A Perspective on the Enhancer Dependent Bacterial RNA Polymerase

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Abstract: Here we review recent findings and offer a perspective on how the major variant RNA polymerase of bacteria, which contains the sigma54 factor, functions for regulated gene expression. We consider what gaps exist in our understanding of its genetic, biochemical and biophysical functioning and how they might be addressed.

Keywords: RNA polymerase; Sigma54; transcription; AAA+ ATPase

1. Introduction

Along with the recognition that bacterial RNA polymerases were heterogeneous with respect to their sigma factor content, came the finding that two classes of sigma factor existed in many different types of bacteria [1]. Unlike the major sigma70 class, the sigma54 class of factor was distinctive in being enhancer dependent and relying on a specialised class of transcription activator which used ATP binding and hydrolysis to catalyse the formation of open promoter complexes (RP O). Closed promoter complexes (RP C) rarely spontaneously isomerised to open complexes, and so sigma54 dependent systems showed a typical major dependence on cognate activators (reviewed in [2,3]). No hard and fast rules allow the prediction of which bacteria will contain an rpoN gene or genes (some organisms have two rpoNs) encoding sigma54, although informatics suggests sigma54 use is rooted in the control of cell envelope functions in response to stress [4]. Because the sigma54 factor controls important bacterial stress response genes in pathogenicity and in agriculture [5–9], there is considerable interest
in working out where the dependence arises in biochemical and structural biology terms. Further, what are the advantages the system may have over conventional repression and activation systems used by the sigma70 class of RNA polymerase holoenzymes?

2. The Sigma54 Factor

Work from Sydney Kustu’s lab provided the first biochemical evidence that the sigma54 factor (encoded by \textit{rpoN}, also called \textit{ntrA}) was a dissociable sigma factor which directed RNA polymerase as a holoenzyme to the $-12$, $-24$ promoter sequences from which it transcribed [10,11]. Sequence analysis of a range of \textit{rpoN} encoded genes was to provide clear indications that the sigma54 protein was unrelated to the major sigma70 class of sigma factors at the level of primary sequence and most likely its fold and tertiary structure. For the most part this lack of relatedness has been upheld by what limited structural data sets for fragments of sigma54 have been obtained to date. The lack of relatedness suggests that sigma54 may direct a different set of structural transitions in taking RPc to RPo to that orchestrated by sigma70 type factors, rather than just changing the kinetics of the same pathway of conformational change.

\textbf{Figure 1.} Domain organisation of \textit{Escherichia coli} $\sigma^{54}$. The DNA binding function is mainly associated with $\sigma^{54}$ Region III, including the DNA crosslinking motif (Xlink), a putative helix-turn-helix motif (HTH) for $-12$ recognition via E378 and the RpoN box for $-24$ binding. Structures of two $\sigma^{54}$ Region III fragments have been obtained (PDB entries: 2K9L and 2O8K).

Sigma54 contains two highly conserved domains (Regions I and III, Figure 1), separated by what may be a flexible linker (Region II). The glutamine- and leucine-rich Region I interacts with activator ATPases, core RNAP and the $-12$ promoter sequence, thus posing an energy barrier to spontaneous isomerization of RPc to RPo. Region I mutations and deletion often resulted in activator-bypass phenotypes [12,13]. Sigma54 Region II is dispensable for RNAP isomerisation and interaction with DNA. Indeed many bacterial sigma54 proteins do not naturally contain this domain, arguing for its ancillary role. Sigma54 Region III is primarily involved in binding to the promoter DNA at several sites, with the strongest interaction being between the RpoN box and the $-24$ promoter element. The RpoN
box forms a winged helix-turn-helix (HTH) motif and inserts the recognition helix into the major groove of the −24 promoter sequence [14,15]. Mutations in the RpoN box have been shown to cause a more than 80% reduction in promoter DNA binding affinity [16].

3. Promoter Recognition

Apparently σ54 RNAP finds promoters by a direct binding route and not sliding, at least in vitro [17]. At its cognate promoters the holoenzyme opens up the A:T base pair 3′ to the promoter −12 consensus GC sequence and so a repressive fork junction structure with which the Region I of σ54 interacts is created. Resolution of this structure by the activator ATPases is a part of the enhancer dependent activation of the RPc to yield an RPo. Details of the −12 recognition problem and how the local −12 proximal un-stacking is achieved in the stable RPo from an unstable prior fully stacked DNA RPc [18] is now a structural biology issue. Genetics implicates a HTH motif E378 residue and specifically also residue L25 in σ54 in the −12 GC recognition, but the specific amino acid sequences in σ54 and in core enzyme needed for base un-stacking just downstream of the −12 GC are unknown although may well map to the Region I of σ54. Similarly DNA melting defective mutants (for RPo) of σ54 have not been obtained, in contrast to σ70, and so quite how σ54 contributes to opening DNA from −10 to −1 is unknown. However mutants in σ54 able to allow RPo formation in vitro without activation suggest the barrier to RPo formation is distinct from determinants that allow formation and maintenance of (at least unstable) forms of RPo. Although not widely surveyed across promoters, it seems that the σ54 holoenzyme forms fewer abortive RNA products before elongation than does the σ70 containing enzyme [19], and so features of σ70 contributing to the frequency of abortive initiation may not have counterparts in σ54.

In contrast to a complex recognition of the promoter −12 intimately linked to maintaining RPc, recognition of the −24 promoter sequence seems relatively well understood, and involves a HTH motif and the RpoN box amino acid sequence, as solved by the Wemmer group by NMR [14]. In contrast to the −35 recognition of σ70 promoters being mediated by the flap domain of the core RNAP, no such dependence seems so for σ54 although other roles for the flap domain are suggested by promoter DNA footprinting experiments with a flapless core and σ54 [20].

4. Activator Remodel of the Sigma54 Holoenzyme

Sigma54 activators (such as NtrC and PspF) belonging to the AAA+ protein family assemble into hexamers and fuel the rearrangement of RPc to RPo. Cryo-EM reconstitutions carried out by the Zhang lab revealed up to three σ54 activators within a hexamer could directly contact the RPc structure for isomerisation [21]. These contacts were made asymmetrically via the GAFTGA loop one motifs to σ54 Region I and the upstream −30 promoter region [22], and were possibly accompanied by the splitting of the hexameric ATPase ring in order to exert directional forces [23]. A bridging density within the holoenzyme was observed to physically block the DNA loading channel formed between the β and β′ subunits prior to activation (Figure 2A). This density was assigned to a part of σ54 Region I and it relocated downstream towards the +1 site in the intermediate complex (RPi) when the ADP-AlFx hydrolysis analogue was added. The DNA melting site was misaligned with the DNA loading site in the RPt in the proposed model, which could constitute an ancillary inhibitory mechanism.
The Stockley and Tuma labs further addressed sigma54 domain movements in relation to promoter DNA and ATP hydrolysis by smFRET analysis (Figure 2B, [24]). Sigma54 Region I moved by approximately 9 Å towards the leading edge of the −10 to −1 transcription bubble in the RP₁ when activated with ADP-AlFx. This downstream movement upon activation fully agreed with the Cryo-EM observations and potentially correlated with blockage removal by the “power stroke” action of activator ATPases. Once ATP was fully hydrolysed, Region I would retract slightly upstream, possibly to accompany the DNA loading event. In contrast, Region III remained rather static with respect to the −24 promoter sequence along the activation pathway for making RPO from RPC.

**Figure 2.** Domain architecture and the proposed mechanism of activation. (A) Cryo-EM reconstitution of the E. coli-PspF-ADP-AlFx with the promoter DNA modelled in [21]; (B) Domain movements of σ₅⁴ Regions I and III during the transcription activation cycle (modified from [24]). The white stars depict fluorophores used in the smFRET experiments.

Overall it is clear that activation for making RPO requires a repositioning of the sigma54 Region I, which occurs through the use of at least two GAFTGA loops of the activator ATPases that bind there. Whether the activator binds a rare state of the holoenzyme in RP₃, or drives the formation of an entirely new functional state is not yet clear. However, the R336A sigma54 variant phenocopies the action of the binding of the activator to RP₃ in that the Region I has moved compared to that in RP₃, and so the barrier to passing from RP₃ to at least an RP₁ if not RPO is not so large. Release of the activators from the sigma54 Region I may be key in passing to RPO from RP₁, as may a contact of the activator with upstream promoter DNA. Kinetic studies on the *glnAP2* promoter using COSMO methodologies indicates that the transition from RP₃ to transcript generation takes around ninety seconds, and activators interact with both RP₃ and RPO [18]. These single molecule studies also lend support to the idea that
an initial form of RPB exists without the DNA base unstacking at $-12$ [18]. Given the heterogeneous nature of nucleotide bound states of the activators, that the hexameric activators may assume an opened form and use a subset of the six possible sigma-contacting mobile surface features [22,25]. Their asymmetric functioning seems a necessary part of the transcription activation mechanism and may reflect the asymmetric target of the $\sigma^{54}$ within the RPB. A processive functioning of the activators may not be necessary for activation, because the activator bound RPI has been shown to possess RPO like characteristics [26]. However quite how and when DNA opening occurs for making the RPO with sigma54 is not known, and could involve other functions of the activators ATPase rather than a direct sigma DNA melting activity. Notably when double stranded DNA is outside of the RNAP in RPI, extensive cross links can be made with the bound activator, which might guide dsDNA into the holoenzyme for making RPO, or indeed act on it directly for making RPO [21]. An interaction of DNA with the activators ATPase domain has been observed [27]. Further, activator binding to RPB (enabled by the use of non-hydrolysable nucleotide analogues such as ADP-AlF$_x$) when promoter DNA from $-10$ to $-1$ is unpaired allows transcription initiation, suggesting the holoenzyme can accept ssDNA and then open up the start site to allow RNA synthesis, without the need for ATP hydrolysis by the activator protein [26].

5. Signaling for Sigma54 Dependent Transcription

Genes under control of the sigma54 are required under specific stress conditions or when a particular C or N source is available. Classical examples include the NtrC dependent activation of genes in response to nitrogen limitation, the use of XylR in expressing genes for catabolism of aromatic hydrocarbons and PspF in activating the psp response for inner membrane stress. Although all contain the critical AAA+ domain needed for remodelling the closed promoter complex, the activators are all signaled to via a range of N-terminal regulatory domains. These fall into a wide range of different classes ranging from the response regulator type aspartic acid phosphorylation target in two component members, to GAF domains in NifA and CARF domains in RtcRs (reviewed in [2]). In many cases the exact ligand interacting with these N-terminal regulatory domains remains unknown.

6. Conclusions and a Final Perspective

Detailed structural insights into RPB, RPI and RPO are now needed to work out quite what it is in structural terms that establishes the barrier to making RPO, and how RPB and RPO interconvert. Comparisons to the use of the sigma70 Region I as a place keeper for DNA and that of sigma54 Region I as a target for activation, maintaining the repressive fork junction structure and escaping the action of certain phage inhibitors of RNAP activity such as T7 phage gp2 will be one valuable outcome from such studies. Similarly, knowing what the functional state the core RNAP is in when bound to sigma54 will provide insights into the importance of the sigma-core interface in gene control and potentially antibiotic action. For example in the presence of sigma54 is the core enzyme in a catalytically competent state, and can smFRET data be reconciled with RNAP clamp opening and clamp closing, and the processive closed state of the core enzyme? Do the sigma54 and its activators take the RNAP down a new set of conformationally distinct changes for making RPO, or is it simply altering the kinetics of a single pathway for making the RPO from the RPB?
Quite where sigma54 came from remains a mystery—it cannot be rooted and seems to have no obvious structural counterpart (although detailed structural analysis may overturn this view, if not for the full protein then for some of its domains). Genomics methods and RNAseq studies will now no doubt offer us perspectives on why have sigma54 at all—is it a relic of a transcription repression mechanism, can it (or has it?) evolve in some cases to activator independence, and does it have repressive functionality alongside its gene activation responsiveness? Currently ChipSeq and RNAseq work with sigma54 and its holoenzyme to define its regulon suggests complexity in the roles of sigma54 [28,29]. For example some RPCs seem not to be served by cognate activators, and amongst these a subset seem to repress transcription by other forms of RNAP holoenzyme. In combination with genomic approaches to better understand roles of sigma54 binding sites on bacterial chromosomes, sufficient knowledge of sigma 54 and sigma70 structure-function relationships will allow us to make orthogonal non-native chimeric sigma factors-to tackle issues of promoter use in synthetic biology and an increased insularity from the bacterial chassis used in such work. Since sigma54 is served by cognate activators of the AAA+ protein family, detailed atomic structures of their co-complexes with RPC will help work out how such AAA+ proteins remodel their targets. Lastly, the full role of sigma54 in bacterial physiology is unknown. Many of its activators respond to signals which are poorly characterised, yet underpin important processes such as RNA repair (the rtcBA genes activated by RtcR) and envelope maintenance (the psp operon activated by PspF). As details of how genes turn on and off in individual cells through transient changes in, for example, local DNA superhelicity to yield the so called bursty behaviours of gene transcription revealed by time series studies, one might wonder if the sigma54 factor with its coupled ATPase overlays some special features to such time series.

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Author Contributions

Nan Zhang and Martin Buck equally contributed to the writing.

Conflicts of Interest

The authors declare no conflict of interest.

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