Effects of substitutions at position 180 in the *Escherichia coli* RNA polymerase σ^{70} subunit

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In order to investigate the role of His180 residue, located in the non-conserved region of the σ^{70} subunit of *Escherichia coli* RNA polymerase, two mutant variants of the protein with substitutions for either alanine or glutamic acid were constructed and purified using the IMPACT system. The ability of mutant σ^{70} subunits to interact with core RNA polymerase was investigated using native gel-electrophoresis. The properties of the corresponding reconstituted holoenzymes, as provided by gel shift analysis of their complexes with single- and double-stranded promoter-like DNA and by *in vitro* transcription experiments, allowed one to deduce that His180 influences several steps of transcription initiation, including core binding, promoter DNA recognition and open complex formation.

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1. Introduction

Transcription is one of the key processes in living cells. In *Escherichia coli*, it is exerted by multisubunit RNA polymerase (RNAP) (subunit composition being $\alpha_2\beta\beta'\omega$), which is known as the core enzyme. During initiation of transcription, the core enzyme requires temporary association with one of the sigma (σ) factors that provides the specific interaction of holoenzyme with promoter DNA, formation of open complex and initiation of RNA synthesis (von Hippel *et al.* 1984). The major σ factor in *E. coli* is σ^{70}. Several functional domains, responsible for interaction with the core enzyme and promoter −10 and −35 consensus elements, have been revealed in its structure mainly by site-specific mutagenesis (Helmann and Chamberlin 1988; Gardella *et al.* 1989; Siegele *et al.* 1989; Lonetto *et al.* 1992; Waldburger and Susskind 1994; Fenton *et al.* 2000; Panaghie *et al.* 2000; Burgess and Anthony 2001) (figure 1a). However, the functional significance of the rather long (245 amino acids) non-conserved region (NCR) between the regions 1.2 and 2.1 was not studied. The corresponding analogues of this region in other related σ subunits exhibit quite a lot of variation both in size and sequence (Malhotra *et al.* 1996). On the basis of the crystal structure of the fragment including 114–448 amino acid residues of σ^{70}, we can deduce that the NCR is composed of a series of α-helices with C- and N-termini of this region in close proximity (Malhotra *et al.* 1996). It was found that the removal of this region resulted in some defects of cell growth but did not influence the basal transcriptional activity of the corresponding holoenzyme *in vitro* (Kumar *et al.* 1995). Recently, by the use of suppression genetics approach, it has been demonstrated that a series of amino acid residues, located in the region 280–371, were involved

Keywords. Non-conserved region; RNA polymerase; σ subunit; site-specific mutagenesis; transcription

Abbreviations used: IMPACT, Intein-Mediated Purification with an Affinity Chitin-binding Tag; NCR, non-conserved region; RNAP, RNA polymerase

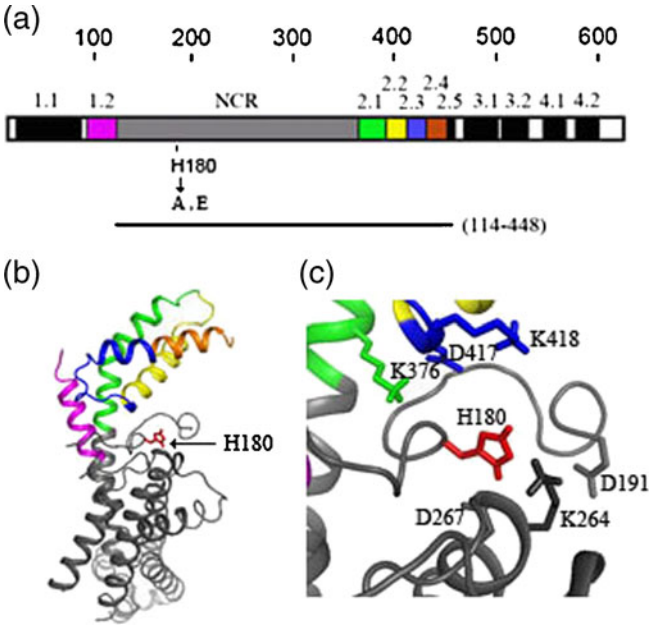

Figure 1. Location of H180 in the structure of *E. coli* RNA polymerase σ^{70} subunit: (**a**) domain organization of σ^{70} subunit; the regions conserved within the σ^{70} family are shown as colored boxes (region 1.2, magenta; 2.1, green; 2.2, yellow; 2.3, blue; 2.4, brown; regions 1.1, 4.1, 4.2, black); NCR (grey) is the non-conserved region; (**b**) ribbon representation of the overall three-dimensional structure of σ^{70} subunit fragment from 114 to 448 residues (underlined in **a**) (PDB code: 1SIG) (Malhotra *et al.* 1996); (**c**) close-up of the region adjacent to the H180 residue. These figures were made using PyMol (DeLano 2002).

in the interaction with the β′ subunit, facilitated promoter escape and hindered early elongation pausing (Leibman and Hochschild 2007), confirming the previous data by Lesley and Burgess (1989) that residues from 361 to 390 are critical for core binding. Investigation of a series of σ^{70} mutants with progressive truncations of the N-terminus indicated the participation of NCR in the modulation of DNA-binding activity of free σ subunits (Dombroski *et al.* 1992, 1993). The experiments on cross-linking of the σ^{70} subunit in complexes of holoenzyme with oligonucleotides, corresponding to non-template promoter strands, allowed one to propose that the phosphate residue at position −12 made contact with either His180 or His242 (Rudakova *et al.* 2000). Taking into account these data, we decided to obtain further information on the role of His180, that being both donor and acceptor of protons can participate as mediator of structural rearrangement at various stages of transcription initiation. His180 is situated in a flexible loop and surrounded by several charged residues, located in the NCR (Asp191, Lys264 and Asp267) and conserved regions 2.2 (Lys376) and 2.3 (Asp417 and Lys418) (figure 1b, c). Moreover, His180 is a single basic amino acid in the vicinity of the highly acidic region 188–209, which was not resolved in X-ray structure and could hypothetically inhibit interaction of the free σ^{70} subunit with DNA (Malhotra *et al.* 1996). In our study, we decided to replace this residue with either a neutral (Ala) or an acidic (Glu) amino acid. The substitutions were introduced by site-specific mutagenesis, and the corresponding mutant variants of the σ^{70} subunit were purified using IMPACT-CN System (New England BioLabs, NEB) (Chong *et al.* 1997) and their properties were assayed in the *in vitro* systems.

2. Materials and methods

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) were from New England Biolabs. *Escherichia coli* RNA polymerase holoenzyme (1.1 μg/μl, 1.2 activity units/μl) was from Sigma, and *Escherichia coli* RNA polymerase core enzyme (0.54 μg/μl, 1 activity units/μl) was from Epicentre Technologies (Madison, WI). [γ-^{32}P]ATP (185 pBq/mol), [α-^{32}P]UTP (148 pBq/mol) were obtained from Isotope (Obninsk, Russia).

Oligonucleotides for PCR, site-directed mutagenesis or RNA-polymerase-binding experiments were synthesized by standard phosphoramidite approach using Applied Biosystems Sythesizer.

2.1 *Strains and plasmids*

Escherichia coli ER1821 was used as the host strain for protein expression and purification.

Construction of the plasmid pC4-d for over-expression of the σ^{70} subunit has been described (Khodak Yu *et al.* 2007).

Plasmids for expression of mutant variants of σ^{70} were prepared from pC4-d by site-directed mutagenesis using the approach described previously (Drutsa and Kaberdin 1992). Oligonucleotides pAGCGTGACCGCGACCTT and pCCTACCGCCACTG(C+A)AGTCGGTTCTGAGC were used as mutagenic primers, and CCCGGAACTGGCTCG and GTTCCGCAAATTTTTCG as auxiliary primers. The complete DNA sequence of rpoD gene in pC4-d and mutated regions in plasmids pC4-d(180A) and pC4-d (180E) was verified by dideoxy sequencing.

Plasmids pKDI and pKDIV-1 have been described previously (Koroleva and Drutsa 1991, Koroleva *et al.* 1997). DNA fragments TI and TIV, containing consensus promoter signals utilized for *in vitro* transcription assays and gel mobility shift experiments (figure 2), were prepared by PCR (Higuchi *et al.* 1988) using the primers AACCTA TAAAAATAGGCGTATCACGA (25-kupl) and CTCTCA TAATTCGCTCCATTAAG (23-kdor) and plasmids pKDI and pKDIV-1 as templates, with subsequent purification by electrophoresis on 8% native polyacrylamide gel.

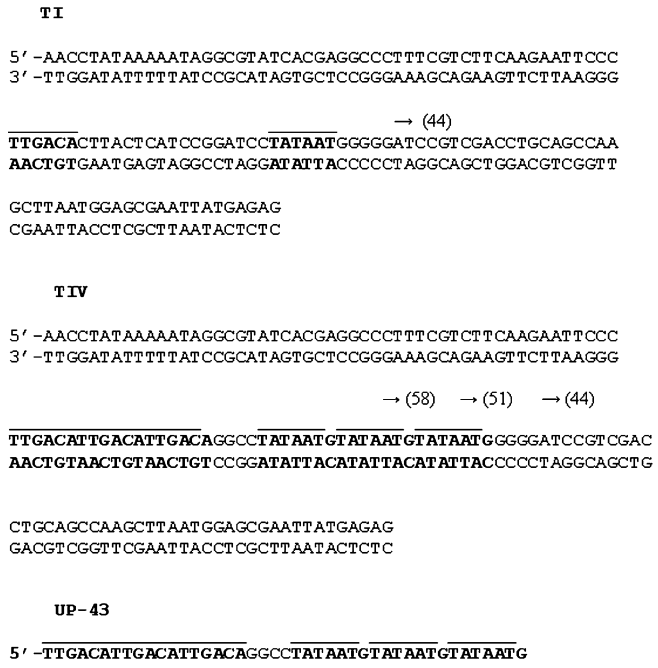

Figure 2. Promoter containing DNA fragments used in this study. Here, −10 (TATAAT) and −35 (TTGACA) elements are marked. The transcription start sites and length of the transcripts, generated from the promoters of templates TI and TIV, are indicated by arrows with numbers.

2.2 *Purification of σ^{70} subunits*

For the expression and purification of wild type (WT) and mutant σ^{70}, we used the IMPACT expression system (NEB), which provides a quick one-step column purification of proteins containing a single extra C-terminal glycine residue. Purifications of WT and mutant variants of σ^{70} were carried out per the NEB protocol (Chong *et al.* 1997) with modifications (Khodak Yu *et al*. 2007). The resulting protein preparations were subsequently analysed using SDS/polyacrylamide gel-electrophoresis (PAGE). Purified natural and mutant σ^{70} proteins were concentrated by ultrafiltration to a final concentration 20–30 μM and stored in 40 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 M dithiothreitol, 50% (v/v) glycerol at −20°C. Protein concentrations were measured by the method of Bradford (1976).

2.3 *Holoenzyme reconstitution*

Reconstitution of active holoenzymes was achieved by incubation of the core enzyme with WT or mutant σ^{70} for 20 min at 30°C in a buffer containing 40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM KCl and 1 mM dithiothreitol with either 100 μg/ml bovine serum albumin or 10% (v/v) glycerol. To investigate the core–σ binding, the concentrations of core RNAP in reaction mixtures (native PAGE analysis of reconstitution mixtures, single-round transcription experiments or gel mobility shift assays) were 10, 25, 50 or 100 nM, and molar ratios of σ^{70} to core varied within the range 0.5–8. In the concentration-dependent experiments, the concentration of the core enzyme was varied from 0.5 to 200 nM, with the σ^{70}–core ratio being 6.

2.4 *Native gel-electrophoresis*

Electrophoresis for analysis of the σ^{70}–core interaction was carried out on a two-layer polyacrylamide gel (4%, 'up' layer and 5%, 'bottom' layer) using Tris-glycine buffer (pH 8.5) at 45 V, 4°C for 4–5 h as described by Ilag *et al.* (2004). The gel was stained with PAGE staining solution (Fermentas, Lithuania).

2.5 *DNA-binding assays*

Binding of RNAP variants with DNA was studied using gel shift technique. Reconstituted RNAP holoenzyme (concentration varied from 5 to 100 nM) was incubated with ^{32}P-labelled DNA fragments (5 or 10 nM) in 10 μl of transcription buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, 50 μg/ml bovine serum albumin) for 20 min at 37°C. Upon addition of 2.5 μl of loading buffer (50% sucrose, 0.025% xylene cyanol, 0.025% bromphenol blue), the reaction mixture was loaded onto a native 4% polyacrylamide gel. In heparin challenge experiments, heparin was added to a final concentration of 100 μg/ml. The gel was run at room temperature at 10 V/cm and upon drying was exposed using a PhosphorImager 400A (Molecular Dynamics) screen. The ratio of bound to free DNA was used to estimate the affinity of the enzyme to DNA.

2.6 *$KMnO_4$ footprinting*

The experiments were carried out basically as described by Sasse-Dwight and Gralla (1989) with minor modifications. The PCR DNA fragment TI (10 nM) and RNAP (200 nM) were incubated for 30 min at 37°C in transcription buffer and then challenged for 10 s with heparin (100 μg/ml) before $KMnO_4$ (10 mM) was added. Upon incubation for 4 min at 37°C, the reactions were quenched by 2-mercaptoethanol (1.2 M) and the DNA precipitated with ethanol and dried. The pellets were treated with 10% aqueous piperidine at 90° C for 30 min. Piperidine was removed by evaporation to dryness. The products were analysed by electrophoresis on denaturing 8% polyacrylamide gel, followed by autoradiog-

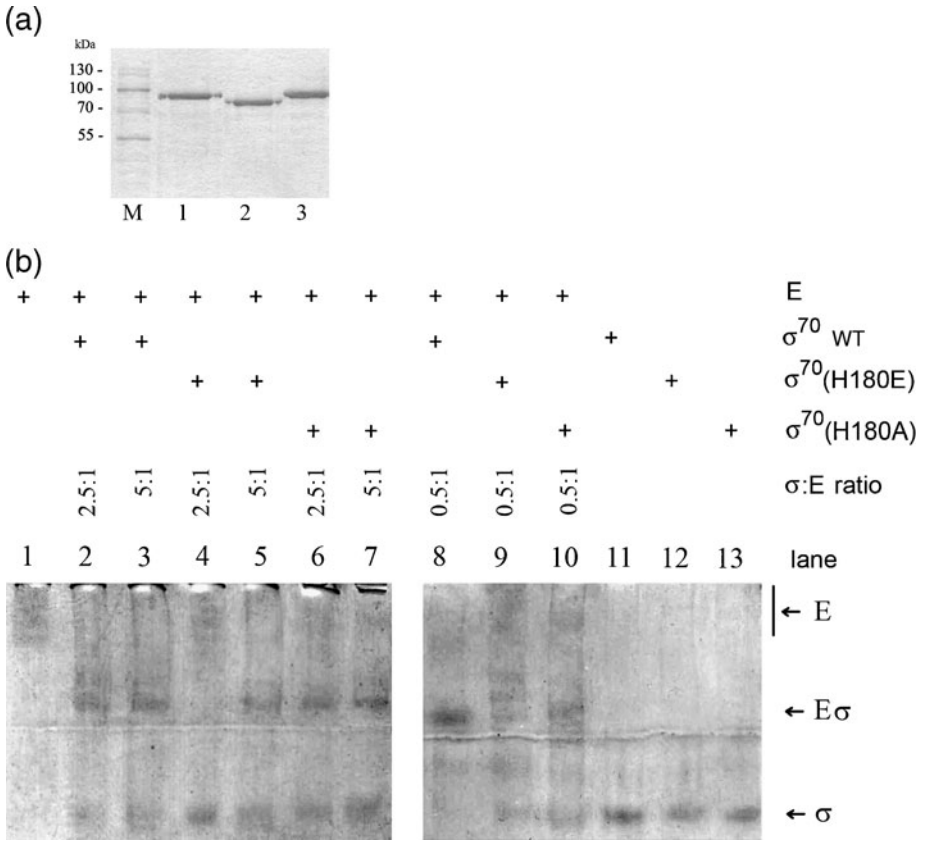

Figure 3. (**a**) SDS/polyacrylamide gel electrophoresis of preparations of WT (lane 1) and mutant variants σ^{70}(H180E) (lane 2) and σ^{70}(H180A) (lane 3). Proteins were visualized by Coomassie blue staining (M, molecular weight ladder). (**b**) Analysis of the binding of WT or mutant variants of σ^{70} to core RNAP using electrophoresis on native two-layer (4% and 5%) polyacrylamide gel. The concentration of core RNAP is 50 nM (lanes 2–7) or 100 nM (lanes 8–10). The positions of core RNAP (E), holoenzyme (Eσ) and σ subunits (σ) are indicated by arrows.

raphy and imaging with PhosphorImager. The sequencing reactions were prepared according to the Maxam–Gilbert protocol (Maxam and Gilbert 1977).

2.7 *Single-round transcription*

Transcription was performed by incubation of RNAP with a DNA template in transcription buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, 100 µg/ml bovine serum albumin) for 5 min at 37°C, followed by the simultaneous addition of ribonucleoside triphosphates (NTPs) and heparin to yield the following final concentrations: 100 µg/ml of heparin, 100 µM (each) NTPs (ATP, GTP, CTP) and 10 µM [^{32}P] UTP (specific activity of 50 cpm/fmol). The final concentration of DNA in the reaction mixture was 5, 10 or 20 nM and that of RNAP varied in the range 5–200 nM (*see* also legends to figures). The total reaction volume was 10 µl. Upon incubation for 10 min at 37°C, the reaction was stopped by adding 5 µl of stop solution (20 mM EDTA, 80% deionized formamide, 0.1% xylene cyanole, 0.1% bromphenol blue) and heating it to 75°C for 30 s. Samples were analysed by denaturing 8% gel electrophoresis and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

3. Results

The starting point in this investigation of the functional role of His180 in the σ^{70} subunit was the construction of two new mutant variants of protein with substitutions: σ^{70}(H180A) and σ^{70}(H180E). The purity of protein preparations, isolated by the IMPACT system (Chong *et al.* 1997), was checked using SDS/PAGE (figure 3a). It should be noted that σ^{70}(H180E) migrates slightly faster than WT σ^{70} subunit. Such a phenomenon was observed previously in the case of some other σ variants with single-point mutations and was attributed to the 'more open structure' of mutant proteins (Gopal *et al.* 1994; Gopal and Chatterji 1997).

To understand the effects of substitutions on the specific steps of transcription initiation, we studied the ability of RNAP, reconstituted with mutant σ^{70} subunits, to bind

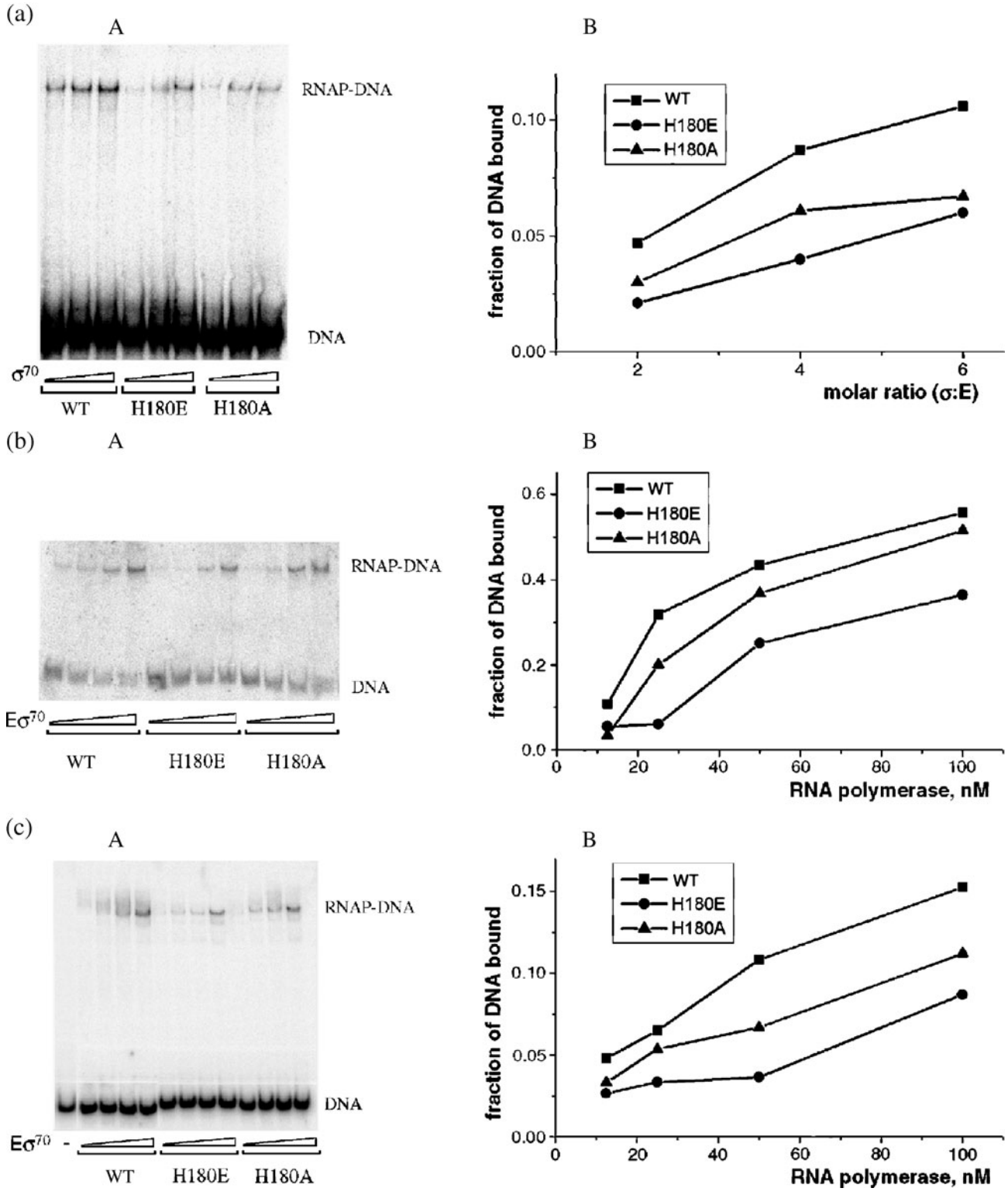
(a)
A
RNAP-DNA
DNA
σ70
WT
H180E
H180A
B
WT
H180E
H180A
fraction of DNA bound
0.00
0.05
0.10
2
4
6
molar ratio (σ:E)
(b)
A
RNAP-DNA
DNA
Eσ70
WT
H180E
H180A
B
WT
H180E
H180A
fraction of DNA bound
0.0
0.2
0.4
0.6
0
20
40
60
80
100
RNA polymerase, nM
(c)
A
RNAP-DNA
DNA
Eσ70 -
WT
H180E
H180A
B
WT
H180E
H180A
fraction of DNA bound
0.00
0.05
0.10
0.15
0
20
40
60
80
100
RNA polymerase, nM

Figure 4 (a–c).

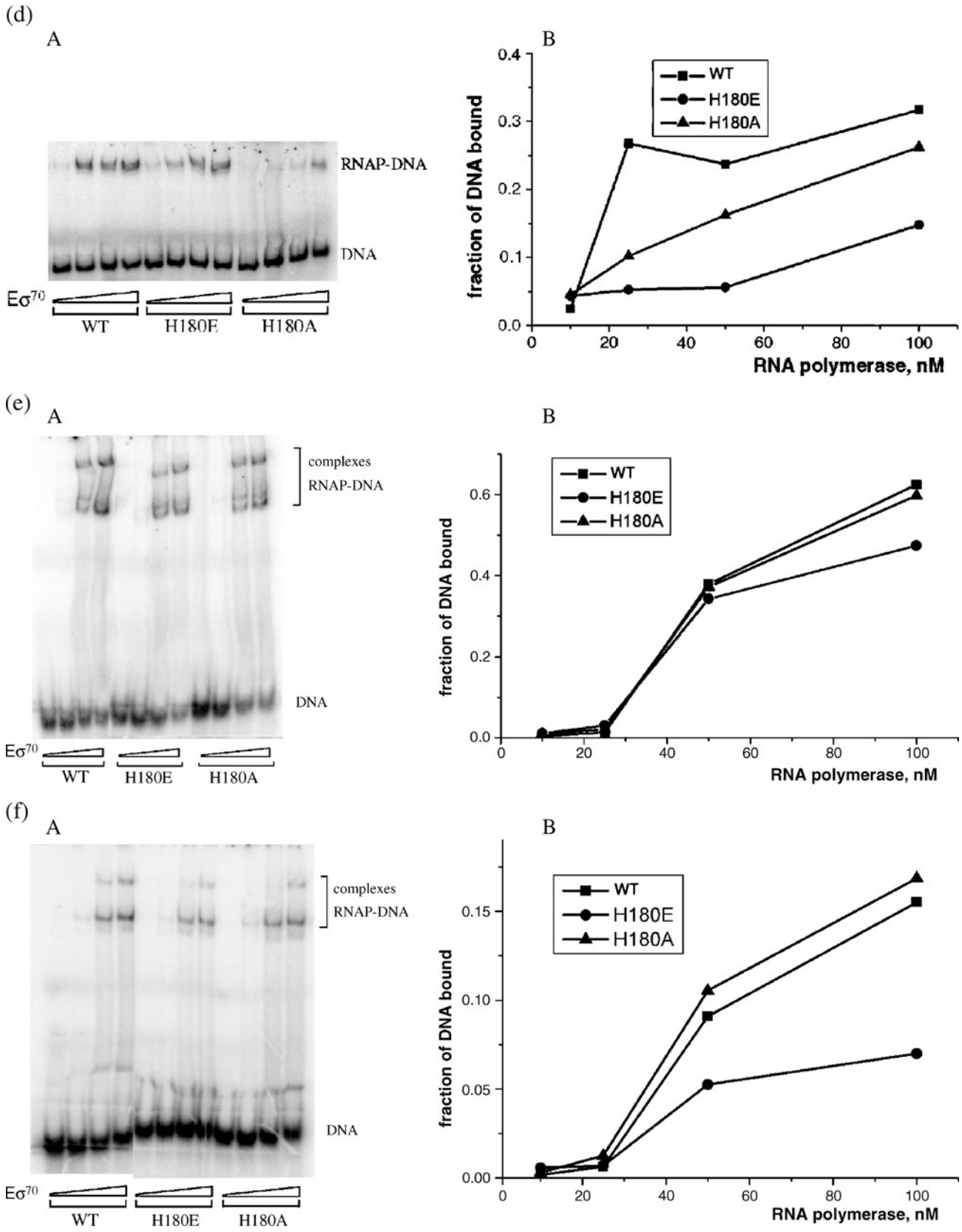

Figure 4 (d–f). For caption, *see* page no. 49.

double-stranded and single-stranded DNA fragments with promoter-like elements and to synthesize *in vitro* run-off transcripts on linear DNA templates containing consensus promoters (figure 2).

First, we investigated the binding of mutant σ subunits σ^{70}(180A) and σ^{70}(180E) to the core enzyme by using native gel shift analysis of the corresponding reconstitution mixtures. The results demonstrated noticeably a lower affinity of mutant σ^{70}(H180E) to core RNAP as compared with WT σ subunit at low σ–core ratios (*see*, for example, lanes 2 and 4 in figure 3b), whereas the behaviour of σ^{70}(H180A) is more similar to that of WT

3.1 *The peculiarities of open complex formation by mutant RNA polymerases*

The gel shift technique was used to study the promoter-binding properties of mutant RNAPs. The binding of holoenzymes to the double-stranded DNA fragment TI, containing a consensus promoter, was carried out under different conditions: either with variation of the σ–core ratio (DNA and RNAP concentrations being constant) or with increase of the concentrations of holoenzymes, saturated with corresponding σ subunits. The reaction mixtures were either challenged with heparin or not and separated on a 4% native polyacrylamide gel (figure 4a–c, panels A). The ratios of DNA bound to total DNA were calculated and presented as a function of σ–core ratio or RNAP concentrations (figure 4a–c, panels B). The results of the first series of experiments (figure 4a) demonstrated the difference in the slopes of σ saturation curves and plateau levels. From the fact that the reduced core–binding affinity of the mutants can lead to a decrease in holoenzyme formation with subsequent decrease in the amounts of closed and hence open complexes, one can conclude that these results indicate the reduced core-binding activities of mutant σ^{70} to the core enzyme, the effects with σ^{70}(H180E) being the strongest.

The next series of experiments with variable concentrations of holoenzymes, saturated with σ subunits demonstrated the reduced DNA-binding affinities of mutant enzymes compared with WT Eσ^{70} both in the absence or presence of heparin (figure 4b, c). The experiments in the presence of heparin characterized the impaired ability of mutant RNAPs, especially Eσ^{70}(H180E), to form promoter-specific open complexes.

To characterize in greater detail the peculiarities of unwinding of promoter DNA in complexes with mutant RNAP, we performed the permanganate footprinting experiments with the use of TI template, ^{32}P-labelled at a lower strand. The general patterns of thymine residues modification within the −10 hexamer region in all cases are similar, indicating the conservation of the overall structure of open complexes (figure 5). Nevertheless, the reactivity of thymines to permanganate is slightly diminished in the case of mutant variants of RNAP compared with WT, which is consistent with gel mobility shift experiments. The fractions of the radioactivity in the bands, corresponding to the cleavage at modified residues, relative to the total radioactivity contained in the columns, for σ^{70}(H180A), σ^{70}(180E) and WT σ^{70} are 0.13, 0.07 and 0.18, respectively.

The same tendency in the behaviour of mutants, although even more pronounced, was observed with the other DNA template TIV (figure 2), which contained three overlapping consensus promoters with different spacers: 16, 17 and 18 b.p. We have previously shown that this construct leads to a higher transcription initiation signal *in vivo* than does the consensus promoter TI, and demonstrates a higher affinity to RNAP (Khodak Yu *et al*. 2007). The results of gel shift experiments (figure 4d) confirmed the assumption that mutant enzymes had defects in the formation of open complexes.

Several studies have shown that RNAP can bind specifically to short single-stranded DNA fragments containing a non-template sequence of the −10 promoter element (Savinkova *et al.* 1988; Marr and Roberts 1997). There are some correlations between the ability of RNAP to bind ssDNA and its ability to form open complexes (Huang *et al.* 1997; Fedoriw *et al.* 1998). To study the effect of mutations on the ability of the corresponding holoenzymes to bind ssDNA, we used 43-mer oligonucleotide UP-43 (figure 2), corresponding to the upper strand of the promoter region of template TIV, which is able to bind efficiently to RNAP with the apparent equilibrium dissociation constant K_d being about 2.0 (±0.2)×10^{-8} M. The data on gel shift experiments (figure 4e and f) demonstrated the ability of all mutant holoenzymes to bind specifically this oligonucleotide although the level of binding varied. (The highest band on the gel is apparently the complex containing 2 molecules of RNAP as a result of aggregation.) The following binding efficiencies (at saturation concentrations of RNAP, 100 nM core enzyme, σ:core=6:1) in the absence and

Figure 4. The effects of σ^{70} mutations on the interaction of RNAP holoenzyme with promoter-like DNA. Gel mobility shift analysis of binding of holoenzymes, reconstituted with WT or mutant σ^{70} variants (indicated), to double-stranded promoter-containing templates TI (**a–c**) and TIV (**d**) and single-stranded oligonucleotide UP-43 (**e, f**) in the absence (**b, e**) and presence (**a, c, d, f**) of heparin. Panels (A): gel mobility shift analysis using non-denaturing 4% polyacrylamide gel electrophoresis. Panels (B): the corresponding curves of titration with σ subunits (**a**) or holoenzymes (**b–f**), reconstituted with WT or mutant σ^{70} subunits (indicated). The DNA concentration was 10 nM and the RNAP concentration was 100 nM (**a**). The σ–core ratio was 6 (**b–f**). Assays were performed two or three times with similar results. Typical data are presented in the figure. Standard deviations do not exceed 12%.

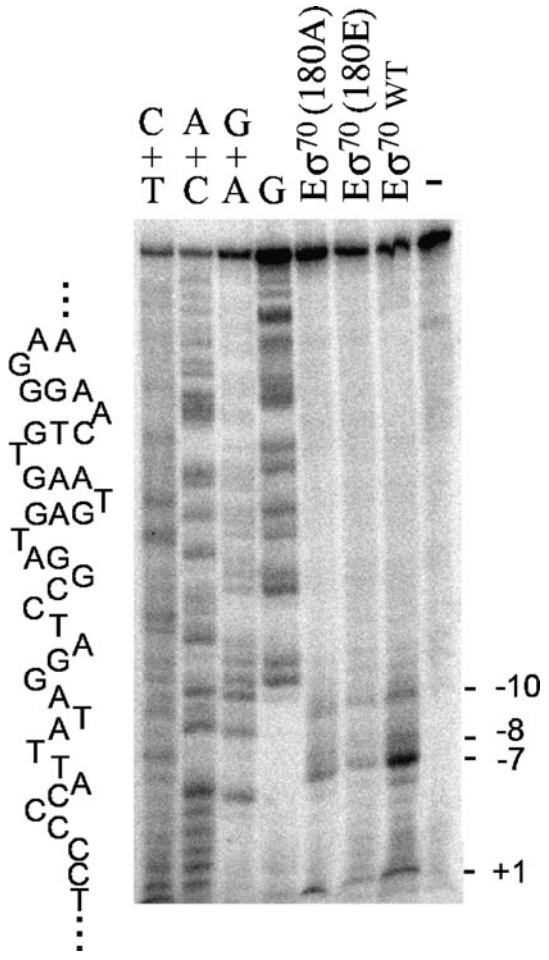

Figure 5. $KMnO_4$ footprints of the open promoter complexes formed by WT or mutant variants of holoenzymes (indicated) on the TI promoter. Lane - is the control experiment in the absence of RNA polymerase. Lanes C+T, C+A, G+A and G are the sequencing ladders.

presence of heparin were observed: about 60–62% and 16% for $E\sigma^{70}$(180A) and WT $E\sigma^{70}$, and 47% and 8% for $E\sigma^{70}$(H180E). The difference in the amounts of complexes in the absence and presence of heparin reflects the ability of RNA polymerase itself to transit from the conformation it has in the primary ssDNA recognition complex (heparin-sensitive) to the conformation it acquires in the open complex (heparin-resistant). Then, in this model system with 'pre-melted' promoter DNA, the mutant $E\sigma^{70}$(H180E) is deficient in this transition.

One can see that $E\sigma^{70}$(H180E) demonstrates less affinity to both dsDNA and ssDNA (in the presence and in the absence of heparin) compared with WT RNAP, and hence it is deficient in promoter binding, in transition to open complex and in specific interaction with the −10 promoter element. $E\sigma^{70}$(H180A) demonstrates less affinity to dsDNA compared with WT but demonstrates no difference in ssDNA binding, and hence, it is deficient only in primary promoter recognition and −10 region melting.

3.2 *Effect of mutations on transcription in vitro*

To obtain further data on the effects of mutations on various stages of transcription, we performed a series of experiments on *in vitro* transcriptions by using TI and TIV DNA templates and mutant holoenzymes. First, to confirm the difference in affinities of σ subunit variants to core RNAP, a series of single-round transcription reactions were carried out, in which the concentrations of DNA (10 nM) and core RNAP (25, 50 or 100 nM) were kept constant while the molar ratio of σ–core varied within the range 0.5–8. Typical examples of these experiments are presented in figure 6 (panels A). (The corresponding quantifications of the full-length transcripts produced are presented in panels B.) The results show that at lower ratios of σ–core, the amount of transcripts, synthesized by $E\sigma^{70}$(H180A) or $E\sigma^{70}$(H180E), is less than in the case of WT But the relative transcription activity of reconstituted holoenzymes under the conditions of enzyme saturation with the σ subunit (at plateau) depends on the RNAP–DNA ratio (compare data in figure 6a–c). The shape of the saturation curves confirmed the reduced affinity of mutant σ subunits to the core enzyme, with the effect being more pronounced for σ^{70}(H180E). The supplementary series of experiments (not presented), in which the concentration of RNAP, saturated with either of σ subunits, was varied over a range of 10 to 100 nM while the concentration of the DNA template was held constant at 10 nM, demonstrated the following general tendency (seen also in figure 6a–c): at low concentrations of RNAP (0.5×10^{-8}M), the quantity of full-length transcripts was reduced for both mutant variants, whereas at elevated concentrations of holoenzymes, the level of transcription for these mutants increased significantly. These data correlate well with those of gel shift experiments, indicating that holoenzymes $E\sigma^{70}$(H180A) and $E\sigma^{70}$(H180E) have lower affinity to promoter DNA, as compared with that of WT RNAP.

The transcription experiments with the use of template TIV support the above conclusions (figure 6d). The accumulation of total amounts of transcripts in each case is in general agreement with the formation of open complexes (figure 4d). It is interesting to note that this construct with three overlapping promoters seems to be very useful in studying the effects of a particular mutations in RNAP on the recognition of promoters with a variable spacer between −10 and −35 elements, because, in some experiments, we observed differences not only in accumulation of three full-length transcripts but also in the pattern of shorter products (*see*, for example, figure 7). The presence of consecutive −10 elements may cause the pausing of RNAP, which had started from the 'distal' of three overlapping promoters, at early elongation step because of the interaction of σ subunit with

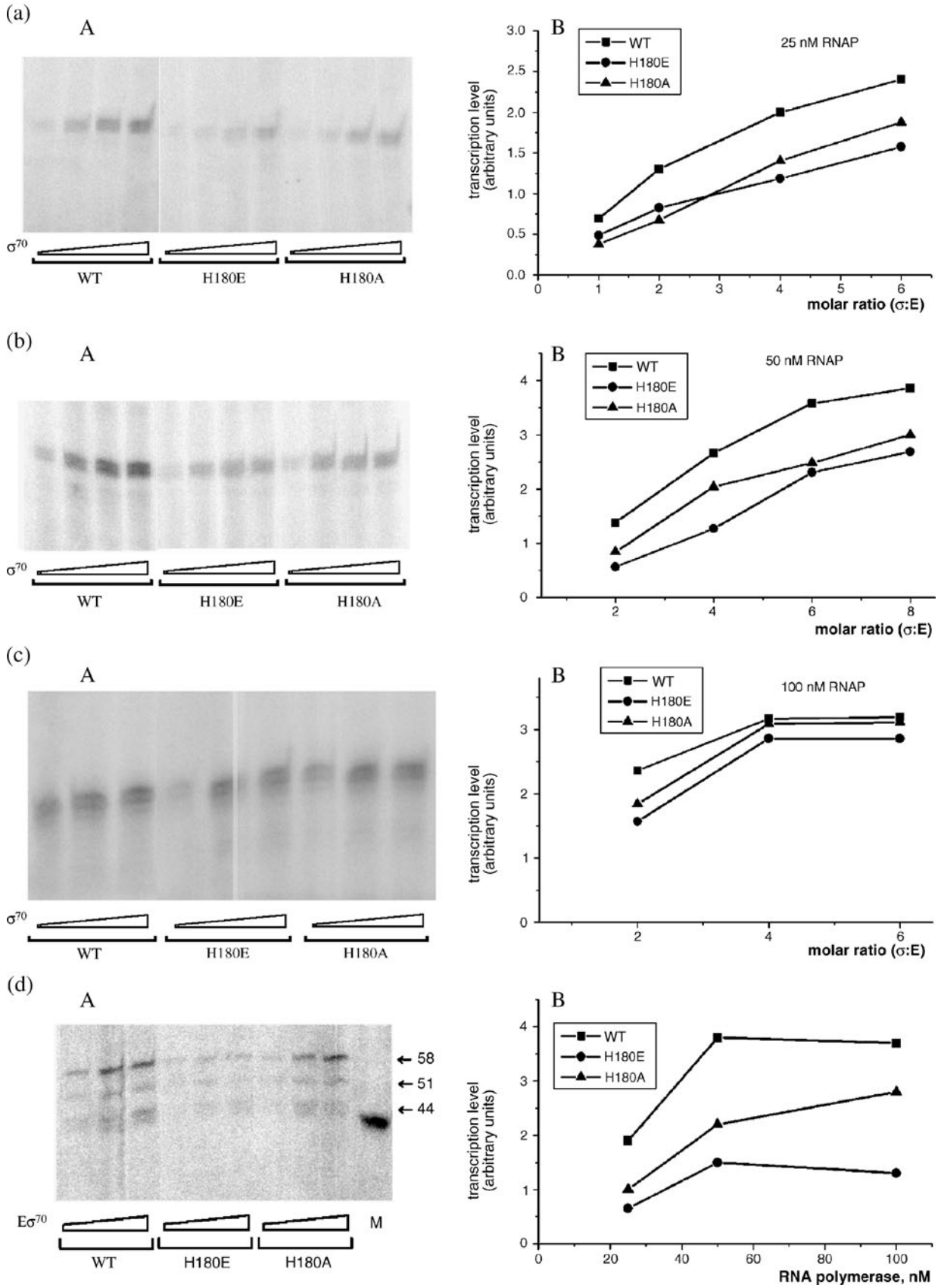

Figure 6. For caption, *see* page no. 52.

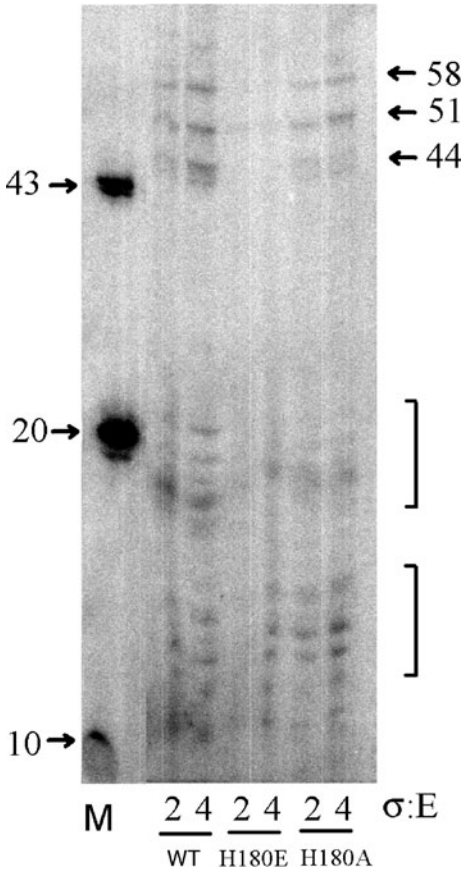

Figure 7. Effects of substitutions in σ^{70} subunit on i*n vitro* transcription, initiated from promoters of the template TIV. Autoradiogram of electrophoretic separation (20% denaturing polyacrylamide gel) of the products, generated with the use of holoenzymes and reconstituted with WT or mutant variants of σ^{70}. DNA concentration was 10 nM, core RNAP concentration was 50 nM. The σ–core ratio is indicated. The incubation time with nucleoside triphosphates is 5 min. Lane M contains oligonucleotide markers. Arrows with numbers on the right indicate the position of the full-length transcripts. The clusters of paused transcripts are indicated by brackets.

downstream −10 elements (Ko *et al.* 1998). Then, with this template one can expect the appearance of the transcripts (besides the abortive ones) in a length range of about 12–20 nucleotides. The ratios of full-length transcripts to paused ones are vary with different holoenzymes under study, possibly indicating the difference in the properties of σ subunits. As of now this aspect is under investigation.

4. Discussion

A major objective of this study was to probe the functional role of His180 located in the NCR of σ^{70} between regions 1.2 and 2.1. We have introduced the following alterations: H180A and H180E. To understand the effects of mutations, the activities of RNA holoenzyme variants were tested in DNA-binding and *in vitro* transcription experiments.

The analysis of σ–core reconstitution mixtures using native gel electrophoresis and the σ saturation experiments on *in vitro* transcription and DNA binding suggests the reduced affinity of σ^{70}(H180A) and σ^{70}(H180E) to the core enzyme as compared with that of WT σ, with a more pronounced effect observed for σ^{70}(H180E). This means that residue His180 can participate in core binding by σ^{70} subunit probably through the interaction with some of the closely positioned residues. Lys376 is one of the candidates for these interactions (figure 1c), because it is located inside the segment of residues 361–390, which, as shown by Lesley and Burgess (1989), is sufficient for the binding of large fragments of σ^{70} to the core enzyme.

The single-round *in vitro* transcription assays with a fixed amount of DNA template and increasing concentrations of different holoenzymes, in conjunction with gel retardation experiments using promoter-containing DNA, showed that the mutant holoenzyme variants have a lower affinity for double-stranded promoter DNA.

The heparin challenge experiments with the use of both double-stranded and single-stranded probes allow one to suppose that residue His180 can influence the isomerization of RNA polymerase to heparin-resistant form and thus affect the formation of open promoter complex. As mentioned above, His180 is located in close vicinity to a highly acidic region (188–209), which presumably inhibits binding of free σ^{70} subunit to DNA (Malhotra *et al.* 1996). Moreover, according to a recently proposed model of σ^{70} location in the RNAP–promoter complex (Hudson *et al.* 2009), this region is situated close to the −10 promoter element. One can suppose that His180 plays a structural role, and maintains the proper orientation of the acidic loop. The mutations under study could result in conformational rearrangements with an accompanying negative effect on DNA binding.

Figure 6. Effect of variation of σ^{70}–core ratio and holoenzyme concentration on single-round *in vitro* transcription using mutant σ^{70}. Panels (A): autoradiograms of electrophoretic separation (8% denaturing polyacrylamide gel) of the transcripts produced from promoters of templates TI (**a**–**c**) and TIV (**d**) with the use of holoenzymes, reconstituted with WT or mutant variants of σ^{70} (indicated). Panels (B): the corresponding curves of titration with σ subunits (**a**–**c**) or holoenzymes (**d**). DNA concentration was 10 nM, and core RNAP concentrations were 25 nM (**a**), 50 nM (**b**) and 100 nM (**c**). The σ–core ratio was 6 (**d**). The yields of transcripts are in arbitrary units. Assays were performed two or three times with similar results. Typical results are presented in the figure. Standard deviations do not exceed 15%. Lane M contains the 43-mer oligonucleotide marker. Arrows with numbers on the right (**d**) indicate the position of the transcripts.

There are also other explanations of the data obtained. Substitution of His180 by glutamic acid resulted not only in noticeable reduction in binding with the double-stranded promoter but also with the non-template strand of consensus promoters. One can speculate that the deprotonated side chain of Glu180 can coordinate Lys414 and Lys418, which are located in close proximity to residue 180 and are known to participate in formation of open complex by non-specific interaction with the phosphate backbone of −10 containing DNA and thus stabilizing the overall structure of 2.3 region, the aromatic residues of which provide DNA melting (Tomsic *et al*. 2001). Formation of H-bonds between amino groups of these lysines and carboxy group of Glu180 can disrupt the interaction of these lysines with DNA. In the case of Ala180, these interactions should be intact; which is why this type of mutation probably did not influence the overall binding with the single-stranded −10 promoter element. At the same time the direct interaction His180 with promoter DNA cannot be excluded.

There are some published data that argue in favour of the above considerations on the structural role of His180. It is well known that the natural σ subunit demonstrates anomalously low mobility in SDS/polyacrylamide gel (Burton *et al*. 1981), and some point mutations, which influence the interaction of N-terminus with DNA-binding domains (regions 2.3–2.4), can significantly alter the σ subunit mobility, reversing it to theoretically expected values (Gopal *et al*. 1994; Gopal and Chatterji 1997). Moreover, it has been suggested that the acidic loop could be responsible for anomalous mobility of proteins (Lowe *et al*. 1979). In our work we also observed that the mutant variant σ^{70}(H180E), which demonstrated the most pronounced effects in all assays, migrates in SDS/polyacrylamide gel slightly faster than WT σ subunit, pointing to participation of residue at position 180 in a local rearrangement of the structure. In a series of other studies of DNA-binding domains occlusion by N-terminal regions, Dombroski *et al.* (1993) have demonstrated that elimination of 180–273 amino acids from the N-terminus results in substantial (an order of magnitude) increase in DNA-binding ability of truncated derivatives compared with that of other mutants with shorter truncations (50–150 amino acids). It is interesting to note that these derivatives are devoid of H180. Thus, this residue is located in the part of NCR involved (in conjunction with region 1) in masking of DNA-binding domains in the free σ subunit.

It should be noted that the alignment of the sequences of NCR to σ^{70} subunits from a series of related bacteria (analysis was done using Basic Local Alignment Search Tool and bacterial database *xbase.bham.ac.uk*; data not shown) demonstrated high conservation of a particular histidine residue in this region.

Taken together, the data presented indicate that residue His180, located in the NCR of σ^{70} subunit, influences (probably in an indirect way) several processes, including core binding, promoter DNA recognition and open complex formation. Close proximity of this residue to several functional regions of the protein allows one to suppose that it can act as a structure-forming component that maintains the proper local conformation of the macromolecule, thus participating in subtle regulation of transcription initiation.

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