Molecular Basis of Reduced Albumin Synthesis in Morris Hepatoma 7777†

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ABSTRACT: The level of albumin mRNA in the normal Buffalo rat liver and Morris hepatoma 7777 was compared by molecular hybridization with albumin complementary DNA (cDNA) and translational assays. Albumin mRNA was found to be 10% of the total rat liver poly(A)-containing RNA population but reduced approximately fourfold in the case of Morris hepatoma 7777. An equivalent decrease of albumin mRNA activity in the hepatoma was detected by translation in a mRNA-dependent cell-free protein-synthesizing system. A proportional increase in total hepatoma poly(A)-containing RNA was not observed, indicating that there was a true fourfold reduction of albumin synthesis in the hepatoma. DNA excess hybridization with albumin cDNA did not reveal any apparent change in albumin gene frequency in the hepatoma compared to normal liver. Complementary DNA copies of total liver and hepatoma poly(A)-containing RNA were synthesized and employed in homologous and heterologous hybridization reactions. Analyses of these reactions showed a high degree of homology between the poly(A)-containing RNA of the liver and hepatoma, with some difference in the relative sequence abundancy. However, qualitative differences were detected in hepatoma 7777 consistent with the concept of alterations in the control of gene expression upon neoplastic transformation.

Albumin biosynthesis and secretion are characteristic functions of the normal rat liver where albumin represents the most prominent protein synthesized by the adult organ (Rothschild et al., 1972; Peters and Peters, 1972; Peters, 1975; Feldhoff et al., 1977). Serum albumin is responsible for the maintenance of the osmotic equilibrium of body fluids and plays an important role in the transport of various essential metabolites and cellular constituents. However, the genetic regulation of albumin synthesis may be altered during hepatic neoplasm. Certain relatively stable, transplantable hepatomas retain the capacity to produce serum albumin but at moderately to greatly reduced levels, as compared to the normal rat liver (Schreiber et al., 1969; Rotermund et al., 1970; Uenoyama and Ono, 1972; Ove et al., 1972; Sell, 1974; McLaughlin and Pitot, 1976). Morris hepatoma 7777, one of these tumors, is classified as a "minimally deviated " hepatoma on the basis of its near-normal karyotype, although it has a relatively fast growth rate and shows a somewhat poor degree of morphological differentiation (Nowell et al., 1967; Morris and Wagner, 1968). Hepatoma 7777 is therefore an attractive experimental model for the study of liver albumin synthesis. By examining the similarities and differences in the mode of albumin synthesis between the normal liver and the hepatoma, it may be possible to decipher the molecular mechanism involved in the control of albumin gene expression. Furthermore, useful information can be gathered on the overall effect of neoplasia upon the regulation of gene expression in the normal rat liver.

This report describes the quantitation of albumin mRNA in the normal rat liver and hepatoma 7777 by molecular hybridization with a complementary DNA (cDNA[1]) probe. In vitro tissue-slice pulse-labeling experiments and translational assays of total mRNAs from both tissues in an mRNA-dependent cell-free rabbit reticulocyte system were also performed. Compared to the normal rat liver, there was approximately a fourfold reduction in the level of albumin mRNA in hepatoma 7777 with a corresponding decrease in albumin mRNA translational activity. Other experiments with albumin cDNA demonstrated that there was not a significant difference in albumin gene frequency between these tissues. During this study, the sequence complexity[1] and diversity of total poly(A)-containing RNA derived from the normal rat liver and hepatoma 7777 were analyzed and compared.

Experimental Procedures

Animals and Hepatomas. Male Buffalo rats bearing hepatoma 7777 were obtained from Dr. H. P. Morris, Howard University College of Medicine, Washington, D.C. A transplanting schedule of 4 weeks was maintained. Transplants were made bilaterally into thigh muscle of normal male Buffalo rats (Simonsen Laboratories, Gilroy, Calif.). All rats were fed water and Purina rat chow ad libitum. Rats with a body weight of 250–300 g were employed. In the case of hepatomas, mature tumors weighing 20–25 g were used and care was taken to remove any small areas of necrotic tissue.

Preparation of mRNA. Rat liver albumin mRNA was purified by specific polysome immunoprecipitation and poly(U)-Sepharose affinity chromatography as described previously (Taylor and Tse, 1976). Total liver RNA was isolated from unfractionated liver homogenates by phenol-chloroform extraction and the total poly(A)-containing RNA

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[1] Abbreviations used: cDNA, complementary DNA; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; R_0t, the product of RNA concentration in moles of nucleotides per liter and the time in seconds; C_0t, the product of DNA concentration in moles of nucleotides per liter and the time in seconds; complexity is defined as the length in nucleotides of the largest composite nonrepeating sequence in a nucleic acid preparation.

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was separated from the total poly(A)-lacking RNA by two successive cycles of affinity chromatography on poly(U)-Sepharose (Keller and Taylor, 1976). Total poly(A)-containing RNA was found to be devoid of ribosomal RNA contaminants when examined by polyacrylamide gel electrophoresis. Corresponding total hepatoma RNA preparations were isolated using the same procedures. An extinction coefficient of 1 A_{260} unit per 50 μg of RNA was employed for all RNA samples.

Synthesis of Complementary DNA. DNAs complementary to albumin mRNA, total liver, and hepatoma poly(A)-containing RNA were synthesized essentially as described previously (Keller and Taylor, 1977), employing an avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase). This enzyme was generously supplied by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, Florida) through the auspices of the Virus Cancer Program of the National Institutes of Health. The cDNA synthesis reaction mixture contained 50 μM [^{3}H]dCTP (23.8 Ci/mmol, New England Nuclear), 200 μM dGTP, 200 μM dATP, 200 μM dTTP, 50 mM Tris-HCl buffer (pH 8.3), 20 mM dithiothreitol, 6 mM $MgCl_2$, 100 mM NaCl, 30 μg/mL oligo(dT) (Collaborative Research), 50 μg/mL actinomycin D (PL Laboratories), 20 μg/mL mRNA, and 100 units/mL of reverse transcriptase. The reaction was performed at 37 °C for 20 min. Following the reaction, the cDNA was collected and its size determined by sedimentation on alkaline sucrose gradients as described (Keller and Taylor, 1977). The yield was approximately 180 ng of cDNA/μg of mRNA with a specific radioactivity of about 20 000 cpm/ng.

Excess RNA–cDNA Hybridization. Hybridization of excess RNA to ^{3}H-labeled cDNA by the rate procedure was performed at 68 °C under paraffin oil in 12 × 75 mm capped polypropylene tubes. The final reaction volume of 10 μL contained: 0.5 M NaCl, 9 μg of yeast tRNA (PL Laboratories, phenol extracted to remove protein), 0.1% sodium dodecyl sulfate, 2 mM EDTA,[1] 25 mM Hepes[1] buffer (pH 7.4), 500–3000 cpm (25–150 pg) of cDNA, and RNA in a concentration range of 0.2–2000 μg/mL, depending on the sample being hybridized. Reactions were terminated at various times by quickly immersing the tubes in an ethanol–dry ice bath.

Hybrids were measured by assaying the completed reactions for S_1 nuclease (Miles) resistant radioactive material. The completed hybridization reaction mixtures were incubated at 37 °C for 60 min in a 0.4-mL solution containing: 100 mM sodium acetate buffer (pH 5.0), 1 mM zinc acetate, 100 mM NaCl, 20 μg of denatured calf thymus DNA (Sigma), and 10 μg of S_1 nuclease. Control assays were performed in the absence of S_1 nuclease. After the incubation, an additional 200 μg of calf thymus DNA was added and the reaction material was precipitated with 12% trichloroacetic acid at 0 °C for 30–60 min. The precipitates were collected on glass-fiber filters (Whatman GF/C) and digested with Protosol, and the radioactivity was measured in a toluene-based scintillation fluid (Keller and Taylor, 1976).

RNA–cDNA hybridization by a titration procedure was performed as described above, except that each final reaction volume of 50 μL contained 50 μg of yeast tRNA, 1500 cpm (75 pg) of albumin cDNA. and an increasing amount of RNA (1–120 ng). Reaction mixtures were incubated at 68 °C for 2 h, and hybrids were measured with 20 μg of S_1 nuclease.

DNA Isolation. Normal rat liver or hepatoma 7777 was homogenized in 5 volumes of 0.25 M sucrose containing 12 mM NaCl, 25 mM $MgCl_2$, and 25 mM Tris-HCl buffer (pH 7.1), and centrifuged at 2500g_{max} for 10 min at 0 °C. The pellet was resuspended in the same buffer, filtered through several layers of sterile gauze, adjusted to 20 μg/mL in α-amylase (Sigma), and incubated at 0 °C for 60 min. After centrifuging at 3000g_{max} for 10 min at 0 °C, the pellet was resuspended in 0.15 M NaCl containing 0.10 M EDTA at pH 8.0, and adjusted to 2% in sodium dodecyl sulfate. After mixing thoroughly, the solution was adjusted to 1.0 M in $NaClO_4$ and stirred for 60 min at 25 °C. DNA was then extracted with 2 volumes of chloroform–isoamyl alcohol (5:1), spooled out of the aqueous phase, and redissolved in 15 mM NaCl containing 1.5 mM sodium citrate buffer at pH 6.8. The DNA solution was adjusted to 20 μg/mL in boiled RNase A (Sigma) and incubated at 37 °C for 60 min; it was then adjusted to 0.5 mg/mL in proteinase K (Merck) and incubated at 37 °C for 60 min. After two more extractions with chloroform–isoamyl alcohol, DNA was spooled out of the aqueous phase and redissolved in 1.5 mM sodium citrate buffer containing 15 mM NaCl at pH 6.8. The $A_{260/280}$ ratio of DNA solutions was typically 1.9 and the $A_{260/230}$ ratio was usually greater than 2.5.

DNA was sheared by two passes through a 40-mL French pressure cell (Aminco) at 36 000 psi. The DNA was then dialyzed against 15 mM NaCl, 1.5 mM sodium citrate buffer at pH 6.8, adjusted to 0.2 M NaCl, and precipitated with 2 volumes of ethanol at −20 °C. The sedimentation coefficient of the sheared DNA was determined by centrifugation in 5–29.4% isokinetic alkaline sucrose gradients (McCarty et al., 1974) containing 0.9 M NaCl and 0.1 N NaOH. Molecular weights were calculated according to Studier (1965), and a mean single-stranded molecular weight corresponding to 450 nucleotides was estimated.

DNA–Albumin cDNA Hybridization. Sheared unfractionated DNA in 0.12 M sodium phosphate buffer containing 2 mM EDTA at pH 6.8 was mixed with [^{3}H]albumin cDNA, and 10-μL aliquots were overlaid with paraffin oil in 12 × 75 mm polypropylene tubes. The tubes were placed in boiling water for 7 min to denature the DNA and then transferred to a 62 °C water bath for incubation. Reaction tubes were removed at various time intervals and frozen in an ethanol–dry ice bath. To the completed reactions was added 0.8 mL of 100 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl and 0.5 mM zinc acetate. The single-stranded DNA in 0.4 mL of the diluted reaction was digested with 10 μg of S_1 nuclease at 37 °C for 60 min. The undigested material was precipitated and radioactivity measured as described for RNA hybridization.

Cell-Free Protein Synthesis in an mRNA-Dependent Reticulocyte System. Nuclease-treated rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (1976). Briefly, the lysate was adjusted to 30 μM in hemin and treated with $CaCl_2$ and micrococcal nuclease (Sigma) to digest endogenous globin mRNA (Shapiro and Baker, 1977). After a 15-min incubation, excess ethylene glycol bis(2-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was added to inactivate the nuclease. Lysates treated this way were used immediately or stored in liquid nitrogen. The translational assays were performed essentially as described for an untreated reticulocyte lysate system (Taylor and Schimke, 1973). Total rat or hepatoma poly(A)-containing RNA at 13 μg/mL was used. Translation was dependent on the addition of exogenous mRNA, resulting in about a sevenfold stimulation above endogenous background. The albumin translation product, labeled with [^{3}H]leucine (60 Ci/mmol, New England Nuclear), was measured immunologically with rabbit anti-albumin. The antibodies were prepared as described (Palmiter et al., 1971). Albumin immunoprecipitates were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described

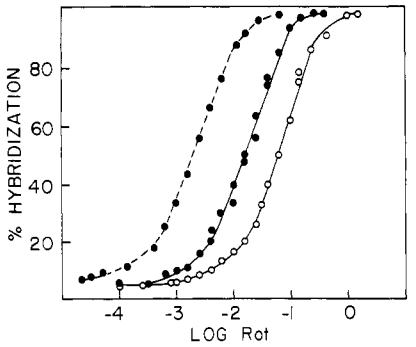

FIGURE 1: Hybridization kinetic curves of total poly(A)-containing RNA with albumin cDNA. Total poly(A)-containing RNA from rat liver (●-●) and hepatoma 7777 (○-○) was hybridized in up to 2500-fold weight excess to 100 pg of albumin cDNA as described under Experimental Procedures. Hybrids were assayed with S_1 nuclease. Excess pure albumin mRNA was also hybridized to the same albumin cDNA preparation (● - - - ●).

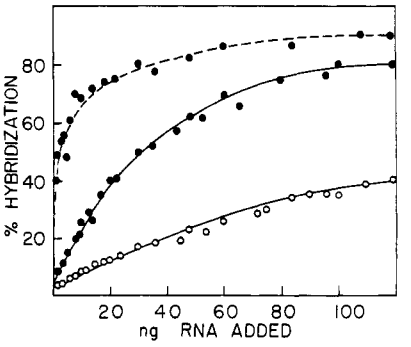

FIGURE 2: Titration of albumin mRNA in total poly(A)-containing RNA. Increasing amounts of total poly(A)-containing RNA from rat liver (●-●) and hepatoma 7777 (○-○) as indicated were incubated with 75 pg of albumin cDNA for 2 h as described under Experimental Procedures. Hybrids were assayed with S_1 nuclease. Increasing amounts of pure albumin mRNA were also incubated with the same albumin cDNA preparation (● - - - ●).

previously (Tse and Taylor, 1977). Total protein synthetic activity of the lysate reaction mixture was determined by examining the trichloroacetic acid precipitable material.

Determination of Albumin Synthesis in Tissue Slices. Rat livers or hepatomas (2.5 g) were cut into 1-mm slices and incubated at 37 °C for 5 min in 13 mL of Hanks' balanced salt solution (Grand Island Biological Co.) containing 6 mM sodium bicarbonate, 7 mM Hepes buffer (pH 7.4), and tenfold concentrated plasma amino acids (Jefferson and Korner, 1967) minus leucine. The incubation was performed in a water bath under 95% O_2/5% CO_2 at a moderate flow rate at a final pH of 7.2. [^{3}H]Leucine was then added to a specific radioactivity of 100 μCi/mL, and the incubation was continued for an additional 5 min. Tissue slices were rinsed thoroughly and then homogenized in 15 mL of cold homogenizing medium as described (Keller and Taylor, 1976). Radioactively labeled albumin and total protein were determined in tissue homogenates as described above.

Quantitation of Cellular RNA and DNA. Rat liver or hepatoma homogenates (17%, w/v) were prepared in 50 mM Tris-HCl buffer (pH 7.1) containing 25 mM NaCl and 5 mM $MgCl_2$ as described (Taylor and Schimke, 1973), and aliquots were treated with 0.5 volume of ice-cold 0.6 N perchloric acid. The acid precipitates were collected by brief centrifugation, washed twice with 0.5 volume of 0.2 N perchloric acid, and hydrolyzed in 0.8 volume of 0.3 N KOH at 37 °C for 60 min. RNA in the hydrolysate was determined by the method of Munro and Fleck (1966) and DNA content was estimated by the diphenylamine assay (Burton, 1956). Tissues from four different animals were examined and assays were performed in triplicate. Mean values ±1 standard deviation were determined.

Results

Quantitation of Albumin mRNA. A complementary DNA (cDNA) against a highly purified albumin mRNA template has been previously prepared and characterized (Keller and Taylor, 1977). Albumin cDNA is employed here as a specific hybridization probe for the quantitation of albumin mRNA in the total poly(A)-containing RNA derived from the normal rat liver and hepatoma 7777. Albumin cDNA hybridized to excess total liver poly(A)-containing RNA about ninefold more slowly than to pure albumin mRNA (Figure 1), indicating that albumin mRNA was 11% of the total liver poly(A)-containing RNA population. The hybridization of albumin cDNA to excess hepatoma RNA (Figure 1) showed

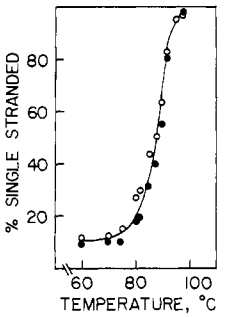

FIGURE 3: Thermal stability of albumin mRNA-cDNA hybrids. Hybridization reaction mixtures containing 50 pg of albumin cDNA and total poly(A)-containing RNA from rat liver, 88 ng (●-●), or from hepatoma 7777, 127 ng (○-○), were incubated for 6 h at 68 °C to obtain maximum hybridization and then quickly frozen as described under Experimental Procedures. Each reaction was heated for 10 min at the indicated temperature and frozen in an ethanol–dry ice bath before analysis with S_1 nuclease.

that albumin mRNA was only 2.8% of the total hepatoma poly(A)-containing RNA population representing an apparent 75% (fourfold) reduction compared to the normal rat liver. In all cases, the hybridization reaction occurred over a R_0t^1 range of 2 log units and went to greater than 95% completion. This reduction was also measured by a titration of albumin cDNA with total poly(A)-containing RNA (Figure 2). A constant amount of albumin cDNA was incubated with increasing amounts of total rat liver or hepatoma poly(A)-containing RNA for a fixed time period. One nanogram of pure albumin mRNA was required to attain a 25% hybridization (within the linear region of the titration curve), whereas 11- and 45-fold more total poly(A)-containing RNA were required in the case of the normal rat liver and hepatoma 7777, respectively. Thus, albumin mRNA comprised about 9% of the total poly(A)-containing RNA population of the normal rat liver and 2.2% of hepatoma 7777. A 76% (fourfold) reduction of albumin mRNA can be calculated for the hepatoma and is in excellent agreement with the above findings.

The thermal stability of hybrids formed between albumin cDNA and albumin mRNA derived from the normal rat liver and hepatoma 7777 was determined (Figure 3). A T_m of 89 °C accompanying a sharp transition in identical melting profiles was observed in both cases, indicative of the formation of faithful homologous hybrids. These findings suggest that there has been essentially no nucleotide change (less than 1%) in the

TABLE I: Quantitation of Albumin mRNA in Various RNA Fractions of Normal Rat Liver and Hepatoma 7777.

RNA fraction	$R_0t_{1/2}$ (mol s L^{-1})	Enrichment (-fold)	Albumin mRNA[a] (%)	Reduction (%)
Liver				
Total unfractionated RNA	0.891	1.0	0.20	
Poly(A)-lacking RNA	2.238	0.4	0.08	
Poly(A)-containing RNA	0.016	55.6	11.00	
Hepatoma				
Total unfractionated RNA	3.548	1.0	0.05	75
Poly(A)-lacking RNA	8.913	0.4	0.02	75
Poly(A)-containing RNA	0.063	56.0	2.80	75

[a] Determination was made by comparing the $R_0t_{1/2}$ values of column 2 with the $R_0t_{1/2}$ value of albumin mRNA–cDNA hybridization shown in Figure 1.

TABLE II: Relative Amount of Albumin mRNA in Normal Liver and Hepatoma 7777.

Measurement	Liver (%)	Hepatoma (%)	Reduction (%)
Hybridization with alb cDNA[a]			
Rate	11	2.8	75
Titration	9.0	2.2	76
Cell-free protein synthesis[b]	10	2.8	72
Incorp of [^{3}H]Leu[b] in tissue slices	8.9	2.9	67

[a] Data are derived from Figures 1 and 2. [b] Measurements were taken as described under Experimental Procedures.

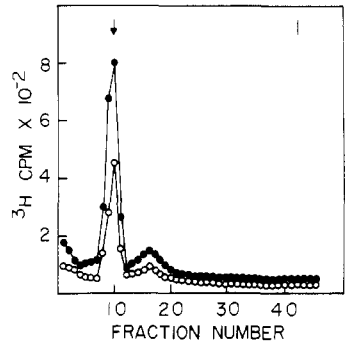

FIGURE 4: Gel electrophoresis of albumin immunoprecipitates from reticulocyte lysate reaction mixture. Translational assays in a mRNA-dependent reticulocyte lysate protein-synthesizing system were performed as described under Experimental Procedures, with 13 μg/mL total rat liver (●-●) or hepatoma 7777 (○-○) poly(A)-containing RNA in the presence of [^{3}H]leucine. The immunoprecipitates formed with rabbit anti-albumin as described previously (Tse and Taylor, 1977) were dissolved in 2% sodium dodecyl sulfate and 1% dithiothreitol and added to 10 μg of ^{14}C-labeled rat serum albumin. The complete mixture was heated for 3 min at 90 °C. Polyacrylamide gel electrophoresis was performed in 12% gel containing 0.1% sodium dodecyl sulfate, and the radioactivity in 2-mm slices was measured (Tse and Taylor, 1977). Migration of the proteins is from left to right with the vertical bar indicating the position of the bromophenol blue tracking dye. The arrow indicates the migration position of a ^{14}C-labeled serum albumin standard.

hepatoma albumin mRNA, at least along its 3′ terminus. Thus, normal liver albumin cDNA is apparently a valid hybridization probe for the quantitation of hepatoma albumin mRNA.

Poly(A)-lacking RNA, material that does not bind to poly(U)-Sepharose, has been reported to have albumin mRNA translational activity (Taylor and Tse, 1976). This material contains albumin mRNA that may possess a short or no poly(A) sequence at its 3′ terminus or a poly(A) sequence that is blocked. The reduction of albumin mRNA in hepatoma 7777 was therefore characterized further with respect to the distribution of albumin mRNA between the poly(A)-containing and poly(A)-lacking RNA population. Hybridizations of albumin cDNA with total unfractionated rat liver phenol-chloroform extracted RNA as well as poly(A)-lacking RNA were performed (Table I). Poly(A)-lacking albumin mRNA was decreased to 0.4-fold, while poly(A)-containing albumin mRNA was enriched 56-fold over the total rat liver RNA. Identical findings were obtained when albumin cDNA was hybridized with the corresponding hepatoma RNA fractions (Table I). Consequently, a 75% (fourfold) reduction in albumin mRNA in hepatoma 7777 could be calculated in all tumor RNA samples. These observations demonstrated that the reduction of albumin mRNA in hepatoma 7777 is not due to an altered distribution of albumin mRNA among various RNA populations with respect to binding to poly(U)-Sepharose.

Measurement of Albumin mRNA Translational Activity. Albumin mRNA activity was assayed by translation of rat liver or hepatoma total poly(A)-containing RNA in a cell-free mRNA-dependent rabbit reticulocyte system followed by immunoprecipitation of the in vitro albumin translation product. The relative albumin mRNA translational activity was reduced 72% (about fourfold) in hepatoma 7777. Gel electrophoretic analysis of the albumin immunoprecipitates isolated from the ^{3}H-labeled reticulocyte reaction mixture was performed in the presence of sodium dodecyl sulfate (Figure 4). The ^{3}H-labeled albumin translation products of normal liver and hepatoma 7777 comigrated with a ^{14}C-labeled serum albumin standard (Taylor and Schimke, 1973). Thus, albumin synthesized by translation of the normal rat liver and hepatoma 7777 mRNAs apparently have similar molecular weight and immunochemical properties. The relative level of albumin synthesis in rat liver and hepatoma was also measured by incubating tissue slices with [^{3}H]leucine. The fraction of radioactively labeled immunoprecipitable albumin relative to total protein was determined in order to normalize possible alterations in the rate of [^{3}H]leucine incorporation. The production of albumin in hepatoma 7777 was found to be reduced by 67% when compared with the normal rat liver. When considered with the results obtained from hybridization reactions (Table II), these observations indicated that the relative level of albumin mRNA translational activity correlated closely with the quantity of albumin mRNA.

Determination of Total Cellular RNA and DNA. The total cellular RNA and DNA content of the normal rat liver and hepatoma 7777 was determined. Normal liver contained 7.1 ± 0.3 mg of RNA and 3.1 ± 0.3 mg of DNA/g of tissue wet weight, whereas the hepatoma contained 6.9 ± 0.7 mg of RNA

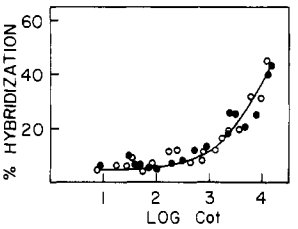

FIGURE 5: DNA excess hybridization to albumin cDNA. Unfractionated rat liver DNA (●-●) and hepatoma 7777 DNA (○-○), sheared to an average length of 450 nucleotides, were hybridized to albumin cDNA as described under Experimental Procedures. Each 10-μL reaction aliquot contained 83 μg of DNA and 27 pg of cDNA. Hybrids were assayed with S_1 nuclease.

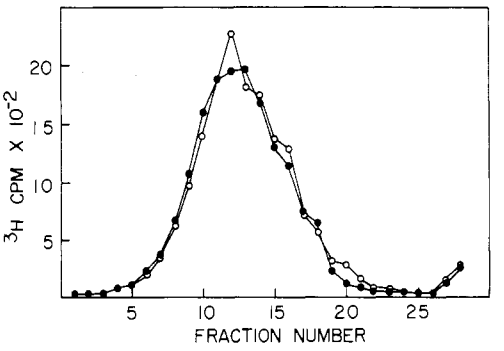

FIGURE 6: Size distribution of total cDNA on alkaline sucrose gradients. Complementary DNAs against total rat liver (●-●) and hepatoma 7777 (○-○) poly(A)-containing RNA were prepared and examined on separate alkaline isokinetic sucrose gradients as described under Experimental Procedures. The direction of sedimentation is from left to right.

and 5.2 ± 0.4 mg of DNA/g of tissue wet weight. Thus, about twofold less RNA per unit DNA was found in the hepatoma as compared to the normal liver. However, the data in Table I indicated that the poly(A)-containing RNA fraction was 1.8% of the total RNA in both tissues. These observations and the results of Table II suggested that there was a true fourfold reduction in the hepatoma albumin mRNA level as compared to normal rat liver.

Examination of Albumin Gene Frequency. To ascertain whether any detectable change in albumin gene frequency had occurred in the tumor, albumin cDNA was hybridized to excess sheared unfractionated DNA from rat liver and hepatoma 7777. The hybridization kinetics of these reactions are essentially the same with either DNA sample (Figure 5) and fall within the range expected for the renaturation of liver unique sequence DNA (Bonner et al., 1974; Colbert et al., 1977). There is no apparent difference in the albumin gene frequency between normal liver and hepatoma 7777. Although albumin mRNA is probably the most abundant mRNA species in both tissues, it is transcribed from unique sequence DNA.

Sequence Complexity of Total Poly(A)-Containing RNAs. Complementary DNA copies of total rat liver and hepatoma 7777 poly(A)-containing RNA were synthesized by means of a viral reverse transcriptase and labeled with [^{3}H]dCTP as described previously (Keller and Taylor, 1977). The size distribution of the cDNAs was examined on alkaline isokinetic sucrose gradients (Figure 6). Essentially identical sedimentation profiles were observed with both cDNA samples, and an average cDNA length of 600 nucleotides was estimated as described by Studier (1965). Hybridization of excess total rat liver poly(A)-containing RNA to its cDNA was performed (Figure 7, upper curve). The hybridization kinetics of this homologous reaction were analyzed to estimate the sequence complexity of the total poly(A)-containing RNA population. The hybridization reaction proceeded to about 95% completion in about five orders of magnitude in R_0t. This result indicated the presence of a heterogeneous population of poly(A)-containing RNA sequences occurring at different frequencies among various abundance classes (Bishop et al., 1974). Determination of the abundance classes and their $R_0t_{1/2}$ values from a somewhat featureless hybridization curve was conducted by computer analysis of the hybridization kinetics according to a program described by Pearson et al. (1977). The best fit for these data indicated the presence of three abundance classes of liver poly(A)-containing RNA (Table III). Examination of the size of liver poly-(A)-containing RNAs by isokinetic sucrose gradient sedimentation and polyacrylamide gel electrophoresis indicated an average length of approximately 1900 nucleotides (Taylor and Conn, unpublished observation), in close agreement with the findings of other studies (Hastie and Bishop, 1976; Colbert et al., 1977). Chicken ovalbumin mRNA, which has a length of 1900 nucleotides (Woo et al., 1975) was therefore used as a kinetic standard for sequence complexity calculations. Ovalbumin cDNA was prepared, characterized, and hybridized to its template, and a $R_0t_{1/2}$ of 4.0×10^{-4} mol s L^{-1} was determined for a cDNA length of 600 nucleotides (Keller et al., manuscript in preparation). Approximately 14% of the liver poly(A)-containing RNA was found to represent a high-abundance class with a corrected $R_0t_{1/2}$ of 5.4×10^{-4} mol s L^{-1} (Table III). This value would correspond to a sequence complexity of 2600 nucleotides, consistent with the estimated length of albumin mRNA (Taylor and Tse, 1976). Furthermore, 12.5% of the cDNA prepared against total liver poly(A)-containing RNA hybridizes to pure albumin mRNA with an $R_0t_{1/2}$ of 6.0×10^{-4} mol s L^{-1}, in agreement with the above observations (Keller and Taylor, manuscript in preparation). In these studies, therefore, the high-abundance class of liver poly(A)-containing RNA was considered to consist of only albumin mRNA. The second abundance class was estimated to contain about 85 sequences, and the third abundance class contained approximately 5500 sequences. Possible poly(A)-containing RNA present at greatly reduced frequencies may not have been detected in our measurements.

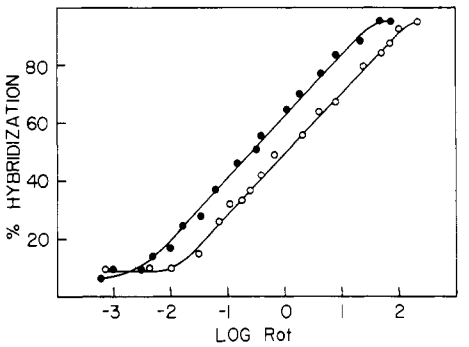

FIGURE 7: Hybridization kinetic curves of rat liver and hepatoma poly(A)-containing RNA to liver cDNA. Total poly(A)-containing RNA from rat liver (●-●) and hepatoma 7777 (○-○) was hybridized in up to 6700-fold weight excess to 150 pg of cDNA prepared against total rat liver poly(A)-containing RNA.

Hybridization of excess total hepatoma poly(A)-containing RNA to its cDNA was also performed (Figure 8, upper curve). Similar to the rat liver RNA-cDNA homologous hybridization, the kinetics of the hepatoma homologous hybridization were complex, with the reaction extending over a range of about 5 R_0t units. The hybridization profile again showed no distinct transitions. With the assistance of computer analysis, the best fit for the hybridization kinetics indicated three

TABLE III: Sequence Complexity of Total Poly(A)-Containing RNA in Normal Rat Liver and Hepatoma 7777.[a]

Tissue	Abundance class	Obsd $R_0t_{1/2}$	Fract of total	Corr $R_0t_{1/2}$	No. of diff sequences[b]
Liver	1	3.9×10^{-3}	0.14	5.4×10^{-4}	1.0
	2	7.9×10^{-2}	0.43	3.4×10^{-2}	85
	3	5.0	0.43	2.2	5500
Hepatoma	1	8.0×10^{-3}	0.08	6.4×10^{-4}	2
	2	0.1	0.46	5.2×10^{-2}	130
	3	8.9	0.46	4.1	10000

[a] Analyses of hybridization kinetics of homologous reactions presented in Figures 7 and 8. [b] Estimates were made by taking the sequence complexity of ovalbumin mRNA to be 1900 nucleotides (Woo et al., 1975) and the $R_0t_{1/2}$ of ovalbumin mRNA-cDNA to be 4.0×10^{-4} mol s L^{-1} for an ovalbumin cDNA of 600 nucleotides in length. The number of different liver mRNA sequences of 1900 nucleotides in length was calculated.

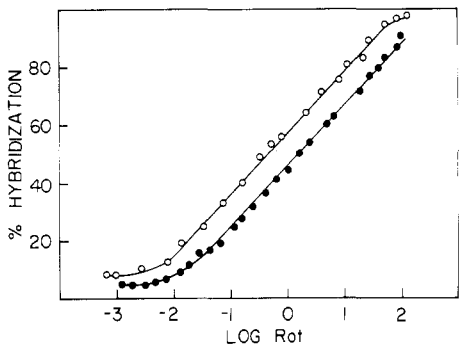

FIGURE 8: Hybridization kinetic curves of hepatoma and rat liver poly(A)-containing RNA to hepatoma cDNA. Total poly(A)-containing RNA from hepatoma 7777 (O-O) and rat liver (●-●) was hybridized in up to 10 000-fold weight excess to 100 pg of cDNA prepared against total hepatoma poly(A)-containing RNA.

abundance classes of hepatoma poly(A)-containing RNA (Table III). The first abundance class was estimated to contain two sequences, which probably correspond to the mRNAs coding for albumin and α-fetoprotein. In addition to the synthesis of serum albumin, hepatoma 7777 has been reported to produce a significant quantity of α-fetoprotein (Wolman et al., 1977; Sell and Morris, 1974). Preliminary experiments indicated that α-fetoprotein production comprises about 4% of the total hepatoma protein synthesis (Liao and Taylor, unpublished observations). The second abundance class represented about 130 sequences, while the third abundance class had a complexity of about 10 000 sequences.

Sequence Homology between the Rat Liver and Hepatoma 7777 Poly(A)-Containing RNA. Heterologous RNA-cDNA hybridization reactions were performed to allow a determination of the degree to which total poly(A)-containing RNA sequences from the normal rat liver and hepatoma 7777 were shared. The lower curve in Figure 7 shows the hybridization kinetics of rat liver cDNA hybridized with total hepatoma poly(A)-containing RNA. Compared to the homologous hybridization, the kinetics of the entire heterologous reaction are shifted to higher R_0t values. This shift indicates that poly(A)-containing RNA species present in the normal rat liver occur less frequently in the hepatoma poly(A)-containing RNA population. Similar to the homologous hybridization reaction, total hepatoma poly(A)-containing RNA saturated 95% of the rat liver cDNA. It appears that almost all of the rat liver poly(A)-containing RNAs distributed among three abundance classes are present in the hepatoma poly(A)-containing RNA population.

To determine what level of hepatoma poly(A)-containing RNA was present in the rat liver, the hepatoma cDNA was hybridized to total rat liver poly(A)-containing RNA (Figure 8, lower curve). Again, compared to the homologous reaction, the heterologous hybridization is shifted to higher R_0t values, indicating that hepatoma poly(A)-containing RNA species are also present in the normal rat liver but at reduced frequencies. In this heterologous hybridization, total rat liver poly(A)-containing RNA reproducibly attained greater than 90% completion, showing only a slight deviation from the homologous reaction. When considered with the significant difference in total sequence complexity (Table III), these findings suggest that most of the relatively abundant hepatoma poly(A)-containing RNA sequences are present in the normal rat liver and that a substantial fraction of the remaining differential sequences, probably of very high complexity and extremely low abundance, may only be found in hepatoma 7777.

Discussion

In this study, the molecular basis of reduced albumin synthesis in hepatoma 7777 has been examined. Quantitation of albumin mRNA in the normal rat liver and hepatoma 7777 was accomplished by two experimental methods of hybridization with albumin cDNA (Table II). Both the rate procedure and the titration method gave essentially the same results. Albumin mRNA was found to be about 10% of the total rat liver poly(A)-containing RNA population and was reduced about fourfold in the case of hepatoma 7777. A fourfold reduction of albumin mRNA in hepatoma 7777 could be determined with various tumor RNA fractions (Table I), and there were no alterations in the binding of hepatoma poly(A)-containing RNA to poly(U)-Sepharose. About 40% of the albumin mRNA population in either tissue failed to bind to the affinity ligand. Indeed, a significant fraction of eucaryotic mRNA in most tissues may contain short or no poly(A) sequences at its 3′-terminus (Rosen et al., 1975; Greenberg, 1976; Nemer et al., 1974; Lewin, 1975).

The nuclease-treated rabbit reticulocyte lysate cell-free system developed by Pelham and Jackson (1976) is dependent upon the addition of exogenous mRNA and is apparently capable of faithful translation of various eucaryotic mRNAs (Pelham and Jackson, 1976; Shapiro and Baker, 1977). Translation of the rat liver and hepatoma mRNAs in this system indicates that albumin mRNA is about 10% of the total rat liver mRNA population and is about fourfold reduced in the case of hepatoma 7777. The results from in vitro tissue-slice [^{3}H]leucine incorporation experiments support these findings (Table II). When considered with the results of hybridization experiments, these observations suggest that albumin synthesis

is a direct function of the quantity of translatable albumin mRNA. A similar result was reported by Keller and Taylor (1976) from their study of albumin synthesis in hypophysectomized rats. Previous studies by Peterson (1976) had also shown a close correlation between the rate of albumin synthesis and quantity of translatable albumin mRNA in hepatomas that produced albumin at different rates.

The normal rat liver and hepatoma 7777 were found to have essentially the same amount of total cellular RNA/g of tissue wet weight, and the poly(A)-containing RNA was 1.8% of the total RNA of both tissues. The fourfold reduction of albumin synthesis in the hepatoma therefore appears to be a result of an actual decrease in the amount of albumin mRNA.

Hybridization of cDNA to excess homologous poly(A)-containing RNA permits us to study the sequence complexity of poly(A)-containing RNA in the normal rat liver and hepatoma 7777. The limitations inherent in this method have been thoroughly discussed (Bishop et al., 1974; Ryffel, 1976; Axel et al., 1976; Young et al., 1976; Levy and McCarty, 1975). Sequence complexity measurements obtained in this manner represent minimum estimates because of difficulties in resolving individual abundance classes in the hybridization kinetics and in accurately determining the $R_0t_{1/2}$ values of the least abundant or the most complex class. Small variations in these $R_0t_{1/2}$ values can lead to large differences in the estimated number of RNA sequences. Data analyses become even more difficult when confronted with somewhat featureless hybridization curves (Figures 7 and 8). These limitations may be minimized to some extent by the availability of an internal mRNA standard (albumin mRNA in this case) and by the use of a computer program in data analysis.

The validity of these experiments rests on the assumption that the poly(A)-containing RNA population is transcribed by reverse transcriptase to yield cDNA in which the individual RNA species are represented with the same frequency as in the total poly(A)-containing RNA population. Our data show that this is apparently true for the rat liver albumin mRNA, since we have shown that albumin mRNA comprises about 10% of the total rat liver poly(A)-containing RNAs (Table II), and approximately 12.5% of the total rat liver cDNAs hybridizes with albumin mRNA. It is difficult, however, to ascertain that less abundant poly(A)-containing RNAs are also represented proportional to their cellular concentration in the cDNA.

Cross-hybridization of cDNA and poly(A)-containing RNA from the normal rat liver and hepatoma 7777 is useful in determining the extent to which these sequences are shared by both tissues. Results in Figures 7 and 8 show a high degree of homology between the poly(A)-containing RNA sequences of the rat liver and hepatomas, except that what is abundant in one tissue occurs less frequently in the other. Apparently, almost all of the liver poly(A)-containing RNA sequences distributed among the three abundance classes are present in the hepatoma. However, some poly(A)-containing RNA sequences of extremely high complexity and low abundance may only be found in the hepatoma as suggested by the degree of cDNA cross-hybridization and the significant difference in sequence complexity (Table III). The findings of other studies are similar, where divergent cell types (Williams et al., 1977; Williams and Penman, 1975; Rolton et al., 1977; Affara et al., 1977) and different tissues (Colbert et al., 1977; Young et al., 1976; Ryffel et al., 1975; Hastie and Bishop, 1977) are found to exhibit extensive homology between their mRNA populations. For instance, 85–90% of polysomal poly(A)-containing mRNA is common to normal and regenerating livers, while 11–14% is unique to either tissue (Colbert et al., 1977). Similarly a great majority of mRNA species in the SV40-transformed human fibroblasts is present in the normal cells (Williams et al., 1977). At most, 3% of the mRNAs in these tumor cells is new. Furthermore, mouse liver, kidney, and embryo each have previously been estimated to contain 7000–12 000 poly(A)-containing RNA sequences, most of these being common to all of these tissues, although there are quantitative differences in their relative abundancies (Young et al., 1976; Ryffel and McCarty, 1975; Hastie and Bishop, 1977). Based upon our findings, it appears that in addition to a fourfold reduction in the albumin mRNA level, hepatic tumorigenesis of hepatoma 7777 is characterized by some changes in the relative abundance of liver poly(A)-containing RNA sequences. The finding of unshared sequences in hepatoma 7777 is not unexpected and is consistent with a general concept of alterations in the control of gene expression during tumorigenesis.

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Light-Induced Free-Radical Reactions of Nucleic Acid Constituents. Effect of Sequence and Base–Base Interactions on the Reactivity of Purines and Pyrimidines in Ribonucleotides†

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ABSTRACT: The reaction with 2-propanol of purines and pyrimidines, induced photochemically with light of $\lambda > 300$ nm and di-*tert*-butyl peroxide as an initiator, was applied to a variety of adenosine-, guanosine-, and uridine-containing ribonucleotides in order to determine the rules which govern the reactivity of the heterocyclic bases of nucleotides. The reactivity of the purine moieties was found to depend on the conformation of the appropriate nucleotide (anti or syn) and on the site of binding of the phosphate group to the ribose moiety. Adenosine moieties (assuming an anti conformation) blocked at their 3′-hydroxyl reacted faster than those blocked at their 5′-hydroxyl. The reactivity of the guanosine moieties (tending to assume a syn conformation) was independent of the site of binding of the phosphate. The uridine moieties of the various nucleotides exhibited a wide range of reactivity. A correlation between the reactivity of the uridines and their involvement in stacking interactions with next- and second-neighboring purines could be made. Thus, the uridine moieties of U-U-U, G-U, U-G, A-U-A, and A-U-G were reactive, while those of A-U and A-U-U were unreactive. The relative reactivity of uridine moieties of nucleotides can, therefore, be used as a measure of the extent of pyrimidine–purine stacking and vice versa.

Exposure of cells to ultraviolet irradiation leads to the induction of a variety of chemical changes in the genetic material. These changes are manifested in biological effects, such as mutations, aging, carcinogenesis, and lethality (see reviews by Setlow, 1968; Burr, 1968; Rahn, 1972; Varghese, 1972; Smith, 1976). The multiplicity of photoproducts interferes with the chemical characterization and the direct assignment of a given chemical change to the consequent biological effect. We have approached this problem through the development of selective photochemical reactions for the various moieties of nucleic acids (Frimer et al., 1976). Accordingly, some selective photochemical modifications of purine and pyrimidine moieties of DNA have been developed (Ben-Ishai et al., 1973; Salomon and Elad, 1974a; Lorberbaum et al., 1976). In a previous publication (Havron et al., 1976), we formulated some rules which govern this selectivity in the reactions of adenine and

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