

## Review Article

# The bacterial enhancer-dependent RNA polymerase

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Transcription initiation is highly regulated in bacterial cells, allowing adaptive gene regulation in response to environment cues. One class of promoter specificity factor called sigma54 enables such adaptive gene expression through its ability to lock the RNA polymerase down into a state unable to melt out promoter DNA for transcription initiation. Promoter DNA opening then occurs through the action of specialized transcription control proteins called bacterial enhancer-binding proteins (bEBPs) that remodel the sigma54 factor within the closed promoter complexes. The remodelling of sigma54 occurs through an ATP-binding and hydrolysis reaction carried out by the bEBPs. The regulation of bEBP self-assembly into typically homomeric hexamers allows regulated gene expression since the self-assembly is required for bEBP ATPase activity and its direct engagement with the sigma54 factor during the remodelling reaction. Crystallographic studies have now established that in the closed promoter complex, the sigma54 factor occupies the bacterial RNA polymerase in ways that will physically impede promoter DNA opening and the loading of melted out promoter DNA into the DNA-binding clefts of the RNA polymerase. Large-scale structural re-organizations of sigma54 require contact of the bEBP with an amino-terminal glutamine and leucine-rich sequence of sigma54, and lead to domain movements within the core RNA polymerase necessary for making open promoter complexes and synthesizing the nascent RNA transcript.

## Introduction

Gene transcription is a pervasive and fundamental process necessary for accessing information in genomes. Transcription of DNA by RNA polymerases is a highly regulated process, underpinning cellular decisions in adaptation and differentiation. RNA polymerases range in complexity from the simpler single subunit phage encoded enzymes to the multi-subunit enzymes found in all three kingdoms of life. All initiate transcription from promoters to achieve patterns of gene-specific expression. Promoter specificity factors directing RNA polymerases to promoters in bacteria fall into two classes, with the sigma54 class requiring specialized AAA+ transcription activator proteins, the bacterial enhancer-binding proteins (bEBPs), for its regulation, whereas the sigma70 class can function in transcription initiation without additional control proteins [1–5]. A range of genetic and biochemical studies have established some special properties of the sigma54 containing RNA polymerase, which distinguish it from the sigma70-type holoenzyme, and which can be attributed directly to the sigma factor itself. These include the ability of the sigma54 factor in the absence of the core RNA polymerase to bind promoter DNA and to be remodelled by its cognate bEBPs in an ATPase reaction, and equivalent activities are not evident with the sigma70 factor [6–20]. The lack of any significant sequence similarity between sigma54 and sigma70 was noted early on and is borne out by their very different molecular structures and differing modes of action within the core RNA polymerase as described below.

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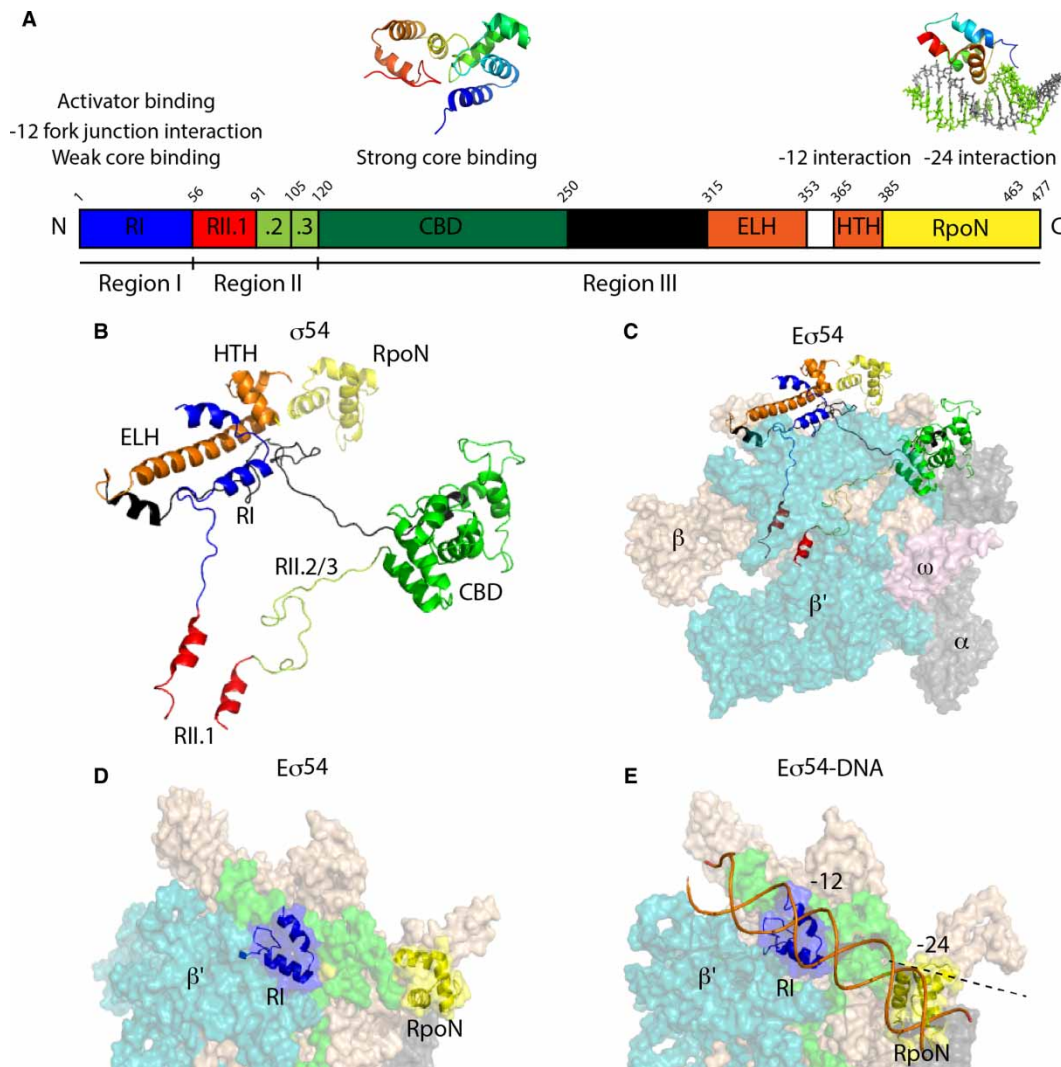
## Structural studies

Until recently, the only high-resolution structural information for bacterial RNAP holoenzymes containing a sigma factor was that for those bound by the primary sigma70-type factors (as recently reviewed in [21]). These structures provide detailed mechanistic insights into promoter recognition and transcription initiation by the primary sigma factors and also the group 2 and ECF sigma factors, which are all related within the sigma70 class. A detailed structure-based mechanistic understanding has been missing for sigma54-dependent transcription initiation and could not be deduced due to the lack of sequence and inferred structural homology of sigma54 with the primary sigma factors [4,22]. Cryo-EM reconstructions of  $\sigma^{54}$ -RNAP holoenzyme in the apo-form and bound to an *Escherichia coli* AAA+ transcription activator protein domain (from PspF, a well-studied bEBP), provided at medium resolution an initial snapshot of the sigma54 holoenzyme in the closed and one nucleotide-dependent activator engaged state. Additionally, NMR structures of the sigma54 core-binding domain and the domain containing the –24 promoter element-binding RpoN box from *Aquifex aeolicus* were obtained in the group of Wemmer and colleagues [6,17,23–25]. However, defining in structural terms the precise interfaces that would exist between sigma54, the RNA polymerase core enzyme and the bEBPs is necessary to help establish its mode of action and ATPase dependence. In particular, a range of functional states of the transcription complex along the transcription initiation pathway need to be structurally defined.

Sigma70 contains four major functional regions: region 1 ( $\sigma_{1.1}$ ) which is located in the downstream DNA channel in the apo-form where promoter DNA is not fully engaged, but is ejected from the channel in the RNAP–promoter DNA complex [26,27]; regions 2 and 3 ( $\sigma_2$  and  $\sigma_3$ ) which are the major core enzyme-binding domains (CBDs) with  $\sigma_2$  also playing an important role in promoter melting through intercalation with DNA bases [28]; and region 4 ( $\sigma_4$ ) which is responsible for –35 promoter recognition [29]. The sigma54 can be divided into three regions based on sequence and function, although they have varying degrees of functional similarities to the regions of sigma70 and no sequence similarity (Figure 1A,B). In sigma54, Region I (RI, residues 1–56, *E. coli* numbering) helps maintain the closed promoter complex by inhibiting the DNA melting reaction, includes the major binding site for the bEBPs, and directs the formation of a DNA fork junction structure at the base pair immediately downstream of the promoter –12 element [19,20,30]. Notably, deletion of RI can bypass the need for the ATPase-driven remodelling, provided that the promoter DNA is melted out [11,31–33], which is correlated with loss of binding tightly to the –12 fork junction structure. Region II (RII, residues 57–120) is acidic and a largely non-essential region without any clear predicted structural features although some repeated acidic residues have been noted [31]. Region III (RIII, residues 120–477) includes the CBD (residues 120–250), a predicted helix-turn-helix motif (HTH, residues 365–385) that interacts with the –12 promoter sequence and the characteristic RpoN domain (residues 386–477), for –24 promoter DNA binding [18,22–25].

## Structural overview of the RNAP holoenzyme containing sigma54

The structure of sigma54 bound to the core RNA polymerase reveals four clear structural domains, and these are connected by long coils and loops that span a large area of the RNAP core enzyme (Figure 1C and ref. [34]). The important N-terminal regulatory RI domain forms a hook comprising two  $\alpha$ -helices. RII also contains two  $\alpha$ -helices (residues 57–91) in addition to coils and loops that are buried inside along the base of the DNA-binding cleft of the RNAP towards the sigma54 CBD (Figure 1B,C). The CBD extends to residue 250 and consists of two  $\alpha$ -helical sub-domains, consistent with the NMR structure of part of this domain. Following on from the CBD, the backbone snakes back towards the upper edge of the main RNAP cleft, which is formed between  $\beta$  and  $\beta'$  subunits, to connect to a long loop and coil region, before an extra long  $\alpha$ -helix (residues 315–353, hereafter called ELH, spanning 50 Å) followed by the HTH domain (Figure 1C). The RpoN domain consists of a three-helical bundle (Figure 1C), consistent with NMR studies of the –24 interacting sequences. It is notable that the sigma54 polypeptide chain snakes back and forth through its loop regions embedded in the RNAP, and so is not co-linear with the RNA polymerase in a promoter 5' to 3' sense (Figure 1C). Consequently, RI interacts with RIII ELH/HTH forming a distinct structural module (Figure 1B) that would correspond to the –12 proximal regulatory centre inferred from biochemical work [20,35–38]. The presence of this regulatory centre also reflects the finding that mutations in RI and RIII can bypass the usual bEBP requirements for making open promoter complexes, presumably through impacts on the networks of interactions made within sigma54, –12 promoter DNA, and their interfaces with the core RNA polymerase.



**Figure 1. Structure of  $\sigma^{54}$ -RNAP.**

(A) Domain organization of  $\sigma^{54}$ . NMR structures of the CBD and RpoN domains are illustrated (PDB entries 2K9L and 2O8K). (B) Crystal structure of  $\sigma^{54}$  (PDB entry 5BYH). Individual domains are coloured as in (A). (C–E)  $\sigma^{54}$ -RNAP holoenzyme in different orientations, coloured by subunits,  $\alpha$  – grey;  $\beta$  – wheat;  $\beta'$  – teal;  $\omega$  – light pink. The dotted line in (E) depicts the trajectory of the  $\sigma^{54}$  RpoN domain in (D) before binding to the promoter.

The –24 interacting RpoN domain, on the other hand, is remote from RI and RIII, an arrangement that can help rationalize the dominant role of –24 recognition in closed promoter complex formation as distinct from promoter melting and bEBP dependence. Overall,  $\sigma^{54}$  spans a region over 100 Å across and 70 Å deep. Its extensive and physically deep interactions with the core RNA polymerase are probably reflected in a complex kinetic association pathway for forming the holoenzyme and may well have implications for establishing a pathway or pathways for its dissociation to occur at transcript initiation and/or elongation.

Key to regulated gene expression is promoter-specific binding by the RNAP holoenzyme. To address this, the sigma54–RNAP crystal structure and a 28 bp duplex DNA model were placed into an 8 Å resolution electron density map to yield a model of a closed promoter DNA complex. In the model, the –24 and –12 elements are in close proximity with RpoN and RIII–HTH, respectively (Figure 1E). Compared with the apo-form of the holoenzyme, the position of the RpoN domain relative to core RNAP was adjusted to fit into the density and to contact the –24 DNA, suggesting that the RpoN domain has mobility during the

transcription cycle, and is dynamic consistent with sigma54 domain separations studied using FRET across the transcription cycle (compare [Figure 1D](#) and [E](#), see below).

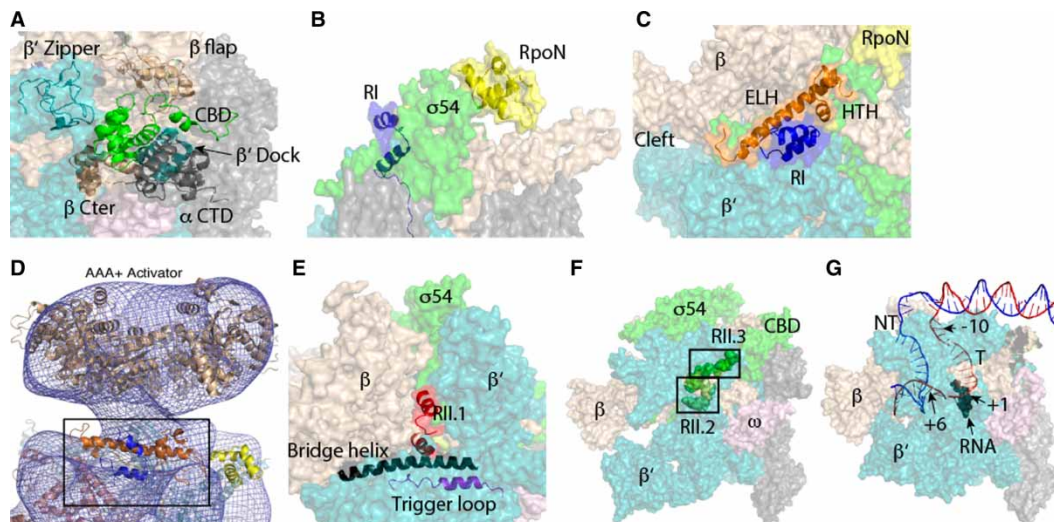
## Functional domains of sigma54 and their interactions with RNAP

All sigma factors contain an RNAP core-binding domain and an upstream promoter element DNA-binding domain, recognizing either the  $-35$  or  $-24$  elements. The CBD of sigma54, the most highly conserved feature among sigma54 proteins from different species, binds towards the upstream face of the RNAP and makes extensive interactions with many features within the RNAP. These include the  $\beta$ -flap (residues 835–937), the C-terminus of the  $\beta$ -subunit (residues 1267–1320), the  $\beta'$  zipper/Zn-binding domain (residues 35–100), the  $\beta'$  dock domain (residues 370–420), and the  $\alpha$ -subunit carboxyl terminal domain ( $\alpha$ -CTD, [Figure 2A](#)), although sigma54-dependent transcription is not typically using the  $\alpha$ -CTD for promoter binding. Where the  $\alpha$ -CTD is used to increase promoter binding, it interacts with the integration host factor [[39–41](#)]. The significance of the  $\alpha$ -CTD to CBD interaction for sigma54-dependent transcription is unclear.

The RpoN domain of sigma54 extends from the main body of RNAP and contacts neither other parts of sigma54 nor core enzyme ([Figure 2B](#)), suggesting that its location can be flexible in the absence of promoter DNA. The RpoN domain is indeed moved relative to the holoenzyme structure when promoter is bound ([Figure 1D,E](#)). This is consistent with the RpoN domain being a dominant and somewhat independent promoter recognition module for sigma54, and contrasts the more complex multi-faceted protein–protein (and protein–DNA) interactions that are involved in  $-12$  promoter recognition. Here, the RIII–HTH interacts with  $-12$  promoter region, in agreement with biochemical and genetic data, suggesting that  $-12$  binding site is a major functional determinant for promoter recognition and stable, closed complex formation and is reorganized during the remodelling that takes place for DNA melting [[36,42–44](#)]. The DNA fork junction that forms just downstream of the  $-12$  promoter CG sequences can be attributed to the DNA interactions directed by the network of RIII–RI interactions at this downstream GC promoter element.

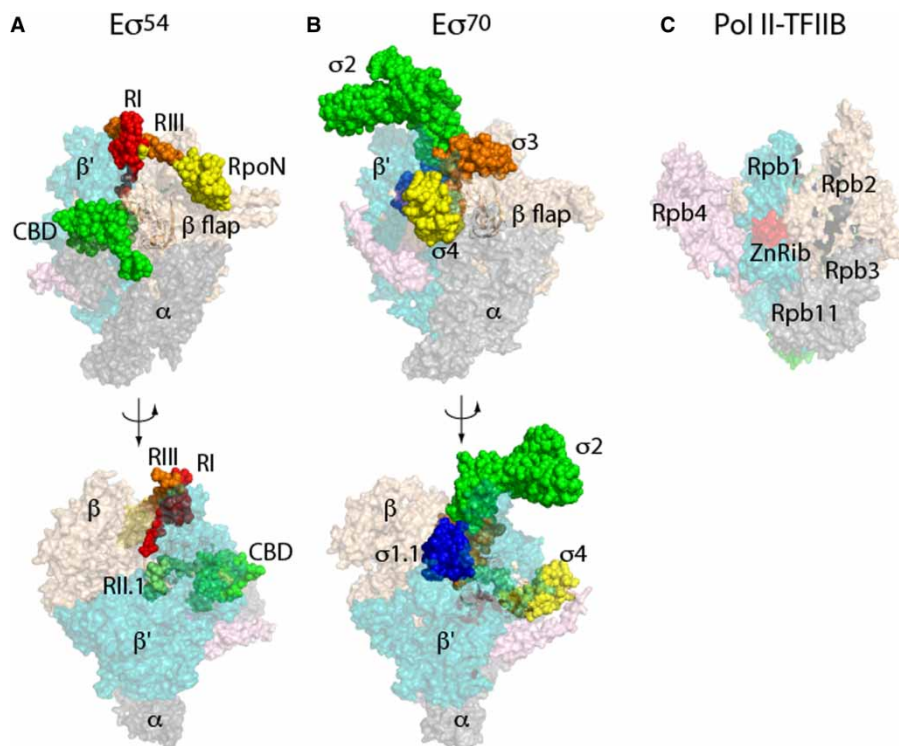
Much of the distinctive bEBP dependence of sigma54 is characterized by its use of its RI, which contains at least two separate contact sites for the bEBPs and plays an inhibitory role to maintain closed promoter complexes prior to bEBP contact [[19,20,31,45](#)]. The RI interacts with RIII, a structural module that lies along the left between  $\beta$  and  $\beta'$  ([Figure 2C](#)). One face of the RI hook is exposed and fitting of the sigma54–RNAP crystal structure into the cryo-EM map of activator-bound holoenzyme positions the RI hook inside the connecting density leading to the hexameric bEBPs ([Figure 2D](#)), in agreement with RI being the major activator-interacting site contacting at least two subunits of the bEBP hexamer through the nucleotide-dependent exposure of its L1 loops [[6,17,19,20](#)]. Interestingly, on the basis of the structure of the sigma70–RNAP in complex with a fork junction DNA, the transcription bubble, starting at  $-12/-11$ , delivers its template strand from this point into the RNAP active site [[46](#)]. The structure, thus, suggests that promoter DNA entry into RNAP is blocked by RI–RIII of  $\sigma^{54}$  and so must be re-organized by the bEBPs ATPase for DNA melting and/or the binding of melted out DNA to take place. This is consistent with FRET data showing that promoter DNA competes for the positioning of the RI relative to upstream and downstream promoter DNA ends [[47](#)].

The RII of sigma54, which has low sequence conservation and has been considered mainly non-essential for transcription and promoter binding, penetrates deeply into the DNA-binding channel, bending across just above the RNAP bridge helix and the trigger loop before travelling upstream ([Figure 2E](#)). The RII can be further divided into three sub-regions based on their locations in the holoenzyme ([Figure 1A,B](#)). RII.1 occupies the downstream DNA-binding cleft of RNAP ([Figure 1C](#)) and so is likely to be a secondary to RI inhibitory sequence. RII.1 connects with the CBD by a flexible linker, which is embedded in the DNA/RNA hybrid binding and the RNA exit channels of RNAP ([Figures 1C](#) and [2F](#)). Interestingly, RII.2 occupies the space of the DNA template strand in the elongation complex (compare [Figure 2F](#) and [G](#)), indicating that RII.2 along with RI has to relocate to permit template-strand DNA access into the RNAP active site for transcription initiation. RII deletions affect promoter DNA binding modestly and the transition from closed to open complex [[31](#)], in agreement with the roles proposed here in inhibiting template DNA delivery and downstream DNA binding. RII.3 lies along the exit path for the RNA in the transcribing complex extending towards the RNA exit tunnel occupied by the CBD ([Figure 2D](#)), indicating that this region may need to relocate as the transcribing RNA reaches a critical length of 7 or 8 nucleotides. Potentially, sigma factor dissociation in the case of sigma54 may be conditioned by the length of the nascent RNA made, and so, the length of RNA could influence rates of



**Figure 2. Unique functional domains and their interaction partners.**

(A) The  $\sigma^{54}$  CBD domain interacts with  $\alpha$ -CTD,  $\beta$ -flap,  $\beta$ -C-terminus (Cter),  $\beta'$ -dock, and  $\beta'$  zipper. (B) The  $\sigma^{54}$  RpoN domain does not seem to interact with other parts of RNAP. (C)  $\sigma^{54}$  RI and RIII ELH/HTH lie directly above the cleft formed between  $\beta$  and  $\beta'$ . (D) Fitting of  $\sigma^{54}$ -RNAP crystal structure into  $\sigma^{54}$ -RNAP-bEBPs cryo-EM contour. RI (and possibly RIII) lies at the connecting density leading to hexameric bEBPs. (E)  $\sigma^{54}$  RII.1 is buried deeply in the DNA-binding channel and directly above the bridge helix and trigger loop. (F and G)  $\sigma^{54}$  RII.2 and 3 (boxed) coincide with the path of the DNA template strand. RII.2 may need to be relocated for RNA extension to occur. The PDB entry 4YLN was used to model the DNA/RNA path in (G).



**Figure 3. Structural comparison of three RNAPs.**

Comparison of  $\sigma^{54}$ -RNAP (PDB entry 5BYH, A),  $\sigma^{70}$ -RNAP (PDB entry 4IGC, B), and Pol II-TFIIB (PDB entry 4BBS, C). ZnRib stands for Zn ribbon domain.

promoter escape into elongation that are separate from the impacts of promoter scrunching within abortive initiation events.

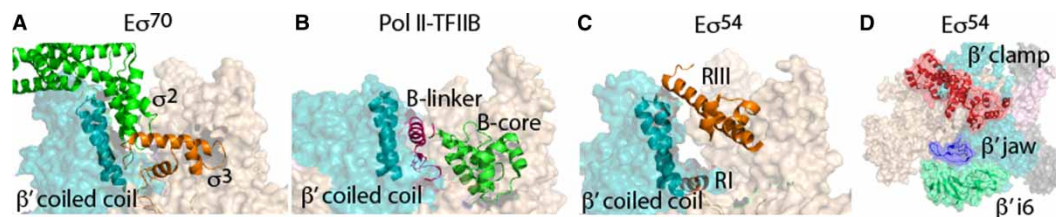
## Homologous functions of sigma54 and sigma70 are achieved by different structural elements

The general functional similarities between the two sigma classes lie in their directing RNAP to the correct promoters. Both contain a CBD and a promoter DNA-binding domain (Figure 3A,B, green and yellow domains, respectively). Strikingly, these two functional domains are in completely different locations in their holoenzymes (compare green and yellow domains in Figure 3A and B, respectively). The CBD of the sigma54 occupies the upstream face (Figure 3A). In contrast, the sigma70 CBD mainly interacts with the  $\beta'$  subunit at the downstream face (Figure 3B). This major structural difference is in agreement with previous biochemical data. Using FeBABE cleavage assays, residue 198 of sigma54 which is within the CBD can be mapped to a region within the  $\beta$ -flap (835–937, ref. [48]). Consistent with a contribution of the  $\beta$ -flap to sigma54 binding in forming the holoenzyme, the  $\beta$ -flap deleted sigma54 holoenzyme showed reduced stability [49,50]. Since the CBD of sigma54 is located near the RNA exit tunnel, it is quite possible that this domain of sigma54 will have to dissociate or relocate on the core RNAP to allow progression from the open complex making short RNAs to promoter escape and full elongation. In contrast, since the sigma70 RNAP CBD ( $\sigma_2$ ) is located in the  $\beta'$  downstream face, it can loosely associate with the RNAP core even during transcript elongation [51–53]. These structural differences might also explain the different abortive transcript patterns noted for the two sigma classes and could impose some sequence selectivity in early transcribed regions to achieve efficient rates of passage into elongation [54].

In contrast, the area on RNAP where the sigma54 CBD binds and where the RNA exits is occupied by the –35 recognition domain  $\sigma_4$  of sigma70 (Figure 3B) and a similar region in Pol II is occupied by the zinc ribbon domain of TFIIB, which interacts with TFIIF [55–57] (Figure 3C). Owing to the extensive interactions between  $\sigma_4$  of sigma70 and the core RNAP, the location of  $\sigma_4$  does not change significantly upon DNA binding although for transcript elongation to occur it has to relocate [58,59]. Notably, the RpoN domain of sigma54 essential for –24 binding is flexible in its location and is in a completely different location from the –35 binding sigma70  $\sigma_4$  (Figure 3A,B).

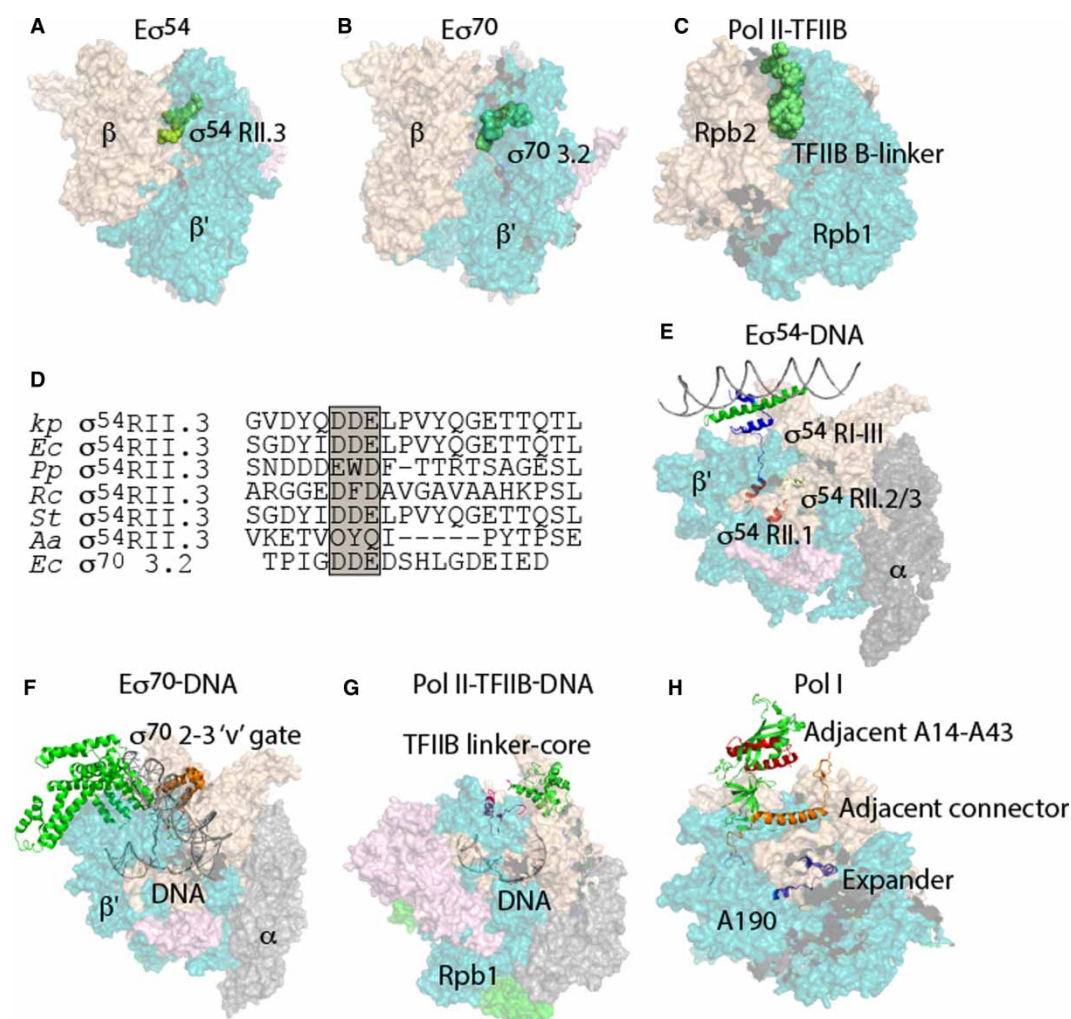
Another major difference between each of the two sigma classes concerns the location of their inhibitory domain. In sigma54, RI interacts with RIII, forming a lid across the RNAP cleft (Figure 3A). In sigma70, the  $\sigma_{1.1}$  is also proposed to have some inhibitory roles, especially evident in combination with the transcription inhibiting phage protein gp2 [60]. The  $\sigma_{1.1}$  is located instead in the downstream DNA channel, overlapping with the sequences in the core RNAP occupied by RII.1 of sigma54 (Figure 3A,B), suggesting that  $\sigma_{1.1}$  and RII.1 might play some homologous regulatory roles in transcription associated with their common placements within the RNAP. However,  $\sigma_{1.1}$  of sigma70 connects with the contiguous  $\sigma_2$  by only a single flexible linker, allowing the  $\sigma_{1.1}$  to move from the RNAP cleft (Figure 3B). For sigma54, on the contrary, RII.1 connects with the CBD by a flexible linker, which is embedded in the DNA/RNA hybrid binding and the RNA exit channels of RNAP (Figure 3A). Owing to its topological restraint, RII.1 cannot relocate easily without affecting the conformation of other parts of the sigma54. RII.1 will thus block downstream DNA entry in closed promoter complexes, but presumably is relocated in intermediate and open promoter complexes following the action of the bEBPs on the RI and the resulting changes in its associated network of interactions with RIII and core RNAP.

Given the two sigmas make distinctly different interactions with the core RNAP, it is perhaps unsurprising that structural differences between the two holoenzymes are not just restricted to the sigmas themselves. Many of the structural modules of the RNAP are likely to be dynamic and have been captured in different positions and conformations across a range of transcription complexes so far studied. We found that significant conformational differences exist in many key modules within the RNAP when aligned on the bridge helix, which is taken as being a defined largely fixed structure, in part, defining the catalytic centre structure of the enzyme where nucleotide addition and phosphodiester bond formation occur. In particular, movements of the  $\beta'$  coiled-coil and the entire  $\beta'$  subunit narrow the downstream DNA channel by up to 5 Å. The clamp can adopt multiple conformations in solution, and this narrowing falls within the range [61]. Interestingly, the  $\beta'$  coiled-coil forms helical bundles with  $\sigma_2$  of sigma70 and helps to stabilize the unpaired non-template DNA strand, and hence the transcription bubble (Figure 4A). B-reader/Finger of TFIIB also interacts with  $\beta'$  coiled-coil



**Figure 4. Interactions with the  $\beta'$ -coiled-coil domain.**

$\sigma^{70}$  2–3 (A) and TFIIB linker core (B) form helical bundles with the  $\beta'$ -coiled-coil domain. In contrast,  $\sigma^{54}$  RI–RIII (C) does not form a helical bundle with the  $\beta'$ -coiled-coil domain. (D) The  $\beta'$  i6 domain is visible in a  $\sigma^{54}$ -RNAP crystal structure. It sandwiches the  $\beta'$ -jaw domain with the  $\beta'$ -clamp.



**Figure 5. Comparison of  $\sigma^{54}$ -RNAP,  $\sigma^{70}$ -RNAP, Pol II–TFIIB, and Pol I (PDB entry 4C2M).**

(A–C)  $\sigma^{54}$  RII3,  $\sigma^{70}$  3.2, and TFIIB linker all occupy similar space within the holoenzyme. (D) Sequence alignment of  $\sigma^{54}$  RII from *Klebsiella pneumoniae* (*Kp*), *E. coli* (*Ec*), *Pseudomonas putida* (*Pp*), *Rhodobacter capsulatus* (*Rc*), *Salmonella typhimurium* (*St*), *A. aeolicus* (*Aa*), and  $\sigma^{70}$  3.2 from *Ec*. The conserved DDE motif is boxed. The DNA passage into the DNA-binding channel is blocked by  $\sigma^{54}$  RI–RIII (E), but allowed by  $\sigma^{70}$  2–3 ‘v’ gate (F) and TFIIB linker core (G). (H) The A14–43 dimer from an adjacent molecule occupies a similar space as does  $\sigma^{54}$  RI–RIII (compare with E). The expander sequence occupies a similar space as  $\sigma^{54}$  RII.2/3.

(Figure 4B). In the sigma54 holoenzyme, the  $\beta'$ -coiled-coil domain does not form a helical bundle with sigma (Figure 4C). Our biochemical data show that disruptions of the RI and RIII–ELH interactions affect open complex formation to a different extent compared with wild-type sigma54 with  $\beta'$  coiled-coil mutants, in agreement with the suggestion that interactions between RI and RIII–ELH, and between RI/RIII–ELH and  $\beta'$ -coiled-coil, might play different roles during open complex formation [34].

The  $\beta'$  trigger loop/helix plays significant roles in nucleotide addition during transcription through a loop-to-helix transition. There is a large insertion (residues 942–1129,  $\beta'$  i6) in the middle of the trigger loop in *E. coli* RNAP, and this insertion plays vital roles in open complex formation, transcription pausing, and termination [62,63]. In all sigma70 holoenzyme structures, this domain is disordered, suggesting a highly flexible nature. In the sigma54 holoenzyme structure, there is clear density for this region, which sandwiches the  $\beta'$  jaw domain (residues 1149–1190) with the  $\beta'$  clamp (residues 131–347 and 1260–1368; Figure 4D). The loss of the  $\beta'$  jaw domain allows core RNAP to utilize certain start site sequence embedded in pre-opened DNA templates, implying that a sigma54-mediated stabilization of the  $\beta'$  insertion is likely to be functionally important for faithful transcription initiation (our unpublished work).

## Regulatory elements in sigma54 and conservation with sigma70, eukaryotic TFIIB, and Pol I

Our structure reveals a diverse range of regulatory strategies on transcription initiation that are utilized by sigma54. These include blocking the template strand from entering the RNAP active cleft by RI–RIII, occupying the downstream DNA channel by RII.1, interference with the template strand, and newly synthesized RNA by RII.2–RII.3. The positions of RI and RIII ELH–HTH within the RNAP play a vital role in these regulatory functions. It seems very likely that as the bEBPs interact with RI, they will relocate RI, RIII, and RII, releasing the inhibition posed by these structural elements [47,64]. Precisely how the bEBPs overcome these multiple modes of inhibition, and the precise roles of ATP binding, hydrolysis and ADP + Pi release in this process remains to be determined. In particular, how distinctive per subunit nucleotide-bound states within hexameric bEBPs co-operate for the initial engagement of the sigma54 RI, its movement or its capture in a transient excursion from the RNAP needs to be established.

Unsurprisingly, some of these regulatory features exist in the eukaryotic Pol II transcription counterpart, TFIIB. For example, like the sigma54 RII.3, region 3.2 in sigma70 (as a  $\sigma$  finger) and the B-linker in TFIIB also insert deep inside the RNAP core and overlap with the space that would be occupied by RNA (Figure 5A–C). Indeed, RII.3 shares sequence homology with  $\sigma$  finger, especially the highly conserved DDE motif (Figure 5D). Residues in the  $\sigma$  finger facilitate template DNA loading at the RNAP active site and RNA separation from DNA, guiding it towards the exit channel [65]. A similar function was proposed for the B-linker in TFIIB [56,66,67], and it is possible that RII.3 also performs similar roles [56,66,67].

RI and RIII form a lid across the RNAP cleft, possibly blocking the template-strand entry (Figure 5E). At similar locations, the domains 2 and 3 ( $\sigma_2$  and  $\sigma_3$ ) of sigma70 form a V-shaped wedge that acts as a gate to allow the DNA template strand to enter the active cleft (Figure 5F) [59]. TFIIB also uses two sub-domains, the core-binding domain and the B-linker, to occupy similar areas (Figure 5G). Furthermore, domain 2 ( $\sigma_2$ ) of sigma70 contains many aromatic residues that are shown to interact via intercalation with the non-template strand, therefore facilitating DNA melting and transcription bubble formation [28,59,68]. In sigma54, there are few conserved aromatic residues in this region (RI and RIII–ELH), and previous biochemical studies show that mutating these residues has little effect on transcription regulation [22]. This is in agreement with data showing that although holoenzyme variants lacking RI can bypass the requirement of bEBPs, they fail to efficiently make open promoter complexes on double-stranded DNA [30,69]. It is possible that the bEBPs may, therefore, be directly required for transcription bubble formation, through an action on DNA which is not that of a classical helicase, but which still promotes DNA strand separation and DNA unwinding.

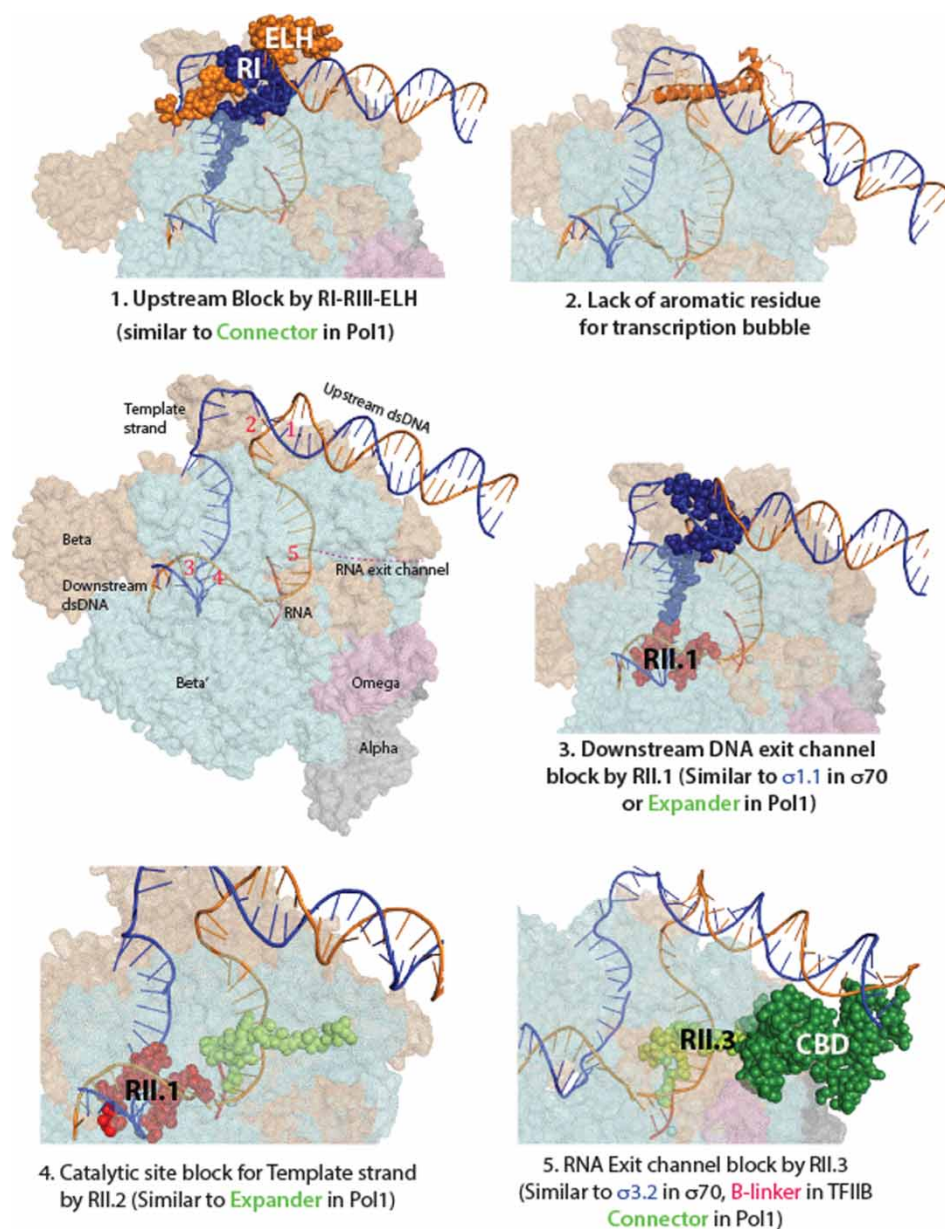
The RII.1 of sigma54 and region 1.1 of sigma70 occupy the downstream DNA channel. RII.2 is located in the channel that is occupied by the template-strand DNA in the transcribing RNAP (compare Figure 2F and G). It appears that sigma70 and TFIIB lack equivalent structural features; however, this area is occupied in eukaryotic Pol I by part of the expander (A190 residues 1337–1440, Figure 5H), a unique insertion in Pol I compared with Pol II [70]. In fact, we find extensive similarities in the regulatory elements between sigma54–RNAP and Pol I. The cleft, covered by the lid formed by RI–RIII in the sigma54 holoenzyme, is capped by subunits at the Pol I dimer interface, A14–A43 (from an adjacent molecule), which inhibits interactions with initiation factors



(Figure 5H). However, it is unclear as this dimer is formed only in the crystal and does not form in solution. Part of the expander in Pol I also coincides with RII.1 (Figure 5E,H), while the connector in Pol I, which is the C-terminus of A43 from an adjacent molecule, localizes like the sigma54 RII.3, in the RNA channel (Figure 5E,H).

## Conserved regulatory hotspots within RNAP and shared regulatory strategies

Although sigma54 and Pol I subunits have no sequence or structural similarity, they employ surprisingly similar regulatory strategies. We suggest the existence of evolutionarily conserved regulatory hotspots within RNAP. There are at least five such hotspots found in the sigma54–RNAP some of which are shared by Pol I and to some extent by sigma70 and TFIIB. The RNAP cleft is capped by a lid formed by RI together with RIII ELH–HTH of



**Figure 6. Transcription regulation hotspots in RNAP and regulations by sigma factors, TFIIB, and those in Pol I.** Those shared by others are in brackets (blue — sigma70; green — Pol I; red — Pol I–II–III–TFIIB).

sigma54 (Figure 6). In Pol I, dimerization in this region occludes binding to initiation factors (Figure 5H). The downstream DNA channel is another highly regulated site (Figure 6). RII.1 of sigma54,  $\sigma_{1.1}$  of sigma70, and the expander in Pol I all occupy this space to regulate RNAP. The template-strand path inside the RNAP cleft is interfered with by RII.2 of sigma70 and the expander in Pol I (Figure 6). A highly conserved regulatory spot is the RNA exit tunnel. RII.3 of sigma54,  $\sigma_{3.2}$  of sigma70, B-linker of TFIIB, and the connector of Pol I occupy this space (Figure 6). Furthermore, available aromatic residues close to the non-template-strand path could also contribute to stabilizing the transcription bubble (Figure 6), with sigma70 acting independently in the DNA melting reaction and sigma54 requiring its ATP-driven bEBPs. The use of an activating protein cofactor in the bEBPs of sigma54 is shared, to some extent, by Pol II–TFIIB that requires TFIIH for activation. Finally, on the basis of the location of RIII ELH–HTH as defined in the DNA complex model, the –12 promoter DNA is located incorrectly for where the template strand should enter RNAP active cleft, and so, this relationship to the promoter DNA requires reorganization to make the open promoter complex. Our structure, therefore, uncovers a wide range of regulatory hotspots within RNAP, which are extensively targeted by sigma54, while subsets of these are targeted as regulatory elements of the transcription complexes in other transcription systems.

## Concluding remarks

Clearly, sigma54 utilizes a multitude of strategies to control transcription initiation and make the process dependent upon the ATPase activity of the bEBPs. How its association with the core RNA polymerase is changed during the remodelling and where its domains locate to within the open promoter complex and during RNA synthesis remain to be established through further detailed structural studies. Strikingly, sigma54 cannot be rooted as it lacks any significant structural or sequence similarity to the sigma70 class or indeed other proteins of known structure, and so, it seems that sigma factor function did evolve at least twice, to account for the two distinct classes of sigma factors present among bacteria. One intriguing finding relates to conserved regulatory hotspots within RNAP and shared regulatory strategies with other RNAPs. The significant structural differences suggest that sigma54, sigma70, TFIIB, and specific subunits in Pol I probably evolved from different ancestors. However, the extensive similarity in regulatory strategies between sigma54 and Pol I as well as shared elements with sigma70 and TFIIB provides clear evidence that regulatory hotspots within RNAP are functionally conserved. Many different transcription factors quite probably interact with these hotspots to regulate the transcription machinery. The reason why sigma54 was not adopted as the primary sigma factor in bacteria may well relate to its elaborate factor — independent silencing of the closed promoter complex. Such a tight off state of the RNA polymerase holoenzyme would not be in line with many housekeeping promoters, necessarily functioning through their intrinsic promoter DNA sequence-directed activities to achieve the required gene expression levels without the use of other factors or through simpler repression mechanisms based on promoter occlusion mechanisms. Turning the pan bacterial genome into an array of enhancer-dependent sigma54-directed transcription units would require massive deployment of resources for the formation of new genes, encoding the new suites of AAA+ activator proteins to direct the transcriptome of cells. Such an expansion might not be of sufficient advantage to warrant its successful evolution, and indeed some bacteria lack the sigma54 system, yet have large numbers (>60) of alternative sigma factors. Rather, it seems that sigma54 is best used when highly regulated processes need to be controlled to obtain a strong expression off state, but also a high on state when open promoter complex formation is coupled to the ATPase activity of the AAA+ activator protein. Potentially, some sigma54 domains may have been part of an early transcription repression system to keep the RNAP in check, even acting globally and in *trans* to genes before the rpoN domain was co-opted for promoter-specific binding and specific gene expression. The alternative scenario in which sigma54 loses its repressive functionality to evolve into an activator-independent sigma factor is formally possible, but is not yet supported by studies examining the *in vivo* phenotypes of such variants for global activator by-pass transcription patterns.

## Abbreviations

bEBPs, bacterial enhancer-binding proteins; CBD, core enzyme-binding domain; ELH, extra long  $\alpha$ -helix; HTH, helix-turn-helix motif; RI, region I; RII, region II; RIII, region III;  $\alpha$ -CTD,  $\alpha$ -subunit carboxyl terminal domain.

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## Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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