A HOMORIBOPOLYNUCLEOTIDE SYNTHETASE IN RAT LIVER NUCLEI ASSOCIATED WITH RIBONUCLEOPROTEIN PARTICLES CONTAINING DNA-LIKE RNA

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1. Introduction

Nuclear heterogeneous DNA-like RNA is currently regarded as the precursor for the cytoplasmic mRNA. The experimental evidence for this concept comes from kinetic data [1, 2], hybridization studies [3, 4] and experiments with virus-transformed cells [5, 6]. The evidence for a precursor role of the heterogeneous DNA-like RNA is further supported by recent experiments on the occurrence and distribution of polyadenylic acid (poly A) in cellular RNA fractions. It has been shown that a segment of poly A of about 100–250 nucleotides in length is covalently linked to both cytoplasmic mRNA and nuclear heterogeneous DNA-like RNA [7–11]. The poly A segment is synthesized in the nucleus independent of transcription and probably is linked to the informational region of the heterogeneous DNA-like RNA either during transcription or after completion of the precursor molecule [12, 13]. The poly A segment associated with the heterogeneous DNA-like RNA appears to be indispensable for the specific processing of DNA-like RNA [13, 14]. For a recent review on poly A in eukaryotic cells see [15]. These poly A sequences are likely to be synthesized by ATP polymerase (EC 2.7.7.19), an enzyme which has been described by Edmonds and Abrams in thymus nuclei 12 years ago [16].

During the course of our investigation on nuclear ribonucleoprotein particles (RNP particles) containing heterogeneous DNA-like RNA [17–20] we have observed the activity of a RNA polymerase in firm association with the nuclear ribonucleoprotein particles. This RNA polymerase preferentially utilizes ATP, the reaction proceeds in the absence of DNA and is strongly stimulated by exogeneous RNA. In some respects the enzyme described here behaves like the ATP-polymerase mentioned above; it might well be that both enzymes are very similar or identical.

2. Methods

Ribonucleoprotein particles sedimenting at 30 S were isolated from purified rat liver nuclei according to Samarina et al. [21] as previously described [18]. Nuclei were first extracted at pH 7.0 with 0.05 M Tris-HCl buffer containing 0.14 M NaCl and 1 mM $MgCl_2$, this was followed by two extractions at pH 8.0 with the same buffer. The combined pH 8.0 extracts were analysed on sucrose gradients as indicated in the figure legends. The peak fractions of the 30 S peak were pooled and used as enzyme preparation, in some cases the peak fractions were dialyzed against 0.065 M Tris-HCl prior to the enzyme assay. Unless stated otherwise the RNA polymerase was assayed in a reaction mixture containing 14–17 μg of protein; 10 μmoles of Tris-HCl pH 7.9; 1.5 μmoles of mercaptoethanol; 1 μmole of Mn^{2+}; 1 μmole of creatine phosphate; 5 μg of creatine phosphokinase and 0.015–0.02 μmoles of labelled ^{3}H-ATP (4 μCi) in a total volume of 150 μl. In some experiments 0.2–0.4 μCi ^{14}C-ATP was used. The reaction was carried out at 37° for 60 min and stopped by the addition of excess unlabelled ATP. The reaction mixture was then precipitated on paper discs in ice cold 5% perchloric acid and the

Table 1
Distribution of RNA polymerase in nuclear extracts.

Conditions	^{14}C-ATP incorporated into RNA per mg of protein (cpm)	Nuclear enzyme activity (% of total)
Extraction at pH 7.0–0.14 M NaCl	2547	18.97
Extraction at pH 8.0–0.14 M NaCl	5300	52.38
Extraction at pH 8.0–0.3 M NACl	7412	28.66
Extraction at pH 8.0–0.3 M NaCl plus ultrasonication	–	–
Extraction at pH 8.0–0.6 M NaCl	–	–

radioactivity measured a previously described [18].

3. Results

Preliminary experiments have shown, that the RNP particle-associated RNA polymerase preferentially polymerizes ATP. Therefore, the enzyme was assayed in a reaction mixture containing ^{14}C or ^{3}H-labelled ATP. Table 1 shows the distribution of enzyme activity in nuclear extracts. At neutral pH, relatively small amounts of RNA polymerase activity are extracted. The major part of enzyme is extractable at pH 8.0 in the presence of 0.14 M NaCl. By raising the NaCl concentration to 0.3 M, an additional 28% of the total enzyme activity is removed from the nuclei. From the remaining nuclear residue no further RNA polymerase is obtained either by treatment with ultrasonics or by elevation of the NaCl concentration to 0.6 M. It is interesting to note that the yield of the RNA polymerase activity obtained by sequential extraction of the nuclei closely parallels the extractability of the nuclear RNP particles containing DNA-like RNA. Thus, both the RNP particles and the RNA polymerase are optimally extracted under the same conditions.

After sucrose gradient centrifugation of the combined nuclear pH 8.0 extracts each fraction of the gradient was assayed for RNA polymerase activity (fig. 1a). It can be seen that the majority of enzyme activity is present as a prominent peak in the central part of the gradient and exactly coincides with the UV-absorbing 30 S RNP particles containing DNA-like RNA. Upon recentrifugation of the 30 S peak fractions on a sucrose gradient almost all RNA polymerase activity remains associated with the 30 S RNP particles (fig. 1b). After high speed centrifugation (300,000 *g* for 3 hr) of the 30 S RNP particles almost all RNA polymerase activity is recovered in the pellet containing the 30 S particles. This suggests that the RNA polymerase is firmly associated with and is an integral part of the 30 S RNP particles.

Some kinetic data of the RNA polymerase are presented in fig. 2. The incorporation of ATP into acid insoluble material proceeds for at least 90 min. reaching a plateau after 2 hr (fig. 2a). The amount of ATP incorporated is directly proportional to the enzyme concentration (fig. 2b). Both mercaptoethanol and Mn^{2+} are required for optimum reaction (fig. 2c) while Mg^{2+} has little effect on the ATP incorporation (fig. 2d). In a control experiment, the nuclear DNA-dependent RNA polymerase (B enzyme) was incubated under the same conditions; this enzyme did not produce detectable amounts of radioactive acid insoluble material.

In table 2 the influence of some compounds on the incorporation of ATP into polynucleotides is listed. The percentage of inhibition of ATP incorporation by the addition of other nucleoside triphosphates are 4% for GTP, 28% for UTP and 54% for CTP. In the presence of orthophosphate and pyrophosphate no ATP is utilized, this might in part be due to the binding of phosphates to Mn^{2+}. RNase A and RNase T_1 are known to not degrade polyadenylic acid, their presence in the incubation medium therefore should not interfere with the polymerisation of ATP [16]. In spite of this, no ATP is incorporated. This suggests that a preexisting RNA other than polyadenylic acid is needed as a primer or template molecule. The addition of DNase I has a

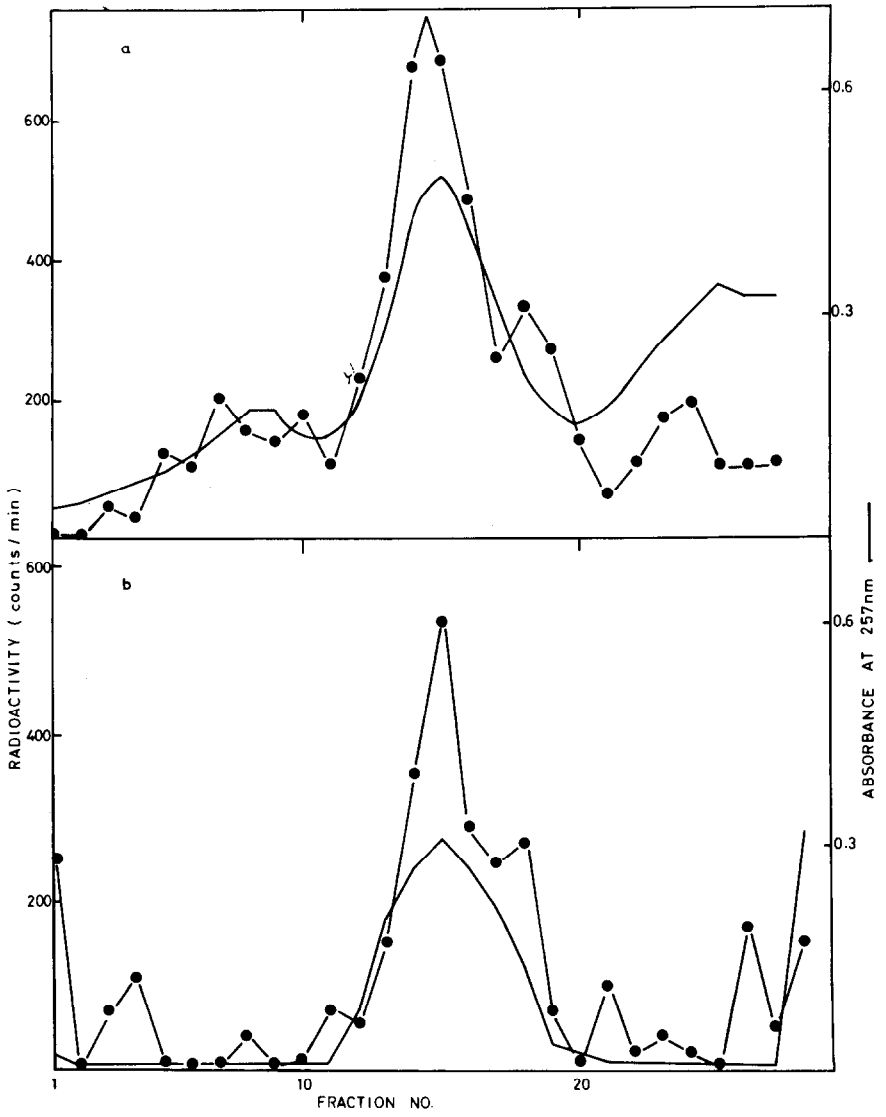

Fig. 1. Demonstration of RNA polymerase activity in the nuclear 30 S RNP particles: a) Nuclear pH 8.0 extracts were analysed on 50 ml of a 15–30% sucrose gradient prepared in pH 8.0-extraction buffer. Ultracentrifugation was carried out in a SW 25.2 rotor of a Spinco L_{II} at 22,000 rpm for 15 hr. Each fraction of the sucrose gradient was measured for RNA polymerase activity. Cpm ^{3}H-ATP incorporated into acid insoluble material (•—•—•); absorbance at 257 nm (——). b) Distribution of RNA polymerase activity after dialysis and recentrifugation of isolated 30 S RNP particles on a sucrose gradient. The conditions of sucrose gradient centrifugation and enzyme assay were the same as in (a).

slight stimulatory effect whereas in the presence of high concentrations of actinomycin D and α-amanitin ATP is incorporated at a slightly reduced rate. Cordycepin (3′-deoxyadenosin) does not inhibit the polymerisation. Nicotinamide mononucleotide is known to strongly stimulate ADP-ribose polymerase [22, 23], its inhibitory effect on the RNA polymerase described here supports, among others, the view that both enzymes are not identical.

The addition of exogeneous RNA's markedly affects the incorporation of ATP (table 3). Poly G reduces the reaction to 18% and poly U to 60% of the

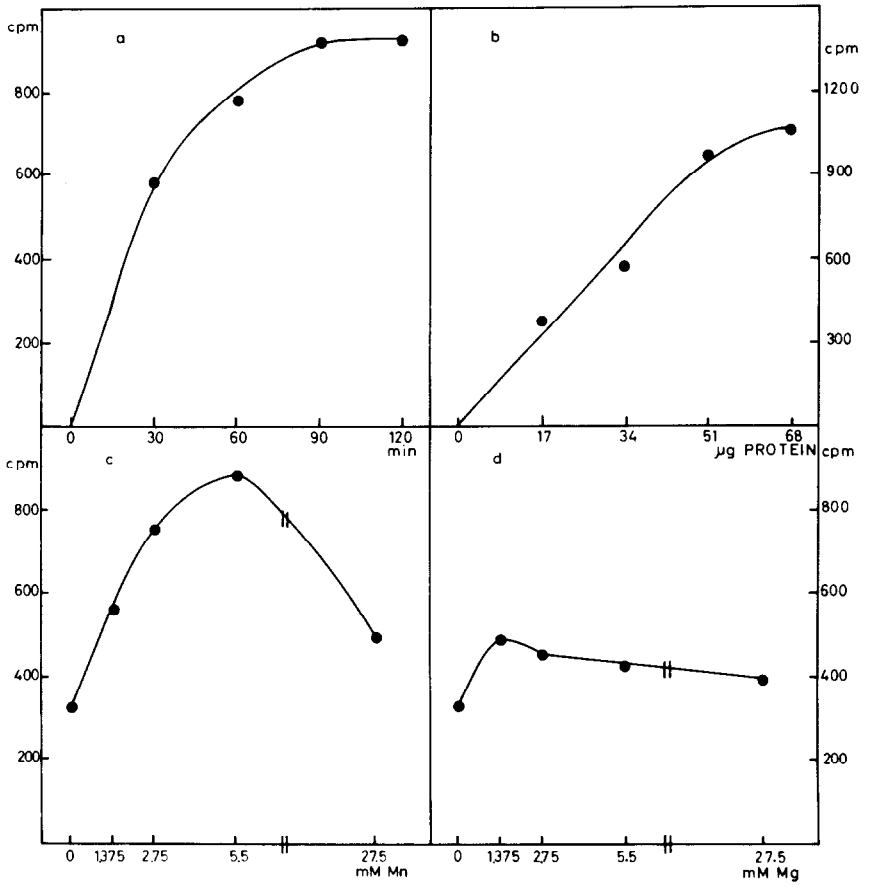

Fig. 2. Some enzymatic characteristics of the RNA polymerase. Cpm ^{3}H-ATP incorporated into acid insoluble material in dependence upon time (a); enzyme concentration (b); Mn^{2+} concentration (c) and Mg^{2+} concentration (d).

control. Poly C and poly A are without significant effect, this is also observed after the addition of rat liver DNA or poly d T.

The complete inhibition of ATP incorporation in the presence of RNase A or RNase T_1 (table 2) suggests that the endogeneous RNA contained in the RNP particles is digested by the RNase and therefore can no longer serve as primer or template molecule for the polymerization of ATP. If this is true, addition of exogeneous RNA should stimulate ATP incorporation. This is indeed the case and depends on the species of RNA added (table 3). The extend of stimulation is rather low if an unrelated RNA species such as tRNA is added. Cytoplasmic rRNA and a nuclear RNA fraction extracted at 37° (mainly ribosomal and some DNA-like RNA, see [2]) stimulate the ATP incorporation to 44% and 54%, respectively. At 65°, a RNA fraction which consists almost exclusively of heterogeneous DNA-like RNA can be extracted from the nuclei [2]; if this RNA is added to the incubation mixture the incorporation of ATP into acid insoluble material is strongly stimulated to 126% over the control value. Partial conversion of the free 3′-terminal hydroxyl groups of the "65°-RNA" to aldehyde by periodate oxidation leads to a significant decrease in the stimulatory action of this RNA on ATP incorporation suggesting that the RNA acts as primer and not as a template.

These results demonstrate that the heterogeneous DNA-like RNA is by far the best primer molecule for

Table 2
Effect of various compounds on the incorporation of ^{3}H-ATP into acid insoluble material.

Conditions	Incorporation of ^{3}H-ATP (% of control)
Control	100
+ GTP (10^{-4} M)	96
+ UTP (10^{-4} M)	72
+ CTP (10^{-4} M)	46
+ Orthophosphate (10^{-2} M)	39
+ Pyrophosphate (10^{-2} M)	1
+ RNase A (66 μg/ml)	2
+ RNase T_1 (1200 units/ml)	14
+ DNase I (66 μg/ml)	120
+ Actinomycin D (200 μg/ml)	80
+ α-Amanitin (0.33 μg/ml)	90
+ Cordycepin (10^{-3} M)	98
+ Nicotinamide mononucleotide (10^{-3} M)	61

Table 3
Effect of exogeneous RNA on the incorporation of ^{3}H-ATP into acid insoluble material.

Conditions	Incorporation of ^{3}H-ATP (% of control)
Control	100
+ Poly A (66 μg/ml)	111
+ Poly C (66 μg/ml)	94
+ Poly U (66 μg/ml)	60
+ Poly G (66 μg/ml)	18
+ Rat liver DNA (130 μg/ml)	111
+ Poly dT (16.5 μg/ml)	100
+ tRNA (200 μg/ml)	123
+ Cytoplasmic rRNA (100 μg/ml)	144
+ Nuclear "37°-RNA" (200 μg/ml)	154
+ Nuclear "65°-RNA" (200 μg/ml)	226

the polymerisation of ATP; and interesting to note, it is also this RNA species to which large segments of polyadenylic acid are added by a non-transcriptive process *in vivo* [12, 13].

The sedimentation profile (not shown) of the RNA which was synthesized *in vitro* and subsequently extracted with SDS phenol, shows a heterogeneous population of labelled RNA's reaching sedimentation values of up to 4 S and also a more rapidly sedimenting RNA fraction.

After extraction with SDS-phenol the RNA synthesized *in vitro* resists digestion with RNase A (3.3 μg/ml for 30 min) to 68% while nuclear control RNA under the same conditions is digested to 95%. On the other hand the RNA synthesized *in vitro* is largely degraded (88%) with snake venom phosphodiesterase. Furthermore, upon alkaline hydrolysis (0.3 N KOH at 37° for 18 hr) of the polynucleotide synthesized *in vitro* more than 95% of the ^{3}H-ATP incorporated was liberated as 2′, 3′-AMP. The low amount of adenosine recovered (4.3%) strongly suggests that in our experiments an internucleotide incorporation of ^{3}H-ATP and no terminal addition of ^{3}H-ATP to RNA had occurred [24]. These results provide additional evidence that the product synthesized by the RNA polymerase described here is polyadenylic acid.

As already mentioned, the RNA polymerase preferentially polymerizes ATP, UTP and GTP is incorporated at a significantly lower rate, whereas CTP is not utilized at all.

4. Discussion

In this communication we have described the occurrence of a DNA-independent ATP polymerizing enzyme in the nuclear RNP particles containing DNA-like RNA. This enzyme probably is identical with the ATP polymerase described by Edmonds and Abrams [16]. However, in contrast to the observations made by these authors the enzyme described here is not stimulated by poly A, it contains a low amount of poly A (2% after digestion with RNase A [25]) and it requires Mn^{2+} instead of Mg^{2+}. Furthermore, the synthesis of poly A by ATP polymerase is not affected by RNase A and is inhibited by exogeneous RNA while in our experiments the synthesis of poly A is completely suppressed in the presence of RNase A and is highly stimulated by nuclear RNA. Further experiments will be needed to clarify these differences.

RNP particles containing DNA-like RNA have been originally described by Samarina et al. [21, 26]. In recent experiments we have further characterized these RNP particles, they sediment between 30 S and 400 S and contain a heterogeneous DNA-like RNA with S values ranging from 5 S to 80 S [19]. The pro-

tein moiety of the RNP particles is characterized by a heterogeneous pattern of polypeptide chains [19, 20, 27, 28] the molecular weights of which have been determined to be between 32,000 and 130,000 [19, 20]. We have previously reported the presence of an endonuclease in the nuclear RNP particles specifically cleaving high molecular DNA-like RNA into smaller RNA chains [18].

The occurrence of an ATP polymerizing enzyme in the same protein fraction which *in vivo* is complexed to heterogeneous DNA-like RNA might well be of significance for the addition of poly A sequences to the informative part of the heterogeneous DNA-like RNA. Accordingly, the following mechanism could be postulated: the proteins of the nuclear RNP particles bind at specific sites to the high molecular DNA-like RNA as it is synthesized on the genome perhaps at the end of each cistron of the polycistronic RNA. After completion of transcription of the high molecular RNA addition of a poly A sequence is accomplished at the free terminal 3′-OH of the molecule by the nuclear particle enzyme. There is evidence that the informative parts of the heterogeneous DNA-like RNA may be located near the 3′-OH terminus [29, 30]. The RNA is then cleaved by the endonuclease of the particles and further poly A sequences may then be added to the informative parts of the cleaved RNA.

The presence of poly A at the 3′-terminus of the RNA could be a factor conferring resistance against degradation by nuclear enzymes and/or a site of attachment of proteins involved in the transport of mRNA from the nucleus into the cytoplasm.

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