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Localisation of the binding site for the initiating substrate on the RNA polymerase from *Sulfolobus acidocaldarius*

Mikhail A. Grachev, Arkadij A. Mustaev, Evgeny F. Zaychikov, Anton J. Lindner* and Guido R. Hartmann*

Limnological Institute, Siberian Division of the Academy of Sciences of the USSR, 664033 Irkutsk, USSR and **Institut für Biochemie, Ludwig-Maximilians-Universität, D-8000 München 2, FRG*

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RNA polymerase from the archaebacterium *Sulfolobus acidocaldarius* was chemically modified with AMP *o*-formylphenyl ester followed by reduction with borohydride. The modified protein catalyzes the labeling of its own largest subunit when incubated with [α-^{33}P]UTP in the presence of poly[d(A-T)]. On cleaving of the labeled protein using cyanogen bromide, hydroxylamine or amino acid-specific endoproteinases for a very brief period, the pattern and size of the radioactive fragments formed are best explained by attachment of the label between Gly^{843} and Met^{895} of the largest subunit. In this region there exists a highly conserved sequence which is also found in other archaebacterial, eukaryotic and prokaryotic RNA polymerases. This suggests that the binding site for the initiating substrate of RNA polymerases has been conserved during evolution.

RNA polymerase; Affinity labeling; Active center; Localization; (*Sulfolobus acidocaldarius*)

1. INTRODUCTION

The application of chemically reactive derivatives of nucleotides as affinity reagents to prokaryotic RNA polymerases has provided valuable information on the topography of the active center. For example, highly selective affinity labeling helped in identifying the subunits forming the active center of *E. coli* RNA polymerase [1]; with the aid of a new analytic technique for localisation of the affinity label on the polypeptide, it has become possible to identify the regions which are immediately adjacent to the active center [2-4].

It is of great interest to compare the topographies of the active center of RNA polymerases from evolutionarily distant organisms. The results obtained from the highly selective affinity labeling of different prokaryotic eukaryotic and archaebacterial RNA polymerases [5-10] suggested that the binding site of the initiating substrate resides on the β-subunit of prokaryotic enzymes, on the second largest subunit of eukaryotic forms and on the B- or B′-subunit of the archaebacterial types. Comparison of the amino acid sequences close to the labeled sites may shed some light on the conservation of these regions during evolution. To date, such sequences have been determined only for the RNA polymerase from *E. coli* [1] and bacteriophages T_7 and Qβ [8,10]. The two sequences identified in the *E. coli* RNA polymerase β-subunit belong to regions of high homology found in RNA polymerase B (II) from *Drosophila* and yeast [11,12]. The affinity-labeled region of T_7 RNA polymerase and Qβ replicase appear to be homologous to sequences in phage T_3, and the mitochondrial enzyme [8,10].

Here, we have identified the region of the B-subunit of *Sulfolobus acidocaldarius* RNA polymerase which becomes labeled in the affinity labeling reaction with AMP *o*-formylphenyl ester. It appeared that, in spite of the limited sequence

Correspondence address: G.R. Hartmann, Institut für Biochemie der Ludwig-Maximilians-Universität, Karlstr. 23, D-8000 München 2, FRG

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homology between the *E. coli* and *Sulfobolus* enzymes (Pühler, G., Lottspeich, F. and Zillig, W., submitted), these labeled regions have a similar primary structure.

2. MATERIALS AND METHODS

2.1. *Affinity labeling*

The reactive nucleotide derivative was added to a solution containing 180 μg/ml *Sulfolobus* RNA polymerase [13], 30 mM Hepes (pH 8.0), 30 mM $MgCl_2$, 30 mM NH_4Cl and 1 mM mercaptoethanol. After incubation for 15 min at 37°C, $NaBH_4$ was added (up to 5 mM), and the mixture maintained for 30 min at 0°C. Template poly[d(A-T)] was added (100 μg/ml), followed by [α-^{33}P]UTP (400 Ci/mmol, Isotop, Tashkent, USSR; up to 2.5 μM), and the mixture incubated for 20 min at 60°C.

2.2. *Limited CNBr cleavage*

The labeled enzyme was denatured by incubation with SDS (1%) for 30 min at 37°C. Subsequently, 1/20 vol. of 1 M HCl, and 1/20 vol. of 1 M CNBr were added, and the cleavage reaction allowed to continue at 20°C. At intervals, aliquots were removed, cleavage stopped by addition of 1/3 vol. stop solution (500 mM Tris-HCl, pH 9.2, 10% mercaptoethanol, 50% glycerol and 1% bromphenol blue) and then heated at 90°C for 3 min.

2.3. *Complete CNBr cleavage*

The affinity-labeled enzyme was denatured with SDS as described above. Subsequently, 1/20 vol. of 1 M HCl, and 1/10 vol. of 1 M CNBr were added. After 5 and 12 h of incubation at 29°C, additional portions of 1 M CNBr (1/10 vol. each) were added. After 24 h the reaction mixture was concentrated by repeated extraction with an equal volume of *n*-butanol. After washing with ether, 1/5 vol. stop solution (section 2.2) was added. The products of the cleavage reaction were separated by electrophoresis in a gradient (10–26%) SDS-polyacrylamide gel. After autoradiography, the region of the gel containing the main product was excised, and the peptide eluted by shaking with water (3 times for 10–15 min). The combined extracts were concentrated to 50–100 μl by extraction with *n*-butanol.

2.4. *Limited hydroxylamine cleavage of labeled enzyme or it CNBr fragment*

An equal volume of a 2 M NH_2OH-0.2 M K_2CO_3 mixture (pH 10) was added to a solution of affinity-labeled protein or its CNBr fragment. After 3–6 h incubation at 37°C, 1/5 vol. of a solution containing 0.1 M sodium acetate (pH 5.0), 5% mercaptoethanol, 5% SDS, 50% glycerol and 0.1% bromophenol blue was added and the products separated by electrophoresis.

2.5. *Enzymatic hydrolysis with endoproteinase Glu-C*

Labeled enzyme was denatured with 1% SDS as above. The detergent was then diluted to 0.5% with $NaHCO_3$ (final concentration 25 mM) and limited cleavage carried out at 37°C with endoproteinase Glu-C from *Staphylococcus aureus* V8 (Boehringer Mannheim) (final concentration 0.1 mg/ml). Hydrolysis was terminated by addition of 1/3 vol. stop solution.

2.6. *Enzymatic hydrolysis with endoproteinase Asp-N*

Limited hydrolysis was carried out as before, but the detergent was diluted to 0.5% SDS with sodium phosphate buffer, pH 7.8 (final concentration 50 mM) and endoproteinase Asp-N from *Pseudomonas fragi* (Boehringer Mannheim) (final concentration 4 μg/ml) was used as enzyme.

3. RESULTS AND DISCUSSION

3.1. *Affinity labeling*

Fig.1 shows the results of affinity labeling of *S. acidocaldarius* RNA polymerase using the *o*-formylphenyl esters of AMP (I), ADP (II) and ATP (III) [1] which were synthesized similarly to the ester of GMP [10], the *p*-formylphenyl esters of AMP (IV), ADP (V) and ATP (VI) [1] and the 4-[*N*-(β-hydroxyethyl)-*N*-methyl]benzaldehyde esters of ADP (VII) and ATP (VIII) [1] as affinity reagents.

Reagents I, III, VI and VIII are the most efficient, whereas all the rest produced only very weak labeling. It is noteworthy that the same reagents are among the most efficient in affinity labeling of RNA polymerase from *E. coli* and wheat germ [1,5].

In all of these cases, the B-subunit, which was also identified earlier [7] as the major target of reagent VI, became labeled. This subunit is immunologically [14] and structurally homologous to the second largest subunit of eukaryotic and prokaryotic RNA polymerases.

3.2. *Localisation of the affinity label*

To localise the position of the affinity label on subunit B, we used the method [2–4] for cleaving the labeled protein statistically only once per molecule at specific sites such as Asp, Glu, Met or Asn-Gly, followed by analysis of the pattern and size of labeled fragments formed by SDS gel electrophoresis. The pattern of fragments was compared with that predicted from the known amino acid sequence of the polypeptide. The polypeptide chain B of *S. acidocaldarius* (127 kDa) consists of 1126 amino acids including 63 Asp and 80 Glu residues distributed more or less evenly over the entire sequence. The 28 Met residues are clustered in three regions of the polypeptide (see scheme, fig.3C). On very brief incubation with one of the amino acid-specific endoproteinase, Glu-C or Asp-N, under conditions where most of subunit B remains uncleaved, a short labeled fragment of ≤ 37

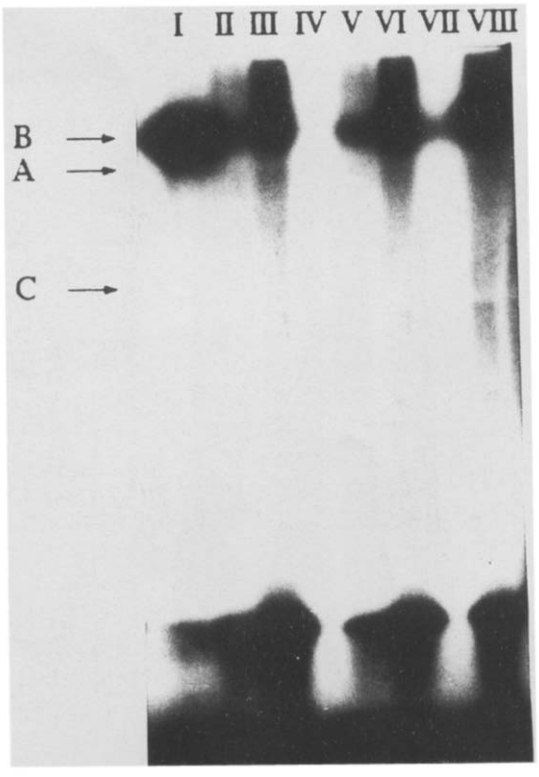

Fig.1. Affinity labeling of *Sulfolobus acidocaldarius* RNA polymerase. Labeling was carried out with 1 mM nucleotide analogue II–VIII (see text) (derivative I: 5 mM). Subsequently, samples were heated for 2 min at 100°C in the presence of 1 % SDS and 1% mercaptoethanol and subjected to electrophoresis in a 7.5–15% gradient polyacrylamide gel containing 0.1% SDS. Lane numbers in the autoradiograph correspond to the nucleotide analogue used. Positions of subunits B, A and C are indicated on the left.

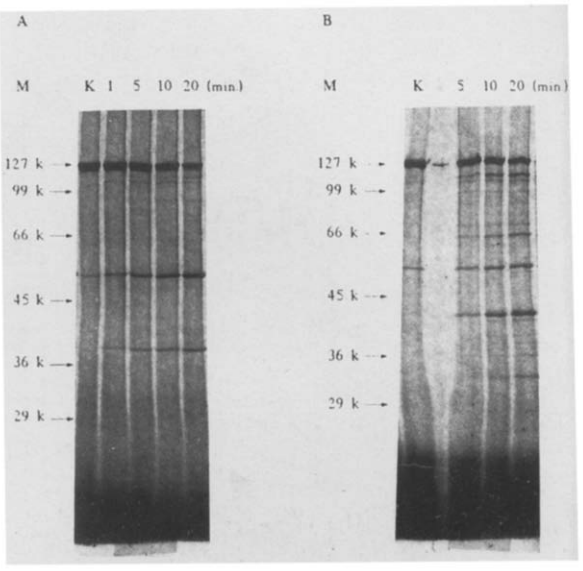

Fig.2. Kinetics of limited hydrolysis of labeled *Sulfolobus* RNA polymerase with endoproteinase Glu-C (A) or endoproteinase Asp-N (B). Time of hydrolysis is indicated above the lanes. Electrophoresis was carried out as in fig.1. M, position of marker proteins (Sigma, Taufkirchen, MW-SDS-706). The labeled band of apparent molecular mass ~ 50 kDa is present already at zero time (lane K) and is not the result of incubation with endoproteinase. For labeling the enzyme, derivative I was used.

kDa is formed in each case (fig.2). This demonstrates that the label must have become attached to the first (N-terminal) or last (C-terminal) quarter of the polypeptide chain.

Labeled subunit B was also cleaved with CNBr for a very limited time at met residues. The predicted pattern of fragments formed by a single cleavage of the polypeptide (fig.3C) is in good agreement with the series of labeled fragments observed (fig.3A) only when the label is attached C-terminally of Met^{747}. The label cannot be bound C-terminally of Met^{895}, since the predicted smaller labeled fragments are not observed. These observations suggest that the label is located between Met^{747} and Met^{895}.

Subunit B contains only 6 Asn–Gly bonds (fig.3C) which are preferentially cleaved by hydroxylamine. The characteristic pattern of fragments formed, particularly of two fragments of 37 and 32 kDa corresponding to products formed by cleavage at Asn^{793} and Asn^{842}, respectively, again shows that the label is attached to the C-terminal region (fig.3B). This conclusion is supported by the absence of a labeled product of about 17 kDa (formed by cleavage at Asn^{153}) which should be formed when the label is in the N-terminal part. The appearance of the two characteristic, smaller fragments of 37 and 32 kDa suggests that the label is located between Gly^{843} and Met^{895}. To confirm this hypothesis, we cleaved the labeled enzyme completely with CNBr. As seen in fig.4 (lane 1), such cleavage leads to a labeled product of about 17 kDa, in accordance with the suggestion that the label resides between Met^{747} and Met^{895}. In order to narrow down the position of the label, the labeled product from complete CNBr cleavage was extracted from the gel and subjected to limited NH_2OH cleavage. The presence of a labeled pep-

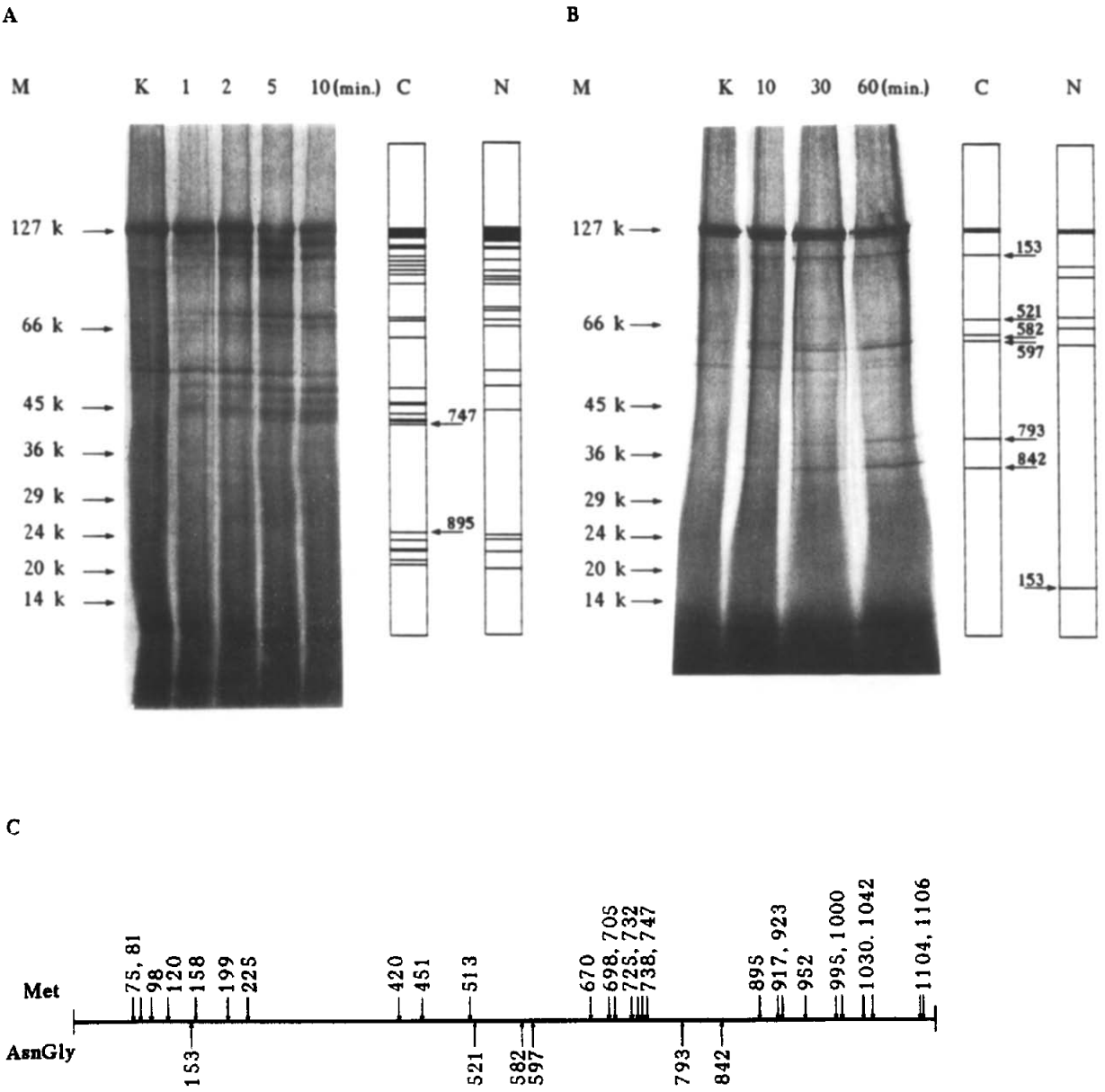

Fig.3. Kinetics of limited cleavage of labeled *Sulfolobus* RNA polymerase with CNBr or NH_2OH. Cleavage with: (A) CNBr; (B) NH_2OH. The time of cleavage is indicated above the lanes. M, position of marker proteins (as in fig.2). Lanes C and N, respectively, indicate the position of the specifically cleaved labeled fragments containing the carboxy- (or amino-) terminus of subunit B as predicted from the sequence (scheme in C) according to Pühler et al. [13] when the label is attached close to the carboxy- (or amino-) terminus. Some cleavage sites are indicated by the position of Met and Gly, respectively. Electrophoresis was carried out in a 10–20% gradient polyacrylamide gel containing 0.1% SDS. For labeling of enzyme, derivative I was used.

tide of about 5 kDa among the two labeled products of this cleavage (fig.4A, lane 2, and B) together with data on limited cleavages of the whole enzyme (fig.3), confirms the notion that the label is located between Gly^{843} and Met^{895}.

Stabilisation of the product by reduction with $NaBH_4$ indicates reaction of the derivative with the ϵ-amino group of Lys [1]. Four Lys residues (Lys^{858}, Lys^{861}, Lys^{875}, Lys^{883}) are present between Gly^{843} and Met^{895}. All belong to a region in the B-

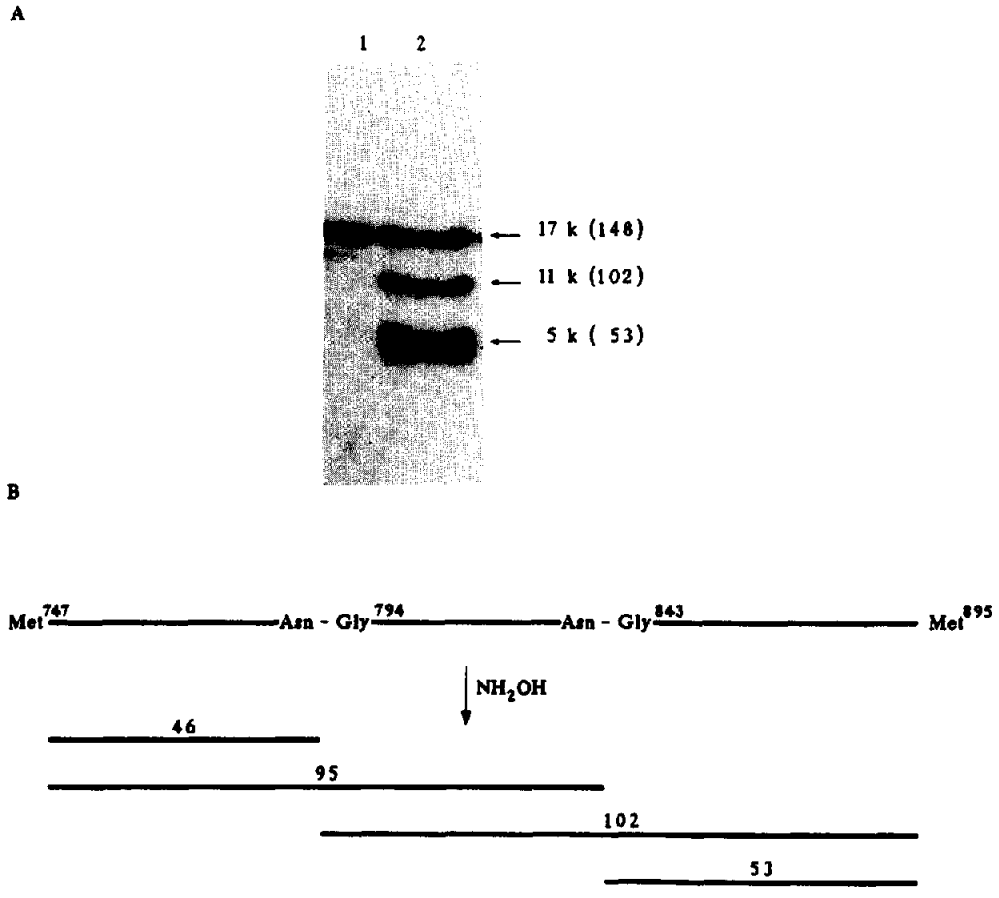

Fig.4. Limited cleavage by hydroxylamine of a labeled 17 kDa peptide obtained by complete CNBr cleavage of labeled *Sulfolobus* RNA polymerase. The labeled fragment (apparent molecular mass 17 kDa) observed in the electropherogram of a reaction mixture where RNA polymerase was completely cleaved by treatment with CNBr (lane 1) was extracted from the gel, treated with NH_2OH for 4 h and analysed by electrophoresis (lane 2). Approximate sizes of fragments are indicated. For labeling of enzyme, derivative I was used. The scheme in B indicates the cleavage pattern as predicted for the labeled Met^{747}-Met^{895} fragment.

S. ACIDOCALDARIUS	857	N K L V K V R V R D L R I P E I G D K F A T R H G Q K G V
YEAST	961	L K F V K V R V R T T K I P Q I G D K F A S R H G Q K G T
DROSOPHILA	865	Y K F C K I R V R S V R I P Q I G D K F A S R H G Q K G T
H. HALOBIUM	348	S K L A K V S V R D E R I P E L G D K F A S R H G Q K G V
MB. THERMOAUTOTROPHICUM	344	S R L A K I R V R E Q R Q P E F I G D K F A S R H G Q K G
E. COLI	1047	L K I V K V Y L A V K R R I Q P G D K M A G R H G N K G V

Fig.5. Comparison of amino acid sequence near the labeled region of RNA polymerase subunit B from *S. acidocaldarius* and subunit β from *E. coli* [15] with similar sequences present in the second largest subunit of RNA polymerase B (II) from yeast [11] and *Drosophila* [12] and in subunit B′ of the enzyme from *H. halobium* [16] and *M. thermoautotrophicum* [17]. Number indicates position of the first amino acid of the sequence.

subunit of archaebacterial RNA polymerase from *S. acidocaldarius* which is highly homologous to a region in the β-subunit of *E. coli* RNA polymerase [15] which becomes labeled by the same reagent (fig.5).

Furthermore, this region is also homologous to a sequence found in subunit B′ of RNA polymerase from the archaebacteria *Halobacterium halobium* [16] and *Methanobacterium thermoautotrophicum* [17] and in the second largest subunit of RNA polymerase B (II) from yeast [11] and *Drosophila* [12]. Sequence differences are mainly due to conservative amino acid substitutions. This suggests that the regions of the polypeptide chain which form the binding sites for the initiation substrate on the RNA polymerases have been conserved during evolution of eubacteria, archaebacteria and eukaryotes.

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