Active Site Labeling of the RNA Polymerases A, B, and C from Yeast*

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Michel Riva, Anton R. Schaffner§, André Sentenac, Guido R. Hartmanng, Arkady A. Mustaev, Evgeny F. Zaychikov, and Mikhail A. Grachev

From the Service de Biochimie, Centre d'Études Nucléaires de Saclay, 91191 Gif-sur-Yvette, France, Institut für Biochemie, Ludwig-Maximilians-Universität, D-8000 München 2, Federal Republic of Germany, and Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Union of Soviet Socialist Republics

Academy of Sciences, 630090 Novosibirsk, Union of Soviet Socialist Republics

RNA polymerases A, B, and C from yeast were modified by reaction with 4-formylphenyl-$\gamma$-ester of ATP as priming nucleotide followed by reduction with NaBH$_4$. Upon phosphodiester bond formation with [$\alpha$-$\text{32P}$]UTP, only the second largest subunit, A$_{125}$, B$_{150}$, or C$_{128}$, was labeled in a template-dependent reaction. This indicates that these polypeptide chains are functionally homologous. The product covalently bound to A, B, and C subunits was observed when the reduction step stabilized the binding of the priming nucleotide was carried out after limited chain elongation. These results illustrate the conservative evolution of the active site of eukaryotic RNA polymerases.

Labeling of the active site of enzymes by chemically reactive substrate analogues is a widely used technique. To discriminate between unspecific association and specific binding to the active center of RNA polymerase a novel methodology has been devised by Smirnov et al. (1981). With new classes of nucleotide derivatives (Knorre, 1983; Grachev et al., 1986b, 1987), this approach has been applied to label the nucleotide binding site of RNA polymerase from Escherichia coli (Grachev et al., 1987) as well as that of the eukaryotic form of enzyme B from wheat germ (Grachev et al., 1986a). These results prompted us to identify the nucleotide binding subunit of the three forms of yeast nuclear RNA polymerases. These enzymes are well characterized at the molecular level (Sen-tenac and Hall, 1982); several of their structural genes have been cloned (Young and Davis, 1983; Allison et al., 1985; Riva et al., 1986) but little is known about the role of their multiple polypeptide components in RNA synthesis or about the functional homology of immunologically related polypeptides (Sentenac, 1985).

In the experiments described in this communication, RNA polymerase A, B, and C from yeast were first reacted with a nonradioactive derivative of ATP esterified at its terminal phosphate group with 4-formylphenol (Grachev et al., 1986b). The aldehyde group is expected to react reversibly (Venegas et al., 1973) with nucleophilic amino groups of the protein, and the Schiff base formed can be stabilized by reduction with sodium borohydride. Specific labeling occurs when active RNA polymerase molecules, containing in their active center the nucleotide derivative in the correct configuration, catalyze phosphodiester bond formation with a radioactive ribonucleoside triphosphate. The product of the enzymatic reaction was found attached exclusively to the second largest subunit of A, B, and C RNA polymerases.

RESULTS

Fig. 1 shows the affinity labeling of yeast RNA polymerases A, B, and C and the identification of the labeled subunit by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In each case, a single band was labeled which corresponds to the second largest subunit B$_{150}$, A$_{125}$, or C$_{128}$, respectively (Fig. 1, lanes 1). Several controls demonstrated the selectivity of this affinity labeling. No discrete labeled band was detected when the ATP derivative was omitted or replaced by ATP or in the absence of template. Labeling of the B$_{150}$ subunit was inhibited by $\alpha$-amanitin (Fig. 2). The $\alpha$-amanitin sensitivity of the labeling reaction was the same as that exhibited by yeast RNA polymerase B in a standard RNA synthesis reaction (i.e. 50 and 90% inhibition at 1 and 20 $\mu$g/ml of toxin, respectively). Labeling of the A$_{125}$ subunit of RNA polymerase A was strongly reduced by $\alpha$-amanitin at high concentrations (0.4–2 $\mu$g/ml) (Fig. 1, lane 8) in keeping with the weak sensitivity of enzyme A to this toxin (Huet et al., 1975). These observations clearly show that this affinity labeling is not due to a nonenzymatic radiolabeling of protein (Schmidt and Hanaa, 1986).

The presence of 0.5 mM ATP during incubation inhibited the labeling of enzyme A but to a lesser extent that of B$_{150}$ or C$_{128}$ (Fig. 1, lanes 7). A 10-fold excess of ATP was required to diminish the labeling of enzyme B (Fig. 2) or enzyme C (not shown). When comparing the three enzymes we found that enzyme A reacted most efficiently with the 4-formylphenyl ester of ATP. However, the labeling of enzyme A was strongly increased and that of enzyme B decreased when using the 2-formylphenyl ester of ATP (not shown). With all derivatives tested, the second largest subunit of the three enzymes was always the only labeled polypeptide.

The radioactivity incorporated was resistant to treatments of the transcription complex by DNase I and RNase A but was sensitive to proteinase K. For characterization of the labeled RNA product, the radioactive subunit B$_{150}$ was eluted

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¶ Recipient of support from Deutsche Forschungsgemeinschaft Grant SFB 304 and the Fonds der Chemischen Industrie. To whom correspondence should be addressed: Institut für Biochemie der Universität, Karlstr. 25, D-8000 München 2, Federal Republic of Germany.

1 The polypeptide components of the RNA polymerases are designated by letter indicating the enzyme form from which it derives and a subscript corresponding to its molecular mass $\times 10^3$.
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from the gel (Grachev et al., 1986a) and degraded with proteinase K in 10 mM NH₄HCO₃, pH 7.8, for 3 h at 37 °C. After inactivation of proteinase K by phenylmethylsulfonyl fluoride, the product was cleaved by acid pyrophosphatase from tobacco and phosphatase, similar to the procedure described by D'Allessio (1982). Upon chromatographic analysis (Mosig et al., 1985), 44% of the total radioactivity migrated with the same rate as the trinucleotide ApApU in four different separating systems; 27% exhibited the mobility of authentic ApU. Its sequence was confirmed by specific enzymatic hydrolysis (Mosig et al., 1985). 9% migrated like free phosphate, and

2 The abbreviation used is: Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

20% of the radioactivity remained at the origin. The formation of the trinucleotide product indicates that yeast enzyme B is capable of catalyzing the formation of two phosphodiester bonds while having the priming nucleotide covalently attached to the active site.

The stable fixation of the radioactive product to RNA polymerase may also be carried out after the enzymatic reaction (Grachev and Zaychikov, 1981). Under these conditions, both large subunits became labeled upon incubation of the ATP derivative with [α-32P]UTP and various combinations of nucleoside triphosphates (i.e., GTP, ATP, and CTP) (Fig. 3). One migrated with the rate of Biso and one slightly above Biso, as seen by superimposing the stained gel and the autoradiography. Both bands were sensitive to pro-
was α-amanitin-sensitive and was not observed when the ATP derivative, the template, or the reduction step were omitted or when the Schiff base was reduced prior to the addition of the nucleotides (data not shown). These results show that after start the nascent RNA chain is not attached to any of the smaller subunits but only to the two largest ones.

**DISCUSSION**

The first important conclusion which can be derived from the affinity labeling of the three forms of yeast RNA polymerase is that in each case the second largest subunit, i.e. B150, A135, or C129, interacts with the priming modified nucleoside triphosphate. Since the labeling procedure requires the catalytic activity of the enzymes, it achieves the unambiguous labeling of the active site. Here the definition of active site also includes a mobile part of the polypeptide backbone which can reach close enough to the amino acids involved in the catalytic process. The second conclusion is that the second largest subunit of enzymes A, B, and C are functionally homologous. They are also homologous in function to the subunit B140 from wheat germ (Grachev et al., 1986a) and to the β-subunit of E. coli RNA polymerase (Smirnov et al., 1981; Grachev et al., 1987). This conclusion is in agreement with previous immunological studies that revealed cross-reaction between yeast B150, A135, and C129 (Sentenac and Hall, 1985) and between yeast B126 and wheat germ B140 (Huet et al., 1982). The structural and functional homology of the second largest subunit of yeast RNA polymerases confirms the previous contention that the three nuclear RNA polymerases must be derived from a common ancestral enzyme after triplication of the genes for the large subunits (Sentenac and Hall, 1982).

Analysis of the oligoribonucleotides covalently attached to RNA polymerase B revealed the presence of the dinucleotide ApU and a large proportion of the trinucleotide ApApU. The elongation of the bound priming nucleotide by an adenine nucleotide in the absence of added ATP may be due to a trace impurity of ATP present in the preparation of γ-substituted ribonucleoside triphosphate. Furthermore, it has been observed that the modified nucleoside triphosphates can serve weakly as substrates for the RNA polymerase from E. coli (Grachev et al., 1980) and for the yeast enzyme B in a poly[d(A-T)]-directed reaction (data not shown). The synthesis of the trinucleotide raises the question of the topology of the polymerase active site. If the attached dinucleotide remains locked in the active site the binding of the third nucleotide and the formation of a second phosphodiester bond implies the existence of a second, functionally equivalent active site (Panka and Dennis, 1985). Alternatively, the translocation of the active center could possibly occur if there is sufficient flexibility of the polypeptide backbone and/or of the spacer attaching the priming nucleotide.

In view of the synthesis of a sizeable proportion of ApU covalently bound to subunit B150, it is interesting to note that α-amanitin suppressed more than 90% this labeling. The same was found for the enzyme B from wheat germ (Grachev et al., 1986a). This indicates that the toxin inhibits the formation of the first phosphodiester bond and not only the translocation step as has been proposed for the enzyme from calf thymus (Vaisius and Wieland, 1982).

Since the largest subunit of eukaryotic RNA polymerase B has been associated with the sensitivity to α-amanitin (Greenleaf, 1983) as well as with the process of chain elongation (Coulter and Greenleaf, 1985; Ruet et al., 1980), it is conceivable that both large subunits contribute to the active site in a broad sense (i.e., including initiation and translocation). This notion is supported by our observation that after limited

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**Fig. 2. Inhibition of affinity labeling of the B150 subunit by a-amanitin (α-Ama.) or ATP.** Left, RNA polymerase B was modified with the ATP derivative as before, and then varying amounts of α-amanitin were added with template and [α-32P]UTP. For quantitative evaluation of the extent of inhibition, the radioactivity incorporated in the bands was determined by counting. Right, modification and labeling of enzyme B was achieved with or without ATP.

**Fig. 3. Affinity labeling of RNA polymerases A and B when reduction was carried out after limited RNA chain elongation.** Left (B), yeast RNA polymerase B (2.1 μg) was incubated for 15 min at 30 °C with the ATP derivative, and then for 30 min in the presence of [α-32P]UTP, GTP (1 μM each), and DNA template. Then the reduction with NaBH₄ was carried out as described in Fig. 1. Lane 1, sample directly loaded on the gel after incubation; lane 2, proteinase K treatment; lane 3, DNase I treatment; lane 4, RNase A treatment; lane 5, reduction step prior to incubation with [α-32P]UTP (0.1 μM) and template. Right (A), similar experiment with RNA polymerase A in the presence of template and various combinations of nucleotides; lane 6, [α-32P]UTP + GTP (1 μM each); lane 7, [α-32P]UTP (0.1 μM), CTP + GTP (1 μM each); lane 8, reduction step prior to incubation with [α-32P]UTP (0.1 μM).
elongation the largest subunit also becomes labeled. The interaction of the largest subunit with the nascent RNA chains is also suggested by its photoaffinity labeling (Bartholomew et al., 1986).

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