A COMPARATIVE STUDY OF "SOLUBLE" RNA POLYMERASE ACTIVITY OF ZAJDELA HEPATOMA ASCITES CELLS AND CALF THYMUS

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1. Introduction

Except for mitochondria, it is admitted that the DNA dependent RNA polymerase activity in animal cells is located in the nucleus. The greater part is attached to the chromatin [1, 2] from which it cannot be easily dissociated; however a probably soluble activity has been directly extracted at low ionic strength from various cells [3–7], in particular from calf thymus [8]. Different RNA polymerase forms have been recently extracted and characterized [9–11], in particular an α-amanitin-resistant activity localized in the nucleolus, and an extranucleolar α-amanitin-inhibited activity.

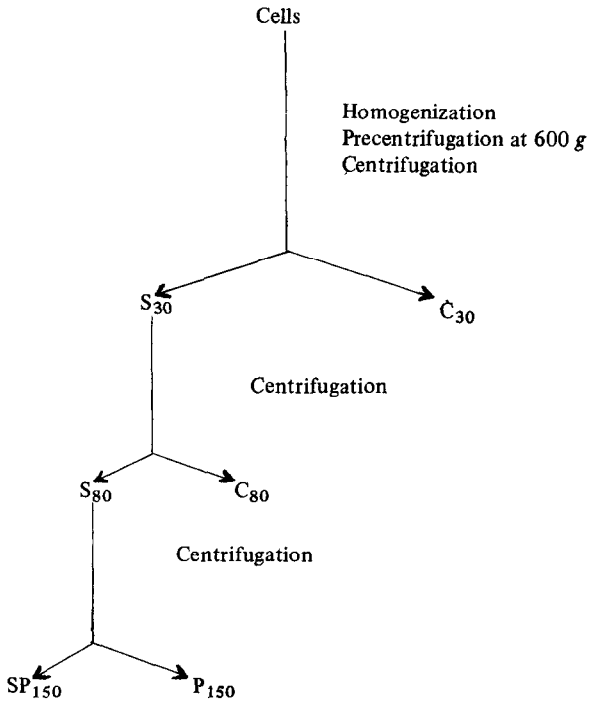
In the present work we describe the characteristics of soluble RNA polymerase activities which, in our conditions of fractionization, are found in the cytoplasm of hepatoma tumour cells and calf thymus and which show characteristics differing according to their origin.

2. Materials and methods

The Zajdela hepatoma ascites cells (10^7 cells) or calf thymus (0.5 g) were suspended in 1 ml of a 50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 0.25 M sucrose medium; 0.5% (v/v) cemulsol NpT6 (Melles-Bezons-France) and 200 μg/ml of collagenase (Bacterial B grade, Calbiochem) were added [13]. After 5 min at 4°, the hepatoma cells were homogenized with an Ultraturrax (Janke–Kunkell) at low speed so that there was practically no breakage of nuclei. Further fractionization was made according to scheme 1. For calf thymus purified nuclei were obtained in high yield, after homogenization with a grinding apparatus for garlic which acts by shearing the tissue and thus eliminates directly collagen fibers. When activity of pellets is to be tested, they are resuspended in a 50 mM Tris HCl pH 8, 5 mM $MgCl_2$ medium.

The reaction mixture (0.650 ml) contained 40 μmoles Tris-HCl pH 8; 2 μmoles $McCl_2$; 4 μmoles NaF; 5 μmoles KCl; 2.5 μmoles phosphoenol pyruvate; 20 μg of pyruvate kinase (Bŏheringer); 1 μmole β-mercaptoethanol; 0.3 μmole each: ATP, GTP, CTP; 13 nmoles UTP (Sigma); 0.4 nmole ^{3}H-UTP (CEA TB 8F 10 Ci/mmole); 80 μg Calf thymus DNA (Choay Laboratory) and 500 μg proteins of different enzymatic forms. In some cases $(NH_4)_2SO_4$ was added. At 37° the reaction time allowed was 20 min. The reaction was stopped by putting the incubation tube in crushed ice and adding successively 2 μmoles UTP, RNA as carrier and 4 ml of a solution of 10% TCA containing 0.1 M $Na_4P_2O_7$ [14]. The precipitate was collected on a Whatman GF/B filters, decontaminated according to Jacob et al. [14]. The dried filters were placed in 1 ml of 1 M NH_4OH for several hours, then 10 ml of scintillator (Toluene, 30% OP 1062 (Ugine Kuhlmann), butyl PBD (Ciba) 8 g/l) added, filters crushed with a mixer and counted in a Tricarb-Packard spectrometer.

Protein determination was performed according to Lowry's method [15] after complete dissolution of pellets with 0.1 ml of a solution of Sarkosyl NL30 (Geigy Laboratories), 2% in 0.3 N NaOH.



Scheme 1. Cell fractionization. RNA polymerase activity is assayed in different fractions. S_{30}, S_{80}, SP_{150} fractions are obtained by centrifugation, respectively: 30 min at 30,000 *g*, 120 min at 80,000 *g* and 150 min at 150,000 *g*. Except for the precentrifugation, all the centrifugations were run in a Spinco 50 rotor.

3. Results

3.1. *Preparation of different fractions*

The S_{30} fractions, prepared according to the scheme 1, show an RNA polymerase activity that we shall term "soluble". For hepatoma cells, the soluble activity stays constant whether the nuclei are preserved or broken during preparation and it cannot be found in isolated nuclei. On the other hand, for calf thymus, the soluble activity is found partly in S_{30} and partly in the respective nuclei.

It is possible to prepare an S_{30} fraction with some activity from nucleoplasm of previously isolated nuclei according to scheme 1. Preliminary experiments suggest that these 2 activities are identical.

Table 1 shows the specific activity of different fractions prepared according to scheme 1. For calf thymus and hepatoma cells, the P_{150} pellet shows the highest specific activity. The important loss of activity during centrifugation at 150,000 *g* cannot be explained, the recombination experiments between P_{150} and SP_{150} fractions not allowing the recovery of this activity.

The P_{150} fraction is only a little contaminated by nucleic acids (< 7% RNA); comparative properties of S_{30} and P_{150} are shown in table 3. In the following, we limit ourselves to a study of the properties of the P_{150} fraction.

Table 2 shows that this RNA polymerase activity requires DNA and 4 ribonucleoside triphosphates. The synthetized product is sensitive to ribonuclease. We have verified, by adding NpT6 to the incubation medium, that the detergent, used during the homogenization of cells, does not modify the enzymatic activity.

The analysis by ultracentrifugation in sucrose density gradient of the P_{150} (fig. 1) obtained from hepatoma cells shows that the activity is to be found in 2 peaks of 22 S and 14 S; minor activities being found in the region of 40 S which contains the greatest part of the P_{150} RNA.

3.2. *Comparative properties of hepatoma cells and calf thymus P_{150}*

In all these experiments, the concentration of DNA is not a limiting factor. The P_{150} hepatoma activity is just slightly higher with double stranded DNA than with denatured, while the calf thymus activity is 4 times lower with denatured DNA as template. It should be observed that with P_{150} derived from thymus or from hepatoma, it is possible to isolate, according to the technique described by Stein et al. [8], a factor with properties similar to factor S.

3.3. *Effects of α-amanitin*

Table 3 shows that at a concentration of 1 μg/ml which inhibits specifically extranucleolar RNA polymerase of eukaryotic cells, the P_{150} hepatoma activity is only slightly affected (10%) while calf thymus derived activity is decreased by 50%. Fig. 2 shows the effects of high concentrations of α-amanitin on RNA polymerase activity of these two enzymes and exhibits a biphasic character. After a dramatic inhibition at low concentration, the RNA polymerase activity decreases quite linearly with α-amanitin concentration, the P_{150} thymus activity staying more sensitive than

Table 1
RNA polymerase activity in different fractions.

	Thymus			Hepatoma		
	Activity (units)	Proteins (mg)	Specific activity	Activity (units)	Proteins (mg)	Specific activity
S_{30}	1.690	9.4	0.18	3.640	7.9	0.46
S_{80}	1.590	7.25	0.22	2.300	5.2	0.44
C_{80}	0.168	0.840	0.20	0.246	0.84	0.27
P_{150}	0.325	0.690	0.47	0.380	0.45	0.83
SP_{150}	0.340	6.8	0.05	0.110	4.1	0.27

Results are given for 1 ml of S_{30}. Incubation conditions are as described under Materials and methods. One unit of activity corresponds to the incorporation of 1 nmole of ^{3}H-UMP in 20 min at 37°.

that of hepatoma. For α-amanitin concentration of 150 μg/ml, the P_{150} thymus activity is nil while that of P_{150} hepatoma is 45% of the initial value.

3.4. *Effects of divalent cations*

Fig. 3 shows the effect of various concentrations of Mg^{2+} and Mn^{2+} on the activity of P_{150} enzyme. The Mn^{2+}/Mg^{2+} activity ratios, at the optimal concentration of each ion, for hepatoma and calf thymus are 2.5 and 10, respectively. These values are similar to those found by Roeder and Rutter [9] for activity of forms III (A_3) and II (B), respectively.

On the other hand, the effect of various concentrations of ammonium sulphate on the P_{150} activity is different with calf thymus and hepatoma enzymes (fig. 4). The P_{150} thymus enzyme exhibits an optimal

Table 2
Characteristics of P_{150} RNA polymerase activity derived from hepatoma cells.

Medium composition	^{3}H-UMP incorporated for 1 mg of P_{150} during 20 min (nmoles)	Activity (%)
Complete	0.88	100
Minus DNA	0.013	1.5
Minus ribonucleoside triphosphate except for ^{3}H-UMP	0.165	19
+ RNAse*	0.045	5

Incubation conditions are as described under Materials and Methods. * 20 min after normal incubation, 3 μg/ml of pancreatic RNase are added and the incubation carried on 5 min.

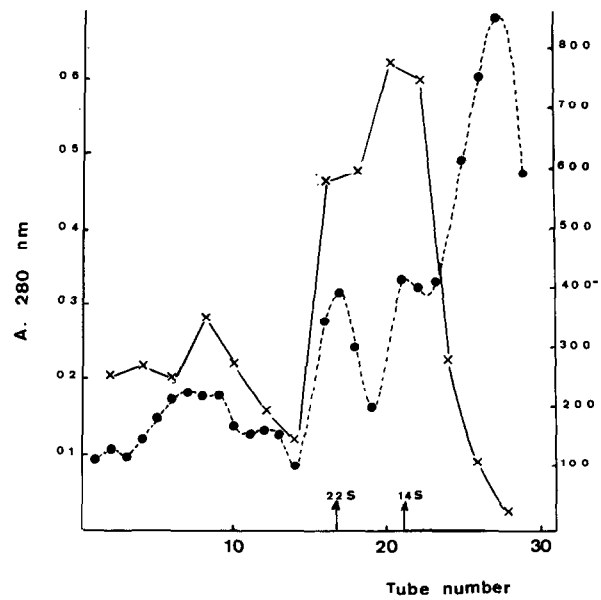


Fig. 1. Ultracentifugation of P_{150} in a sucrose density gradient. 0.1 ml of P_{150} (500 μg) suspension was layered on 5 ml of a sucrose gradient 5–20% in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ medium, in a Spinco SW 50 rotor cellulose tube. Centrifugation at 48,000 rpm for 100 min. Gradient collected by bottom puncture of the tube. (●---●---●): Absorbance 280 nm; (x–x–x): Cpm ^{3}H-UMP incorporated.

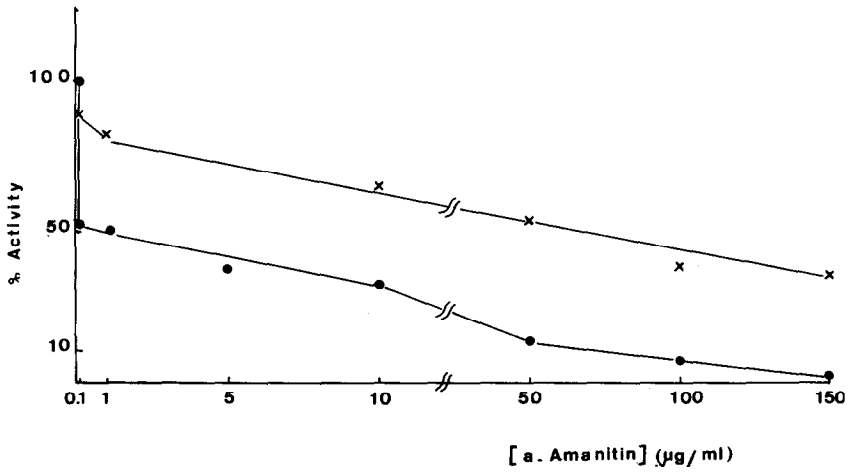


Fig. 2. Effects of α-amanitin on P_{150} RNA polymerase activity. The conditions of incubation are the same as indicated in Materials and methods. (x–x–x): P_{150} derived from hepatoma cells. (●–●–●): P_{150} derived from calf thymus.

activity at 0.1 M salt (as B form extracted from nuclei) while the P_{150} hepatoma enzyme shows a practically constant activity up to 0.16 M (as that of form III (A_{III}).

It is interesting to note that the points of inflexion obtained for concentrations of 0.03 M and 0.1 M, respectively, with thymus and hepatoma derived P_{150} are significant.

4. Discussion

In hepatoma cells, fractionated according to our method, a very low RNA polymerase soluble activity is found in nuclei, while the cytoplasmic fraction S_{30} shows significant activity. On the other hand, for calf thymus the soluble RNA polymerase activity is found in equal amounts in the nuclei and in the cytoplasmic

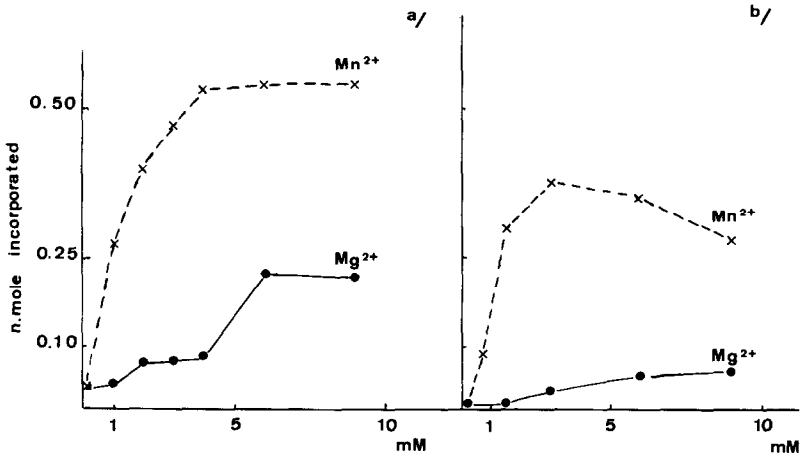


Fig. 3. Dependence of P_{150} activity on Mn^{2+} and Mg^{2+} ions. Conditions of incubation are the same as indicated in Materials and methods except that there is no NaF and there is only Mn^{2+} or Mg^{2+} at the indicated concentration. a) P_{150} of hepatoma cells; b) P_{150} of calf thymus.

Table 3
Properties of RNA polymerase activities of S_{30} and P_{150} derived from calf thymus and hepatoma cells.

	Fractions	Native DNA (%)	Denatured DNA (%)	α-Amanitin (1 μg/ml)
Thymus	S_{30}	100	19.5	12
	P_{150}	100	26.5	48
Hepatoma	S_{30}	100	89	90
	P_{150}	100	100	95

Activities are shown as a percentage of the nmoles of ^{3}H-UMP incorporated in the normal assay for each fraction. Specific activities of different fractions are those shown in table 1.

fraction S_{30}. It is possible that, for hepatoma, the whole soluble enzyme goes out of the nuclei during fractionization of the whole cell, while for thymus the soluble enzyme stays partly in the nuclei. It is of interest to note that for thymus, the repartition of soluble activity between cytoplasm and nuclei is not affected by various concentrations of Mg^{2+} from 2–20 mM in the preparation medium. Yet we cannot exclude the hypothesis of a cytoplasmic localisation for these soluble enzymes prior to fractionization, more especially as such an assumption has been made for rat liver DNA polymerase [17].

Our fractionization procedure, which avoids precipitation with ammonium sulphate or protamine sulphate, produces enzyme preparations more or less without nuclei acid contamination and with specific activities similar to that obtained by other authors after the same precipitations [11, 16]. The purification and characterization of these enzymes by chromatography will allow us to compare them with other forms previously described (A and B). Our results, especially with hepatoma cells, show that RNA polymerase extraction from isolated nuclei does not necessarily lead to the characterization of all the enzyme forms present in the cell.

According to the effects of various concentrations of α-amanitin on our enzyme forms, it is difficult to integrate them in the classification proposed by Kedinger et al. [12]. Preliminary experiments of P_{150} thymus fractionization on DEAE-cellulose according to Chambon did not allow us to separate an α-amanitin-resistant activity and an α-amanitin-inhibited activity [10]. Moreover, the fact that some of this activity is inhibited only by a high concentration of α-amanitin is still inexplicable. It is possible that these soluble enzymes become α-amanitin-resistant by reaction with cytoplasmic components, or that they represent a new form of α-amanitin-resistant nucleolar enzyme (A).

Moreover, the properties of soluble cytoplasmic enzymes are clearly different for calf thymus and hepatoma cells where α-amanitin-resistant forms prevail. Mandel and Chambon [16] have shown that the relative amount of enzyme forms BI and BII is different in extracted and purified enzymatic preparations from calf thymus and from isolated nuclei prepared

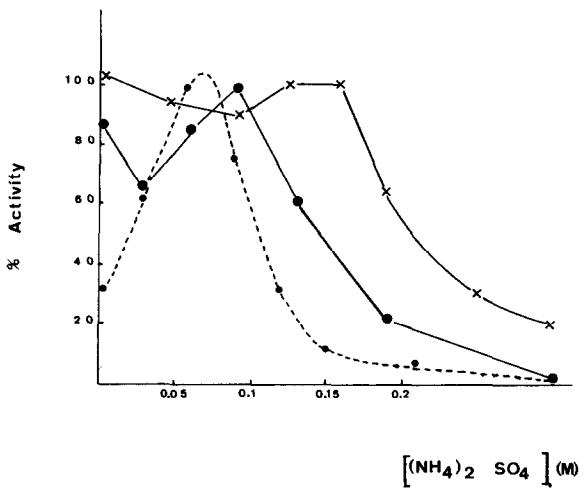


Fig. 4. P_{150} activity as a function of $(NH_4)_2\,SO_4$ concentration. Conditions of incubation are the same as in Materials and methods except for $(NH_4)_2SO_4$ concentration. (•---•---•): RNA polymerase activity extracted from hepatoma nuclei; (x–x–x): P_{150} activity of hepatoma cells; (•–•–•): P_{150} activity of calf thymus.

from rat liver. In this case it is difficult, as these authors emphasize, to decide if this fact is a characteristic of the tissue or if it is due to a specific loss during purification, or to a proteolytic process giving 2 enzyme forms from a single one [18]. On the other hand, in our experiments, it seems that the qualitative repartition of soluble activities is different according to the tissue, and sets the problem of knowing if in hepatoma cells or in mitotic active cells, the α-amanitin-resistant form predominates. This line of investigation is being pursued.

Acknowledgements

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