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Characterisation of holoenzyme lacking σ^N Regions I and II

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ABSTRACT

The sigma-N (σ^N) protein associates with bacterial core RNA polymerase to form a holoenzyme that is silent for transcription in the absence of enhancer-binding activator proteins. Here we show that the acidic Region II of σ^N from *Klebsiella pneumoniae* is dispensable for polymerase isomerisation and transcription under conditions where the inhibited state of the holoenzyme is relieved by removal of σ^N Region I sequences. Holoenzymes lacking Region I or Regions I+II were equally susceptible to the order of addition-dependent inhibition or stabilisation of DNA binding afforded by *in trans* Region I sequences. Region I+II-deleted σ formed a holoenzyme with a DNA-binding activity more susceptible to inhibition by non-specific DNA than that lacking Region I. Region II sequences appear more closely associated with formation of a holoenzyme and σ proficient in DNA binding than with changes in holoenzyme conformation needed for unmasking a single-strand DNA-binding activity used for open complex formation. Region II may therefore function to optimise DNA interactions for an efficient σ cycle.

INTRODUCTION

Regulated transcription depends upon the association of RNA polymerase with a variety of factors that can direct promoter binding and control initiation rates. In bacteria the RNA polymerase (RNAP) containing the σ^N factor functions in enhancer-dependent transcription of genes associated with various stress and growth limiting conditions (1,2). The σ^N transcription mechanism is distinct from that using the major σ^{70}-type factor. Activation of σ^{70} transcription often involves a recruitment mechanism to increase promoter occupancy, whereas activation of the σ^N-holoenzyme depends upon accelerating post-binding steps. Activators of the σ^N-holoenzyme catalyse formation of transcription-competent open promoter complexes in a reaction requiring γ–β bond hydrolysis of nucleoside triphosphates (3–5). Response to activator requires the N-terminal Region I of σ^N and removal of Region I allows the holoenzyme to isomerise and to productively interact with DNA templates in which the start site proximal sequences are melted out (6–9). Region I maintains the holoenzyme in an inhibited state (8–10) and activator is believed to overcome Region I inhibition (7). Region I interacts with core RNAP, indicating that it may exert activity through core RNAP (11,12).

We have now extended our analysis of the function of Region I of *Klebsiella pneumoniae* σ^N (9) to examine dependence of Region I activity upon Region II sequences, an acidic part of σ^N that lies between Regions I and III (see ref. 2; Fig. 1A). Region III contains sequences important for binding promoter DNA and core RNAP (13–16). Protein footprints implicate Region II sequences in core RNAP binding (11,17), but whether the interaction is direct or indirect is not clear (11,12). Deletion and insertion mutagenesis implicates Region II in promoter melting (18). Hence Region II appears to contribute to core-dependent activities of the holoenzyme, of potential significance to the regulated opening of the promoter DNA.

We have compared the properties of holoenzymes assembled from Region I-deleted (RI$\Delta\sigma^N$) and Region I+II-deleted (RI+II$\Delta\sigma^N$) σ and assayed for the activator-independent isomerisation of the holoenzyme that occurs when Region I is deleted (9). Response of the Region I+II-deleted σ to Region I sequences presented *in trans* was determined to avoid complications associated with changing the *cis* relationship of Region I. The results show that Region II is dispensable for RNAP isomerisation, interaction with pre-melted DNA and activator-independent transcription from supercoiled DNA templates. However, Region II contributes to the DNA-binding activity of the holoenzyme, possibly reducing core–σ dissociation, and appears important for promoter-specific binding.

MATERIALS AND METHODS

Proteins

The *K.pneumoniae* σ^N protein (amino acids 1–477) and derivatives RI$\Delta\sigma^N$ (amino acids 57–477) and RI+II$\Delta\sigma^N$ (amino acids 107–477) were prepared as before (14,17,19; Fig. 1A). Purified σ^N N-terminal sequences 1–56 (Region I, RI) were obtained by overproducing an N-terminal histidine-tagged fragment as a soluble sequence in *Escherichia coli* (9,12; Fig. 1A). A purified C-terminal-deleted form of activator, PspFΔHTH, was used in activation assays (17,20,21). Purified core RNAP was from *E.coli* (a generous gift from A. Kolb, Pasteur Institute, Paris). Working

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protein solutions were stored at –20°C, stocks at –70°C, in 10 mM Tris–HCl pH 8.0, 50% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT (TGED) containing 50–250 mM NaCl.

DNA heteroduplex formation

Mismatched *Rhizobium meliloti nifH* promoter DNA heteroduplexes (Fig. 1B) were prepared by annealing radioactively 5'-^{32}P-end-labelled oligonucleotides (9). The extent of non-duplex DNA from –10 to –1 was chosen by reference to the known start of DNA melting at the *nifH* promoter (14,22) and previous work with the σ^N-dependent *glnA*p$_2$ promoter (3,10,23). Oligonucleotides were size purified on 8% denaturing urea–polyacrylamide gels (detection was by UV shadowing) prior to kinasing. Pairs of DNA strands with either the unlabelled strand present at a 2-fold molar excess (10 pmol in 20 µl) over [^{32}P]DNA or, for preparing unlabelled heteroduplex template DNA for transcription assays, with both strands at equal concentration were heated at 95°C for 3 min in 10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$ and then rapidly chilled in iced water for 5 min to allow heteroduplex formation.

DNA homoduplex formation

For DNA footprinting, homoduplex template DNA was prepared by extending *R.meliloti nifH* M13mp19 single-stranded DNA with 5'-^{32}P-labelled universal primer as described previously (14). Fully complementary end-labelled oligonucleotides for preparing –60 to +28 homoduplex DNA (Fig. 1B) were annealed together as described above.

Native gel complex formation assays

A gel shift assay (17,24) was employed to detect σ^N and its holoenzyme bound to a radioactively labelled *R.meliloti nifH* homoduplex or heteroduplex promoter DNA fragment. Typical holoenzyme interaction assays included 100 nM core RNAP plus 200 nM σN or RIΔσ^N or 600 nM RI+IIΔσ^N and 1.6 or 16 nM homoduplex or heteroduplex DNA plus, where necessary, nucleotide (see figure legends) in STA buffer (25 mM Tris–acetate, pH 8.0, 8 mM magnesium acetate, 10 mM KCl, 1 mM DTT, 3.5% w/v PEG 8000). Experiments with RI+IIΔσ^N employed a ratio of six σ to one core RNAP to ensure full holoenzyme formation. For activation 4 µM PspFΔ-HTH activator protein (17,21) and 1–4 mM nucleotide (see figure legends) were also added. Core RNAP, σ proteins and DNA were pre-incubated at 30°C for 10 min and then, if necessary, nucleotide and activator were added for 10 min followed by a glycerol bromophenol blue loading dye (final concentration 10% glycerol) and, if required, heparin (final concentration 100 µg/ml). Samples were then loaded onto 4.5% native polyacrylamide gels to separate free and bound DNA which were detected by autoradiography. Quantitative data were from phosphorimager analyses. To enhance the reliability of our data, binding assays were replicated two to three times each and representative data are shown.

In vitro transcription assays

Heteroduplex promoter DNA fragments (16 nM) and holoenzyme (100 nM) were incubated in a 40 µl reaction for 10 min at 30°C in STA buffer containing 40 U of RNase inhibitor (RNasin; Promega) prior to the addition of 1 mM GTP or 1 mM ATP (for [α-^{32}P]UTP and [γ-^{32}P]GTP transcript assays, respectively) and 4 µM PspFΔHTH activator protein to allow open complexes to form. After a further 10 min, heparin (100 µg/ml) and elongation mix [ATP, CTP and UTP at 0.1 mM and 12.5 µCi [α-^{32}P]UTP (800 Ci/mmol; Amersham) or GTP, CTP and UTP at 0.1 mM and 10 µCi [γ-^{32}P]GTP (5000 Ci/mmol; Amersham)] were added to allow synthesis of transcripts, which were allowed to accumulate for 10 min before phenol extraction and ethanol precipitation. Samples were run on 15% denaturing polyacrylamide gels and transcripts were visualised by autoradiography. For transcription from supercoiled DNA, template DNA (10 nM) was plasmid pMKC28 containing the *R.meliloti nifH* promoter fragment from pMB210.1 (22,25) cloned into pTE103 (26). After holoenzyme and nucleotide pre-incubations as indicated in the figure legends, heparin (100 µg/ml) plus remaining nucleotides (0.1 mM each) and 3 µCi [α-^{32}P]UTP were added for 10 min. RNA was precipitated and analysed on 6% sequencing gels. Detection was by autoradiography and phosphorimaging.

DNA footprints

Footprints using S1 nuclease were conducted as described before (9). Briefly, end-labelled linear homoduplex template DNA (1.6 nM) prepared by primer extension of a single-strand M13mp19 clone of the *R.meliloti nifH* promoter (14,27) was incubated with either 2 µM σ or 100 nM holoenzyme in STA buffer for 10 min at 30°C. For activation 4 mM GTP and 4 µM PspFΔHTH were added for a further 10 min. Binding reactions (25 µl) were then exposed to 700 U of S1 nuclease (Pharmacia) for 5 min before termination by the addition of 10 mM EDTA and rapid phenol extraction. DNA was recovered by ethanol precipitation and analysed on a 6% sequencing gel. Markers were generated by chemical cleavage of the template DNA with piperidine following partial methylation using dimethylsulphate. Detection was by autoradiography.

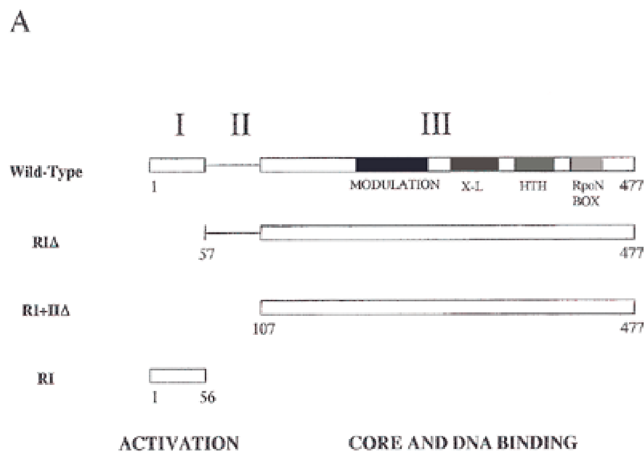
RESULTS

The stable association of the σ^N-holoenzyme with pre-melted DNA can occur independently of enhancer binding activator protein if the σ N-terminal Region I sequences are deleted (9). To evaluate the contribution of Region II sequences to the association of holoenzyme with melted DNA we used a σ Region I+II deletion protein lacking the first 106 residues (107–477, RI+IIΔσ^N) and compared its properties with holoenzyme containing σ sequences 57–477 (RIΔσ^N) (Fig. 1A). We measured the interaction of holoenzymes with wild-type homoduplex and heteroduplex *R.meliloti nifH* promoter DNA (Fig. 1B).

Region II is not required for holoenzyme association with melted DNA templates, but does assist σ DNA binding

We compared the DNA binding affinities of RI+IIΔσ^N, RIΔσ^N, wild-type σ^N and their holoenzymes for heteroduplex 1 DNA using a gel mobility shift assay (9; Fig. 1B). Results show that the RI+IIΔ- and RIΔ-holoenzymes bind the heteroduplex DNA with similar affinities (Fig. 2A). The affinity of the wild-type σ^N-holoenzyme for heteroduplex DNA was less than that of the RI+IIΔ- and RIΔ-holoenzymes, consistent with a less extensive DNA contact (9).

Comparing the affinities of the σ proteins for the heteroduplex DNA we observed that RI+IIΔσ^N bound the hetero-



B

-25 -14 +1

Wild-type Homoduplex
-60---CTGGCAC---TTTTGCACGATCAGCCCTGGGCGCG--+28
---GACCGTG---AAAACGTGCTAGTCGGGACCCGCGC--

Heteroduplex 1
-60---CTGGCAC---TTTTGCAC*TCGACTAAAG*GGGCGCG--+28
---GACCGTG---AAAACGTGCTAGTCGGGACCCGCGC--

Heteroduplex 2
-60---CTaaCAC---TTTTGCAC*TCGACTAAAG*GGGCGCG--+28
---GAttGTG---AAAACGTGCTAGTCGGGACCCGCGC--

Figure 1. (**A**) *Klebsiella pneumoniae* σ^N sequences assayed. Full-length σ (residues 1–477) is divided into Regions I–III (2) based on sequence alignments. The N-terminal Region I is required for enhancer responsiveness and also inhibits the productive interaction of σ^N-holoenzyme with melted DNA (9,23,30). Region II is acidic and variable amongst σ^N class members. Region III contains core RNAP-binding sequences and DNA-binding determinants which include a site for promoter DNA cross-linking (X-L), a helix-turn-helix motif (HTH) and a conserved eight residue sequence termed the RpoN box, together with sequences that modulate DNA binding (13,19,35,36). Purified *K.pneumoniae* σ sequences 1–477, 57–477, 107–477 and 1–56 were used in this work (9,14). (**B**) Homoduplex and heteroduplex linear DNA sequences comprising nucleotides –60 to +28 of the *R.meliloti nif*H promoter bearing a near consensus σ^N-binding site were used for σ and holoenzyme DNA-binding experiments. Heteroduplex 1 carries a –10 to –1 top strand mismatch (9) and control heteroduplex 2 a GG→AA change in the –24 consensus promoter element in addition to the –10 to –1 mismatch sequence.

duplex DNA poorly (Fig. 2B). Clearly, although Region II of σ^N is not required for association of holoenzyme with heteroduplex promoter DNA, formation of the RI+IIΔσ^N–melted DNA complex is somewhat Region II dependent. However, diminished promoter DNA binding of RI+IIΔσ^N to template DNA is not specific to melted DNA targets, since weak binding to homoduplex DNA is observed (14,28; see also below). Possibly core to melted DNA contacts help heteroduplex binding of the RI+IIΔ-holoenzyme (9).

Heparin-stable complexes form with melted DNA independently of Region II

The σ^N-holoenzyme closed promoter complexes are disrupted by the polyanion heparin (3,29) whereas activator-dependent σ^N-holoenzyme open promoter complexes are heparin resistant. Removal of Region I of σ^N allows the holoenzyme to form stable heparin-resistant complexes on pre-melted DNA without activator (9). It was also shown that the initiating nucleotide at the *R.meliloti nifH* promoter, GTP, increased stability of the RIΔ-holoenzyme–heteroduplex DNA complexes in a heparin challenge assay and that phosphodiester bond formation was not required (dideoxy GTP stabilised equally well; 9).

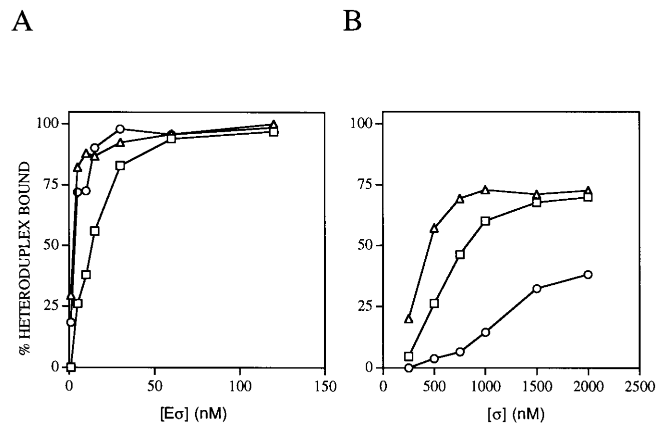


Figure 2. Holoenzyme (**A**) and σ (**B**) binding to heteroduplex 1 DNA. Increasing amounts of full-length σ^N (open square), RIΔσ^N (open triangle) and RI+IIΔσ^N (open circle) or their holoenzymes [core (E):sigma (σ) at 1:2 for full-length σ^N and RIΔσ^N, 1:6 for RI+IIΔσ^N] were combined with heteroduplex 1 (1.6 nM) in a gel shift DNA-binding assay. Bound and unbound DNA was separated by native gel electrophoresis and the percentage of bound DNA quantified by phosphorimager analysis (9).

We therefore examined whether Region II contributed to the formation of activator-independent heparin-resistant complexes on pre-melted heteroduplex DNA. Additionally, the effect of nucleoside triphosphates (GTP, CTP, ATP and UTP), dGTP, ddGTP and non-hydrolysable GTP analogues (GTP-γ-S and 5'-guanylyl-imidodiphosphate) upon complex stability was measured (Fig. 3 and data not shown). Assays allowed the binding of the RI+IIΔ-, RIΔ- and wild-type σ^N-holoenzymes to heteroduplex 1 promoter DNA either in the presence or absence of nucleotide. Subsequently, complexes were challenged with heparin, separated by native gel electrophoresis and the number of holoenzyme-bound DNA complexes surviving the challenge measured with time (Fig. 3).

With the wild-type σ^N-holoenzyme and in the absence of nucleotide the heparin challenge resulted in a rapid decay of complexes representing the destabilisation of closed complexes and the presence of a few very stable complexes (Fig. 3, GTP panel, open squares). Nucleotides and GTP analogues had no effect on the heparin stability of wild-type σ^N-holoenzyme–heteroduplex DNA complexes (9; data not shown). The stability of the RI+IIΔ- and RIΔ-holoenzyme complexes on heteroduplex DNA in the absence of nucleotide was greater than those formed by σ^N-holoenzyme (Fig. 3, GTP panel, open symbols). All potential initiating nucleotides (GTP, ddGTP or the GTP analogues GTP-γ-S and 5'-guanylyl-imidodiphosphate) increased the stability of RI+IIΔ- and RIΔ-holoenzyme–heteroduplex DNA complexes (Fig. 3, closed symbols, and data not shown). Nucleotides that are not efficient initiating nucleotides (ATP, UTP, CTP and dGTP) at the *R.meliloti nifH*

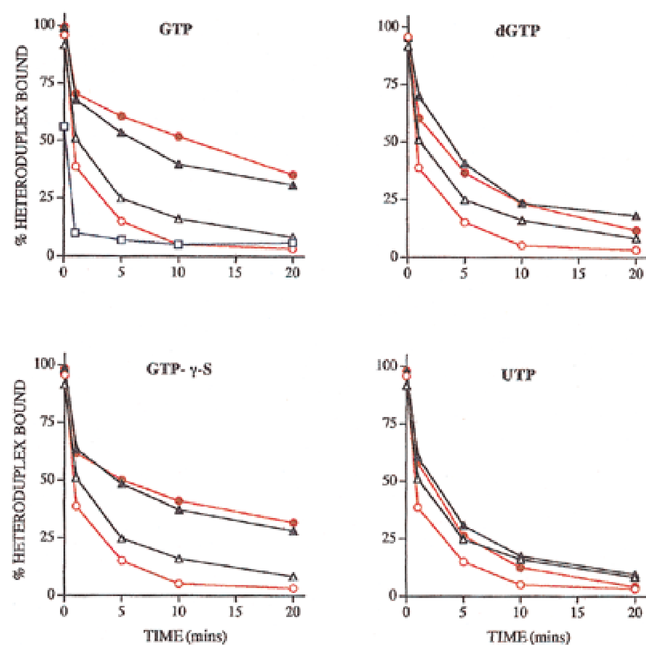


Figure 3. Holoenzyme stability on heteroduplex DNA. Full-length σ^N- (square), RIΔ- (triangle) and RI+IIΔ-holoenzymes (circle) (100 nM, E:σ as in Fig. 2) were incubated with heteroduplex 1 DNA (16 nM) without (open symbols) or with (filled symbols) a nucleotide or nucleotide analogue (1 mM GTP, dGTP, GTP-γ-S or UTP) for 10 min prior ($t = 0$) to a 100 µg/ml heparin challenge. Samples were removed 1, 5, 10 and 20 min after the addition of heparin and immediately run on native gels (9). Following phosphorimager analysis the percentage of heparin-resistant bound DNA complexes remaining were plotted against time.

promoter did not stabilise (Fig. 3 and data not shown; see also refs 3,8). As expected from the selectivity of RNAP (3,8), dGTP did not stabilise complexes (Fig. 3). In all cases examined the stability and number of heparin-stable complexes formed by the RI+IIΔ- and RIΔ-holoenzymes were very similar. A control experiment with the mutant –24 heteroduplex promoter fragment (heteroduplex 2; see Fig. 1B) with conserved GG residues changed to AA showed that stable complex formation required the consensus promoter sequence (less than 1% ddGTP-stablised holoenzyme complexes were detected after an 8 min challenge with heparin; data not shown).

We conclude that stable complex formation with heteroduplex DNA occurs largely independently of Region II sequences. The stabilising effects of GTP and related nucleotides suggests that Region II does not control the selection of initiating nucleotides nor properties of the holoenzyme that are required for their stabilising effects. The inference that stabilisation by initiating nucleotides would correlate with an ability to make transcripts was subsequently confirmed (see below).

Region II influences σ and holoenzyme interactions with homoduplex DNA

Substitution of heteroduplex 1 DNA by the 88mer homoduplex DNA fragment showed that the formation of heparin-stable

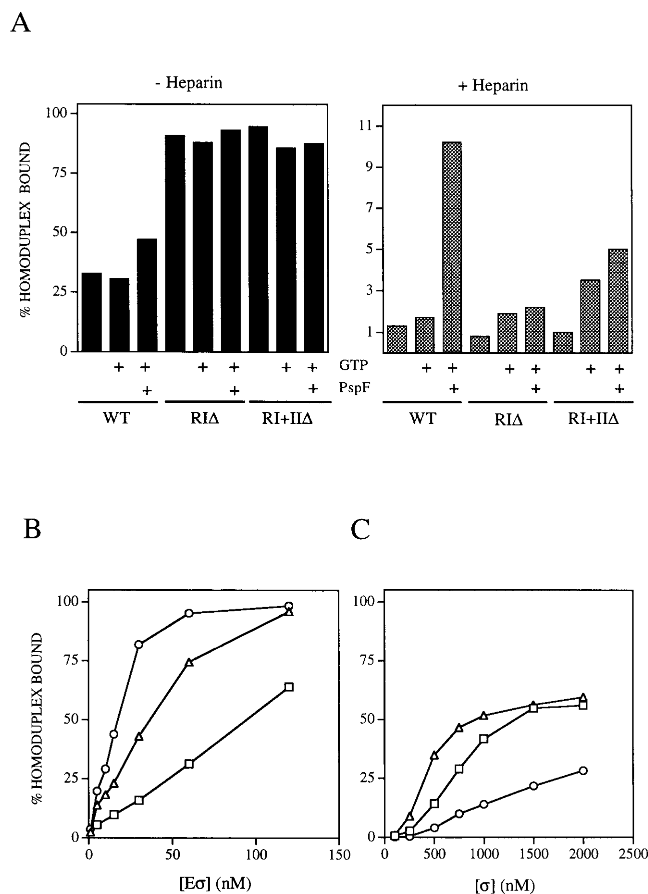


Figure 4. Heparin-resistant complex formation (**A**) and binding of holoenzymes (**B**) and σ proteins (**C**) with homoduplex DNA. For (A), wild-type, RIΔ- and RI+IIΔ-holoenzymes (250 nM, E:σ as in Fig. 2) were incubated with homoduplex DNA (16 nM) in the absence or presence of activator PspFΔHTH (PspF, 4 µM) and/or GTP (4 mM) as indicated (+). Prior to gel loading, heparin (100 µg/ml) was added to one half of the sample for 5 min. The number of initial bound complexes (minus heparin, black bars) and those surviving the heparin challenge (grey bars) are shown in the histograms. For (B) (holoenzyme, Eσ) and (C) (sigma, σ), titrations were carried out as described in Figure 2 except that homoduplex DNA (1.6 nM) was used. Full-length σ^N (open square), RIΔσ^N (open triangle) and RI+IIΔσ^N (open circle). In (A), for σ^N-holoenzyme, ~30% DNA was bound by free σ^N.

complexes with the RI+IIΔ- and RIΔ-holoenzymes largely required the pre-melted –10 to –1 DNA segment (Fig. 4A). The number of initial complexes forming with the RI+IIΔ- and RIΔ-holoenzymes and homoduplex DNA were similar, but they were rapidly destroyed by heparin (<5% of initial complexes survived 5 min with heparin) regardless of the presence of activator PspFΔHTH and/or GTP (Fig. 4A). This result contrasts with the stable complex formation between the RI+IIΔ- and RIΔ-holoenzymes and heteroduplex DNA (Fig. 3). Heparin-resistant complexes with homoduplex DNA were formed with the wild-type σ^N-holoenzyme in the presence of activator and GTP (Fig. 4A). These results are consistent with prior data indicating that closed complexes with RI+IIΔ- and RIΔ-holoenzymes do not respond to activator and nucleotide to form stable open complexes on conventional linear DNA templates (9).

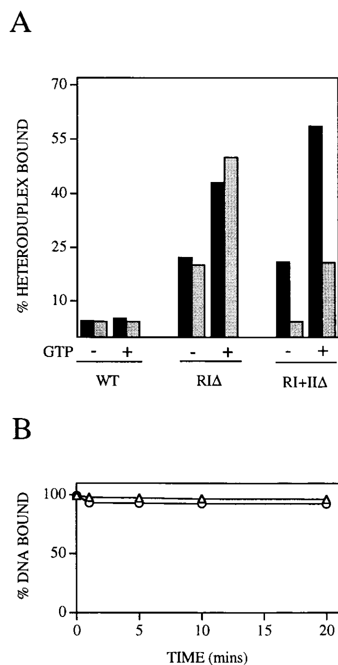


Figure 5. The effect of non-specific DNA on stable complex formation. (**A**) Binding of wild-type σ^{N}-, RIΔ- and RI+IIΔ-holoenzymes (100 nM, E:σ as in Fig. 2) to heteroduplex 1 DNA (16 nM) in the absence (black bars) or presence (grey bars) of non-specific salmon sperm DNA (455 ng/µl). GTP (1 mM) is included in the assay where indicated (+). The number of complexes surviving an 8 min heparin challenge are shown. (**B**) Challenge of pre-bound RIΔ- (open triangle) and RI+IIΔ-holoenzymes (open circle) (100 nM, E:σ as in Fig. 2) with salmon sperm DNA (455 ng/µl). After binding to heteroduplex DNA (16 nM) for 10 min in the absence of any nucleotide a sample was taken ($t = 0$) and salmon sperm DNA was added. The number of remaining complexes was then followed with time.

The σ^{N}-holoenzyme formed fewer initial complexes (minus heparin) with homoduplex DNA than did the deleted σ proteins (Fig. 4A). Titrations of the binding of holoenzymes to the homoduplex DNA showed that the N-terminal-deleted σ proteins assembled into holoenzymes with greater DNA binding activity than the wild-type σ^{N}-holoenzyme (Fig. 4B). Consistent with this, DNA footprints showed that removal of Region I and Regions I+II increased the interaction of holoenzyme with promoter DNA (see below). In titrations exceeding 100 nM σ^{N}-holoenzyme, the free σ^{N} bound the DNA (data not shown) limiting the amount of DNA available to bind holoenzyme, explaining why 250 nM σ^{N}-holoenzyme (Fig. 4A) does not bind more DNA than 120 nM (Fig. 4B).

RI+IIΔσ^{N} had lower affinity for homoduplex (Fig. 4C; ref. 14) and heteroduplex DNA (Fig. 2B) than wild-type σ^{N} or RIΔσ^{N}, suggesting that Region II contributes to σ DNA-binding activity regardless of whether the DNA is in a melted state. However the RI+IIΔ-holoenzyme bound homoduplex DNA slightly better than the RIΔ-holoenzyme (Fig. 4B), contrasting with the heteroduplex DNA results (Fig. 2A), where both holoenzymes bound similarly. The absence of Region II appears to be associated with a modest shift towards favouring holoenzyme binding to the unmelted DNA (compare Figs 2A and 4B).

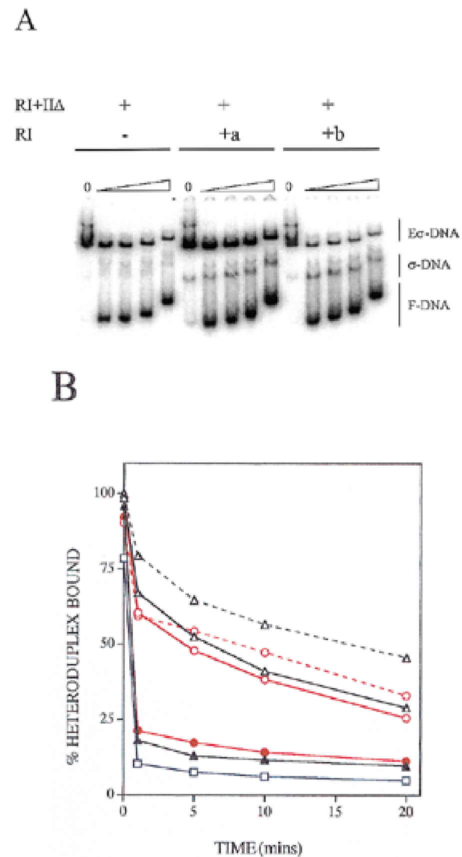


Figure 6. Region I *in trans* influences the stability of holoenzyme DNA complexes. (**A**) Gel mobility shift assay showing the effect of Region I on the stability of RI+IIΔ-holoenzyme–heteroduplex DNA complexes. +a, Region I added after DNA binding; +b, Region I added before DNA binding. Region I of σ^{N} (residues 1–56, 2 µM) was incubated with RI+IIΔ-holoenzyme (100 nM) for 5 min prior to adding 16 nM heteroduplex 1 DNA (+b) or for 10 min with preformed holoenzyme–DNA complexes (+a). GTP (1 mM) was added for 1 min after which ($t = 0$) a sample was taken, heparin (100 µg/ml) was then added and further samples taken with time and loaded onto a running native gel. Holoenzyme– and σ–DNA complexes (Eσ–DNA and σ–DNA) and free DNA (F-DNA) are indicated. Triangles above the lanes indicate increasing time with heparin (1, 5, 10 and 20 min); lanes 0, minus heparin. (**B**) Stability curves for the RI+IIΔ-holoenzyme as in (A) and equivalent experiment with RIΔ holoenzyme. The percentages of holoenzyme–DNA complexes surviving the heparin challenge are shown. A stability curve for the wild-type σ^{N}-holoenzyme in the absence of Region I is shown for comparison. Full-length σ^{N}- (square), RIΔ- (triangle) and RI+IIΔ-holoenzymes (circle). Controls were holoenzymes in the absence of Region I (solid lines, open symbols). Region I added to holoenzymes prior to adding heteroduplex DNA (solid lines, filled symbols) for 5 or 10 min after holoenzyme DNA binding (dashed lines, open symbols).

Holoenzyme formation is influenced by Region II

We confirmed previous work (14) showing that at least twice as much RI+IIΔσ^{N} than RIΔσ^{N} was needed to saturate core RNAP, suggesting that Region II plays a role in stabilising the holoenzyme (data not shown). When we conducted heteroduplex DNA binding assays in the presence of non-specific DNA (holoenzyme was pre-formed without DNA, then added to a mixture of heteroduplex DNA and salmon sperm DNA),

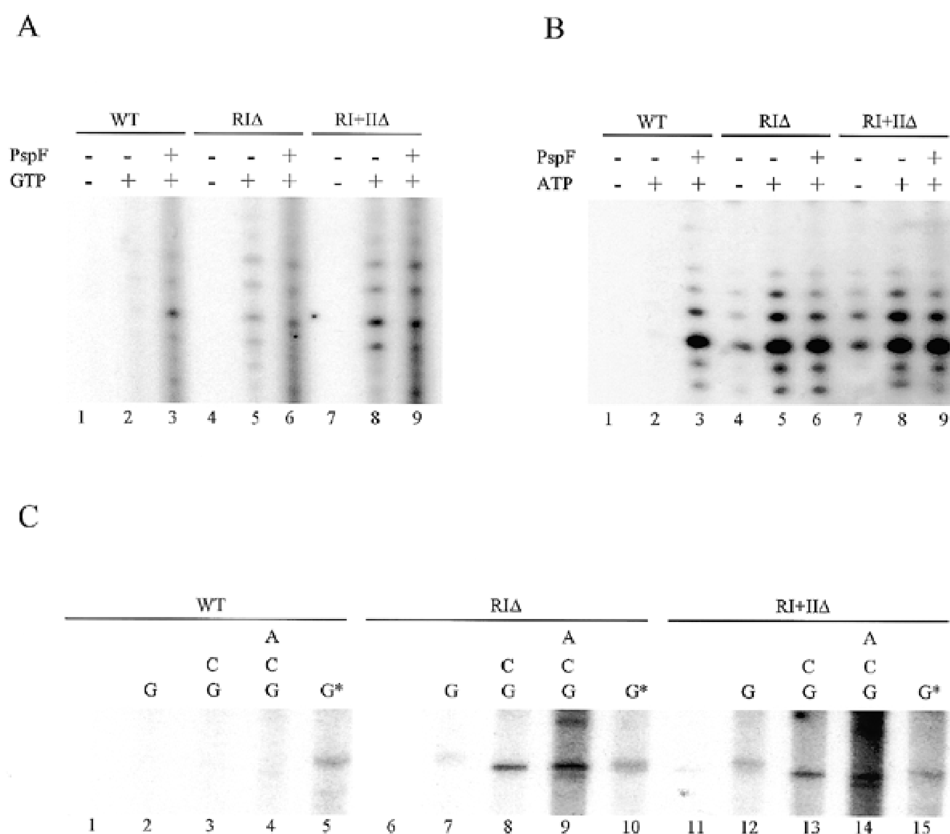


Figure 7. Activator-independent transcription activity of the RIΔ- and RI+IIΔ-holoenzymes. (**A** and **B**) [α-^{32}P]UTP- and [γ-^{32}P]GTP-labelled transcripts from heteroduplex DNA with GTP or ATP (for A and B, respectively) as the hydrolysable nucleotide used by the activator. Holoenzymes (100 nM, E:σ as in Fig. 2) with GTP or ATP at 1 mM in the presence or absence of PspFΔHTH (4 µM) were used to transcribe the 88mer heteroduplex 1 template DNA (16 nM). Adding ATP, CTP and UTP (A) or GTP, CTP and UTP (B) started transcripts. (**C**) Transcripts labelled using [α-^{32}P]UTP and supercoiled pMKC28 *R.meliloti nifH* DNA. Holoenzymes (100 nM, E:σ as in Fig. 2) alone or with nucleotide(s) (1 mM of each) or 4 mM GTP when used with 4 µM PspFΔHTH (G*, lanes 5, 10 and 15) were incubated with template DNA (10 nM) prior to heparin challenge and the addition of remaining rNTPs plus label. Wild-type σ^{N}-holoenzyme was used as a control for activated transcription.

the number of heparin-stable complexes formed with the RI+IIΔ-holoenzyme was 4- to 5-fold less than in the absence of non-specific DNA, a result contrasting with the relatively small effect of non-specific DNA upon stable complex formation by the RIΔ-holoenzyme (Fig. 5A). Salmon sperm DNA reduced the number of heparin-resistant RI+IIΔ-holoenzyme complexes regardless of the presence of GTP. Measurement of the number of initial complexes (minus heparin) forming between the RI+IIΔ- and RIΔ-holoenzymes showed that the non-specific DNA had the effect of diminishing them 4- to 5-fold for the RI+IIΔ-holoenzyme, but only by ~20% for the RIΔ-holoenzyme (data not shown). To test whether non-specific DNA preferentially disrupted the RI+IIΔ-holoenzyme, pre-formed RI+IIΔ- and RIΔ-holoenzymes were added to increasing amounts of salmon sperm DNA (25–455 ng/µl). Both holoenzymes behaved similarly and formed a non-specific complex with salmon sperm DNA (data not shown). This implies that the effect of non-specific DNA upon heteroduplex DNA binding by the RI+IIΔ-holoenzyme is at the level of its DNA-binding properties rather than the σ–core interaction *per se*.

When the RIΔ- and RI+IIΔ-holoenzymes were pre-formed in the absence of competitor DNA and then bound to heteroduplex DNA, a challenge with non-specific DNA showed them to be equally stable (Fig. 5B). Overall, the results show that Region II specifies some properties of the holoenzyme that suppress non-specific DNA binding.

The RI+IIΔ-holoenzyme responds to Region I *in trans*

The number of heparin-stable complexes between the RIΔ-holoenzyme and heteroduplex DNA is reduced by Region I provided *in trans* (9). We used the heparin challenge assay to determine if Region II sequences were required for the *in trans* inhibition and measured heteroduplex DNA binding and stability by the RI+IIΔ-holoenzyme in the presence or absence of added purified Region I. Region I was incubated with the RI+IIΔ-holoenzyme before or after heteroduplex DNA binding. Following challenge with heparin, samples were analysed on a native gel (Fig. 6A) and the fraction of holoenzyme–DNA complexes surviving the heparin challenge with time calculated (Fig. 6B). When Region I was present *in trans* (Fig. 6A, compare 0 lanes) a σ–DNA complex was detected. This

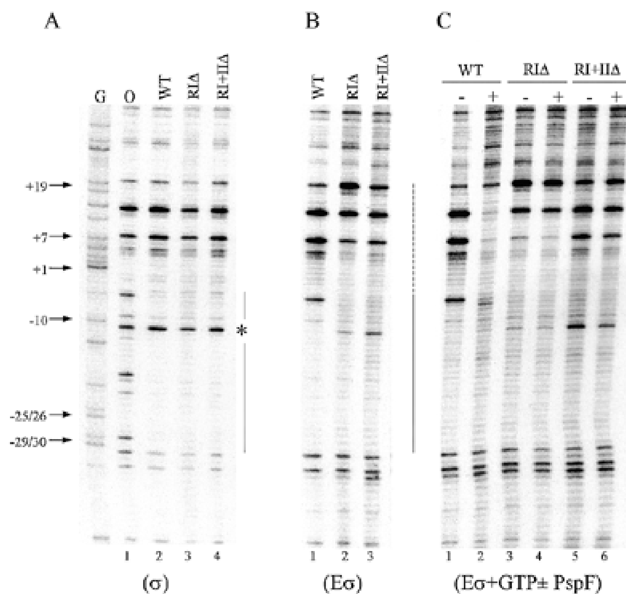


Figure 8. S1 nuclease footprints on linear homoduplex *R.meliloti nifH* promoter DNA. (**A**) Wild-type σ^N, RI$\Delta\sigma^N$ and RI+II$\Delta\sigma^N$ footprints. (**B**) Holoenzyme footprints. (**C**) Holoenzyme footprints in the presence of GTP with (+) or without (–) activator PspFΔHTH. The extent of the footprint seen with the holoenzymes (B and C) or σ proteins (A) is indicated by a solid line, broken to indicate the weaker footprint between +7 and +20. The star indicates the –10 cutting lost on holoenzyme binding. Reactions contained 1.6 nM template DNA, σ proteins at 2 µM, holoenzymes at 100 nM (core:sigma as in Fig. 2), activator PspFΔHTH at 4 µM and GTP at 4 mM. Chemical cleavage of template DNA was used to generate a G sequencing ladder as a marker (A, lane G).

implies that Region I *in trans* can assist binding of RI+II$\Delta\sigma^N$, but whether binding is via a holoenzyme intermediate is not clear. With the RIΔ-holoenzyme and Region I *in trans* a σ–DNA complex was not seen (data not shown).

When Region I was added to the RI+IIΔ-holoenzyme prior to heteroduplex DNA binding, the number of initial DNA complexes did not diminish but the number of heparin-stable complexes was significantly reduced to a level seen with the wild-type σ^N-holoenzyme alone (Fig. 6B). Adding Region I after engagement of the RI+IIΔ-holoenzyme with heteroduplex DNA resulted in a modest stabilisation of complexes (Fig. 6B). For comparison, the results for an equivalent experiment with the RIΔ-holoenzyme and Region I *in trans* are also shown (Fig. 6B). Similar results were observed with the RIΔ-holoenzyme (Fig. 6B; 9).

We conclude that Region I exerts its inhibitory effect upon the RI+IIΔ- and RIΔ-holoenzymes before assembly onto the heteroduplex DNA and that once engaged on melted DNA Region I helps to stabilise the holoenzyme complexes. Effects of Region I *in trans* occur independently of Region II.

Activator-independent transcription activity does not require Region II

The σ^N-holoenzyme only efficiently transcribes from heteroduplex DNA templates when activated whereas the RIΔ-holoenzyme transcribes without activator (9,10). With the RI+IIΔ-holoenzyme we observed that transcripts labelled with [α-^{32}P]UTP (for internal labelling) or [γ-^{32}P]GTP (for 5'-end labelling) formed independently of activator as they did for the RIΔ-holoenzyme (Fig. 7A and B). The pattern of transcripts seen with the holoenzymes assembled with the deleted σ proteins was indistinguishable from activated σ^N-holoenzyme transcripts (Fig. 7A and B, lanes 3). Clearly, activator-independent transcription activity on heteroduplex DNA is independent of Region II. Furthermore, patterns of transcripts indicate that the selection of the start site is not influenced by Region II (Fig. 7B). The latter is consistent with the restricted set of nucleotides that stabilise complexes on heteroduplex DNA (Fig. 3). The size and intensity of the transcripts strongly indicates that Region II makes little contribution to the catalytic activity of the RNAP or selection of initiating nucleotide and, by inference, the location of the catalytic centre of the polymerase over pre-melted DNA.

The absence of Region I allows the RIΔ-holoenzyme to transcribe independently of activator from the *R.meliloti nifH* promoter provided as a supercoiled DNA template (Fig. 7C; 8). Transcripts probably arise from holoenzyme that contacts with transiently melted DNA (8,9). However, the initiated complex must be formed (by the addition of GTP) to survive the heparin challenge step of the single cycle transcription assay (the transcript initiates 5'-GGG) (see below). When the RI+IIΔ-holoenzyme was assayed for activator-independent transcription it was found to transcribe to a similar level as the RIΔ-holoenzyme (Fig. 7C). A control with the wild-type σ^N-holoenzyme showed that activator and GTP was required to form transcripts when Region I was present *in cis* (Fig. 7C, lane 5). With the RIΔ-and RI+IIΔ-holoenzymes, to detect transcripts initiating nucleotide (GTP) or early incorporated nucleotides (G+C, G+C+A) had to be present prior to the heparin challenge (Fig. 7C, compare lanes with NTPs). This contrasts with the use of heteroduplex DNA templates, where complexes survive the heparin challenge prior to any nucleotide addition (Figs 7A and B and 3), presumably because the strands are stably separated. Interestingly, different combinations of nucleotides added prior to the heparin challenge resulted in slightly different sized activator-independent transcripts with both N-terminal deleted σ proteins (pre-incubation with GTP producing the largest transcript). Possibly the spatial relationship between transiently melting DNA sites and the catalytic centre of the holoenzyme is not fixed. However, in each case transcription occurs for the RIΔ- and RI+IIΔ-holoenzymes via a heparin-sensitive intermediate that does not require Region II for its formation. Clearly, Region II sequences are not needed for the productive association of holoenzyme with the melted DNA required for making transcripts (Fig. 7) nor for the inhibition of this association by Region I (Fig. 6). Additionally. the catalytic function of the polymerase does not rely upon Region II sequences.

Extended S1 nuclease footprints of the RI+IIΔ-holoenzyme

Removal of Region I of σ^N reveals a single-strand DNA-binding activity in the holoenzyme that suggests how the holoenzyme can transcribe from stably melted (heteroduplex DNA) or transiently melted (supercoiled DNA) templates and why such complexes are accessible to the kinetic stabilisation afforded by initiating nucleotides in heparin challenge assays (9; this paper). S1 nuclease footprinting assays have shown that the RIΔ-holoenzyme has isomerised and has a DNA foot-

print that extends beyond the transcription start (9). Extra holoenzyme–DNA contacts upon removal of Region I are also consistent with improved DNA binding by the RI+IIΔ- and RIΔ-holoenzymes (Figs 2 and 4).

To assess the contribution of Region II sequences to RNAP isomerisation we used S1 nuclease footprinting. RI+IIΔσ^N had a footprint from around –33 to –5 equivalent to that of RIΔσ^N and wild-type σ^N, with the sequence around –10 being susceptible to cleavage by S1 nuclease (Fig. 8A). In contrast, the wild-type σ^N-, RI+IIΔ- and RIΔ-holoenzymes all protected the –10 residue from cleavage but differed in their footprints near to start site proximal bases (Fig. 8B; 9). Compared to the σ^N-holoenzyme, the RI+IIΔ-holoenzyme produced an extended footprint similar to that of the RIΔ-holoenzyme with the residue at +20 showing increased reactivity to cleavage (Fig. 8B). We conclude that the RI+IIΔ-holoenzyme has undergone the same change as the RIΔ-holoenzyme and that the extended footprint represents the isomerised state of the holoenzyme (9).

The RI+IIΔ-holoenzyme consistently produced a weaker footprint than the RIΔ-holoenzyme (Fig. 8B, lanes 2 and 3, and C, lanes 3 and 5). The footprints did not change with activator or nucleotide (compare Fig. 8B, lanes 2 and 3, with C, lanes 3–6). The latter is consistent with requirements for Region I sequences to allow enhancer responsiveness. Indifference of the footprint to nucleotide likely reflects few initiated complexes on linear DNA. Activated σ^N-holoenzyme also shows an extended footprint to +20 (previously we had seen partial protection to +7 but with 4-fold less GTP as the initiating nucleotide; 9) with the +7 to +20 sequence being strongly protected from cleavage (Fig. 8C, lane 2). Presumably the extended core-dependent footprint seen in the absence of Region I or Regions I+II or upon activation of the σ^N-holoenzyme reflects, through polymerase isomerisation, the unmasking of determinants in the core polymerase that bind the single-strand DNA of the melted out segments of the heteroduplex templates to achieve heparin-stable complex formation.

We conclude that Region II sequences are unimportant for maintaining a conformation of the holoenzyme that allows an extended interaction with DNA.

DISCUSSION

Current models for transcription initiation by the σ^N RNAP implicate Region I in determining activator responsiveness (8,9) and Region II in aspects of DNA melting, possibly conformational changes in the holoenzyme needed for stable strand separation (18). Properties of altered σ^N proteins in which Region I sequences are deleted or substituted suggest that the inhibited state of the holoenzyme is determined by Region I (6,9,30). It is clear that the RIΔ- and RI+IIΔ-holoenzymes show many similar promoter interaction properties and that Region II is not necessary for stable complex formation with pre-melted DNA templates, positive and negative responses to Region I sequences presented *in trans* or the activator-independent initiation of transcription from templates where melting occurs either transiently (supercoiled DNA) or is stable (heteroduplexes).

We conclude that RNAP isomerisation and the initiation of transcription on pre-melted or transiently melting DNA templates does not require Region II sequences. The stability of holoenzymes lacking Region II on pre-melted DNA argues that the isomerised state of the holoenzyme does not require Region II, at least when Region I sequences are removed by deletion. However, it seems that Region II sequences may contribute to activities of the holoenzyme important for DNA melting since *in vivo* under activating conditions the rate of DNA melting at the *glnA*p_2 promoter is changed when Region II sequences are deleted or duplicated (18). Possibly some effects of changing Region II are indirect and mediated through Region I-dependent conformational changes associated with inhibiting polymerase isomerisation prior to activation and the subsequent stabilisation of isomerised holoenzyme on melted DNA (8,9; this paper). These positive and negative effects of Region I could be changed in magnitude by deleting or inserting Region II sequences. Other effects of changing Region II, as discussed below, may reflect a contribution of Region II to a σ–core interaction that suppresses holoenzyme non-specific DNA binding.

Negligible differences in the stabilities or transcription activities of RIΔ- and RI+IIΔ-holoenzyme complexes on heteroduplex DNA were observed, providing little evidence that the Region II sequences contribute to properties of the DNA-bound holoenzyme on melted DNA. However, evidence suggests that Region II sequences contribute to core RNAP interactions. First, more RI+IIΔσ^N is required to saturate the core; second, DNA binding of the RI+IIΔ-holoenzyme is sensitive to non-specific competitor DNA. Possibly non-specific DNA interacts with a site on the core normally made unavailable by Region II. The removal of Region II correlated with a modest holoenzyme preference for binding homoduplex rather than heteroduplex DNA (Figs 2 and 4), whereas RI+IIΔσ^N was generally a poorer DNA-binding protein than either RIΔσ^N or intact σ^N. DNA-binding properties of σ^N and its holoenzyme are therefore influenced by Region II sequences. For σ^{70}, acidic sequences are suggested to inhibit DNA binding (31). For σ^N, the opposite is observed when Region II is removed, suggesting a positive role for Region II in a σ cycle where σ is DNA bound at melted sequences without the core (32). Some of the core-dependent changes in protein footprints in Region II of σ (11,17) may reflect core–σ interactions involving Region II that stabilise the holoenzyme prior to promoter binding. A changed interaction of RI+IIΔσ^N with the core and DNA may account for the weaker holoenzyme binding seen in the S1 footprints (Fig. 8). However, a major core-binding determinant lies between residues 120 and 215 outside Region II, suggesting that the role of Region II in core binding is ancillary (12). Possibly, where two non identical σ^N proteins are present in an organism differences in Region II sequences could contribute to increased affinities for core RNAP (33,34) and selection of promoter DNA prior to DNA melting. However, it is clear that determinants for maintaining the isomerised state or the inhibited state of the holoenzyme lie mainly outside Region II and within Regions III and I, respectively. Although Region II function appears associated with a peripheral contribution to activities whose major determinants lie within Regions I and III, results show that Region II assists σ binding to homoduplex and heteroduplex DNA and reduces competition by non-specific DNA. These contributions likely favour productive interactions between σ and DNA in the σ cycle. The lack of conservation of Region II implies that none of its activities are essential.

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