Disruption of substrate binding site in *E. coli* RNA polymerase by lethal alanine substitutions in carboxy terminal domain of the β subunit

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Abstract Alanine substitution of four amino acids in two evolutionarily conserved motifs, PSRM and RFGEME, near the carboxy terminus of the β subunit of *E. coli* RNA polymerase results in a dramatic loss of the enzyme's affinity to substrates with no apparent effect on the maximal rate of the enzymatic reaction or on binding to promoters. The magnitude and selectivity of the effect suggest that the mutations disrupt the substrate binding site of the active center.

Key words: RNA polymerase; Site-directed mutagenesis; Substrate binding center

1. Introduction

RNA polymerase (RNAP) is a multifunctional multisubunit enzyme which is central to gene expression and regulation. Its functional cycle constitutes a succession of basic steps such as specific promoter binding, local melting of DNA double helix, catalytic formation of short abortive transcripts, promoter clearance, processive elongation of RNA and termination [1]. The principal functional determinants and general architecture of RNAP must be the same in all organisms as is evident from evolutionary conservation of its two largest subunits [1]. Direct structural approaches to RNAP have so far produced only low-resolution models which do not yet permit detailed structure functional interpretations [2]. We developed a molecular genetic approach to identify localities involved in the basic reactions of RNAP [3–5]. Using this approach we constructed mutant RNAP with triple alanine substitution of Asp residues in β′ subunit evolutionary invariant NADFDGDA motif and demonstrated that these residues were involved in coordinating Mg^{2+} ions directly participating in catalysis [6]. Here we report the construction and characterization of mutant RNAPs with quadruple substitutions in two highly conserved motifs of the β subunit which pinpointed the regions involved in binding of nucleotide substrates.

2. Materials and methods

2.1. Engineering and phenotypic analysis of mutations REME and PSRM

The expression plasmid pMKSe2 that carries rifampicin-sensitive allele of *rpoB* gene [7] was used for genetic construction of REME. The mutation was engineered by oligonucleotide-directed mutagenesis [8] in 526 bp *Eco*RI-*Sph*I fragment of *rpoB* that spans the distance between codons 1134 and 1319. This fragment was cloned into the multiple cloning site of the M13mp18 vector. The engineered sequence was GGT-CAa-gcT-TTC-GGG-GcG-gcG-Gca-GTG, with the mutated nucleotides shown in lower-case letters. After verification by DNA sequencing, the 282 bp *Bst*EII-*Sph*I M13 fragment that carries the mutation was recloned into the recipient plasmid pMKSe2. The samples were then transformed into rifampicin-resistant HB101 host carrying the pLacI^{Q} expression plasmid to insure tight suppression of potentially toxic *rpoB* allele. The candidates were screened for the possession of unique *Hin*dIII site introduced in the mutagenesis and by appearance of the inducible full-size β polypeptide on SDS gel electrophoresis. The resulting REME mutant had the following amino acid residues substituted for alanines: R^{1269}, E^{1272}, M^{1273}, and E^{1274}.

For the construction of *PSRM* mutation a derivative of pMKA92 plasmid [3] with added unique *Xma*I site in the codon 1063 was used (pMKA92BalVAn). On the first stage of this PCR based mutagenesis following primers were used: (1) the 47 bp PCR primer overlapping mutagenized region carried the desirable sequence and unique *Ssp*I site; (2) the 17 bp primer was complementary the region of 1033–1039 codons of the β subunit. On the next stage the amplified 240 bp fragment was used as primer for the second PCR. The other 17 bp primer covered unique *Sph*I site at the very 3′ end of the β subunit. The *Xma*I-*Sph*I fragment of the 850 bp PCR product was cloned into the pMK92BalVAn plasmid. The samples were then transformed into HB101 host carrying the pLacI^{Q} expression plasmid. The candidates were screened for the possession of unique *Ssp*I site introduced in the mutagenesis and by appearance of the inducible full-size β polypeptide on SDS gel electrophoresis. The engineered sequence confirmed by DNA sequencing was GTA-gCG-gCT-gcG-AAt-ATt-GGT, with the mutated nucleotides shown in lower-case letters. The resulting PSRM mutant had the following amino acid residues substituted for alanines: P^{1104}, S^{1105}, R^{1106}, and M^{1107}. The phenotypic analysis of the plasmid-born *rpoB* alleles performed as described in [3] demonstrated that *REME* was dominant while *PSRM* was recessive lethal mutation.

2.2. RNAP and DNA templates

The wild-type and mutant RNAPs were reconstituted from individually overexpressed subunits as described [9]. The reconstituted enzymes contained a stoichiometric amount of σ factor after two steps of chromatography.

The following DNAs were used for in vitro transcription: the 130 bp fragment containing the T7A1 promoter [10]; the 110 bp *Eco*RI-*Bam*HI fragment of the pHC624lac plasmid that carried *lac*UV5 promoter [11]; the 202 bp *Eco*RI-*Hin*dIII fragment of the pRLG930 plasmid carrying *rrnB* P1 promoter [12].

2.3. In vitro transcription

The T4 DNA transcription assay was performed as described in [3]. All other assays, unless specified otherwise, were performed under the following general conditions. The standard 10 μl reaction contained 0.1 pmole of DNA and 0.5 pmole of RNAP in 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 100 mM KCl, 7 mM β-mercaptoethanol, 0.5 mM dinucleotide primer, and 0.1 mM of specified NTPs, one of which carried α-^{32}P (3 Ci/mmole) as indicated. Incubation was at 37°C for 15 min. The reactions were stopped by the addition of 15 μl of 8 M urea, and samples were applied to an 8 M urea PAGE (21% acrylamide, 2% *N*,*N*′-methylene-bis-acrylamide), which were run and radioautographed as described earlier [13]. The amount of radioactivity in bands was quantified on Betascope 603 blot analyzer.

In the experiment on the accumulation of GpApA, a His-tagged RNAP was pre-incubated with the *lac*UV5 promoter fragment for 10 min at 37°C to form an open promoter complex. Complexes then were transferred to the room temperature and the substrates

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Abbreviations: RNAP, RNA polymerase

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were added in the saturating concentrations (wild-type RNAP: 0.5 mM GpA, 0.1 mM ATP, REME RNAP: 2 mM GpA, 2.5 mM ATP).

3. Results

3.1. Mutations selectively reducing RNAP affinity to substrates

The mutant RNAP holoenzyme carrying β^{REME} and β^{PSRM} were reconstituted from individually overexpressed subunits [9]. As the control, the wild-type enzyme carrying β specified by the parent plasmid was assembled in the same way. For experiments requiring immobilized RNAP, $(His)_6$-tagged β′ subunit [14] was used for reconstitution. The reconstituted mutant enzymes turned out to be completely inactive in the standard in vitro assay on the bacteriophage T4 DNA template.

The activity of the reconstituted enzymes was then determined on the 130 base-pair DNA fragment carrying the bacteriophage T7A1 promoter (Fig. 1). Priming dinucleotide CpA was present in the system and the reaction was allowed to proceed either in the presence of all four ribonucleoside triphosphate (lanes 1, 2, 5, 6) permitting formation of the runoff transcript, or in the presence of [α-^{32}P]UTP (lanes 3, 4, 7, 8) when only the abortive trinucleotide CpA**p**U (here and later bold type face designates radioactive phosphate) could be formed. Two concentrations of nucleoside triphosphate were used, 0.1 and 1.0 mM (odd and even numbered lanes, respectively). It can be seen that the mutant polymerase was completely deficient in the formation of the runoff transcript, and could only form the abortive trinucleotide at the elevated NTP concentration, and at a slower rate than the wild-type. Similar type of defect was found in the PSRM mutant. The activity of the mutant enzymes represented stable open promoter complexes since it displayed linear time dependence in the presence of DNA competitor heparin (data not shown).

Fig. 1. in vitro activity of the REME mutant RNAP. The gel autoradiogram shows the products made by wild-type (lanes 1–4) and REME (lanes 5–8) RNAPs in steady-state transcription reaction on 130 bp T7A1 promoter fragment in the presence of either all four transcription substrates (lanes 1, 2, 5, 6) or only UTP. In both cases [α-^{32}P]UTP was included. Odd lines represent the activities of RNAPs at standard concentrations of triphosphates (0.1 mM), even lines at elevated (1 mM). The transcription reactions were initiated with CpA. The major abortive product and the runoff transcript are identified.

The dependence of CpA**p**U synthesis on substrate concentration was studied in more detail in the experiment of Fig. 2. As can be seen, the REME RNAP required dramatically higher concentrations of both CpA and UTP for activity. Calculation of apparent K_M for the substrates [15] for the mutant REME enzyme gave the values of >3500 μM (CpA) and 540 μM (UTP) which are by two orders of magnitude higher than the parameters of the wild-type enzyme (32 μM and 5.6 μM, respectively). Remarkably, at high concentrations of the substrates, the rate of the trinucleotide formation by the mutant was reproducibly higher than by the wild-type (compare panels D and B). Thus, the mutation drastically decreased the enzyme's affinity to both substrates without substantively affecting the catalytic rate. Calculated K_m values for the PSRM mutant were 1000 mM CpA and 625 mM UTP. Other experiments were carried out only using the REME mutant with the strongest phenotype.

3.2. Enhanced loss of a trinucleotide product by REME polymerase

As is evident from Fig. 1, lane 6, the mutant RNAP failed to extend RNA beyond the joining of the two added substrates on the T7A1 promoter, an observation that was confirmed on several other *E. coli* promoters (data not shown). Such promoters are known to abort nascent oligonucleotides from the catalytic complex. Presumably, the mutation decreased the stability of the nascent product of the first reaction in the complex so that it was lost from the active center before it could be elongated, notwithstanding the high concentration of the next nucleotide.

We were interested to compare the wild-type and the *REME* mutant enzymes for their ability to retain trinucleotides in the ternary complex. In most systems, product release occurs too fast to be measured directly. In some cases, however, nascent oligonucleotide release is sufficiently slow to permit measurement using the technique of His-tagged RNAP immobilized on a solid support where one can rapidly fractionate free and enzyme-associated reaction products by centrifugation. The experiment of Fig. 3 presents the time course of accumulation of GpA**p**A synthesized on the *lac*UV5 promoter in free and enzyme-bound form. As can be seen, a substantial fraction of the trinucleotide is retained by the wild-type enzyme pellet in this steady-state system whereas no retention was detected in the case of the mutant enzyme. This result qualitatively demonstrates that *REME* polymerase looses trinucleotides significantly faster than the wild-type polymerase.

3.3. REME polymerase requires increased substrate concentration during elongation

REME RNAP can not be forced to leave the promoter and

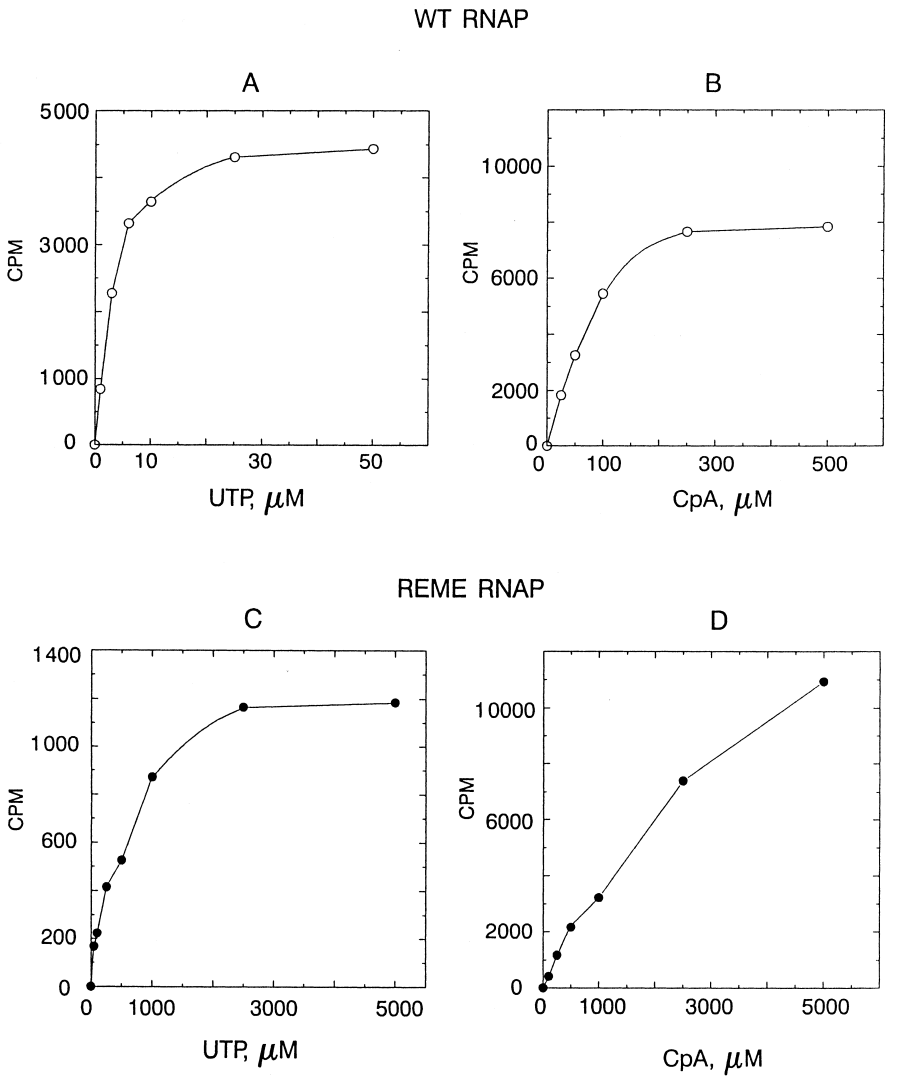

Fig. 2. The dependence of the abortive CpApU synthesis on substrate concentration. Plots show the accumulation of the radioactive CpApU product on T7A1 promoter in 15 min at 37°C by wild-type (panels A and B) and REME (panels C and D) RNAPs as a function of the transcription substrate (CpA and UTP) concentrations. The dependence of CpApU formation on UTP concentration (panels A and C) was observed at 500 mM CpA for both enzymes. The measurement of the CpApU synthesis rate as function of CpA concentration (panels B and D) was performed at 100 mM of UTP for WT RNAP and 2500 mM for the mutant RNAP. The data represent quantification of ^{32}P radioactivity in individual gel bands.

start elongation even in the presence of high nucleotide concentration on most studied promoters. In order to synthesize longer RNA with *REME* RNAP, and to study chain elongation in a stable ternary complex we took advantage of an unusual mode of initiation that is not associated with the loss of the nascent product. For this purpose we employed the *rrnB* P1 promoter which has the initial transcribed sequence $C_{-4}A_{-3}C_{-2}C_{-1}A_{+1}C_{+2}U_{+3}G_{+4}$. In the presence of CpA and CTP, the trinucleotide CpApC (−1,+1,+2) is not aborted but slips three positions upstream and is extended by CTP yielding the tetranucleotide CpApCpC (−4,−3,−2,−1). The tetranucleotide product is stably locked in the promoter complex and can be extended to longer chains without being lost from the active center [16]. *REME* RNAP was inactive on this promoter at the standard NTP and primer concentrations. However, at elevated substrate concentrations, transcripts were made by the mutant extending down to the template positions +9 and +10 (Fig. 4, lanes 1–3). These positions are 'high apparent K_M' sites for the wild-type RNAP which stops at +9 when the NTP concentration is 1 μM (lane 5) but resumes elongation when the substrate concentration is increased (lane 6). The requirement for relatively high NTP concentrations at these positions by the wild-type enzyme may explain why *REME*, a high K_M mutant, does not elongate past these points at all.

Stable ternary complex carrying the radiolabeled hexamer transcript CpApCpC**p**ApC can be generated on the *rrnB* P1 promoter in the presence of CpA, CTP and [α^{32}P]ATP. Such complexes could be 'chased' to form the 7-mer transcript by adding the next nucleotide UTP. We measured the concentration of UTP required for the chase of 50% of the material from 6-mer to 7-mer in a 2 min reaction. The half transition was observed at 0.05 μM and 3 μM of UTP for the wild-type and *REME* mutant, respectively (Fig. 5, panels A and C).

A similar experiment was then performed for the extension of the 7-mer into the 8-mer transcript by adding GTP. The 50% transition was observed at 0.08 μM and 4.6 μM of GTP for the wild-type and the mutant, respectively (Fig. 5, panels B and D). Thus the dramatically higher K_M of the *REME* mutant was demonstrated for the elongation reaction at two defined sequence positions involving the addition of a pyrimidine and a purine nucleotide.

Experiments on pyrophosphorolysis of 6-mer transcripts formed on *rrnB* P1 promoter demonstrated that the PSRM and REME RNAPs required more than ten-fold higher concentration of pyrophosphate to make shorter 5- and 4-mer transcripts as efficiently as the wild-type RNAP (data not shown).

4. Discussion

The REME and PSRM polymerases have a dramatically increased K_M for substrates, and at the same time, display unchanged maximal catalytic rate, promoter selectivity and binding. The magnitude and selectivity of the effect suggest that the mutations disrupt the sites involved in binding of initiating and elongating substrates but do not affect the catalytic and promoter binding centers. Besides increase in K_M, the REME and PSRM mutants display severe defects at stages following the first phosphodiester bond formation. These post initiation defects can not be completely compensated by increasing substrate concentration in the case of most promoters studied. This suggests that the RFGEME and PSRM motifs are also involved in RNAP functions other than substrate binding.

Both ourselves and other authors have previously reported increased apparent K_M for substrates in a number of RNAP mutants with substitutions of evolutionary conserved amino acid residues [17–19] and with a deletion in an evolutionary variable region [20]. However, in these reports the extent of the effect was mush lower than in the case of *REME*. A limited set of mutants with substitutions of individual residues in the RFGEME motif has recently been described in literature but again none of these mutations affected RNAP function as dramatically as *PSRM* and *REME* mutations [21–23]. Comparison of the properties of these mutants with those of the REME mutant suggests that different amino acid residues of the motif may participate in different interactions within the active center.

Our previous data suggest that the PSRM motif is located no farther than 1 nm from the catalytic center [24] while RFGEME motif is at the edge of a segment that can be crosslinked to DNA downstream of the active center [25]. The data obtained in this study however do not prove that the PSRM and RFGEME motifs are directly involved in contacts with substrates. Testing this notion would require detailed study of a large set of mutants with substitutions of individual residues in these motifs in combination with crosslinking experiments.

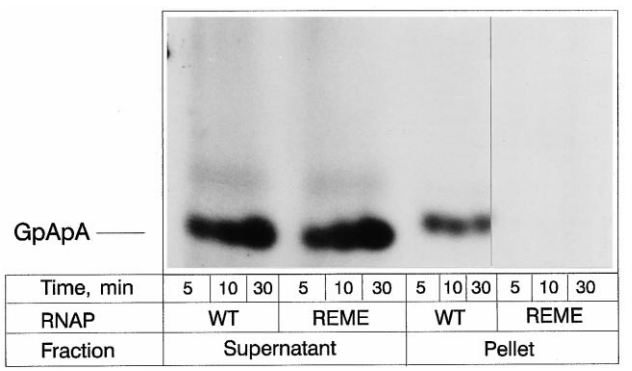

Fig. 3. Accumulation of GpApA in free and enzyme-bound forms on the *lac*UV5 promoter. The autoradiogram shows the time course of GpApA synthesis by wild-type and mutant REME RNAPs. The assay was carried out under saturating concentrations of the substrates (GpA and ATP) for both His-tagged enzymes. Aliquots of the reaction mixture were taken at the times indicated above. The abortive GpApA was found in the supernatant while enzyme-bound fraction of the trinucleotide retained in the pellet after centrifugation.

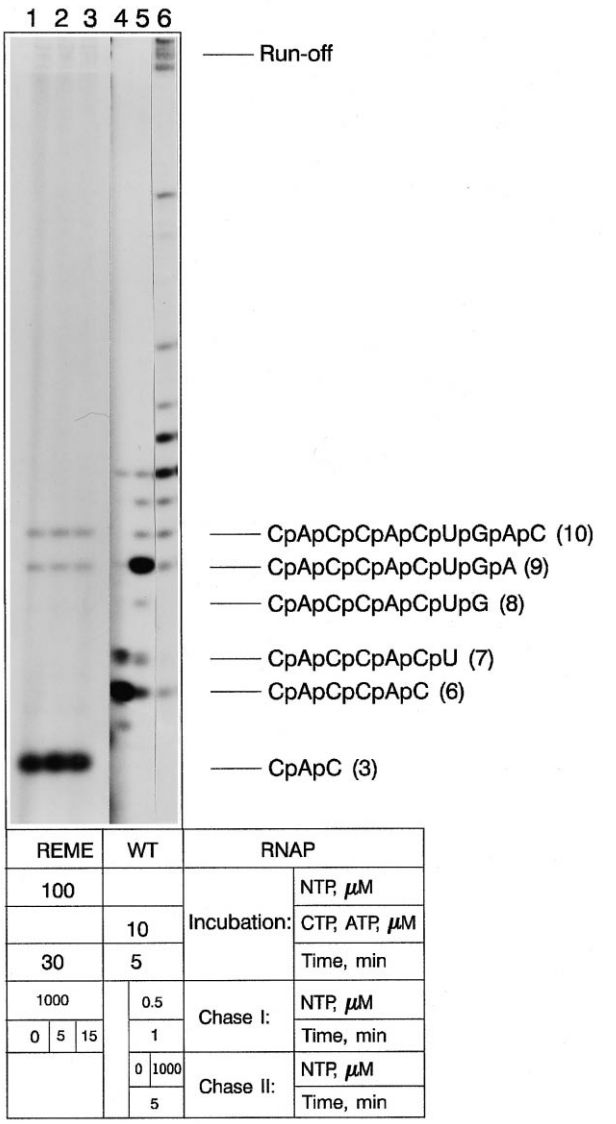

Fig. 4. Transcription activity of the REME RNAP on the ribosomal *rrnB* P1 promoter. The gel autoradiogram shows the products made by the REME RNAP when primed with CpA (1 mM) in the presence of all four nucleotides (lanes 1–3). Lanes 4–6 demonstrate the activity of the wild-type RNAP after the initiation that involves the transcript slippage. All transcription complexes were purified through G-50 columns before being chased (Chase I only).

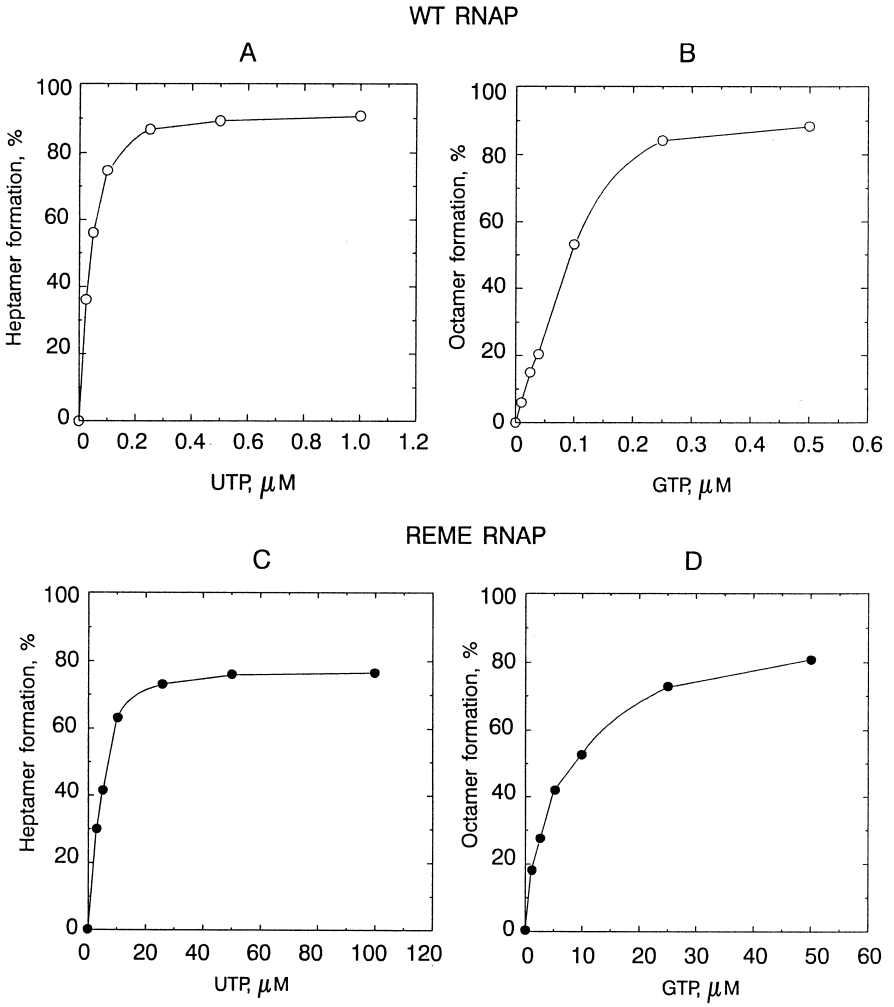

Fig. 5. Effect of NTP concentration on hexa- and heptamers elongation by the wild-type and the REME RNAPs on the ribosomal *rrnB* P1 promoter. The plots show the kinetics of the RNA products formation in the single nucleotide addition reaction by wild-type and the mutant enzymes. Ternary complexes containing 6 base (panels A, C) and 7 base (panels B, D) long transcripts were purified through G-50 columns and incubated for 2 min at 37°C in presence of the next nucleotide. The data represent quantification of ^{32}P radioactivity in individual gel bands.

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