The relationship between the activity of DNA-dependent RNA polymerase I and the rate of synthesis of rRNA in hepatoma cells in culture

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Cell culture lines were established from the transplantable mouse hepatomas H6 and H129. Both cell lines had a doubling time of about 30 h when maintained in medium containing 5% foetal bovine serum. H6 cells contained about 3–4 times more DNA-dependent RNA polymerase I (Pol I; ribonucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6) than did H129 cells. Moreover, the H6-cell enzyme was more heat-labile than that from H129 cells. Steady-state contents of 28S rRNA were measured in both cell lines. Exponentially growing cultures of H6 cells contained about 6.5 pg of 28S rRNA/cell, and similar cultures of H129 cells contained about 5.8 pg/cell. Stationary cultures of both cell lines contained about 2 pg of 28S rRNA/cell. By two different techniques, the half-time for turnover of 28S rRNA was estimated to be 16–17 h for both H6 and H129 cells. Knowing the turnover rate and the steady-state concentration, one may calculate that both H6 and H129 cells synthesize 28S rRNA at a rate of about 0.25 pg/h per cell. The amount of template-bound Pol I activity was similar in nuclei isolated from H6 and H129 cell cultures. These data indicate that, although H6 cells contained 3–4 times more Pol I than did H129 cells, both cell lines synthesized rRNA at about the same rate.

Mitotic stimulation of cells is frequently associated with an increased rate of synthesis of rRNA. Transcription of rDNA genes is catalysed by DNA-dependent RNA polymerase I (Pol I) and the activity of this enzyme also increases as a result of mitotic stimulation (Mainwaring *et al.*, 1971; Blatti *et al.*, 1971; Sajdel & Jacob, 1971; Smuckler & Tata, 1971; van den Berg *et al.*, 1974; Jaehning *et al.*, 1975; Guilfoyle *et al.*, 1975). These observations are consistent with the hypothesis that increased rates of proliferation require increased amounts of rRNA. It has been calculated that mouse liver cells contain Pol I sufficient to saturate only about 10% of the rDNA cistrons, whereas rapidly growing plasmacytoma cells contain much more Pol I (Schwartz *et al.*, 1974). This had led to the suggestion that increased synthesis of rRNA may be regulated, in some cases, by the activity of Pol I.

In our laboratory, experiments have been undertaken to study the relationship between Pol I activity and the rate of rRNA synthesis in normal and neoplastic cells. A number of observations have been made, and those most relevant to the data described

in this paper will be briefly reviewed. Pol I amounts have been compared in five transplantable mouse hepatomas and livers of three inbred host strains. Two classes of tumours were observed, those having very high Pol I activity and those having Pol I activity similar to that of normal liver. Pol I in all tumour strains was chromatographically distinguishable from liver Pol I, suggesting that neoplastic liver cells may contain an altered form of the enzyme. Two hepatoma lines were selected for detailed study, H6 and H129. Hepatoma H6 is the most rapidly growing of all tumours studied in our laboratory. Homogenates of H6 tumours contain about 5–8 times more Pol I activity (per mg of protein) than normal liver and about 3–4 times more Pol I activity than a slower-growing tumour, H129. Hepatoma H129 has Pol I activity slightly higher than that of host liver. In addition, thermal-denaturation studies indicate that Pol I from normal liver and that from H129 tumours are similar in heat-stability, whereas Pol I from H6 tumours is much more heat-labile. Homogenates of H6 and H129 tumours have been mixed and Pol I has been partially purified from such mixtures. Recovery of enzyme was additive, indicating that low activity in H129 tumours does not result from loss of activity during

Abbreviation used: Pol I, DNA-dependent RNA polymerase I (EC 2.7.7.6, eukaryotic type I).

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extraction or from substances that degrade or inhibit the enzyme. Moreover, the presence of two forms of different heat-stabilities was demonstrated in such mixtures, indicating that heat-lability of H6-tumour Pol I does not result from modification during the extraction procedure. This suggests that H6 tumours contain an altered form of Pol I, whereas H129 tumours contain an enzyme which more closely resembles the normal liver enzyme (A. H. Cavanaugh & E. A. Thompson, unpublished work). Pol I from H129 and H6 tumours was purified to apparent homogeneity, and the specific activities of the purified enzymes are similar (A. H. Cavanaugh & E. A. Thompson, unpublished work). This indicates that elevated Pol I activity in H6 tumours is due to the presence of an increased number of Pol I holoenzyme molecules.

Cell-culture lines from hepatomas H6 and H129 have been established. Such lines facilitate studies designed to elucidate the mechanisms which govern Pol I activity. The experiments described below were designed to compare rates of synthesis of rRNA in these two lines to determine to what extent increased Pol I activity in H6 cells is accompanied by increased production of 28S rRNA. Steady-state amounts of rRNA have been determined in exponential-phase and stationary cultures of H6 and H129 cells. Rates of degradation of 28S rRNA have been measured, as well as Pol I activity and cell doubling times of exponential-phase cells. These data indicate that, even though H6 cells contain 3–4 times more Pol I than do H129 cells, both cell lines synthesize 28S rRNA at similar rates.

Experimental

Establishment and maintenance of cell culture lines

Hepatoma H6 was obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and was maintained in A-strain mice by serial subcutaneous transplantation. A mouse bearing a small subcutaneous tumour (about 0.5g) was killed and immersed in 70% (v/v) ethanol. The tumour was excised, washed with sterile phosphate-buffered saline (2.7mM-KCl, 0.9mM-KH₂PO₄, 8.0mM-Na₂HPO₄·7H₂O and 0.14M-NaCl), and minced in a 6.0cm Petri dish. Then 2ml of sterile 0.25% trypsin containing 0.2g of EDTA/litre (Gibco, Grand Island, NY, U.S.A.) was added and the tumour was disaggregated by gentle swirling at room temperature. A suspension of single cells and small chunks of tumour was placed in a 6.0cm dish containing 4ml of Waymouth's medium MAB 87/3 (Gibco) with 5% foetal bovine serum (KC Biologicals, Kansas City, MO, U.S.A.), 1000 units of penicillin/ml and 1mg of streptomycin/ml (Gibco). Cells which adhered to the plate were maintained by serial subculturing by standard cell-culture techniques.

After 20 passages, H6-cell cultures were used for experiments.

Hepatoma H129 was obtained from the National Cancer Institute through the Mason Research Institute (Frederick, MD, U.S.A.). This line was maintained in C3H-strain mice by serial transplantation of either subcutaneous or ascites tumours. The cell-culture line used in the experiments below was of ascites origin. Cells were aspirated from the peritoneal cavity and placed in Waymouth's medium MAB 87/3 with 5% foetal bovine serum, 1000 units of penicillin/ml and 1mg of streptomycin/ml. H129 cells grew in suspension. Two separate culture lines were established from subcutaneous H129 tumours, and these also grew in suspension. The H129 cell-culture line was serially subcultured for 20 passages before experiments were begun. Both cell lines were maintained at 37°C in an atmosphere of air/CO₂ (19:1).

Cell-doubling times were determined for H129 cells by withdrawing, at various times, small samples of cells from several flasks that had been inoculated with a known number of cells. H6 cells from replicate flasks were removed by trypsin treatment. Cells were counted in a haemocytometer.

Measurement of steady-state concentration of rRNA

H129 cells were grown for 24h in the presence of 1μCi of [³H]uridine (41Ci/mmol; New England Nuclear, Boston, MA, U.S.A.)/ml. Labelled cells were centrifuged for 10min at 500g, then washed once in TSM medium, containing 10mM-Tris/HCl (pH 7.4), 10mM-NaCl and 1.5mM-MgCl₂. Cells were resuspended in TSM medium to a final concentration of 10⁷ cells/ml, frozen at -20°C, and thawed. The suspension was homogenized by a 5s burst from an Ultrasonics Sonifier with a microprobe at minimum amperage. The homogenate was centrifuged at 4500g for 5min, and 0.5ml of the supernatant was layered on a linear 14ml sucrose gradient consisting of 15–30% sucrose in TSM medium with 1% sodium dodecyl sulphate. The gradients were centrifuged at 25°C for 17h in a SW40Ti rotor at 26000rev./min in a Beckman L5-50B ultracentrifuge. An ISCO fractionator (Instrument Specialities Co., Lincoln, NB, U.S.A.) was used to fractionate the gradients and 18S and 28S rRNA were identified by absorption at 254nm. Pooled 18S and 28S rRNA were precipitated with 2vol. of 95% ethanol at -20°C for 16h. The precipitates were resuspended in TSM medium and RNA content was measured by the method of Warburg & Christian (1941). The A_{260}/A_{280} ratio of these preparations was 2.0. The specific radioactivity of 18S rRNA was 7.7×10^6 c.p.m./mg and that of 28S rRNA was 7.5×10^6 c.p.m./mg. These were used as internal standards to monitor recovery of 18S and 28S rRNA in subsequent experiments.

Cells from stationary or exponential-phase cultures were suspended in TSM medium to a final concentration of 10^7 cells/ml. Before homogenization $15000\text{ }^3\text{H}$ c.p.m. of 18S rRNA and/or $39000\text{ }^3\text{H}$ c.p.m. of 28S rRNA were added. Suspensions were frozen, thawed and homogenized as described above. RNA from a known number of cells (about 5×10^6 cells/gradient) was resolved by sucrose-density-gradient centrifugation, and 18S and 28S rRNA were identified and precipitated with ethanol. All steps were identical with those described for preparation of labelled 18S and 28S rRNA. The ethanol-precipitated RNA was resuspended in TSM medium and RNA content was measured. A sample was counted for radioactivity (as described below) to determine percentage recovery of labelled 18S and 28S rRNA. RNA content was corrected for contribution of labelled RNA to total mass (about 16%) and all values were normalized to 100% recovery. In 10 such experiments the mean ratio of A_{260}/A_{280} for 28S rRNA was 1.95 (s.d. = 0.10).

Preliminary experiments revealed that degradation products of tritiated exogenous 28S rRNA sedimented in sucrose gradients at about 18S. Recovery of 18S rRNA was therefore overestimated to an extent which varied inversely with the recovery of 28S rRNA. For this reason it was assumed that any observations made about 28S rRNA could be generalized, and no further attempt was made to measure steady-state concentrations or rates of turnover of 18S rRNA.

Estimation of the rate of turnover of rRNA

Radioactive methionine was used to label rRNA for turnover studies in culture. This compound was chosen for several reasons. Initially, since methylation occurs after transcription, re-utilization was avoided by the use of methyl-labelled methionine as a precursor. Perhaps more important is the fact that the cell-culture medium contains large amounts of unlabelled methionine. This assures that the rate of incorporation of label will not be influenced by changes in the size of the exogenous precursor pool.

Cells were incubated with either [$Me\text{-}^3\text{H}$]-methionine ($5\mu\text{Ci/ml}$, 80Ci/mmol ; New England Nuclear) or [$Me\text{-}^3\text{H}$]-methionine ($5\mu\text{Ci/ml}$) plus [$Me\text{-}^{14}\text{C}$]-methionine ($0.5\mu\text{Ci/ml}$, 52mCi/mmol ; New England Nuclear). At various times labelled cells were homogenized as described above, and homogenates were extracted with an equal volume of phenol saturated with TSM medium. Phenol extraction was essential to eliminate potential artifacts caused by adventitious co-purification of proteins labelled with methionine. Phenol-extracted RNA was resolved on sucrose gradients and precipitated with ethanol. Ethanol precipitates were redissolved in TSM medium and extracted with 2×1 vol. of chloroform to remove residual phenol. RNA con-

tent and specific radioactivity were measured as described above.

In experiments in which double-labelling techniques were used to measure turnover of rRNA, purified 28S rRNA was incubated with proteinase K (E.M. Biochemicals, Darmstadt, Germany) at a concentration of $65\mu\text{g}$ of the enzyme/ml in 10mM-Tris/HCl (pH 8.0)/ $10\text{mM-EDTA}/10\text{mM-NaCl}/0.5\%$ sodium dodecyl sulphate (Gross-Bellard *et al.*, 1973). After 1 h at 37°C , 0.1 ml of the digest was mixed with cold 5% (w/v) trichloroacetic acid and the precipitate was collected on a 2.5 cm Whatman GF/C filter. The filters were washed with 2×20 ml of cold 5% trichloroacetic acid, followed by 10 ml of 95% ethanol. Precipitated RNA was hydrolysed by incubation with 0.5 ml of Protosol (New England Nuclear) for 30 min at 60°C and radioactivity was measured after the addition of 15 ml of Demilume 30 (Packard Instruments, Downers Grove, IL, U.S.A.). Additional information about counting parameters is given below.

Measurement of Pol I activity in cultured cells

About 2×10^8 cells were grown in multiple 75cm^2 flasks. The cells were harvested, washed once with 50 ml of phosphate-buffered saline containing 5% (v/v) foetal bovine serum, and then with 50 ml of phosphate-buffered saline. The pellet from the second wash (about 1 ml of packed cells) was resuspended in 3.0 ml of TGED buffer, which contained 50mM-Tris/HCl (pH 7.9), 25% (v/v) glycerol, 0.1mM-EDTA and $1.5\text{mM-dithiothreitol}$ (Schwartz *et al.*, 1974). Samples were homogenized by four strokes with a glass-Teflon homogenizer. The salt concentration was adjusted to 0.32M by the addition of $4\text{M-(NH}_4)_2\text{SO}_4$, pH 7.9 (Schwarz/Mann, Orangeburg, NY, U.S.A.). Homogenates were sonicated for 4×15 s with 2 min cooling periods. The sonicated mixture was centrifuged at 4°C for 50 min at 40000 rev./min in a Beckman type 65 rotor. After centrifugation, TGED medium was added to the supernatant to adjust the final salt concentration to $0.1\text{M-(NH}_4)_2\text{SO}_4$. The resulting suspension was centrifuged again at 4°C in the type 65 rotor for 50 min at 50000 rev./min , and the supernatant was diluted with an equal volume of TGED and centrifuged again under identical conditions. The supernatant from the final centrifugation was designated F-3. F-3 fractions were stored in liquid N_2 until chromatography was performed. Protein concentration was measured by the method of Bradford (1976).

Pol I was resolved by chromatography on DEAE-Sephadex A25. This was washed with 0.1M-NaOH and then distilled water until pH 7.0 was reached, and then was equilibrated with TGED medium containing $0.05\text{M-(NH}_4)_2\text{SO}_4$. Columns

(6.5 cm × 1.0 cm) were prepared immediately before use. About 8 ml of F-3 fraction was loaded on the column, which was then washed with 20 ml of TGED medium containing 0.05 M-(NH₄)₂SO₄. Pol I was eluted in a stepwise fashion with TGED containing 0.15 M-(NH₄)₂SO₄. Identity of the enzyme was confirmed by titration with α -amanitin (Weinmann & Roeder, 1974). In no experiment was any detectable Pol II or Pol III eluted together with Pol I. By titration of F-3 fractions with α -amanitin it could be estimated that Pol I recovery was about 88–100%. Pol I recovery from conventional salt-gradient-elution chromatography (Roeder & Rutter, 1970) was always considerably lower in our experience.

Pol I activity was measured in a final volume of 100 μ l. Samples (50 μ l) of column fractions were mixed with 50 μ l of a solution containing 0.1 M-Tris/HCl (pH 7.9), 5 mM- β -mercaptoethanol, 1 mM-ATP, -CTP and -GTP, 3.4 mM-MnCl₂, 0.1 M-(NH₄)₂SO₄, 20 μ g of native calf thymus DNA, 16 μ M-UTP and 1.9 μ Ci of [³H]UTP (35 Ci/mmol; New England Nuclear) (Roeder & Rutter, 1970). Throughout the range of activities reported in this paper, incorporation of [³H]UMP was a linear function of the amount of enzyme added to the reaction mixture. The reaction was carried out for 15 min at 37°C and was terminated by spotting the incubation mixture on a 2.5 cm DE-81 filter (Whatman) (Weaver *et al.*, 1971). Filters were washed twice with 5% (w/v) Na₂HPO₄ containing 20 mM-NaH₂P₂O₇, three times with 5% Na₂HPO₄, twice with distilled water and finally with 95% ethanol. Filters were dried and counted for radioactivity to measure incorporation of [³H]UMP. One unit of enzyme activity is defined as incorporation of 1 pmol of UMP in 15 min at 37°C.

Thermal denaturation of Pol I

Pol I from DEAE-Sephadex was mixed with TGED medium containing bovine serum albumin to a final concentration of 3 mg of protein/ml. Glass tubes (10 mm × 75 mm) were preincubated at 40°C. Enzyme (50 μ l) was added to each tube, and these were withdrawn from the water bath at appropriate intervals and placed in an ice/water slurry. Enzyme activity was then measured as described above.

Measurement of transcriptional activity in isolated nuclei

Hepatoma cells were homogenized in 4 vol. of 2.3 M-sucrose containing 1 mM-dithiothreitol. Homogenates were centrifuged at 39 000 g for 1 h, and the pellet was resuspended in 0.34 M-sucrose containing 1 mM-dithiothreitol. After a second centrifugation at 2000 g for 10 min, the nuclear pellet was resuspended in 2 vol. of 0.24 M-sucrose containing 1 mM-dithiothreitol. All procedures were performed at

4°C. The nuclear suspension was stained with Cytodiachrome (Regis Chemical Co., Morton Grove, IL, U.S.A.) and examined microscopically. Few broken nuclei or intact cells were observed. Such a suspension could be stored for 1–2 weeks in liquid N₂ with no appreciable loss of polymerase activity. RNA polymerase activity in isolated nuclei was assayed in a reaction mixture lacking exogenous DNA but otherwise identical in composition with that described above. α -Amanitin and heparin were added as indicated in the text. Suspensions were incubated for 15 min at 37°C and the reaction was terminated by the addition of 50 μ l of 5% sodium dodecyl sulphate, followed immediately by 1.0 ml of cold 5% trichloroacetic acid. Precipitated nucleic acids were filtered on a Whatman GF/C filter, washed with 2 × 20 ml of cold 5% trichloroacetic acid, rinsed with 10 ml of cold 95% ethanol, dried, and counted for radioactivity as described below. DNA content of isolated nuclei was assayed by the procedure of Burton (1956).

Liquid-scintillation counting

For measurement of Pol I activity, [³H]RNA adsorbed to DE-81 filters was counted for radioactivity in 3.5 ml of toluene containing 5 g of 2,5-diphenyloxazole/litre. Counting efficiency was 8%. In order to measure specific radioactivity of labelled 28S rRNA, a sample of the purified material containing a known amount of RNA was counted for radioactivity in 3.5 ml of Aquasure (New England Nuclear) with an efficiency of 42%. The ratio ³H/¹⁴C was measured in 15 ml of Demilume 30 under conditions in which spill-over of ¹⁴C into ³H was 7.5%. Efficiency for ³H was 28% and ¹⁴C efficiency was 68%. The ratio of d.p.m. was calculated by using conventional techniques. A Beckman LS 3133P liquid-scintillation spectrometer was used for all samples.

Results

Although precise growth experiments have not been performed, it has been observed that hepatoma H6 grows much more rapidly *in vivo* than does hepatoma H129. For example, injection of 0.2 ml of a suspension of finely chopped H6 tumour will result in the formation, within 10–12 days, of a tumour having a mass of about 1 g. A similar inoculum of H129 cells requires 25–30 days for formation of a 1 g tumour. However, cell-culture lines derived from H6 and H129 tumours have similar doubling times in culture; in the presence of 5% foetal bovine serum, the generation times were about 31 h and about 27 h respectively. In 10% foetal bovine serum, H6 cells doubled every 18 h, and H129 cells every 20 h. For reasons of economy, all subsequent experiments were performed in 5% foetal bovine serum.

The relative growth rates of H6 and H129 cells were not the same *in vivo* and in culture. Pol I activity has been linked to growth rate, and there was some concern that Pol I activities in cultured cells might differ from those previously measured in subcutaneous H6 and H129 tumours. Cultures of each line were prepared at a density of 10^5 cells/ml in six 75 cm² flasks, each of which contained 50 ml of medium. After three doublings about 2×10^8 cells were recovered from six flasks. These cells were homogenized and F-3 extracts were prepared as described in the Experimental section. Then 25 mg of F-3 protein was applied to a DEAE-Sephadex A25 column and Pol I was eluted with 0.15 M-(NH₄)₂SO₄. Representative chromatograms are shown in Fig. 1.

Fig. 1. Step elution chromatography of Pol I from H6 (■) and H129 (●) cells

About 1 ml of packed cells of each line were homogenized and polymerase was extracted as described in the Experimental section. The F-3 extracts were loaded on DEAE-Sephadex A25 columns and eluted with 0.15 M-(NH₄)₂SO₄. Fractions (1 ml) were collected and assayed.

In this particular experiment, the specific activity of the partially purified H6-cell Pol I was about 220 units/µg of protein. The specific activity of the partially purified enzyme from H129 cells was about 60 units/µg of protein. In both cases, enzyme activity in the presence of 50 µg of α -amanitin/ml was greater than 98% of control activity, indicating that Pol I was completely resolved from Pol II and Pol III. In a previous series of experiments the specific activity of H6-cell Pol I, extracted from subcutaneous tumours and resolved by step-elution chromatography, was about 200 units/µg, and that of H129-cell Pol I was about 60 units/µg. Thus cell-culture lines of these two hepatomas resemble subcutaneous tumours with respect to amount of Pol I activity.

Differences in heat-lability have also been reported for Pol I from hepatomas. The enzyme from subcutaneous H6 tumours was denatured at 40°C with a half-time of about 2 min, whereas that from H129 tumours was denatured with a half-time of about 7 min. Pol I was resolved by step-elution chromatography of F-3 extracts of cultured cells. Heat-denaturation studies were carried out at 40°C, and the results obtained are shown in Fig. 2. The half-time of denaturation of H6-cell Pol I was about 2–3 min and that of H129-cell enzyme was about 7 min.

The data presented above indicate that cell-culture lines of H6 and H129 tumours contain different amounts of Pol I activity. The thermal stability of the two enzymes is different, indicating that the two cell lines contain distinct forms of Pol I. Experi-

Fig. 2. Heat-denaturation of Pol I from H6 (■) and H129 (●) cells

Pol I was resolved by step elution as described in the text and in Fig. 1. Bovine serum albumin, dissolved in TGED medium, was added to a final protein concentration of 3 mg/ml. This mixture was incubated at 40°C for the intervals indicated, after which polymerase activity was measured. All time points are the means of triplicate assays.

Table 1. Amounts of 28S rRNA in H6 and H129 cells in culture

Amounts of 28S rRNA were measured as described in the Experimental section; ^3H -labelled 28S rRNA was used as a recovery standard. Mean recovery from H6 cells was 56 (± 11)% from exponential-phase cultures and 57 (± 30)% from stationary cultures. Mean recovery of ^3H -labelled 28S rRNA from H129 cells was 68 (± 6)% from exponential-phase cultures and 61 (± 14)% from stationary cultures. Where appropriate, standard deviations were calculated and indicated in parentheses.

Cell line	Growth state	No. of flasks	$10^{-5} \times$ Cell density (cells/ml)	28S rRNA (pg/cell)
H6	Exponential	1	1.8	8.4
	Exponential	1	8.2	8.4
	Exponential	6	9.8–10.2	6.5 (± 1.0)
	Exponential	1	13.8	6.6
	Stationary	3	28.8	2.0 (± 1.0)
H129	Exponential	1	0.8	6.2
	Exponential	6	6.0–6.5	5.8 (± 0.2)
	Exponential	1	8.7	10.2
	Stationary	6	24.2	2.0 (± 0.2)

ments were undertaken to determine if cultured cells of H6 and H129 tumours synthesize rRNA at different rates. As a first step, steady-state concentrations of 28S rRNA were measured in exponential-phase cells. As shown in Table 1, concentrations of 28S rRNA were similar at culture densities in the range of 1×10^5 – 14×10^5 cells/ml, indicative of steady-state conditions with respect to 28S rRNA content. Exponential-phase H6 cells contained about 6.5 pg of 28S rRNA/cell, and exponential-phase H129 cells contained about 5.8 pg of 28S rRNA/cell. Cells of both lines grow very slowly at high densities, and cultures become essentially stationary at densities greater than about 2×10^6 cells/ml. RNA concentrations were measured in stationary cultures. As shown in Table 1, slowly growing H6 and H129 cell cultures contain about 2 pg of 28S rRNA/cell. By Student's *t*-test analysis, the observed steady-state concentrations of 28S rRNA in exponential-phase H129 and H6 cells were not significantly different. However, differences between stationary and exponential-phase cultures were highly significant ($P < 0.0005$).

To estimate rates of turnover of 28S rRNA, two approaches were employed. The first of these involved measurement of the time course of incorporation of the [^3H]methyl group of [$\text{Me-}^3\text{H}$]methionine. Cells were incubated with medium containing [^3H]methionine ($5 \mu\text{Ci/ml}$). At various times, 28S rRNA was extracted and purified. The specific radioactivity ($^3\text{H d.p.m./mg}$) of 28S rRNA is shown in Fig. 3 as a function of labelling time. These results show that both H6 and H129 cells approach radioisotopic equilibrium at the same rate. Furthermore, equilibrium is not achieved at labelling times less than 48 h. Assuming that equilibrium has been reached at 48 h, one may estimate that about half of the 28S rRNA within the cell is replaced every 16 h.

Fig. 3. Incorporation of [^3H]methionine into 28S rRNA of H6 (■) and H129 (●) cells

Flasks were inoculated at a density of 10^5 cells/ml. After 24 h, $5 \mu\text{Ci}$ of [^3H]methionine/ml was added; this is 0 h in the Figure. At the intervals indicated, cells were harvested and ^3H -labelled 28S rRNA was purified and determined.

A double-labelling technique was employed to measure more precisely the turnover rate of 28S rRNA. Cultures of H6 and H129 cells were maintained for 72 h in 'double-labelled' medium,

Table 2. Estimation of the rate of synthesis of 28 S rRNA

All manipulations were carried out as described in the text. Half-time of turnover was calculated from least-squares regression analysis of the data shown in Fig. 4. Steady-state concentrations of 28S rRNA were taken from the experiment, depicted in Table 1, in which six replicate flasks were assayed simultaneously. There was no statistically significant difference between the values indicated for steady-state concentrations or half-times of turnover.

Cell line	Steady-state concentration of 28S rRNA (c, pg/cell)	Half-time of turnover (t ₁ , h)	Rate constant of degradation (k _d , h ⁻¹)	Synthesis rate (RS _{28S} , pg/h per cell)
H6	6.5	16	0.043	0.28
H129	5.8	17	0.041	0.24

containing 5 μ Ci of [³H]methionine/ml and 0.5 μ Ci of [¹⁴C]methionine/ml. To ensure that radioisotopes and/or nutrients were not depleted, the ‘double-labelled’ medium was changed at 48 h. At the end of 72 h, the cells were washed and fresh medium was added which contained only [¹⁴C]-methionine (0.5 μ Ci/ml). Cells were harvested at various times and 28S rRNA was resolved by sucrose-gradient centrifugation. RNA was precipitated with ethanol, redissolved in TSM medium, and the ³H/¹⁴C d.p.m. ratio was determined. In Fig. 4, the percentage of maximum ³H/¹⁴C ratio is plotted as a function of time, with the zero-time value (the time at which [³H]methionine was withdrawn) being 100%. The half-time of ³H/¹⁴C decay was about 16 h for H6 cells and about 17 h for H129 cells. These data indicate that both cell lines replace about 50% of their 28S rRNA every 16–17 h.

Knowing the steady-state concentrations and the rates of turnover of 28S rRNA, one may calculate synthesis rates from the formula

$$RS = Ck_d$$

where RS = rate of synthesis, C = steady-state concentration and k_d = rate constant of degradation (in time⁻¹). The constant k_d may be determined from the half-time of turnover (t₁) from the relationship

$$k_d = \frac{\ln 2}{t_1}$$

These manipulations have been carried out and the data are summarized in Table 2. No significant difference was observed in the synthesis rates of 28S rRNA in H6 and H129 cells in culture.

The rate of transcription of rDNA may also be estimated by measuring polymerase activity in isolated nuclei in the presence of high concentrations of α -amanitin and heparin. The only active form of RNA polymerase is Pol I bound to the template in a transcriptionally active manner, and by measuring polymerase activity under these condi-

Fig. 4. Turnover of 28 S rRNA in H6 (■) and H129 (●) cells

Flasks were inoculated at a density of 10⁵ cells/ml of ‘double-labelled’ medium. ‘Double-labelled’ medium was changed at 48 h. After 72 h, ‘double-labelled’ medium was replaced with medium containing 0.5 μ Ci of [¹⁴C]methionine/ml. This point is indicated as 0 h in the Figure. Cells were harvested at the times indicated, 28S rRNA was purified, and ³H/¹⁴C ratios were measured. To ensure that ³H/¹⁴C ratios were not influenced by proteins which co-purified with phenol-extracted 28S rRNA, samples were digested with proteinase K and ³H/¹⁴C ratios of trichloroacetic acid-precipitated proteinase K-resistant material were measured. In no experiment was the ³H/¹⁴C ratio altered by this procedure. The data plotted are ratios measured before proteinase digestion. Least-squares regression analysis indicated that the half-time for turnover of H6-cell 28S rRNA was 16 h and that of H129-cell 28S rRNA was 17 h. However, analysis of variance indicated that the slopes of the lines were not significantly different, and the curve drawn here is the least-squares regression line calculated for all data obtained from both H6 and H129 cells.

tions one may obtain an estimate of the rate of transcription that was going on before isolation of the nuclei (Yu, 1975; Fuhrman & Gill, 1975; Cox, 1976). Nuclei were isolated from H6 and H129 cells and the rate of incorporation of [³H]UMP was

determined in the absence of exogenous DNA and in the presence of 100 μ g of α -amanitin/ml and 5 mM-heparin. Template-bound Pol I of H6 cells incorporated a mean of about 5.0×10^4 c.p.m. of [^{3} H]UMP/mg of DNA. Under identical conditions Pol I from H129 cells incorporated about 5.4×10^4 c.p.m./mg of DNA. This experiment has been performed three times and the means were not significantly different, as determined by Student's *t* test.

Discussion

As discussed in the introduction to this paper, subcutaneous H6 tumours contain 3–5 times more Pol I than do H129 tumours. The enzyme from H6 tumours is also more heat-labile than that of H129 tumours or normal liver (A. H. Cavanaugh & E. A. Thompson, unpublished work). These properties are maintained in cell-culture lines established from the hepatomas; H6 cells contain a heat-labile form of Pol I and about three times more enzyme activity than H129 cells. Many studies have indicated that differences in heat-stability result from structural variation (Langridge, 1968; Lusis & Paigen, 1975; Free & Schimke, 1978). The data reported above and other results (A. H. Cavanaugh & E. A. Thompson, unpublished work) indicate that Pol I in H6 cells is structurally different from the enzyme in H129 cells. Whether the difference is genetic or post-translational in origin cannot be ascertained at present.

The experiments described in this paper were undertaken to determine if differences in Pol I activity and structure were reflected in the rates of synthesis of rRNA. The most reliable estimates are obtained by exploiting the fact that steady-state concentrations of any macromolecules are determined by the rate of synthesis and the rate of degradation, and may be predicted from the relationship $RS = Ck_d$ (Schimke & Doyle, 1970). The steady-state concentration (*C*) may be measured directly, and the rate constant of degradation or turnover (k_d) may be calculated from the half-life of turnover of the compound of interest.

The concentration of 28S rRNA was measured in cultures containing various concentrations of cells. When flasks were inoculated at a density of about 10^5 cells/ml, steady-state concentrations of 28S rRNA were maintained for four or five doublings. No significant difference was observed in steady-state concentrations of 28S rRNA in H6 and H129 cells in cultures that were undergoing exponential growth. Both cell lines contained about 6 pg of 28S rRNA/cell. At cell densities higher than about 2×10^6 cells/ml, cultures of H6 and H129 cells ceased to grow rapidly. These are referred to as

stationary cultures, even though cells will continue to increase in number in such cultures at a lower rate. The concentration of 28S rRNA in stationary cultures was about 2 pg/cell, significantly lower than that observed in cultures growing exponentially. In stationary phase and in exponential phase, H129 and H6 cells contained similar amounts of 28S rRNA.

The rate of turnover of 28S rRNA was measured. Turnover includes both degradation and dilution owing to cell division. The rate of turnover may be estimated by adding a radioactive precursor and measuring the length of time required for the specific radioactivity of RNA to become constant. At this point, one may assume that virtually all of the RNA within the cell has been synthesized after the addition of the labelled precursor. This defines the period of time required to replace all of the cellular RNA with newly synthesized material. An estimate of the turnover rate may be derived in this manner if one ignores potential discrepancies caused by size and rate of equilibration of precursor pools. Both H6 and H129 cells required about 48 h to reach equilibrium with respect to the specific radioactivity of 28S rRNA. A half-time of turnover of about 16 h may be estimated for both cell lines.

A more precise estimate of turnover has been achieved by labelling cells to equilibrium in the presence of a precursor labelled with both ^{3} H and ^{14} C. Methionine was selected primarily to avoid reutilization, which may lead to underestimation of the turnover rate when labelled uridine is employed as a tracer. Cells were grown in the presence of [^{3} H]- and [^{14} C]-methionine for 72 h. Beyond this point specific radioactivity of either [^{3} H]- or [^{14} C]-RNA did not increase as a function of time. Thereafter the medium was replaced with fresh medium containing only [^{14} C]methionine and the turnover of 28S rRNA was measured as a function of decay of the ^{3} H/ ^{14} C d.p.m. ratio. Starting from a maximum ^{3} H/ ^{14} C ratio of 10, the ratio decreased to 5 in about 16 h. This confirms the original estimate that half of the cellular RNA is replaced every 16–17 h. The estimated turnover of 28S rRNA was significantly more rapid than would be predicted on the basis of cell doubling times (28–30 h). Therefore both H6 and H129 cells must actively degrade rRNA.

H6 and H129 cells contained similar steady-state concentrations of 28S rRNA. Likewise, both cell lines appeared to have similar turnover rates for 28S rRNA. From this it is concluded that both H6 and H129 cells synthesize 28S rRNA at similar rates, about 0.26 pg/h per cell.

The rate of synthesis of 28S rRNA may be determined by the rate of processing of the 45S precursor as well as the rate of transcription. Owing to the low amount and rapid turnover of 45S RNA, it is difficult to measure the rate of synthesis of the

primary transcript. It is possible, however, to estimate the rate of transcription by measuring template-bound Pol I activity in isolated nuclei. Data from such experiments suggest that the rates of transcription from rDNA are similar in nuclei isolated from H6 and H129 cells. Although such data must be regarded as preliminary, they are consistent with the observation that both cell lines synthesize 28S rRNA at the same rates.

In conclusion, H6 cells contain high Pol I activity, relative to H129 cells. Furthermore, H6-cell Pol I is structurally different, as determined by comparison of thermal denaturation rates. However, differences in structure and amount of Pol I activity are not reflected in the rate of transcription of those cistrons coding for rRNA. These data are consistent with the hypothesis that H6-cell Pol I is transcriptionally less efficient and that the cell is compelled by compensatory mechanisms to maintain the amount of this enzyme at elevated values. Alternatively, regulation at the level of the ribosomal promoters could account for the observation that both cell lines synthesize rRNA at the same rate. However, such a mechanism does not account for differences in amounts and properties of Pol I in H6 and H129 cells. Experiments to compare the structural and transcriptional properties of Pol I, purified to homogeneity, from H6 and H129 cells and from normal liver should help elucidate those mechanisms involved in regulation of transcription of rDNA in normal and neoplastic cells.

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