The transcription inhibitor lipiarmycin blocks DNA fitting into the RNA polymerase catalytic site

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Worldwide spreading of drug-resistant pathogens makes mechanistic understanding of antibiotic action an urgent task. The macrocyclic antibiotic lipiarmycin (Lpm), which is under development for clinical use, inhibits bacterial RNA polymerase (RNAP) by an unknown mechanism. Using genetic and biochemical approaches, we show that Lpm targets the σ^{70} subunit region 3.2 and the RNAP β' subunit switch-2 element, which controls the clamping of promoter DNA in the RNAP active-site cleft. Lpm abolishes isomerization of the 'closed'-promoter complex to the transcriptionally competent 'open' complex and blocks σ^{70} -stimulated RNA synthesis on promoter-less DNA templates. Lpm activity decreases when the template DNA strand is stabilized at the active site through the interaction of RNAP with the nascent RNA chain. Template DNA-strand fitting into the RNAP active-site cleft directed by the β' subunit switch-2 element and the σ^{70} subunit region 3.2 is essential for promoter melting and for *de novo* initiation of RNA synthesis, and our results suggest that Lpm impedes this process.

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Introduction

DNA-dependent RNA polymerase (RNAP) is an essential enzyme for gene expression in living organisms from all kingdoms of life. The bacterial RNAP holoenzyme consists of a catalytic core (composed of subunits 2α , β , β' and ω) and a σ subunit required for promoter recognition, promoter opening and transcription initiation (Gross *et al.*, 1998; Helmann and deHaseth, 1999). Structural studies have revealed that the β' and β subunits of the RNAP core form the pincers (or jaws) of a crab claw-like structure in which the active site is buried deeply in the cleft between the jaws

(Zhang *et al.*, 1999). The mobile clamp domain, formed mainly by the β' subunit pincer, serves as a lock of the active-site cleft and a docking site for the σ subunit. In the elongation complex, the 8–9 bp DNA–RNA hybrid occupies the RNAP active-site cleft, whereas the ~ 15 bp DNA duplex downstream of the start site (downstream DNA) is fixed by the pincers in the downstream channel (Vassilyev *et al.*, 2007). The nascent RNA chain exits the active-site cleft through the RNA exit channel formed by the elements of the β' clamp and β subunit. The σ subunit region 3.2 forms a linker between the σ regions 2 and 4 that fill the RNA exit channel in the holoenzyme (Vassilyev *et al.*, 2002; Murakami *et al.*, 2002b). The linker is ejected out of the channel as soon as 10–11 nucleotides (nt) of RNA are formed.

In *Escherichia coli*, the σ^{70} subunit is responsible for transcription initiation for most promoters during exponential growth. During promoter recognition, evolutionarily conserved regions 2 and 4 of σ^{70} bind the -10 and -35 elements of the promoter, respectively; this interaction leads to the formation of the initial 'closed'-promoter complex. Initiation of RNA synthesis requires the formation of the 'open'-promoter complex, in which ~ 13 bp of promoter DNA around the transcription start site are melted to form a transcription bubble. The transition from the 'closed'- to 'open'-promoter complex includes several intermediates and proceeds through nucleation of melting in the -10 element, followed by the downstream propagation of the transcription bubble towards and beyond the transcription start site (Buc and McClure, 1985; Spassky *et al.*, 1985; Sclavi *et al.*, 2005; Rogozina *et al.*, 2009; Schroeder *et al.*, 2009).

Structural and biochemical data suggest that full open-complex formation requires clamping of the downstream part of the promoter DNA duplex (positions -5 to $+20$) by the RNAP β' clamp and β pincer (Craig *et al.*, 1998; Murakami *et al.*, 2002a; Sclavi *et al.*, 2005). The β' switch-2 element, which links the clamp domain to the RNAP body, likely has an important function in the positioning of template DNA in the RNAP active site (Cramer *et al.*, 2001; Naji *et al.*, 2008). Recently, the β' switch-2 element was shown to be a target for the antibiotic myxopyronin, which prevents melting of the promoter transcription start site and consequently inhibits transcription (Mukhopadhyay *et al.*, 2008; Belogurov *et al.*, 2009).

Transcription inhibitors are of great interest as tools to dissect the molecular mechanisms of transcription initiation and elongation. In addition, RNAP, a target for a large body of antibiotics, remains an attractive target for antibacterial drug discovery (Villain-Guillot *et al.*, 2007). Currently, rifamycins (rifampicin and its analogues) remain a first line of antibiotic treatment for tuberculosis and are the only clinical inhibitors targeting RNAP. However, use of rifampicin is strongly compromised now because of the spread of resistant forms of bacteria. For this reason, discovery of new inhibitors and their targets has become an urgent goal in medical research.

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After rifampicin, the second inhibitor of RNAP proposed for use in the clinic is lipiamycin (Lpm) (Figure 1A). This drug is a narrow-spectrum, macrocyclic antibiotic produced by *Actinoplanes deccanensis* (Coronelli *et al*, 1975). Lpm (also known as fidaxomicin tiacumicin B, OPT-80 or PAR-101) is currently in phase III clinical trials against *Clostridium difficile* infections and has been shown to be effective against rifampicin-resistant forms of *Mycobacterium tuberculosis* (Kurabachew *et al*, 2008). The mechanism of Lpm action is not understood. It was suggested that Lpm does not affect promoter binding, but instead blocks the formation of the first phosphodiester bond during the initiation of RNA synthesis (Sonenshein and Alexander, 1979). In addition, it was shown that the σ -like factors of SPO1 bacteriophage make *Bacillus subtilis* RNAP resistant to Lpm (Osburne and Sonenshein, 1980).

The probable-binding site for Lpm on the RNAP surface can be defined by the location of amino-acid changes that confer resistance in *B. subtilis* (Sonenshein *et al*, 1977;

Gualtieri *et al*, 2006), *M. tuberculosis* (Kurabachew *et al*, 2008) and *Enterococcus faecalis* (Gualtieri *et al*, 2009). The Lpm^R substitutions, which mapped exclusively in the β and β' subunits, are clustered at the N-terminal region of the β' subunit (comprising evolutionarily conserved regions A, B and C) and the C-terminal region of the β subunit in proximity to region I (Supplementary Figure S1). Positioning of the Lpm^R mutations on the *Thermus thermophilus* RNAP structure revealed that the antibiotic targets several functional elements of the RNAP mobile clamp domain: the β' subunit Zn-finger (*E. coli* R⁹⁹→P,G), β' Lid (*E. coli* P²⁵¹→R), β' switch-2 (*E. coli* R³³⁷→L,Q) and β switch-3 (*E. coli* Q¹²⁵⁶→K,H,E) (Figure 1B). The β' subunit Zn-finger and the β' Lid are located in the 'clamp core' close to the RNA exit channel, whereas the β' switch-2 and β switch-3 connect the clamp to the RNAP body (Cramer *et al*, 2001; Murakami *et al*, 2002a,b). Potentially, Lpm could affect the function of any of these elements during transcription initiation or could interfere with the growing RNA chain. Characterizing the structure of the RNAP-Lpm complex is necessary to fully understand the mechanism of inhibition. However, transcription experiments have shown that *Thermus* RNAPs, the only existing models suitable for structural studies, are resistant to Lpm (Supplementary Figure S2). This fact precludes us from performing crystallography experiments. To shed light on the mechanism of Lpm action, we used biochemical and genetic approaches in combination with structural modelling using the available structure of *T. thermophilus* RNAP (Vassilyev *et al*, 2002). We analysed the effect of Lpm on transcription initiation at the *lacUV5* promoter and on promoter-independent initiation on short synthetic DNA templates (scaffold). Our experiments reveal a new mechanism of transcription inhibitor action that differs from any mechanism described before. We show that Lpm blocks promoter melting and likely traps RNAP at one of the closed intermediates on the pathway to the transcriptionally competent open-promoter complex. To our knowledge, Lpm is the first example of an antibiotic whose activity strongly depends on both the σ subunit structure and the β' switch-2 element implicated in positioning of the template DNA strand at the RNAP catalytic-site cleft.

Results

Lpm blocks de novo and primed RNA synthesis at the *lacUV5* promoter

Earlier, it has been suggested that Lpm targets formation of the first phosphodiester bond during transcription initiation (Sonenshein and Alexander, 1979). To test this possibility, we analysed the effect of Lpm addition on the [³²P]-UTP- and ATP-initiated synthesis of the dinucleotide pppApU at the T7A1 promoter and the tetranucleotide pppApApUpU at the *lacUV5* promoter (Figure 2A). Indeed, high-resolution gel analysis showed that synthesis of the dinucleotide and the tetranucleotide was inhibited by Lpm. If Lpm functions by blocking the first phosphodiester bond formation, then addition of a dinucleotide RNA primer, which bypasses the first catalytic step, should abrogate the inhibition. To test this assumption, abortive transcription at the *lacUV5* promoter was initiated by the addition of three NTPs (UTP, ATP and GTP) either with or without the 100 μ M ApA primer complementary to the positions +1 and +2 of the template

Figure 1 Modelling of the mutations conferring resistance to Lpm in the structure of the *T. thermophilus* RNAP. (A) Chemical structure of Lpm. (B) Structure of the core RNAP in complex with scaffold DNA (Vassilyev *et al*, 2007) with the amino acids substitutions conferring resistance to Lpm shown as CPK. Numbering corresponds to *T. thermophilus* and *E. coli* (in brackets) enzymes. The β' switch-2 (magenta), β switch-3 (cyan), β' Lid (orange), β' Zn-finger (brown) and β clamp (purple) elements of the clamp are shown as ribbons. DNA is shown in red (template strand) and dark blue (non-template strand), and RNA is indicated in yellow. Position of the active site is marked by magnesium ion (red sphere). RNAP subunits: β' —khaki, β —blue, α —gold, ω —grey. Molecular graphics images were produced using the UCSF Chimera package (Pettersen *et al*, 2004).

Figure 2 Lpm inhibits abortive RNA synthesis at the T7A1 and *lacUV5* promoters. **(A)** Inhibition of dinucleotide (AU) and tetranucleotide (AAUU) synthesis by 100 μM of Lpm. Transcription was initiated at the T7A1 and *lacUV5* promoters. The [³²P]-RNA products separated in a 30% PAGE-7M urea gel are shown. **(B)** Effect of ApA primer on the inhibition by Lpm. Transcription initiated at the *lacUV5* promoter in the presence of U, G, and A or U, G, A and ApA in the presence of increasing concentrations of Lpm. The [³²P]-RNA products separated in a 24% PAGE-7M urea gel are shown.

(Figure 2B). Under these conditions, RNAP synthesizes abortive RNA products up to 9 nt and forms the promoter-proximal elongation complex containing a 16mer RNA (Brodolin *et al*, 2004). No difference in inhibition of abortive-products synthesis or elongation-complex formation was observed between *de novo* initiation (IC₅₀ ~9.2 ± 2.6 μM) and initiation in the presence of ApA (IC₅₀ ~12.5 ± 3 μM). Thus, we concluded that Lpm does not affect the first catalytic step *per se*. The inhibition concentration (IC₅₀) values obtained for the *lacUV5* promoter correspond well to the earlier reported IC₅₀ for *E. coli* RNAP of 8 μg/ml (Sergio *et al*, 1975) and the equilibrium dissociation constant value for Lpm-RNAP complex (*K*_d ~12–23 μM; Talpaert *et al*, 1975). As earlier reported, the inhibition of RNA synthesis by Lpm exhibited an ‘order-of-addition’ effect: it was observed if the drug was added to RNAP only before, not after, the formation of an open-promoter complex (Supplementary Figure S3A) (Sergio *et al*, 1975; Sonenshein and Alexander, 1979; Sarubbi *et al*, 2004).

Lpm targets the β' switch-2 element of the *E. coli* RNAP

Mutations conferring Lpm resistance in *E. coli* have not been characterized biochemically yet. To explore the mechanism of Lpm action, we focused our study on the β' switch-2 element, in which one of the spontaneous mutations conferring the Lpm^R phenotype to *E. faecalis*, *B. subtilis* (Gualtieri *et al*, 2006, 2009) and *M. tuberculosis* (Kurabachew *et al*, 2008) was localized. We proposed that Lpm also targets the *E. coli* RNAP β' switch-2 element function. To test this possibility, we compared the ability of Lpm to inhibit transcription initiated at the *lacUV5* promoter by wild-type *E. coli* RNAP or mutant RNAP^{R337A} with a substitution of the conserved arginine residue in the β' switch-2 element (Belogurov *et al*, 2009). Single-round transcription was initiated by the addition of four NTPs and heparin (Figure 3A). The experiment showed that Lpm effectively inhibited synthesis of full-length RNA transcripts by wild-type RNAP (IC₅₀ ~15 μM), whereas transcription initiation by the mutant RNAP^{R337A} was much less sensitive to Lpm (IC₅₀ ~168 μM). Thus, we conclude that

Lpm may hinder the β' switch-2 element function, whereas the substitution R337A decreases the affinity of Lpm to its binding site.

Deletion of residues 513–519 in the σ⁷⁰ region 3.2 makes RNAP resistant to Lpm

Analysis of the *Thermus* RNAP holoenzyme structure showed that the σ region 3.2 filling the RNA exit channel is located close to the Lpm-binding determinants (Figure 3B). Furthermore, the region 3.2 hairpin loop (σ^{3.2HL}) contacts the β' switch-2 element and the DNA template strand. Thus, Lpm may affect σ-core RNAP interactions, or σ may affect the Lpm-binding site formed by the residues of core RNAP. Indeed, core RNAP-driven transcription was less sensitive to Lpm than holo RNAP-driven transcription (see below and Sonenshein *et al* (1977)). However, no difference in inhibition efficiency was observed if the drug was added to core RNAP before or after the σ⁷⁰ subunit, and no change in affinity of the σ⁷⁰ subunit to core RNAP was observed in the presence of 100 μM of Lpm (Supplementary Figure S3B).

To explore the influence of the σ structure on inhibition, we compared RNA synthesis initiated at the *lacUV5* promoter by RNAP containing either wild-type σ⁷⁰ or σ⁷⁰ with a deletion of the residues 513–519 (σ^{Δ3.2HL}) (Figure 3B). The RNAP-bearing mutant σ^{Δ3.2HL} is deficient in *de novo* initiation on promoters, but is fully active when a dinucleotide primer is used for initiation (Kulbachinskiy and Mustaev, 2006). Transcription initiation at the *lacUV5* promoter in the presence of an ApA primer and increasing concentrations of Lpm was analysed. The result of this experiment showed that, when RNAP with the σ^{Δ3.2HL} was used, the synthesis of run-off and abortive RNAs was resistant to 100 μM of Lpm, whereas the wild-type enzyme was completely inhibited by Lpm. We propose that region 3.2, because of its proximity to the Lpm-binding determinants, can either contribute to the formation of the binding site and, thus, increase its affinity to Lpm or can modulate the function of the β' switch-2 in the positioning of template DNA at the active site. The mutant σ^{Δ3.2HL} in combination with an RNA primer used for initiation makes transcription resistant to Lpm.

Lpm abolishes *lacUV5*-promoter melting

If Lpm blocks transcription by hindering the β' switch-2 function, then it should affect DNA-protein interactions in the open-promoter complex. To test this assumption, we explored whether Lpm blocks the formation of the heparin-resistant open-promoter complex, the step preceding the initiation of RNA synthesis (Figure 4A). First, RNAP binding to the fluorescein-labelled *lacUV5*-promoter fragments in the presence of 100 μM Lpm and heparin was analysed by the electrophoretic mobility shift assay (EMSA) (Figure 4B). The assay allows us to detect a mixed population of stable, heparin-resistant open (RP_o) or intermediate complexes (RP_i) formed on the *lacUV5* promoter (Straney and Crothers, 1985). The results showed that formation of the heparin-resistant RP_o or RP_i was strongly abolished by Lpm, whereas closed complex (RP_c) formation was not affected. Little inhibition of the RP_o/RP_i formation was observed when Lpm-resistant RNAP^{R337A} was used in the experiment (Figure 4B). To test further whether the open-complex formation is the target of Lpm, we tested whether RNAP can melt promoter DNA in the presence of the inhibitor.

Figure 3 Influence of the mutation in the β' switch-2 element and the σ^{70} region 3.2HL deletion on the inhibition of transcription at the *lacUV5* promoter. (A) Inhibition of transcription initiated at the *lacUV5* promoter by wild-type RNAP (WT) and mutant RNAP^{R337A} (R337A) in the presence of increasing concentrations of Lpm. The run-off [³²P]-RNA products separated in a 24% PAGE-7M urea gel and quantification of the experiment are shown. The quantity of run-off RNA synthesized by wild-type (black circles) or mutant (open circles) RNAPs is plotted as a function of inhibitor concentration. (B) Modelling of the σ^A subunit region 3.2 from the *T. thermophilus* holoenzyme (Vassilyev *et al*, 2002) on the structure of scaffold-RNAP complex (Vassilyev *et al*, 2007). The σ subunit is shown as green ribbons with the deletion of amino acids 513–519 in the region 3.2 as CPK. The elements of the RNAP clamp domain-bearing Lpm^R mutations are shown as ribbons, and the mutated residues as spheres. Colour codes are as described in Figure 1B. Dinucleotide RNA primer is shown in yellow. (C) Inhibition of transcription initiated by RNAP carrying either wild-type σ^{70} (WT) or mutant $\sigma^{\Delta 3.2HL}$ ($\Delta 3.2HL$) in the presence of increasing concentrations of Lpm. [³²P]-RNA products separated in a 24% PAGE-7M urea gel are shown. Transcription was performed in the presence of ApA primer. (D) Quantification of the experiment shown in (C). The quantity of run-off RNA synthesized by wild-type (black circles) or mutant (open circles) RNAPs is plotted as the function of the inhibitor concentration. Mean values and s.d. from two independent experiments are presented.

Complexes of wild-type or mutant RNAP and *lacUV5* promoter labelled at the template DNA strand were probed by KMnO₄, which reacts with the thymines at the open (melted) DNA regions (Figure 4C). The result of the experiment performed with wild-type enzyme shows that the thymines at positions –11, –9, –8 and +1 of the promoter DNA were not accessible to KMnO₄ in the presence of Lpm; therefore, the transcription bubble is closed. Little inhibition of promoter melting was observed for the mutant RNAP^{R337A} (Figure 4C, lanes 4 and 5), or if Lpm was added to RNAP after the RP₀ formation (Figure 4C, lanes 3 and 6). As Lpm fully abolishes the formation of the transcription bubble at the *lacUV5* promoter, we conclude that Lpm blocks isomerization of the closed-intermediate complex (RP_i) to the open complex.

Taking into account that *lacUV5* is a mutant promoter with non-optimal 18 bp spacing between the –10 and –35 consensus elements, we explored if Lpm inhibits melting of other promoters as well. Similar to the *lacUV5*, complete inhibition of promoter melting was observed with two natural promo-

oters: the –10/–35 consensus λ P_R promoter and the 'extended –10' consensus *galP2* promoter (Supplementary Figure S4A and B) suggesting that mechanism of inhibition is general.

Lpm does not prevent binding of promoter DNA to the RNAP β' and β pincers

Two possible mechanisms of inhibition by Lpm can be inferred from the above experiments: (1) the downstream segment of promoter DNA (positions –5 to +20) cannot enter the RNAP active-site cleft, as was suggested in the case of myxopyronin (Mukhopadhyay *et al*, 2008), and, therefore, the promoter melting was abolished; (2) the contacts with downstream DNA were formed, but the melting was abolished, as was observed in the case of the RNAP-promoter complexes formed at low temperature (Spassky *et al*, 1985; Craig *et al*, 1998). To discriminate between the two mechanisms proposed above, we probed DNA-protein interactions between RNAP and *lacUV5* promoter using DNase I footprinting (Figure 4D and E). The experiment revealed no significant change in the upstream protection region

Figure 4 Lpm blocks formation of the *lacUV5*-promoter-open complex. (A) Kinetic steps in transcription initiation at the *lacUV5* promoter (Buc and McClure, 1985). P—promoter, R—RNAP, RP_c—closed complex, RP_i—intermediate complex, RP_o—open complex. (B) EMSA of the complexes of RNAP (WT—wild-type, R337A—mutant RNAP^{R337A}) and fluorescein-labelled *lacUV5*-promoter fragment formed either in the presence of Lpm or heparin or both. Complexes were resolved in native 5% PAGE. The position of the open complex (RP_o), closed complex (RP_c) and non-bound DNA (Free DNA) are shown. (C) KMnO₄ probing of the *lacUV5*-promoter complexes formed by the wild-type RNAP (WT) and mutant RNAP^{R337A} (R337A) in the presence of 50 μM of Lpm (lanes 2, 3, 5 and 6). Star indicates Lpm added after the RP_o formation (lanes 3 and 6). DNA was labelled by fluorescein at the 5' end of template strand. The positions of thymines reactive to KMnO₄ in the open complex are indicated. M—A + G sequencing marker. Profiles on the right show the scan of the lanes for RP_o (red) and RP_o + Lpm (green). (D, E) DNase I footprinting of the *lacUV5*-promoter complexes in the presence of 100 μM of Lpm. DNA labelled either at non-template (D) or template (E) strands. Profiles on the right show the scan of the lanes for free DNA (blue), RP_o (red) and RP_o + Lpm (green). (F) The bar graph shows quantification of the DNase I footprinting result for template DNA strand. The ratio of peak area values for indicated bands within the promoter region protected by RNAP in RP_o in the presence or without Lpm were normalized to the peak area values for corresponding bands of free DNA. [T-45] and [T-35] designate the sum of signals in the positions -45 to -39 and the positions -34 to -30 correspondingly. The peak areas values of the bands within the each lane were normalized to the area values of the bands not protected by RNAP within the same lane (positions -48, -50). An average and standard errors of two experiments are shown. The quantified bands are indicated by the red stars in panel E. The RNAP holoenzyme domains interacting with the DNA bases at the indicated positions of promoter in RP_o are shown schematically beneath the bar graph.

(non-template positions -45 to -27 and template positions -50 to -28) in the presence of Lpm. Therefore, the RNAP α subunit C-terminal domain and the σ^{70} subunit region 4.2 remain bound to the promoter DNA in the presence of the drug. In addition, DNase I hypersensitive sites (non-template positions -24 and -23 and template positions -25 , -35 and -36) reflecting bending of DNA in the open complex remained unchanged. The most dramatic changes were observed downstream the -35 element, at the promoter DNA segment that enters into the active-site cleft and the channel formed by the β' , β pincers. Quantification of the template DNA-strand bands intensity showed only 20–40% of protection from DNase I in the presence of Lpm at the positions -14 and -10 (contacted by the σ^{70} region 3) and positions -3 and $+15$ (contacted by the β' , β pincers) (Figure 4F). However, ~ 50 – 60% of protection of the DNA bases at positions $+1$ and $+8$ persist in the presence of Lpm. This result suggests that interactions between RNAP and downstream DNA were destabilized, but not inhibited completely. Consistent with this conclusion, no effect of Lpm on the downstream DNase I protection border was observed for the strong λP_R promoter (Supplementary Figure S4C), whereas promoter melting was fully blocked (Supplementary Figure S4A). In addition, some enhancement in the accessibility of DNase I was observed in the region between positions -20 and $+10$ of the λP_R . Stronger inhibition by Lpm of the *lacUV5* downstream DNase I protection compared with that of λP_R likely reflects a difference in the structure and stability of the kinetically significant intermediates formed on these two promoters (Saecker *et al*, 2002).

To provide additional proof that RNAP remains bound to promoter DNA in the presence of Lpm, we performed Exonuclease III (ExoIII) digestion of promoter complexes (Supplementary Figure S5). The results of the experiment showed that the upstream and downstream borders of protection (ExoIII stops around the position -45 and the position $+18$) remained unchanged. A slight variation in downstream protection likely reflects the destabilization of the downstream DNA binding and correlated with the DNase I footprinting results. Finally, the results of the EMSA and footprinting experiments suggest that Lpm does not affect the interaction with the upstream part of the *lacUV5* promoter (including the -35 element), but destabilizes binding of downstream DNA to the RNAP clamp as reflected by higher accessibility to endonucleolytic digestion by DNase I in the presence of Lpm. On the basis of the above results, we propose that promoter melting is blocked by Lpm because of the inability of RNAP to bind the single-stranded DNA template (ssDNA) at the RNAP active-site cleft.

Lpm inhibits σ^{70} -dependent RNA synthesis initiated at minimal DNA templates

Lpm can function either by occluding the downstream DNA from binding to the channel formed by the RNAP pincers or simply by preventing the ssDNA fitting (entry) at the active-site cleft. To explore further the mechanism of inhibition, we used a promoter-less synthetic DNA scaffold (Figure 5A) comprising a 10 nt single-stranded tail and a 9 nt DNA duplex, analogous to the one used for crystallization (Campbell *et al*, 2001; Vassilyev *et al*, 2007). The scaffold mimics the downstream segment of promoter DNA (template DNA

positions -8 to $+11$; non-template DNA positions $+3$ to $+11$) that enters the RNAP channel formed by the β and β' pincers in the open-promoter complex. Promoter-independent transcription initiation at the scaffold is strongly stimulated by the σ^{70} subunit (Figure 5B) in the same manner as the stimulation observed with the M13 origin DNA (Zenkin and Severinov, 2004). The 13mer RNA was the major product synthesized during initiation on the scaffold in the presence of all NTPs and the CpGpC RNA primer (pRNA3). This finding suggests that transcription starts from the one preferable site. Lpm efficiently inhibited *de novo* RNA synthesis and pRNA3 extension on the assembled scaffold or ssDNA (Figure 5B; Supplementary Figure S6). Furthermore, stronger inhibition was observed on ssDNA ($IC_{50} \sim 16 \mu M$) than on the scaffold ($IC_{50} \sim 35 \mu M$). This result indicates that interactions between the downstream DNA duplex and RNAP antagonized the inhibition.

We also observe that transcription initiated by core RNAP at the scaffold or ssDNA is less sensitive to Lpm than transcription initiated by the holoenzyme (compare core RNAP $IC_{50} \sim 70 \mu M$ with holo RNAP $IC_{50} \sim 34 \mu M$) (Figure 5B and D). This finding supports the idea that the σ^{70} subunit contributes to the formation of the Lpm-binding site. Primed RNA synthesis on ssDNA showed a similar sensitivity to Lpm as the sensitivity observed for *lacUV5*-promoter-dependent transcription (see Figure 2). Thus, the inhibition on the scaffold and on the promoter likely involves the same mechanism. To prove that inhibition of RNA synthesis at the scaffold also occurs by targeting the β' switch-2, we performed a pRNA3 primer extension assay by mutant holo and core RNAP^{R337A} at increasing Lpm concentrations (Figure 5C and D). The synthesis of RNA was not affected by Lpm in the case of the mutant RNAP. This result indicates that Lpm inhibited transcription on the scaffold through the same mechanism as it did on promoter DNA, likely by targeting switch-2-mediated loading of the ssDNA (positions -8 to $+1$) to the active-site cleft.

Long RNA–DNA hybrid suppresses Lpm activity

According to the structure of the RNAP-scaffold complex, the interactions with the first 6 nt of the RNA–DNA hybrid comprise most of the contacts formed by the RNAP active-site cleft in the elongation complex (Vassilyev *et al*, 2007). Indeed, formation of a 7-nt RNA–DNA hybrid leads to significant stabilization of the scaffold-RNAP complex (Korzheva *et al*, 1998; Sidorenkov *et al*, 1998). If Lpm impedes template DNA positioning at the RNAP active-site cleft, then stabilization of the template by a long DNA–RNA hybrid should suppress the inhibition. To explore this possibility, we compared the efficiency of inhibition of UTP addition to RNA primers of 2 (pRNA2), 3 (pRNA3), 7 (pRNA7) and 13 (pRNA13) nt in length (Figure 5E; Supplementary Figure S6). Addition of [³²P]-UTP to RNA primers was performed in the presence of $100 \mu M$ Lpm (Figure 5E) or with increasing concentrations of Lpm (Supplementary Figure S6). The experiments showed that Lpm strongly inhibited nucleotide addition to the pRNA2 and pRNA3, whereas no (or weak) inhibition was observed for pRNA7 and pRNA13. Furthermore, the sensitivity to Lpm was stronger in the case of *de novo* initiation ($IC_{50} \sim 6 \mu M$) than in the case of initiation from pRNA2 or pRNA3 ($IC_{50} \sim 15$ and $35 \mu M$, correspondingly). Thus, Lpm targets RNAP determinants that

Figure 5 Lpm inhibits σ^{70} -dependent RNA synthesis from promoter-less templates. (A) The sequences of the minimal DNA scaffold and the annealed 3 nt RNA primer (pRNA3) are shown. Nucleotides are numbered with respect to the site of the first nucleotide addition (+1). (B) Synthesis of RNA initiated from pRNA3 by core RNAP (lanes 1–10) or holoenzyme (lanes 11–20) in the presence of increasing concentrations of Lpm. Transcription was initiated from either single-stranded template (ssDNA, lanes 1–5, 11–15) or assembled scaffold (scaffold, lanes 6–10, 16–20). The insert on the right shows the low exposure of the gel for lanes 16–20. The lengths of the RNA chains are indicated on the right. (C) Synthesis of RNA initiated from pRNA3 at the scaffold by the mutant holoenzyme or core RNAP^{R337A} in the presence of increasing concentrations of Lpm. (D) Quantification of the experiments shown in (B) and (C). The quantity of 9–12 nt RNA was plotted as a function of the inhibitor concentration. (E) The RNA primer length affects the sensitivity of RNA synthesis to Lpm. A scheme of the assembled scaffold and the 7 nt RNA primer (pRNA7) is shown. The panel on the right shows the RNA products produced after the addition of [³²P]-UTP to the RNA primers (pRNA3 and pRNA7) in the presence of Lpm. The length of the synthesized RNA is indicated.

are required mainly for *de novo* initiation of RNA synthesis, but are dispensable after the transition to the elongation mode when a stable RNA–DNA hybrid is formed. We propose that long RNA primers confer resistance to Lpm on the scaffold because of stabilization of ssDNA in the active site.

Lpm disturbs contact between RNAP and ssDNA

The results obtained on the *lacUV5* promoter and on the scaffold suggest that Lpm affects interactions of RNAP with the downstream part of the promoter (positions –11 to +15). To test whether Lpm directly blocks binding of the scaffold

Figure 6 UV cross-linking and EMSA to probe the DNA-protein interactions in the RNAP-scaffold complex in the presence of Lpm (A, B). The UV cross-linked RNAP-scaffold complexes formed without or in the presence of 100 μ M of Lpm were resolved by EMSA (A) or SDS-PAGE (B). (C) UV cross-linking of the oligonucleotides used in scaffold assembly (ssDNA) and assembled scaffold to σ^{70} and holoenzyme. The position of the label either on template (T) or non-template (NT) DNA strands is indicated. Cross-linked complexes resolved on SDS-PAGE are shown.

to RNAP, EMSA and UV cross-linking experiments were performed in parallel (Figure 6). The EMSA experiment showed that formation of the RNAP-scaffold complex was not significantly affected by 100 μ M Lpm at conditions in which transcription is inhibited completely (Supplementary Figure S5A). UV cross-linking was used to test the close interactions between scaffold DNA and RNAP at equilibrium conditions in solution. The cross-links formed between the scaffold (or ssDNA) and RNAP were to core RNAP (β' or β subunit) and the σ^{70} subunit. Control experiments showed that cross-links were specific to the template DNA strand (labelled by fluorescein at the 5' end), and no cross-link to the non-template strand was formed (Figure 6C). Lpm abolished core-specific cross-links (but not the σ^{70} cross-link) (Figure 6B), but did not prevent RNAP-scaffold complex formation in the EMSA experiment (Figure 6A). These results suggest that Lpm blocks the formation of the contacts between the template DNA strand and core RNAP, or changes the conformation of the DNA-binding site without significantly affecting the overall stability of the complex.

Discussion

Lpm blocks promoter melting driven by the RNAP β' clamp modules

There are two principal findings of this study: (1) the antibiotic Lpm inhibits transcription by blocking formation of a transcriptionally competent open-promoter complex, and (2) the inhibition requires the integrity of the σ^{70} region 3.2. Structure analysis has shown that Lpm targets several important regulatory elements of the RNAP (the base of β' Zn-finger, the β' Lid, the β' switch-2 and β switch-3) located in the mobile clamp domain. We suggest that the inhibitory effect of Lpm is mainly because of targeting of the β' switch-2 element and the σ^{70} region 3.2, because the antibiotic inhibits RNA synthesis started at the synthetic DNA scaffold, which is independent from other elements of the clamp (Naryshkina *et al*, 2006; Touloukhonov and Landick, 2006; Kent *et al*,

2009). Indeed, it was shown that the β' switch-2 controls fitting (or loading) of template DNA into the active-site cleft. This process is required for melting of the promoter transcription start site during open-complex formation (Naji *et al*, 2008; Belogurov *et al*, 2009). According to the RNAP structure, σ^{70} region 3.2 contacts both the β' switch-2 and template DNA; therefore, it can also participate in template fitting at the active-site cleft (Murakami *et al*, 2002a; Touloukhonov and Landick, 2006). Our data suggest that Lpm does not block the entry of downstream DNA duplex into the channel formed by the RNAP β' and β pincers, but it completely blocks the formation of the transcription bubble. Furthermore, using a synthetic DNA scaffold, we show that Lpm impedes the interaction between the RNAP core and the template DNA strand. Thus, we suggest that Lpm abolishes β' switch-2-dependent fitting of template DNA (positions -10 to +1) to the active-site cleft. This fitting is required for open-complex formation and initiation of RNA synthesis.

Two models based on different sets of data have been proposed for the mechanism of promoter melting during open-complex formation. An early model for open-complex formation suggests that RNAP forms contacts with the downstream part of the promoter (between promoter positions -5 and +25) before the formation of the open transcription bubble (promoter positions from -11 to +2) (Buc and McClure, 1985; Spassky *et al*, 1985; Craig *et al*, 1998; Davis *et al*, 2007). According to the new model based on real-time kinetics analysis (Rogozina *et al*, 2009; Schroeder *et al*, 2009), promoter melting can occur before the formation of RNAP contacts with the downstream part of the promoter DNA. Thus, melting and downstream DNA binding by the RNAP pincers can be uncoupled. Our data fit to the model in which binding of downstream DNA to the RNAP pincers are not sufficient for promoter melting, and active participation of the core RNAP clamp modules is required. Lpm hinders the function of the clamp and blocks the isomerization of the closed intermediate (RP_i) to the open-promoter complex.

Lpm acts before myxopyronin in the pathway to the open complex

Recently, new antibiotics (myxopyronin, corallopyronin and ripostatin) targeting the β' switch-2 region have been characterized (Mukhopadhyay *et al*, 2008; Belogurov *et al*, 2009). Two models of transcription inhibition for the drugs targeting β' switch-2 have been proposed: one model suggested that the inhibitor precludes loading of the template DNA strand into the RNAP active-site cleft (Belogurov *et al*, 2009); another suggested that myxopyronin blocks the RNAP clamp in a partially closed conformation that prevents entry of downstream DNA to the channel formed by the β , β' pincers (Mukhopadhyay *et al*, 2008). According to the 'clamp-closure' model, the inhibitor should not work on ssDNAs. Our results show that Lpm inhibits transcription from an ssDNA more efficiently than from a scaffold DNA with a double-stranded DNA duplex. Hence, Lpm directly targets the ssDNA loading at the RNAP active-site cleft. This finding excludes the myxopyronin-like model of 'clamp closure' and favours the 'template loading block' model. Furthermore, we propose that Lpm can lock the clamp in an open or partially open conformation (or it can prevent clamp closure) because the downstream part of the promoter DNA becomes partially protected from DNase I in the presence of the antibiotic. This result may reflect the weak binding of DNA at the RNAP active-site cleft and subsequent destabilization of the promoter complex.

Therefore, despite the closely positioned binding sites of Lpm and myxopyronin, there are striking differences in the mechanisms of inhibition. Both molecules act at the promoter-melting step; however, the effect of Lpm is much stronger because it completely abolished the transcription bubble formation, whereas myxopyronin only prevented melting of the promoter transcription start site. Therefore, Lpm blocks the transition from closed to open complex, whereas myxopyronin targets the open complex at the final step of promoter melting.

The stronger inhibition of melting induced by Lpm than by myxopyronin is likely due to the concerted targeting of several elements of the RNAP clamp and the σ^{70} subunit region 3.2. Indeed, blocking only the β' switch-2 function prevents melting of the start site, but not the upstream part of the transcription bubble (Belogurov *et al*, 2009). Among the elements targeted by Lpm, only the β' Lid was shown to be required for stable open-promoter-complex formation (Touloukhonov and Landick, 2006). However, deletion of the β' Lid has no effect on promoter melting (Naryshkina *et al*, 2006). Deletion of the β' Zn-finger has no effect on promoter-complex stability (King *et al*, 2004), whereas the β switch-3 is implicated in the maintenance of the RNA-DNA hybrid in elongation complexes (Kent *et al*, 2009). Future studies should explore the effect of Lpm^R substitutions in the Lid, switch-3 and Zn-finger on the promoter melting and transcription initiation.

Template strand fitting links open-complex formation and *de novo* initiation of RNA synthesis

We have shown that Lpm can efficiently inhibit $\sigma^{3.2HL}$ -stimulated (Brodolin K, unpublished) transcription initiation on the synthetic scaffold templates and ssDNA in a β' switch-2-dependent manner. Thus, template fitting is not only required for promoter melting, but also for efficient binding

of NTPs at the active site. Indeed, it was shown that three elements targeted by Lpm, β' switch-2 (Naji *et al*, 2008), σ^{70} region 3.2 (Zenkin and Severinov, 2004; Kulbachinskiy and Mustaev, 2006) and the β' Lid (Naryshkina *et al*, 2006; Touloukhonov and Landick, 2006), are required for *de novo* initiation of RNA synthesis. As these elements do not make direct contact with the RNAP NTP-binding site, whereas they interact with the template DNA, we suggest that the stabilization of the template stimulates the initiation of the first phosphodiester bond synthesis or NTP binding. These regions become dispensable as soon as a sufficiently long RNA is synthesized on a promoter or long RNA primers are used for initiation of transcription on promoters or promoterless templates (Zenkin and Severinov, 2004; Naryshkina *et al*, 2006). Such RNA primers likely contribute to the stabilization of the template at the active site leading to promoter melting. Indeed, as was shown in the case of mutant ($\beta^{\Delta 186-433}$) RNAP, lacking one of the pincers (β lobe1), the melting of the *lacUV5*-promoter transcription start site can be detected during the synthesis of long (>6 nt), but not short (<4 nt), RNAs (Brodolin *et al*, 2005).

Taken together, these results suggest that formation of the open-promoter complex and *de novo* initiation of RNA synthesis are tightly linked events subjected to regulation through the template DNA and the β' switch-2. The best known examples are the ribosomal RNA promoters for which stable open-complex formation is stimulated by the first initiating nucleotides (Gourse, 1988; Ohlsen and Gralla, 1992; Gaal *et al*, 1997). Indeed, it was shown that the β' switch-2 element is implicated in regulation of transcription of ribosomal promoters by DksA/ppGpp (Rutherford *et al*, 2009).

Lpm senses the structure of the σ factor region 3.2

We have shown that transcription inhibition by Lpm depends on the $\sigma^{3.2HL}$, which is implicated in the stabilization of incoming NTP at the active site. Two possible mechanisms can be deduced from this finding: (1) $\sigma^{\Delta 3.2HL}$ contributes to the formation of the Lpm-binding site by interacting with the RNAP clamp domain and (2) $\sigma^{\Delta 3.2HL}$ regulates the β' switch-2 function targeted by Lpm. Indeed, transcription initiation on scaffold DNA showed that core RNAP is less sensitive to Lpm (and less active in transcription) than holoenzyme. Hence, σ^{70} modulates Lpm activity, but it is not mandatory for Lpm binding.

It is tempting to speculate that Lpm abolishes function of the $\sigma^{3.2HL}$ by preventing the subunit's communication with the β' switch-2 element or its interaction with the template DNA. Indeed, if Lpm targets the $\sigma^{3.2HL}$, then transcription that starts from a dinucleotide or a longer RNA primer (when $\sigma^{3.2HL}$ is dispensable) should be resistant to Lpm. We observed such a resistance for transcription initiation at the *lacUV5* promoter carried by RNAP with the mutant $\sigma^{\Delta 3.2HL}$. This finding suggests that the $\sigma^{3.2HL}$ either affects the conformation of the Lpm-binding site or stabilizes the active conformation of the β' switch-2 or both. Interestingly, a similar effect of the deletion in the $\sigma^{3.2HL}$ (residues 507–519, overlapping with the deletion studied in our work) on sensitivity of transcription to rifamycins was earlier reported, and it was suggested that $\sigma^{3.2HL}$ acts allosterically through the template DNA strand (Artsimovitch *et al*, 2005). Thus, the drugs targeting different non-overlapping binding sites may

inhibit transcription through a similar mechanism. Meanwhile, an alternative 'steric-occlusion' model of rifampicin action was also proposed (Feklistov *et al*, 2008).

As the binding site of Lpm is close to the σ subunit region 3.2 that is highly variable between the alternative σ factors, we propose that Lpm inhibition activity should vary between RNAPs bearing alternative σ factors. In support of this proposal, it was shown that transcription of the phage SPO1 later genes driven by the *B. subtilis* RNAP and the phage σ -like factors is resistant to Lpm (Osburne and Sonenshein, 1980). These findings open the way to design σ -specific drugs targeting specific transcription pathways (e.g. activation of virulence genes). Finally, Lpm is the first example of a transcription inhibitor blocking the early step of open-promoter-complex formation and provides an excellent tool to study this complex process. Clarifying the mechanism of inhibition by Lpm is an essential step in future drug design and awaits new models suitable for crystallographic studies.

Materials and methods

Proteins, DNA templates and RNA

E. coli RNAP core enzyme was purchased from Epicentre. Mutant *E. coli* core RNAP-bearing substitution R337A was a generous gift from Dr Irina Artsimovitch. The wild-type σ^{70} subunit cloned in the pET21 vector was expressed in BL21(DE3) strain and purified by affinity chromatography according to Qiagen protocols. The σ^{70} -bearing deletion of amino acids 513–519 was purified as described in Kulbachinskiy and Mustaev (2006). The 116 bp *lacUV5*-promoter fragment (promoter positions from –59 to +58) was produced by PCR with the primers 5'-CTCACTCATTAGGCACCCAGGC-3' and 5'-CCAGCGGTGAAGGGCAATCAGC-3' (Sigma). To label DNA either at the template or non-template strand, fluorescein was introduced at the 5'-end of the primer. DNA scaffolds were assembled from two oligonucleotides: 5'-ACAATGCA-3' and 5'-TGCAATTGTCAGCGATC TA-3' or 5'-TGCAATTGTCATCCGCTCA-3' in the transcription buffer (TB) containing 40 mM HEPES pH 8.0, 50 mM NaCl, 5 mM MgCl₂ and 5% glycerol. Oligonucleotides were heated at 65°C for 5 min and then annealed by lowering the temperature to 16°C for 30 min. The RNA primers pRNA2 (5'-CG-3'), pRNA3 (5'-CGC-3'), pRNA7 (5'-GAGCGGA-3') and pRNA13 (5'-CGCUAGAUGUUA-3') were purchased from Eurogentec.

Transcription at the *lacUV5* and scaffold DNA

Transcription at the *lacUV5* promoter was performed in 10 μ l TB. RNAP holoenzyme was reconstituted by the mixing of σ^{70} subunit (0.5 μ M final) and 80 nM core RNAP and then incubating for 5 min at 24°C. Next, 1/10 reaction volume of Lpm solution in 50% methanol was added and incubated with RNAP at 37°C for 10 min. The mixture was incubated with *lacUV5* DNA fragment (13 nM) at 37°C for 5 min to form an open complex. Transcription initiated by addition of ATP, GTP and CTP (50 μ M each) and 3 μ Ci [α -³²P] UTP was performed for 5 min at 37°C in the presence of 0.1 mg/ml heparin. Transcription at the scaffolds was performed as follows:

80 nM RNAP, 200 nM scaffold and 1 μ M RNA primer were incubated at 16°C for 5 min then at 24°C for 5 min and supplemented with either [α -³²P] UTP or 4 nt as above. Transcription was carried out for 5 min at 24°C. The reactions were stopped by adding 90% formamide. RNA was analysed on 24% PAGE-7MUrea denaturing gel.

DNA-protein cross-linking and EMSA

UV cross-linking of the scaffold-RNAP complexes was performed by 2-min irradiation of the complexes in open Eppendorf tubes placed under the UV-lamp (BLX-E254) at 254 nm. Afterwards, cross-linked complexes were resolved by 6% SDS-PAGE. For EMSA experiments, the RNAP-*lacUV5*-promoter complexes, formed as described above, were incubated with 100 μ g/ml of heparin for 2 min at 37°C and loaded on a 5% native 0.5 $\times$ TBE-PAGE. RNAP-scaffold complexes assembled at 16°C were incubated at room temperature for 5 min and loaded on 6% native 0.5 $\times$ TBE-PAGE.

KMnO₄ probing and DNase I footprinting

For KMnO₄ probing, reactions were performed in 10 μ l TB. RNAP (400 nM) was incubated with 100 μ M of Lpm for 10 min at 37°C. Fluorescein-labelled *lacUV5* promoter (75 nM final) was added to the reaction, which was incubated for another 10 min at 37°C. The samples were treated with 5 mM KMnO₄ for 30 s at 37°C. The reaction was stopped with the addition of a $\frac{1}{2}$ volume of 1.5 M potassium acetate and 1 M β -mercaptoethanol. DNA was precipitated by ethanol and incubated with 100 μ l 0.5 M piperidine at 90°C for 15 min. Then DNA was precipitated with n-butanol and washed with 70% ethanol. Dried samples were dissolved in 90% formamide and analysed on 10% sequencing gel. For DNase I footprinting experiments, *lacUV5*-promoter complexes were formed as described for permanganate probing. The samples were treated with 5 U/ml DNase I (Promega) for 1 min. The reactions were stopped by addition of 5 mM EDTA pH 8 and 200 ng poly(dA-dT). Samples were ethanol precipitated, dried and dissolved in 90% formamide. DNA fragments were analysed on 10% sequencing gel. Gels were scanned with Typhoon 9400 Imager (GE Healthcare) and quantified using ImageQuant software (Molecular Dynamics). Peak quantification and data processing were performed as described in Sclavi *et al* (2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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