Waldburger *et al.* (1990), found that a Gln to His change at position 437 of σ^{70} , within region 2.4, suppressed the effects of a T→A to C→G 'down' mutation at position -12 in the -10 hexamer of the *ant* and *lac* promoters. Similarly, changing Thr to Ile at position 440 increases the activity of promoters containing a G or C instead of T at this position (Siegele *et al.*, 1989; see also Zuber *et al.*, 1989; Daniels *et al.*, 1990; Tatti *et al.*, 1991). Recently, the crystal structure of a σ^{70} fragment that includes region 2.4 was solved (Malhotra *et al.*, 1996), showing that region 2.4 contains an amphipathic α -helix, with residues 437 and 440 positioned such that they can both contact bases at the -12 position.

A number of activator-independent promoters have now been reported where specific -35 hexamer contacts are not required for transcription initiation (reviewed by Bown *et al.*, 1997). Transcription initiation at these promoters is dependent on a supplementary sequence element, 5'-TG-3', located one base upstream of the -10 hexamer. This results in an 'extended' -10 element, 5'-TGnTATAAT-3', which appears to create alternative contact points for RNAP, most likely via the σ subunit, such that transcription can be initiated in the absence of specific -35 region contacts (Ponnambalam *et al.*, 1986; Keilty and Rosenberg, 1987; Burns *et al.*, 1996). In support of this, Kumar *et al.* (1993) showed that RNAP containing an altered form of σ lacking the C-terminal 84 amino acids (including region 4.2, which is responsible for contacting the -35 hexamer) could initiate transcription at a consensus extended -10 promoter (but was unable to initiate transcription at a typical promoter with -10 and -35 regions resembling the consensus but without the 5'-TG-3' extension). In this work we describe an experiment designed to identify the segment of the σ^{70} subunit involved in making contact with the 5'-TG-3' extension. Our strategy was to start with a promoter where activity was completely dependent on a 5'-TG-3' extension, to inactivate the promoter by making base changes in the extension, and then to search for mutant σ^{70} derivatives that allowed transcription initiation at the inactivated promoters. This experiment identifies a region of σ^{70} immediately adjacent to region 2.4 which is involved in the recognition of the 5'-TG-3' motif. We report both *in vivo* and *in vitro* data to support our conclusions.

	-50	-30	-10	+1
KAB-TG	CGTCTTCAAGAATTCTAGACAGCTGCATGCATCTTTGTTATGGTTATTTTCATACCATAAGC			
KAB-TC	CGTCTTCAAGAATTCTAGACAGCTGCATGCATCTTTCTTATGGTTATTTTCATACCATAAGC			
KAB-TT	CGTCTTCAAGAATTCTAGACAGCTGCATGCATCTTTTATGGTTATTTTCATACCATAAGC			
Consensus		TTGACA	TATAAT	

Fig. 1. Base sequence of the KAB-TG, KAB-TC and KAB-TT promoters used in this work. The -10 and -35 hexamers are highlighted in bold: the 5'-TG-3' extension at position -14/-15 is underlined.

Results

Isolation and characterization of σ^{70} mutants

The *E. coli* *galP1* promoter is an example of an extended -10 promoter, having a -35 region with little homology to the consensus (Ponnambalam *et al.*, 1986) and mutations in the 5'-TG-3' motif render *galP1* inactive (Chan *et al.*, 1990). Both methylation protection and interference studies suggest that RNAP makes direct contact with the G-C base pair at position -14 of *galP1* (within the 5'-TG-3' motif) (Minchin and Busby, 1993). The starting point of this work was the KAB set of three semi-synthetic promoters, based on the *galP1* sequence (Figure 1). Activity of the KAB-TG promoter is dependent on a 5'-TG-3' motif (at positions -14 and -15) upstream of the -10 hexamer, 5'-TATGGT-3'. In the KAB-TC and KAB-TT promoters, the 5'-TG-3' motif is changed to 5'-TC-3' and 5'-TT-3' respectively, and this greatly reduces promoter activity.

Our aim was to identify σ^{70} mutants that would permit RNAP to serve the KAB-TC and KAB-TT promoters. To do this, the *rpoD* gene (cloned in plasmid pKB σ^{70}) was amplified by error-prone PCR, using conditions such that, on average, each amplified DNA fragment encoding *rpoD* contained one base pair substitution. These fragments were then cloned into pKB σ^{70} , generating a library of σ^{70} mutants, which was then screened for σ^{70} derivatives that permitted increased transcription initiation at the KAB-TC and KAB-TT promoters. To facilitate this screen, the three promoters KAB-TG, KAB-TC and KAB-TT were each fused to *lacZ*. As expected, cells carrying the KAB-TG::*lacZ* fusion exhibited a Lac⁺ phenotype (red colonies on MacConkey indicator plates) whilst cells carrying the KAB-TC::*lacZ* or KAB-TT::*lacZ* fusions exhibited a Lac⁻ phenotype (white colonies on MacConkey indicator plates). Candidates from the σ^{70} mutant library were screened for the ability to confer a red/pink Lac⁺ phenotype on cells carrying the KAB-TC::*lacZ* or KAB-TT::*lacZ* fusions. Thirty thousand colonies from 10 independent PCR libraries were screened and two mutant σ candidates (from different libraries) were isolated. We confirmed that both of these candidates made stable functional sigma by checking their ability to complement the *rpoD* temperature-sensitive mutation in the *E. coli* strain 285c.*recA*, during growth at 42°C. The complete nucleotide sequence of *rpoD* for both candidates was determined. Both contained the same single base change, an A-T to G-C transition at position 1373 of the *rpoD* sequence, which alters a GAG codon to GGG, and results in the substitution of a glutamic acid for glycine at position 458 of σ^{70} .

Specificity of E σ^{70} -EG458

The activity of RNAP holoenzyme carrying σ with the EG458 substitution (E σ^{70} -EG458) at the three promoters

Fig. 2. The activity of the KAB-TG, KAB-TC and KAB-TT promoters with σ^{70} (shown in black) and σ^{70} -EG458 (shown in grey) *in trans*. *EcoRI-HindIII* fragments carrying the promoters were cloned into the *lacZ* expression vector, pRW50 and β -galactosidase levels are taken as a direct measure of the promoter activity. In the histogram, activity levels are expressed as a percentage of the wild type activity at each promoter. The activity (nmole ONPG hydrolysed per min per mg dry weight bacteria) of E σ^{70} at KAB-TG, KAB-TC and KAB-TT was 2570, 51 and 49 respectively.

KAB-TG, KAB-TC and KAB-TT was assessed, exploiting the *lac* fusions described above. Measurements were made in the strain CAG20177 which carries host σ^{70} under the control of the repressible *trp* promoter. Plasmid pKB σ^{70} encoding either wild type or σ^{70} -EG458 was introduced and β -galactosidase assays were performed on cells grown in conditions in which the host-encoded σ^{70} was repressed. Thus, the level of transcription from each promoter as a result of specific initiation by either E σ^{70} or E σ^{70} -EG458 could be determined. The results, presented in Figure 2, show that the EG458 substitution significantly affects the activity of all three promoters. The activity of promoter KAB-TG is reduced by 15% with E σ^{70} -EG458. In contrast, the activity of the mutant promoter derivatives, KAB-TC and KAB-TT is increased by 2.5- to 3-fold with E σ^{70} -EG458 (compared with E σ^{70}). As a control, we made two further derivatives of promoter KAB-TG. In one, the activity of the promoter was reduced >50-fold by the introduction of a single change in the -10 hexamer (from 5'-TATGGT-3' to 5'-CATGGT-3') and, in the other, a similar effect was produced by altering the -35 hexamer element (from 5'-TAGACA-3' to 5'-TAGGTA-3'). The effects of these promoter mutations could not be sup-

Fig. 3. An autoradiograph of gel mobility shift assays to separate promoter fragments bound to RNA polymerase from free fragments. RNA polymerase was pre-incubated with labelled fragment carrying KAB-TG (lanes 1–4), KAB-TC (lanes 5–8) and KAB-TT (lanes 9–12). Experiments were performed with 100 nM core enzyme (lanes 1, 5 and 9), 100 nM Eσ⁷⁰ (lanes 2, 6 and 10), 100 nM Eσ⁷⁰-EG458 (lanes 3, 7 and 11) and labelled fragment alone (lanes 4, 8 and 12). The samples were heparin challenged before loading on the gel. The faint retarded bands seen with core enzyme are likely to be due to a trace contamination of σ⁷⁰.

pressed by the introduction of plasmid pKBσ⁷⁰ encoding σ⁷⁰-EG458. This shows that the effect of the EG458 substitution is position specific.

Improved interaction of Eσ⁷⁰-EG458 with the KAB-TC and KAB-TT promoters

The binding of Eσ⁷⁰ and Eσ⁷⁰-EG458 to promoters can be assessed simply using gel mobility shift assays. Labelled DNA fragments carrying the promoters, KAB-TG, KAB-TC or KAB-TT were incubated with either purified core enzyme (E), purified Eσ⁷⁰ or purified Eσ⁷⁰-EG458 at 37°C for 30 min to allow open complex formation. The resulting complexes were challenged with heparin (to test the stability of the RNAP–DNA complexes) before separation on a native polyacrylamide gel. The assays (Figure 3) clearly show that core enzyme (E) is unable to form stable complexes with any promoter DNA (lanes 1, 5 and 9) and that the binding of holo-RNAP (Eσ⁷⁰) to KAB-TG is greater than to KAB-TC or KAB-TT (lanes 2, 6 and 10). Comparing the binding of Eσ⁷⁰ and Eσ⁷⁰-EG458, at both KAB-TC and KAB-TT in this assay, Eσ⁷⁰ binds relatively poorly, whereas Eσ⁷⁰-EG458 has a greater affinity (compare lanes 6 and 10 with lanes 7 and 11). The assay shows that Eσ⁷⁰-EG458 can still bind to the KAB-TG promoter (compare lanes 2 and 3), suggesting that EG458 must be a relaxed specificity mutant of σ⁷⁰.

Characterization of open complexes formed by Eσ⁷⁰ and Eσ⁷⁰-EG458

Since DNA unwinding is an important step in transcription initiation, we studied the interactions of Eσ⁷⁰ and Eσ⁷⁰-EG458 in the open complexes by probing with potassium permanganate (as described by Chan *et al.*, 1990). Open complexes were formed by preincubating DNA fragments carrying the KAB-TG, KAB-TC and KAB-TT promoters

Fig. 4. An autoradiograph of a gel to show the pattern of cleavage resulting from potassium permanganate and piperidine treatment of *Pst*I–*Hind*III fragments carrying KAB-TG (lanes 1, 4, 7 and 10), KAB-TC (lanes 2, 5, 8 and 11) and KAB-TT (lanes 3, 6, 9 and 12). Experiments were performed with 100 nM core enzyme (lanes 1–3), 100 nM Eσ⁷⁰ (lanes 4–6), 100 nM Eσ⁷⁰-EG458 (lanes 7–9) and labelled fragment alone (lanes 10–12). Lane M is a calibration marker made by the Maxam–Gilbert G-specific sequence reaction on one of the promoter constructions. The gels are calibrated with the transcription start point as +1.

with E, Eσ⁷⁰ and Eσ⁷⁰-EG458. Single-stranded T residues in the open complexes could be modified by KMnO₄ and the points of modification were detected by cleavage with piperidine and analysis on a denaturing polyacrylamide gel. The results (Figure 4) show that all open complexes form at the same position on the DNA, confirming that RNAP is binding to the promoters as expected. Moreover, the pattern and location of unwinding in the open complexes formed by both Eσ⁷⁰ and Eσ⁷⁰-EG458 are the same at the KAB-TG, KAB-TC and KAB-TT promoters, with DNA opening confined to positions –11 to +3. The degree of promoter opening by the two forms of RNAP holoenzyme, as detected by KMnO₄, corroborates the β-galactosidase and gel mobility shift assay data. Wild type holoenzyme, Eσ⁷⁰, readily forms open complexes at KAB-TG, a very small amount of opening is observed at KAB-TC, and no opening is found at KAB-TT (compare lanes 4, 5 and 6). In contrast, with Eσ⁷⁰-EG458, open complexes form at all three promoters, confirming that the EG458 substitution confers a relaxed specificity on σ⁷⁰ and allows RNAP to initiate transcription at promoters carrying ‘down’ mutations in the 5′-TG-3′ motif of extended –10 promoters.

The region between conserved regions 2.4 and 3 in σ⁷⁰: effects of the HA455 substitution

Figure 5 shows an alignment of amino acid sequences around E458 in σ⁷⁰ with the corresponding sequence in nine bacterial σ factors. E458 is located in the spacer between conserved regions 2.4 and 3. However, it is clear from the figure that a number of amino acids in this region (including E458) are well conserved between different σ factors. For example, the histidine residue at position 455 is particularly conserved. We investigated the effect of changing this residue in σ⁷⁰ to alanine on the activity of the three KAB promoters, together with the effects of alanine substitutions at the neighbouring less-conserved

Fig. 5. A PILEUP [Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin] of amino acid sequences of bacterial σ factors between region 2.4 and the start of region 3. The location of E458 and H455 is indicated by open boxes. The position of region 2.4 and the start of region 3 is indicated by shaded boxes. The lineup includes: *E. coli* σ^{70} (RP70_ECOLI), *Salmonella typhimurium* σ^{70} (RP70_SALTY), *Pseudomonas aeruginosa* σ^{70} (RP70_PSEAE), *Myxococcus xanthus* σ^{80} (RP80_MYXXA), *Bacillus subtilis* σ^A (RPSA_BACSU), *Chlamydia trachomatis* σ^{70} (RP70_CHLTR), *Streptomyces coelicolor* σ^{HrdB} (HRDB_STRCO), *Anabaena* sp. σ^A (RPSA_ANASP) and *Streptomyces aureofaciens* σ^A (RPSA_STRAU).

isoleucine 457 and threonine 459. The mutants σ^{70} -HA455, σ^{70} -IA457 and σ^{70} -TA459 were constructed in plasmid pKB σ^{70} and their ability to drive transcription initiation at the KAB-TG, KAB-TC and KAB-TT promoters was assayed in strain CAG20177 as described above. The results (Figure 6) show that alanine substitutions at either isoleucine 457 or threonine 459 have little or no effect on the ability of RNA polymerase to initiate transcription. In contrast, substitution of histidine 455 results in a 50% reduction in expression from the KAB-TG promoter and smaller effects at the KAB-TC and KAB-TT promoters. We conclude that histidine 455 of σ^{70} is also involved in interactions with the 5'-TG-3' motif at extended -10 promoters.

Discussion

It is well documented that the σ subunits of bacterial RNA polymerases are primarily responsible for making sequence-specific contacts with promoter DNA. In previous studies, particular amino acids in regions 2.4 and 4.2 were shown to be responsible for contacting the -10 and -35 hexamers respectively. At extended -10 promoters, contacts between region 4.2 of σ^{70} and the -35 region appear to be unimportant for promoter activity, and these promoters function because σ makes supplementary contacts with a 5'-TG-3' motif just upstream from the -10 hexamer. To identify the part of σ^{70} that interacts with the 5'-TG-3' motif of extended -10 promoters, we developed a genetic screening system for selecting and analysing a mutant form of σ^{70} that suppressed 'down' mutations in the G-C base pair at position -14 of the extended -10 region of the KAB-TG promoter. The entire *rpoD* gene which encodes a product of 613 amino acids was randomly mutated, and yet the screening system twice generated the same σ^{70} mutant carrying a single amino acid substitution at position 458. This substitution partially restores the activity of the KAB-TC and KAB-TT promoters, containing a C-G or T-A base pair at position -14 respectively. The activities of these promoters is increased 2.5- to 3-fold in the presence of σ^{70} -EG458 compared with σ^{70} , whilst, at the starting KAB-TG promoter, activity is reduced to ~85% of that with σ^{70} . The mutant σ^{70} was stable, retained its core binding affinity and no difference in electrophoretic mobility could be detected. The simplest conclusion is that Glu458 of σ^{70} is involved in recognition of the G-C bp of the 5'-TG-3'

Fig. 6. The activity of the KAB-TG, KAB-TC and KAB-TT promoters with σ^{70} (shown in dark grey), σ^{70} -HA455 (shown in black), σ^{70} -IA457 (shown in white) and σ^{70} -TA459 (shown in light grey). *EcoRI-HindIII* fragments carrying the promoters were cloned into the *lacZ* expression vector, pRW50. The β -galactosidase level was measured and is taken to be a direct measure of the activity of the cloned promoter. In the histogram, levels are expressed as a percentage of the wild type activity at each promoter.

motif. This conclusion was corroborated by the gel mobility shift assays shown in Figure 3, and potassium permanganate footprints shown in Figure 4.

Glu458 of σ^{70} lies in a region just downstream of conserved region 2.4 (amino acids 434-453) which is known to be involved in recognition of the -10 hexamer of promoters (Helmann and Chamberlin, 1988; Siegel *et al.*, 1989; Daniels *et al.*, 1990). Since Glu458 and other residues in this region are well conserved between different σ factors (Figure 5), we suggest that this region, which we propose to call region 2.5, has a function, which is to recognize 5'-TG-3' elements upstream of promoter -10 hexamer elements. We conclude that extended -10 regions are recognized by an extension of region 2 of σ (region 2.5). One interesting exception, where Glu458 is not conserved, is σ^{32} , the heat shock σ factor, where this region has a different function and is involved in

Fig. 7. The amino acid sequence of σ^{70} with the four conserved regions highlighted by shading. Regions 1, 2, 3 and 4 are divided into sub-regions. The figure also shows a consensus promoter and indicates that the -10 and -35 promoter elements are recognized by regions 2.4 and 4.2 respectively. Region 2.5 is shown making direct interaction with the 5'-TG-3' motif located one base pair upstream of the -10 element. Note that the precise boundaries of different regions are arbitrary: here, we define region 2.5 as the entire sequence between region 2.4 and region 3.1.

chaperone-mediated post-translational regulation of heat shock σ levels (Yura, 1996).

It should be noted that a mutant σ^{70} subunit carrying an amino acid change at position 458 had previously been isolated by Waldburger *et al.* (1990), as a suppressor of mutant *ant* promoters. The P_{ant} promoter contains a consensus -35 hexamer and a -10 hexamer that differs from consensus at only one position. The σ mutant, *rpoD*-EK458, was isolated as a suppressor of a down mutation at -12, and also as a suppressor of down mutations at -10 and -9. EK458 causes a small increase in the activity of 16 P_{ant} promoters with down mutations at different positions in both the -10 and -35 hexamers, and it was suggested that the effects of EK458 on promoter recognition were position independent. Our result provides a possible explanation for the observations of Waldburger *et al.* (1990). The P_{ant} promoter is not an extended -10 promoter, and all the promoters used in their study carried a 5'-TA-3' at position -15/-14. It is likely that the EK458 substitution is 'suppressing' the effect of having 5'-TA-3' rather than 5'-TG-3' upstream of the -10 hexamer.

How could region 2.5 of σ make contact with the 5'-TG-3' element? Analysis of protein-DNA hydrogen bonds in 28 regulatory protein complexes solved by X-ray crystallography revealed that nine out of ten hydrogen bonds involving glutamic acid involved interactions with cytosine (Mandel-Gutfreund *et al.*, 1995). This preferred binding of glutamic acid to cytosine suggests that the protein side chain of Glu458 of σ^{70} interacts with cytosine rather than the guanine at position -14 of our test promoters. Presumably the substitution of other bases, as at the KAB-TC and KAB-TT promoters, leads to electrostatic repulsion that is alleviated by the EG458 substitution. Note that interactions between Gly458 and other base pairs could occur through interactions involving the protein

backbone, accounting for how E σ^{70} -EG458 can drive transcription at the KAB-TC and KAB-TT promoters. Malhotra *et al.* (1996) recently solved the crystal structure of a σ^{70} fragment from amino acid 114 to 448. This fragment contains part of conserved region 1.2 and all but the C-terminal five residues of conserved region 2. Region 2.4 was found to form an α -helix with the amino acids involved in recognition of the -10 hexamer being solvent exposed on one face of the helix. Figure 7 is a model showing the orientation of region 2.4 with respect to the DNA (Malhotra *et al.*, 1996), and also the predicted orientation of region 4.2. The model predicts that amino acids involved in contacting the 5'-TG-3' motif would be in the region defined by EG458 (which we dub region 2.5). Finally, extended -10 promoters are ubiquitous in nature, occurring in most bacteria. Indeed, nearly half of the total promoters in some Gram-positive microorganisms can be classified as extended -10 promoters (Helmann, 1995). We conclude that region 2.5 of σ plays an important role in setting the promoter preferences of many bacterial RNA polymerases.

Materials and methods

Strains, promoters and plasmids

The *E. coli* K12 host strains for this work were: the Δlac , *recA* strain DH5 α (Hanahan, 1983), the *rpoD* (ts) strain 285c.*recA* donated by R. Hayward (Harris *et al.*, 1978), the *rpoD* repressible strain CAG20177 (donated by M. Lonetto) and the BL21 (λ DE3) strain which carries a copy of the inducible gene for T7 RNAP on its chromosome (Studier and Moffatt, 1986). The promoters KAB-TG, KAB-TC and KAB-TT were cloned as *EcoRI*-*HindIII* fragments in either the low copy number *lacZ* reporter vector pRW50 (Lodge *et al.*, 1992) or the *galK* fusion vector, pAA121 (Kelsall *et al.*, 1985). These promoters, shown in Figure 1, are described in detail by Chan and Busby (1989). Derivatives of KAB-TG carrying further substitutions in the -10 or -35 hexamer were made by site-directed mutagenesis and cloned as *EcoRI*-*HindIII*

fragments into pRW50. By convention, promoter sequences are numbered with the transcription start site as +1, with upstream sequences prefixed with a '-' sign. The plasmid, pKB σ , encoding σ^{70} was constructed using megaprimer PCR (Barne, 1997; details can be found on <http://www.biochemistry.bham.ac.uk/sdm/pkbsigma.html>). The construct was checked by its ability to complement the *E. coli* *rpoD* temperature-sensitive strain 285c.*recA* for growth at 42°C. To confirm the construct, the complete base sequence of the insert was determined using the Pharmacia T7 Sequencing Kit. All preparations of plasmid DNA, restriction endonuclease treatments and ligations were performed as described by Sambrook *et al.* (1989). Synthetic oligos were purchased from Alta Bioscience at The University of Birmingham.

In vitro mutagenesis of *rpoD* and isolation of altered specificity mutants of σ^{70}

PCR reaction mixes were set up in a final volume of 50 μ l with various MgCl₂ concentrations (1–10 mM), primers (1 μ M) (upstream primer: 5'-AGGCGTATCACGAGGCCCT-3' and downstream primer: 5'-GGG-GAAGCTTTTAATCGTCCAGGAAGCTACGC-3'), template DNA (pKB σ) (50–100 pM), dNTPs (200 mM) (Pharmacia) and *Taq* DNA polymerase (1 unit) (Boehringer Mannheim). The cycle profile was: 39 cycles of 30 s at 94°C, 30 s at 58°C, 2 min at 72°C; 1 cycle of 30 s at 94°C, 30 s at 58°C, 4 min at 72°C. The PCR product carrying randomly mutagenized *rpoD* was purified on a 2% agarose gel, digested with *Eco*RI and *Hind*III and recloned into pKB σ . Mutants were isolated by screening against KAB-TC/pRW50 or KAB-TT/pRW50 in DH5 α . Transformants were selected on MacConkey indicator plates (Difco Laboratories) supplemented with lactose, ampicillin (80 μ g/ml) and tetracycline (30 μ g/ml). Altered or relaxed specificity mutants of σ^{70} were identified as red/pink colonies on the indicator plates. Plasmid DNA was recovered from candidates and introduced back into the screen to check phenotypes. The full *rpoD* nucleotide sequence of each candidate was determined.

Construction of σ^{70} -HA455, σ^{70} -IA457 and σ^{70} -TA459

For these mutants, the appropriate base changes were introduced into plasmid pKB σ using PCR. The base changes were introduced in an initial round of PCR during the synthesis of a megaprimer. These megaprimers were then used in a second PCR reaction to incorporate a downstream restriction site to aid cloning of the DNA fragments into pKB σ . The primers used were 5'-GATGGTCTCAATCATAGCCACCG-GAATACGGAT-3', 5'-CTTGTTGATGGTCTCAGCCATATGCACCG-GAAT-3' and 5'-GTTGAGCTTGTGATGGCCTCAATCATATG-CAC-3' for the construction of *rpoD*-HA455, *rpoD*-IA457 and *rpoD*-TA459 respectively (the DNA modifications are highlighted in bold). These primers were used with an upstream primer (5'-GCAACATCGGT-CTGA-3') flanking a unique *Bam*HI restriction site in the template pKB σ . The PCR products were gel purified and then used as primers in a second round of PCR in conjunction with a 'downstream' primer 5'-GCTTCACGCGCGGTACAG-3' that flanks a unique *Xho*I restriction site. The final PCR products were gel purified, restricted with *Bam*HI and *Xho*I, cloned into plasmid pKB σ and the entire σ sequence was checked.

Determination of promoter activity in vivo

β -galactosidase expression in cells carrying different promoter::*lacZ* fusions cloned in pRW50, and different σ^{70} mutants cloned in pKB σ , was measured as described by Lodge *et al.* (1992) and was taken to reflect the activity of the cloned promoters. Assays were performed in the *E. coli* strain CAG20177, carrying *rpoD* under the control of the repressible *trp* promoter, which allows the level of transcription initiation in the absence of host σ^{70} to be determined (M.Lonetto, V.Rhodus and C.Gross, personal communication). Pre-cultures were grown in Lennox broth plus ampicillin (80 μ g/ml), tetracycline (30 μ g/ml) and 3- β -indoleacrylic acid (0.2 mM). Assay cultures were grown in Lennox broth plus ampicillin (80 μ g/ml) and tetracycline (30 μ g/ml) (in the absence of 3- β -indoleacrylic acid, expression of chromosomal *rpoD* is suppressed in strain CAG20177). Each assay was performed independently at least six times and activities were reproducible to within 10%. To exclude the possibility that some effects were due to changes in plasmid copy number, plasmids were checked by gel electrophoresis. Assays were also performed in the *E. coli* DH5 α . Assayed in this strain, both the mutant σ and the wild type chromosomal σ were present. These assays resulted in the same conclusions as assays in strain CAG20177.

Reconstitution of RNA polymerase

Plasmids pGEMAX185, pGEMBC and pGEMD, which enable over-production of the RNAP α , β and β' , and σ subunits respectively were

given by Akira Ishihama. Plasmid pGEMD-EG458, encoding σ^{70} -EG458, was constructed by digesting the pKB σ derivative encoding *rpoD*-EG458 with *Bam*HI and *Xho*I and cloning the resulting 444 bp fragment carrying *rpoD*-EG458 into pGEMD. *E. coli* BL21 (Δ DE3) cells were transformed with the different plasmids for overproducing RNAP subunits: over-expression and purification of the individual subunits and reconstitution into functional RNAP was performed as detailed in Igarashi and Ishihama (1991).

Electromobility shift assays

Band shift assays were performed on labelled *Eco*RI-*Hind*III promoter fragments carrying the KAB-TG, KAB-TC and KAB-TT promoters (Figure 1). Typically, labelled fragments (1 nM) were incubated at 37°C with different preparations of RNAP (100 nM) in transcription buffer (200 mM HEPES pH 8.0, 50 mM MgCl₂, 500 mM K-glutamate, 10 mM DTT, 250 μ g/ml BSA) for 30 min to allow open complex formation. Loading buffer (3 μ l containing 0.1% bromophenol blue, 0.01% xylene cyanole FF, 50% glycerol and 160 μ g/ml heparin in 1 $\times$ transcription buffer) was added immediately prior to loading on a 4.5% polyacrylamide gel. After electrophoresis, gels were visualized by autoradiography.

DNA footprinting assays using permanganate

DNA footprinting experiments were performed on *Pst*I-*Hind*III promoter fragments (purified from pAA121 derivatives) carrying the KAB-TG, KAB-TC and KAB-TT promoters. To examine the lower strand, the *Hind*III ends were labelled with [γ -³²P]ATP (DuPont NEN) using T4 polynucleotide kinase (New England Biolabs). Typically, labelled DNA fragments (1 nM) were preincubated at 37°C with different RNAP preparations (100 nM) in transcription buffer (as above) before addition of freshly prepared KMnO₄ (protocols were as previously used by Chan *et al.*, 1990). The reaction was stopped after 4 min by the addition of KMnO₄ stop solution (50 μ l containing 3 M ammonium acetate, 0.1 mM EDTA, 1.5 M β -mercaptoethanol). After phenol/chloroform extraction and ethanol precipitation the samples were cleaved by treatment with piperidine. Resulting fragments were electrophoresed on calibrated 6% sequencing gels (Sequagel, National Diagnostics) that were visualized by autoradiography.

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