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Multiple active centers of multi-subunit RNA polymerases

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The active center of multi-subunit RNA polymerase consists of two modules, the Mg^{2+} module, holding the catalytic Mg^{2+} ion, and a module made of a flexible domain, the Trigger Loop. Uniquely, the TL module can be substituted by alternative modules, thus changing the catalytic properties of the active center.

Keywords: RNA polymerase, transcription, active center, hydrolysis, transcription factor

Abbreviations: RNAP, RNA polymerase; TL, trigger loop; EC, elongation complex

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RNA Polymerase Active Center

Multi-subunit RNA polymerase (RNAP), like all nucleic acid polymerizing enzymes, uses a two metal ion (in this case Mg^{2+}) mechanism.[1] Only one Mg^{2+} ion is held tightly in the active center; another one is bound weakly and, during RNA synthesis, is introduced by the incoming NTP. Besides phosphodiester bond formation (and pyrophosphorolysis, its direct reversal), the active center of RNAP can catalyze hydrolysis of phosphodiester bonds of the nascent RNA. However, during hydrolysis, the affinity of the labile Mg^{2+}II to the active center of RNAP is much lower than during synthesis. Such lability of one of the catalytic Mg^{2+} is apparently used by RNAP as a simple way of control of unwanted hydrolysis of RNA. Specific mechanisms exist to increase affinity for the labile Mg^{2+} when hydrolysis becomes required for the resolution of backtracked or misincorporated complexes (see below).[2,3] Thus, the active center can be modulated by changing the affinity for Mg^{2+}II according to the needs of RNAP. However, a much greater flexibility of regulation of the active center exists, and not only via affinity to the Mg^{2+}II ion.

Some time ago, we showed that a flexible loop, the trigger loop (TL), in the vicinity of the catalytic Mg^{2+} ions plays a crucial role in catalysis.[4] Crystallographic studies showed that the TL alternates between folded (closed) and unfolded (open) conformations.[5,6] Folding of the TL brings its catalytic residues (in bacterial RNAP, β' R1239 and H1242; *T. aquaticus* numbering) close to the catalytic Mg^{2+} ions, where they stabilize the transition state of the phosphodiester bond synthesis[6-9] or act as general acid-base during phosphodiester bond hydrolysis.[10] In the open state of the TL, these catalytic residues are too far from the Mg^{2+} ions to participate in reactions, and the active center looks too "open" to be able to properly orient the reactants and to exclude unwanted water molecules (**Fig. 1A**). In this sense, the active center can be viewed as consisting of two essential parts: the static "Mg^{2+} module" and the dynamic TL module (**Fig. 1C**).

The intrinsic hydrolysis of the penultimate phosphodiester bond can be assisted by the transcript's 3' NMP, which coordinates Mg^{2+}II and may be involved in coordinating the attacking H_2O molecule.[2,10] Besides direct participation as an acid-base, the TL may also be involved in the transcript-assisted part of the cleavage by positioning the 3' NMPs in the active center.[10] Multiple functions of the TL hint at different conformations the folded TL can adopt in various circumstances and reveal the versatility it brings to the RNAP active center. A unique feature of the RNAP active center, however, is that the flexible TL can be displaced and substituted for by transcription factors and small molecules. In other words, the TL module of the active center can be substituted for by "alternative modules," thus changing the identity of the active center. This phenomenon will be discussed below.

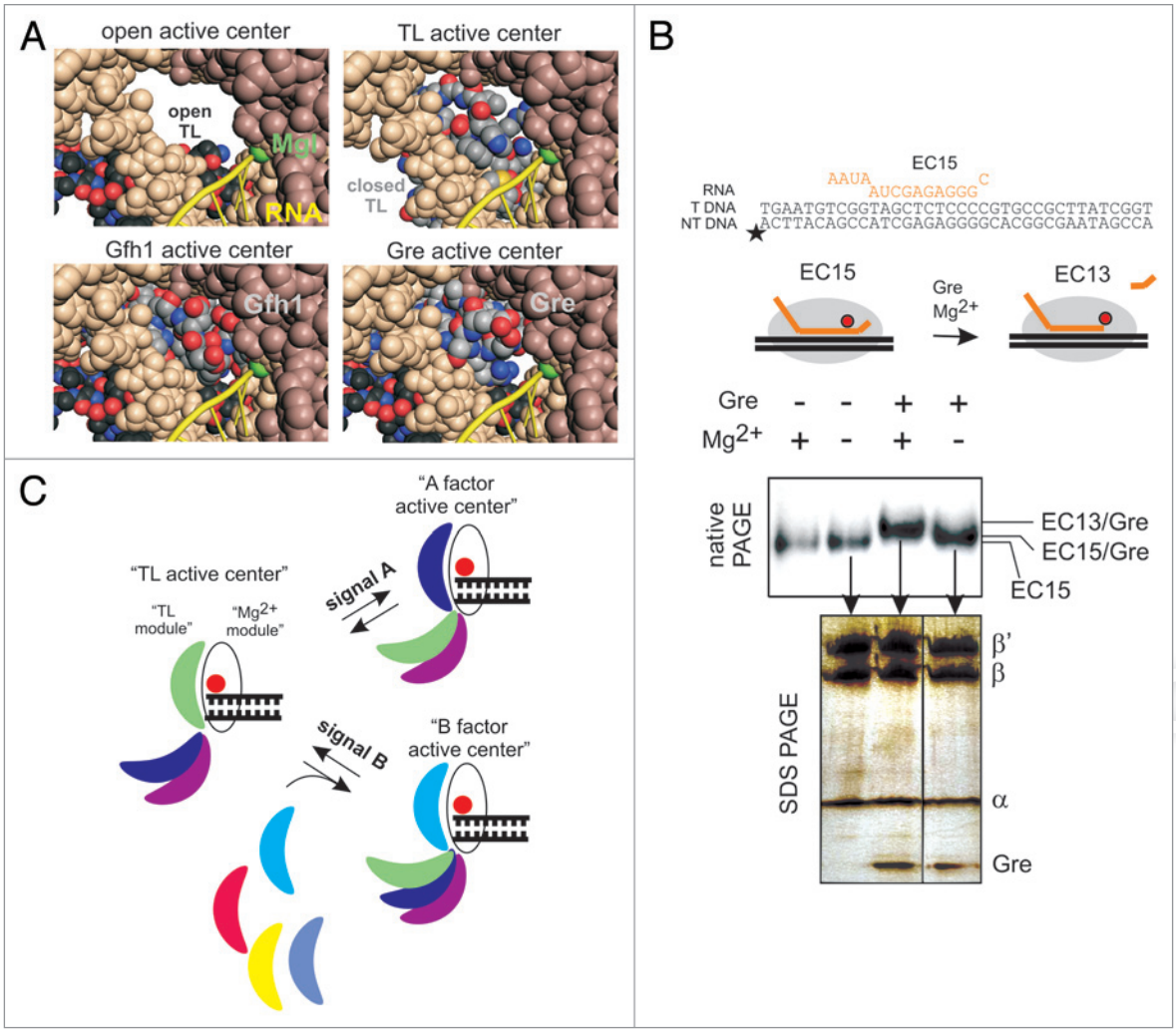

Figure 1. Switching of RNAP active centers. (A) Substitution of the flexible TL in the active center of RNAP by Gfh1 and Gre factors. Left top: the "TL module" is absent from the active center when the TL (dark gray) adopts its open conformation (PDB 2BE5; parts of β and β' forming "Mg^{2+}" module of the active center are in beige and brown spacefill, respectively; RNA is yellow; Mg^{2+}I is green sphere). Right top: the "TL active center" is formed when the TL (gray) adopts its closed conformation (PDB 2O5J). Left bottom: the "Gfh1 active center" (PDB 3A0H) is formed when Gfh1 (light gray) substitutes for the TL (dark gray from PDB 2O5J) in the active center. Right bottom: "Gre active center" is formed when the TL (dark gray) is substituted by Gre factor (light gray). The structure of *E. coli* GreB (PDB 2P4V) was fitted into the structure of Gfh1 bound to the elongation complex (PDB 3A0H). (B) The Gre factor stays bound to the elongation complex after the cleavage reaction is completed (for protein purification, oligonucleotides, elongation complex assembly see ref. 14). Gel at the top: electrophoretic mobility shift assay of elongation complex EC15 assembled with *T. aquaticus* RNAP, with radioactively labeled non-template strand (black asterisk). Heparin was added to reactions to 100 μg/mL final concentration. Where specified, $MgCl_2$ was added to 10 mM final concentration. *T. aquaticus* Gre was added in a two molar excess over the fully assembled elongation complexes for 1 min (concentration of assembled elongation complex was determined by quantification of the amount of the non-template strand that was incorporated in the complex). Gre in the presence of Mg^{2+} converts EC15 to EC13, which is resistant to Gre.[14] Complexes were supplied with 10% glycerol, resolved in a 6% Tris-glycin polyacrylamide gel (19:1), and revealed by PhosphorImaging (GE Healthcare). The mobility of EC13/Gre was slightly different from that of EC13 (not shown). The presence of RNAP and factors in the complexes was confirmed by separation of the cut out complexes by 4–15% gradient SDS PAGE (bottom part). After electrophoresis, the gel was silver stained. (C) Switching of the active centers of RNAP. In response to some signals, the "TL module" (green) of the active center can be substituted by various "alternative modules" thus converting "TL active center" into corresponding "alternative active centers" (crescents of different colors). "Alternative modules" may stay permanently associated with RNAP, or bind RNAP from solution in response to various signals.

"Alternative Active Centers" Gfh1 and Gre

The crystal structure of *T. thermophilus* transcription factor Gfh1 (a homolog of transcription cleavage factor Gre) bound to RNAP[11] revealed that, while Gfh1 inserts its coiled-coil domain into the secondary channel and reaches the very active center, it displaces the TL away from the active center, thereby disabling it to adopt a catalytically active folded conformation. Using the above terminology, the "Gfh1 module" replaces the "TL module," thus converting the "TL active center" into the "Gfh1 active center" (**Fig. 1A**). While the "Mg^{2+} module" remains intact, the specific activity of the active center, determined by

the "flexible modules," has now changed. It should be noted, however, that the only known specific activity for the Gfh1 is the absence of activity.[12,13] Therefore, the switch to the "Gfh1 module" leads to inhibition of RNAP. It is possible, however, that the specific activity of Gfh1 manifests itself in response to some particular signal, as in the case of Gre (see below), which is yet to be discovered.

Transcription factor Gre assists hydrolysis of the transcript in the backtracked and misincorporated complexes. Recently, we showed that Gre (*T. aquaticus*, used in this study, has only one Gre factor, as opposed to GreA and GreB of *E. coli*) can displace the TL from the active center, thus switching off the TL-dependent activities.[14] Instead, it imposes a new, highly effective cleavage activity. This can be seen as if the "TL module" of the active center, efficient in synthesis and not efficient in hydrolysis, is replaced by the "Gre module," inactive in synthesis but effective in hydrolysis. In other words, a "polymerase" active center is converted into a "proofreading" one (**Fig. 1A**). Interestingly, such a swap of active center modules takes place only in response to a particular signal. The "TL module" is substituted by the "Gre module" only when RNAP undergoes backtracking or misincorporates erroneous NMP (which also causes backtracking by 1 base pair). After the hydrolysis reactivates the elongation complex, the "Gre module" is displaced back by the "TL module."[14]

In the active (not backtracked) complex, the "Gre module" does not displace the "TL module," ensuring that processive transcription is not interrupted or affected by hydrolysis of the nascent transcript.[14] It, however, seems that Gre stays bound to RNAP even when the "TL module" occupies the active center. We performed an electrophoretic mobility shift assay (EMSA) of *T. aquaticus* elongation complexes containing 15 nucleotide long RNA with mispaired 3' end NMP (EC15, which mimics a 1 base pair backtracked complex; scheme in **Fig. 1B**). The nontemplate strand was radiolabeled to ensure that only fully assembled complexes are monitored. Addition of Gre slowed migration of the elongation complex (**Fig. 1B** and top part), which is consistent with our previous data that it interacts with backtracked complexes.[14] The presence of Gre in the complex was confirmed by separating the proteins from the EMSA band by SDS PAGE (**Fig. 1B** and bottom part). Note, that, in the absence of Mg^{2+}, Gre cannot cleave RNA. Addition of Mg^{2+}, which allowed Gre to perform cleavage of the dinucleotide, converted EC15 to "transcriptionally competent" EC13, which is largely resistant to Gre.[14] Interestingly, however, Gre still stayed bound to EC13 as was confirmed by separation of the proteins present in the EMSA band (**Fig. 1B** and bottom part). This suggests that Gre alternates with the TL without dissociation from the elongation complex (scheme in **Fig. 1C**).

"Alternative Active Centers" of Eukaryotic RNAPs

A similar situation with hydrolysis in backtracked and misincorporated complexes is observed in eukaryotes. The TL of eukaryotic RNA polymerase II (RNAP II) does not catalyze hydrolysis, as does bacterial TL. For phosphodiester bond hydrolysis, RNAP II uses an external factor, TFIIS. TFIIS is structurally unrelated to Gre but also has an extended domain, which reaches the catalytic Mg^{2+} through the secondary channel. As in the case of Gre,[14] the functional TL is not required for TFIIS cleavage.[15] Consistently, as evidenced from the crystal structure of the RNAP II backtracked elongation complex, TFIIS substitutes for the TL in the active center.[16] It is not known if the "TFIIS module" can stay bound to the elongation complex after cleavage, in the same way the Gre factor does. Interestingly, however, homologs of TFIIS of RNAP I and RNAP III (A12.2 and C11, respectively) are actual subunits of these polymerases, i.e., are permanently attached to them. In other words, "A12.2 module" and "C11 module" alternate with the "TL modules" of their respective RNAPs without dissociation from the elongation complex (**Fig. 1C**).

Multiple Active Centers of RNAP

A plethora of factors and small molecules that bind to RNAP in the secondary channel suggests that the on/off switching and exchanging of the activities via substitutions of active centers might be a common way of RNAP regulation. A Gre-like structure of some bacterial transcription factors strongly implies that they may as well act via substitution of the "TL module" of the active center. The number of such potential "alternative modules" of bacterial RNAP active center is astonishing—GreA, GreB, Gfh1, DksA, TraR, Rnk and YacL. In eukaryotes, multiple (up to 5 in *Paramecium tetraurelia*) TFIIS-like proteins were found,[17] also suggesting the existence of multiple "alternative modules" of RNAP II active center. TFIIS was recently found to be associated with RNAP III,[18] suggesting that, besides the hydrolytic "C11 module" TFIIS may be another "alternative module" of the RNAP III active center. Some small molecules may also act by replacing the "TL module." In the currently available structures of RNAP in complex with alarmone ppGpp[19] or inhibitor Tagetitoxin,[20] both small molecules are bound in the vicinity of the catalytic Mg^{2+} and would prevent the TL from adopting its normal folded conformation. Such substitution of the "TL module" with "Tagetitoxin module" or "ppGpp module" may just inhibit the RNAP. However, it is possible that it may also somehow modify RNAP activities. Peptide antibiotic MicrocinJ25 binds in the secondary channel of RNAP, and may inhibit RNAP via substitution of the TL in the active center. Interestingly, MicrocinJ25 increases the life time of the pauses during elongation, but does not affect elongation between pauses,[21] suggesting that it may respond to particular states of the elongation complex in a manner similar to Gre.

Recent works revealed at least partial redundancy of the functions of some of the "alternative modules." GreB, if overexpressed, can complement the loss of DksA in control of rRNA transcription in *E. coli*.[22] *E. coli* GreA can mimic DksA effect on preventing replication fork arrest.[23] Interestingly, just one change of aspartic acid residue at position 44 of *E. coli* GreA to the glutamic acid, which is specific for DksA, converts GreA to DksA-like factor.[24] TraR can compensate for defects in growth caused by DksA deletion.[25] Despite this partial redundancy in

function, it is possible (and is a fact in the case of Gre and TFIIS) that some "alternative modules" may bring distinct activities to the RNAP active center. It cannot be excluded that they may target some transient conformation of the active center, which has not been captured yet. As we have shown, Gre replaces TL only in misincorporated and backtracked elongation complexes. Very recently, DksA was proposed to recognize paused elongation complexes.[26] If other potential "alternative modules" of the RNAP active center respond to specific signals and if they bring any specific activity to the RNAP active center remains to be established.

Inhibition or activation of enzymatic activity by changing the amino acid "landscape" of their active centers by other proteins is a common way of regulation of proteinaceous enzymes. For example, large number of NTP hydrolyzing enzymes use a conserved arginine residue, the "arginine finger," to stabilize the transition state and catalyze cleavage of the γ-phosphate of the NTP. This arginine does not reside in the active center of the enzyme, but rather on a distinct domain of the enzyme or on a separate activator protein,[27] and has to be delivered to the active center for catalysis to take place. In some cases, activator proteins even have dual specificity—they do not only bear an "arginine finger" but also an "asparagine thumb," each of which is able to activate a different class of GTPases.[28] This situation resembles a detachable "modules" of RNAP active center. However, to the best of our knowledge, the existence multiple interchangeable modules of the single active center of RNAP is a unique phenomenon among proteinaceous enzymes.

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