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Transcriptionally active RNA polymerases from Morris hepatomas and rat liver

ELUCIDATION OF THE MECHANISM FOR THE PREFERENTIAL INCREASE IN THE TUMOUR RNA POLYMERASE I

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The amount and/or activity of DNA-dependent RNA polymerases I, II and III from resting liver, regenerating liver and a series of Morris hepatomas (5123D, 7800, 7777, 3924A) were determined after extraction of the enzymes from whole tissue homogenates and subsequent fractionation by DEAE-Sephadex column chromatography. When compared with resting liver, the tumours exhibited a characteristic enzyme pattern in which polymerase I, but not II, was increased. The increase in RNA polymerase I was proportional to the tumour growth rates. Alterations in polymerase III were confined to the most rapidly proliferating hepatomas. By contrast, all classes of RNA polymerase were found to be increased during liver regeneration. Relative to resting liver, the fastest growing tumour, 3924A, exhibited the highest activities and/or amounts of RNA polymerase I (8-fold) and III (5-fold) per g of tissue. These alterations in the tumour RNA polymerases were reflected in corresponding increases in the transcriptionally active (bound or chromatin-associated) enzyme population. The mechanisms underlying the augmented synthesis of RNA *in vitro* by bound polymerase I from hepatoma 3924A were elucidated by product analysis. The results indicated that, relative to liver RNA polymerase I, the tumour enzyme produced more nascent RNA chains and elongated these chains at a faster rate. The number of 3′-termini, as measured by incorporation into uridine, was higher in the hepatoma even under conditions which prevented re-initiation, suggesting increased amount of transcriptionally active RNA polymerase I in the tumour.

Since the discovery of multiple species of RNA polymerases (EC 2.7.7.6) in eukaryotic cells (for reviews see Jacob, 1973; Chambon, 1975; Roeder, 1976; Jacob & Rose, 1980), elucidation of the probable regulatory roles of these enzymes in the synthesis of discrete classes of RNA has been a challenging problem. Increased production of a particular RNA species is not always accompanied by an increase in the corresponding transcribing enzyme. There are nonetheless several reports which indeed suggest a role for the RNA polymerases in control of gene expression. For example, the amount and/or activity of RNA polymerase I has been shown to be dramatically altered in response to various physiological stimuli which result in increased synthesis of precursor rRNA (Sajdel & Jacob, 1971; Sebastian *et al.*, 1973; Guilfoyle *et al.*, 1975; Jänne *et al.*, 1976; Lin *et al.*, 1978). Similar increases have also been observed in rapidly growing tumours (for a review, see Jacob & Rose, 1978). To date, the lack of appropriate control tissues has limited comparison of the RNA polymerases during normal and neoplastic growth. Previous studies in our laboratory have shown that RNA polymerase I purified from the isolated nuclei of the rapidly proliferating Morris hepatoma 3924A was increased 3–6-fold relative to the corresponding enzyme from resting or host liver (Rose *et al.*, 1976). Only polymerases I and II were thoroughly examined, since the bulk of RNA polymerase III, by virtue of its loosely bound state, was released during the preparation of pure nuclei.

The present investigation was undertaken (*a*) to develop a method for increased recovery of RNA polymerase III in the extracted enzyme, (*b*) to determine whether, as in other rapidly growing control tissues (Yu, 1975*b*; Jaehning *et al.*, 1975; Benz *et al.*, 1977), the amounts and/or activities of

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all three RNA polymerases were altered in the hepatomas, (*c*) to demonstrate if the observed alterations in RNA polymerases were related to the growth of the tumours, and (*d*) to elucidate the probable mechanisms underlying the changes in the activity of hepatoma RNA polymerase I observed *in vitro*.

Methods

Animals

Male rats bearing Morris hepatomas which had been implanted bilaterally into thigh muscle were used at various times after transplantation. Rats carrying the rapidly growing tumours 7777 (Buffalo strain) or 3924A (ACI strain) were decapitated 25–30 days after implantation, and those bearing the slow-growing hepatomas 5123D and 7800 (both Buffalo strain) were killed between 35 and 40 days. Since preliminary experiments revealed no quantitative differences in the major classes of RNA polymerases in livers of the tumour-bearing hosts or male Sprague–Dawley rats (results not shown), the latter strain was utilized as the source of both resting and regenerating liver. Tumour-bearing and control rats were matched as closely as possible with respect to age.

Extraction of RNA polymerases from whole tissue homogenates

Livers and hepatomas were quickly removed and exsanguinated in cold 0.25M-sucrose/0.9% NaCl. Necrotic and connective tissues were dissected off the tumours. Tissue (10g) was minced and homogenized directly in alkaline sonication buffer (5ml/g of original tissue): 50mM-Tris/HCl (pH 8.9), 1mM-$MgCl_2$, 0.1mM-EDTA, 2mM-dithiothreitol, 50mM-KCl, 0.5mM-phenyl-methanesulphonyl fluoride and 40% (v/v) glycerol. DNA-dependent RNA polymerases were then extracted by the procedure previously described for purified nuclei (Rose *et al.*, 1976). Briefly, the initial homogenate was sonicated in the alkaline buffer followed by precipitation of the enzymes with $(NH_4)_2SO_4$. The extract was centrifuged and the pellet obtained was suspended (2ml/g of original tissue) in Buffer A (Rose *et al.*, 1976), which consists of 50mM-Tris/HCl (pH 7.9), 5mM-$MgCl_2$, 0.1mM-Na_2EDTA, 0.5mM-dithiothretiol and 50% (v/v) glycerol, and dialysed overnight against the same buffer. The suspension was then centrifuged (80000**g** for 45min) and the supernatant retained. The viscous residue was taken up in a Buffer A without glycerol and sonicated. Glycerol was added to a final concentration of 30% (v/v) and the mixture was incubated at 37°C for 30min and re-centrifuged as above. The resultant supernatant was combined with that previously retained and subjected to DEAE-Sephadex column chromatography as described in the legend to Fig. 1 to resolve individual classes of RNA polymerase. The incubation step was found to be essential for complete extraction of the enzymes from whole tissue homogenates. The polymerase activity of the residual fraction remaining after the final centrifugation was negligible, which indicated virtually complete recovery of the enzymes from whole tissue by this procedure.

Extraction of bound RNA polymerases from whole tissue

The 'bound' enzyme fraction (Yu, 1975*a*) was obtained essentially by techniques previously reported (Lin *et al.*, 1976; Leonard & Jacob, 1977). Tissues were homogenized (8ml/g of tissue) in SKTM buffer [25mM-sucrose, 50mM-Tris/HCl (pH 7.6 at 20°C), 25mM-KCl, 10mM-$MgCl_2$], followed by centrifugation at 1000**g** for 10min to recover the crude nuclear pellet. This fraction was then gently resuspended in SKTM buffer, re-centrifuged, and the polymerases were extracted from the resulting pellet by the method outlined for whole tissue. This enzyme corresponds to the chromatin-associated polymerase population (Lin *et al.*, 1976). Since the amount of protein in this fraction was less than that for whole tissue extract, the sonication volume was decreased to 2–3ml/g of tissue and bovine serum albumin (0.5mg/ml) was added to the preparation before dialysis to ensure enzyme stability.

Assay of DNA-dependent RNA polymerase activity

RNA polymerase activity was determined by using assay conditions described earlier (Rose *et al.*, 1976) with native calf thymus DNA (Worthington) as template. Unless otherwise indicated, all samples were incubated at 30°C for 15min. Column fractions were assayed in the absence and presence of α-amanitin (0.5μg/ml). The polymerase activity of the enzymes pooled after DEAE-Sephadex chromatography was determined in the absence of α-amanitin and at low (0.5μg/ml) and high (133μg/ml) concentrations of the toxin in order to assess the contribution of each polymerase class. Enzyme activity insensitive to α-amanitin was attributed to polymerase I, and polymerases II and III were identified by complete inhibition at low and high concentrations of the toxin, respectively (Jacob *et al.*, 1970; Weinmann & Roeder, 1974). RNA polymerase I was measured in the presence of 30mM-$(NH_4)_2SO_4$, whereas polymerases II and III were assayed at 100mM-$(NH_4)_2SO_4$. Since enzymes extracted from hepatoma 3924A exhibited different salt optima (Rose *et al.*, 1976; B. W. Duceman & S. T. Jacob, unpublished work), the concentration of $(NH_4)_2SO_4$ in the assay of these enzymes was adjusted to 12.5mM for polymerase I or 50mM for polymerases II and III.

Estimation of the average chain length of RNA synthesized in vitro

RNA was synthesized by RNA polymerase I in the standard assay with [^{3}H]UTP as the labelled substrate. The reaction was terminated by the addition of 10% (w/v) trichloroacetic acid, followed by successive washings with 5% (w/v) trichloroacetic acid until no radioactivity could be detected in the supernatant. After the acid had been thoroughly drained off, RNA in the acid-insoluble fraction was hydrolysed for 18h in 0.3M-KOH. The labelled UMP and uridine residues in the hydrolysate were separated by t.l.c. on poly(ethyleneimine)–cellulose (Randerath & Randerath, 1967) as described by Rose & Jacob (1976), by using glass-distilled water in the first direction and 1.8M-LiCl in the second.

Determination of protein and DNA

The DNA content of whole tissue homogenates was determined by Burton's (1956) modification of the diphenylamine procedure. The inclusion of organic-solvent extractions to remove possible interferring substances (Munro & Fleck, 1966) from whole tissue homogenates did not alter the results. Protein content was determined by the modification of Lowry's procedure described by Bennett (1967).

Results

DNA-dependent RNA polymerases from whole-tissue homogenates

DNA-dependent RNA polymerases were isolated from 'resting' livers, regenerating livers at 18h after hepatectomy, and from Morris hepatomas 5123D, 7800, 7777 and 3924A as described in the Methods section. Fig. 1 represents the DEAE-Sephadex column-chromatographic profiles of RNA polymerases extracted from equivalent amounts of liver and hepatoma whole-tissue homogenates. As expected, both liver and tumour contained three classes of enzyme activity. These were detected by assay of column fractions in the presence or absence of α-amanitin (0.5μg/ml), thus allowing the emergence of class-III polymerase (see insets in Fig. 1).

The column profiles showed that, relative to resting liver, the proliferating tissues exhibited altered patterns of polymerase activity. Particularly striking was the extent of the changes in RNA polymerase I. For each growing tissue, the total measurable activity of polymerase I was elevated above that observed for the same enzyme isolated from equivalent amounts of resting liver. In addition, correlation between the amount and/or activity of this enzyme and tumour growth rate was evident. Thus, RNA

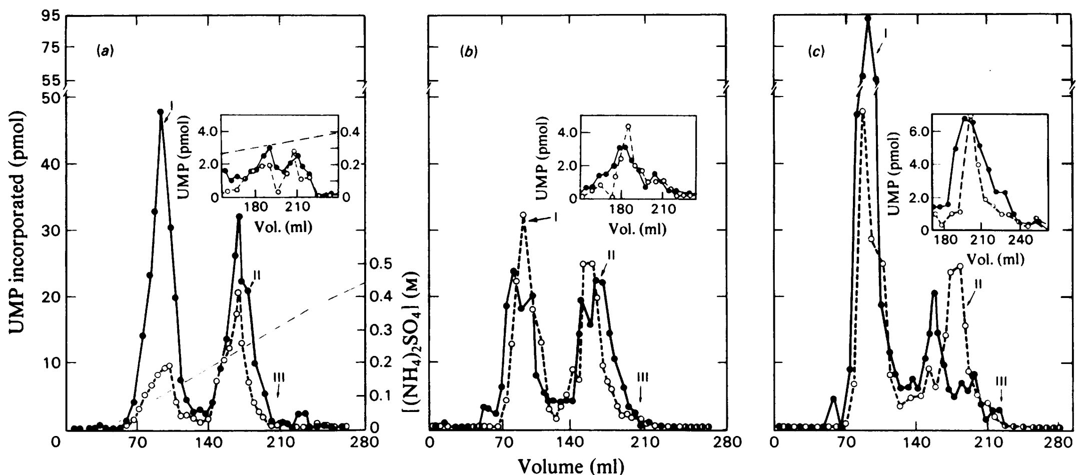

Fig. 1. *DEAE-Sephadex chromatography of RNA polymerases from Morris hepatomas*
RNA polymerases were extracted from 10g of tumour or control tissue (see the Methods section), and a portion of each preparation (equivalent to 2.5g of tissue) was fractionated by chromatography on a DEAE-Sephadex column (2.4cm x 14cm). The column was washed with 2 column bed volumes of Buffer A (Rose *et al.*, 1976) containing 10mM-$(NH_4)_2SO_4$, and the polymerase enzymes were eluted with a linear gradient (6 column volumes) of 10–550mM-$(NH_4)_2SO_4$ in Buffer A; 3ml fractions were collected and 40μl samples assayed as described in the Methods section. Polymerase-III activity (insets) was determined in the presence of α-amanitin (0.5μg/ml). (*a*) ○, Control liver; ●, regenerating liver. (*b*) Hepatomas 5123D (○) and 7800 (●). (*c*): Hepatomas 7777 (○) and 3924A (●).

polymerase I was greater in the more rapidly growing tumours 7777 and 3924A than in the more slowly growing hepatomas 5123D and 7800. Similar alterations in polymerases II or III were not evident from the column scans. In order to quantify enzyme activities further, fractions corresponding to each class of polymerase activity were pooled separately, dialysed and re-assayed at optimal salt concentrations (see the Methods section).

The results in Table 1 demonstrate that each tumour exhibited a substantial increase in RNA polymerase I, relative to liver, when results were expressed either per g of tissue or per mg of DNA. Furthermore, consistent with the column profiles (Fig. 1), polymerase I was highest in the most rapidly growing tumours, 7777 and 3924A (3- and 8-fold higher than in liver per g of tissue, respectively). When assayed under conditions of optimal ionic strength (see the Methods section), the class-III enzyme also exhibited considerable variation. On normalization to DNA content of the respective tumour, polymerase III was found to be increased in the fastest-growing hepatomas (approx. 2-fold) relative to 'resting' liver. On the contrary, there were no significant changes in the class-II RNA polymerase from any of the tumours examined. This was evident either from the column-chromatographic profiles or when enzyme activity was expressed per g or per mg of DNA. (The low value obtained for tumor 3924A most probably reflects the markedly higher DNA content of this hepatoma; Lea *et al.*, 1966; Nowell *et al.*, 1967). The lack of changes in polymerase II was particularly interesting when comparison was made with a normally proliferating tissue, regenerating liver. In that rapidly growing control tissue, all RNA polymerases extracted from whole-tissue homogenate were increased at 18h after hepatectomy (Table 1). This observation confirmed and extended the results for enzymes extracted from isolated liver nuclei after hepatectomy (Yu, 1975*b*). These results thus indicate that the pattern of polymerase response in a normally growing tissue was significantly different from that observed in several hepatomas.

Separation of 'bound' RNA polymerases from resting liver and from Morris hepatomas 3924A and 5123D

It has been demonstrated that DNA-dependent RNA polymerases exist in two populations within the nucleus (Yu, 1975*a*). One fraction, designated the 'bound' or template-engaged enzyme, is considered to represent the transcriptionally active RNA polymerase. A second fraction, 'free' enzyme, is not associated with chromatin and is therefore thought to be functionally inactive (Matsui *et al.*, 1976). The two populations of RNA polymerase I have been shown to exhibit independent responses to carcinogen administration (Leonard & Jacob, 1977) as well as to physiological stimuli (Yu, 1975*b*; Hentschel & Tata, 1977; Tillyer & Butterworth, 1978; Büning, 1978). Therefore it was decided to determine whether the increases in polymerase enzymes observed in Morris hepatomas relative to liver reflected changes in the functionally active population.

A crude nuclear residue which contained the bound polymerase population was isolated from liver and tumour tissues (see the Methods section). The enzymes in this fraction were solubilized and

Table 1. *DNA-dependent RNA polymerases from whole-tissue homogenates*

The DEAE-Sephadex column fractions containing RNA polymerases I, II or III (Fig. 1) were pooled separately, dialysed overnight against Buffer A (Rose *et al.*, 1976) and assayed as described in the Methods section. RNA polymerase activity was measured in triplicate at two concentrations of each enzyme. The results for resting liver and hepatoma 3924A were compiled from four separate experiments and the data for hepatoma 7800 from two extractions. One unit of enzyme activity corresponds to 1 nmol of UMP incorporated in 15 min.

Tissue	Activity (units/g of tissue)			Activity (units/mg of DNA)		
Polymerase ...	I	II	III	I	II	III
Resting	2.7	7.0	0.55	1.6	3.6	0.28
Regenerating liver	11.8	14.7	1.1	7.0	8.7	0.63
Morris hepatomas						
5123D	8.1	8.5	1.0	3.2	3.3	0.39
7800	6.1	7.4	0.56	2.7	3.2	0.26
7777	8.8	7.0	1.2	4.0	3.1	0.52
3924A	21.4	7.8	2.7	3.9*	1.5*	0.51*

* Hepatoma 3924A contains an average of 73 chromosomes, which results in higher DNA content/cell (Nowell *et al.*, 1967).

fractionated by DEAE-Sephadex column chromatography. It was evident (Fig. 2) that the increase in RNA polymerase I observed with whole-tissue extracts of the hepatomas (Table 1) reflected changes in the bound or active enzyme-I fraction. To quantify polymerase activities further, the enzymes were individually pooled and assayed at optimal salt concentrations (see the legend to Fig. 1 for details). Table 2 shows that the bound RNA polymerase I was indeed increased in hepatomas relative to liver (3–10-fold per g of tissue), the highest value being accorded to the fastest-growing tumour. As demonstrated for enzyme from whole-tissue extracts, polymerase-II activity in the bound population from the hepatomas was almost identical with that from the resting liver. On the other hand, only the more rapidly proliferating tumour 3924A showed an increase (3-fold) in the class-III enzyme activity over the control value. It should be noted that the DNA recovery did not vary appreciably between the experimental groups. This, coupled with similar RNA polymerase-II amounts/activities in liver and the hepatomas, rules out the possibility that differential recoveries of nuclei may contribute to the increased RNA polymerase I in hepatomas. These results therefore confirm and extend the observations drawn from estimation of the relative amounts and/or activities of the polymerases in whole tissue (Table 1). It could thus be concluded that (1) the pattern of activity of polymerases I, II and III in hepatomas differed from that observed in resting or growing livers, (2) the extent of the alterations measured in tumours was related to growth rate and (3) the increased hepatoma enzyme observed in whole-tissue homogenates reflected elevations in the activity and/or amount of the transcriptionally active RNA polymerase fraction.

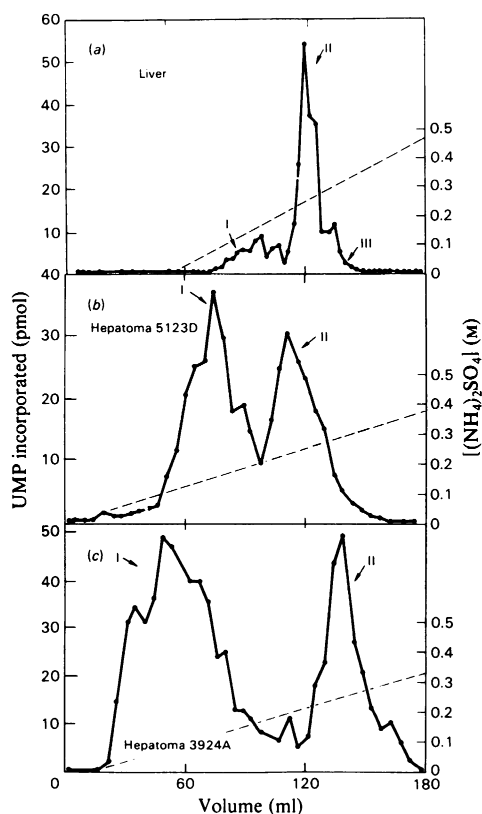

Fig. 2. *DEAE-Sephadex chromatography of the bound polymerase fraction from rat liver and Morris hepatomas*
Enzyme preparation extracted from 10g of tissue was applied to a DEAE-Sephadex column (1.5cm x 15cm). The column was washed as described in the legend to Fig. 1 and polymerases were fractionated with a linear gradient (12 column volumes) of 10–550mM-$(NH_4)_2SO_4$ in Buffer A (Rose *et al.*, 1976). Fractions were collected, assayed and pooled as described in the legend to Fig. 1. (*a*), Control liver; (*b*), hepatoma 5123D; (*c*), hepatoma 3924A.

Mechanisms for the increase in bound RNA polymerase I in Morris hepatoma 3924A

The increase in bound polymerase I in the hepatomas could be due either to an increased number of enzyme molecules or to activation of pre-existing enzyme. Activation, in turn, might result in an enhanced rate of re-initiation or the production of longer transcripts *in vitro* by the class-I enzyme. RNA synthesized under standard assay conditions, with $[^3H]UTP$ as the radiolabelled precursor will, on hydrolysis, yield labelled uridine (3′-termini) and uridine monophosphates (internal residues). The ratio of labelled uridylate to uridine will be an indication of the average RNA chain length. Assuming random termination, the number of nucleoside residues can provide a measure of the number of chains formed (Cox, 1976). To distinguish between these possibilities, RNA synthesized *in vitro* by bound polymerase I from resting liver or hepatoma 3924A was acid-precipitated and subjected to alkaline hydrolysis. Uridine and UMP were separated by t.l.c. on poly(ethyleneimine)–cellulose (see the Methods section). The results are presented in Table 3.

The average length of RNA chains syntheszied by the hepatoma RNA polymerase I was 4 times that formed by the corresponding enzyme from resting liver (327 and 79 nucleotides respectively). Several observations eliminate the possibility that contaminating ribonculeases or other factors in either polymerase-I preparation contribute to the differences in chain lengths of RNA synthesized *in vitro*. First, control and tumour polymerase-I preparations were allowed to synthesize RNA under

Table 2. *'Bound' DNA-dependent RNA polymerases from resting liver and from Morris hepatomas 3924A and 5123D*
Fractions containing the respective RNA polymerases obtained after a single DEAE-Sephadex column chromatography (Fig. 2) were pooled separately, dialysed, and assayed at optimal salt concentrations (see the Methods section). Control and 3924A-hepatoma values represent results from three separate experiments; the values for tumour 5123D are from two different experiments.

Tissue	Polymerase ...	Activity (units/g of tissue) I	II	III	Recovery of DNA (%)
Resting liver		0.40	2.64	0.09	57
Hepatoma 5123D		1.21	2.11	0.11	36
Hepatoma 3924A		4.25	2.53	0.27	51

Table 3. *Size of RNA synthesized in vitro by RNA polymerase I from resting liver and Morris hepatoma 3924A*
RNA was synthesized *in vitro* by bound RNA polymerase I from Morris hepatoma 3924A or resting liver. For these measurements the specific radioactivity of [^{3}H]UTP in the standard assay was adjusted to 150c.p.m./pmol. RNA was then hydrolysed with KOH and chain lengths were determined as described in the Materials section. Values for hepatoma 3924A represent the average of three determinations (S.D. $\pm$54 nucleotides), and the results for resting liver represent two determinations, which yielded identical results. Values in parentheses are average chain lengths determined under conditions that prevent re-initiation.

Tissue	Incorporation (pmol/g of tissue) UMP	Uridine	Estimated chain length $\left(\frac{\text{UMP} + \text{uridine}}{\text{uridine}}\right)$
Resting liver	390	5	79 (84)
Hepatoma 3924A	4240	13	327 (278)

standard assay conditions (see the Methods section) for 15min. Further reaction was halted by the addition of actinomycin D (40μg/ml) and incubation was continued for an additional 30min. Relative to the activity measured immediately on addition of actinomycin D, no decrease in the UMP incorporated into RNA could be detected after incubation in the presence of the inhibitor. Second, when portions of tumour-3924A or liver enzyme preparations were mixed and then incubated together for 30min in the RNA-synthesizing system, the resultant incorporation of UMP into product was found to equal the theoretical value obtained by summing the activities of both enzymes when determined alone. For example, samples (20μl) of control and hepatoma-3924A RNA polymerase I preparations (equivalent to 3 and 5munits respectively) when mixed under standard assay conditions yielded 8.6munits of activity. This observation indicates the absence of stimulatory or inhibitory factors in the enzyme preparations, which might account for the differences in measured chain length. Finally, it should be noted that the size of the average RNA chain synthesized by liver polymerase I (79 nucleotides), obtained after a single DEAE-Sephadex column chromatography, is essentially identical with the length measured (85 nucleotides) when a highly purified enzyme preparation, which contained no detectable ribonuclease activity (Leonard & Jacob, 1977), was used. The higher activity (10-fold as expressed per g of tissue) of RNA polymerase I from the hepatoma could conceivably have resulted from a preferential transcription of adenosine-rich sequences in the DNA template by that enzyme. To rule out this possibility, polymerase I activity was measured by using each of the four radioactive nucleoside triphosphates. The DNA-dependent incorporation of [^{3}H]-ATP, [^{3}H]GTP and [^{3}H]CTP into RNA by the tumour (3924A) enzyme was 10–11-fold higher than that observed for the liver polymerase. This was consistent with the relative activities obtained with [^{3}H]UTP. Moreover, the ratio of the tumour RNA polymerase-I activity to the liver polymerase-I activity was maintained when the reaction time was increased from 15min to 30min, which further indicates the lack of ribonuclease activity in the control and tumour enzyme preparations.

The 10-fold increase in the bound polymerase I activity observed in hepatoma 3924A relative to liver (Table 2) cannot be entirely accounted for by the increase in the average product size (4-fold). It

therefore seemed plausible that the augmented RNA synthesis *in vitro* by the hepatoma enzyme might also be due to formation of more RNA chains. Accordingly, the relative amounts of radioactive precursor incorporated as 3′-termini of RNA chains by enzyme from both groups was estimated (see the Methods section). Expressed per g of tissue, hepatoma enzyme yielded 13 pmol of uridine compared with 5 pmol of uridine for bound polymerase I from resting liver. The total RNA synthesis *in vitro* can be considered to be the product of chain initiation and elongation. Consequently, the extent of the increases in the number of 3′-termini (>2-fold) and chain length (4-fold) can entirely account for the 10-fold elevation of tumour polymerase activity (Table 2).

Although a greater number of RNA chains may correspond to an increase in the amount of actively initiating polymerase molecules, it may alternatively be due to a higher capacity of the hepatoma enzyme for RNA-chain re-initiation. To assess the latter possibility, enzyme-activity measurements were made after one round of initiation as described below.

Average RNA chain length and 3′-termini measured after one round of initiation

High salt concentrations have been successfully used to inhibit completely re-initiation of RNA synthesis by the prokaryotic RNA polymerase (Hyman & Davidson, 1970). This same approach has been adapted to block re-initiation by eukaryotic polymerases I (Leonard & Jacob, 1977) and II (Cedar, 1975). The method consists of two stages; first, initiation complexes are allowed to form under standard assay conditions in the absence of UTP and CTP, then the pyrimidines are added to the reaction mixture simultaneously with relatively high concentrations of $(NH_4)_2SO_4$. Elongation of RNA chains is allowed to proceed during subsequent incubation, although the presence of a high $(NH_4)_2SO_4$ concentration effectively inhibits any further initiation. Preliminary experiments, in agreement with the results of Leonard & Jacob (1977), confirmed that the addition of $(NH_4)_2SO_4$ (160 mM) was sufficient to prevent initiation completely by bound enzyme I from either liver or hepatoma 3924A. Interestingly, initiation by the class-I enzyme from tumour and liver demonstrated a marked dependence on salt concentration, with optimal activation observed at 10–15 mM. The formation of initiation complexes was also time-dependent. Highest values were obtained after 10–20 min incubation before the addition of pyrimidines and salt. Incubation beyond 20 min caused a decrease in the amount of initiation observed, probably owing to dissociation of complexes (results not shown). The time course of RNA synthesis was

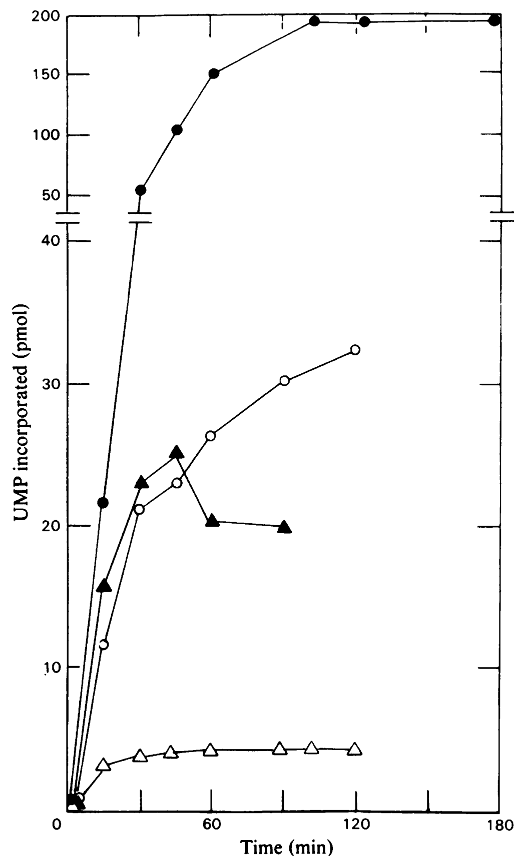

Fig. 3. *Time course of RNA synthesis under standard assay conditions and in the absence of re-initiation*
RNA synthesis by bound polymerase I from Morris hepatoma 3924A and from liver was measured under conditions of the standard assay, in the presence of α-amanitin (0.5 μg/ml), as described in the Methods section and under conditions that limited initiation to one round. The procedure used to prevent re-initiation was essentially that described by Leonard & Jacob (1977). Initiation complexes were allowed to form for 10 min in the standard assay mixture (100 μl total volume), modified as follows: UTP and CTP were deleted; $(NH_4)_2SO_4$ and α-amanitin were added to concentrations of 12.5 mM and 0.5 μg/ml respectively. After preincubation (30°C), $(NH_4)_2SO_4$, CTP and [^{3}H]UTP (130–140 c.p.m./pmol) were added to final concentrations of 160 mM, 0.64 mM and 20 μM respectively. The reaction mixture (120 μl total volume) was further incubated (30°C) for the times indicated and the reaction terminated by chilling in ice and by adding 100 μg of unlabelled UTP. The incorporation of UMP was determined as described by Rose *et al.* (1976). ▲ and ●, Control and tumour RNA polymerase I, respectively, under standard assay conditions; △ and ○, control and tumour RNA polymerase I, respectively, measured in the absence of re-initiation.

measured under the standard assay conditions (see the Methods section) and after one round of initiation (Fig. 3). Under the former conditions, RNA synthesis by the hepatoma RNA polymerase I continued for nearly 2h, whereas the liver-enzyme activity reached a plateau by about 45min. On the other hand, after initiation was limited to one round, liver enzyme elongated RNA for only 15–20min, whereas the elongation of RNA chains by the tumour polymerase continued for the same time period (2h) as observed under the standard assay conditions. These data indicate that enhanced re-initiation is not responsible for the increased number of 3′-termini (uridine residues) in the RNA synthesized by the tumour enzyme. To substantiate this conclusion further, measurements were made of the average RNA chain length and the number of 3′-termini synthesized by tumour and control enzymes after re-initiation was blocked. The lengths of the average chains formed by either enzyme preparation were not substantially altered by preventing re-initiation. RNA products exhibiting average lengths of 84 and 278 nucleotides were synthesized after 30min of elongation by the bound polymerase I from resting liver and hepatoma 3924A respectively (Table 3). The extent of the increase (>3-fold) was similar to that observed under standard assay conditions (4-fold). Although the number of 3′-termini in the RNA synthesized by both the liver and hepatoma enzyme was decreased after inhibition of re-initiation, the relative increase in the incorporation of labelled precursor into uridine residues by tumour polymerase did not differ from that observed under the standard assay conditions (Table 3).

Discussion

The present studies demonstrate an altered pattern of DNA-dependent RNA polymerases I, II and III from a series of Morris hepatomas with different growth rates as compared with the corresponding enzymes from appropriate control tissues. Unlike the normally growing tissues (Yu, 1975*b*; for review see Rutter *et al.*, 1974), in which all three polymerases are increased, the tumours exhibit a preferential increase in RNA polymerase I, with significant increase in the class-III enzyme observed only in the most rapidly proliferating hepatomas. The polymerase II from all tumours in the present investigation is relatively constant, in agreement with reports for mouse myeloma (Schwartz *et al.*, 1974) and lympholeukaemic cells (Verovskii & Gorbacheva, 1978).

Extraction of the enzymes directly from whole tissue homogenates, followed by chromatography on DEAE-Sephadex, allows rapid and quantitative assessment of the respective classes of RNA polymerase from relatively small amounts of tissue, and facilitates accurate comparison of the cellular activities of the enzymes from different sources. Using this technique, we have been able to determine the contribution of each class of polymerase, using as little as 2g of tissue. This approach has the added advantage of recovering all of the RNA polymerase III, which is not always retained in isolated nuclei (Lin *et al.*, 1976). Further, it is possible to eliminate the extensive chromatographic fractionation required for the separation of RNA polymerases from large quantities (derived from 100g of tissue) of isolated hepatoma nuclei (Rose *et al.*, 1976). This method has been successfully employed by us (Lin *et al.*, 1978) in determining the responses of all three RNA polymerases in mouse kidney to androgens and progestins. Extensive purification of the enzyme for the present studies was not warranted for two reasons. First, differential stabilities of the enzymes from the various tissues might result in significant differences in recovery. Second, the polymerases at this stage of purification did not contain nuclease activities. Mixing experiments, as described previously for RNA polymerases from the bound fraction, were performed with enzymes from whole tissues. Many combinations of the enzymes from resting liver and different hepatomas were studied, and in all cases the observed incorporation was within 10% of predicted values. The increased ratio of RNA polymerase I to RNA polymerase II in the tumours, is also reflected in the ratio of enzymes extracted from nuclei prepared in hyperosmotic sucrose. Thus the RNA polymerase I extracted and extensively purified from the hepatoma 3924A nuclei is 3–6-fold higher (in units/mg of DNA) than that of the corresponding enzyme from liver (Rose *et al.*, 1976).

It must be emphasized that alterations in the total cellular amounts and/or activities of RNA polymerases may not be indicative of changes in the functional activity of the enzymes, since a significant proportion of each polymerase class exists in the free or inactive form (Yu, 1975*a*; Lin *et al.*, 1976; Tillyer & Butterworth, 1978). However, the persistence of the augmentation of RNA polymerases I and III in the 'bound' (transcriptionally active) fraction suggests that the alterations in these enzymes in the whole tissue extract indeed reflect the enzymes in functional state. However, these data do not rule out increases in the free enzymes as well.

The increase in the number of 3′-termini, even under conditions that do not favour re-initiation (Leonard & Jacob, 1977), and the longer average chain length of the product synthesized by the hepatoma RNA polymerase (Table 3), indicate that, in addition to an increased number of enzyme molecules, the tumour polymerase might also have undergone activation, perhaps by phosphorylation

(Hirsch & Martello, 1976). The present studies do not unequivocally establish that both these changes have occurred simultaneously in tumorigenesis. The suggested increase in the number of enzyme molecules, however, is consistent with similar changes in liver after a single injection of the chemical carcinogen thioacetamide (Leonard & Jacob, 1977). Enlargement of the nucleolus, the site of synthesis of rRNA (Busch *et al.*, 1963), in both tumour 3924A and thioacetamide-treated liver (Busch & Smetana, 1970) suggests a common mechanism for the augmented rRNA synthesis in these tissues. Since there has been no evidence for enzyme activation at early time points after thioacetamide treatment (Leonard & Jacob, 1977), such changes may occur at later stages of tumour induction.

The tumour-specific increase in the chromatin-bound population of RNA polymerase I agrees well with the report that rRNA genes are amplified in a rat hepatoma line (Miller *et al.*, 1979). If this is the case, the cell might be able to meet the demand for transcribing the available templates by providing more 'bound' enzyme molecules. This could be manifested either by actual synthesis of new molecules, by stabilization of existing enzyme molecules, or by shifting the free population of the enzyme to the bound state. Calculations of the number of 3′-termini (uridine residues) indicate that a greater proportion of the tumour enzyme must exist in the transcriptionally active state, thereby meeting the cellular demand for rRNA synthesis in these proliferating tissues.

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