

The highly conserved glutamic acid 791 of Rpb2 is involved in the binding of NTP and Mg(B) in the active center of human RNA polymerase II

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ABSTRACT

During transcription by RNA polymerase (RNAP) II, the incoming ribonucleoside triphosphate (NTP) enters the catalytic center in association with an Mg²⁺ ion, termed metal B [Mg(B)]. When bound to RNAP II, Mg(B) is coordinated by the β and γ phosphates of the NTP, Rpb1 residues D481 and D483 and Rpb2 residue D837. Rpb2 residue D837 is highly conserved across species. Notably, its neighboring residue, E836 (E791 in human RNAP II), is also highly conserved. To probe the role of E791 in transcription, we have affinity purified and characterized a human RNAP II mutant in which this residue was substituted for alanine. Our results indicate that the transcription activity of the Rpb2 E791A mutant is impaired at low NTP concentrations both *in vitro* and *in vivo*. They also revealed that both its NTP polymerization and transcript cleavage activities are decreased at low Mg concentrations. Because Rpb2 residue E791 appears to be located too far from the NTP–Mg(B) complex to make direct contact at either the entry (E) or addition (A) site, we propose alternative mechanisms by which this highly conserved residue participates in loading NTP–Mg(B) in the active site during transcription.

INTRODUCTION

RNA polymerase (RNAP) II, the enzyme that synthesizes mRNA in mammals, is composed of 12 subunits, termed Rpb1–Rpb12 (1,2). Crystal structures of yeast RNAP II

(3–7) have confirmed that the two largest subunits, Rpb1 and Rpb2, form the enzyme's catalytic center. These structures also revealed the presence of a cleft at the surface of the enzyme, which buries this active center. The cleft is formed, on one side, by a mobile 'clamp' which is connected to the protein by a series of five 'switch' regions. In the elongation complex, the DNA enters the cleft through a pair of 'jaws' and the DNA helix is opened before the active center. The template strand binds to switches 1 and 2 and the 'bridge' helix. Beyond the bridge, the template strand and the nascent RNA form a 9 bp duplex. Beneath the active site and in line with the bridge is found a 'pore' or secondary channel; the space formed by the cleft at the surface of the protein being the main channel. The secondary channel widens towards the bottom of RNAP II, forming an inverted 'funnel'. In the past, both channels have been proposed as privileged routes for NTP entry, and pyrophosphate exit (3,4,8).

DNA and RNAPs catalyze the nucleophilic attack of the 3' hydroxyl of a first nucleotide on the α -phosphate of a second incoming NTP, leading to phosphodiester bond formation and the release of a pyrophosphate molecule. Based on crystal structures of T7 DNA polymerase (9) and *Bacillus stearothermophilus* DNA polymerase (10), a two metal ion mechanism has been proposed (11,12). Through this mechanism, a first Mg²⁺ ion [metal A or Mg(A)] lowers the affinity of the 3' oxygen for the hydrogen thereby facilitating the O⁻ attack on the 5' α -phosphate. The second Mg²⁺ ion [metal B or Mg(B)] facilitates the release of the pyrophosphate. High-resolution crystal structures of yeast RNAP II revealed the position of the two Mg²⁺ ions in the catalytic site of the enzyme (3,4,13). In the yeast RNAP II structure, Mg(A) is coordinated by three aspartate residues of Rpb1, D481, D483 and D485, contained within the strictly conserved motif NADFDGD (3,4,13). Mg(B) was recently localized near residue D481 and D483

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of Rpb1 as well as the highly conserved residue D837 of Rpb2 (13). However, previous studies have suggested that the highly conserved residue E836 is also implicated in the coordination of Mg(B) in the active site (3,4). A number of studies have also proposed that Mg(B) would necessitate the presence of the incoming NTP for its complete coordination required for the polymerization process (3,4,8,13). However, biochemical data in support of the role of E836, D837 and NTP as Mg(B) coordinating residues is currently lacking.

In addition to their polymerization activity, DNA-dependent RNAPs can also catalyze the 3' endonucleolytic cleavage of transcripts to stimulate elongation (14,15). First discovered in *Escherichia coli* (16,17), this cleavage activity takes place when RNAP has backtracked at pause and arrest sites (18,19). Weakly associated DNA-RNA hybrids can induce backtracking of the enzyme to a more stable register (20). To resume elongation from this more stable position, RNAP needs to cleave the 3' end of the transcript that now extrudes from the catalytic site through the pore. This intrinsic nuclease activity is stimulated at basic pH, strongly suggesting a hydrolytic mechanism rather than pyrophosphorolysis (21). The 3' cleavage activity is also enhanced by specific factors, GreA and GreB in bacteria (22,23) and TFIIS in eukaryotes (18,19). TFIIS was first identified as an elongation factor that helps RNAP II to elongate through arrest sites *in vitro* (24,25). TFIIS greatly stimulates the intrinsic nuclease activity of RNAP II (26–28). The crystal structure of TFIIS in a complex with RNAP II (29) provided insight into the mechanism by which this factor enhances transcript cleavage. Specifically, it revealed that two conserved acidic residues in TFIIS essential for its activity (30), are located in the vicinity of the polymerase Mg(B) and could participate in its coordination. Therefore, it was suggested that a two metal ion mechanism is involved in the 3' endonucleolytic activity of RNAP II. A two metal ion mechanism is consistent with the requirement for divalent cations for the cleavage activity (27). In addition, recent studies on GreB, the prokaryotic homolog of TFIIS, have also provided evidence for a two metal ion mechanism for the 3' transcript cleavage activity of bacterial RNAP (31,32).

In order to investigate the role of the highly conserved residues E791 and D792 of Rpb2 (equivalent to E836 and D837 in yeast RNAP II), we have studied mutant RNAP II having alanine substitutions in these residues. We found that the D792A substitution in Rpb2 impairs the assembly of RNAP II, thereby preventing the study of its effect during transcription. In contrast, the E791A substitution does not affect assembly of the enzyme but rather makes its polymerization activity more sensitive to low NTP concentrations and both its polymerization and cleavage activities more sensitive to low Mg²⁺ concentrations. Accordingly, we found that the equivalent substitution in yeast Rpb2, E836A, confers sensitivity to low nucleotide concentrations *in vivo*, while D837A is lethal either alone or in combination with E836A. Our results demonstrate for the first time that amino acid E791 (E836 in yeast) participates in the binding of Mg(B) in the active site and is required both for the polymerization and cleavage reactions. They also support the hypothesis that this second Mg²⁺ ion enters the catalytic center with the incoming NTP. We propose, for the polymerization reaction, that Rpb2 E791 interacts with the NTP-Mg(B) complex and participates in its loading to the active site prior to catalysis.

MATERIALS AND METHODS

Protein factors

Recombinant yeast TBP (33), human TFIIB (34), RAP74 and RAP30 (35), TFIIE56/α and TFIIE34/β (36–38), TFIIF (39,40), calf thymus RNAP II (41) and GST-TFIIS (42) were purified as previously described.

Tandem affinity purification of wild-type and mutant RNAP II

TAP-tagged wild-type and mutant RNAP II subunits were expressed in human EcR-293 cells and purified as previously described (43,44).

Gel mobility shift assay

Gel mobility shift assays were performed as previously described (45) with the following modifications. Complexes were assembled using highly purified TBP (40 ng), TFIIB (120 ng), RAP30 (200 ng), RAP74 (400 ng), TFIIE34 (80 ng), TFIIE56 (110 ng) and wild-type or mutant RNAP II (450 ng).

Run-off transcription assay

Transcription reactions were performed as described previously (42). TBP (30 ng), TFIIB (30 ng), RAP30 (30 ng), RAP74 (65 ng), TFIIE34 (40 ng), TFIIE56 (60 ng), TFIIF (4 ng) and wild-type or mutant RNAP II (165 ng) were incubated with 500 ng of linearized DNA template containing the adenovirus major late (AdML) promoter from nt -50 to +10 fused to a G-less cassette. Reactions were performed at various concentrations of NTP specified in the figure legends. Under these conditions a 391 nt run-off transcript is produced.

Transcription assay on immobilized template

A biotinylated template containing the AdML promoter from nt -50 to +10 fused to a G-less cassette and modified to contain a G at position +24 was immobilized on Dynabeads (Dyna, Oslo, Norway) according to the manufacturers' recommendations. The biotinylated template (225 ng) was pre-incubated with TBP (15 ng), TFIIB (15 ng), RAP30 (15 ng), RAP74 (30 ng), TFIIE34 (20 ng), TFIIE56 (30 ng), TFIIF (2 ng) and RNAP II (83 ng) for 30 min at 30°C in transcription buffer (12 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 1 mM DTT). Mix 1 was added (100 μM ATP, 100 μM CTP and 5 μCi of 0.08 μM [α-³²P]UTP, 3 mM EGTA, 12.5 mM MgCl₂, 1 U/μl RNase inhibitor) and transcription was allowed to proceed for 30 min at 30°C. Beads were then washed sequentially with 10 vol of 2× transcription buffer containing 0.3% sarkosyl and 1 mg/ml BSA, 5 vol of 2× transcription buffer containing 1 mg/ml BSA and 5 vol of 1× transcription buffer containing 1 mg/ml BSA. Beads were resuspended in 1× transcription buffer and incubated for 30 min at 30°C with Mix 2 (variable concentrations of ATP, CTP and UTP, 3 mM EGTA, 12.5 mM MgCl₂ and 1 U/μl RNase inhibitor; see figure legends). Transcription was stopped by adding 200 μl of stop solution (0.1 M sodium acetate, 0.5% SDS, 2 mM EDTA, 100 μg/ml tRNA). After phenol/chloroform extraction and ethanol precipitation, transcripts were analyzed on an 18% polyacrylamide, 9% urea denaturing gel.

Yeast strains and manipulations

The *Saccharomyces cerevisiae* strain yBC-1, which conditionally expresses Rpb2 under the control of the Gal1 promoter, was constructed in two steps. First, strain W303-1A (*MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) was transformed with the plasmid pGAL-*RPB2* (*URA3*). Second, the chromosomal *RPB2* allele of this merodiploid strain was disrupted with *LEU2*. The resulting strain, yBC-1, is dependent on galactose for growth. To test the phenotype associated with the substitutions E836A, D837A and the double substitution E836A D837A, plasmids encoding these different *rpb2* alleles (cloned into pFL39 [*TRP1*, *CEN*, *ARS*]), or wild-type *RPB2* as a control, were introduced into yBC-1 and transformants selected on galactose-medium lacking tryptophan. These transformants were then streaked onto three different media, either synthetic galactose-medium or glucose-medium lacking or containing 6-AU (50 µg/ml) and grown at 30°C for 5 days. In a separate experiment, the different mutant *rpb2* alleles were also introduced into the wild-type yeast strain W303-1A in order to test if they confer a dominant-lethal phenotype. Transformants were selected on synthetic glucose-medium lacking tryptophan. Yeast transformations were performed as previously described (46). Details on the construction of these plasmids will be provided upon request.

Cleavage assay

RNAP II complexes stalled at nucleotide +24 were formed as described above with Mix 2 containing 100 µM of non-radioactive ATP, CTP and UTP, 3 mM EGTA, 12.5 mM MgCl₂ and 1 U/µl RNase inhibitor. Beads were washed three times with 5 vol of 1× transcription buffer containing 1 mg/ml BSA. Beads were resuspended either in 20 µl of cleavage buffer pH 9.5 (70 mM CAPS-KOH, pH 9.5, 100 mM KCl, 5mM MgCl₂, 5% glycerol and 1 mM DTT) and incubated for 1 h at 30°C, or in 20 µl of TFIIS containing buffer pH 8 (70 mM Tris, pH 8, 100 mM KCl, 5 mM MgCl₂, 5% glycerol, 1 mM DTT and 0.42 ng/µl TFIIS) and incubated for 5 min at 30°C. In the experiments shown in Figure 3D, various concentrations of MgCl₂ were used in the cleavage buffer pH 9.5 as indicated. Cleavage reactions were stopped by the addition of 200 µl of stop solution and transcripts were processed as described above.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (47,48) with the modifications implemented by Jeronimo *et al.* (44). Cell lines expressing the following RNAP II mutants were analyzed: wild-type RNAP II, MB E791A/D792A, MB E791A and MB D792A. A mock-transfected cell line was used as a negative control. Quantitative PCR (Q-PCR) experiments were performed as previously described (49). Primer pairs were designed for both the promoter and transcribed regions of *GNB2L1* (−200 to +55 and +998 to +1165, respectively), *FTL* (−157 to +33 and +955 to +1176, respectively) and *ENO1* (−90 to +91 and +1143 to +1292, respectively) genes. The position of the promoter and transcribed regions was selected based on the major transcription start site listed in the DataBase of Transcription Start Site (DBTSS, April 2003 version, http://dbtss.hgc.jp/samp_home.html) and the Mammalian Gene

Collection (MGC, March 2003 version, <http://mgc.nci.nih.gov/>). Oligonucleotide sequences will be made available upon request. Relative immunoprecipitation efficiency was calculated by dividing the amount of DNA fragments spanning the promoter or coding region of the specified genes by the amount of DNA fragments from a conserved gene-less region located in the 13q21.33 band (50) in the immunoprecipitated sample. Data points originate from two independent sets of duplicate Q-PCR using chromatin obtained from three independent immunoprecipitations, giving a total of 12 data points for each immunoprecipitation.

Molecular modeling

Modeling was performed using the crystallographic structure of elongating yeast RNAP II (4,13) and the program Swiss PDB viewer.

RESULTS

RNAP II mutant MB E791A is sensitive to low NTP concentrations

The Rpb2 mutants MB E791A, MB D792A and MB E791A/D792A were designed to experimentally assess whether these highly conserved residues are involved in the coordination of Mg(B) in the active center, as predicted by early crystallographic data (3,4). Only MB E791A assembled correctly in the cell extract (Figure 1A) and was able to form a preinitiation complex with the general initiation factors on promoter DNA *in vitro* (Figure 1B). Both MB D792A and MB E791A/D792A lacked some of the small RNAP II subunits. As expected from their assembly defect, both MB D792A and MB E791A/D792A were also defective in run-off transcription reactions (data not shown). In contrast, the correctly assembled mutant MB E791A was able to synthesize an accurately initiated 391 nt run-off transcript, albeit less efficiently than the wild-type enzyme (Figure 2A).

Previous reports have shown that the substitution equivalent to E791A in *E.coli* (51) and in the archae *Methanococcus jannaschii* (52) impaired the ability of these enzymes to transcribe at low NTP concentrations. As shown in Figure 2A, the human mutant MB E791A is also sensitive to low NTP concentrations *in vitro*. Increasing the concentration of non-radioactive UTP in the assay partially alleviated the failure of MB E791A to produce run-off transcripts, but had no effect on the wild-type enzyme. Because the same amount of radioactive UTP (0.08 µM, 0.25 µCi) was used in all of the assays, increasing the concentration of unlabeled UTP from 2.5 µM UTP to 10 µM resulted in less incorporation of the labeled NTP in the 391 nt transcript synthesized by the wild-type enzyme, as indicated by the lower intensity of that band. Further increasing the concentration of non-radioactive UTP >40 µM led to a decrease in signal for both the wild-type and the mutant enzyme.

To confirm the sensitivity of MB E791A to NTP concentration, we immobilized the biotinylated template on streptavidin coated beads. The template used contained the AdML promoter that directed the synthesis of a 24 nt transcript in the absence of GTP as it contains a G residue at position +24 (see Figure 2B for a schematic representation). Wild-type

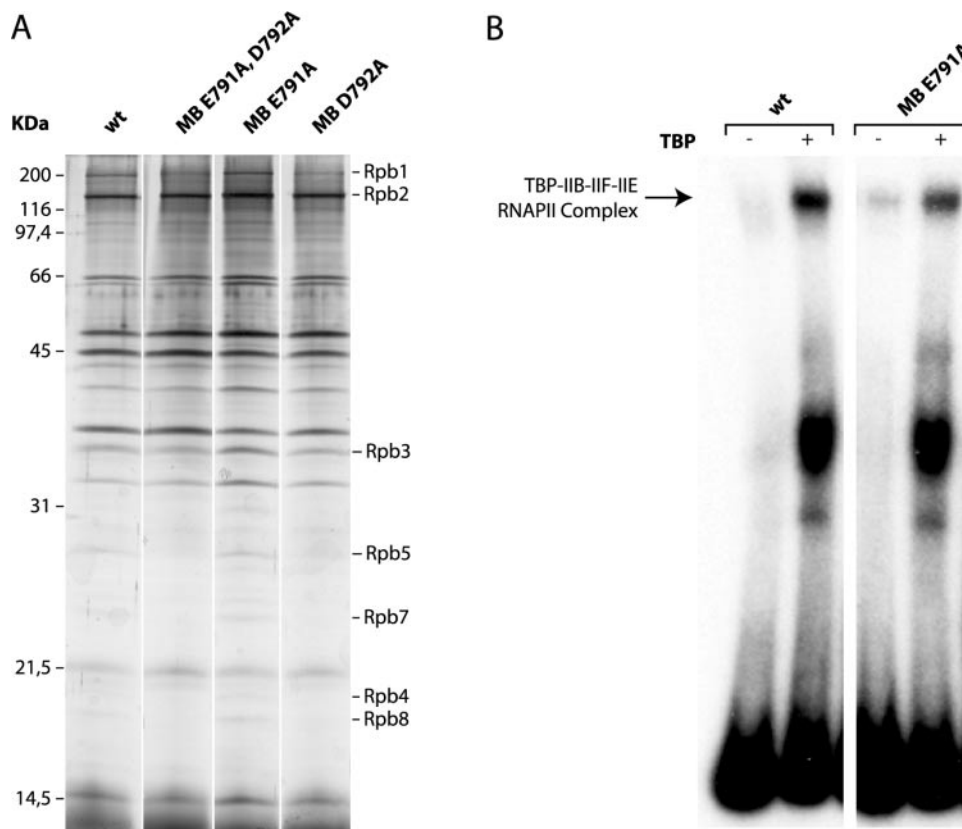


Figure 1. Purification of wild-type and metal B mutant RNAP II and characterization of their ability to form a pre-initiation complex. (A) The TAP purification procedure was used to isolate wild-type (wt) and mutant (MB E791A, D792A; MB E791A; MB D792A) RNAP II. The purified RNAP II were visualized on a SDS gel stained with silver. The position of molecular weight markers and RNAP II subunits are indicated. (B) An EMSA was performed using either wild-type RNAP II or MB E791A in the presence of TBP, TFIIB, TFIIF and TFIIIE. A control reaction assembled in the absence of TBP is included in each case. The position of the TBP-IIB-IIF-IIE-RNAP II-promoter complex is indicated.

RNAP II or MB E791A were pre-assembled with the GTF on the immobilized template. Transcription was allowed to proceed for 30 min in the presence of a mix of NTP (Mix 1). Mix 1 contained 100 μ M of ATP and CTP and 5 μ Ci of 0.08 μ M [α - 32 P]UTP and allowed the synthesis of transcripts of various lengths (Figure 2B, lanes 1 and 6). Under these conditions, the wild-type enzyme was able to predominantly produce the 24 nt transcript. In contrast, the majority of the MB E791A enzyme was stalled at around +10, indicating that the mutant enzyme is impaired in its ability to transcribe at limiting NTP concentrations. Increasing the concentration of non-radioactive NTP in Mix 2 stimulated production of the 24 nt transcript by the mutant polymerase (compare lanes 7–10 with lanes 2–5). At 0.64 μ M of NTP, the mutant was as active as the wild type enzyme.

We then wished to determine the phenotypes associated with the Rpb2 substitutions E836A (E791A), D837A (D792A) and the double substitutions E836A D837A (E791A D792A), in *S.cerevisiae*. To do so, the mutant *rpb2* alleles as well as the wild-type allele, all expressed from the endogenous *RPB2* promoter and cloned into a centromeric plasmid (*TRP*, *CEN*, *ARS*), were introduced into yeast strain yBC-1. In this strain, the chromosomal *RPB2* allele has been disrupted (*Rpb2::LEU2*) and is complemented by a plasmid expressing a galactose-inducible *RPB2* gene (*pGal-Rpb2*). On galactose-containing medium, both the wild-type Rpb2

encoded by the *pGal-Rpb2* allele and the mutant or wild-type Rpb2 under the control of the endogenous *RPB2* promoter, are expressed. However, on glucose-containing medium transcription from the *pGal* promoter is repressed and only the wild-type or mutant Rpb2 allele transcribed from the endogenous promoter is expressed. Figure 2C shows that transformation of yBC-1 with the *RPB2* D837A expressing vector has a dominant-negative effect on cellular growth and/or viability. This dominant effect, which was also observed in the wild-type strain W303-1A (data not shown), suggests that the Rpb2 D837A subunit interferes with the function of wild-type RNAP II. In contrast, expression of the mutant Rpb2 E836A and E836A D837A had no dominant effect on the growth of yBC-1 on galactose (Figure 2C). Viable yBC-1 transformants expressing these mutant proteins were then streaked on glucose-containing medium (Figure 2D) to repress expression of the *pGal-Rpb2* allele and reveal the recessive phenotypes associated with these mutant subunits. While the Rpb2 E836A subunit could support yeast growth on glucose medium, the double mutant Rpb2 E836A D837A was found to be lethal. Similar results were obtained in a strain lacking TFIIS (data not shown).

We then tested if Rpb2 E836A could support cell growth in the presence of 6-azauracil (6-AU), an inhibitor of IMP-dehydrogenase, which leads to a depletion of the intracellular pool of GTP and UTP (53). Most yeast mutants with

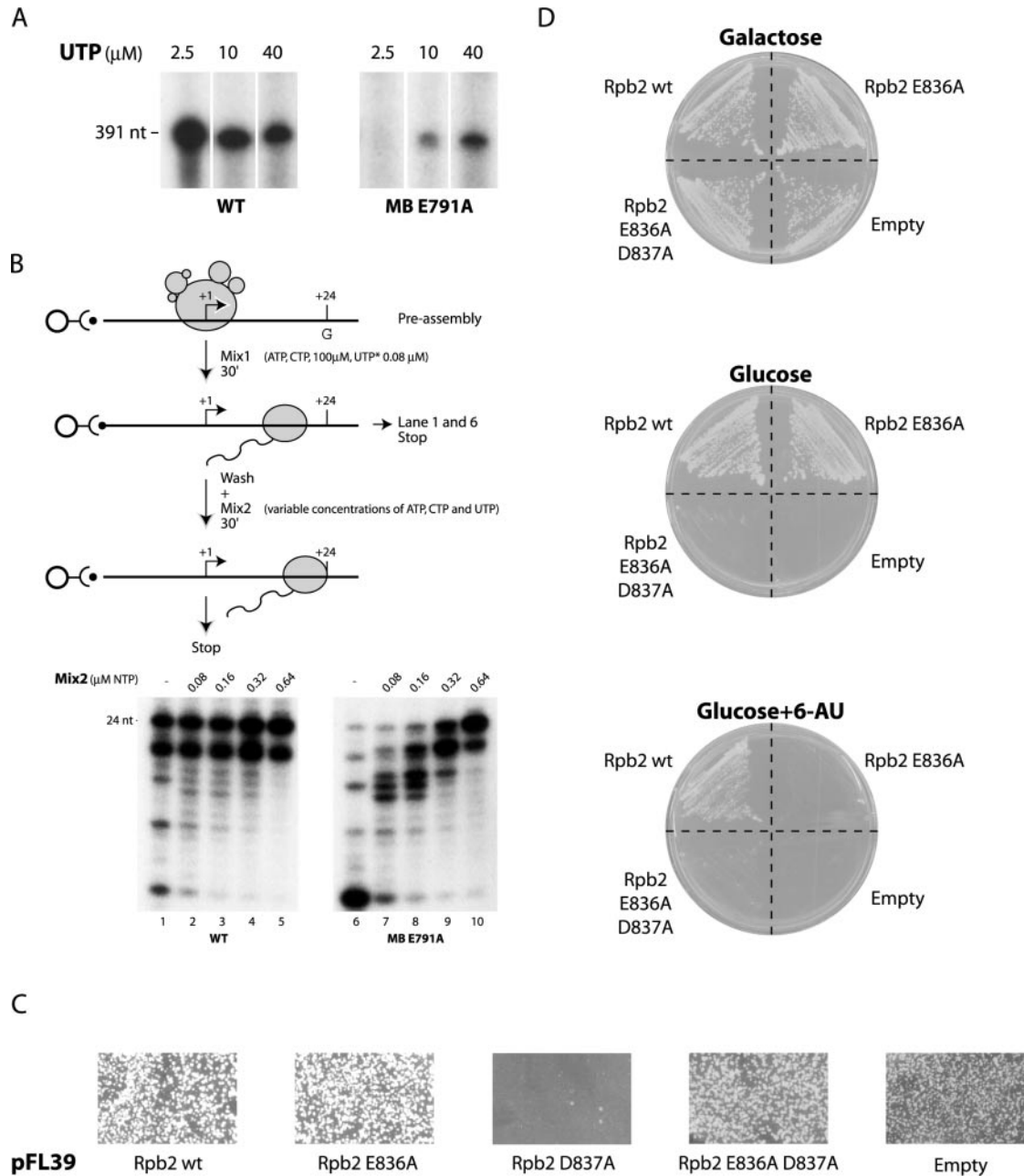


Figure 2. Mutant MB E791A is sensitive to NTP concentrations during polymerization. (A) *In vitro* transcription reactions were reconstituted with either wild-type RNAP II or MB E791A in the presence of TBP, TFIIB, TFIIF, TFIIIE and TFIIH. The linearized AdML template directs the synthesis of a 391 nt transcript. The reaction contained 750 μM ATP and CTP, 2.5 μCi of 0.04 μM [α - ^{32}P]UTP and increasing amounts of non-radioactive UTP, as indicated in the figure. (B) *In vitro* transcription reactions on immobilized template. A scheme of the transcription procedure is presented. Transcriptional complexes were assembled with wild-type RNAP II or MB E791 mutant in the presence of TBP, TFIIB, TFIIF, TFIIIE and TFIIH on the immobilized template. The AdML template directs the synthesis of a 24 nt transcript in the absence of GTP. (C) The *S.cerevisiae* strain yBC-1 (*RPB2::LEU2*, *pGAL-RPB2*) was transformed with either the wild-type or mutant E836A, D837A or E836A D837A *RPB2* allele cloned into a centromeric plasmid (pFL39; *TRP1*, *CEN*, *ARS*) and transformants selected on synthetic galactose-medium lacking tryptophan at 30°C. The pictures show the growth of Trp⁺ transformants expressing the indicated *RPB2* allele 3 days post-transformation. (D) The yBC-1 transformants from (C) were streaked on synthetic galactose medium, as well as on glucose medium containing or lacking 6-AU (50 $\mu\text{g}/\text{ml}$), and grown at 30°C for 5 days.

a defect in transcriptional elongation show a growth defect on 6-AU containing medium (54). This was also found to be the case for cells expressing Rpb2 E836A, which showed severely reduced growth on glucose-medium supplemented with 50 $\mu\text{g}/\text{ml}$ 6-AU, as compared with wild-type cells (Figure 2C). We found that the 6-AU sensitivity imposed by the E836A substitution in Rpb2 was more pronounced

than that associated with the previously reported *rpo21-18* or *rpo21-24* mutations in Rpb1 (data not shown), which affect the binding of TFIIS to RNAP II (55). These results suggest that the yeast E836A RNAP II is sensitive to low nucleotide concentrations *in vivo*, consistent with the *in vitro* transcription results obtained for the human RNAP II counterpart (E791A).

MB E791A is more sensitive than wild-type RNAP II to $MgCl_2$ concentrations both during NTP polymerization and transcript cleavage

To test the hypothesis that E836/E791 is involved in the binding of the second Mg^{2+} ion, we looked at the ability of MB E791A to transcribe at different $MgCl_2$ concentrations. Figure 3A and B show that MB E791A is more sensitive to low $MgCl_2$ concentrations than wild type when assayed for the production of the 24 nt transcript from immobilized templates. These results strongly suggest that Rpb2 amino acid E791 is involved in the binding of Mg(B) during NTP polymerization.

Since a two metal ion mechanism was also proposed for the cleavage activity of RNAPs (30–32), we tested the ability of the mutant MB E791A to cleave the 3' end of a transcript. In the experiments shown in Figure 3C and D, wild-type and mutant RNAP II were stalled at G + 24 using our transcription assay on immobilized templates, with a Mix 2 containing 100 μ M ATP, CTP and UTP. Complexes were washed and incubated at pH 9.5 in the absence of NTP for 1 h. It has been shown that in a stalled complex, RNAP II is able to cleave the 3' end of a transcript at a basic pH in the absence of TFIIS (21). At pH 8, intrinsic cleavage is almost completely abolished, but increasing the pH to 9.5 stimulates this RNAP II activity. The cleavage activity of RNAP II can also be greatly stimulated by the addition of TFIIS. Figure 3C shows that MB E791A is

able to cleave the 3' end of a transcript as efficiently as the wild type. At pH 9.5, the wild-type and the mutant cleave the RNA at two major sites, one at +22 and the other at +18. The addition of TFIIS stimulates both the wild type and the mutant to cleave, favoring the first (+22) site.

Previous studies using an *E.coli* mutant RNAP (E813A/D814A) analogous to our double mutant MB E791A/D792A have suggested that these two residues are not involved in the coordination of Mg(B) in the catalytic site (56). Specifically, using a cleavage assay similar to the one used in Figure 3C, Goldbarb and colleagues have shown that the ability of the mutant enzyme to cleave the 3' end of a transcript was equivalent to that of the wild-type enzyme at all $MgCl_2$ concentrations tested. We therefore determined the effect of decreasing the $MgCl_2$ concentration on the cleavage activity of MB E791A at pH 9.5 (Figure 3D). We measured the disappearance of the 24 nt band for the wild type and the mutant at different $MgCl_2$ concentrations (*x*-axis). The *y*-axis represents the ratio of the intensity of the cleaved band to the uncleaved band. In contrast to Sosunov *et al.* (56) the graph shows that at low $MgCl_2$ concentrations, MB E791A is less active than wild type. At higher $MgCl_2$ concentrations (5 mM), the mutant is as efficient as the wild type. Together, these results show that Rpb2 amino acid E791 is involved in the binding of Mg^{2+} in the active site of RNAP II for both the polymerization and cleavage reactions.

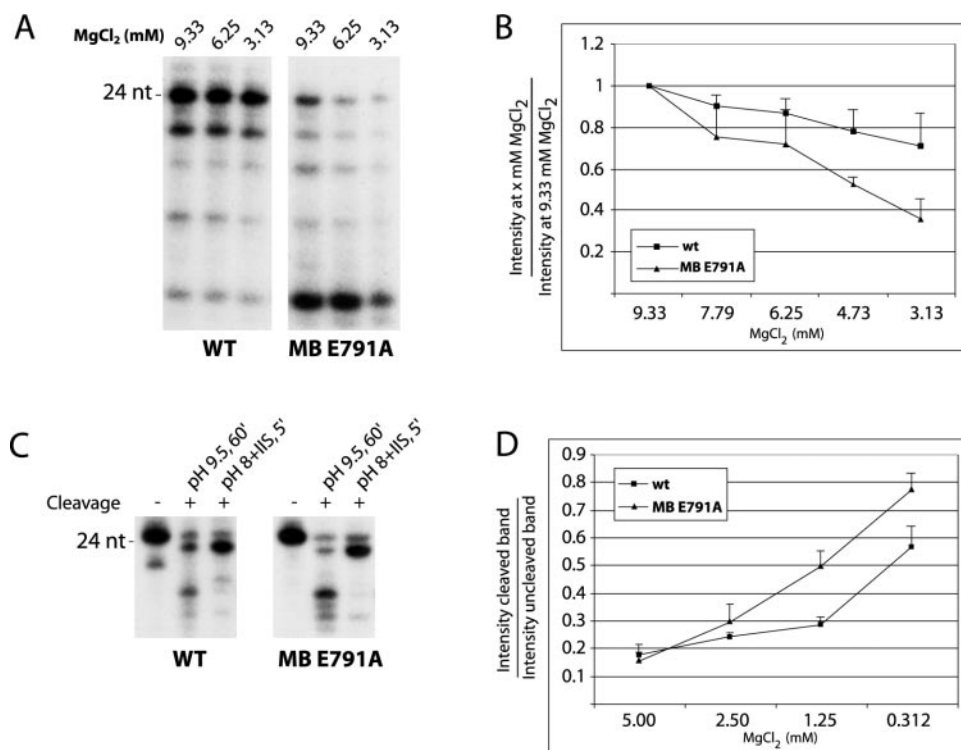


Figure 3. Mutant MB E791A is sensitive to $MgCl_2$ concentrations during both NTP polymerization and transcript cleavage. (A) *In vitro* transcription reactions on immobilized template at different $MgCl_2$ concentrations in Mix 1. Reactions were stopped 30 min after addition of Mix 1. (B) The intensity of the 24 nt transcript band obtained at each $MgCl_2$ concentration was quantified using a phosphorimager and normalized to the intensity of the band obtained at 9.33 mM $MgCl_2$. These relative values are reported on the *y*-axis of the graph and are the mean of three independent experiments. (C) Cleavage reactions were performed as described in Figure 4B except that Mix 2 contained 100 μ M ATP, 100 μ M CTP and 100 μ M UTP. After 30 min incubation with Mix 2, complexes were washed and incubated for 1 h at pH 9.5 or for 5 min at pH 8 in the presence of 8.2 ng of TFIIS. (D) The intensity of the 24 nt transcript band was quantified using a phosphorimager from cleavage reactions performed for 1 h at pH 9.5 in a buffer containing the indicated concentrations of $MgCl_2$. The intensity of each band was divided by the intensity of the uncleaved band (reaction stopped after 30 min of incubation with Mix 2) and reported on the *y*-axis of the graph. The ratios calculated are the mean of three independent experiments.

TFIIS stimulates transcription by both wild-type and MB E791A RNAP II at limiting NTP concentrations *in vitro*

In yeast, the TFIIS gene (*DST1*) is not essential for viability (57,58). However, knocking out the gene confers sensitivity to 6-AU (57,58). *In vitro* experiments have shown that reducing the concentration of NTPs causes RNAP II to pause and overall elongate less efficiently (26,27,59). Since MB E791A is sensitive to low nucleotide concentrations, we decided to test the effect of TFIIS on transcription by this mutant *in vitro*. Figure 4A shows that at 10 μ M UTP, TFIIS stimulates synthesis of the 391 nt transcript by MB E791A but has no effect on transcription by the wild-type enzyme. Reducing UTP concentrations from 2.5 to 0.625 μ M causes a decrease in the synthesis of the run-off transcript by the wild-type enzyme and causes the enzyme to arrest at different sites (Figure 4B). At these limiting concentrations of NTP, TFIIS now stimulates transcription by the wild-type RNAP II. These *in vitro* results confirm that TFIIS helps RNAP II to transcribe at low NTP concentrations, as suggested by previous *in vivo* experiments (57,58). We hypothesize that TFIIS helps RNAP II to resume arrest at low NTP concentrations by stimulating its 3' transcript cleavage activity, possibly by stabilizing the binding of Mg(B) in the active site.

Metal B mutant E791A is defective for transcription *in vivo*

In order to determine the transcriptional defects of the human MB E791A mutant *in vivo*, ChIP experiments were performed using IgG beads to precipitate the different TAP-tagged RNAP II bound to chromatin (Figure 5). Q-PCR was used to determine the enrichment of wild-type and mutant RNAP II on the promoter and coding region of 3 actively transcribed genes relative to an untranscribed region. The three transcribed genes used in these studies were: the ferritin light polypeptide gene (*FTL*), the guanine nucleotide binding protein beta polypeptide 2-like 1 gene (*GNB2L1*) and the enolase 1 (alpha) gene (*ENO1*).

As we previously described (44), wild-type TAP-tagged RNAP II was found at the promoter and in the transcribed region of the three genes tested, in substantially greater amounts than in the negative control (mock-transfected cell line). Consistent with the observation that MB E791A is able to form a pre-initiation complex *in vitro*, this mutant was

present at the promoter of *FTL*, *GNB2L1* and *ENO1* genes *in vivo*. Our results indicate that MB E791A is also located in the coding region of all three genes, albeit at a lower level than wild type. For the *FTL* and *GNB2L1* genes, the MB E791A mutation had a greater effect on the density of RNAP II in the coding region than at the promoter. For the *ENO1* gene, the mutant is similarly affected in both regions. Taken together, our ChIP results indicate that the E791A mutation has a more profound impact on transcript elongation than promoter recruitment *in vivo*.

We propose that a similar situation takes place in yeast cells. In the yeast strain carrying the E836A mutation, the RNAP II mutant is probably altered in its elongation efficiency on some genes but to an extent that does not affect growth under normal conditions. The addition of the drug 6-AU has a dramatic effect on growth of the mutant since it further reduces the altered enzyme's capacity to elongate by lowering the concentration of nucleotides in the cell (Figure 2C). The finding that mutant MB E791A is less efficiently recruited to promoters and less present in the transcribed regions *in vivo* cannot be explained solely by the *in vitro* defects presented in this article. We speculate that, *in vivo*, other factors that regulate transcription interact with the mutated polymerase and affect its ability to associate with promoters and to transcribe.

DISCUSSION

In this study, we have purified and characterized a human RNAP II mutant in which the highly conserved residue E791 of Rpb2 has been substituted for an alanine. Our results indicate that the Rpb2 E791A substitution in the human enzyme alters both its polymerization activity at low NTP concentrations (*in vitro*, see Figure 2A and B; *in vivo*, see Figure 2C) or at low Mg concentrations (Figure 3A and B) and its transcript cleavage activity at low Mg concentrations (Figure 3C and D). Although previous reports have shown that the substitution equivalent to E791A in the RNAP of *E.coli* (51) and the archae *M.jannaschii* (52) impaired the ability of these enzymes to transcribe at low NTP concentrations, our results provide, what is to our knowledge, the first biochemical evidence that the E791A substitution reduces transcription at low Mg concentrations. Indeed, previous studies using an *E.coli* mutant RNAP (E813A/D814A) analogous to our double mutant MB E791A/D792A have suggested that these two

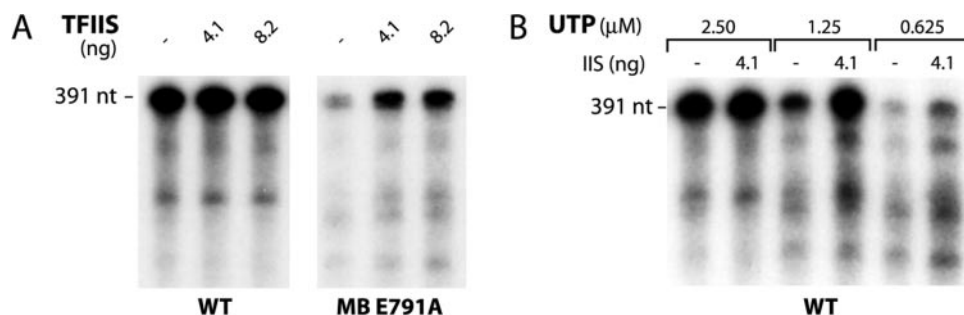


Figure 4. TFIIS stimulates transcription of both wild-type RNAP II and mutant MB E791A at low NTP concentrations. (A) *In vitro* transcription reactions were reconstituted using wild-type or mutant RNAP II in the presence of TBP, TFIIB, TFIIF, TFIIE, TFIIH and various amounts of TFIIS where indicated. The reaction contained 750 μ M ATP and CTP, 10 μ M UTP and 2.5 μ Ci of 0.04 μ M [α - 32 P]UTP. (B) Reactions were performed as in (A) for the wild-type RNAP II with the exception that reactions contained various amounts of non-radioactive UTP and 4.1 ng of TFIIS, where indicated.

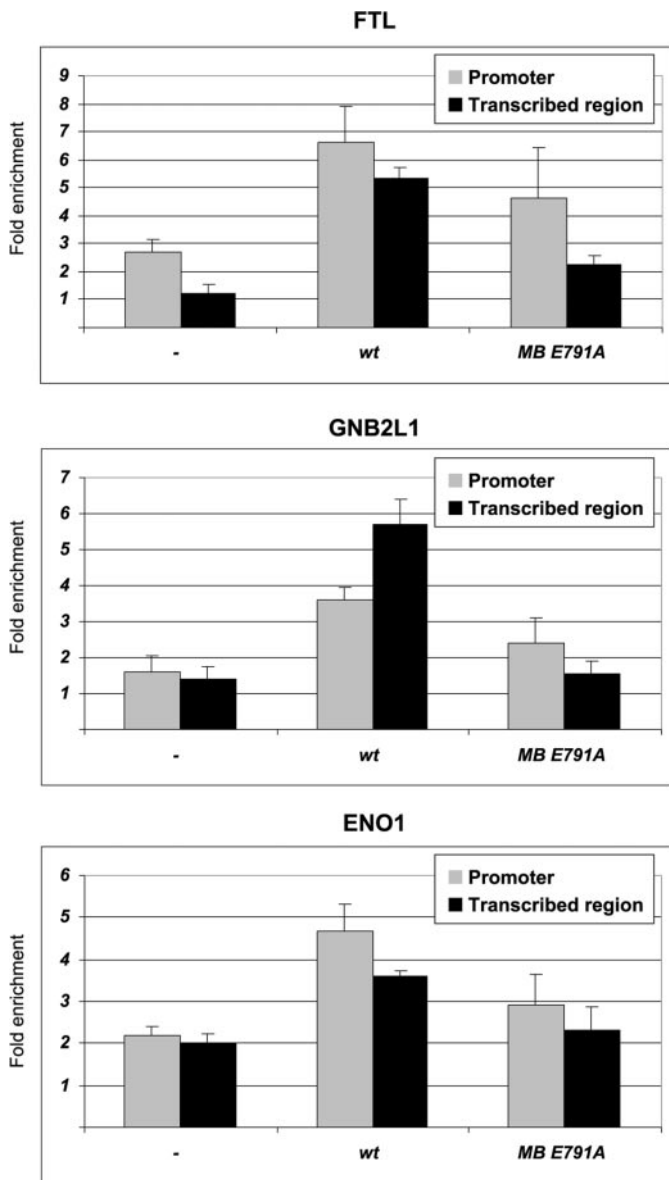


Figure 5. Association of wild-type and mutated RNAP II with chromatin *in vivo*. Fold enrichment over the control region of both the promoter and the transcribed region of *FTL*, *GNB2L1* and *ENO1* genes following immunoprecipitation of chromatin fragments was determined using Q-PCR. Enrichment was normalized to a conserved gene-less region located in the 13q21.33 band (see Materials and Methods). Data points originate from two independent sets of duplicate Q-PCR using chromatin obtained from three independent immunoprecipitations, giving a total of 12 data points for each immunoprecipitation. The negative control is a mock transfected cell line.

residues are not involved in the coordination of Mg(B) in the catalytic site (56). Specifically, using a cleavage assay similar to the one used in Figure 3C, Goldfarb and colleagues have shown that this mutant enzyme cleaves the 3' end of a transcript as efficiently as the wild-type enzyme at all MgCl₂ concentrations tested. The discrepancy between our results and those of Goldfarb and colleagues might be accounted for by the fact that MgCl₂ concentrations <5 mM were not tested by Sosunov *et al.* (56).

At high NTP concentrations, TFIIIS was found to stimulate elongation of the Rpb2 E791A mutant, but not that of

wild-type RNAP II (Figure 4). Stimulation of the wild-type enzyme by TFIIIS was only observed when the NTP concentration was made limiting. These data support previous *in vivo* results showing that TFIIIS helps RNAP II to transcribe at low NTP concentrations (57,58), presumably by increasing NTP-Mg(B) binding in the enzyme's active site. Our finding that TFIIIS is able to stimulate transcript elongation at high NTP concentrations by a RNAP II having the Rpb2 E791A substitution supports a role for this residue in NTP-Mg(B) binding. A lower density of the mutated RNAP II along transcribed genes, as determined by ChIP experiments *in vivo* (Figure 5), is also consistent with the role of E791 in transcript elongation.

Recently, Kornberg and co-workers (13) have published the crystal structure of yeast RNAP II bound to a DNA-RNA duplex and a nucleotide positioned at the *i* + 1 site, poised for bond formation. In this structure, Mg(B) was found to be located in proximity to Rpb1 residues D481 and D483 and Rpb2 D837. On this basis, the authors proposed that these amino acids are implicated in Mg(B) coordination at the active site. At this step of the polymerization process, Rpb2 E836 is located too far from Mg(B) to interact with it. The structure of a RNAP II with a mismatched nucleotide revealed the existence of a second binding site for the incoming NTP, called the 'entry' or E site. It was suggested that the NTP-Mg(B) complex enters the active site through the pore and first binds to the E site in an inverted orientation, followed by a rotation and subsequent binding to the so-called 'addition' or A site.

To account for our results showing that the mutant E791A is sensitive to NTP and MgCl₂ concentrations during the polymerization reaction, we propose that Rpb2 E836/791 is implicated in loading the NTP-Mg(B) into the E site. We can envisage two distinct mechanisms by which this could occur. First, it is possible that the Rpb2 E791 side chain is essential for specifying the proper topology of the E site. In this case, substituting E791 for alanine could conceivably modify the configuration of the E site, for example, by affecting the precise positioning of residue D792, such as to reduce its binding affinity for the incoming NTP-Mg(B). Alternatively, it is possible that Rpb2 E791 defines a third and distinct NTP-Mg(B) binding site within the active center, which we will refer to as the 'pre-loading' (P) site. Figure 6A depicts how an incoming NTP-Mg(B) entering the active site through the pore would first interact transiently with the P site (panel 1), before being loaded in the E site at a position where metal B can interact with Rpb2 D837, Rpb1 D481 and D483 and the NTP β and γ phosphates, as described (13) (panel 2). The nucleotide is next flipped and positioned at the A site for catalysis (panel 3). Consistent with the existence of a P site, we note that E836/E791 is the acidic residue within the vicinity of the active site that is the most accessible to an incoming NTP-Mg(B) entering from the pore.

In a recent series of papers, Burton and colleagues have proposed that the incoming NTPs access the active center through the main channel (8,60,61). Furthermore, these authors have proposed an NTP-driven translocation model in which the incoming NTP pairs with the DNA base at the *i* + 2 site. According to this model, this pairing is necessary to induce translocation of the NTP at the *i* + 1 site (A site) for catalysis and allows substrate selection prior to arrival into the catalytic site. Our data is also consistent with the idea that the NTPs access the catalytic center through the main channel.

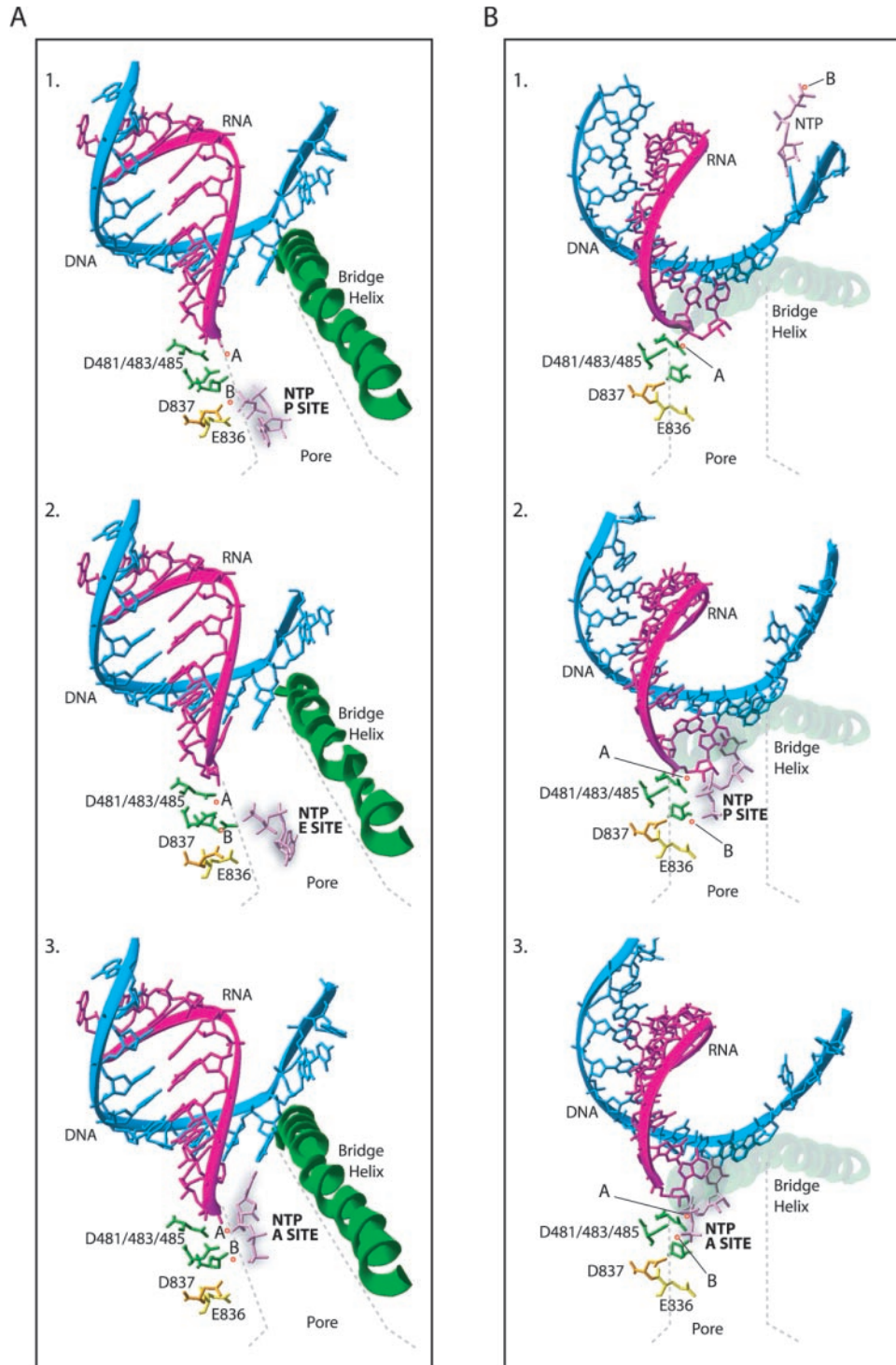


Figure 6. Proposed models for the NTP polymerization reaction. (A) The NTP–Mg(B) accessing the active center from the pore first binds to a ‘Pre-entry’ (P) site where it can contact Rpb2 residue E836/E791 and other residues that remained to be identified. From the P site, the NTP–Mg(B) moves to the E site before rotating to access the A site where it is paired with the template DNA. (B) The incoming NTP–Mg(B) can alternatively enter the catalytic center through the enzyme’s main channel and associate with E836/E791 in the P site. The subsequent steps are as in (A). The exact location of the P site is speculative. See text for details. Incoming NTP: pink; Rpb1 D481, D483, D485, bridge helix: green; Rpb2 E836: yellow; Rpb2 D837: orange; RNA: magenta; DNA: blue; and metal A and B: red.

This would imply, as depicted in Figure 6B, that Mg(B) is implicated in an interaction with E836 (panel 2) during translocation of the NTP–Mg(B) from the $i + 2$ site (panel 1) to the A site (panel 3). The interaction of E836 with NTP–Mg(B)

would play a crucial role in loading the NTP or retaining it in the active site for catalysis. If this model is correct, the position of the E site would be more consistent with it being a site for expulsion and subsequent exit of a mismatched nucleotide.

The mutant MB E791A is also sensitive to MgCl₂ concentration in the cleavage reaction, suggesting that E791 is implicated in binding Mg(B) for this activity of the polymerase. High-resolution structures of RNAP II engaged in transcript cleavage will be required to determine the molecular basis of the interaction between E836 and Mg(B) during the rescue of backtracked transcripts.

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