A NEW FORM OF MAMMALIAN DNA-DEPENDENT RNA POLYMERASE AND ITS RELATIONSHIP TO THE KNOWN FORMS OF THE ENZYME

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1. Introduction

Extraction of mammalian cell nuclei with a buffer of high salt concentration together with sonication yields the form I (α-amanitin resistant) and II (α-amanitin sensitive) DNA-dependent RNA polymerase in approximately 1:2 proportions [1–3]. We report here that low salt extraction yields mainly form I polymerase. A low salt (fig. 1) and a high salt (fig. 2) extraction and purification procedure have been devised based on previously reported methodology [1–4]. The form I polymerase obtained by low salt extraction can be separated into two species, Ia and Ib, of roughly equal activity by phosphocellulose chromatography. The two forms differ in their relative activities with Mg^{2+} and Mn^{2+} ions and on native and denatured DNA. Form Ia is the form I enzyme found previously [1, 2]. Form Ib is present in only small amounts in unextracted nuclei but this level is enhanced up to almost that of Ia during low salt extraction. The implications of this finding with respect to interconversion between the various polymerase forms are investigated further and a model of the nuclear transcription system presented which explains these and other reported results.

2. Materials and methods

RNA polymerase was assayed, normally for 10 min at 37°, as by Chesterton and Green [5] with 0.04 mM ^{3}H-UTP (56.7 cpm/pmole) and using Whatman GF-C filters to trap the RNA product. The low salt (form I) assay contained 6 mM $MgCl_2$ and 0.18 M KCl whilst in the high salt (form II) assay these were replaced with 1 mM $MnCl_2$ and 0.13 M $(NH_4)SO_4$. α-Amanitin was used at 0.1 μg/ml. When assaying nuclei, the filters were digested in 0.5 ml hyamine for 20 min at 65° before counting for radioactivity. One unit of enzyme incorporates 1 pmole of UTP into RNA per min. Separation and purification of the polymerases from nuclei was carried out as shown in figs. 1 and 2 using the methods reported by others [1–4, 6].

3. Results and discussion

3.1. *RNA polymerase forms extracted from nuclei at high and low salt concentration*

A comparison of the properties of the various polymerase forms purified via the schemes in figs. 1 and 2 with those of the polymerase activity in the crude low salt nuclear extract (table 1) shows that low salt extraction yields mainly form I polymerase. Small amounts of form II (less than 10% of total ac-

Table 1
Properties of the dialysed low salt nuclear extract and of the various forms of purified RNA polymerase.

Enzyme	Low salt (form I) assay						High salt (form II) assay					
	Mg^{2+} (%)	Mn^{2+} (%)	Ratio (Mn^{2+}/Mg^{2+})	No DNA (%)	Denatured DNA (%)	Plus α-amanitin (%)	Mg^{2+} (%)	Mn^{2+} (%)	Ratio (Mn^{2+}/Mg^{2+})	No DNA (%)	Denatured DNA (%)	Plus α-amanitin (%)
Dialysed low salt extract	100	139	1.4	4.0	7.7	99.5	48.4	124	2.6	10.8	125	77.5
Ia from low salt extract	100	109	1.1	0.4	4.0	98.5	4.2	44.1	10.4	0.5	8.3	43.3
Ib from low salt extract	100	68.2	0.7	0.9	10.2	97.7	10.9	67.3	6.2	0.6	12.2	69.5
II from high salt extract	9.4	33.8	3.6	0.8	8.6	0	17.7	100	5.6	0.3	182	1.3
Ia from high salt extract	100	460	4.6	0.4	9.0	98.9	5.1	43.4	8.5	0	6.1	41.7
Ib from high salt extract	100	196	2.0	0	39.5	111	10.4	48.4	4.7	1.6	21.5	51.9

Activities are shown as a percentage of the pmoles UTP incorporated in the normal assay for each fraction. This value, reading from top to bottom, was as follows: 46.7; 230; 275; 7.24; 12.2 and 11.5. The Mg^{2+} or Mn^{2+} ions were each used alone in the absence of the other. Low and high salt assays contained Mg^{2+} and Mn^{2+} ions, respectively, where not indicated.

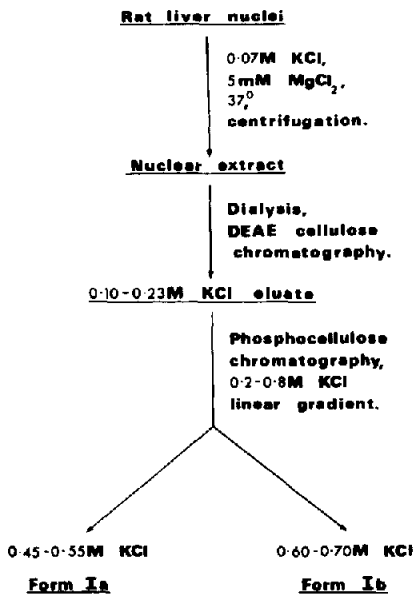

Fig. 1. Low salt concentration extraction and the further purification of nuclear RNA polymerases. Buffers all contain 0.01 M tris-HCl, pH 8, and 1 mM dithiothreitol. Forms Ia and Ib are purified 75 and 400 fold, respectively, over the nuclear level.

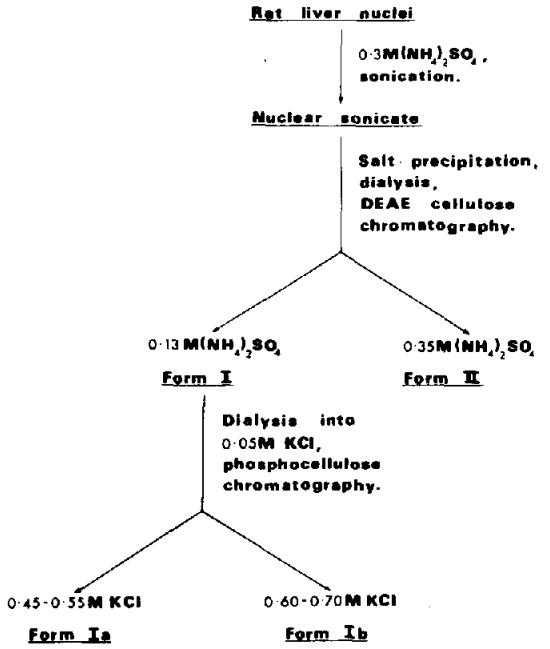

Fig. 2. High salt concentration and further purification of nuclear RNA polymerases. Forms Ia, Ib and II are purified 130, 40 and 20 fold, respectively, over the nuclear level.

tivity) can be detected in the extract by DEAE cellulose chromatography as reported by Roeder and Rutter [2]. In contrast, the high salt extraction method (fig. 2) yields more form II than I (fig. 3A).

Separation of form I polymerase into two species has not been reported before. The two enzymes, termed Ia and Ib, elute from phosphocellulose with 0.45–0.55 M KCl and 0.6–0.7 M KCl respectively, whereas form II remains as a discrete component in this system (fig. 4). Low salt extraction yields equivalent amounts of Ia and Ib (fig. 4a) whilst high salt extraction gives a form I fraction containing only a low level of Ib (fig. 4D, 0 min). Form Ia therefore is the form I enzyme reported previously [1, 2]. The two form I polymerases have small but reproducible differences, form Ia being more active with Mn^{2+} ions but less active on denatured DNA than Ib (table 1). All forms as purified require added DNA and all form I enzymes are most active on native DNA in contrast to form II which prefers denatured DNA.

High salt extraction of separated nucleoli and nucleoplasm followed by DEAE cellulose chromatography of the polymerases (fig. 3) confirmed an earlier report that nucleoli contain only form I and that form II is restricted to the nucleoplasm [2]. The form I found in the nucleoplasm could be due to the presence of nucleolar fragments. Analysis of the nucleolar and nucleoplasmic form I enzymes on phosphocellulose showed that form Ib is present at a low level (15% of total form I activity) in the nucleolus (fig. 4C).

3.2. *The possibility of interconversion*

High or low salt extraction, then yields mainly form Ia plus either II or Ib respectively. That this phenomenon could be due to interconversion was investigated further. It was found that form Ib was produced during low salt extraction (fig. 4D) whilst the overall levels of forms I and II remained high and roughly constant (table 2). In these experiments equal suspensions of nuclei in the low salt extraction buffer were extracted and purified by the high salt method (fig. 2) after incubation for various periods at 37°. The proportion of Ib in the form I fraction increased from 10% to 35% in 20 min and remained unchanged thereafter. Although the levels of forms I and II fluctuate, the fact that form II does not decrease below

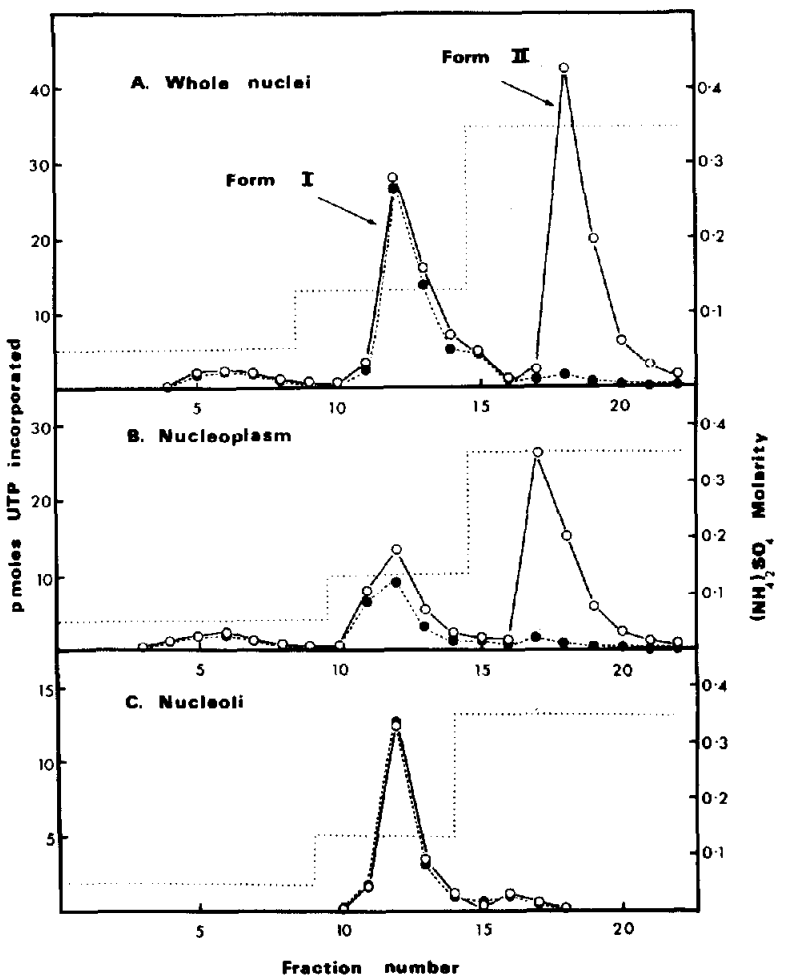

Fig. 3. Intranuclear localisation of form I and II. The polymerases in TGMED buffer (2) were separated on a 1.5 X 4 cm DEAE cellulose column, collecting 2.5 ml fractions of which 0.05 ml was assayed. The 0.05 M and 0.13 M $(NH_4)_2SO_4$ eluates and the 0.35 M $(NH_4)_2SO_4$ eluate were assayed by the low salt (using 0.04 M $(NH_4)SO_4$ in place of KCl and high salt methods, respectively. o——o, no α-amanitin; •- - - - - - - - - - •, plus α-amanitin.

the zero time level and that the total amount of form I has not changed after 20 min extraction indicate that, unless Ib is formed from an inactive source and that Ia is destroyed concominantly, interconversion between the two form I species occurs.

As shown above (table 2), form II remains at a high level during low salt extraction if the nuclear suspension is extracted finally at high salt concentration. However, if the intranuclear activity of form II is assayed directly during low salt incubation (fig. 5) an 80% drop in activity is seen over the first 30 min. This loss might represent conversion to Ib. We have found that form II is stable at high salt concentrations which inactivate purified form I enzymes. Possibly therefore, form II is regenerated from Ib during high salt extraction, thereby explaining the results in table 2. Incubation for 60 min in the low salt extraction buffer results in a decrease of 280 and 1210 enzyme units of intranuclear form I and II respectively, whilst 460 units of form I are extracted. Thus the loss of form I does not balance that extracted. Form II exhibits about 10% activity under form I assay conditions (table 1) hence a 10 fold

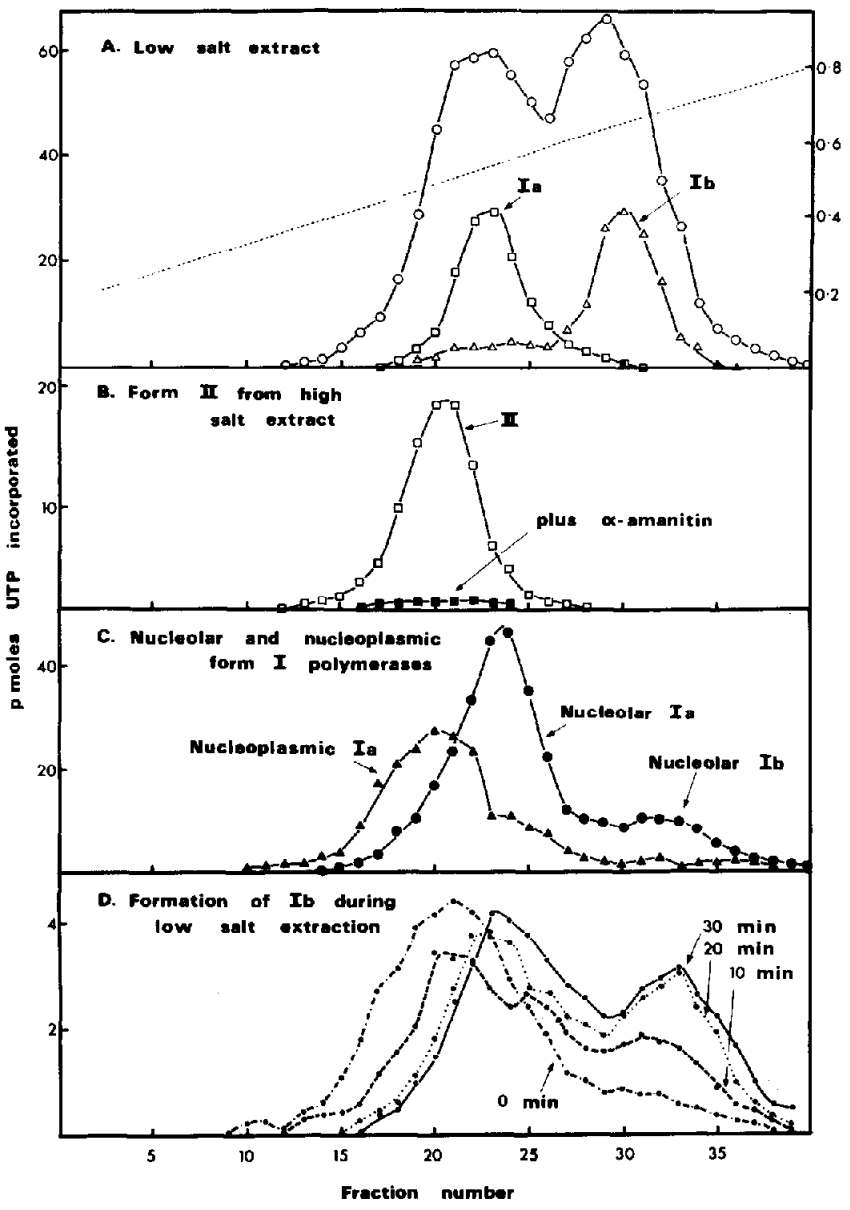

Fig. 4. Phosphocellulose column separation of forms Ia, Ib and II. The polymerases in 0.01 M tris-HCl, pH 8, 0.1 mM EDTA, 1 mM dithiothreitol, and 40% glycerol were eluted from a 1.5 X 6 cm column of phosphocellulose with a linear gradient of KCl, collecting 1.5 ml fractions of which 0.02 ml were assayed. Closed symbols indicate that α-amanitin was included in the assay. A) Initial separation of forms Ia and Ib as indicated in fig. 1 and rechromatography of the peak fractions. B and C) As indicated. D) Separation of form I polymerases extracted by the high salt method from equal volumes of low salt nuclear suspensions incubated for various periods at 37°.

reduction of activity might occur on conversion to Ib. Making this assumption, the transformation of all the form II to Ib can be accounted for in terms of the form I extracted. This mechanism would account for the fact that high or low salt extraction yields form Ia together with II or Ib respectively, although no direct

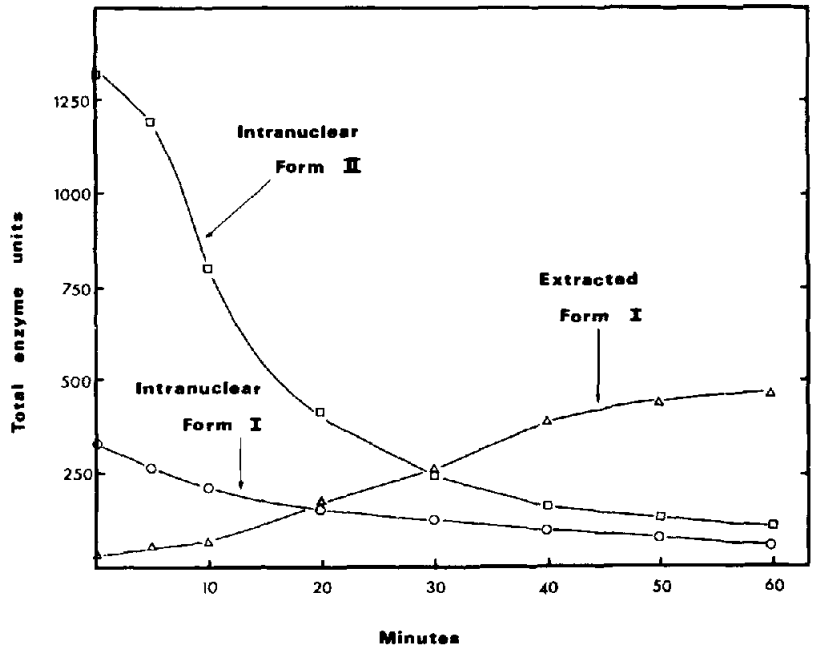

Fig. 5. The levels of active intranuclear forms I and II and extracted form I during low salt extraction of nuclei.

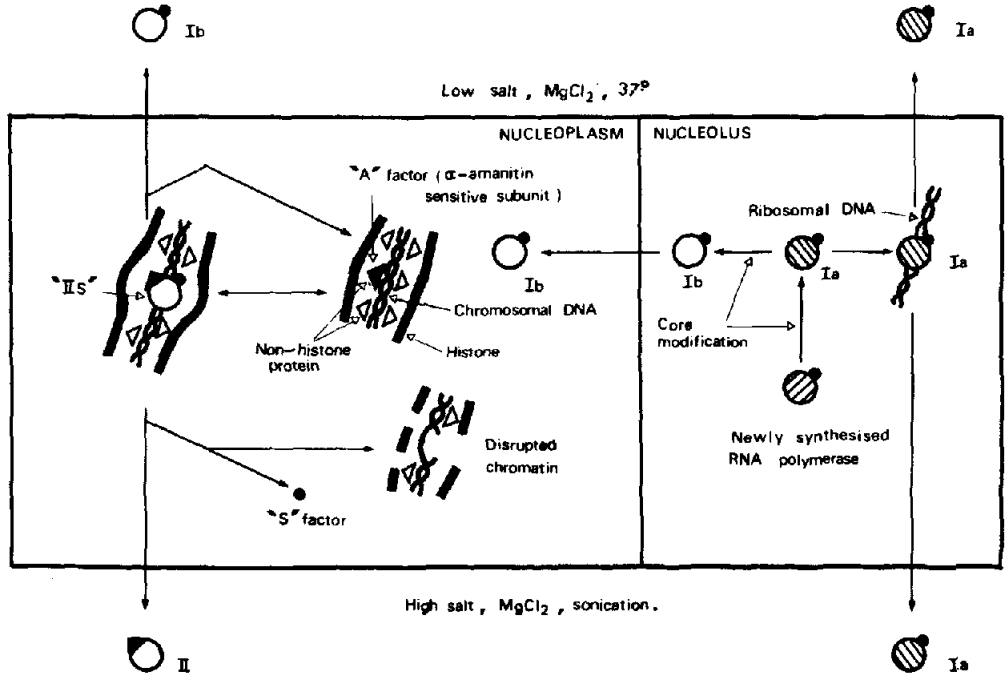

Fig. 6. Suggested model for the rat liver nuclear DNA transcription system and its response to low and high salt extraction.

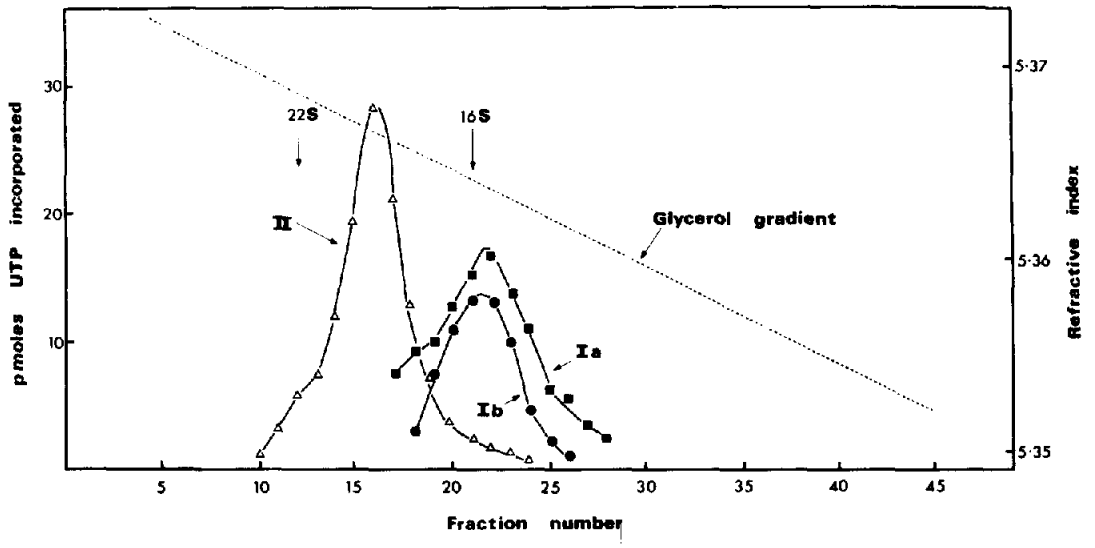

Fig. 7. Sedimentation behavior of forms Ia, Ib and II in glycerol gradients. Gradients (15 ml) were centrifuged for 37 hr at 75,000 *g*. Markers were the two forms of *Escherichia coli* β-galactosidase.

evidence for interconversion has been obtained as yet.

3.3. *Role of the 'S' factor*

The 'S' factor was isolated by Stein and Hausen [7] from calf thymus and was shown to combine with form II and stimulate transcription by this enzyme on native DNA by up to 10 fold. This could be interpreted as restoring normal form I properties to the form II enzyme which *in vitro* preferentially transcribes denatured DNA (table 1). Since, in the nucleus form II must transcribe native DNA, *in vivo* the enzyme probably functions as form 'IIS' (form II with bound 'S' factor). The factor has also been isolated from rat liver [8]. It stimulates form II but not form I [8].

3.4. *Explanatory model*

These results may be summarised as follows: (a) low salt extraction of nuclei yields mainly form Ia plus Ib whilst high salt extraction gives form Ia plus II; (b) during low salt extraction, form Ib is generated probably from Ia and also possibly from form II; (c) form I enzymes are specific for native DNA whereas form II prefers denatured DNA. Although other explanations are possible, taken together with the discovery of the 'S' factor and its selective stimulation of form II, these findings correlate well with the speculative model shown in fig. 6 which is self-explanatory.

The α-amanitin sensitive subunit ('A' factor) of form II, of which there are probably many species, is shown as a non-histone protein. Gilmour and Paul [9] have shown that the activity of chromosomal cistrons can be restricted specifically by such proteins. Perhaps they may also exert a positive control as indicated. That α-amanitin inhibits elongation of growing RNA chains rather than their initiation *in vitro* [3] shows that the 'A' factor is involved in the process of RNA synthesis as well as possible gene activation. It is also suggested that form I polymerases, because of their preference for native DNA (table 1) and their lack of interaction with the 'S' factor [8] actually contain this factor as does the enzyme transcribing nucleoplasmic genes ('IIS'). The factor, however, is lost from 'IIS' when form II is detached from the chromatin by the high salt conditions. The fact that the high salt extracted form I enzymes have more than double the activity of the low salt extracted form I polymerases on denatured DNA indicates that some of these enzyme molecules may also have lost the 'S' subunit (table 1). This might account for their high activity with Mn^{2+} ions, a property character-

Table 2
Effect of incubation at 37° on the levels of form I and form II polymerases, as measured after purification by the high salt extraction method, in suspensions of nuclei in the low salt extraction buffer.

Polymerase	UTP incorporation (pmoles) Extraction time (min)				
	0	5	10	20	30
Form I	372	352	440	400	335
Form II	702	861	797	758	736

Batches of nuclei from 36 g liver in 18 ml suspension were extracted for each time point and the polymerase forms separated on DEAE cellulose. Total UTP incorporation/fraction/min is shown. Forms I and II were measured as α-amanitin resistant and sensitive activities, respectively.

istic of form II which does not contain the 'S' subunit (table 1). In contrast, low salt extraction allows the reversible interchange between chromatin-bound form 'IIS' and free Ib.

As form Ib lacks the predicted 'A' factor it should be smaller than form II. Sedimentation of the enzymes in glycerol gradients showed this to be correct (fig. 7), forms Ia, Ib and II having S values of 16, 16 and 18.5 respectively. Since the 'S' factor is a small heat stable protein [7], its presence or absence might not affect the sedimentation rates to a measurable extent. This data therefore indicates the 'A' factor to be a large protein. Preliminary subunit analysis by SDS-polyacrylamide gel electrophoresis of the form II enzyme (purified 900-fold from nuclei on DEAE-cellulose, phosphocellulose and a glycerol gradient) agrees with the structure that might be predicted from the sedimentation data and the model. The major polypeptides detected, by comparison with marker proteins [11], have molecular weights of 60,000, 100,000, 160,000 and 200,000 in a molar ratio of 1.92:1.04:2.0:0.86, respectively. The overall molecular weight (740,000) agrees with that expected from the S value (about 750,000). This structure resembles that of *Escherichia coli* RNA polymerase [10] except for the presence of a unique mammalian high molecular weight subunit which is probably the 'A' factor. The three lower molecular weight subunits are analogous to the α, σ and β polypeptides, respectively, of the bacterial enzyme. Definition of the common subunits possessed by the different mammalian RNA polymerases awaits complete purification of forms Ia, Ib and II.

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