Control of Transcriptional Fidelity by Active Center Tuning as Derived from RNA Polymerase Endonuclease Reaction*

Ekaterina Sosunova†, Vasily Sosunov†, Vitaly Epshtein†‡, Vadim Nikiforov†‡, and Arkady Mustaev†‡

From the †Public Health Research Institute Center, New Jersey Medical School, Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103 and the ‡Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia

Background: Factor-assisted co-transcriptional proofreading and precise selection of NTP substrates provide high transcription fidelity.

Results: Both processes can be achieved through active center tuning (ACT) from the inactive to catalytic state in response to establishing recognition contacts of the reaction substrates.

Conclusion: High transcription fidelity can be explained by ACT.

Significance: Suggested ACT mechanism represents an exceptional example of substrate recognition coupling to catalysis.

Precise transcription by cellular RNA polymerase requires the efficient removal of noncognate nucleotide residues that are occasionally incorporated. Mis-incorporation causes the transcription elongation complex to backtrack, releasing a single strand 3′-RNA segment bearing a noncognate residue, which is hydrolyzed by the active center that carries two Mg2+ ions. However, in most x-ray structures only one Mg2+ is present. This Mg2+ is tightly bound to the active center aspartates, creating an inactive stable state. The first residue of the single strand RNA segment in the backtracked transcription elongation complex strongly promotes transcript hydrolytic cleavage by establishing a network of interactions that force a shift of stably bound Mg2+ to release some of its aspartate coordination valences for binding to the second Mg2+ thus enabling catalysis. Such a rearrangement that we call active center tuning (ACT) occurs when all recognition contacts of the active center-bound RNA segment are established and verified by tolerance to stress. Transcription factor Gre builds on the ACT mechanism in the same reaction by increasing the retention of the second Mg2+ and by activating the attacking water, causing 3000–4000-fold reaction acceleration and strongly reinforcing proofreading. The unified mechanism for RNA synthesis and degradation by RNA polymerase predicts that ACT also executes NTP selection thereby contributing to high transcription fidelity.

Transcription of DNA by RNA polymerase (RNAP)3 is a highly accurate templated synthesis. The fidelity of transcription relies on both efficient initial substrate selection and on proofreading that removes mis-incorporated residues.

Addition of each nucleotide by RNAP yields two principal states (Fig. LA) such that the enzyme moves forward along the DNA template from the pretranslocated state (I) to the post-translocated state (II), which vacates the active center for the next NTP substrate. The enzyme can also backtrack (state III), which releases the 3′-RNA segment from the RNA/DNA hybrid. Backtracking is strongly promoted when noncognate nucleotide residues are incorporated. The disengaged single strand RNA segment in a backtracked complex can be removed by RNAP in its endonucleolytic mode (1, 2), whereas the pretranslocated complex can release the 3′-terminal RNA residue (3) following exonuclease reaction (Fig. LA). Intrinsic endonuclease activity of RNAP, strongly augmented by prokaryotic GreA and GreB proteins (4–6) and by eukaryotic SII factor (7, 8), is believed to provide proofreading that removes RNA segments containing a noncognate residue (9, 10), thereby enabling the enzyme to continue transcription from the newly formed RNA 3′ end.

All these reactions operate through a two-metal mechanism (3, 6) at the same active center (11) located at the intersection of the main channel that encloses the nucleic acid scaffold of transcription complex and the secondary channel, which supplies NTP substrates and accommodates a 3′-disengaged RNA segment in backtracked TEC. Also, a long α-helix of the β′ subunit (F-bridge or bridge helix) separating the main and secondary channels near the downstream edge of the RNA/DNA hybrid together with a β′ G-loop (trigger loop) have been implicated in catalysis and RNAP translocation (12–16). Exactly how the two-metal mechanism operates in the case of endonuclease reaction and the role of various active center elements in catalysis remain obscure. Studies on Thermus aquaticus RNAP proposed direct involvement of the functional groups of the disengaged +2 RNA residue in endonuclease reaction through...
activation of the attacking water and coordination of catalytic Mg-II (17). However, molecular modeling shows that this is inconsistent with $S_{n2}$ reaction mechanism that strictly defines the position of two catalytic Me$_2$H$_{11001}$ ions, scissile phosphodiester group, and the attacking water.

$S_{n2}$ mechanism (3, 6, 11, 18–22) relies on two Mg$_2$H$_{11001}$ ions coordinated by the aspartate triad ($\beta'$ Asp-460, $\beta'$ Asp-462, and $\beta'$ Asp-464) of the active center (Fig. 1B). One of the Mg$_2$H$_{11001}$ ions (Mg-I) is coordinated by all three Asp residues, whereas the second Mg$_2$H$_{11001}$ ion (Mg-II) is coordinated by only two residues (Asp-460 and Asp-462). The latter are called “bridging” because they connect both Mg$_2$H$_{11001}$ ions. During RNA synthesis (Fig. 1B, left panel), Mg-I activates the 3’-attacking hydroxyl of the terminal RNA residue and the $\alpha$-phosphate of an NTP substrate for nucleophilic attack; Mg-II facilitates release of pyrophosphate. In RNA hydrolytic cleavage (Fig. 1B, right panel), Mg$_2$H$_{11001}$ ions switch their role. In this reaction Mg-II activates the attacking water, whereas Mg-I promotes release of the leaving group. One of the ions (Mg-I) is bound with high affinity and the other (Mg-II) with low affinity and has to be stabilized by additional coordination with the following: (i) two terminal phosphates of incoming NTP in polymerization reaction; (ii) the same phosphates of NTP bound to an E site (which is believed to be involved in substrate loading (3, 20)) in exonuclease transcript cleavage, and (iii) carboxylate side chains of Gre or TFIIS proteins in factor-assisted RNA endonuclease cleavage (6, 18–21).

In the majority of x-ray structures of multisubunit RNAP, only one Mg$_2$H$_{11001}$ ion (Mg-I) is seen bound to all three aspartates of the active center. According to quantum mechanics calculations (23), this Mg$_2$H$_{11001}$ ion forms strong coordination bonds with the aspartates, which makes this state stable. However, all reactions of RNAP require two Mg$_2$H$_{11001}$ ions for catalysis, suggesting that this inactive stable state has to be tuned to a catalyt-
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To achieve this, Mg-I must be pushed aside to free the coordination valences of the bridging Asp residues so they can bind Mg-II for catalysis (Fig. 1C). The rearrangement involves repositioning of all three Asp side chains and a drastic change in Mg-I coordination geometry, causing rotation of the bridging aspartates to co-linear orientation (Fig. 1C, right panel). This assumption finds support in recently resolved x-ray structures (3GTG and 3PO3 (24, 25)) of TEC in which endonuclease reaction occurs. Analysis of these structures and our biochemical data show that the above rearrangement of the active center can be promoted by the recognition contacts of substrates in both RNA hydrolytic proofreading and RNA polymerization reactions. It is this rearrangement that we refer to herein as active center tuning (ACT).

In the living cell, endonuclease transcription cleavage is assisted by Gre factors that strongly increase the reaction rate. We find that these factors build on the ACT mechanism by further increasing Mg-II retention and by activating the attacking water for hydrolytic reaction.

The above findings in combination with crystallographic data extend the two-metal active center model to a new level of understanding of unified catalytic mechanism of RNA synthesis and degradation by RNAP. The suggested mechanism for ACT involves the loading (in hydrolytic RNA cleavage) or proper positioning (in RNA synthesis reaction) of catalytic Mg-II.
**EXPERIMENTAL PROCEDURES**

**Reagents and Equipment**

Unless otherwise stated, all chemistry reagents were purchased from Sigma, Acros Organics, TCI America, or Fluka. Nucleic acid enzymes were from New England Biolabs and United States Biochemical Corp. NTP substrates were from Amersham Biosciences; radioactive NTPs were from MP Biomedicals; oligonucleotide RNA primers were from Oligos, Etc. Preparative ion-exchange column chromatography was conducted on DE-53 cellulose and Dowex-1 (40 mg/ml) resin. The radioactive RNA products resolved by electrophoresis were quantified by phosphorimagery using a Molecular Dynamics device (GE Healthcare) and Storm 60 software. Molecular modeling was performed using WebLab ViewerLight 4.0 (Molecular Simulations Inc.).

**Modified Substrate Analogs**

**α-Methyl-ATP**

Because the published synthetic procedure (28) did not yield sufficient amounts of the material, we developed new and more efficient and fast protocol. Twenty six milligrams (100 μmol) of dry adenosine were suspended in 90 μl of trimethylphosphate dried over barium oxide. The mixture was supplemented with 13 μl (20 mg, 150 μmol) of methylphosphonic dichloride and agitated for 1–1.5 h until the suspension became transparent. The residue was centrifuged, re-dissolved in water, and subjected to preparative TCL in an acetonitrile/water (3:1) development system. The fractions containing pure α-methyl-ATP were eluted with water and evaporated to dryness under reduced pressure. Yield was 35–40%. As expected, the product had UV absorption spectrum identical to CMP and displayed resistance to alkaline phosphatase.

**Cytidine 5'-Phospho-3'-methylphosphate**

Cytidine 3'-methylphosphate (40 μmol) was dissolved in 1 ml of polynucleotide kinase buffer and supplemented with 0.3 μl of 0.3 M ATP, pH 8.0. Fifty microliters of T4 phage polynucleotide kinase (Bio-labs) was added, and the mixture was kept at 37 °C. After 30 h of incubation, TLC analysis revealed nearly complete conversion of the starting material to reaction product, which was purified by column chromatography on Dowex-1 × 2 and isolated as described above for α-methyl-ATP. Yield was 90%.

**Cytidine-5'-Triphospho-3'-methyl Phosphate**

It was obtained from cytidine-5'-phospho-3'-methyl phosphate using the procedure described previously (29) and purified on Dowex-1 × 2 as described above.

**Transcriptional Complexes**

Defined TECs were obtained by RNAP “walking” on the 5'-biotinylated T7A1 promoter fragment immobilized on neutravidin-coated agarose (Pierce). The 370-bp DNA promoter fragment with 5'-biotin tag was generated by PCR amplification from promoter-containing pTZ19 plasmid. The open promoter complex was obtained by mixing 1 μl of neutravidin-agarose suspension with immobilized T7A1 promoter with 1 pmol of RNAP in 20 μl of transcription buffer (TB) containing 50 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl2, 1 mM 2-mercaptoethanol. The mixture was incubated for 10 min at 37 °C and washed with TB (four times by 1 ml). To prepare TEC-13A with labeled penultimate residue, 10 μl of “start” mixture (10 μM ApUpC RNA primer and ATP, GTP 13 μM each) was added to obtain first TEC-11A stalled for the absence of the next substrate. After 2.5 min of incubation at 37 °C, the suspension was washed with cold TB (six times by 1 ml) and 1 μl of [α-32P]CTP (3000 Ci/mmol) was added to the reaction mixture. After 15 min of incubation at 0 °C, the beads were washed with cold TB without MgCl2, followed by addition of ATP and MgCl2 (to final concentrations of 30 μM and 1 mM, respectively) and incubation for 15 min at 0 °C. To remove the excess of ATP the mixture was washed by TB (four times by 1 ml). In the case of the internal labeling of RNA, the washed beads containing open complex were supplemented with 10 μl of the start mixture (see above) containing 1 μl of [α-32P]ATP (3000 Ci/mmol) and 13 μM GTP followed by incubation (2.5 min at 37 °C) to obtain TEC-11A. For characterization of the exonuclease reaction, the [α-32P]NTP was introduced into 3'-terminal RNA position of TEC-13A and TEC-21U.

**Incorporation of Modified Nucleotides in TECs**

A330s and A330s were introduced in TEC-13A and TEC-14C at 300 μM substrate concentration upon incubation of the relevant TEC at 0 °C for 30 min. TEC-VI of Fig. 7A was obtained by incubation of TEC-11A with [α-32P]dCTP (3000 Ci/mmol, 3 μM) followed by elongation with 50 μM ATP for 20 min at 0 °C, whereas elongation of TEC-12dC with 150 μM ATP αCH3 (20 min at 37 °C) was used to generate TEC-VII of Fig. 7A. TEC-III was synthesized by elongation of natural TEC-11A with 150 μM...
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ATP αCH₃ for 20 min at 0 °C. Incubation of TEC-11A internally labeled at adenylate residues (see above) with 300 μM CTP-3’-methyl phosphate for 20 min at 37 °C yielded TEC-V of Fig. 7. The last four TECs were obtained in the presence of 1.5 mM MnCl₂ and 10 mM MgCl₂ to facilitate modified substrate incorporation. The modified TECs were washed with cold TB (without MnCl₂ or MgCl₂) and used in further experiments.

**Intrinsic and Gre-assisted RNA Cleavage in TEC**

For endonuclease cleavage, the sample of stalled ³²P-labeled TEC in 10 μl of TB without MgCl₂ was diluted by 10 μl of TB, containing various MgCl₂ or MnCl₂ concentrations, and the mixture was incubated at 21 °C. GreA- and GreB-induced cleavage was performed using an equimolar ratio of a factor to TEC. The cleavage was stopped by adding 1 volume of the loading buffer (7 M urea, 20 mM EDTA, bromphenol blue and xylene cyanol, 0.25% each), and reaction products were resolved on a 20% PAGE (20:3 acrylamide/N,N’-methylenebisacrylamide ratio). In the case of TECs with ATP αCH₃ and CTP-3’-methylphosphonate, the reaction products were resolved on 30% PAGE (20:3 acrylamide/N,N’-methylenebisacrylamide ratio, 6 M urea, 1 × TBE). The Arrhenius coefficient of 5.6 for endonuclease cleavage reaction was determined by measuring the rate of the reaction at 21 and 37 °C. Details of reaction conditions with PPi and dNTP/dNTP are presented in the figures. For testing the pH dependence of endonuclease cleavage, the stalled ³²P-labeled TEC was washed (four times by 1 ml) with cold buffer (1 mM Tris-Cl, pH 7.9, 100 mM KCl, and 1 mM 2-mercaptoethanol), and then 10 μl of the samples were mixed with the equal volumes of various pH buffers. The final concentrations of the buffers were as follows: 50 mM Pipes-Cl, pH 6.0, 50 mM Tris-Cl, pH 7.0–9.0, 50 mM CAPS-NaOH, pH 10.0. Other components were as in TB.

**Hydroxyl Radical RNA Cleavage in TECs Mediated by Active Center-bound Fe²⁺**

A stalled TEC in 10 μl of TB without MgCl₂ was supplemented with 20 μM Fe(NH₄)₂(SO₄)₃ and 10 mM DTT. The reaction mixture was left at 21 °C. In 10 min, the reaction was stopped by adding of 1 volume of the loading buffer (7 M urea, 20 mM EDTA, bromphenol blue and xylene cyanol 0.25% each) and analyzed by PAGE.

**Site-directed Mutagenesis of RNA Polymerase**

Mutational substitutions in RNAP were introduced using the QuikChange mutagenesis kit following the supplier’s protocol.

**RESULTS**

**Structural Analysis of RNAP Suggests the Mechanism for Active Center Tuning in the Reaction of Hydrolytic RNA Cleavage**—We analyzed the 14 available RNAP structures and found that in most cases the coordination geometry of Mg-I corresponds to the inactive state to which Mg-II cannot bind (data not shown); but in two of the structures the carboxylate residues are in the bridging orientation allowing coordination of Mg-II (which was modeled onto the structures as shown in Fig. 1C, right panel). Of particular note is that both of these exceptions represent backtracked RNAP complexes (24, 25). In Fig. 3A, we compare one of these, the backtracked complex of yeast pol II (Protein Data Bank 3GTG), with the closely related pretranslocated complex of that enzyme (PDB 116H (30)). In the backtracked complex, the contacts of +1 ribose residue with the active center side chains are established, and carboxylate residues Asp-460 and Asp-462 are in the active bridging orientation (Fig. 3, A and C, right panel). These ribose contacts are not established in pretranslocated complex of yeast pol II, in which the active center carboxylates are in inactive, nonbridging orientations (Fig. 3, A and C, left panel). This comparison points to a possible causal relationship between establishment of the ribose recognition contacts and catalytic orientation of bridging carboxylate residues. As seen from Fig. 3B, the disengaged i + 2 residue of 3’-RNA forms additional contacts with the active center that facilitate +1 ribose recognition interactions. In particular, the phosphate group of the +2 disengaged RNA residue forms hydrogen bonds with Rpb1 N479 (β’ Asn-458 in E. coli) and Rpb1 Q1078 (E. coli β’ Gln-929) side chains (24). Another interaction in yeast pol II includes hydrogen bonding with Rpb1 Asn-1082 (E. coli β’ Arg-933), which suggests a salt bridge with the phosphate in the bacterial enzyme (as determined from homology modeling). These additional interactions apparently bring the 2’-hydroxyl group of +1 RNA residue to hydrogen bonding distance with the guanidinium group of Rpb1 Arg-446 (E. coli β’ Arg-425), which has been implicated in catalysis of RNA synthesis (15). This movement of the +1 RNA residue associated with establishment of the recognition contacts (Fig. 3C) forces the shift of Mg-I, because it is tethered to the moving 5’-phosphodiester group of the +1 ribose residue through coordination. Movement of Mg-I is accompanied by re-orientation of two bridging carboxylates that remain bound to Mg-I through coordination but acquire free valences for binding of Mg-II, which is required for catalysis of subsequent hydrolytic reaction (Fig. 3C, right panel). We named the above rearrangement “active center tuning” because it sets the active center for catalysis. Next, we set up to find experimental support to ACT mechanism.

**ACT Explains High Efficiency of Endonuclease Transcript Cleavage Compared with Exonuclease Reaction**—The active center catalytic state apparently seen in backtracked TEC predicts that the endonuclease reaction (that proceeds in the backtracked complex) could be more efficient compared with exonuclease reaction occurring in a pretranslocated complex in which the active center is not tuned. For quantitative characterization of exo- and endonuclease hydrolytic activities, we examined the status of RNAP translocational equilibrium in various TECs assembled on the T7 A1 promoter using RNA cleavage by hydroxyl radicals generated by active center coordinated Fe²⁺, which substitutes Mg-I (6, 13, 21, 31, 32). The cleavage occurs between the RNA residues occupying the i and i + 1 site as shown in Fig. 4, A and B. Notably, the presence of 3’-terminal phosphate group increases electrophoretic mobility of the cleavage products (compare the size markers on the right and left of the Fig. 4A) because of two extra negative charges. As seen from the cleavage pattern in TEC-13A, RNA truncation to 12 and 11 nucleotides occurs with equal efficiency (Fig. 4, A and B) indicating that pretranslocated and backtracked by 1-nucleotide states are equally represented, thereby
creating equal opportunities for exo- and endonuclease reactions. However, the efficiency of endonuclease reaction in this TEC is 50 times higher than that of exonuclease cleavage (which releases 3'-terminal pA residue) as determined from quantitation of corresponding reaction products (Fig. 4, lane 4). In accordance with the prediction of the ACT mechanism, the bulk of this effect was due to enhanced Mg-II retention in the backtracked TEC (Table 1) as a result of freeing of the active center carboxylate coordination valences promoted by RNA residue (as shown in Figs. 1B and 3B). High Me-II association with backtracked TECs was also seen for endonuclease cleavage of TEC-14C, TEC-16G, and TEC-22A assembled at the same promoter (data not shown) as in TEC formed of other DNA templates (33).

Effect of RNA Modifications on Transcript Cleavage and Pyrophosphorolysis Is Consistent with ACT—To explore further the ACT model predictions, we determined how various RNA substitutions (shown in Fig. 5A) affect the endonuclease reaction as summarized in Tables 1 and 2. The substitution of the R oxygen of phosphate group, which participates in tuning through hydrogen bonding with Asn-458 of the active center (Figs. 2B and 3B) by bulkier and less electronegative sulfur caused a 6-fold reduction in endonuclease cleavage rate (Table 2). Methyl substitution of the same oxygen resulted in a 40-fold cleavage rate reduction evidently due to complete loss of the group’s hydrogen bonding (Table 2). Deletion of the entire nucleoside part of the disengaged +2 RNA residue (while preserving +2 phosphate) decreased the reaction rate by 20-fold. However, the most severe effect, a 300-fold rate reduction, was observed by 2'-deoxy substitution in the +1 RNA residue, which according to the model eliminates two hydrogen bonds with Asn-458 and Arg-425, involved in the tuning. This points to a crucial contribution of the 2'-hydroxyl group interactions to ACT, whereas the other RNA contacts involved in this mechanism play an auxiliary role. The implications of this finding in RNAP catalytic mechanism are presented under “Discussion.”

As expected, the above deoxy-substitution also greatly affected pyrophosphorolysis (Fig. 4, lanes 9 and 10). As estimated, the substitution caused a 100–400-fold drop in pyrophosphorolysis rate, supporting the involvement of ACT in this reaction.

Importantly, the effect of the RNA substitutions was not due to the shift in backtracking equilibrium (see Fig. 5B). The above observations are in accord with our expectations from the ACT model. As suggested by x-ray structure of yeast pol II backtracked TEC, the other residues of disengaged 3'-RNA segment do not make interactions with RNAP. In agreement with this
observation, introduction of phosphorothioate substitution in the +3 position of RNA did not affect RNA cleavage in backtracked TECs (data not shown).

**Effect of \( \beta'N458D \) Mutation Supports ACT Mechanism**—Important evidence for ACT is provided by the properties of a \( \beta'N458D \) substitution (Fig. 6). According to the model, it was expected that this mutation would suppress backtracking by reinforcing the interactions of the 3'-terminal RNA residue with recognition protein side chains that trigger ACT. In detail, the \( \beta'N458D \) substitution introduces an ionized oxy-group in

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**TABLE 1**

Reaction rates and Mg-II dissociation constants for hydrolytic RNA cleavage in TECs with nonmodified RNA

| TEC          | Exonuclease | Endonuclease | Factor-assisted cleavage |
|--------------|-------------|--------------|--------------------------|
|              | Intrinsic   | Endonuclease | GreA,B                   | GreA | GreB |
|              | cleavage    |              |                          |      |      |
|              | \( K_d \) M\(^{2+} \) | \( k_{app} \) min\(^{-1} \) at 10 mM M\(^{2+} \) | \( K_d \) M\(^{2+} \) | \( k_{app} \) min\(^{-1} \) at 10 mM M\(^{2+} \) | Relative cleavage rate | \( K_d \) M\(^{2+} \) | \( k_{app} \) min\(^{-1} \) | Relative cleavage rate | \( k_{app} \) min\(^{-1} \) | Relative cleavage rate |
|--------------|-------------|--------------|--------------------------|------|------|
| TEC-13A (pCpA\(^a\)) | NA\(^b\) | NA | \( >100 \) (9–11\(^c\)) | 0.042 | 1    | 0.3–0.5 | 1.5  | \( <10^{-3} \) | \( <6.7 \times 10^{-4} \) | 0.09 | 0.03 |
| TEC-12C (pGpApC\(^a\)) | NA | NA | \( >100 \) (8–12\(^c\)) | 2.3 \( \times 10^{-3} \) | 0.055 | NA    | ND   | \( <10^{-3} \) | \( <3.2 \times 10^{-4} \) | 1    | 1    |
| TEC-13A (pA\(^a\)) | ND\(^d\) | 0.8 \( \times 10^{-3} \) | NA              | NA | ND | ND | ND | ND | ND |

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\(^{a}\) 3'-terminal cleavage product.

\(^{b}\) NA means not applicable.

\(^{c}\) Done at pH 10.0.

\(^{d}\) ND means not detectable.
place of a neutral amide that is expected to affect crucially the hydrogen bonding network in TEC. An Asp residue cannot stabilize the backtracked state because it cannot hydrogen bond to a phosphate group (Fig. 6A). Instead, an Asp residue can form strong hydrogen bonds simultaneously with the 2'- and 3'-hydroxyl groups of the +1 3'-terminal RNA residue in the TEC pretranslocated state (Fig. 6B), because the oxo-group of Asp is a better hydrogen bond acceptor than the amide of Asn. Both of the above factors should stabilize the pretranslocated state. Indeed, the substitution strongly suppressed backtracking in TEC-27 (Fig. 6C) as seen from the reduced rate for accumulation of arrested fraction of the TEC. As a result, this mutant displayed only the exonuclease type of activity typical of pretranslocated TEC. The most striking effect of the substitution was observed in the exonuclease reaction, which compared with WT had a higher reaction rate and a lower dissociation constant for Mg-II (10 mM versus 100 mM) (Fig. 6C) as expected from the ACT model. Remarkably, control N458D substitution displayed an even slightly opposite effect on exonuclease cleavage compared with the WT enzyme.

**TABLE 2**

Reaction rates and Mg-II dissociation constants for hydrolytic RNA cleavage in TECs with modified RNA

| TEC          | Intrinsic cleavage | Factor-assisted cleavage |
|--------------|-------------------|-------------------------|
|              | Exonuclease       | Endonuclease            | GreA,B | GreA | GreB |
|              | $K_d$ Mg$^{2+}$   | $k_{app}$ min$^{-1}$ at 10 mM Mg$^{2+}$ | $K_d$ Mg$^{2+}$ | $k_{app}$ min$^{-1}$ | Relative cleavage rate |
| II$^b$ TEC-13p(S)A (pCp(S)A)$^c$ | ND$^d$ | ND | $>100$ | $7 \times 10^{-3}$ | 0.17 | $0.3-0.5$ | 1.3 | 0.87 | 1.3 | 0.42 |
| III TEC-13p(Me)A (pCp(Me)A) | ND | ND | $>100$ | $10^{-3}$ | 0.024 | $0.3-0.5$ | 0.09 | 0.07 | 0.09 | 0.03 |
| V TEC-12pCp(OMe) (pCp(OMe)) | ND | ND | $>100$ | $2.2 \times 10^{-3}$ | 0.053 | $0.3-0.5$ | 1.4 | 0.93 | 0.4 | 0.13 |
| VI TEC-13pCdPpIA (pCdPpIA) | ND | ND | $>100$ | $1.3 \times 10^{-3}$ | $3.0 \times 10^{-3}$ | ND | 0.01 | $6.7 \times 10^{-3}$ | 0.01 | $3.2 \times 10^{-3}$ |

$^a$ Data are as compared with nonmodified RNA in TEC-13.

$^b$ Numbering is as in Fig. 5A.

$^c$ 3'-Terminal cleavage product.

$^d$ ND mean not detectable.
Mg$^{2+}$ binding by introduction of a residue capable of additional coordination of Mg-II. However, considering the large distance (9.2 Å) from Mg-II catalytic position to N458D carboxylate group, this hypothesis looks unlikely. Therefore, the N458D substitution properties also support the ACT mechanism predictions.

**FIGURE 6. Effect of β′N458D substitution on hydrolytic RNA cleavage.** A, inability of β′ Asp-458 residue of the active center to hydrogen bond to the phosphate of 3′-disengaged +2 RNA residue destabilizes backtracked state and shifts the equilibrium to pretranslocated state, which is stabilized by strong hydrogen bonding of terminal RNA residue to β′ Asp-458. This enhanced ribose contacts promote ACT that increases the Mg-II retention and the reaction rate.

**Adenine Residue in +2 Position of Disengaged RNA Segment in Backtracked TEC Is Strongly Preferred in Endonuclease Cleavage—**To further investigate the mechanism of endonuclease reaction in *E. coli* TEC, we explored the sensitivity of endonuclease hydrolysis to the size and sequence of 3′-disengaged RNA segment in backtracked TEC. For these experiments, we used the same set of TECs assembled on the T7 A1 promoter containing RNAs 12–16 nucleotides long (Fig. 4). As seen from Fig. 4A, hydroxyl radicals generated by active center bound Fe$^{2+}$ cut the 3′-terminal nucleoside residue in TEC-12C, indicating that this complex is pretranslocated. TEC-13A was truncated by one and by dinucleotide segments with the hydroxyl radicals suggesting that this TEC exists in pretranslocated and backtracked by one nucleotide states. RNA cleavage patterns in TEC-14C and TEC-16G revealed the states with different degrees of backtracking (Fig. 4, A and B) along with the pretranslocated state. Surprisingly, despite the variety of backtracked states randomly populated in the tested TECs, in all these TECs the cleavage occurred between A11 and C12 of the RNA transcript (data not shown), pointing to sequence preference of endonuclease reaction. The cleavage preference is not due to the identity of RNA residues occupying $i$ and $i+1$ sites of the active center, because the cleavage between A13 and C14 in TEC-16G (allowed by backtracking equilibrium) is also expected should the above be the case. Another possible specificity determinant could be a residue in the register 2 (first 3′-unpaired single strand RNA residue of backtracked TEC). To test this proposal, we introduced tubercidin residue (containing a –CH segment instead of –N at position 7 of the purine base as shown in Fig. 5A, structure IV) instead of adenine in position 13 of TEC-13A. Importantly, this substitution does not change the base pairing interactions in DNA scaffold of the TEC, thereby excluding the influence of RNA/DNA sequence on RNAP translocational equilibrium and the cleavage. In agreement with this expectation, the efficiency of endonuclease cleavage in the modified TEC was significantly (6–7-fold) reduced (Fig. 4D). As follows from Fe$^{2+}$ affinity cleavage (Fig. 5B, lanes 19–21), this was not due to the change in backtracking status of the TEC. The above sequence preference of endonuclease cleavage is also observed in the other TECs in which adenosine residue is the first in disengaged RNA segment. The examples are TEC-12C (Table 1), arrested TEC-21U and TEC-27U (cleavage between A11 and C12 (3)), as well as TEC-22A, in which the cleavage occurs between A20 and U21 (data not shown).

**Modeling of Endonuclease Reaction for E. coli RNAP—**Strong adenine preference (>30-fold) of the cleavage reaction described above as well as our previous cross-linking data (34) could not be explained by x-ray structure of yeast pol II backtracked TEC, suggesting different orientation of +2 RNA residue in *E. coli* TEC.
We modified the x-ray model (3GTG) by placing a base of +2 RNA residue in the active center cleft in the vicinity of the residues βArg-678, βArg-1107, and β’Asp-460–Asp-464 segment (as suggested by cross-linking (34)), while preserving the orientation of +2 phosphate seen in x-ray structure as shown in Fig. 2, A and B. This was achieved through the rotation of +2 RNA residue around P–5’O and 5’O–5’C bonds using the above cross-linking constraints for E. coli RNAP as well as those imposed by the apostructure of the active center (Fig. 2A). This allowed unambiguous placing of the nucleoside residue in the narrow pocket at the interface of β and β’ subunits formed by evolutionary invariant residues βLys-1073, βArg-678, βArg-1106, βAsp-814, and the catalytic β’DDD-loop of the active center (Fig. 2, A and B), which we named B site (for base binding). In this arrangement, the 6-amino group of adenine in a residue on endonuclease and exonuclease reactions (Fig. 2, A and B). Indeed, dramatic differences in response of these reactions to the same mutations (Table 3). In particular, mutation of βAsp-814, βLys-1073, and βArg-1106 resulting in a significant drop of endonuclease activity should weaken hydrogen bonding with adenine of +2 RNA residues in the B site (Fig. 2B), affecting the residue orientation crucial for positioning of the attacking water. In our previous studies (3), stimulatory effect of βArg-1106 substitution on exonuclease cleavage was attributed to increased retention of Mg-II in the mutant due to additional coordination by βAsp-814 carboxylate (which is not available for coordination in WT enzyme due to engagement in salt bridge with βArg-1106 as shown in Fig. 2B). The same effect of the mutation on Mg-II retention was seen in exonuclease reaction (Table 3). However, this stimulatory effect was apparently negated by elimination of water positioning through disruption of adenosine interactions in the B site caused by mutation. As a result, the substitution increased the competing exonuclease pathway in TEC-13 so that the rates of both hydrolytic reactions became comparable (data not shown). The lower Mg-II dissociation constant for the mutant in endonuclease reaction (1 mM) compared with exo-cleavage (10 mM) can be explained by the additional synergistic effect of ACT.

Another substitution that increased Mg-II retention in endonuclease reaction was β’R731A, whose influence can be similar to that of βR1106A (see above). Smaller magnitude of the effect can be explained by the larger distance of β’Arg-731 residue from βAsp-814 (4.5–5.6 Å in different structures). The other example of striking difference is represented by the effect of βR678A substitution. The enhanced activity of the mutation in the exo-cleavage (Table 3) does not allow simple explanation, because the substitution did not change Mg-II retention. One of the possible effects could be Mg-II re-positioning to a more optimal location due to elimination of electrostatic repulsion between the metal and positively charged Arg side chain. In the model for endonuclease reaction (Fig. 2, A and B), the βArg-678 residue is completely shielded from the active center by the base of +2 RNA residue, which could reduce the influence of the substitution on Mg-II re-positioning.

Transitions in the active center caused by pH have been previously demonstrated for exo- and endonuclease reactions (2, 3). It is envisioned that deprotonation of the attacking water at elevated pH should generally stimulate nuclease activity. Such stimulation should plateau out upon the pH approaching system’s pH value. Instead, like in the case of exonuclease reaction (3), the pH dependence curve for wild-type enzyme in endonuclease reaction as explained by the model for endonuclease reaction (Fig. 2, A and B). Indeed, dramatic differences in response of these reactions to the same mutations (Table 3). In particular, mutation of βAsp-814, βLys-1073, and βArg-1106 resulting in a significant drop of endonuclease activity should weaken hydrogen bonding with adenine of +2 RNA residues in the B site (Fig. 2B), affecting the residue orientation crucial for positioning of the attacking water. In our previous studies (3), stimulatory effect of βArg-1106 substitution on exonuclease cleavage was attributed to increased retention of Mg-II in the mutant due to additional coordination by βAsp-814 carboxylate (which is not available for coordination in WT enzyme due to engagement in salt bridge with βArg-1106 as shown in Fig. 2B). The same effect of the mutation on Mg-II retention was seen in exonuclease reaction (Table 3). However, this stimulatory effect was apparently negated by elimination of water positioning through disruption of adenosine interactions in the B site caused by mutation. As a result, the substitution increased the competing exonuclease pathway in TEC-13 so that the rates of both hydrolytic reactions became comparable (data not shown). The lower Mg-II dissociation constant for the mutant in endonuclease reaction (1 mM) compared with exo-cleavage (10 mM) can be explained by the additional synergistic effect of ACT.

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cleavage displayed the abrupt upturn in the range 9–10 (Fig. 7A), suggesting that the additional pH-dependent factor contributes to catalysis. Similarly to exonuclease hydrolysis (3), the upturn can be explained by deprotonation of β-Arg-1106, which destroys its salt bridge with β-Asp-814, enabling additional Mg-II coordination by the latter (see above). This is supported by the upturn absence for βE813A/D814A and βR1106A mutants in exonuclease reaction. Indeed, the first mutation eliminates additional Mg-II coordination by β-Asp-814, thus making Mg-II binding pH-independent as does the second substitution by eliminating the salt bridge.

As seen from Fe2+–mediated hydroxyl radical RNA cleavage in TEC-13A, most of the tested mutations did not change significantly the pretranslocated-to-backtracked state ratio (Fig. 5C). Notably, β’N458A substitution increased the ratio to 4:1 from 1:1 in WT TEC, suggesting an important contribution of the residue to stabilization of the backtracked state. The change in the equilibrium can be explained by loss of the hydrogen bonding of the side chain with the +2 RNA residue phosphate in backtracked state, whereas in pretranslocated state the β’Asn-458 residue does not contact RNA (30). Substitution β’R933S suppressed backtracking, re-directing the main RNA cleavage site in TEC-26A from between A11 and C12 to between A20 and U21 (data not shown). This change can be explained by stabilization of the backtracked state in wild-type RNAP through hydrogen bonding and the salt bridge of β’-Arg-933 side chain with the phosphate of the +2 RNA residue as suggested by homology modeling (Figs. 2B and 3B).

Molecular Modeling of Gre-assisted Transcript Cleavage Reaction—Gre-assisted endonuclease RNA hydrolysis is believed to provide co-transcriptional proofreading in the bacterial cell, because this reaction is much more efficient compared with intrinsic cleavage. For the absence of x-ray structure of TEC with Gre factors and to explain our results, we built a working model, which compiles high resolution structures of T. aquaticus RNAP and GreB factor modeled into the EM structure for the GreB–T. aquaticus RNAP complex (19), as well as the structure of nucleic acid scaffold and active center DDD loop of yeast Pol II elongation complex (24), in which factor-assisted reaction proceeds. The modeling was accomplished by: i) pasting of the DNA/RNA scaffold of backtracked transcription complex of yeast Pol-II onto a high resolution model of Gre/Taq RNAP transcription complex derived from low-resolution EM image (19); ii) placing of β’-DDD-loop segment of yeast Pol-II backtracked TEC (PDB 3GTG) into Taq RNAP structure of Gre complex (19); iii) fitting of catalytic Mg-II (not seen in yeast Pol-II x-ray image) and the attacking water into the active center using constraints of Sn2 hydrolytic mechanism, and iv) adjusting of the resulting model through GreB moving towards the active center by ~0.5 nm to enable coordination of Mg-II by two carboxylate residues of Gre tip as suggested in previous studies (6, 19, 21).

For the model shown in Fig. 8, docking of the Gre factor into the RNAP secondary channel caused minor clashes with the RNAP protein, suggesting structural adjustment in the complex (19), also seen in the co-crystal of Gfh1, Gre-like inhibitor with T. thermophilus RNAP (35). Remarkably, no clashes were observed at the active center. In the model Gre tip contacts the β’-DDD-loop of the active center (residues 458–464), β’ F-bridge residues 781–785, β’ 729–736 segment (GARG motif), β regions 677–681 (NRAP motif), and 1104–1107 (PSRM motif). Such placement of the factor is supported by biochemical evidence. In particular, cross-linking data reflect ~1 nm distance of Gre factor tip from the RNA base in i + 1 site of the active center (6) consistent with the model. Yet binding of the factor to TEC causes protection of all of the above listed active center sites against hydroxyl radicals generated by active center coordinated Fe2+ (21). The important conclusion from the Gre docking model was displacement of the nucleoside part of disengaged +2 RNA residue from the B site due to steric clashes of the residue with the Glu-44 side chain of the Gre tip that has to coordinate Mg-II. This prediction is in accord with the loss of adenine specificity of Gre-stimulated reaction observed in nonstimulated cleavage (6, 36). The same conclusion follows from the absence of the effect of +2 RNA base elimination on GreA-mediated cleavage (Table 2, TEC-V). Remarkably, the spatial arrangement of the +2 RNA phosphate residue catalytically competent in intrinsic cleavage reaction is allowed in the Gre complex, which is consistent with the observed effects of ACT (mediated through the phosphate res-
In contrast to intrinsic reaction, substitution of (Table 3) was also observed in the Gre-assisted reaction (Fig. 9). Chains adjacent to catalytic DDD-loop of the active center endonuclease reaction in TEC to the substitutions of the side cleavage, as explained by the model.

Sensitivity of the intrinsic cleavage affecting both Mg-II binding and ionization of the attacking water. To test this prediction, we examined the efficiency of intrinsic and Gre-assisted RNA cleavage at various pH values. Previously (3), we observed that pH had a dual effect on nonassisted RNA cleavage (37), which also coordinates Mg-II (Table 8).

Effects of RNAP Active Center Mutations on Gre-stimulated RNA Cleavage Reveals General Base Catalysis Predicted by the Model—An additional factor involved in catalysis of hydrolytic RNA cleavage could be activation of the attacking water. Indeed, in the exonuclease active center of DNA polymerase (DNAP) the activation is achieved through H-bonding of the water with the carboxylate group of Glu-357 (37), which also coordinates Mg-II (Fig. 8C). As seen from our model (Fig. 8B), Glu-44 of Gre could assume the same orientation as Glu-357 in DNAP allowing simultaneous coordination of Mg-II and activation of the attacking water. To test this prediction, we examined the efficiency of intrinsic and Gre-stimulated RNA cleavage at various pH values. Previously (3), we observed that pH had a dual effect on nonassisted RNA cleavage affecting both Mg-II binding and ionization of the attacking water. To exclude the effect of pH on Mg-II retention as a reference, we used βR1106A mutant enzyme in which the binding of magnesium is pH-independent (3). As seen from Fig. 7B, the response of the reaction rates to pH was strikingly different. Specifically, pH elevation from 6 to 10 caused an ∼100-fold increase in the rate of the intrinsic reaction, although the rate of Gre reaction increased only five times, suggesting that attacking water ionization is a much less crucial factor in Gre-stimulated reaction. Indeed, the apparent pK_a value for the
water deduced from the pH dependence was 8.9 and 7.8 (for intrinsic and Gre-assisted cleavage respectively) reflecting 10–15-fold water activation by the Gre factor. In accordance with this mechanism, the mutational substitution of the Glu-44 residue in Gre simultaneously decreased retention of Mg-II (6, 21) and restored intrinsic cleavage activity of RNAP (in Gre-TEC complex), manifested by high pH dependence (21), thereby reflecting elimination of the attacking water activation by the substitution. These data are in agreement with our model, because the loss of coordination contact of the mutated residue with Mg-II allowed the base binding in the B site in the mode competent for intrinsic reaction. The above observations support the constructed structural model for Gre-assisted catalysis.

**DISCUSSION**

**Role of ACT Mechanism in Substrate Selection by RNA Polymerase**—The common $S_{\text{n}2}$ mechanism views RNA hydrolysis and pyrophosphorolysis as nucleotidyl transfer reaction operating in reverse, suggesting that the same substrate recognition principles (through ACT) could operate in both RNA synthesis and RNA degradation. This idea is supported by the strong negative effect of +1 RNA residue deoxy-substitution on both pyrophosphorolysis and endonuclease reaction rate (200–400-fold), which is comparable with the discrimination factor (120–1200-fold) between ribo- and deoxy-NTP incorporation by RNAP (15, 33). The common mechanism paradigm would posit that in the first step of substrate selection the nucleoside part of the NTP occupies the same position as the 3'-terminal +1 RNA residue in the pretranslocated complex (Fig. 10, A and B) in which the pairing with a DNA base is in place, but the ribose contacts are not established and the active center is not tuned. At the next selection step, the ribose recognition contacts of NTP substrate must be established so that the nucleoside part of NTP will assume its orientation as in the +1 RNA residue of the backtracked complex in which both base pairing and ribose recognition contacts are in place and the active center is tuned (Fig. 10, A and B). In the backtracked complex, simultaneous formation of a base pair and ribose recognition contacts causes significant angling of the base pair compared with the pretranslocated complex (Fig. 10A). This angling weakens hydrogen bonds and affects stacking interaction of the bases, thereby creating a stress that challenges both base pairing and ribose contacts so that noncomplementary or imperfectly base-paired NTPs are rejected (due to loss of the contacts with a template DNA base), as NTPs with inappropriate ribose (e.g. 2'-, or 3'-deoxy-NTPs). Therefore, tuning of the catalytic part of the active center is possible only when all recognition contacts of the NTP substrate are established and tested by tolerance to the stress. In support of this selection mechanism, introduction of a bulkier substituent to the part of the F-bridge (bridge helix) that crashes into the base pair ($^T790V$) increased the fidelity of transcription (33) by imposing more stress (Fig. 10, A and B). The suggested ACT mechanism for substrate selection is fundamentally different from that proposed for DNAP, whereby discrimination between deoxy- and ribo-substrate is achieved through strict fitting requirements for the sugar (38) rather than through active center rearrangement. Remarkably, in the DNAP active center carboxylates stem from rigid scaffolds, whereas in multisubunit RNAP they reside in a flexible loop (DDD-loop). As follows from Ramachandran plots (data not shown), ACT is accompanied by significant re-shaping of the DDD-loop, which underscores the importance of the active center flexibility in multisubunit RNAP catalytic mechanism. Such re-shaping would not be possible in DNAP.

**Species-specific Mechanisms for RNA Cleavage in Various RNAP**

Possible Effect of RNAP Trigger Loop Transitions on Endonuclease Cleavage—Structural and biochemical studies have implicated the transitions of the $\beta'$-trigger loop in the catalytic mechanism of RNA polymerization (13, 14, 16, 17, 39, 40). The absence of an effect of $\alpha$-amanitin (which blocks trigger loop transition), on endonuclease cleavage in yeast pol II (41), as well as insensitivity of the same reaction to trigger loop deletion in
Catalytic Mechanism of Endonuclease Transcript Cleavage in E. coli—This study demonstrates a significant difference in the features of exo- and endonucleolytic RNA cleavage in TEC despite apparent mechanistic similarity of the reactions. This difference is evidenced by the following: (i) absolute reaction rate, which is 20–50 times higher for endonuclease hydrolysis; (ii) response to active center mutations, which generally have the opposite effect on the reaction rates, and (iii) response to noncognate NTP, which greatly stimulates exonuclease cleavage, while having no effect on endonuclease.

Our data attribute the observed differences between the reactions to the influence of the +2 RNA residue in backtracked TEC. This conclusion is strongly supported by the crystal structure of yeast pol II backtracked TEC in which only this residue of disengaged transcript segment is seen in defined orientation (24), suggesting specific interactions with active center side chains.

Although the x-ray structure is consistent with the results of biochemical studies on yeast pol II, fitting the data obtained for E. coli RNA required adjustment of the structural model. Thus, re-positioning of the nucleoside part of +2 RNA residue from the orientation seen in yeast pol II into the active center cleft (B site) between DDD-loop and the “basic rim” was required.

Besides ACT, other factors that can increase the efficiency of endonuclease reaction are positioning and/or activation of the attacking water. Both modes involve hydrogen bonding of the water’s hydrogen atom with an acceptor group. In the positioning mode, establishing the hydrogen bond results in stabilization of the attacking water, whereas in the activation process, an acceptor group (general base) also lowers the apparent $pK_a$ value of the attacking water facilitating ionization, which can be detected by measuring the reaction rate at various pH values. Such testing performed in this study did not reveal activation of the attacking water in intrinsic endonuclease reaction. However, water “positioning” in this reaction is consistent with the deleterious effect of active center substitutions that are expected to disrupt the positioning by loosening the contacts with the adenine nitric base of +2 RNA residue, which in turn hydrogen bonds with the attacking water through the enocyclic oxygen (Fig. 2B). Surprisingly, we observed activation of the attacking water in a Gre-stimulated reaction.

Implications for Transcript Cleavage Mechanisms in Yeast pol II and RNA from Thermophiles—Despite the common origin, the multisubunit RNAP evolved to develop specific mechanisms for regulation of RNA synthesis and transcription-coupled RNA processing, associated with RNAP endonuclease activity. Endonuclease reaction of RNA degradation in TEC displayed by all cellular RNAP is an intrinsic property of the enzymes. However, there is a striking difference in the relative rates of cleavage, which are ~1, 10², and 10³ for yeast pol II,
RNAP Active Center Tuning Controls Transcription

E. coli, and T. aquaticus RNAP, respectively (17, 41, and this study). Contrary to E. coli, the endonuclease reaction in yeast pol II enzyme can yet proceed through the pyrophosphorolytic pathway (27). The biological significance of this pathway is unclear (due to low intracellular pyrophosphate concentration); however, conceptually, the absence of this reaction in E. coli points to the important difference in the structural mechanism of hydrolytic RNA cleavage. The model for the reaction suggested in this study attributes this phenomenon to steric block of the pyrophosphate-binding site by the base of +2 RNA residue in E. coli, although the x-ray structure of yeast pol II TEC allows pyrophosphate binding (Fig. 2C). Obstruction of B site of the active center by +2 RNA residue also explains the absence of the stimulatory effect of noncognate NTP on endonuclease in E. coli (3) and T. aquaticus (17). A higher reaction rate by E. coli RNAP compared with yeast enzyme could be explained by the influence of the base of +2 RNA residue, which according to our model facilitates the reaction by positioning the attacking water (Fig. 2, A and B). The difference in the reaction mechanism in yeast and E. coli is surprising given high evolutional conservation of the active center. The possible explanation involves stabilization of the orientation of the +2 RNA residue seen in x-ray structure by hydrogen bonding with the side chain of Rpb2 Tyr-679 highly conserved in eukaryotes. The counterpart of Tyr-679 in E. coli RNAP is methionine (βMet-681) that cannot hydrogen bond. Another explanation for this phenomenon is impossibility of the interactions of G or U residues in the +2 RNA position with the adenine-specific B site in the crystal of the yeast pol II complex, resulting in alternative orientations of the residue. The issue can be addressed by crystallization of the appropriate complexes.

Specific effects of +2 RNA residue based on Mg-II retention in T. aquaticus RNAP (17) and not observed in the E. coli enzyme underscore the difference in catalytic mechanisms. Unlike in the E. coli RNAP N-7 substitution in the +2 adenine base did not change the reaction rate in T. aquaticus TEC, but it significantly reduced the binding constant for Mg-II. Other variations of the structure of the +2 nucleoside also affected Mg-II retention in T. aquaticus TEC, although in E. coli TEC the change of the whole nucleoside residue to the methyl group did not alter the $K_d$ values for Mg-II. Yet, pH values increasing from 9 to 10 only marginally stimulated endonuclease reaction in the T. aquaticus case, whereas in E. coli TEC the abrupt upturn of pH dependence curve is seen in this range (Fig. 7A). The upturn in E. coli TEC is explained by elimination of the salt bridge of βAsp-814 with βArg-1106 (which enables Mg-II coordination) due to deprotonation of the latter. These facts suggest higher occupancy of the coordination site by Mg-II in T. aquaticus than in E. coli TEC, which is consistent with the higher endonuclease activity of parent T. thermophilus TEC (26) and confirmed by direct measurement of Mg-II $K_d$ values in T. aquaticus TEC (17).

Stronger retention of Mg-II in T. aquaticus RNAP can be explained by the higher degree of ACT in this enzyme. Another explanation involves the contribution of the structure seen in T. aquaticus core RNAP (43), in which the βArg-1106–βAsp-814 salt bridge is broken, thereby facilitating Mg-II binding. Along with the above differences, there are also remarkable similarities in the catalytic mechanism of E. coli and T. aquaticus enzymes, as evidenced by the same effect of thio-substitution at +2 RNA residue on the reaction rate and by the fact that +2 adenylate is the most catalytically proficient residue in endonuclease reaction for both T. aquaticus (17) and E. coli TEC.

**Catalytic Mechanism for Transcript Cleavage Factors—Gre factors, found in all known bacteria, are required for normal growth. They execute proofreading and facilitate propagation of RNAP along DNA during RNA synthesis by both preventing transcriptional arrest and rescuing arrested TEC. This is achieved through hydrolytic cleavage of the disengaged RNA segment in reversibly backtracked and/or arrested transcription complexes. Gre factors strongly (~3000–4000-fold) stimulate intrinsic RNase H type activity of RNAP by increasing retention of Mg-II required for catalysis (6, 19, 21). However, the magnitude of the enhancement of Mg$^{2+}$ retention is only ~300-fold (6), pointing to another catalytic factor, which according to this work is 10–15-fold activation of the attacking water that when combined with Mg$^{2+}$ retention enhancement factor accounts for the whole magnitude of the catalytic Gre effect. These findings clearly demonstrate how Gre factors build up on the RNAP active center to enhance the enzyme’s rudimentary RNA cleavage activity to the level of typical nuclease.

Variations in the mechanism of RNA cleavage by cellular RNAP can account for different arsenals of auxiliary protein factors assisting this reaction in the living cell. Indeed, in yeast and E. coli intrinsic transcript cleavage activity of RNAP is low and has to be stimulated by protein factors TFIIS and GreA and GreB. The importance of Gre factors for E. coli is underscored by poor fitness of a bacterial strain lacking both factors (2). Notably, in T. thermophilus enzyme basal endonuclease activity is nearly 2 orders of magnitude higher than in E. coli, which could be sufficient on its own to maintain the vital function of transcript cleavage. This concept is supported by the absence of GreB-like factor in T. aquaticus and T. thermophilus. Interestingly, the stimulation effect of the GreA-like factor from T. thermophilus on T. thermophilus RNAP is only ~30-fold (26), which is about 2 orders of magnitude less than that for E. coli RNAP with GreB, which makes the rate of factor-stimulated RNA cleavage nearly equal for both enzymes.

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