Highly selective affinity labelling of RNA polymerase B (II) from wheat germ

M.A. Grachev, G.R. Hartmann*+, T.G. Maximova, A.A. Mustaev, A.R. Schäffner*, H. Sieber* and E.F. Zaychikov

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the USSR Academy of Sciences, Novosibirsk 90, USSR and **Institut für Biochemie der Ludwig-Maximilians-Universität, D-8000 München 2, FRG*

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DNA-dependent RNA polymerase B (II) from wheat germ was modified by incubation with 4-[*N*-(β-hydroxyethyl)-*N*-methyl]benzaldehyde esters of AMP, ADP or ATP, followed by reduction with $NaBH_4$. Reaction of the modified enzyme with [α-^{32}P]UTP in the presence of various DNA templates led to a highly selective affinity labelling of the subunit with M_r 140 000 by covalently linked ApU. Labelling was inhibited by 1 μg/ml α-amanitin.

RNA polymerase B (II) *(Wheat germ)* *Affinity labeling*
4-[N-(β-Hydroxyethyl)-N-methylamino]benzaldehyde *Dinucleotide synthesis*

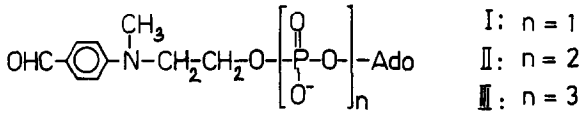
1. INTRODUCTION

The functional role of the subunits of eukaryotic RNA polymerase B (II) (EC 2.7.7.6), in particular of the subunits constituting the active center, has not yet been clarified [1]. This problem can be addressed by using reactive derivatives of nucleotides for affinity labelling studies and was successfully applied for the study of the bacterial RNA polymerase from *Escherichia coli* (review [2]). A new method for highly selective affinity labelling of the active center of RNA polymerase exploits the catalytic activity of the modified enzyme to synthesise dinucleotides according to eqn 1 [3,4]:

$$XpA + UTP \longrightarrow XpApU + PP \qquad (1)$$

where X = enzyme covalently linked to the priming nucleotide pA.

This approach employs reactive nucleotide derivatives such as cyclic adenosine 5′-trimetaphosphate, which are first bound covalently to the active center and to other sites of the RNA polymerase-DNA complex. When a radioactively labelled substrate is added it will react with the priming substrate in the active center of the enzyme to yield labelled dinucleotide covalently attached to the protein. Reactive nucleotide derivatives bound to other sites will not become labelled. The enzyme-DNA complex can subsequently be dissociated with detergents. Only the subunit to which the dinucleotide was bound covalently will then be labelled. Apart from cyclic adenosine 5′-trimetaphosphate other reactive nucleotide derivatives such as I–III may also be used.



I: n = 1
II: n = 2
III: n = 3

These derivatives contain a reactive aldehyde group which can react easily with free amino groups of the polypeptide chain, forming Schiff bases which can be stabilised by mild reduction with $NaBH_4$. We have described previously that RNA polymerase B (II) from wheat germ also

+ To whom correspondence should be addressed

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catalyses dinucleotide synthesis [5] and have therefore employed the new method for specifically affinity labelling of this eukaryotic enzyme. This communication shows that the subunit with M_r 140000 of wheat germ RNA polymerase B (II) is specifically labelled during conditions of dinucleotide synthesis when adenine nucleotide derivatives I–III are used as priming substrate and [α-^{32}P]UTP as second substrate.

2. MATERIALS AND METHODS

2.1. *RNA polymerase*

The enzyme from wheat germ was purified as in [5]. Analysis of the preparation by electrophoresis in the presence of dodecyl sulfate revealed the standard subunit pattern [6]. The largest subunit appeared to be partially degraded to polypeptides with $M_r \geqslant 160000$.

2.2. *Synthesis of adenine nucleotide derivatives*

40 μl 2,2-dimethoxypropane, 40 μl 1,3-propanediol and 5 μl F_3C-COOH were added to 20 mg 4-[*N*-(β-chloroethyl)-*N*-methylamino]benzaldehyde in 100 μl dimethyl sulfoxide. The mixture was kept at 95°C for 2 min, hereby converting the aldehyde group into the acetal, which increases the reactivity of the β-chloroethylamino group in the subsequent alkylation reaction. The mixture was neutralized with 20 μl triethylamine and diluted with water to 1.5 ml. The precipitate formed was pelleted by centrifugation, washed with 1 ml water and dissolved in 0.2 ml dimethylformamide. 10 μl triethylamine and 10 mg of one of the nucleotides AMP, ADP or ATP (as triethylammonium salts) dissolved in 0.1 ml water were added, and the mixture was then kept at 56°C for 3 h. Under these conditions alkylation occurs at the terminal phosphate group. Excess alkylating agent was removed by extracting 3 times with 1 ml peroxide-free ether. The reaction mixture was then acidified with acetic acid to pH 5 and incubated at 56°C for 20 min to regenerate the aldehyde group. The product was isolated by chromatography on Merck silica gel thin-layer plates (no.5738) with a mixture of acetonitrile and water (4:1) as solvent. Spots were visualised under UV light. The product was eluted with water (3 times 1 ml), concentrated by evaporation to 10 mM and kept at 0°C. The yield was up to 80%. The homogeneity of the product was confirmed by high-performance liquid chromatography on a Merck LiChrosorb-NH_2 column with a gradient of potassium phosphate, pH 7.5, in 7 M urea. The product showed two absorption maxima at 258 and 350 nm. The latter disappeared upon reduction with 0.01 M $NaBH_4$ for 5 min at 25°C. The net negative charge of the product is one unit smaller than that of the starting nucleotide as shown by micro-ion-exchange chromatography at pH 7.5 [4] (to be published).

2.3. *Affinity labelling*

1 μl of one of the adenine nucleotide derivatives I, II or III was added to 8 μl of a solution containing 2–4 μg wheat germ RNA polymerase B (II) and DNA template such as plasmid pUC18 (0.6 μg) or poly[d(A-T)$_{100-500}$] (0.07 μg), 11 mM Hepes-NaOH, pH 7.9, 0.9 mM $MnCl_2$, 66 mM KCl, 0.3 mM dithiothreitol and 5% glycerol. This mixture was incubated for 30 min at 37°C. Then 1 μl of 100 mM $NaBH_4$ was added and the incubation continued for 30 min. Subsequently 1 μl of 6 μM [α-^{32}P]UTP (spec. act. 400 or 3000 Ci/mmol) was added and the mixture kept at 37°C for another 30 min. The enzyme was then dissociated into subunits by adding 2.5 μl of 5% dodecyl sulfate containing 5% mercaptoethanol, 50% glycerol and 0.5% bromophenol blue and heating to 95°C for 2 min. Subunits were separated by electrophoresis at 75 V for 14 h in gels with a linear gradient of 3–18% acrylamide in the presence of 0.1% dodecyl sulfate. The proteins were stained with Coomassie brilliant blue G-250, and the dried gels subjected to autoradiography.

3. RESULTS

RNA polymerase B (II) was modified with one of the adenine nucleotide derivatives I, II or III, stabilised by reduction and incubated with [α-^{32}P]UTP as described in section 2. A comparison of the protein staining pattern after gel electrophoresis under denaturing conditions with the autoradiography revealed that during incubation exclusively the subunit with M_r 140000 (designated as p140) became heavily labelled. Larger polypeptides remained unlabelled. Some weak radioactivity is observed in the region of the smaller polypeptides with M_r below 30000 (fig.1, tracks 2–4). It is unspecific since it is also observed

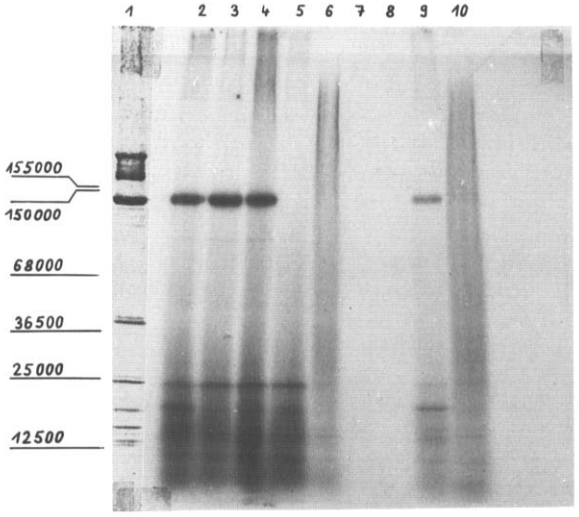

Fig.1. Gel electrophoretic analysis of the affinity modification of wheat germ RNA polymerase B (II). The experiments were carried out as described in section 2 with plasmid pBR322 containing as insert fragment d from the nopaline dehydrogenase gene [5]. (1) Gel stained with Coomassie brilliant blue; (2) autoradiography of the same experiment with derivative III; (3) with derivative II; (4) with derivative I; (5) without derivative; (6) without derivative but with 1 mM ATP; (7) with derivative III but without DNA template; (8) with derivative III but without enzyme; (9) as 2 but with 1 μg/ml α-amanitin; (10) with derivative III and equimolar amounts of ATP (1 mM each).

upon incubation with [α-^{32}P]UTP in the absence of the nucleotide derivative as priming substrate (fig.1, track 5) (see also [7]). Subunit p140 was not labelled when the nucleotide derivatives were replaced by ATP (fig.1, track 6) or when the DNA template or the enzyme were omitted from the reaction mixture (fig.1, tracks 7,8). In the presence of 1 μg/ml α-amanitin – an inhibitor of dinucleotide synthesis by the wheat germ enzyme [5] – labelling of subunit p140 is strongly reduced (fig.1, track 9). In an equimolar mixture of the adenine nucleotide derivative III and ATP the unmodified substrate should compete successfully with the synthetic ATP derivative. Indeed, no labelling is observed (fig.1, track 10). Upon incubation with pronase, but not with pancreatic ribonuclease, the radioactive label at the position of subunit p140 disappears (not shown). It indicates that the radioactive label is not due to absorbed RNA and is attached covalently to the protein.

To identify the phosphodiester bond in the product, use was made of the preferential acid lability of the phosphoanhydride bond. The enzyme was modified with the ADP ester II, and the reaction carried out as described above. After electrophoretic separation of the subunits the crushed region of the gel containing the radioactively labelled subunit was treated 3 times with 50 mM HCl containing 0.1% dodecyl sulfate for 3 h at 56°C. The neutralised solution was incubated with alkaline phosphatase to remove external phosphate groups. During gel electrophoresis part of the radioactive material obtained after partial acid hydrolysis migrated with the same mobility as ApU. This was confirmed by chromatography on poly(ethyleneimine)-cellulose thin-layer foils [5]. Additional evidence was obtained by incubation of this product with ribonuclease T_2 [5] which resulted in the formation of radioactive 3′-AMP from A[^{32}P]pU.

Labelling of subunit p140 is not dependent on the source of the template. Plasmid pBR322 containing fragment d from the nopaline dehydrogenase gene [5] between the *Bam*HI and *Hin*dIII site, the isolated fragment d [5], plasmid pUC18 or pUC18 containing the 251 bp *Taq*XI fragment of the major late promoter of adenovirus 2, and even poly[d(A-T)] were equally effective as templates. Circular DNAs were as good as linearised template DNAs.

4. DISCUSSION

So far the catalytic activities of the subunits of RNA polymerase B (II) have been studied mainly by interference of monoclonal antibodies with individual reactivities of the enzyme [8–11]. The results obtained do not allow unequivocal interpretations. The study of the binding of α-amanitin pointed to the second largest subunit as target [12,13]. In experiments where radioactive DNA was crosslinked to RNA polymerase B (II) the two largest subunits became labelled [14]. In our study the strong labelling of the second largest subunit p140 of the wheat germ enzyme depends strictly on the conditions for oligonucleotide synthesis in the presence of enzyme containing covalently bound nucleotide as priming substrate. In fact, the forma-

tion of an internucleotide 3′,5′-phosphodiester bond is clearly demonstrated by the transfer of a radioactive phosphate residue from 5′-[α-^{32}P]UTP to 3′-AMP during the hydrolysis of the product with ribonuclease T_2. This observation, as well as the inhibitory effect of α-amanitin, shows that the modified enzyme is still catalytically active. Furthermore, these observations suggest that the affinity label must be linked to the enzyme close to its active center. It was shown previously that RNA polymerase B (II) from wheat germ, like the bacterial enzyme [15,16], exhibits a broad substrate specificity with respect to the residues at the 5′-terminus of the priming substrate [5]. In agreement with these results the reactive nucleotide derivatives I–III, which differ in the number of phosphate residues, all proved to be active in the specific labelling reaction. This observation suggests a certain flexibility of the spacer attached to the priming substrate. If this were not so one would have to assume several amino acids with nucleophilic groups in the side chain to be located at appropriate distance around the active center and to function as acceptors for primers I–III to accommodate their different lengths. The successful affinity modification opens a new way for the elucidation of the topography of the active site of eukaryotic RNA polymerase B (II).

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