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Localisation of a Region on Subunit B' Neighboring the Active Center of the RNA Polymerase from *Methanobacterium thermoautotrophicum* Strain W

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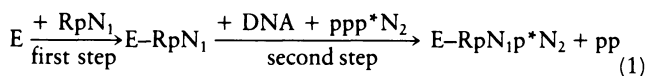
Summary

The immediate neighborhood of the active center of the DNA-directed RNA polymerase from the archaeobacterium *Methanobacterium thermoautotrophicum* is radioactively labeled upon incubation of the enzyme with one of 15 different chemically reactive nucleotide analogs which function as initiating substrate and with [α -³³P]UTP as elongating substrate in presence of poly[d(A–T)] as template. All analogs lead to the labeling of subunit B'. With analogs containing an alkyl chloride as the chemically reactive group, subunit B'' also becomes labeled. This suggests that regions of subunit B'' must also be located close to the active center. In the case of subunit B' the label is attached between tyr²⁷³ and met³⁸³. Comparison of the amino acid sequence of the labeled region with that of labeled regions in RNA polymerase from other sources indicates a strong conservation of the active center during evolution.

Key words: Archaeobacteria – *Methanobacterium thermoautotrophicum* – RNA polymerase – Active Center – Affinity labeling – Evolution

Introduction

The binding site of the initiating nucleotide in the active center of the DNA-directed RNA polymerase from archaeobacteria can be labeled highly specifically (Thomm et al., 1988) using the method designed by Grachev and Mustaev (1982). This method is based on the reaction sequence



(RpN₁, chemically reactive analog of the initiating nucleotide; E, DNA-directed RNA polymerase; ppp^{*}N₂ [α -P]radioactively labeled nucleoside triphosphate). In non-methanogenic, thermophilic archaeobacteria, such as *Sulfolobus acidocaldarius*, the largest subunit designated B becomes radioactively labeled (Thomm et al., 1988). The labeled region of this subunit contains an amino acid sequ-

ence (Grachev et al., 1989b) which is also found in highly conserved form in the labeled region of subunit B₁₅₀ of RNA polymerase B (II) from *Saccharomyces cerevisiae* (M. Riva, C. Carles, A. Sentenac, M. A. Grachev, A. A. Mustaev, E. F. Zaychikov, submitted) and in the labeled region of subunit β from *Escherichia coli* (Grachev et al., 1989a). The second and third components, B' and B'', of the RNA polymerase from methanogens and extreme halophiles are immunologically related to different parts of subunit B from sulfur-dependent archaeobacteria (Schnabel et al., 1983). When RNA polymerases of this type are used as enzyme in the labeling procedure with the 4-hydroxybenzaldehyde ester of ATP as the chemically reactive analog of the initiating nucleotide, subunit B' becomes labeled (Thomm et al., 1988). We were interested in investigating whether the labeling of subunit B' depends on the exact chemical structure of the analog of the initiating substrate. Furthermore, we wanted to localize the region of subunit

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B' to which the radioactive label becomes attached. As the amino acid sequence of subunit B' of this enzyme is known (Berghöfer et al., 1988), the mapping of the attachment site can be carried out by the method developed by Grachev et al. (1989a).

Materials and Methods

RNA polymerase from *Methanobacterium thermoautotrophicum* strain W was purified as described (Thomm et al., 1986) and the preparation divided into 15 μ l aliquots, sealed under N₂/H₂ (95 : 5) and stored in liquid nitrogen.

Synthesis of the reactive adenine nucleotide analogs. Analogs I to VI were synthesized similarly as described for the GMP analog by Grachev et al., 1989a (see also Grachev et al., 1986) with the triethylammonium salts of the corresponding adenine nucleotides. Similarly, analog VII was synthesized from ATP and vanilline (Grachev et al., 1987b). The synthesis of VIII and of the imidazolides X and XI is described by Grachev et al. (1987a). The same procedure is applied for the synthesis of the ATP derivative IX. XIII is synthesized as XII (Grachev et al., 1987a).

Synthesis of analog XIV. 5 μ l N-methylimidazole, 15 mg triphenylphosphine and 15 mg 2,2'-dipyridyldisulfide were added to 5 mg AMP (triethylammonium salt) dissolved in 50 μ l dimethylsulfoxide and the mixture incubated for 10 min at room temperature. Then 10 mg methyl-2-chloroethylamine hydrochloride and an equimolar quantity of triethylamine were added and the incubation continued for 10 min. The product was precipitated with 1 ml ether and washed several times with ether until the yellow color had disappeared. XIV was purified by thin layer chromatography on silica gel 60 F₂₅₄ plates (Merck, Darmstadt), with acetonitrile/water (4 : 1) as solvent (R_F: 0.33). The purified compound is homogeneous as judged by thin-layer chromatography on silica gel 60 F₂₅₄ plates with dioxane/concentrated ammonia/water (6 : 4 : 1) as solvent as well as by microcolumn liquid chromatography on DEAE-cellulose (Baram et al., 1983). The latter procedure indicates -1 as the charge of the compound. The UV spectrum is identical to that of AMP. Treatment with 0.01 M HCl at 37°C for 10 h produces AMP (charge -2 as revealed by microcolumn chromatography) and the corresponding amine.

Synthesis of analog XV. 10 mg ATP (triethylammonium salt) was dissolved in 60 μ l dimethylformamide, mixed with 10 mg 4-formylbenzylbromide and 5 μ l diisopropylethylamine and heated at 60°C for 15 min. The product was precipitated with 1 ml acetone containing 1% NaI, washed with acetone and purified by chromatography on silica gel 60 F₂₅₄ thin layer plates with acetonitrile/water (4 : 1) as solvent (R_F: 0.25). It gave an orange color upon staining with 2,4-dinitrophenylhydrazine. The compound was eluted with water and dried *in vacuo*. It is homogeneous as judged by thin layer chromatography described for analog XIV. The mobility is not altered after treatment with alkaline phosphatase. Microcolumn liquid chromatography on DEAE-cellulose indicates a charge of -3 of the product (Baram et al., 1983). Its UV spectrum exhibits a maximum at 258 nm and a minimum at 229 nm ($A_{290}/A_{258} = 0.12$). After reduction with 0.05 M borohydride (5 min at 20°C) the spectrum changed to a maximum at 262 nm, and a minimum at 235 nm.

Affinity labeling. The reaction mixture contained 20 mM HEPES, pH 7.9, 10 mM MgCl₂, 0.1 M KCl, 0.1–0.3 mg/ml RNA polymerase (from a freshly opened vial) and 1 mM nucleotide analog except for III (2.5 mM) and XII or ATP (0.5 mM). The mixture was incubated for 15 min at 37°C. Sodium borohydride (up to 10 mM) was then added and the mixture incubated for

another 15 min at 0°C. In some experiments analogs XII and XIII were first reduced by treatment with borohydride (5 mM reagent, 50 mM NaBH₄, 5 min, 20°C) and then immediately incubated with RNA polymerase for 30 min at 37°C followed by elongation. With IX–XI and XIV the reduction step was omitted. Here the time of incubation was 30 min at 37°C. For the elongation step 0.1 mg/ml poly[d(A-T)] (Sigma) and approximately, 1 mCi/ml [α -³³P]UTP (Isotop, Tashkent, 2000 Ci/mmol) were added and the incubation continued for 30 min at 56°C. ³³P labeled material was used as it yields sharper bands in the autoradiography due to the much lower radiation energy. Then 0.25 mg/ml RNase A (Serva, Heidelberg) was added and the incubation continued for 30–45 min at 37°C.

Electrophoretic analysis. The mixture was heated for 15 min at 56°C in presence of 1% SDS and 1% mercaptoethanol and subjected to electrophoresis in a polyacrylamide gradient (10–25%) slab gel (13 × 18 × 0.05 cm) in presence of 0.1% SDS. For the isolation of the separated labeled polypeptides the piece of gel containing the polypeptide was cut out, washed with 0.5 ml water for 2 min, then crushed and eluted two times in 0.2 ml water containing 0.1% SDS and 50 μ g/ml bovine serum albumine for 15 min. The eluates were combined, freeze-dried and dissolved in water.

Cleavage of the labeled enzyme. After RNase treatment SDS was added to 1% of the reaction mixture and the incubation continued for 30 min at 37°C or 15 min at 56°C.

Limited cleavage with H₂NOH was carried out as described (Grachev et al., 1989b). The control was performed identically but without H₂NOH (2.5 h incubation).

Limited and long time (22 h) cleavage with BrCN was carried out as described (Grachev et al., 1989b). For the control the labeled enzyme was also incubated at pH 1–2 but without addition of BrCN. Here the incubation period was identical with that of the longest incubation with BrCN. Under the acid conditions cleavage at asp-pro bonds (55/56; 127/128) may occur.

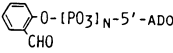
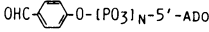
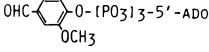
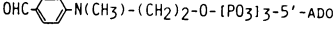
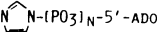
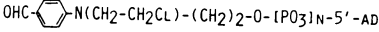
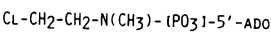
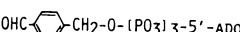
Cleavage with bromine-water was carried out similarly as described (Maximova et al., 1989). The solution of the isolated BrCN fragment from the B' subunit was acidified with HCl to pH 1–2 and 1/8 volume of a 1 : 10 diluted saturated solution of bromine in water was added. After 5 min incubation at room temperature, the cleavage was stopped by addition of 1/4 volume of a solution containing 5% mercaptoethanol, 0.5 M triethanolamine, pH 9, 0.2% bromophenol blue, 50% glycerol. The control was treated identically but without bromine-water. A gel gradient containing 20–30% acrylamide was used for electrophoretic analysis in this experiment.

Results

Influence of the substrate analog

The highly specific labeling of the RNA polymerase from *Methanobacterium thermoautotrophicum* strain W was carried out with a large variety of chemically reactive analogs of the initiating nucleotide (Table 1) in presence of poly[d(A-T)] as template and [α -³³P]UTP as second substrate. These analogs differed from each other in the distance of the chemically reactive group from the nucleoside monophosphate moiety or in the nature of the chemically reactive group (either an aldehyde, imidazole or alkylchloride group). Two of them (XII and XIII) were bifunctional since they carried two different reactive groups. With all derivatives at least a weak labeling of the second largest subunit B' was achieved similar to that shown pre-

Table 1. Chemically reactive nucleotide analogs used in the highly specific labeling procedure

SUBSTRATE ANALOG	ABBREVIATION
	I: N=3; II: N=2; III: N=1
	IV: N=3; V: N=2; VI: N=1
	VII
	VIII
	IX: N=3; X: N=2; XI: N=1
	XII: N=3; XIII: N=1
	XIV
	XV

viously (Thomm et al., 1988) with analog IV (data not shown). However, with analog XIV which carries an alkylating group, the third largest subunit B'' in addition to B' became labeled (Fig. 1; A).

The labelling of subunit B'' is dependent on the presence of template and substrate analog and disappears after incubation with proteinase K (Fig. 1; B, C, D). The same has been observed in all previous labeling experiments using this method (Hartmann et al., 1988). A strong labeling of subunit B'' is also observed when the reduced form of analogs XII and XIII (obtained by NaBH₄ reduction of the aldehyde group with concomitant activation of the alkylating activity of the ethylchloride group) is incubated with the enzyme (Fig. 1; E, G). When the aldehyde group of XII and XIII is not reduced prior to the reaction with the enzyme the labeling of subunit B' is increased and that of B'' strongly decreased (Fig. 1; F, H.). It suggests that the labeling of subunit B'' is mainly due to alkylation whereas subunit B' is available to both the ethylchloride and to the aldehyde group of the affinity reagents.

Mapping of the attachment site for analog III

When substrate analog III is used in the labeling procedure, exclusively subunit B' consisting of 604 amino acids

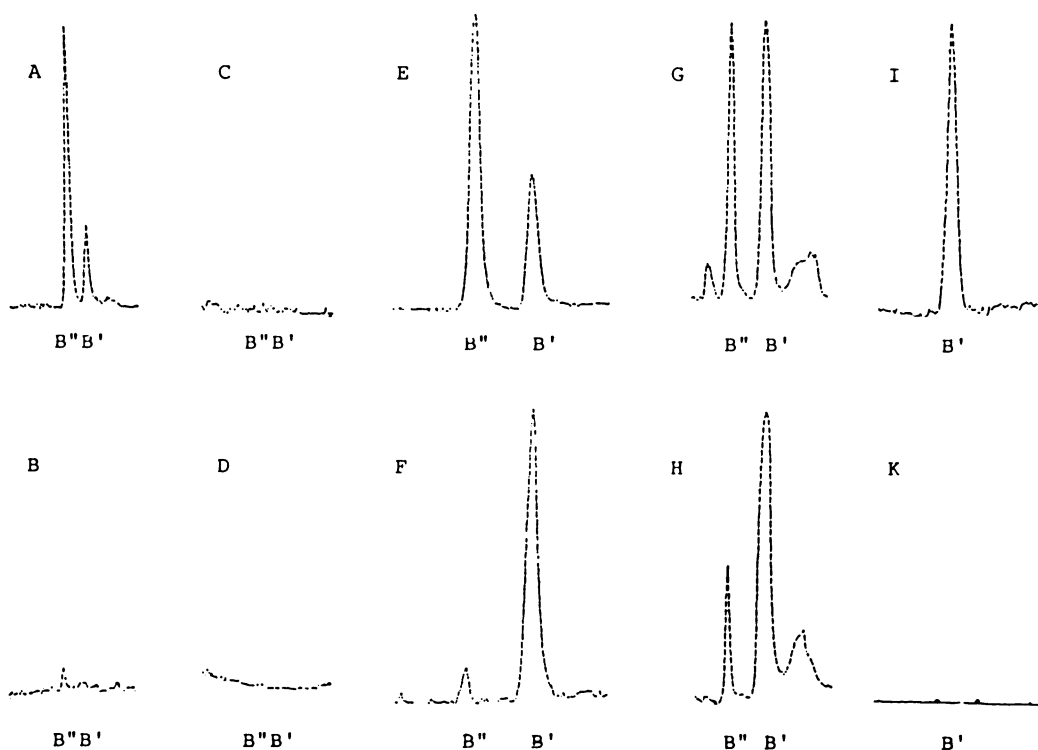


Fig. 1. Affinity labeling of *Methanobacterium thermoautotrophicum* RNA polymerase with different nucleotide analogs. The densitometric tracing of the autoradiographies of the gel electrophoretic analyses is shown. B' and B'' indicate the position of the subunits. A-D: alkylating analog XIV; A: complete; B: without template; C: without analog; D: complete with subsequent 15 min digestion by proteinase K (Merck, Darmstadt); E, F: bifunctional analog XII, borohydride reduction before or after incubation with the enzyme. G, H: bifunctional analog XIII, reduction before or after incubation with the enzyme. I: analog III, complete with reduction by NaBH₄; K: complete without analog but with 1 mM ATP.

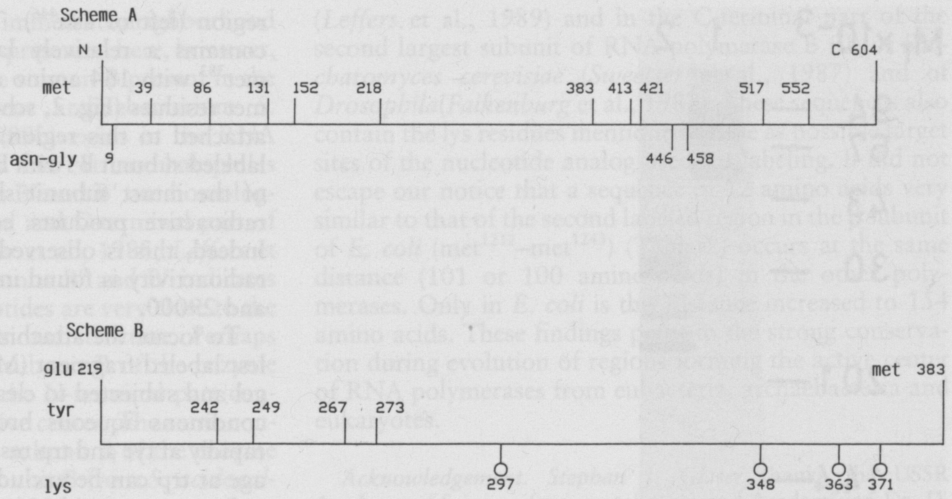


Fig. 2. *Scheme A*. Position of met residues and asn-gly peptide bonds in the amino acid sequence of subunit B' from *M. thermoautotrophicum* (Berghöfer et al., 1988). Met-ser (at 112, 211, 405) and met-thr (156, 482) have been omitted for clarity. *Scheme B*. Position of tyr between glu 219 and met 383. Position of lys between tyr 273 and met 383.

(M_r 68000), becomes labeled (Fig. 1; I, K). The polypeptide contains three asn-gly bonds (at the asn positions 9, 446 and 458; Fig. 2, scheme A) (Berghöfer et al., 1988). They may be cleaved rather specifically by hydroxylamine at pH 10 (Bornstein et al., 1977) yielding two large polypeptides with M_r 51000 and 52000 and two small polypeptides with 18000 and 17000. Indeed, a limited cleavage leads to the appearance of two adjacent labeled bands with $M_{r, app}$ of approximately 52000 and 55000 (Fig. 3, lane 2) in addition to the radioactive zones already present in the control (Fig. 3; lane 1). No radioactive bands with $M_{r, app}$ between 17000 and 18000 can be seen (corresponding to the fragments gly⁴⁴⁷ or ⁴⁵⁹-C-terminus⁶⁰⁴). This clearly demonstrates that the label must be attached to the region left of asn⁴⁴⁶ (see scheme A).

BrCN cleaves polypeptides rapidly at met residues except when they are followed by thr and ser (Schroeder et al., 1969). Following incubation of the labeled enzyme with BrCN under conditions where only a small part of subunit B' is cleaved (single hit conditions: in the average less than one cleavage per polypeptide chain (Grachev et al., 1989a), several labeled fragments appear in addition to those present in the control (Fig. 4, lanes 1-4). The smallest fragment formed has a size of about 45000. Subunit B' contains 10 met residues not followed by thr or ser which are distributed rather evenly over the polypeptide chain, particularly over the N- and C-terminal region (Fig. 2, scheme A). The absence of products smaller than 45000 is evidence that the radioactive label is not attached to the two terminal regions but must be located in the middle

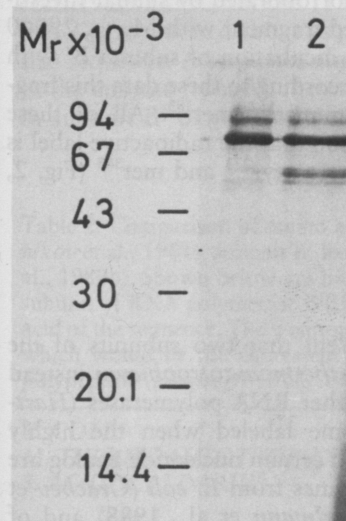


Fig. 3. Limited cleavage of labeled *M. thermoautotrophicum* RNA polymerase with H_2NOH (lane 2). Control (lane 1): incubation in absence of H_2NOH .

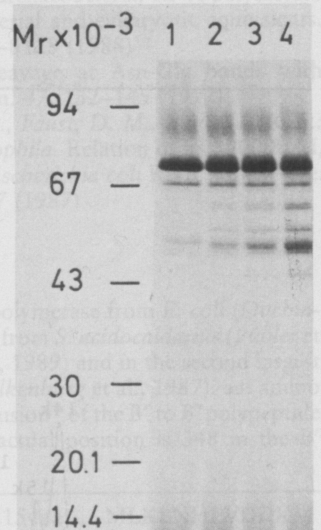


Fig. 4. Limited cleavage of labeled *M. thermoautotrophicum* RNA polymerase with BrCN. Lane 1: control, lanes 2, 3, 4: 1, 2 and 5 min incubation with BrCN, respectively. The formation of the labeled bands with $M_{r, app}$ 63000 and 55000 in the control is due to cleavage at the asp-pro bonds at positions 55 and 127 under the prevailing acid conditions.

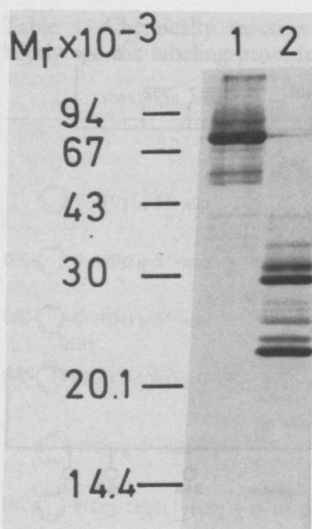


Fig. 5. Long term cleavage of labeled *M. thermoautotrophicum* RNA polymerase with BrCN. 22 h incubation without (lane 1) and with (lane 2) BrCN.

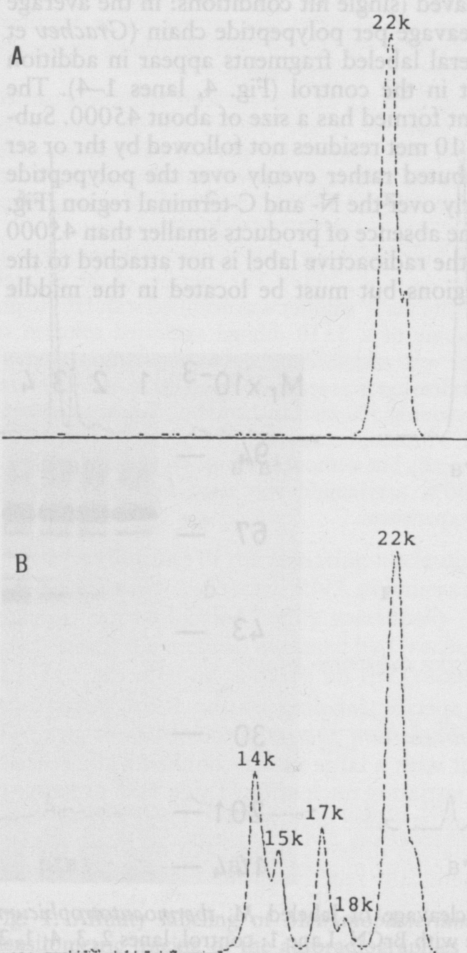


Fig. 6. Limited cleavage by bromine of the labeled 22000 fragment obtained after the long time incubation of the labeled enzyme with BrCN. A: control B: 5 min incubation.

region (left of asn^{446}). The middle region of subunit B' contains a relatively large region between met^{218} and met^{383} with 164 amino acids (M_r 18700) which is free of met residues (Fig. 2, scheme A). If the label ($M_r \sim 800$) is attached to this region, a longer incubation time of the labeled subunit B' with BrCN leading to the disappearance of the intact subunit should result in the formation of radioactive products equal to or larger than 19500. Indeed, this is observed (Fig. 5, lane 1, 2). Most of the radioactivity is found in fragments with $M_{r, \text{app}}$ of 22000 and 29000.

To locate the attachment site more precisely, the smallest labeled fragment ($M_{r, \text{app}}$ 22000) was eluted from the gel and subjected to cleavage with bromine. Under acidic conditions aqueous bromine cleaves polypeptides most rapidly at tyr and trp residues (Spande et al., 1970). Cleavage at trp can be excluded here since subunit B' contains only one trp residue (at position 88) far away from the middle region. At a low concentration of bromine, only a limited cleavage occurs (condition of single hit, Grachev et al., 1989a) since the main part of the BrCN fragment present remains intact (Fig. 6). Four degradation products with different intensities and characteristic mobilities can be detected ($M_{r, \text{app}}$ 18000, 17000, 15000, 14000). None is smaller than 14000. This suggests that the label is attached to a region of that size which is free of tyr. There exists only one region of this size bordered by either tyr only or tyr and met in the middle part of subunit B' which fulfills these conditions. This is the region between tyr^{273} and met^{383} (scheme B). Since three more tyr residues (at positions 267, 249, 242) occur before the next met residue at position 218, a single hit cleavage (Grachev et al., 1989a), by bromine at these residues should result in the formation of three additional radioactive fragments with the size of 14000, 16000 and 17000. This characteristic pattern of radioactive fragments was observed (Fig. 6). Cleavage at tyr^{242} seems to be the rarest since the intensity of this product is the smallest.

These observations are corroborated by similar investigations of the eluted labeled fragment with $M_{r, \text{app}}$ 29000 obtained after a long term incubation of subunit B' with BrCN (data not shown). According to these data this fragment comprises the region met^{152} – met^{383} . All of these results support the conclusion that the radioactive label is attached to the region between tyr^{273} and met^{383} (Fig. 2, scheme B).

Discussion

It is not without precedent that two subunits of the RNA polymerase from *M. thermoautotrophicum* instead of only one, as in many other RNA polymerases (Hartmann et al., 1988), become labeled when the highly specific labeling method and certain nucleotide analog are applied. In case of the enzymes from *E. coli* (Grachev et al., 1987a), *Anabaena* (Hartmann et al., 1988) and of DNA primase from yeast (Foiani et al., 1988), two subunits (beta and sigma or the primase subunits p48 and p58, respectively) also become labeled. This suggests that

more than one subunit forms the immediate neighborhood of the active center. The example presented here, however, is of particular interest. The large subunit B present in the enzyme of the sulfur dependent archaebacterium *S. acidocaldarius* (Pühler et al., 1989b) exists in the RNA polymerases of the methanogens and extreme halophiles in a split form. The two subunits B'' and B' are homologous in sequence to the N-terminal and C-terminal part of subunit B, respectively (Berghöfer et al., 1988; Leffers et al., 1989). Labeling of both subunits B'' and B' indicates that regions of these two polypeptides are very close to the active center in the three-dimensional structure. Perhaps not only the C-terminal part of subunit B of the enzyme from *S. acidocaldarius* but also the N-terminal part contains regions neighboring the active center. The same concept may be applied similarly to subunit β of the enzyme from *E. coli* which resembles subunit B from *S. acidocaldarius* in sequence (Pühler et al., 1989a).

In the case of the enzyme from *E. coli* (Ovchinnikov et al., 1981), the label becomes attached to either one of two regions (ile¹⁰³⁶-met¹⁰⁶⁶ or met¹²³²-met¹²⁴³) (Grachev et al., 1989a) of subunit β . In subunit B from *S. acidocaldarius* the label is bound to the region between gly⁸⁴³ and met⁸⁹⁵ (Grachev et al., 1989b) whereas in subunit B' of the enzyme from *M. thermoautotrophicum* the label is fixed to the region between tyr²⁷³ and met³⁸³. The attachment site of a nucleotide analog with an aldehyde group is most probably a lys residue (Grachev et al., 1987a). In the labeled region four lys residues occur at the position 297, 348, 363 and 371. Comparison of the amino acid sequences of this labeled region with the labeled region of subunit B of the enzyme from *S. acidocaldarius* (Grachev et al., 1989b) and of subunit β from *E. coli* (Grachev et al., 1989a) immediately reveals several regions with high sequence similarity. Particularly striking is a stretch of 16 amino acids in which in *S. acidocaldarius* only a single ser residue is replaced by thr. Even in the less closely related *E. coli* enzyme only six amino acids are replaced by rather conservative substitutions (Table 2). The lys residues 363 and 371 mentioned above as possible targets are present in this sequence. A sequence almost identical to that labeled in the enzyme from *M. thermoautotrophicum* also occurs in subunit B' of the enzyme of *Halobacterium halobium*

(Leffers et al., 1989) and in the C-terminal part of the second largest subunit of RNA polymerase B (II) of *Saccharomyces cerevisiae* (Sweetser et al., 1987) and of *Drosophila* (Falkenburg et al., 1987). These sequences also contain the lys residues mentioned above as possible target sites of the nucleotide analog used for labeling. It did not escape our notice that a sequence of 12 amino acids very similar to that of the second labeled region in the β subunit of *E. coli* (met¹²³²-met¹²⁴³) (Table 2) occurs at the same distance (101 or 100 amino acids) in the other polymerases. Only in *E. coli* is this distance increased to 154 amino acids. These findings point to the strong conservation during evolution of regions forming the active center of RNA polymerases from eubacteria, archaebacteria and eukaryotes.

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Table 2. Comparison of amino acid sequences within the labeled regions of the β -subunit of RNA polymerase from *E. coli* (Ovchinnikov et al., 1981), subunit B' from *M. thermoautotrophicum* (Berghöfer et al., 1988) and subunit B from *S. acidocaldarius* (Pühler et al., 1989b). Shown below are homologous sequences in subunit B' from *H. halobium* (Leffers et al., 1989) and in the second largest subunit of RNA polymerase B (II) from *S. cerevisiae* (Sweetser et al., 1987) and *D. melanogaster* (Falkenburg et al., 1987). aa: amino acid of the sequence. The position given for *M. thermoautotrophicum* is for an artificial head to tail "fusion" of the B' to B'' polypeptide which would be the equivalent of the subunit in organisms with an uncleaved B subunit. The actual position is 348 in the B' polypeptide (Berghöfer et al., 1988).

<i>E. COLI</i>	(1051) KVYLAVKRRIQ-PGDKMAGRHHGNGKGVIS ... 154aa ... MLKLNHLVDDKM
<i>M. THERMOAUTOTROPHICUM</i>	(863) KIRVREQRQPEFIGDKFASRHGQKGVVG ... 100aa ... YQKLHHMTTDRI
<i>S. ACIDOCALDARIUS</i>	(861) KVRVRDLRIPE-IGDKFATRHHGQKGVVG ... 101aa ... YQKLHHMVADKM
<i>H. HALOBIUM</i>	(875) KVSVRDERIPE-LGDKFASRHGQKGVVG ... 100aa ... YHKLHYHMVSNKL
<i>S. CEREVISIAE II</i>	(965) KVRVRTTKIPQ-IGDKFASRHGQKGTIG ... 100aa ... YQRLRHMVDDKI
<i>DROSOPHILA MELANOGASTER II</i>	(869) KIRVRSVRIPQ-IGDKFASRHGQKGTTCG ... 100aa ... YQRLKHMVDDKI

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