INCORPORATION OF [γ^{32}P] ATP BY EUKARYOTIC RNA POLYMERASE A

Trevor J.C. BEEBEE
*School of Biology, University of Sussex, Falmer, Brighton, England**

Received 13 June 1973

1. Introduction

Since the original discovery [1] that RNA synthesis is initiated at the 5′ terminus of the molecule, studies of this aspect of transcription *in vitro* have been facilitated by measuring the incorporation of γ-labelled [^{32}P]nucleoside triphosphates into RNA. Such experiments have shown, using prokaryotic RNA polymerases, that purine nucleotides are used exclusively in this process though the relative amounts of ATP and GTP vary between DNA templates [2, 3].

Following the isolation of multiple eukaryotic RNA polymerases from a variety of sources (e.g. [4–8]), attempts have been made to determine whether initiation by these enzymes is similar to that of the prokaryotic type (e.g. [9–12]). However, most of these measure γ^{32}P incorporation and assume it is due to initiation. The results of the experiments in this paper demonstrate that in the case of the rat liver form A RNA polymerase most of the observed incorporation of γ^{32}P by the DEAE purified enzyme is independent of DNA and exhibits characteristics unlike those of an initiation reaction. Previous results with the form A enzyme at this stage of purification [9–11] are therefore questionable. It is suggested that a protein which cochromatographs with RNA polymerase A on DEAE-Sephadex contains a kinase activity.

2. Materials and methods

RNA polymerase A and B were purified from rat liver nuclei to the stage of DEAE-Sephadex chromatography by the method of Roeder and Rutter [13]. In some experiments RNA polymerases AI and AII were prepared by leaching nuclei and purification of the extracted protein fraction on DEAE-cellulose and phosphocellulose as described by Chesterton and Butterworth [14]. Rat liver nuclei were prepared by the method of Blobel and Potter [15], and RNA polymerase activity was assayed as described previously [16].

Protein was estimated by the method of Lowry [17], and DNA by that of Burton [18].

3. Results and discussion

RNA polymerase from rat liver nuclei were eluted from a column of DEAE-Sephadex as shown in fig. 1A. RNA polymerase A activity, totally resistant to α-amanitin, was eluted at about 0.12 M ammonium sulphate and the α-amanitin sensitive B enzymes at about 0.28 M. The ability of the various fractions to incorporate [γ^{32}] ATP is also shown. Peaks of incorporation occurred in the region of the A enzyme and at about 0.18 M ammonium sulphate. No incorporation of the γ-label by the B enzymes was ever observed, even when denatured DNA was used as a template (unillustrated results). The reason for this was not determined; possibly too little enzyme was present for the measurement of initiation by this technique, or alternatively nucleotides other than ATP may have been utilised in this system. Fig. 1B shows that preincubation of the mixed RNA polymerase (fraction 4) with a small amount of [γ-^{32}P]ATP prior to chromatography resulted in a similar pattern of insoluble radioactivity in the eluted fractions as was incorporated by those of fig. 1A; the elution profile of the A enzyme activity was slightly altered.

* Present address: Department of Biochemistry, University College, Gower Street, London WC1 6BT, England.

North-Holland Publishing Company – Amsterdam

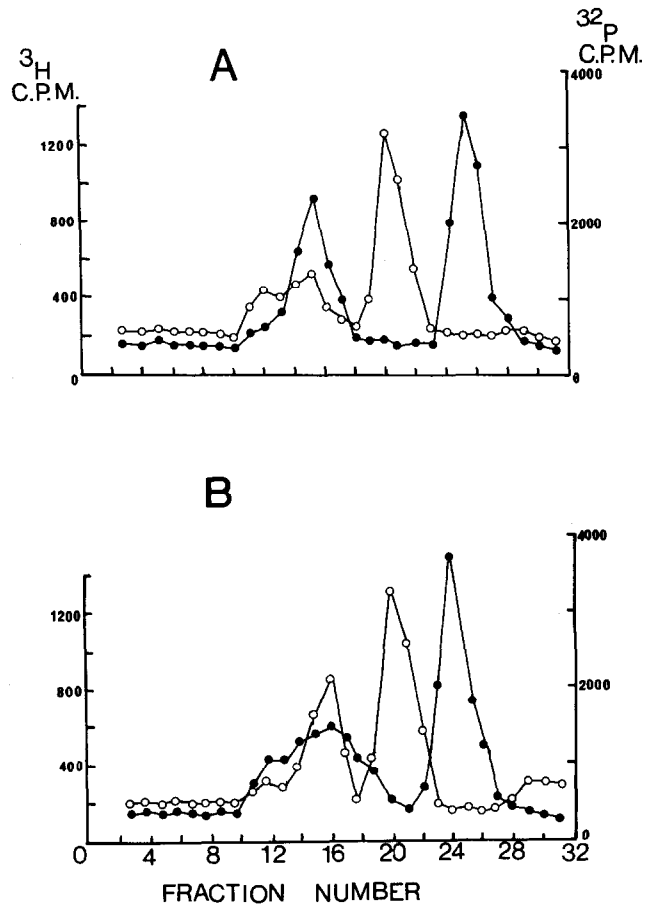

Fig. 1. γ-^{32}P-incorporation by rat liver RNA polymerases. A) 3.5 mg of mixed soluble RNA polymerases were adsorbed on a 1.3 X 3 cm column of DEAE-Sephadex A-25 which had been previously equilibrated with TGMED and 0.05 M ammonium sulphate [13]. The column was eluted with a linear gradient of 0.05–0.5 M ammonium sulphate in 20 ml, and 0.5 ml fractions collected. 50 μl aliquots of each fraction were tested for RNA polymerase activity in incubations containing, in 125 μl: 50 mM Tris-HCl pH 8.0; 2 mM β-mercaptoethanol; 2 mM $MnCl_2$; 25 μg native calf thymus DNA; 0.75 mM ATP, GTP and CTP; 1 μCi [^{3}H]UTP, specific activity 1 Ci/mmole. Assays were for 10 min at 30°C. In addition, 50 μl aliquots were assayed under similar conditions but with 0.75 mM unlabelled UTP and 0.5 μCi [γ-^{32}P]ATP (4 Ci/mmole). Assays were terminated and prepared for radioactivity counting as described previously [16]. B) 3.5 mg of mixed soluble RNA polymerases were incubated in 0.5 ml for 2 min at 30°C in the presence of 2 μCi [γ-^{32}P]ATP. This incubation did not affect the elution profile of the enzymes if the ATP was omitted. The proteins were then chromatographed as described above, and fractions assayed for RNA polymerase activity. The remainder of each fraction was added to 3.0 ml 5% (w/v) trichloroacetic acid, allowed to stand for 15 min at 0°C, and filtered on Oxoid cellulose acetate filters. These were then washed with a further 15 ml of the precipitant solution prior to preparation for radioactivity counting. (•—•—•) [^{3}H]UMP incorporation. (○—○—○) γ^{32}P incorporation.

[γ-^{32}P]ATP incorporation by DEAE-purified A enzyme was further investigated in the absence of DNA. Fig. 2 shows the kinetics of incorporation in the presence of either magnesium or manganese, at the concentrations normally used in assays. The activity was clearly independent of DNA, and maximally stimulated by magnesium. The latter property is a feature of protein kinases known to occur in nuclei [19].

The effects of DNA on [γ-^{32}P]ATP incorporation are shown in fig. 3. Preincubation of A enzyme and DNA reduced the consequent incorporation when the ATP was added later, in the presence of manganese.

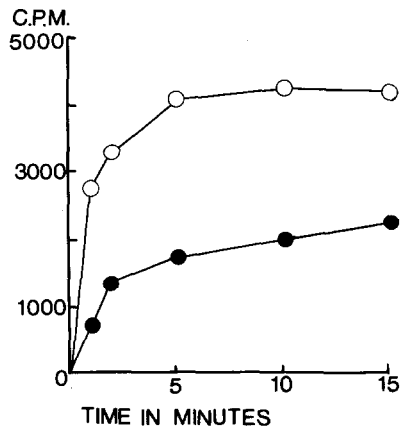

Fig. 2. Cation dependence of γ-^{32}P incorporation by RNA polymerase A. Assays, each in 1.0 ml, contained the usual concentrations of reactants (see legend to fig. 1) including 0.75 mM CTP, GTP and UTP and 4 μCi [γ-^{32}P]ATP but without DNA. Duplicate 50 μl aliquots were removed from the incubations at the times indicated, precipitated with trichloroacetic acid and prepared for radioactivity counting as described previously. (●—●—●) Assays containing 2 mM $MnCl_2$; (○—○—○) Assays containing 4 mM $MgCl_2$.

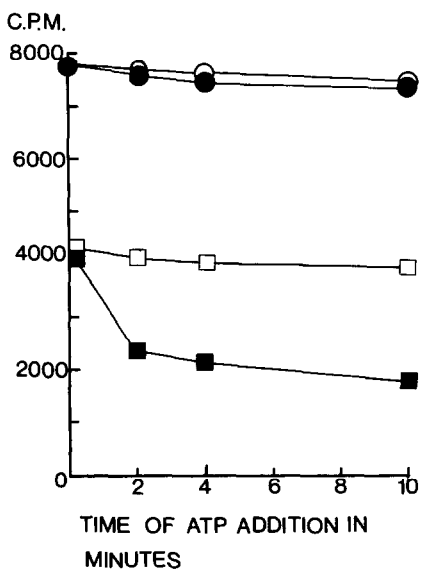

Fig. 3. Effects of DNA on γ-^{32}P incorporation. 0.5 μCi [γ-^{32}P]ATP were added to assays at the times shown, and incubated for 5 min at 30°C prior to precipitation. (○—○—○) In the presence of 4 mM $MgCl_2$ and without DNA. (●—●—●) In the presence of 4 mM $MgCl_2$ and 20 μg calf thymus DNA. (□—□—□) In the presence of 2 mM $MnCl_2$ and without DNA. (■—■—■) In the presence of 2 mM $MnCl_2$ and 20 μg calf thymus DNA.

Table 1
Effects of nucleoside triphosphates on γ-^{32}P incorporation and enzyme activity of RNA polymerase A.

Nucleotides in preincubation	^{32}P incorporated (cpm)		[^{3}H]UMP incorporated (pmoles)	
	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}
None	3290	6046	210	162
0.5 mM GTP	2876	1231	192	115
0.5 mM CTP	2977	2961	212	120
0.5 mM UTP	3040	2063	–	–
0.5 mM ATP	–	–	198	122
0.5 mM ATP, GTP and CTP	–	–	118	33
0.5 mM UTP, GTP and CTP	2666	858	–	–

Assays were: (a) In the absence of DNA and with 3 μg RNA polymerase A, 0.5 μCi [γ-^{32}P]ATP and other nucleotides as shown for 5 min at 30°C; or (b) 12 μg RNA polymerase A were preincubated with the nucleotides shown for 2 min at 30°C, after which enzyme activity was measured in the usual way after addition of 25 μg DNA and the other assay constituents. Either 2 mM $MnCl_2$ or 4 mM $MgCl_2$ were present throughout the assays, as indicated in the table. The effects of higher concentrations of the labelled nucleotide being utilised in the 2 types of assay were not examined due to the complications of isotope dilution and reduction of observable incorporation.

DNA had no significant effect in the presence of magnesium, suggesting that the specificity of the incorporation may depend on the cation present.

The results of preincubating RNA polymerase A with various combinations of nucleoside triphosphates on the subsequent γ-^{32}P incorporation and RNA polymerase activity are summarised in table 1. Preincubation with nucleotides markedly reduced γ-^{32}P incorporation in the presence of magnesium, but there was little effect with manganese. Activity of the A enzyme was also reduced by preincubation with nucleotides in the absence of DNA, and again the effect required the presence of magnesium. The nucleotides differed in their abilities to instigate these responses; GTP was apparently more efficient than the pyrimidine nucleotides. Preincubation of RNA polymerase B with nucleotides did not affect the subsequent activity (unillustrated results). It is not clear from these data whether the nucleotides inhibited γ-^{32}P incorporation or competed with the ATP for

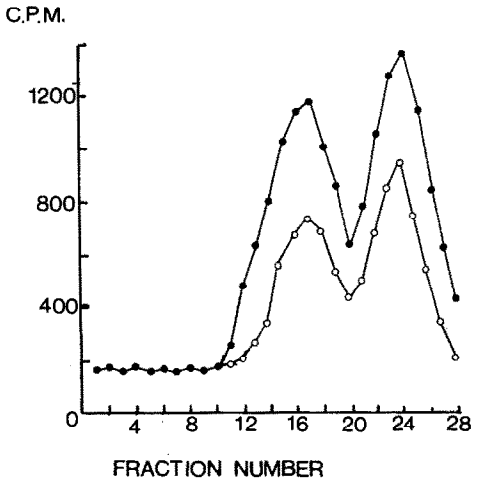

Fig. 4. Effects of ATP on the 2 forms of RNA polymerase A. 3 mg aliquots of RNA polymerase A proteins prepared by nuclear leaching to the stage of DEAE cellulose chromatography [14] were incubated alone or with 0.5 mM ATP for 5 min at 30°C in the presence of 2 mM $MnCl_2$ and 4 mM $MgCl_2$. The 2 aliquots were then chromatographed on identical 1 X 3 cm columns of phosphocellulose and eluted with linear gradients of 0.2–0.8 M KCl in 20 ml. 0.5 ml fractions were collected, and aliquots from each tested for RNA polymerase activity in the usual way. (o—o—o) Enzyme preincubated alone. (•—•—•) Enzyme preincubated with 0.5 mM ATP.

the reaction. The effects on enzyme activity favour the latter explanation.

One possible mechanism of action for the effects observed here was an interconversion of the 2 forms of RNA polymerase A. The enzyme was prepared by a method which yields approximately equal amounts of AI and AII [14]. This activity, which was capable of incorporating γ-^{32}P in a similar manner to that prepared by the sonication technique [13], was incubated with ATP prior to chromatography on phosphocellulose. Fig. 4 shows that both AII and AI activities were depressed equally by this treatment, suggesting that no interconversion was mediated by the nucleotide.

These results emphasise the dangers of using γ-^{32}P labelled nucleotides to measure initiation by partly purified RNA polymerases. The properties of the incorporation catalyzed by the rat liver RNA polymerase A indicate the presence of an additional activity causing the incorporation of γ-phosphate and under certain circumstances inhibiting the enzyme activity. It follows that estimation of the lengths of RNA molecules synthesised by this enzyme using a ratio of ^{32}P : ^{3}H incorporation [11] is an unreliable method at this stage of purification. That the ^{32}P incorporation was not on the complete nucleotide was confirmed by the observation that nucleoside triphosphates labelled with ^{3}H on the ribose moiety were not incorporated by the A enzyme in the absence of DNA and the three complementary nucleotides. The observations are compatible with the presence in the enzyme preparation of a protein kinase activity, an impurity which can be removed by phosphocellulose chromatography (S.J. Flint, personal communication) meaningful studies of the rat liver form A RNA polymerase therefore require this further purification step and removal of the contaminant.

References

[1] Bremer, H. and Konrad, M. (1964) Proc. Natl. Acad. Sci. U.S. 51, 801.
[2] Maitra, U. and Hurwitz, J. (1965) Proc. Natl. Acad. Sci. U.S. 54, 815.
[3] Naono, S. and Tokuyama, K. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 375.
[4] Roeder, R. and Rutter, W. (1969) Nature 224, 234.
[5] Kedinger, C., Gnaidowski, M., Mandel, J., Gissinger, F., and Chambon, P. (1970) Biochem. Biophys. Res. Commun. 38, 165.
[6] Mondal, H., Mandal, R. and Biswas, B. (1970) Biochem. Biophys. Res. Commun. 40, 1194.
[7] Ponta, H., Ponta, U. and Wintersberger, E. (1971) FEBS Letters 18, 204.
[8] Doenecke, D., Pfeiffer, C. and Sekeris, C. (1972) FEBS Letters 21, 237.
[9] Chambon, P., Gissinger, F., Mandel, J., Kedinger, C., Gnaidowski, M. and Meilhac, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 693.
[10] Varrone, V., Ambesi-Improbato, F. and Macchia, V. (1972) FEBS Letters 21, 99.
[11] Smuckler, E. (1972) Biochem. Biophys. Res. Commun. 49, 473.
[12] Seifart, K., Juhasz, P. and Benecke, B. (1973) European J. Biochem. 33, 181.
[13] Roeder, R. and Rutter, W. (1970) Proc. Natl. Acad. Sci. U.S. 65, 675.
[14] Chesterton, C. and Butterworth, P. (1971) European J. Biochem. 19, 232.
[15] Blobel, I. and Potter, V. (1966) Science 154, 1662.
[16] Beebee, T., Korner, A. and Bond, R. (1972) Biochem. J. 127, 619.
[17] Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 193, 265.
[18] Burton, K. (1956) Biochem. J. 62, 315.
[19] Langan, T. (1966) in: Regulation of Nucleic Acid and Protein Synthesis (Koningsberger, V. and Bosch, L., ed.), p. 233, Elsevier, Amsterdam.