EVIDENCE FOR THE EXISTENCE OF TWO DIFFERENT POLY(A) POLYMERASES AND A NOVEL RIBOHOMOPOLYMER POLYMERASE IN RAT LIVER NUCLEI.

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Summary: Enzymes catalysing the non-transcriptive polymerisation of ATP,UTP,GTP and CTP to the corresponding ribohomopolymers have been isolated from rat liver nuclei.By chromatography on agarose and phosphocellulose two different poly(A) polymerases,a Mg^{++}-dependent and a Mn^{++}-dependent poly(A) polymerase,have been resolved.A Mn^{++}-dependent enzyme system synthesizing poly(U),poly (G) and poly(C) from UTP,GTP and CTP is co-purified with the Mn^{++}-dependent poly(A) polymerase.For the synthesis of the ribohomopolymers a primer-RNA,but not DNA,is essential.The polymerases described have a molecular weight of 41,ooo daltons;they are not identical with the DNA-dependent RNA polymerase or polynucleotide phosphorylase.

Poly(A) segments of 15o - 2oo nucleotides in length are covalently linked to the 3'-terminus of the heterogeneous nuclear RNA (HnRNA) and cytoplasmic mRNA (1-3).There is evidence that poly(A) is added to the HnRNA by a non-transcriptive process after completion of the precursor molecule (4).In the cell nucleus the synthesis of poly(A) probably is mediated by poly(A) polymerase (EC 2.7.7.19).A Mg^{++}-dependent poly(A) polymerase has first been detected in calf thymus nuclei (5) and extensively purified from whole calf thymus (6).

Recently we have described (7,8) the occurance of a Mn^{++}-dependent and a Mg^{++}-dependent poly(A) polymerase in rat liver nuclei.On the basis of their different enzymatic characteristics with respect to requirements of ions and mercaptoethanol,their different susceptibility to AF/o13 and unlabelled nucleosidetriphosphates as well as on the basis of the different chain lengths of poly(A) synthesized in vitro,we have concluded that the Mn^{++}-dependent and the Mg^{++}-dependent poly(A) polymerase very probably represent two different enzymatic entities (8).In this communication we wish to report experiments which confirm our earlier

suggestion: there are two different poly(A) polymerases in the cell nucleus;in addition to the poly(A) polymerases an apparently novel enzyme system catalysing the synthesis of poly(U),poly(G) and poly(C) independent of a DNA template is described.

Methods: Rat liver nuclei were prepared by a modification of the procedure of Chauveau et al. (9).Nuclear extracts were obtained by a method which has successfully been employed for the isolation of the DNA-dependent RNA polymerase from rat liver nuclei (1o). After ultrasonication of the nuclear suspension the proteins were precipitated by the addition of solid ammonium sulfate to 7o% saturation,dissolved in buffer A (1o mM Tris-HCl pH 7.9,o.25 mM EDTA, 2 mM $MgCl_2$,2 mM ß-mercaptoethanol,2o% glycerol and NH_4Cl at a concentration of o.2 M or o.4 M) and dialysed against the same buffer. The extract was clarified by centrifugation and submitted to gel filtration.The incorporation of radioactivity and enzyme activity was measured as previously described (8) except that the reaction mixture contained Mn^{++} at a concentration of 7.5 mM and nuclear primer-RNA was added to the saturating concentration of 5o ug/ml. The activity of the DNA-dependent RNA polymerase was measured as described in Ref.1o.

Results: Chromatography of nuclear extracts on a Bio-Gel A-1.5 m column equilibrated with buffer A at low ionic strength (o.2 M NH_4Cl) and subsequent measurement of poly(A) polymerase activity in the presence of either Mn^{++} or Mg^{++} reveals almost identical elution profiles (Fig.1a).If nuclear extracts were submitted to gel filtration under the same experimental conditions except that the NH_4Cl concentration was o.4 M instead of o.2 M a completely different and heterogeneous elution pattern is obtained for the poly(A) polymerase assayed in the presence of Mg^{++} (Fig.1b).The main peak of enzyme activity elutes with a specific volume which corresponds to a molecular weight of about 6oo,ooo daltons;in addition to the main peak three smaller peaks can be discerned.The elution point of the Mn^{++}-dependent poly(A) polymerase remains unaffected by variations of the salt concentration.The extent of aggregation of the Mg^{++}-dependent poly(A) polymerase at high salt varied somewhat from experiment to experiment.Due to the rapid inactivation of the Mg^{++}-dependent poly(A) polymerase upon storage in o.4 M NH_4Cl the enzyme activity had to be assayed immediately after fractionation on the Bio-Gel column or had to be dialysed against low salt buffer.The differences in the elution profile of the Mg^{++}-activated poly(A) polymerase at low and high salt can be explained by a specific,salt mediated aggregation of the polymerase as has also been described for other enzymes (11).If the eluate fractions from the Bio-Gel column are also assayed for enzymatic

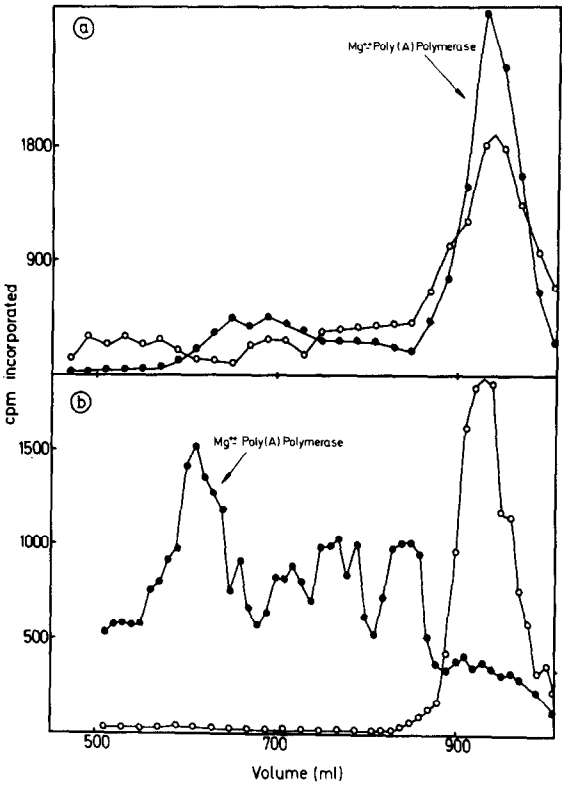

Fig.1 Effect of salt on the elution pattern of poly(A) polymerases from an agarose column.(a) chromatography in the presence of o.2 M NH_4Cl.(b) chromatography in the presence of o.4 M NH_4Cl. Nuclear extracts (2o ml) were dialysed against buffer A containing either o.2 or o.4 M NH_4Cl and chromatographed on a 4 x 8o cm Bio-Gel A-1.5 m column.Fractions of 1o ml were collected at a flow rate of 15 ml/hr.Mn^{++}-dependent poly(A) polymerase O——O.

activities capable of polymerizing UTP,GTP and CTP (8) a very similar chromatographic behavior compared with that of the poly(A) polymerases is evident (Fig.2a and 2b).In this experiment the elution profile of the DNA-dependent RNA polymerase has also been determined as a marker enzyme and in order to demonstrate that the DNA-dependent RNA polymerase apparently is not related to the ribohomopolymer polymerases investigated here (Fig.2b).

The elution profiles presented in Fig.2 suggest that the molecular weights of the poly(A) polymerases and those of the polymerases which incorporate UTP,GTP and CTP into acid-insoluble material are very similar.Chromatography of nuclear extracts on a gel with high resolving power (Sephadex G-2oo superfine) shows almost identical elution pattern for the enzymatic activities of

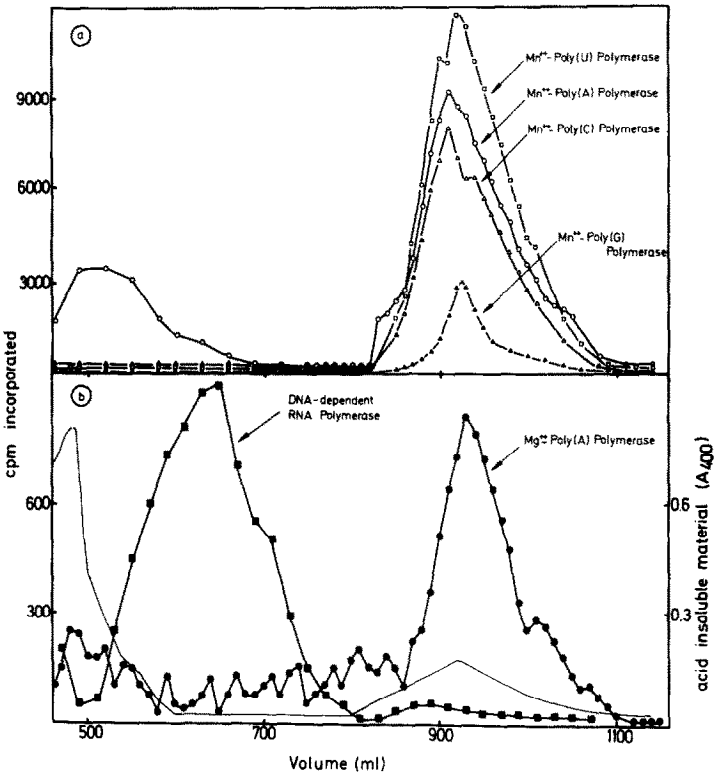

Fig.2 Chromatography of nuclear extracts on an agarose column. The conditions of chromatography were the same as described for Fig.1a.(a) Mn^{++}-dependent polymerases.(b) Mg^{++}-dependent poly(A) polymerase and DNA-dependent RNA polymerase.

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the different polymerases (Fig.3b).Employing standard markers as references (Fig.3a) a molecular weight of 41,000 was estimated for the main elution point of the polymerases from a plot of molecular weight versus elution volume.We have presented evidence that the Mg^{++}-dependent and the Mn^{++}-dependent poly(A) polymerase are two different enzymatic entities.We could not,however,demonstrate whether the Mn^{++}-dependent polymerases represent a single enzyme capable of synthesizing poly(A),poly(U),poly(G) and poly(C) or if each of these ribohomopolymers is synthsized by a separate enzyme molecule.Fig.3b shows a heterogeneous elution profile of the Mn^{++}-activated poly(A) polymerase in the 40,000 - 50,000 molecular weight region.Two poorly resolved peaks can be discerned.This profile is characteristic for the Mn^{++}-dependent poly(A) polymerase; if the conditions of chromatography on Sephadex G-200 superfine are changed (chromatography on a 3 x 200 cm column in the pre-

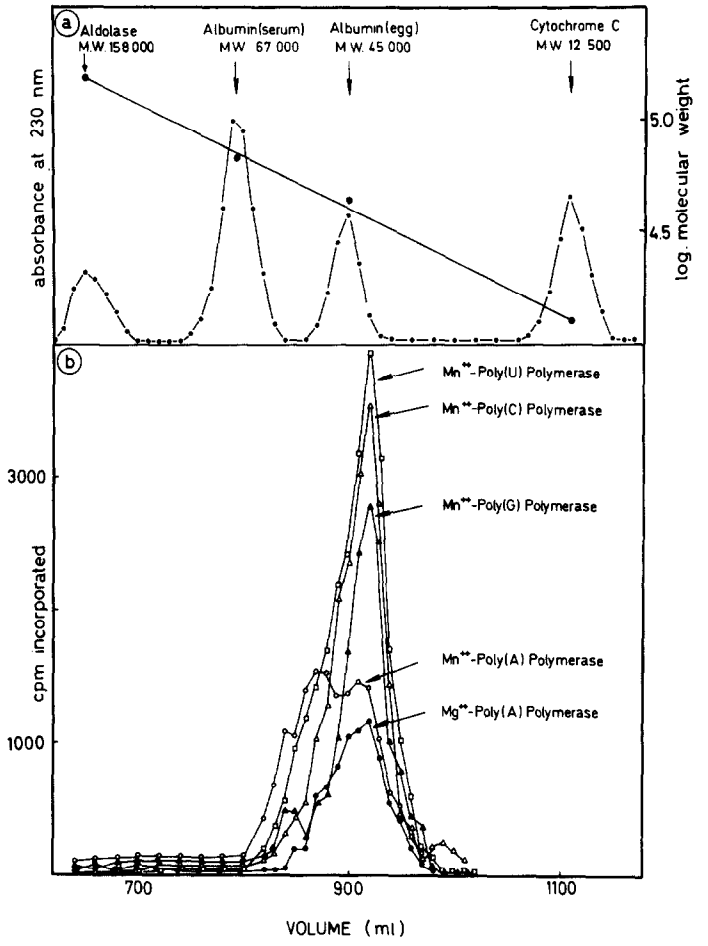

Fig.3 Molecular weight determination of nuclear ribohomopolymer polymerases on a Sephadex G-2oo superfine column. Nuclear extracts (5 ml) were dialysed against buffer A containing o.2 M NH_4Cl and submitted to gel filtration on a calibrated 3.5 x 14o cm Sephadex G-2oo superfine column. (a) elution profile of marker proteins. (b) elution profile of ribohomopolymer polymerases.

sence of o.6 M NH_4Cl) the two peaks of enzyme activity are well separated (unpublished obsrevation). Furthermore, the yield of each of the Mn^{++}-dependent ribohomopolymer polymerases after extraction from the nuclei varies considerably from one experiment to another (compare Figs.2a, 3b and 4). On the basis of these data and taking into account that in general a diversity of catalytic functions does not reside with enzymes of low molecular weight, we tentatively assume that separate enzymatic entities are responsible for the synthesis of the different ribohomopolymers.

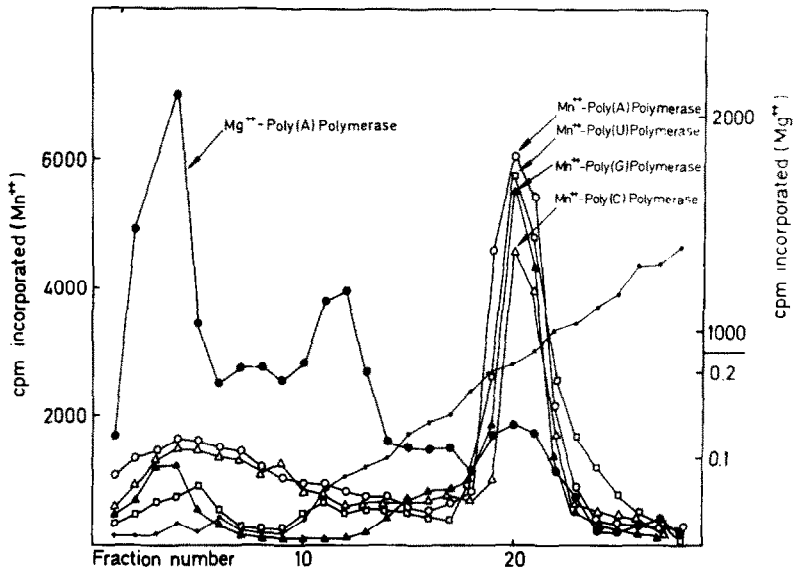

Fig.4 Phosphocellulose chromatography of nuclear ribohomopolymer polymerases on a 2 x 4 cm column. After chromatography of nuclear extracts on Bio-Gel the enzyme fractions were pooled, concentrated and dialysed against o.o1 M Tris-HCl pH 7.9 containing 1 mM ß-mercaptoethanol and 2o% glycerol. The enzymes were adsorbed to phosphocellulose, the column was washed with 1o ml of buffer and then connected to 6o ml of a o - o.4 M NH_4Cl gradient. Fractions of 2.5 ml were collected.

Further support for the conclusion that there are two different poly(A) polymerases comes from the different elution pattern of both enzymes from a column of phosphocellulose (Fig.4). After gel filtration of nuclear extracts on Bio-Gel the enzymatic activities were pooled and submitted to chromatography on phosphocellulose. The major part of enzyme activity of the Mg^{++}-dependent poly(A) polymerase is bound with low affinity to the phosphocellulose and is washed out or eluted at a lower concentration of NH_4Cl than the Mn^{++}-dependent poly(A) polymerase which eluted at o.21 M NH_4Cl together with the other Mn^{++}-dependent polymerases (Fig.4).

We have already presented evidence that the ribohomopolymer polymerases described here are not related to the DNA-dependent RNA polymerase (Ref.8 and Fig.2). The data shown in Table I exclude the possibility that the polymerases in question are identical with the polynucleotide phosphorylase; UDP, GDP and CDP are not utilized at all. ADP is incorporated to a limited extent, i.e. 31%

Table I Substrate and primer - dependence of nuclear ribohomopolymer polymerases.

Substrate	Ionic conditions of assay	Conditions (cpm incorporated, % of control)			
		+RNA (1o ug)	-RNA	-RNA +DNA (1o ug)	+RNA $-Mn^{++}$ $+Mg^{++}$
(^{3}H)ATP	(Mg^{++})	1oo	7.5	2.2	1oo
(^{3}H)ADP	(Mg^{++})	31.4	-	-	-
(^{3}H)ADP +creatinphosphate +$_3$kinase	(Mg^{++})	136.8	-	-	-
(^{3}H)ATP	(Mn^{++})	1oo	8.o	1.2	2.8
(^{3}H)ADP	(Mn^{++})	4o.3	-	-	-
(^{3}H)ADP +creatinphosphate +$_3$kinase	(Mn^{++})	117.2	-	-	-
(^{3}H)UTP	(Mn^{++})	1oo	7.8	1.2	o.o
(^{3}H)UDP	(Mn^{++})	o.o	-	-	-
(^{3}H)GTP	(Mn^{++})	1oo	o.o	11.o	8.3
(^{3}H)GDP	(Mn^{++})	o.o	-	-	-
(^{3}H)CTP	(Mn^{++})	1oo	7.o	5.4	6.4
(^{14}C)CDP	(Mn^{++})	o.o	-	-	-

of the control for the Mg^{++}-dependent poly(A) polymerase and 4o% of the control for the Mn^{++}-dependent poly(A) polymerase. Formation of (^{3}H)ATP from (^{3}H)ADP by the addition of creatinphosphate and creatinphosphokinase restores the synthesis of poly(A) and even exceeds that of the control (Table I). The synthesis of ribohomopolymers is dependent on the presence of exogeneous RNA; DNA is not accepted as primer-polynucleotide. Furthermore, the incorporating activity of the Mn^{++}-dependent polymerases is almost completely blocked if Mn^{++} is replaced by Mg^{++} (Table I). Depending on the type of ribohomopolymer synthesized in vitro, the average chain lengths varied from 1o - 7o nucleotides (not shown); these results are very similar to those reported previously (8).

It was the aim of this investigation to confirm and extend our previous observations (8) which had inferred by indirect evidence the existence in nuclear extracts of a Mg^{++}-dependent poly(A) polymerase and a Mn^{++}-dependent enzyme system capable of synthesizing poly(A), poly(U), poly(G) and poly(C). The Mn^{++}-dependent

ribohomopolymer polymerases have to our knowledge not yet been described and may represent a novel enzyme system.Apart from the ribohomopolymers synthesized by the Mn^{++}-dependent polymerases the formation of ribocopolymers may also be possible (8).At present it can only be speculated about the functional significance of the Mn^{++}-dependent polymerases,a possible involvement in the actinomycin - resistant RNA synthesis in the nucleoplasm (12,13) is conceivable.

It remains to be determined which of the poly(A) polymerases is responsible for the addition of poly(A) to the HnRNA;if both enzymes are involved,this process might be more complicated than suggested so far.

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