Comparison of the Multiple Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase Forms of Whole Rat Liver and a Minimal-Deviation Rat Hepatoma Cell Line

By C. JAMES CHESTERTON and STUART M. HUMPHREY

*Department of Virology, Royal Postgraduate Medical School,
Hammersmith Hospital, London W.12, U.K.*

and PETER H. W. BUTTERWORTH

*Department of Biochemistry, University College London,
Gower Street, London W.C.1, U.K.*

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To investigate the possibility that the pattern of multiple DNA-dependent RNA polymerases of an animal cell exerts a controlling influence on its nature, the activities of these enzymes were compared in differentiated rat liver and in a rapidly growing minimal-deviation rat hepatoma cell line by using established techniques of enzyme extraction, separation and determination. Relative to the DNA content of the tissues, RNA polymerase activities of forms AI, AII and B were approx. ninefold, twofold and twofold higher respectively in the cell line than in the liver. Tests indicated that these results could not be explained by differences in extraction efficiency or by the presence of unbound inhibitors or stimulators of polymerase activity in the final enzyme preparations. New forms of the enzyme were not detected in either tissue. The significance of these findings with respect to the possible role of multiple RNA polymerases in the control of cellular activities is discussed.

Multiple DNA-dependent RNA polymerases (nucleoside triphosphate–RNA nucleotidyltransferase; EC 2.7.7.6) have been detected in several differentiated animal tissues (Blatti *et al.*, 1970; Chambon *et al.*, 1970; Chesterton & Butterworth, 1971*a,b*). Five different forms have been reported. By the nomenclature proposed by Keding *et al.* (1971), these are termed AI, AII, AIII, BI and BII. The form A enzymes are insensitive to the drug α -amanitin whereas the B polymerases are inhibited in its presence. Forms AI and AII are located in the nucleoli of rat liver nuclei and have optimum activity in low-salt conditions with Mg^{2+} ions (Roeder & Rutter, 1970; Chesterton & Butterworth, 1971*b*). The others are found in the nucleoplasm and, except for the AIII enzyme, prefer high salt concentrations and Mn^{2+} ions (Roeder & Rutter, 1970; Lindell *et al.*, 1970). The properties of the mammalian AIII polymerase have not been reported. The physiological significance of the different ionic strength and bivalent ion concentration optima of the A and B polymerases is not yet known. Possibly the ionic environment of the nucleoli and nucleoplasm in respect of these enzymes reflects the differences observed *in vitro*. The various forms of the enzyme can be separated by DEAE-cellulose, DEAE-Sephadex and phosphocellulose chromatography (Chesterton & Butterworth, 1971*a*; Lindell *et al.*,

1970; Keding *et al.*, 1971; Mandel & Chambon, 1971).

Recent work indicates that the AI and B polymerases have different gene specificities. In the *Xenopus* oocyte system, nucleolar ribosomal DNA appears to bind form AI polymerase but not the form B polymerases (Tocchini-Valentini & Crippa, 1970) suggesting that form AI is involved specifically in rRNA synthesis. Rat liver chromatin is transcribed by the B polymerases but not by form AI or AII polymerases (Butterworth *et al.*, 1971) indicating that the expression of nucleoplasmic genes is mediated by the form B enzymes alone. These findings would be expected from the known locations of the enzymes within the nucleus. Hence, it seems feasible to postulate that the nature of an animal cell may be controlled directly or indirectly by the activities of the various multiple DNA-dependent RNA polymerases which it possesses.

If this view is correct, it would be expected that differentiated cells and rapidly growing cells from the same source would have a different pattern of multiple polymerases. With the development of extraction and separation methods for these enzymes, this point is now open to experiment. We have therefore extracted and separated the DNA-dependent RNA polymerases from whole rat liver and from a hepatoma cell line. It is reported that although no new forms

of the enzyme were detected in the growing cells, they did contain a substantially higher amount of the form AI polymerase than is found in differentiated liver.

Experimental

Techniques are not yet available to separate the BI and BII polymerases from rat liver (Mandel & Chambon, 1971). Also, we have been unable to detect the AIII enzyme reported by Lindell *et al.* (1970). The separation procedures used here yield the AI, AII and combined B enzymes from rat liver. Materials and experimental technique were as described by Chesterton & Butterworth (1971*a,c*).

To give statistically significant results, the polymerases were extracted from five batches of rat liver and from five batches of minimal-deviation-hepatoma cells. The activities of the purified enzymes from each batch were determined separately. Each batch of rat liver weighed approx. 25g and was obtained from two male Wistar rats. Each batch of minimal-deviation-hepatoma cells (approx. 5×10^8 cells) was grown to near confluency in 2 litre glass roller bottles by using Eagle's BHK medium supplemented with 10% foetal calf serum (Reel & Kenney, 1968). The hepatoma cells are a cell line (H4-II-E) derived from the Reuber H-35 hepatoma (Pitot *et al.*, 1964). They retain many of the characteristics of normal liver cells and will induce the Reuber H-35 hepatoma when injected into rats. They were kindly supplied by Dr. I. Freshney (Beatson Institute for Cancer Research, Glasgow, U.K.).

The polymerases were extracted and separated as described below and in Scheme 1. Rat liver (approx. 12g batches) was cut up into small pieces and homogenized in 36ml of 0.32M-sucrose–3mM-MgCl₂–0.1mM-dithiothreitol by using glass homogenizers with a Teflon pestle (clearance was 0.15–0.25mm). This and subsequent procedures were carried out at 0°–4°C. Six strokes of the pestle were used to homogenize each batch with the pestle rotating at 1450rev./min. The homogenate was filtered through 3 layers of absorbant gauze, diluted 1.6 times with the homogenizing medium, and diluted 1.28 times with water. It was separated into nuclear and supernatant (extra-nuclear) fractions by centrifugation at 700g for 10min.

The nuclear pellet was resuspended in a volume of sonication buffer (0.01M-tris–HCl, pH8, 1M-sucrose, 5mM-MgCl₂, 1mM-dithiothreitol) equal to 0.5 times the weight of liver from which the nuclei were derived. Portions of 11ml were adjusted to 0.3M-(NH₄)₂SO₄ by addition of 0.9ml of 4M-(NH₄)₂SO₄ and were then sonicated 6 times for 10s, allowing 20s for cooling between sonications. A Branson Sonicator fitted with a medium probe was used at full power. Each portion was diluted with 24ml of TGMED

Scheme 1. Extraction and separation of the forms of DNA-dependent RNA polymerases

The methodology is described in the Experimental section and by Chesterton & Butterworth (1971*a*).

buffer [0.05M-tris–HCl buffer, pH8, 25% (v/v) glycerol, 5mM-MgCl₂, 0.1mM-EDTA and 1mM-dithiothreitol] and centrifuged at 30000g for 30min to remove precipitated nucleoprotein. The supernatant was adjusted to 100%-satd. (NH₄)₂SO₄ by the slow addition of 0.42g of solid (NH₄)₂SO₄/ml of supernatant with stirring under N₂. After stirring for a further 30min, the precipitated protein was sedimented at 30000g for 30min, redissolved in the minimal volume of TGMED buffer containing 0.04M-(NH₄)₂SO₄, and dialysed against 4 litres of this buffer overnight. The dialysed solution, which contained precipitated protein, was cleared by centrifugation at 30000g for 30min. This solution was termed the extract.

The supernatant (extra-nuclear fraction) to the nuclear pellet was adjusted to the composition of the sonication buffer by the addition of the relevant volumes of 2.4M-sucrose, 1M-tris–HCl buffer, pH8, 1M-MgCl₂ and 1M-dithiothreitol before sonication at 0.3M-(NH₄)₂SO₄; the subsequent procedure was as described above.

Each batch of hepatoma cells, after release from

the glass with 0.02% (w/v) EDTA at 37°C, was harvested by light centrifugation and resuspended in 5ml of the sonication buffer. This gave a total volume of approx. 10ml, which was sonicated in 0.3M-(NH₄)₂SO₄. The sonication procedure and the method for the subsequent extraction of polymerases were as described above for the liver nuclear pellet.

Each preparation was loaded on a column (6cm × 2.5cm for liver extracts and 6cm × 1.5cm for hepatoma cell extracts) of DEAE-cellulose. The exchanger was equilibrated before use with TGMED buffer containing 0.04M-(NH₄)₂SO₄. After the column had been washed with this buffer system until no further protein was eluted, the form A and B polymerases were eluted successively with TGMED buffer containing 0.13M- and 0.35M-(NH₄)₂SO₄ respectively (Chesterton & Butterworth, 1971b). The column-chromatographic technique was as described by Chesterton & Butterworth (1971a,c). After dialysis for 3h against two changes (each of 2 litres) of 0.01M-tris-HCl buffer, pH8, 25% (v/v) glycerol, 1mM-EDTA, 1mM-dithiothreitol and 0.05M-KCl, the AI and AII polymerases were separated by phosphocellulose chromatography as described previously (Chesterton & Butterworth, 1971a). All preparations could be stored at -70°C without loss in activity after adjustment to 40% (v/v) glycerol.

The enzymes were assayed in duplicate under optimum conditions (Chesterton & Butterworth, 1971b) for 10min at 37°C with rat liver DNA as template by the standard method reported previously (Chesterton & Butterworth, 1971a). One unit of enzyme incorporates 1pmol of UMP into RNA/min. DNA and protein were determined by the methods of Burton (1956) and Lowry *et al.* (1951) respectively.

Results and Discussion

By using the ion-exchange systems shown in Scheme 1, all the detectable RNA polymerase activity extracted from the hepatoma cells was fractionated into forms AI, AII and B, indicating that no new forms of the enzyme are present in the hepatoma cells. The extraction technique when applied to liver nuclei gave apparent recoveries of both form A and B polymerases of about 30%. With hepatoma cells these values were 1500 and 1900% respectively. The significance of such values is obviously dubious since it is difficult to assess the integrity of enzyme assays carried out with crude systems such as liver nuclei and cell sonicates.

Fig. 1 shows that the corresponding forms of the polymerase from liver and hepatoma cells sedimented through a glycerol gradient to similar positions relative to the 13S marker, *Escherichia coli* DNA-dependent RNA polymerase (kindly provided by Dr. L. V. Crawford, Imperial Cancer Research Fund

Laboratories, London W.C.2). Form B from both sources appeared to be slightly heavier than the form A enzymes, which sedimented at almost the same rate as the marker. In agreement with earlier reports (Chambon *et al.*, 1970; Blatti *et al.*, 1970), these results indicate that the mammalian polymerases have a molecular weight similar to that of the bacterial enzyme, i.e. about 500000. The hepatoma cellular polymerases exhibited similar properties with respect to bivalent ion and salt concentrations to their counterparts in rat liver (Chesterton & Butterworth, 1971b). Also, the effects of substituting denatured DNA for native DNA and of including α -amanitin (kindly provided by Professor T. Wieland, Max-Planck Institute, Heidelberg) in the enzyme assay mixture were similar for corresponding enzymes from cells and liver (Table 1). These findings indicate that no new forms of DNA-dependent RNA polymerases are present in the hepatoma cells, although the presence of different members of each type (AI, AII and B), especially with respect to the B polymerases, cannot be eliminated until samples pure enough for subunit-structure analysis are available.

The liver extranuclear fraction yielded significant amounts of RNA polymerase activity only in the form AI and B preparations. Tests showed that whereas the liver nuclear and hepatoma cellular suspensions yielded enzymes that were dependent for activity on added DNA, required the presence of all four nucleoside triphosphates, and were inhibited by actinomycin D and by pre-treatment of the template with deoxyribonuclease, the liver extranuclear enzyme preparations contained substantial amounts of assayable activity that was independent of these factors (Table 1). In common with the product of the other enzymes, that of the contaminating activity was sensitive to alkali and ribonuclease. Possibly the DNA-independent activity was catalysed by some form of homopolyribonucleotide polymerase (Klempner, 1963a,b). Since the DNA-dependent RNA polymerases present in the extranuclear fraction have almost certainly been washed from the nucleus, it was not deemed necessary in the present investigation to purify them from the contaminating activity to examine their properties in isolation.

The activity of each DNA-dependent RNA polymerase extracted from rat liver and hepatoma cells, relative to the amount of DNA present in the original liver homogenate or cell suspension, is shown in Table 2. The relative activities of the form AII and B enzymes are about twofold enhanced in hepatoma cells and that of the AI polymerase is over eightfold higher in the hepatoma cell line than in rat liver. Whereas the difference between the respective form AII and form B activities in liver and cells can be accounted for by the experimental error inherent in the determinations, that between the form AI activities cannot be similarly dismissed and is highly

Fig. 1. Relative sedimentation rates of the RNA polymerase forms

(a) Rat liver nuclear enzymes. Each gradient contained approx. 60 enzyme units of the polymerase under test. (b) Minimal-deviation-hepatoma cellular enzymes. Each gradient contained approx. 30 enzyme units of the polymerase under test. The enzymes were sedimented in parallel 6ml 10–30% glycerol gradients containing 0.05M-tris-HCl buffer, pH 8, 0.1mM-EDTA, 1mM-dithiothreitol and 0.1M-(NH₄)₂SO₄ for 7h at 260000g in the MSE 3 × 6.5ml titanium swing-out rotor of the MSE 65 ultracentrifuge. One gradient of the three that could be centrifuged simultaneously always contained *E. coli* DNA-dependent RNA polymerase as a 13S marker (Richardson, 1966). Fractions (0.2ml) were collected and assayed for polymerase activity. The total enzyme units per fraction are shown. ●, Form AI; ■, form AII; ○, form B; □, *E. coli* RNA polymerase; ----, refractive index.

significant. It is noteworthy that whereas the form A polymerase fraction from purified rat liver nuclei contains only about 10% of form AII (Chesterton & Butterworth, 1971b), in the crude rat liver nuclear fraction this percentage is about 35 (Table 2). The difference is probably due to the fact that form AII is lost from the nuclei at a faster rate than form AI under low salt conditions (C. J. Chesterton & P. H. W. Butterworth, unpublished work). Hence during the further purification of nuclei probably more form AII than form AI is washed out and lost.

In an effort to check whether or not the extraction technique might artifactually generate apparent differences in the enzyme contents, rat liver nuclei and hepatoma-cell sonicates were assayed, by using differential salt and bivalent-ion conditions (Chesterton & Butterworth, 1971b) and α -amanitin, which inhibits only form B, to select for either form A or B activity. The A/B activity ratio was 0.75 in rat liver nuclei and 3.3 in sonicated hepatoma cells. These values are 0.76 and 3.6 respectively when calculated for the separated enzymes (Table 2).

Table 1. *Properties of the RNA polymerase forms extracted from rat liver and minimal-deviation-hepatoma cells*

The test incubations, which were carried out as described previously (Chesterton & Butterworth, 1971a), contained the following amounts of the relevant enzyme: liver nuclear AI, AII and B, 7.0, 6.0 and 11.0 units respectively; liver extranuclear AI and B, 0.95 and 6.8 units respectively; hepatoma cellular AI, AII and B, 5.6, 0.54 and 2.3 units respectively.

RNA polymerase activity of enzyme preparations								
Conditions	Liver nuclear			Liver extranuclear		Hepatoma cellular		
	AI	AII	B	AI	B	AI	AII	B
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Normal	100	100	100	100	100	100	100	100
Minus ATP	1	1	11	45	50	9	3	10
Minus CTP	1	0	3	41	76	0	5	3
Minus GTP	3	1	3	29	50	0	3	3
Minus DNA	0	0	0	60	300	7	10	15
Minus DNA, plus denatured DNA	9	40	200	120	650	19	25	580
Plus deoxyribonuclease	0	0	0	41	80	4	12	2
Plus ribonuclease	3	5	12	4	6	4	3	9
Plus alkali	0	0	0	0	0	0	0	0
Plus actinomycin D	0	0	4	42	74	5	8	3
Plus α -amanitin	99	111	2	98	100	101	95	11

Table 2. *Total DNA-dependent activity of the RNA polymerase forms extracted from rat liver and minimal-deviation-hepatoma cells*

The mean value for five experiments is shown for each enzyme together with the s.d. The weight of liver tissue and number of hepatoma cells taken for each experiment were as described in the Experimental section. The values were calculated relative to the amount of DNA present in the original liver homogenate or cell suspension and therefore are comparable. As described in the text, it is not possible to measure reliable recoveries of enzyme activity. The mean DNA and protein contents of the liver batches were 44 ± 7.7 mg and 1320 ± 350 mg respectively. These values for the hepatoma cell batches were 11.0 ± 2.1 mg and 321 ± 144 mg respectively.

Source	RNA polymerase activity (enzyme units/10mg of DNA)		
	AI	AII	B
Liver nuclear fraction	66 ± 14	35 ± 7	132 ± 38
Liver extranuclear fraction	25 ± 6	low*	low*
Hepatoma cells	787 ± 187	67 ± 38	237 ± 98
Ratio hepatoma/liver	8.6	1.9	1.8

* Small amounts may be present but are difficult to measure accurately owing to the presence of DNA-independent activity (see Table 1).

Taking into consideration the doubtful validity of polymerase assays carried out in crude systems, the similarity of the respective ratios does suggest that the results in Table 2 do reflect to some extent the amounts *in vivo* of the polymerase forms.

Since the enzyme preparations are not pure, the apparently different AI activities could be produced by unknown inhibitors (such as nuclease) or stimulators of detectable RNA polymerase activity. To investigate this possibility, form B purified 4800-fold from rat liver nuclei (Chesterton & Butterworth, 1971c) and shown to be free of ribonuclease and deoxyribonuclease (Butterworth *et al.*, 1971) was assayed in the presence and absence of each enzyme preparation (Table 3). None of the enzyme preparations stimulated the activity of the added form B, but a small degree of inhibition was observed in the mixtures containing the AI and B preparations from both cells and liver. This could be caused by traces of contaminating ribonuclease. To test the possibility that the difference between the liver and cell AI activities is caused by the presence of a factor acting specifically on the AI enzyme, form AI preparations from the above sources were assayed independently and in a mixed incubation (Table 3). Again only a small degree of inhibition of the additive activities was observed. These experiments show that the results in Table 2 cannot be explained in terms of factors affecting RNA polymerase activity unless they bind tightly to the polymerase and will not interact with the added enzyme. The

Table 3. Activity of mixtures of RNA polymerases from rat liver and from minimal-deviation-hepatoma cells

The enzymes were assayed under identical conditions alone and mixed with a second polymerase as indicated. All incubation mixtures involving form B contained 1 mM-MnCl₂ as the only bivalent ion present and 0.08 M-KCl and 0.08 M-(NH₄)₂SO₄. The AI polymerase incubation mixture contained 6 mM-MgCl₂ and 0.04 M-(NH₄)₂SO₄. The activity of the mixed enzymes is shown as a percentage of the sum of their activities alone. The amount of enzyme present per incubation was as follows: purified rat liver nuclear form B, 6.0 units; liver nuclear AI, AII and B, 1.9, 0.44 and 1.2 units respectively; hepatoma cellular AI, AII and B, 2.2, 0.65 and 1.0 units respectively. The mixture of form AI enzymes (line 3) contained 1.1 units of liver AI polymerase and 0.82 unit of hepatoma cellular AI polymerase.

Enzyme mixture	RNA polymerase (% of combined activities)		
	AI	AII	B
Liver nuclear polymerases plus purified form B	90	99	70
Hepatoma cellular polymerases plus purified form B	83	89	70
Hepatoma cellular form AI plus liver nuclear form AI	92	—	—

sigma factor, which has a strong affinity for the 'core' *E. coli* RNA polymerase and which allows the enzyme to transcribe certain bacteriophage T4 genes (Burgess *et al.*, 1969), is an admirable precedent for such a stimulating factor. A low-molecular-weight protein that enhances the activity of the B polymerases on native DNA has been isolated from calf thymus (Stein & Hausen, 1970) and from rat liver (Seifart, 1970). This, however, has no effect on the form A enzymes and cannot be demonstrated to bind tightly to the B polymerases (Seifart, 1970). Another possible explanation of the results is that the supposed factor is present at a concentration too low to produce a detectable stimulation or inhibition in the mixture but yet can still exert its effect in the preparation. This seems unlikely, however, when the difference in AI enzyme activities is almost an order of magnitude. The concentration of factor required to produce a ninefold stimulation or 90% inhibition would almost certainly affect any added polymerase.

The findings reported above show that, although

new types of DNA-dependent RNA polymerase activity cannot be detected in the actively growing cells tested, differences in the contents of the known polymerase forms present in rat liver and hepatoma cells do exist, particularly with respect to the form AI enzyme. Hence it is possible that multiple polymerases do play a role in controlling the nature of the cell. Since there is some evidence that the form AI enzyme binds specifically to ribosomal DNA in the nucleolus (Tocchini-Valentini & Crippa, 1970), a difference in the AI polymerase contents may only reflect different rates of rRNA synthesis. However, the following questions must then be posed. (a) Does the content of form AI control the rate of rRNA synthesis? (b) How does the rate of such synthesis affect the nature of the animal cell? It is noteworthy that the DNA-dependent RNA polymerase activity that is lost in rat prostate tissue immediately after castration is of the form A type (Mainwaring *et al.*, 1971). The activity is regained quickly if androgenic hormones are administered to the animals. This suggests that form A polymerase activity can be rapidly altered in response to environmental changes and, together with the results presented here, indicates that form AI may be an integral part of the cell's machinery for the control of its functions.

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