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Antibiotics trapping transcription initiation intermediates

To melt or to bend, what's first?

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Promoter DNA melting, culminating in the loading of the single-stranded DNA template into the RNA polymerase active site, is a key step in transcription initiation. Recently, the first transcription inhibitors found to block distinct steps of promoter melting were characterized. Here, the impact of these studies is discussed with respect to the current models of transcription initiation.

Key words: σ subunit, switch-2 region, transcription inhibitors, promoter melting, lipiarmycin, myxopyronin

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Bacterial RNA polymerase (RNAP) is a complex molecular machine, composed of the catalytic core (subunits 2αββ'ω) and one of the promoter-specific σ factors directing promoter recognition and melting. Transcription initiation, governed by the interplay between a panoply of promoter sequences and a number of σ factors, is modulated by numerous transcriptional activators, repressors and small regulatory molecules. Such a network provides the basis for the fine-tuning of bacterial gene expression. Understanding transcriptional regulation requires the characterization of the intermediates and checkpoints in the initiation pathway leading to a transcriptionally active RNAP-promoter complex, generically referred to as RP_o. The antibiotics lipiarmycin (Lpm) and myxopyronin (Myx), which target two distinct steps in forming RP_o,[1-3] open up new perspectives for fundamental studies of transcriptional regulation and for medical research to identify new drug target sites.

Three Steps to the Open Promoter Complex

Transcription initiation starts with the reversible promoter binding that results in the formation of the "closed complex" (RP_c or I_1), containing straight double-stranded DNA (**Fig. 1A**). The closed complex isomerizes to form the transcriptionally competent "open complex" (RP_o), in which the promoter DNA is bent ~90° and ~13 bp of DNA (positions -11 to +2) around the transcription start site are melted to form a transcription bubble. The formation of the transcription bubble starts from the nucleation of melting at the -10 element, followed by the downstream propagation toward position +1.[4-6] The slow isomerization from RP_c to RP_o involves several intermediate complexes (RP_i or I_2) and includes large scale conformational changes in RNAP.[7-10] Kinetic studies performed primarily using the two promoters *lac*UV5 and λP_R indicate that there is a general three-step scheme for open complex formation (**Fig. 1A**). It should be kept in mind that the structures of the kinetically significant intermediates for these two schemes are different: for the *lac*UV5 promoter, the RP_i is "closed" and stable,[4] whereas the RP_i complex for the λP_R promoter is "open" and unstable.[10]

Role of the RNAP Clamp in Loading of Template into the Active Site

X-ray studies of multi-subunit RNAPs provide a framework for understanding the transcription mechanics. The structure

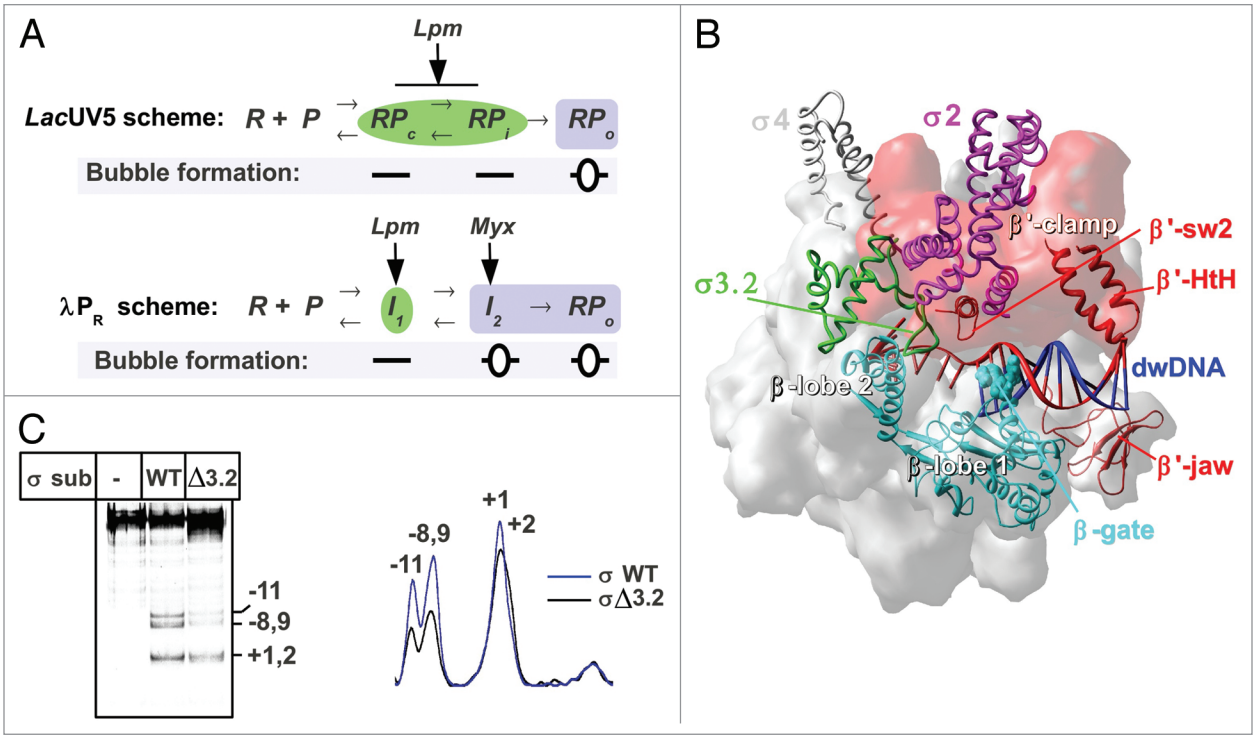

Figure 1. (A) Kinetics scheme of RP_o formation on the *lac*UV5,[7] and λP_R promoters.[9] R: RNAP, P: promoter. Complexes with open DNA marked by blue boxes; closed complexes marked by green ellipses. (B) Structural model of RNAP in complex with dwDNA fragment.[15] The structure of *T. thermophilus* RNAP[12] is shown as a molecular surface colored in gray. The β lobes (in cyan), β' switch-2 (sw2) (Ser602-Lys621), β' jaw (Arg1266-Gly1328) and β' B-helix-turn-helix (HtH) (Leu469-Pro506)[18] are shown as ribbons, the β-gate loop (Arg243-Pro248)[12] is shown in CPK. Numbering corresponds to *T. thermophilus*. The β' clamp part of the RNAP surface is colored in red. DNA is shown in red (template) and blue (non-template). The σ subunit is shown as ribbons with the structural domains 2, 3 and 4 in magenta, green and gray, respectively. (C) Influence of the $\sigma_{3.2HL}$ deletion on *lac*UV5 promoter melting. $KMnO_4$ probing of the open complexes formed between end-labeled *lac*UV5 promoter and the RNAP carrying either wild type σ^{70} or σ^{70} with deletion of the region 3.2 hairpin loop (Asp513-Leu519).[3] Positions of the template strand thymines reactive to $KMnO_4$ are indicated. The scan of the gel is shown on the right.

of bacterial RNAP resembles a crab claw, with the pincers (or jaws) formed by the clamp domain (primarily the β' subunit) and β subunit lobes[11-13] or the rope-swing region (*T. thermophilus* βAla132-Ser387),[14] (**Fig. 1B**). The active site marked by the magnesium ion is deeply buried in the cleft between the pincers. The clamp domain is linked to the core by the five switch regions: β' switch-1, -2 and -5 and β switch-3 and -4.[11] The β' clamp serves as a docking site for the σ subunit. The weakly conserved region 3.2 of σ forms an unfolded linker between the σ promoter recognition regions 2 and 4 and fills the RNA exit channel in the holoenzyme.[13] It is likely that the σ region 3.2 hairpin loop ($\sigma_{3.2HL}$, *E. coli* σ^{70} residues Glu508-His519) can function as a sensor of RNA length that triggers the initiation-to-elongation transition as soon as 10–11 nucleotides of RNA have been synthesized, but the mechanism of this transition is not yet understood.

To form the catalytically competent open complex, the +1 base of the single-stranded template DNA of the transcription bubble must be placed in the active site cleft, whereas the ~15 bp DNA duplex downstream of the start site (dwDNA) should be locked by the pincers in the downstream channel.[15] The β' switch-2 element contacts the template at positions -2 and -3 and assists in positioning the template in the RNAP active site.[11,16] Additional elements of the β' clamp (B-helix-turn-helix and "jaw," **Fig. 1B**) form a network of DNA-protein contacts that stabilizes the promoter and elongation complexes.[15,17-19]

However, in the holoenzyme, the active site cleft is too narrow (14–17 Å) to allow the entry of the DNA duplex (~22 Å), but it can accommodate single-stranded DNA.[1,13] Access to the active site is blocked by the "β-gate loop"[12] and σ subunit region 1.1 (**Fig. 2B**). To bypass this barrier, several scenarios are possible: (1) the cleft opens (clamp or lobes swinging), (2) the DNA melts or (3) σ is displaced. In support of the "clamp swinging" mechanism, the comparison of bacterial and eukaryotic RNAP structures has revealed different positions of the clamp.[11,13]

Figure 2 (See page 62). (A) Chemical structures of Myx and Lpm. (B) Overlap of the Lpm and Myx binding sites. Amino acids substitutions conferring resistance to Lpm[27] are shown in CPK colored in cyan for β subunit (*T. thermophilus* Q^{1018}, V^{1087}, N^{1064}) and magenta (R^{613}) or dark blue (R^{87}, P^{526}) for β' subunit, $\sigma_{3.2HL}$ is shown as green ribbons and Myx is shown in ball-and-stick and orange. R^{613} is shown in two conformations—as in holoenzyme and as in the RNAP-Myx complex.[1] (C) Model of the mechanism of Lpm and Myx action. RNAP core is shown as a gray ellipse. The σ subunit is shown in green, region 3.2 is shown as a green triangle, and the β' switch-2 is shown as a blue rectangle. The +1 base of the promoter is indicated by a red circle.

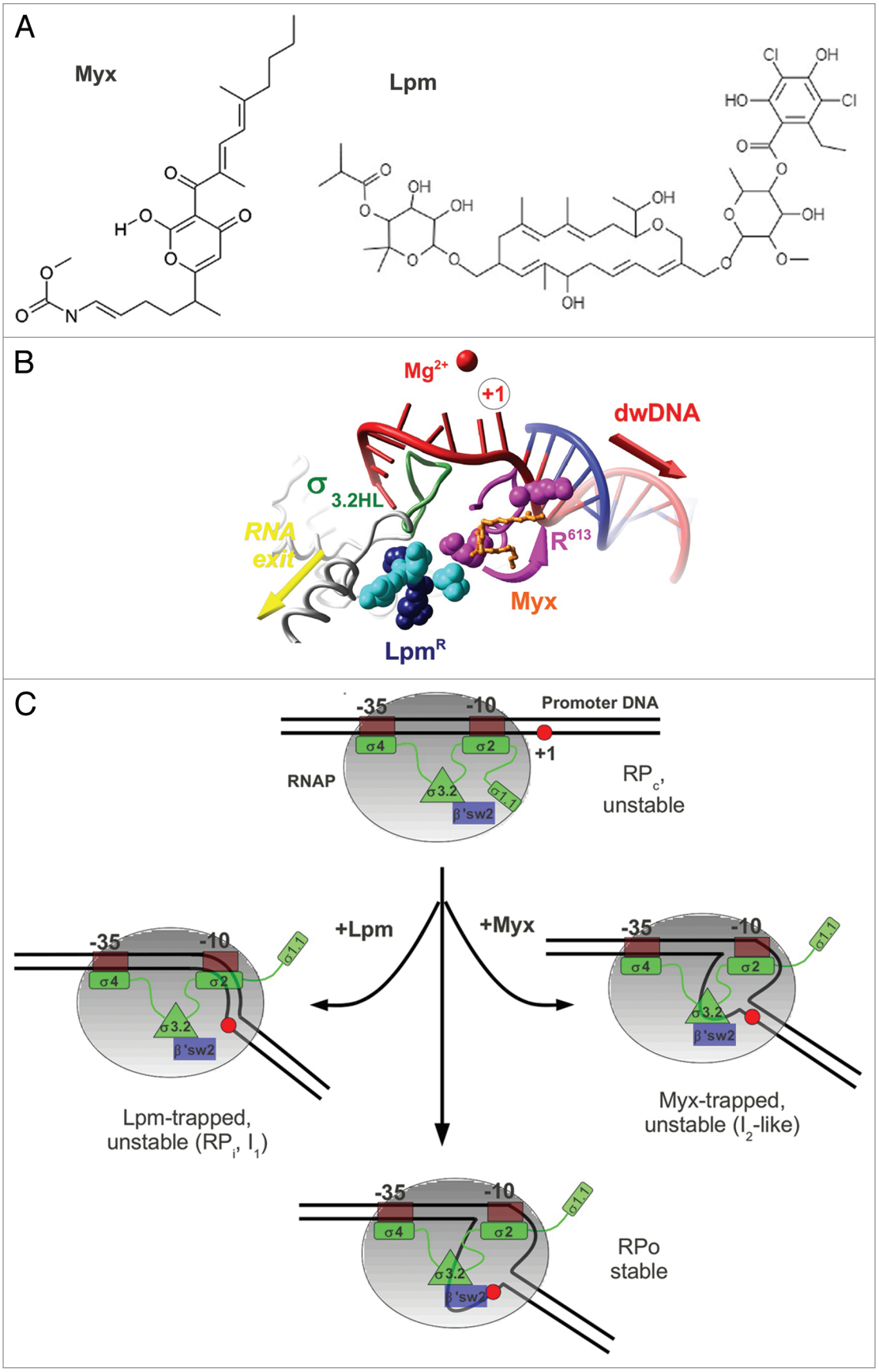

Figure 2. For legend see previous page.

Recently, an alternative model was suggested, in which the β subunit lobes must be transiently displaced to allow the dwDNA to enter to the dwDNA binding channel while the clamp remains static.[14] Additionally, replacement of the region 1.1 by the dwDNA takes place during isomerization from RP_i to RP_o.[20]

The "Bend-Load-Open" and "Open-Bend-Load" Models of RP_o Formation

Two models have been proposed to explain the mechanism of template DNA loading in the RNAP active site cleft. In the first model, referred to as "bend-load-open," the downstream part of the promoter (positions from -5 to +25) is bent over the σ region 2 and then loaded to the active site cleft before the formation of the fully open transcription bubble.[10] The interaction of the RNAP clamp with dwDNA promotes the conformational transition of the core RNAP and the melting of the transcription start site within the active site cleft.[8] This model is supported by the footprinting and cross-linking studies of the putative intermediate complexes formed at low temperature on the *lac*UV5, λP_R, T7A1 and *gro*E promoters.[4,21-23] Whereas the equivalence of the "temperature-trapped" complexes to the real transcription intermediates remains in question, the real-time footprinting studies on λP_R showed that the contacts with dwDNA are formed in closed intermediate RP_c before the bubble opening occurs in RP_i.[10,24]

The second model, referred to as "open-bend-load," is based on real-time kinetic measurements on the T7A1 promoter[5] and on the -10/-35 synthetic consensus promoter.[6] In this model, melting precedes (or is even required for) the entry of the downstream DNA segment into the RNAP jaws. Computer-based Brownian dynamics simulations of the $RP_c \rightarrow RP_o$ transition have also suggested that DNA melts before entering the cleft.[14] The apparent contradiction between the above models may indicate that the exact sequence of the events depends on the promoter type.[5]

$\sigma_{3.2HL}$ Forms a Single Functional Module with the β' Switch-2 Element

Several studies have suggested that the β lobe and the β' clamp play an active role in bubble propagation.[25] Indeed, deletions in the β subunit lobe,[26] or mutations in the β' switch-2,[1,16] block the propagation of melting toward the transcription start site. Thus, RNAP acts as a helicase during the isomerization from RP_c to RP_o, unwinding the promoter DNA that was pre-melted by the σ subunit. The β' switch-2 contacts $\sigma_{3.2HL}$, which likely forms a single functional module implicated in the RP_o formation and template fitting at the active site. Accordingly, the substitutions in the *E. coli* RNAP β' switch-2 or the deletion of $\sigma_{3.2HL}$, increase the K_m for initiating nucleotides.[16] Additionally, the deletion of $\sigma_{3.2HL}$ changes the relative $KMnO_4$ sensitivity of the thymines within the *lac*UV5 promoter transcription bubble (**Fig. 1C**). Thus, even if $\sigma_{3.2HL}$ is not essential for melting, it can affect the template fitting at the active site.

β' Switch-2 and σ Region 3.2 are the Targets for Transcription Inhibitors

Transcription inhibitors are valuable tools to study the molecular mechanism of transcription. Most known inhibitors target the RNAP active site functions, and no inhibitors acting at the promoter complex formation step were known until recently. The first class of such inhibitors to be characterized was the β' switch-2-targeting antibiotics, produced by myxobacteria: Myx from *Myxococcus fulvus*, corallopyronin A from *Corallococcus coralloides* and ripostatin A from *Sorangium cellulosum*.[1,2] Among these molecules, Myx was shown to block transcription start site melting.[1] Recently, Tupin et al. have demonstrated that the antibiotic Lpm from actinomycetes (*Actinoplanes deccanensis*) represents a new class of RNAP inhibitor that blocks isomerization from the closed to the open promoter complex.[3] Genetic analysis showed that Lpm targets not only β' switch-2 but also other functional regions of RNAP (the β' Zn-finger, the β' Lid and β switch-3).[27] Importantly, the activity of Lpm displays strong dependence on the integrity of the $\sigma_{3.2HL}$ structure.[3] Because $\sigma_{3.2HL}$ is not essential for Lpm binding, it was proposed that Lpm might disrupt communication between β' switch-2 and $\sigma_{3.2HL}$.[3] As a consequence, fitting and stabilization of template DNA in the active site cleft is abolished. Notably, $\sigma_{3.2HL}$ is highly variable[28] and is absent in some alternative σ factors (σ^E) or has insertions (e.g., σ^{32}); thus, the prediction is that the inhibition mediated by Lpm is highly selective and depends on the promoter. Indeed, bacteriophage σ-like proteins gp28 and gp34 lacking the region 3.2, renders RNAP resistant to Lpm.[29]

The putative binding site for Lpm is located upstream of the Myx binding site (relative to the +1 base of template), at the entry to the RNA exit channel (**Fig. 2B**). β' switch-2 refolding in the Myx-RNAP complex was proposed as the basis of inhibition.[1] This refolding results in repositioning the R^{613} (*E. coli* R^{337}) residue toward the active site (the distance change ~12 Å), creating additional space in the cleft. Notably, the refolded conformation of β' switch-2 is more compatible with the size of Lpm (volume ~940 $Å^3$) that is larger than Myx (volume ~390 $Å^3$). A plausible hypothesis would be that the β' switch-2 is refolded in a similar manner in presence of Lpm.

Myx Mechanism and the Final Tuning of RP_o Structure

Myx hinders the β' switch-2 function and prevents correct fitting of the template DNA strand into the RNAP active site cleft. So, which step of the open complex formation is targeted by the Myx? The Myx-induced block of the λP_R promoter start-site melting correlated with the lack of a DNase I footprint at promoter positions +18 to +22.[1] However, the major part of dwDNA can still be loaded into the cleft in the presence of Myx. Hence, the Myx-trapped promoter complex likely corresponds to an intermediate immediately preceding the first fully open unstable complex I_2 observed at λP_R[10] (**Figs. 1A and 2C**). The lack of protection between +18 and +22 in the Myx-trapped complex could reflect the lack of "final tuning" in the RP_o

structure observed in real-time kinetics studies.[5] This transition may include folding of the RNAP jaw domains[8,19] or scrunching of the DNA due to bubble formation.[14]

Lpm Mechanism and RP_i Isomerization

Strikingly, while targeting the β' switch-2, Lpm induces much more dramatic effects than Myx. Indeed, Lpm completely blocks the formation of the transcription bubble during RP_o formation on different types of promoters[3] and likely inhibits RNAP through a "multiple-hit" mechanism. Firstly, Lpm prevents the $\sigma_{3.2}$/β' switch-2-dependent fitting of the +1 base into the active site (major mechanism) (**Fig. 2C**). Secondly, Lpm targets the β' Lid and β switch-3. Hence, these elements can also participate in the stabilization of the transcription bubble. Finally, Lpm may keep the β' clamp in an open position that prevents the stabilization of dwDNA binding. Indeed, a strong effect of Lpm on internal protection from DNase I (positions -20 to +15) was observed at *lac*UV5 but not at the λP_R promoter.[3] At the same time, no change in the border of downstream protection (+18) from exonuclease III on *lac*UV5 was observed in Lpm-trapped complexes. Hence, promoter dwDNA duplex is loaded to the active site cleft in presence of Lpm, while bubble formation is blocked. Transient opening of the RNAP pincers must occur to allow this loading.

Considering the kinetic schemes, the Lpm-trapped complex resembles the closed RP_c complex on λP_R scheme (dwDNA protection up to +20) and closed intermediate complexes (RP_c or RP_i) formed on *lac*UV5 at 14°C (**Fig. 1A**). The latter were characterized by the dwDNA footprint up to +20 and fast mobility on native gel electrophoresis.[23,30] Since the kinetically significant RP_i complex on *lac*UV5 is stable,[4] the Lpm-trapped complex could correspond to the unstable closed intermediate that is transient on *lac*UV5 but kinetically significant on λP_R. The instability likely reflects the dysfunction of β' switch-2.[16] Real-time footprinting experiments are required to position the Lpm-trapped complexes in the pathway to RP_o.

Conclusions and Perspectives

The studies on Lpm and Myx support the "bend-load-open" model and suggest that dwDNA can enter the active site channel in double-stranded form. Subsequent bubble formation takes place inside the channel and can be uncoupled from dwDNA binding.

Identifying antibiotics that act at the promoter-opening step in a σ-dependent manner raises a question about the role of these molecules in nature. It is tempting to speculate that such molecules cannot simply be killer molecules but might function as transcriptional regulators, repressing or even activating gene expression.[31] Indeed, the effect of Lpm or Myx on the open complex resembles the effect of DksA/ppGpp on stringent promoters.[32] Future structural and biochemical studies of different promoters and with alternative σ factors are required to understand the detailed mechanism of action and functional role of these molecules.

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