Changes in Expression of Albumin and α-Fetoprotein Genes during Rat Liver Development and Neoplasia†

José M. Sala-Trepat,* John Dever,‡ Thomas D. Sargent, Kelwyn Thomas, Stewart Sell, and James Bonner

ABSTRACT: Albumin mRNA was isolated and purified from rat liver polysomes by a combination of immunoprecipitation of specific polysomes, poly(U)–Sepharose 4B chromatography, and fractionation of the resulting poly(A)-containing RNA on a sucrose gradient. α-Fetoprotein (AFP) mRNA was isolated from Morris hepatoma 7777 by a similar procedure. The purity of the mRNA preparations was determined by analytical gel electrophoresis under denaturing conditions, analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the polypeptides synthesized in a wheat germ cell-free system, and the kinetics of hybridization to cDNA transcribed from albumin mRNA and AFP mRNA. The albumin mRNA possessed a chain length of ~2265 nucleotides and the AFP mRNA possessed a length of ~2235 nucleotides when examined under stringent denaturing conditions on agarose gels containing 10 mM methylmercury hydroxide. Analysis of poly(A) content by a hybridization assay with [^{3}H]poly(U) revealed the presence in albumin mRNA of a poly(A) region containing approximately 100 adenosine residues. The AFP mRNA preparation was found to contain an average poly(A) tract of approximately 190 bases. Thus, albumin mRNA appears to contain ~330 untranslated nucleotides, and AFP mRNA appears to contain a similar number (~285) of noncoding, nonpoly(A) bases. The purified albumin and AFP mRNAs were used as templates for synthesis of full-length cDNA hybridization probes. Both of the probes selectively hybridized to their templates with kinetics expected for single RNA species the sizes of albumin and AFP mRNA. R_0t analysis was used to quantitate albumin and AFP mRNA sequences during normal liver postnatal development and liver oncogenesis. The number of polysomal AFP mRNA molecules per liver was found to drastically decrease during the first weeks of postnatal life, concomitant with a decline in the AFP synthetic capacity of the livers and in the serum concentrations of AFP. During this period, the concentration of albumin mRNA molecules per cell in the liver remained at high, approximately constant levels. In Morris hepatoma 7777, the concentration of AFP-specifying sequences was at least 10^3-fold higher than that found in normal adult liver, whereas the content of albumin mRNA was four- to fivefold lower. These changes in concentration of albumin and AFP mRNA sequences closely correlated with a parallel variation in the specific protein synthetic capacity of the tissues.

Albumin and α-fetoprotein (AFP)[1] are two major plasma proteins synthesized by the mammalian liver and which are under developmental control (Abelev, 1971). Although these two proteins are similar in physicochemical properties, their serum concentrations have a reciprocal relationship. AFP is the major serum protein in the developing fetus, where it is synthesized by the yolk sac and embryonic liver (Gitlin et al., 1972; Sell & Skelly, 1976). During early postnatal life, serum levels of AFP drastically decrease to extremely low concentrations, only detectable by radioimmunoassay in normal, nonpregnant adults (Gitlin & Boesman, 1966; Sell et al., 1974b). Albumin serum concentrations follow an opposite pattern. They increase from low levels early in fetal development to high, approximately constant levels after birth and in adult life (Abelev, 1971; Van Furth & Adinolfi, 1969). Phenotypic expression of AFP is resumed in the adult rat during regeneration of the liver following partial hepatectomy, as well as after chemically induced necrosis of hepatocytes, and during exposure to chemical hepatocarcinogens (Abelev, 1971; Watabe, 1971; Sell et al., 1976). Highly elevated levels of serum AFP in hepatoma-bearing animals have been widely documented (Abelev, 1968; Sell et al., 1976). Most of the processes which lead to AFP reexpression cause albumin synthesis to diminish. Thus, a reduced rate of albumin synthesis in relation to that of total protein has been reported in regenerating liver (Schreiber et al., 1971) and in different hepatomas (Schreiber et al., 1969; Sell et al., unpublished experiments).

Albumin, the dominant protein species in adult sera, has many functions including binding and transport of metabolites and metabolic effectors and control of intravascular volume (Peters, 1977). The similarity of the chemical properties of albumin and AFP, and the demonstration of amino acid sequence homology betweeen these proteins (Ruoslhati & Terry, 1976), as well as immunogenic similarities in their denatured states (Ruoslhati & Engvall, 1976), suggests that AFP possibly serves as a fetal albumin. Other functions including an immunoregulator role (Lester et al., 1976) and a protective role of the fetus from the effects of maternal estrogens have also been proposed for this fetal protein

Understanding of the mechanisms that control the expression of the albumin and AFP genes may provide insight into the molecular basis of ontogenic development and malignant transformation. The mechanisms involved are being explored (Koga & Tamaoki, 1972; Innis & Miller, 1977). We report studies of the control of expression of these two proteins during ontogeny and liver carcinogenesis. We have purified to apparent homogeneity the mRNAs which code for rat albumin and for rat AFP. Full-length, specific cDNAs to both

† From the Division of Biology, California Institute of Technology, Pasadena, California 91125, and the University of California at San Diego Medical School, La Jolla, California 92093. *Received November 8, 1978.* This work was supported by the U.S. Public Health Service (Grant GM-13762), the National Cancer Institute, the National Institutes of Health (Grant CA-22227), and the French Research Council (C.N.R.S.).

* On leave of absence from Laboratoire d'Enzymologie, C.N.R.S., 91190-Gif-sur-Yvette, France.

‡ Present address: Department of Biology, Fort Lewis College, Durango, CO 81301.

[1] Abbreviations used: AFP, α-fetoprotein; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary DNA; AMV, avian myeloblastosis virus; poly(A), polyadenylic acid; poly(U), polyuridylic acid; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; NT, nucleotides; poly(A)$^+$ mRNA, polyadenylic acid containing mRNA; poly(A)$^-$ RNA, RNA lacking poly(A); R_0t, the product of the total RNA concentration in moles of nucleotide per liter and the time in seconds.

0006-2960/79/0418-2167$01.00/0

mRNAs have been synthesized and used as molecular hybridization probes to quantitate albumin and AFP mRNA sequences in rat liver at different stages of neonatal development and in Morris hepatoma 7777. We have correlated these results with those obtained from complementary studies on the albumin and AFP synthetic capacities of the tissues and with the albumin and AFP levels circulating in the serum of the same animals.

Experimental Procedures

Materials and General Procedures. The materials and general procedures used have been previously reported (Sala-Trepat et al., 1978). Poly(U)–Sepharose 4B and cyanogen bromide activated Sepharose 4B were obtained from Pharmacia. Methylmercury hydroxide was from Alfa Chemicals, Ventron Corporation, Beverly, MA.

Animals and Hepatomas. Male rats of the Sprague–Dawley strain were used for isolation of albumin mRNA and for preparation of liver RNA fractions from animals at different stages of development. Morris hepatoma 7777 was maintained and transplanted into both gastroenemius muscles of mature male Buffalo rats as described elsewhere (Sell et al., 1974a). Only exponentially growing tumors were used and special care was taken to remove any necrotic or infected tissue.

Preparation and Purification of Antigens and Antibodies. Rat serum albumin was purified from Faction V (Sigma) according to published procedures (Taylor & Shimke, 1973). The final albumin preparation was shown to be homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis (see Figure 4) and double-diffusion Ouchterlony analysis. Analytical amounts of immunochemically pure rat AFP were originally obtained from rat amniotic fluid by repeated fractionation on an isoelectric focusing column (Sell et al., 1972). This preparation was used to raise monospecific antibodies in rabbits. The immunoglobulin G (IgG) fraction from the resulting antisera was coupled to a cyanogen bromide activated Sepharose 4B (Cuatrecasas, 1970) and used as an immunoadsorbent for large-scale preparation of rat AFP. Preparative isolation of rat AFP from Morris hepatoma 7777 ascitic fluid was carried in four steps: affinity chromatography of crude ascitic fluid on the anti-AFP–Sepharose 4B column; passage over a second immunoadsorbent column to which the IgG fraction of a goat antirat albumin serum was attached; Sephadex G-200 chromatography; and isoelectric focusing. The protein prepared in this manner was shown to contain two electrophoretic variants (Figure 4) by $NaDodSO_4$–polyacrylamide gel electrophoresis and was in agreement with previous reports (Watanabe et al., 1975; Aussel et al., 1973). Aliquots of purified albumin and AFP were radioiodinated in vitro with lactoperoxidase and ^{125}I (Amersham/Searle) Shimke et al., 1974).

The purified antigens were also used to raise antibodies in goats. The IgG fraction prepared from the crude antisera by precipitation with 40% saturated $(NH_4)_2SO_4$ and subsequent chromatography on CM-cellulose and DEAE-cellulose (Shimke et al., 1974) was then applied to an albumin–Sepharose 4B column. The adsorbed antibody was eluted with sterile 0.2 M glycine adjusted to pH 2.7 with HCl. The eluant was neutralized, $(NH_4)_2SO_4$ precipitated, and dialyzed against 50 mM Tris-HCl, pH 7.6, buffer containing 150 mM NaCl and 5 mM $MgCl_2$. Highly purified goat antirat AFP was prepared from crude goat antisera following a similar procedure. Double-diffusion Ouchterlony analysis showed that the final antibody preparations were monospecific.

The purified specific antibodies were coupled to Sepharose 4B via cyanogen bromide activation to generate affinity columns for isolation of the respective antigens.

A nonimmune goat IgG fraction was injected subcutaneously into a burro. The burro (antigoat IgG) IgG was then prepared by $(NH_4)_2SO_4$ fractionation and made RNase free by chromatography on CM-cellulose and DEAE-cellulose (Shimke et al., 1974).

Preparation of Polysomes. Polysomes were prepared from normal rat liver (for isolation of albumin mRNA and other RNA fractions) or Morris hepatoma 7777 (for isolation of AFP mRNA) essentially as described by Schimke et al. (1974). Seventeen milliliters of the above postmitochondrial supernatant thus prepared was layered onto 1 mL of 0.5 M sucrose in buffer C (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, and 0.5 mg/mL heparin) over 15 mL of 1 M sucrose in buffer C on the top of 4 mL of 2.5 M sucrose in the same buffer. The polysome preparation was centrifuged on a discontinuous sucrose gradient at 25 000 rpm for 4 h at 4 °C. The polysomes which banded at the 1–2.5 M sucrose interface were removed in about 5 mL of heavy sucrose by puncturing the side of the tube with a sterile syringe. Polysomes were used immediately either for isolation of albumin (or AFP) specific polysomes by immunoprecipitation or for extraction of total polysomal RNA.

Isolation of Specific Polysomes by Immunoprecipitation. The polysomes removed from the heavy-sucrose interface were dialyzed overnight against a large volume of 50 mM Tris-HCl, pH 7.6, buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 100 μg/mL heparin. The dialysate was centrifuged in a Beckman SW 27 rotor for 10 min at 11 000 rpm to remove aggregates. The resulting polysome supernatant usually contained between 10 and 15 A_{260} units/mL. For isolation of albumin-synthesizing polysomes, polysome solutions obtained from normal adult liver were incubated with 74 μg/mL of pure goat antialbumin antibody for 45 min at 0 °C. The antibody–nascent chain–polysome complex was then precipitated by incubation with a 48-fold weight excess of RNase-free burro antigoat IgG for an additional 90 min at 0 °C with occasional gentle agitation. The entire mixture was then layered on a discontinuous sucrose gradient of 7.5 mL of 0.5 M sucrose over 14 mL of a 1 M sucrose pad, both containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, and 1% (w/v) each of Triton X-100 and sodium deoxycholate, and centrifuged at 11 000 rpm for 16 min on a SW 27 rotor at 2 °C. The sucrose solution containing the nonprecipitated polysomes was removed and saved for RNA extraction; the pellet was washed by suspending once in the same buffer and recentrifuged through the discontinuous sucrose gradient.

AFP-synthesizing polysomes were isolated in a parallel manner from polysome solutions prepared from Morris hepatoma 7777, except for the use of 50 μg/mL of monospecific goat antirat AFP antibody and a 40-fold excess of burro antigoat IgG.

Extraction of RNA from Polysomes. Extraction of RNA from the immunoprecipitate pellets and other polysomal preparations was carried out as previously described (Sala-Trepat et al., 1978).

Sucrose Density Gradient Analysis of RNA Preparations. Analysis of RNA samples on 5–20% linear sucrose gradients containing 50% dimethyl sulfoxide (Me_2SO) was performed as described elsewhere (Sala-Trepat et al., 1978).

Purification of Albumin mRNA and AFP mRNA. Ethanol-precipitated RNA extracted from albumin (or AFP) immunoprecipitated polysomes was collected by centrifugation, washed with 66% ethanol, dried by lyophilization, and dissolved

in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1% $NaDodSO_4$. The RNA was heated at 65 °C for 10 min and then chilled in an ice bath. The poly(A) RNA was then collected on a poly(U)–Sepharose 4B column. The bound RNA was eluted with 90% formamide containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% $NaDodSO_4$. The eluate was adjusted to 0.2 M NaCl and ethanol-precipitated at −20 °C overnight. Rechromatography of the RNA preparations over a second poly(U)–Sepharose 4B column was generally carried out to remove remnant rRNA contamination.

Albumin and AFP mRNAs were further purified from the poly(A)-containing RNA preparations by sedimentation in linear 5–20% sucrose gradients containing 50% Me_2SO. The ethanol-precipitated poly(A)-containing RNA samples were collected by centrifugation, dissolved in 600 μL of 40 mM Tris-HCl, pH 7.5, buffer containing 50% Me_2SO, 0.1 M LiCl, 5 mM EDTA, and 0.5% $NaDodSO_4$, and heated at 40 °C for 10 min. The samples were then loaded on 12 mL of 50% Me_2SO–sucrose gradients. Centrifugation was for 20 h at 41 000 rpm in the SW 41 Beckman rotor.

Isolation of Other RNA Fractions. Whole-cell RNA was prepared from normal adult rat liver and from Morris hepatoma 7777 as described (Sala-Trepat et al., 1978). Total polysomal RNA from either the rat liver or the hepatoma tissue, and the corresponding poly(A)-containing RNA fractions, was isolated as described above.

Determination of the Poly(A) Content of RNA Preparations. The content of poly(A) sequences in the different RNA preparations was assayed by a hybridization assay with [^{3}H]poly(U) as previously described (Sala-Trepat et al., 1978).

Cell-Free Translation in Wheat Germ Extracts and Immunoprecipitation of Proteins Synthesized in Vitro. RNA samples were translated in vitro in the wheat germ cell-free extract essentially as described (Sala-Trepat et al., 1978), except for the use of either [^{3}H]leucine (58 Ci/mmol; Amersham/Searle) or [^{35}S]methionine (400–550 Ci/mmol; Amersham/Searle) as the labeled amino acid. Aliquots of the in vitro translation reaction mixtures after incubation were adjusted to contain 37 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 1% Triton X-100, and 1% sodium deoxycholate, followed by the addition of 4 μg of rat serum albumin or rat AFP carrier and enough antialbumin or anti-AFP antibodies to ensure quantitative precipitation of the carrier proteins. After 90 min at room temperature, samples were incubated overnight at 4 °C. The immunoprecipitates were purified through a 1 M sucrose cushion containing 1% Triton X-100 and 1% sodium deoxycholate as described by Peterson (1976). The precipitation of 4 μg of bovine γ-globulin added to control samples by antibovine γ-globulin was used as a control for nonspecific precipitation. This value was subtracted from the antialbumin or anti-AFP precipitable counts.

Analysis of Cell-Free Translation Products by $NaDodSO_4$–Polyacylamide Gel Electrophoresis and Fluorography. Total ^{35}S-labeled cell-free translation products were prepared and analyzed on discontinuous $NaDodSO_4$–polyacrylamide slab gels as described (Sala-Trepat et al., 1978).

Electrophoresis of RNA on Agarose Gels under Denaturing Conditions. Electrophoresis of RNA in 1.2% agarose slab gels (3 mm × 15 cm) containing 10 mM methylmercury hydroxide was as described by Bayley & Davidson (1976). Electrophoresis was carried out for 9 h at 60 V. After electrophoresis, the gels were rinsed with 1 M ammonium acetate and stained in a 1 M ammonium acetate solution containing 1 μg/mL of ethidium bromide for 45 min at room temperature. The gels were then observed in ultraviolet light.

Synthesis of Complementary DNA to Albumin mRNA and AFP mRNA. cDNAs were synthesized with AMV reverse transcriptase (gift of Dr. J. Beard, Life Sciences, Inc., St. Petersburg, FL) in 25–100 μL reaction mixtures.

Size Analysis of cDNAs. [^{3}H]cDNA samples were made 0.1 M NaOH and 0.9 M in NaCl and loaded on 11.7-mL 5–20% sucrose gradients containing 0.1 M NaOH, 0.9 M NaCl, and 10 mM EDTA. Centrifugation was at 40 000 rpm for 24 h in a SW 41 Beckman rotor. Sheared sea urchin DNA of known size (6 and 13.1 S; gift of M. Chamberlin) was used as internal optical density markers. Molecular weights were calculated from the corresponding $s_{20,w}$ values by using the relationship $s_{20,w} = 0.0528^{0.4}$ (Studier, 1965).

[^{32}P]cDNAs were analyzed on alkaline-agarose slab gels, prepared as described by McDonnell et al. (1977). *Hind* III digests of PM2 DNA (gift of Horace B. Gray, University of Houston), labeled in vitro with [γ-^{32}P]ATP and T_4 polynucleotide kinase, served as DNA markers.

RNA Excess Hybridizations to Albumin [^{3}H]cDNA and to AFP [^{3}H]cDNA. RNA excess hybridizations with the [^{3}H]cDNAs were prepared in 10–50 μL reaction mixtures containing 10 mM Tris-HCl, pH 7.4, 0.18 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, single-stranded [^{3}H]cDNA (3000 cpm), and varying amounts of RNA. The reaction mixtures, taken up and sealed in siliconized tubes, were incubated at 68 °C for varying times up to 72 h. Hybrid formation was detected by treatment of samples with single-stranded specific S_1 nuclease (Savage et al., 1978). The data are plotted as the percent hybridization (percentage of S_1 resistant counts) as a function of the log of the product of molar concentration of RNA nucleotides and incubation time in seconds. Data were analyzed and plotted by using a computer program (Pearson et al., 1977) designed to fit the data according to the equation

$$c/C_0 = 1 - \exp[(-\ln 2)(R_0t/R_0t_{1/2})]$$

where c/C_0 represents the fraction of [^{3}H]cDNA in hybrid form at time t, R_0t = mol s L^{-1} of nucleotides RNA, and $R_0t_{1/2}$ = mol s L^{-1} of nucleotides RNA at 50% hybridization.

Measurement of Protein Synthesis in Liver and Hepatoma Explants. Small tissue fragments (0.3-mm square) were cultured in leucine-free Dulbecco's modified Eagle's minimum essential medium containing [^{3}H]leucine (5–10 μCi/mL) for 20 h at 37 °C in 5% CO_2. The culture supernatants and cell lysates were precipitated with 10% trichloroacetic acid to determine total protein synthesis and specifically precipitated with antialbumin or anti-AFP to determine specific protein synthesis. An antibovine γ-globulin (BGg)–carrier BGg system was used to coprecipitate nonspecific proteins prior to specific precipitation.

Determinatioin of Albumin and AFP Levels in Serum. The serum concentrations of albumin were determined colorimetrically by binding of bromocresol green and of AFP were determined by using a specific radioimmunoassay (Sell & Gord, 1973).

Results

Isolation and Purification of Albumin mRNA from Rat Liver Polysomes. The method used for the isolation of albumin mRNA is based on the selection of polysomes involved in the synthesis of this protein by indirect immunoprecipitation of the nascent polypeptide–polysome complex with affinity-purified monospecific antibodies raised against it (Shapiro et al., 1974; Schimke et al., 1974). Although this technique has been used as the only specific step in the isolation of several eukaryotic mRNAs for several specific proteins (Shapiro &

Table I: Purification of Albumin mRNA from Rat Liver

fraction	RNA (mg)	total translational act.[b] (cpm/μg)	sp albumin mRNA act.[b] (cpm/μg)	albumin mRNA/total mRNA (%)[c]	purificn[d] (x-fold)	yield[e]	poly(A)[f] (%)
total polysomal RNA	110[a]	63 380	5170	7.8	1	100	0.21
polysomal RNA from immunoprecipitate supernatant	91	58 330	680	1.1	0.13	11	0.25
polysomal RNA from immunoprecipitate	12.37	55 430	37 690	68	7.28	81	0.12
poly(A)$^+$ fraction	0.230	2.2×10^6	1.35×10^6	61	261	55	5.5
poly(A)$^-$ fraction	11.2	21 580	11 650	54	2.25	25	<0.0001
sucrose gradient purified (peak fractions only)	0.056	1.9×10^6	1.9×10^6	100	367	18.7	4.4

[a] Isolated from 80 g of rat liver. [b] Cell-free translation assays in the wheat germ system were carried out at different RNA concentrations. Values given were determined from the linear parts of the translational activity–RNA concentration plots. [c] Indicates the percentage of total translational products represented by albumin, as determined by immunoprecipitation of total synthesized proteins by using a monospecific antibody against albumin. [d] Fold purification was calculated from the specific albumin mRNA activity of the corresponding fraction. [e] Yield was calculated from the total albumin specific activity in each fraction. [f] Poly(A) content was determined by molecular hybridization with [^{3}H]poly(U) as described under Experimental Procedures.

Schimke, 1975; Schechter, 1974; Taylor & Tse, 1976; Innis & Miller, 1977), nonspecific adsorption of other polysomes to the antibody–polysome complex has, in other instances (Strair et al., 1977; Groner et al., 1977; Lee et al., 1978), limited the efficiency of the purification. The results shown in Figure 1 suggested that the RNA extracted from our albumin immunoprecipitates was contaminated by other mRNA species. The sedimentation pattern of total rat liver polysomal RNA in a sucrose gradient (Figure 1A) shows that albumin mRNA activity is associated with a peak in the 17S region of the gradient. The size distribution of poly(A)-containing RNA molecules (corresponding to the main part of the total population of mRNAs) was analyzed by hybridizing an aliquot from each fraction of the gradient with [^{3}H]poly(U). Figure 1B shows that the polysomal RNA extracted from the immunoprecipitate is highly enriched in poly(A)-containing molecules migrating in the position of albumin mRNA but that poly(A)-containing RNA is also found in significant proportions in other regions of the gradient. The results of the in vitro translation assays shown in Table I show that only 68% of the ^{3}H-labeled translation products depicted by this immunoprecipitated first-step purified RNA was immunoprecipitable by monospecific antialbumin. Residual rRNA species were removed from the crude polysomal RNA by further chromatography (2X) on a poly(U)–Sepharose 4B column. The poly(A)-containing RNA fractions were pooled and sedimented on a 5–20% sucrose gradient in 50% Me_2SO (Figure 2). The main absorbance peak comigrates with albumin mRNA activity. The fractions comigrating with the albumin mRNA activity peak were pooled and constituted our purified albumin mRNA. As can be seen in Table I, the immunoprecipitation step afforded a 7.28-fold purification. Poly(U)–Sepharose 4B chromatography yielded another 35-fold purification, followed by a 1.4-fold increase on the sucrose gradient. The overall purification for the peak fractions of albumin mRNA was approximately 367-fold from total rat liver polysomal RNA. Our final preparation appears to be nearly pure by the immunoprecipitation assay of total in vitro translated products. It is noteworthy that a significant proportion of the albumin mRNA activity is associated with the flow-through fraction of the poly(U)–Sepharose 4B step. This fraction is shown to contain undetectable amounts of poly(A). It would seem that more than 25% of the albumin mRNA molecules in rat liver is not polyadenylated or contains very short poly(A) segments not detectable by our molecular hybridization assay. A similar finding has been reported by other authors (Taylor & Tse, 1976; Strair et al., 1977). The poly(A) content of our pure albumin mRNA is 4.4%. This

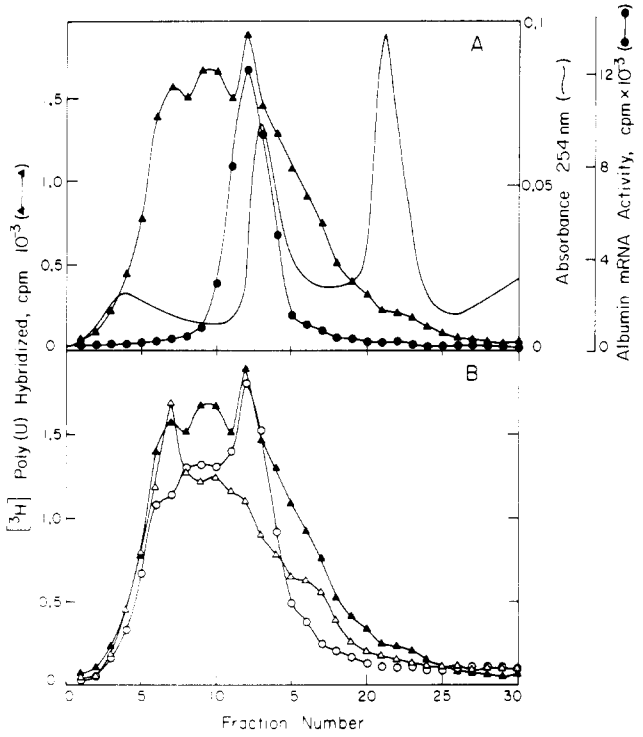

FIGURE 1: Distribution of poly(A)-containing RNA molecules and albumin translational activity in rat liver polysomal RNA samples after sedimentation in Me_2SO–sucrose gradients. (A) Approximately 75 μg of total polysomal RNA from rat liver was heated in the presence of Me_2SO and sedimented on a 50% Me_2SO–sucrose gradient. The gradient was fractionated and monitored for absorbance at 254 nm (—). Poly(A) content was determined on 85-μL samples from each fraction by molecular hybridization with [^{3}H]poly(U) (▲). The remaining sample from each fraction was ethanol-precipitated, washed with 70% ethanol, lyophilized, and translated in vitro in a wheat germ extract. Specific albumin mRNA (●) activity was determined by immunoprecipitation of total translation products as described under Experimental Procedures. (B) After immunoprecipitation of total rat liver polysomes with goat antialbumin and burro antigoat IgG, polysomal RNA was extracted from both the supernatant (△) and the immunoprecipitate (○) fractions. Sedimentation of RNA samples and analysis of poly(A) content on each fraction of the gradients were carried out as indicated above. In all cases, centrifugation was for 20 h at 41 000 rpm in the SW41 Beckman rotor. Sedimentation is from left to right.

Table II: Purification of AFP mRNA from Hepatoma 7777

fraction	RNA (mg)	total translational act. (cpm/μg)	sp AFP mRNA act. (cpm/μg)	AFP mRNA/ total mRNA (%)	purificn (x-fold)	yield	poly(A) (%)
total polysomal RNA	83[a]	43 918	4874	11.1	1	100	0.20
polysomal RNA from immunoprecipitate supernatant	69	45 918	317	0.7	0.065	5.4	0.18
polysomal RNA from immunoprecipitate	7.3	49 373	30 117	61	8.5	55	0.25
poly(A)$^+$ fraction	0.140	1.56×10^6	1.05×10^6	67	216	36.5	9.5
poly(A)$^-$ fraction	6.9	12 703	7621	60	2	13	<0.004
sucrose gradient purified (peak fractions only)	0.033	1.95×10^6	1.93×10^6	99	396	16	8.5

[a] Isolated from 10 exponentially growing 777 Morris hepatomas. Other details are as indicated in Table I.

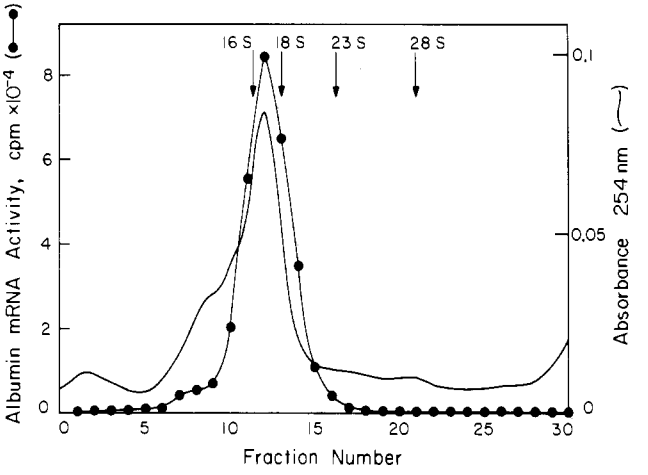

FIGURE 2: Purification of albumin mRNA on Me_2SO-containing sucrose gradients. The poly(A)-containing RNA extracted from the immunoprecipitate enriched in albumin-synthesizing polysomes was fractionated on a 5–20% linear sucrose gradient containing 50% Me_2SO. Centrifugation was for 20 h at 41 000 rpm in a SW41 Beckman rotor. Gradients were fractionated and the absorbance at 254 nm (—) was determined by using an ISCO absorbance monitor. Following ethanol precipitation of each fraction, identical aliquots were spun down, washed with 70% ethanol, lyophilized, and dissolved in H_2O. Determination of albumin mRNA activity (●) was performed by using the wheat germ translation assay described under Experimental Procedures. The arrows denote the position of 16- and 23S *E. coli* and 18- and 28S rat liver rRNA markers run on a parallel gradient.

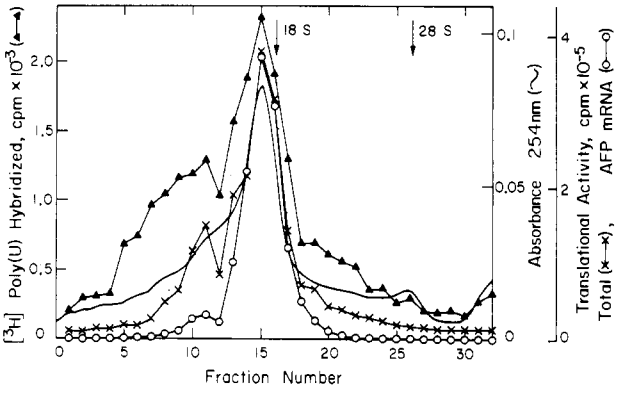

FIGURE 3: Purification of AFP mRNA by sedimentation of poly(A)-containing RNA isolated from the immunoprecipitate of AFP-synthesizing polysomes on Me_2SO-containing sucrose gradients. Experimental conditions were identical with those described in Figure 2. Following fractionation of the gradients 1-μL aliquots were removed from each fraction for assay of poly(A) content (▲). The determination of total mRNA translational activity (×) and AFP mRNA activity (O) was carried out in the wheat germ translation assay described under Experimental Procedures. The arrows denote the position of 18- and 28S rat liver polysomal RNA run on a parallel gradient.

is about half the average content of poly(A) in the total population of poly(A)-containing RNA molecules from rat liver polysomes (Sala-Trepat et al., 1978).

Purification of AFP mRNA from Morris Hepatoma 7777. Isolation of AFP mRNA from Morris hepatoma 7777 polysomes was achieved by a procedure similar to that used for the isolation and purification of albumin mRNA from rat liver. Immunoprecipitation and sedimentation analysis of the immunoprecipitated polysomal RNA on sucrose gradients gave results similar to those shown in Figure 1. The polysomal RNA preparation extracted from the immunoprecipitate was found to be only 61% pure by the in vitro translation assay. It was further purified as described above for albumin mRNA (Figure 3). The size distribution of poly(A)-containing RNA molecules on the gradient together with the results of the translation assays and the absorbance profile confirms the presence of non-AFP mRNA species in the immunoprecipitate poly(A)$^+$ fraction. The absorbance profile shows only a slight contamination by 28S rRNA. A main absorbance peak in the 5–20% sucrose gradient (Figure 3) cosediments with the high AFP mRNA activity containing fractions. These fractions, which appear by translation assay to be composed of pure AFP mRNA, represent our final AFP mRNA preparation. The final purification for the peak fractions of AFP mRNA gradient was approximately 396-fold. This purification (Table II) is similar to that achieved for albumin mRNA from rat liver polysomes (Table I). The mass fraction of AFP mRNA molecules in polysomal RNA from hepatoma 7777, as determined by translation assay, is of the same order as the albumin mRNA content in rat liver polysomal RNA. It can also be seen that, paralleling the results obtained for albumin mRNA, a significant amount of AFP translational activity is associated with the poly(A)$^-$ fraction of the immunoprecipitate polysomal RNA. However, the poly(A) content of the purified AFP mRNA is much higher than that of the albumin mRNA preparation (8.5% as opposed to 4.4%).

Analysis of the in Vitro Translation Products by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The purity of the sucrose gradient purified albumin mRNA and AFP mRNA preparations was further assessed by analysis of the cell-free translation products on discontinuous Laemmli slab gels (Laemmli, 1970). Figure 4 shows a fluorogram of total in vitro translated products synthesized from purified albumin mRNA and AFP mRNA together with the products resulting from rat liver and hepatoma 7777 poly(A)-containing polysomal RNA directed translation mixtures. Migration of in vitro radioiodinated samples of native albumin and AFP on

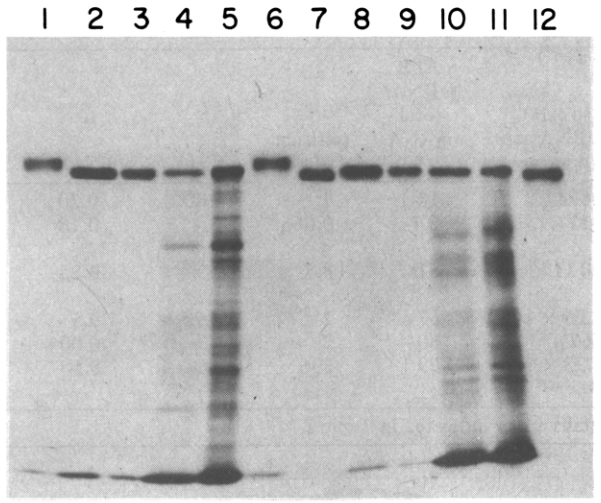

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of products synthesized in the wheat germ system directed by different mRNA preparations. Translations were carried out in 25-μL reaction mixtures with [^{35}S]methionine as the labeled amino acid. Aliquots of total cell-free translation products were analyzed on a 9% acrylamide slab gel with a 4.5% stacking gel, as described under Experimental Procedures. Slots 2 and 3 show the reaction products synthesized by the sucrose gradient purified AFP mRNA; slots 4 and 5 contain the products derived from total poly(A)-containing polysomal RNA from Morris hepatoma 7777; slots 8 and 9 show the products obtained from the synthesis directed by highly purified albumin mRNA (sucrose gradient peak); slots 10 and 11 depict the proteins synthesized by poly(A)-containing RNA isolated from total rat liver polysomes; slots 1 and 6 were loaded with native rat AFP labeled in vitro with ^{125}I; slots 7 and 12 show the migration of authentic rat serum albumin radioiodinated in vitro with ^{125}I. A photograph of a fluorogram of the dried gel is shown. The preflashed Kodak RP X-omat film was exposed for 20 h.

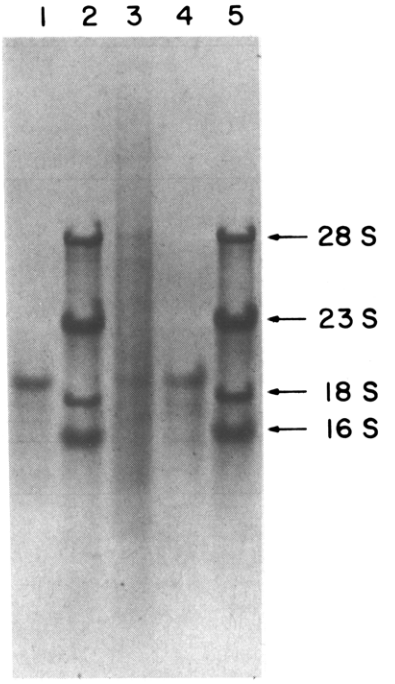

FIGURE 5: Electrophoresis of purified albumin and AFP mRNAs on a methylmercury–agarose slab gel. Various amounts of different RNA samples were applied to the slots of the 15-cm slab gel containing 1.2% agarose and 10 mM methylmercury hydroxide. In slot 1, the migration of 0.75 μg of highly purified AFP mRNA (sucrose density gradient peak) is shown. Slots 2 and 5 were loaded with 4 μg of a mixture of 16- and 23S *E. coli* rRNA markers together with 3 μg of 18S + 28S rat liver ribosomal RNA. Slot 3 contained 4.2 μg of poly(A)-containing RNA samples isolated from total rat liver polysomes. Slot 4 shows the sucrose density gradient purified albumin mRNA (0.7 μg). After electrophoresis, the gel was stained with ethidium bromide and photographed. Migration is from top to bottom.

the same gel is also shown for comparative purposes. Albumin and the AFP mRNA translation products are seen to migrate as single polypeptide bands coincident with prominent bands in the products synthesized by total poly(A)-containing polysomal RNA from rat liver and Morris hepatoma 7777, respectively. These results agree with the data of Tables I and II. The cell-free product of albumin mRNA is shown to migrate slightly slower than native albumin. This is in agreement with previous findings reported by Strauss et al. (1977) concerning the size of the preproalbumin polypeptide synthesized in vitro. Native rat AFP can be seen to have two electrophoretic variants, as previously shown by other authors (Watanabe et al., 1975; Aussel et al., 1973). The in vitro translation products of AFP mRNA directed reaction mixtures appear to migrate in the same positions as the preproalbumin but with a slightly faster mobility than both native AFP variants. The slower migration of native AFP probably reflects the inability of the wheat germ system to form the final glycosylated polypeptide, which is characteristic of the in vivo synthesized AFP (Nishi, 1970; Watanabe et al., 1975). We have made no attempts to provide evidence for a possible in vitro synthesized precursor of the AFP polypeptide chain found in serum.

Comparative Size Analysis of Albumin mRNA and AFP mRNA. Complete denaturation of RNA is a primary requisite for accurate molecular weight determination by gel electrophoresis. Gel electrophoresis on agarose gels containing 10 mM methylmercury hydroxide has been shown to fully denature even those RNAs containing very GC rich, helical regions (Bayley & Davidson, 1976; Lehrach et al., 1977). We have determined the molecular weights of the purified albumin mRNA and the AFP mRNA by their electrophoretic mobility on such agarose-denaturing gels by using rat liver and *Escherichia coli* rRNAs as markers. Both albumin and AFP mRNA migrate essentially as single bands behind the 18S rRNA (Figure 5). Trace amounts of 18S rRNA might, however, still be present in those preparations. A sample of total poly(A)-containing polysomal RNA from rat liver was also electrophoresed on the same slab gel. A discrete band comigrating with albumin mRNA can be readily observed. This is in agreement with the translation results shown in Table I and Figure 4, which indicate that albumin mRNA accounts for as much as 8% of the total mRNA activity in rat liver polysomal RNA. The discrepancy between the migration behavior of the albumin and AFP mRNA species relative to the 18S rRNA on sucrose gradients and gels might be due to incomplete denaturation of rRNA molecules, rich in GC, in 50% Me_2SO sucrose gradients.

Data averaged from three independent electrophoretic runs yielded a linear plot of the log of the molecular weight vs. the distance of migration. An average value of 770000 is obtained for albumin mRNA and 760000 is obtained for AFP mRNA. The calculated mRNA lengths in nucleotides are given in Table III. From the length of the mRNA molecules and the data on poly(A) content obtained by molecular hybridization with [^{3}H]poly(U) (Tables I and II), the length of the poly(A) segment at the 3′ end of the molecule can be calculated. A value of 102 3′-adenylate residues per molecule of albumin mRNA and 190 residues for AFP mRNA molecules is obtained. These values have been confirmed by direct determination of the number-average poly(A) length, after exhaustive digestion of albumin and AFP mRNA samples by a mixture of ribonucleases A and T_1 (Sala-Trepat et al., 1978).

Table III: Size Estimates for Albumin mRNA and AFP mRNA

protein	length of secreted product (no. of amino acids)	length of precursor synthesized in polysomes (no. of amino acids)	size of the mRNA[d] (in nucleotides)	size of poly(A) (in nucleotides)	expected coding bases	probable noncoding nonpoly(A) bases[f]
albumin	584[a]	584 + 24[c]	2265	102	1824	333
AFP	585[b]	585 + ?	2235	190	1755[e]	284

[a] From known amino acid sequence of human serum albumin (Behrens et al., 1975). [b] From preliminary primary structural data of human AFP (Parmelee & Deutsch, 1978). [c] From published data on rat liver preproalbumin, the initial product of in vitro translation of albumin mRNA (Strauss et al., 1977). [d] Calculated from Figure 6, by using 340 daltons per nucleotide. [e] Minimum estimate. [f] Determined by subtracting the number of coding nucleotides per six nucleotides for initiation and termination plus the number of adenine nucleotide residues at the 3′ end of the molecule from the total length of the mRNA.

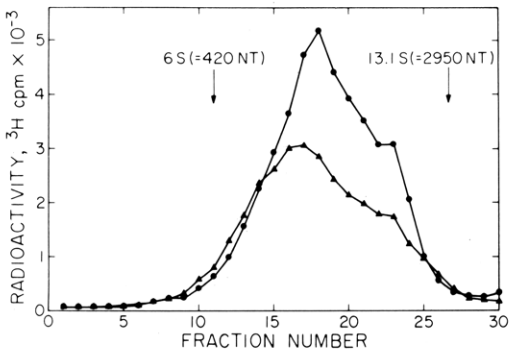

FIGURE 6: Size of albumin and AFP [^{3}H]cDNAs determined by alkaline sucrose gradient centrifugation. Albumin and AFP [^{3}H]-cDNAs were synthesized from highly purified albumin and AFP mRNAs, respectively, by using AMV reverse transcriptase as indicated under Experimental Procedures. cDNA samples were adjusted to 0.1 M NaOH, 0.9 M NaCl, and 10 mM EDTA and loaded onto 12 mL of 5–20% sucrose gradients containing the same buffer. Centrifugation was at 40 000 rpm for 24 h in a SW41 Beckman rotor. The arrows indicate the position of the internal sea urchin sheared DNA standards. (●) Albumin [^{3}H]cDNA; (▲) AFP [^{3}H]cDNA.

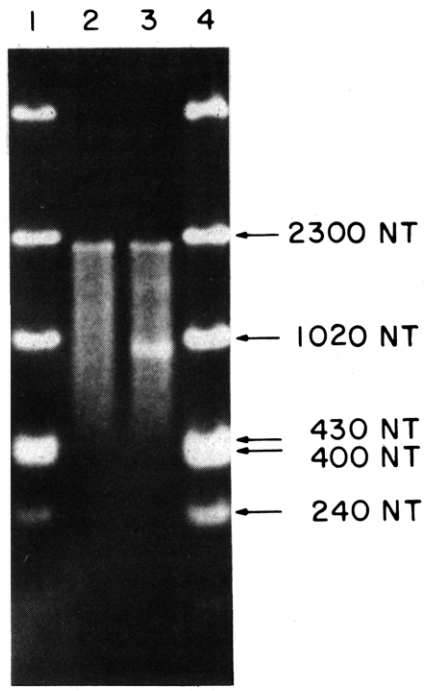

FIGURE 7: Autoradiogram of albumin and AFP [^{32}P]cDNAs following electrophoresis on an alkaline-agarose slab gel. Albumin and AFP [^{32}P]cDNAs were synthesized by using AMV reverse transcriptase and [^{32}P]dCTP as the labeled substrate. cDNA samples containing approximately 8000 cpm were electrophoresed on a 1% agarose gel containing 30 mM NaOH and 2 mM EDTA and autoradiographed as described under Experimental Procedures. Slot 1 and slot 4: PM2 DNA markers (in vitro ^{32}P-labeled *Hind* III fragments of PM2 DNA). Slot 2: AFP [^{32}P]cDNA. Slot 3: albumin [^{32}P]cDNA. Migration is from top to bottom.

The total maximum number of nontranslated, nonpoly(A) bases is calculated to be 333 nucleotides for albumin mRNA and 284 nucleotides for AFP mRNA (Table III). Those calculations take into account the size of the preproalbumin polypeptide, precursor of the final secreted product. Whether the AFP protein is also synthesized as a slightly larger precursor is not yet known.

Synthesis and Characterization of cDNA Probes. Preliminary studies were carried out to optimize conditions of synthesis of cDNA for maximum yield and size of transcripts by using total rat liver poly(A)-containing mRNA as template. Optimal conditions finally adopted were as indicated in Experimental Procedures. By use of total poly(A)-containing mRNA as template approximately 30–40% of the input template was transcribed into cDNA product. When albumin or AFP mRNA was used, overall transcription efficiencies of the order of 20–25% were obtained. A size analysis of the [^{3}H]cDNAs synthesized from albumin and AFP mRNA on alkaline sucrose gradients is shown in Figure 6. Both cDNAs show a similar size distribution with modal peaks in the region of 8.8–9.2 S (~1150 nucleotides) and a shoulder coincident with an expected size of 700 000 daltons (~2100 nucleotides). The cDNA molecules sedimenting in the high molecular weight region of the gradient probably represent full-length transcripts. In order to ascertain whether full-length products were synthesized under our reaction conditions, cDNAs to albumin and AFP mRNA were made by using [^{32}P]dCTP as the labeled substrate, and the reaction products were analyzed on alkaline agarose gels by using *Hind* III fragments of PM2 DNA as markers (Figure 7). The autoradiogram shows that, from both templates, a certain proportion of the ^{32}P-labeled products migrates as a discrete band, slightly faster than the 2300-nucleotide band of the PM2 marker. In addition, the ^{32}P-labeled transcripts of albumin mRNA show another discrete, very intense band migrating near the 1020-nucleotide band of the PM2 marker. We have analyzed different preparations of albumin and AFP [^{32}P]cDNAs on alkaline agarose gels with similar results. Synthesis of incomplete transcripts of defined lengths has been reported by Efstratiadis et al. (1975) for globin and chorion mRNAs. These may indicate the existence of particular base sequences or regions of extensive secondary structure within the mRNA molecules which may block further enzyme action.

The ^{32}P-labeled *Hind* III fragments of PM2 DNA provide calibration for the nucleotide length over the desired range. The length of the top band of different albumin and AFP [^{32}P]cDNA preparations averages 2180 nucleotides for albumin cDNA and 2120 nucleotides for AFP cDNA. These lengths are in agreement with the expected values for full-length cDNA transcripts if cDNA synthesis is initiated near

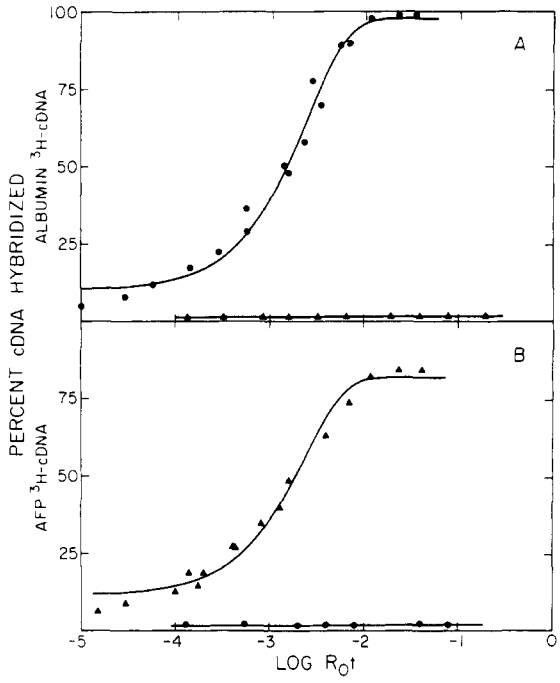

FIGURE 8: Specificity of the albumin [^{3}H]cDNA and AFP [^{3}H]cDNA probes. RNA excess hybridization reactions were performed with highly purified albumin mRNA (●) and AFP mRNA samples (▲). (A) Hybridizations to albumin [^{3}H]cDNA. (B) Hybridizations to AFP [^{3}H]cDNA. Hybridization conditions and S_1 nuclease assay procedures are described under Experimental Procedures.

the 3′ end of mRNA poly(A) tracts.

The albumin and AFP [^{3}H]cDNA probes were hybridized back to their templates, and the kinetics of the reactions are shown in Figure 8. Reactions between albumin [^{3}H]cDNA and albumin mRNA attained 98% S_1 nuclease resistance (Figure 8A) and those between AFP [^{3}H]cDNA and its template went to 80–85% completion. The kinetics of both reactions were approximately those expected from a single first-order reaction, indicating that each cDNA was transcribed from a single species of mRNA. These data provide an additional proof for the purity of our mRNA preparations. The $R_0t_{1/2}$ value for the albumin mRNA reaction is 1.738×10^{-3} mol s L^{-1} and that for the AFP mRNA hybridizations is 1.65×10^{-3} mol s L^{-1}. By comparison with the $R_0t_{1/2}$ (1.43×10^{-3} mol s L^{-1}) obtained for ovalbumin mRNA (~2180 nucleotides) under similar hybridization conditions (Shapiro & Schmike, 1975), we calculate a kinetic complexity of the order of 2500 nucleotides for our mRNAs. This is in good agreement with the actual length values determined from the electrophoretic mobilities on methylmercury hydroxide–agarose gels (~2250 nucleotides for both mRNAs).

Figure 8 also shows the specificity of our [^{3}H]cDNA probes. Under our stringent hybridization conditions, there was no reaction between albumin cDNA and AFP mRNA nor between AFP cDNA and albumin mRNA. Since a certain degree of amino acid sequence homology has been reported to exist between human albumin and AFP, we have made a preliminary study of any possible homology between albumin and AFP mRNA. Hybridization of albumin [^{3}H]cDNA to AFP mRNA was carried out under conditions of high salt and low temperature (1 M NaCl reaction mixtures at 49 °C) which favor formation of mismatched hybrids. Even under these conditions, no significant hybridization occurs after incubation to a R_0t of 1.

The fidelity of the hybrids formed between the cDNA probes and their mRNA templates is demonstrated in the thermal denaturation experiment shown in Figure 9. The sharp transition and the high T_m (91–92 °C) observed in the melting of both hybrids indicate little mismatch.

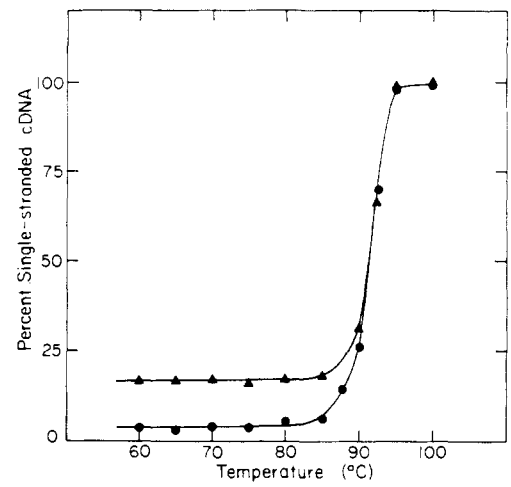

FIGURE 9: Thermal denaturation of albumin [^{3}H]cDNA–albumin mRNA (●) and AFP [^{3}H]cDNA–AFP mRNA (▲) hybrids. Hybrids of albumin [^{3}H]cDNA and AFP [^{3}H]cDNA (100 000 cpm) with their respective templates were formed by incubating the 300-μL reaction mixtures to a R_0t of 7×10^{-2}. The hybridization mixtures were then diluted into 120 μL of 0.18 M NaCl hybridization buffer and divided into 20 × 20 μL capillaries. The thermal stability of the hybrids was assessed by treatment with S_1 nuclease following 10-min equilibration at the designated temperature.

Table IV: Determination of Albumin mRNA Specific Sequences in Rat Liver

RNA fraction	$R_0t_{1/2}$	content of albumin mRNA (%)	purifcn to polysomal RNA
purified albumin mRNA	1.73×10^{-3}	100	456
whole-cell RNA	2.11	0.082	0.37
polysomal RNA	7.75×10^{-1}	0.22	1
poly(A)$^+$ polysomal RNA	2.47×10^{-2}	7.03	32
poly(A)$^-$ polysomal RNA	5.45	0.031	0.14
immunoprecipitate supernatant polysomal RNA	7.77	0.022	0.1
poly(A)$^-$ fraction from immunoprecipitate polysomal RNA	2.64×10^{-1}	0.658	2.99

Determination of Albumin and AFP mRNA Content in Different Cellular RNA Fractions from Adult Rat Liver and Morris Hepatoma 7777. The cDNA probes were used to determine the albumin and AFP mRNA content of different RNA fractions. This was done by RNA excess hybridization. The R_0t curves are shown in Figure 10; the $R_0t_{1/2}$ values of the reactions were obtained by using a computer program as indicated under Experimental Procedures. A direct calculation of the percentage of the RNA present as albumin or AFP mRNA in each preparation can be obtained from the $R_0t_{1/2}$ of the pure mRNAs and the $R_0t_{1/2}$ of the reaction of the corresponding RNA preparation ($100R_0t_{1/2}$ pure mRNA/$R_0t_{1/2}$ given RNA). The data are compiled in Tables IV and V. Albumin mRNA represents about 7% of the total mass of the poly(A)$^+$ polysomal RNA fraction from rat liver, and AFP mRNA comprises a similar fraction of the poly(A)-containing RNA preparation from Morris hepatoma 7777. These results and the final purification data obtained for the immunoprecipitate supernatant polysomal RNA and the poly(A)$^-$ fraction from the immunopurified RNA are in good agreement with the results shown in Tables I and II, which

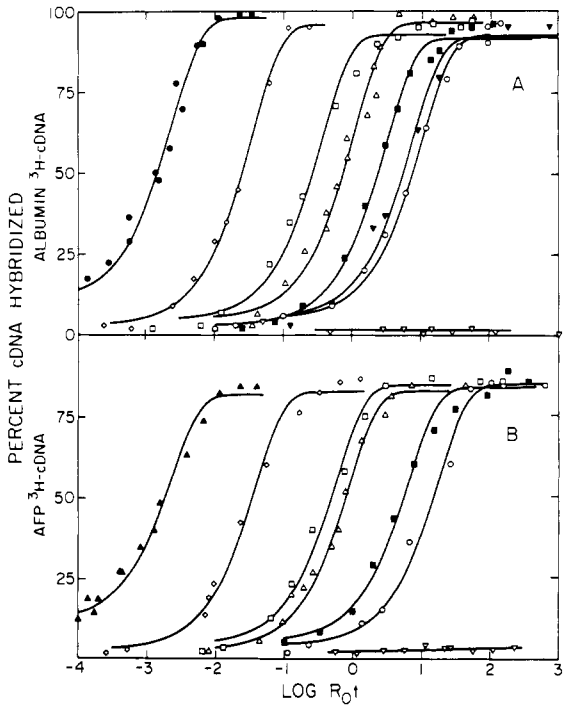

FIGURE 10: Hybridization kinetics of albumin [^{3}H]cDNA with different RNA fractions from rat liver (A) and of AFP [^{3}H]cDNA with Morris hepatoma 7777 RNA fractions (B). RNA excess hybridizations were carried out as described under Experimental Procedures. (●) Purified albumin mRNA; (▲) purified AFP mRNA; and (△) rat kidney polysomal RNA. The following symbols represent equivalent RNA fractions from rat liver (A) and from Morris hepatoma 7777 (B): (■) whole-cell RNA; (△) total polysomal RNA; (◊) total poly(A)-containing polysomal RNA; (▼) total poly(A)$^-$ polysomal RNA; (□) poly(A)$^-$ fraction from polysomal RNA isolated from the immunoprecipitate of specific polysomes; and (○) polysomal RNA isolated from the supernatant after immunoprecipitation of the albumin (A) or AFP (B) synthesizing polysomes.

Table V: Determination of AFP mRNA Specific Sequences in Morris Hepatoma 7777

RNA fraction	$R_0t_{1/2}$	content of AFP mRNA (%)	purificn to polysomal RNA
purified AFP mRNA	1.65×10^{-3}	100	385
whole-cell RNA	2.65	0.062	0.238
polysomal RNA	6.3×10^{-1}	0.26	1
poly(A)$^+$ polysomal RNA	2.74×10^{-2}	6.02	23
immunoprecipitate supernatant polysomal RNA	13.02	0.0126	0.048
poly(A)$^-$ fraction from immunoprecipitate polysomal RNA	4.15×10^{-1}	0.3975	1.52

are derived from in vitro translation experiments. No hybridization of albumin [^{3}H]cDNA or AFP [^{3}H]cDNA was detected when total polysomal RNA from rat kidney was used in the reactions. All these results taken together show that our cDNA probes are very specific for albumin and AFP mRNA sequences.

From the $R_0t_{1/2}$ of the reactions driven by whole-cell RNA from Morris hepatoma 7777 (Figures 10B and 11A), we estimated the albumin mRNA content to be 0.0165% of the total hepatoma RNA and the mass fraction represented by AFP mRNA to be 0.062%. Thus, AFP mRNA sequences are three to four times more abundant than albumin mRNA in hepatoma 7777. As shown in Table IV, albumin mRNA represents 0.082% of the total-cell mRNA in rat liver. Although our values for albumin mRNA content are somewhat

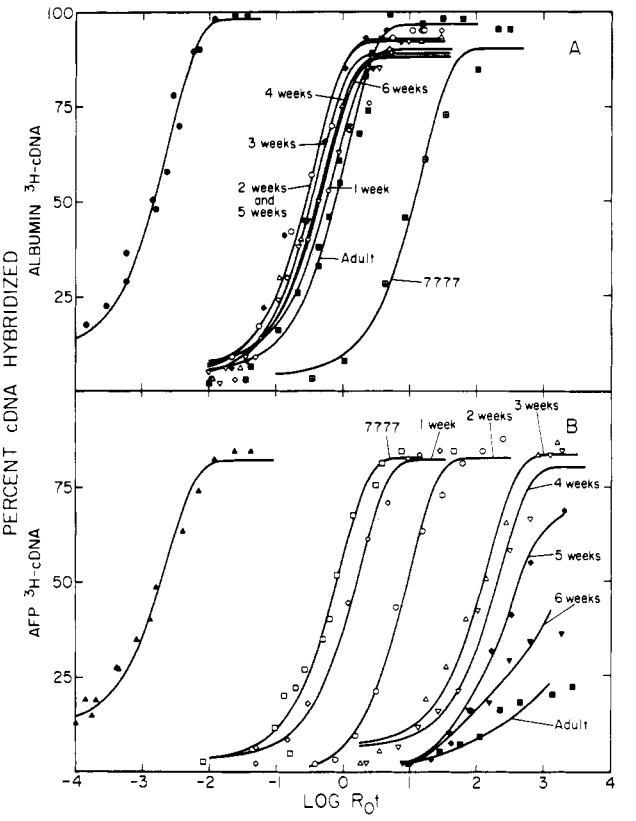

FIGURE 11: Quantitation of albumin (A) and AFP (B) mRNA sequences in rat liver polysomal RNA during early neonatal and adult life and in Morris hepatoma 7777 RNA. Polysomes were prepared from the livers of normal adult rats and of animals sacrificed at different stages of early postnatal life. Total polysomal RNA was extracted from 1-week-old rats (◊), 2-week-old rats (○), 3-week-old rats (△), 4-week-old rats (▽), 5-week-old rats (♦), 6-week-old rats (▼), and adult (3-month-old) animals (■). Whole-cell RNA (□) and total polysomal RNA (□) were prepared from exponentially growing Morris hepatoma 7777. RNA excess hybridization reactions were performed as indicated under Experimental Procedures. The R_0t curves for pure albumin mRNA (●) and pure AFP mRNA (▲) are also included for comparison.

lower than those recently reported by Tse et al. (1978), our results confirm the conclusion drawn by these authors concerning the reduction (four- to fivefold) of albumin mRNA sequences in hepatoma 7777 when compared to normal rat liver. In experiments in which AFP [^{32}P]cDNA was reacted with whole-cell RNA from adult rat liver (not shown), we detected no significant hybridization up to a R_0t of 10^3 mol s L^{-1}. Therefore, the AFP mRNA content of adult rat liver total RNA is less than 0.00015% by mass, which shows the dramatic increase in AFP mRNA sequences in Morris hepatoma 7777 (0.062% of total RNA, or more than 10^2–10^3-fold increase).

The albumin and AFP synthetic capacities of adult rat liver and hepatoma 7777 were measured simultaneously in explants of portions of the tissues excised for isolation of the RNA used for hybridization experiments. Albumin represented about 10.9 and 2.3% of the total proteins synthesized by rat liver and hepatoma 7777, respectively. AFP comprised about 10.3% of all the protein synthesized by hepatoma 7777 and less than 1% of that produced by rat liver. Thus, the decrease in albumin synthesis observed in Morris hepatoma 7777 correlates very well with the reduced content of albumin-specifying sequences when compared to rat liver.

Quantitation of Albumin and AFP mRNA Molecules in Normal Rat Liver during Early Stages of Postnatal Life. R_0t analysis was also used to quantitate albumin and AFP mRNA

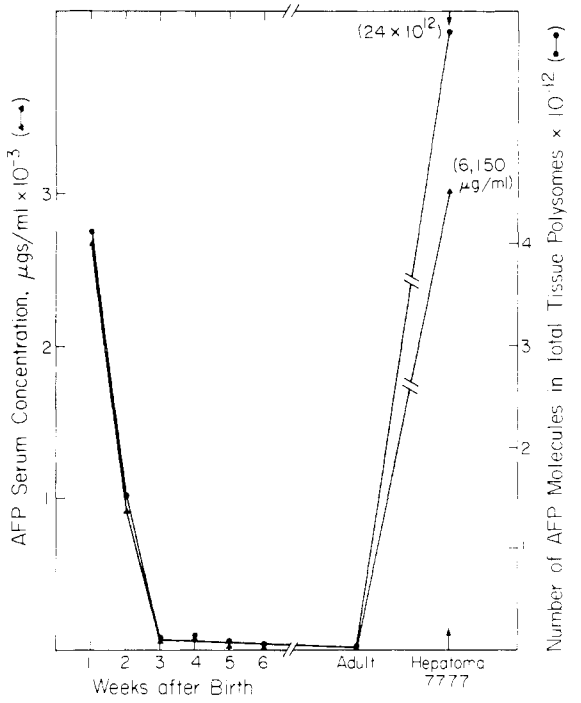

FIGURE 12: Comparison of the changes in AFP concentration in the serum with the content of AFP mRNA sequences in liver and hepatoma tissues during neonatal development and liver carcinogenesis. The serum concentrations of AFP in the developing rats or the tumor-bearing animals (▲) were determined by using the radioimmunoassay described elsewhere (Sell & Gord, 1973). The amount of AFP mRNA sequences in the polysomes (●) was measured by R_0t analysis as described in the text.

sequences in polysomal RNA preparations isolated from the livers of rats at different stages of postnatal development. When the RNA preparations were reacted with AFP [^{3}H]-cDNA, a series of parallel hybridization curves was generated which displayed progressively slower rates of hybridization from 1 week after birth to adult life (Figure 11B). This indicates a continued decrease in functional AFP mRNA sequences in the liver. In reactions with albumin [^{3}H]cDNA, the hybridization curves from the different RNA preparations clustered within a limited range of R_0t values (Figure 11A). This indicates no important variation in albumin mRNA content during liver postnatal life. By use of the $R_0t_{1/2}$ values of the reactions shown in Figures 11A and 11B and the molecular weight of the mRNAs, the number of albumin and AFP mRNA molecules in polysomal RNA per gram of tissue was calculated. This calculation takes into account the following data: (a) the DNA content per gram (wet weight) of normal rat liver is 1.7 mg and (b) the ratio of total polysomal RNA to DNA is 2.3 (Blobel & Potter, 1967). It also assumes that there is no significant variation in this ratio value in livers from different stages of postnatal development. Values obtained for the number of polysomal AFP mRNA molecules per total liver (or hepatoma tissue) are plotted in Figure 12 as a function of development. The value for Morris hepatoma 7777 assumes that, in tumor tissue, the polysomal RNA content per gram of tissue is not markedly different from that of normal rat liver. In this figure are also plotted the concentrations of AFP in the serum of the animals from which the polysomal RNAs were extracted. AFP determinations were carried out by radioimmunoassay (Sell & Gord, 1973). Changes in AFP concentration in the serum displayed a very good correlation with the levels of functional AFP mRNA molecules per total tissue. Reasonably good correlations were also obtained between the latter values and the AFP synthetic capacity of the different tissues, as determined by specific immunoprecipitation of the total proteins synthesized in 20-h tissue explants (not shown). However, the limited sensitivity of the immunoprecipitation technique makes it unsuitable for accurate estimation of AFP synthesis in livers excised from animals older than 2 weeks.

The number of polysomal albumin mRNA molecules per liver cell varied between 55000 in newborn animals (1 week old) and 110000 in rats 2 and 5 weeks old. The content of polysomal albumin mRNA molecules in normal adult animals was determined in a different experiment and found to be around 38000 molecules per cell. The variation did not follow a definite pattern. We do not know whether the relatively small differences in the number of polysomal albumin mRNA molecules are significant or whether they reflect variation between different individuals. The albumin serum concentrations found in the same animals varied between 19 mg/mL in 1-week-old rats and 40 mg/mL in the 6-week-old animals.

Discussion

The genes coding for the plasmatic proteins albumin and AFP constitute a particularly interesting system for exploration of the molecular events underlying changes in gene expression during both embryonic development and liver tumorigenesis. As a first step in the analysis of such a system, we have isolated, purified, and characterized the mRNAs encoding these two proteins. Immunochemical methods have recently been described for the isolation of albumin mRNA (Taylor & Tse, 1976) and AFP mRNA (Innis & Miller, 1977). In our hands the albumin and AFP mRNA preparations obtained after poly(U)–Sepharose 4B chromatography of the immunopurified RNAs were substantially contaminated by other mRNA species. Similar results have been reported by Strair et al. (1977) when immunoprecipitation methods alone were used for the isolation of albumin mRNA. We have isolated and purified both albumin and AFP mRNAs to an essentially homogeneous state by a combination of immunoprecipitation of specific polysomes, poly(U)–Sepharose 4B chromatography, and fractionation of the resulting poly(A)-containing RNA fraction on a 50% Me_2SO sucrose gradient. Albumin mRNA was purified 300–400-fold from total polysomal RNA from rat liver, and AFP mRNA was purified 400–450-fold from Morris hepatoma 7777 polysomal RNA. These values are those approximately expected for pure preparations considering the content of albumin-specifying sequences in the rat liver total mRNA population and the mass fraction of AFP mRNA in the hepatoma 7777 polysomal RNA.

Our in vitro translation studies with the poly(A)$^-$ fractions of the immunopurified RNAs suggest that significant proportions of albumin mRNA and AFP mRNA molecules are not polyadenylated or they lack poly(A) sequences of more than 10–15 adenosine residues. It seems unlikely that this observation could be attributable completely to a possible artifactual degradation of the poly(A)-containing mRNAs during the isolation procedure since the poly(A)$^-$ fractions can synthesize intact albumin (or AFP) polypeptides in the wheat germ system (not shown). Whether these deadenylated albumin and AFP mRNA molecules reflect aging of the mRNA molecules (Sheiness & Darnell, 1973) or the lack of poly(A) addition to some of the initial mRNA transcripts (Milcareck et al., 1974) remains to be studied. These observations are consistent with the findings reported from other laboratories for totally deadenylated globin mRNA (Bard et al., 1974), ovalbumin mRNA (Doel & Carey, 1976), and casein mRNA (Rosen et al., 1975) detected in various systems. Comparative analysis of poly(A) content in the purified preparations of

albumin and AFP mRNA showed that the average length of poly(A) in AFP mRNA molecules (~190 residues) is much larger than that of albumin mRNA (~102 residues). At the present time, the function of poly(A) and the significance of its size are unclear.

We have estimated the purity of our albumin and AFP preparations by several methods: (a) specific immunoprecipitation of the total proteins synthesized from the mRNAs in a wheat germ cell-free translation system; (b) examination of the total in vitro made polypeptides in $NaDodSO_4$–polyacrylamide gels; (c) electrophoresis of the mRNAs under fully denaturing conditions; and (d) molecular hybridization analysis. By all of these measures all mRNAs are essentially pure.

The purified albumin and AFP mRNAs have been copied with AMV reverse transcriptase. Under optimal conditions a substantial proportion of the transcripts are full-length cDNAs, as determined by electrophoresis on alkaline agarose gels. The upper band of the cDNA products has approximately the same size as that of the mRNAs used as templates. This provides an independent estimate of the molecular weight of the albumin and AFP mRNA molecules.

We have shown that under our standard reaction conditions the albumin and AFP [^{3}H]cDNA probes do not cross-react with the heterologous mRNA templates. Furthermore, when the cDNAs were hybridized to total rat kidney polysomal RNA, no reaction was observed up to high R_0t values. This lends support to our contention that the radiolabeled albumin and AFP cDNA probes are highly specific for albumin and AFP mRNA sequences, respectively. Even when the [^{3}H]-cDNAs were reacted with the heterologous templates under conditions which favor mismatch duplex formation, we could not detect significant levels of hybridization. This finding suggests that no substantial degree of sequence homology exists between the albumin and the AFP mRNA molecules. However, since we used the S_1 nuclease assay for hybrid detection, a small degree of sequence homology might not have been detected. Ruoslahti & Terry (1976) have demonstrated a certain degree of sequence homology between the human albumin and the AFP polypeptides but the comparison extended only to 59 residues (out of 580–585), and, therefore, the extent of homology could not be estimated. Recent results obtained by Peters et al. (1978) have shown that the N-terminal sequences and the C-terminal residue of murine albumin and AFP do not show homology. This is in line with our hybridization studies.

The albumin and AFP [^{3}H]cDNA probes have been used to explore two aspects of the expression of the albumin and AFP genes. First, we analyzed the changes in expression of albumin and AFP occurring in liver neoplasia. Then we studied the molecular mechanisms responsible for the decline in AFP synthesis during postnatal development. Normal adult liver synthesizes negligible amounts of AFP, whereas albumin represents as much as 8–10% of all the proteins synthesized. In agreement with a recent report by Tse et al. (1978), we have found that the albumin synthetic capacity of Morris hepatoma 7777 is reduced four- to fivefold when compared to that of the rat liver. On the contrary, AFP production by the hepatoma tissue is dramatically increased up to levels representing about 6–10% of the total proteins synthesized by the tissue. We have shown that these changes in albumin and AFP synthesis correlate well with the cellular content of albumin- and AFP-specifying sequences.

In neonatal rats the concentration of AFP in the serum was shown to drastically decrease during the first weeks of postnatal life, whereas albumin levels remained approximately constant during the same period. The albumin and AFP synthetic capacities of the tissues manifested similar patterns. A selective decrease in the number of AFP mRNA molecules in the liver polysomes has been shown to coincide with the kinetics of AFP phenotypic extinction in the same animals. We found no drastic variation in the number of polysomal albumin mRNA molecules in the liver of these rats. These results taken together suggest that the level of expression of albumin and AFP in mammalian liver and Morris hepatoma 7777 is mainly regulated by modulating the steady-state concentration of the corresponding functional mRNAs. Thus, translational control does not appear to be a major factor in the control of gene expression of albumin and AFP in rat liver.

Acknowledgments

We thank Dr. Harold P. Morris of the Department of Biochemistry, Howard University College of Medicine, Washington, D.C., who graciously furnished the tumor-bearing rats with which we established the 7777 line. We also thank R. F. Murphy for help in the computer analysis of the hybridization data. The skillful technical assistance of John Rising is gratefully acknowledged.

References

Abelev, G. I. (1968) *Cancer Res. 28*, 1344–1350.
Abelev, G. I. (1971) *Adv. Cancer Res. 14*, 295–358.
Aussel, C., Uriel, J., & Mercier-Bodard, C. (1973) *Biochimie 55*, 1431–1437.
Bard, E., Efron, D., Marcus, A., & Perry, R. P. (1974) *Cell 1*, 101–106.
Bayley, J. M., & Davidson, N. (1976) *Anal. Biochem. 70*, 75–85.
Behrens, P. Q., Spiekerman, A. M., & Brown, J. R. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 591p.
Blobel, G., & Potter, V. R. (1967) *J. Mol. Biol. 26*, 279–292.
Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.
Doel, M. T., & Carey, N. H. (1976) *Cell 8*, 51–58.
Efstratiadis, A., Maniatis, T., Kafatos, F. C., Jeffrey, A., & Vournakis, J. N. (1975) *Cell 4*, 367–378.
Gitlin, D., & Boesman, M. (1966) *J. Clin. Invest. 45*, 1826–1838.
Gitlin, D., Pernicelli, A., & Gitlin, G. M. (1972) *Cancer Res. 32*, 979–982.
Groner, B., Hynes, N. E., Sippel, A. E., Jeep, S., Huu, M. C. N., & Shutz, G. (1977) *J. Biol. Chem. 252*, 6666–6674.
Innis, M. A., & Miller, D. L. (1977) *J. Biol. Chem. 252*, 8469–8475.
Koga, K., & Tamaoki, T. (1974) *Biochemistry 13*, 3024–3028.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, D. C., McKnight, G. S., & Palmiter, R. D. (1978) *J. Biol. Chem. 253*, 3494–3503.
Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.
Lester, E. P., Miller, J. B., & Yachnin, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4645–4648.
McDonnell, M. W., Simon, M. N., & Studier, P. W. (1977) *J. Mol. Biol. 110*, 119–146.
Milcareck, C., Price, R., & Penman, S. (1974) *Cell 3*, 1–11.
Nishi, S. (1970) *Cancer Res. 30*, 2507–2513.
Parmelee, D. C., & Deutsch, H. F. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1618p.
Pearson, W. R., Davidson, E. H., & Britten, R. J. (1977) *Nucleic Acids Res. 4*, 1727–1735.

Peters, T. (1977) *Clin. Chem. (Winston-Salem, N.C.) 23*, 5–12.
Peters, E. H., Nishi, S., & Tamaoki, T. (1978) *Biochem. Biophys. Res. Commun. 83*, 75–82.
Peterson, J. A. (1976) *Nucleic Acids Res. 3*, 1427–1436.
Rosen, J. M., Wood, J. L. C., & Comstock, J. P. (1975) *Biochemistry 14*, 2895–2903.
Ruoslahti, E., & Terry, W. D. (1976) *Nature (London) 260*, 804–805.
Ruoslahti, E., & Engvall, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4641–4644.
Sala-Trepat, J. M., Savage, M., & Bonner, J. (1978) *Biochim. Biophys. Acta 519*, 173–193.
Sala-Trepat, J. M., Sargent, T. D., Sell, S., & Bonner, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 695–699.
Savage, M. J., Sala-Trepat, J. M., & Bonner, J. (1978) *Biochemistry 17*, 462–467.
Schechter, I. (1974) *Biochemistry 13*, 1875–1885.
Schimke, R. T., Palacios, R., Sullivan, O., Kiely, M. L., Gonzalez, C., & Taylor, J. M. (1974) *Methods Enzymol. 30*, 631–648.
Schreiber, G., Rotermund, H. M., Maeno, H., Weigand, K., & Lesch, R. (1969) *Eur. J. Biochem. 10*, 355–361.
Schreiber, G., Urban, J., Zahringer, J., Reutles, W., & Frosch, W. (1971) *J. Biol. Chem. 246*, 4531–4538.
Sell, S., & Gord, D. (1973) *Immunochemistry 10*, 439–442.
Sell, S., & Skelly, H. (1976) *J. Natl. Cancer Inst. 56*, 645–648.
Sell, S., Jalowayski, I., Bellone, C., & Wepsic, H. T. (1972) *Cancer Res. 32*, 1181–1189.
Sell, S., Wepsic, H. T., Nickel, R., & Nichols, M. (1974a) *J. Natl. Cancer Inst. 52*, 133–137.
Sell, S., Nichols, M., Becker, F., & Leffert, H. (1974b) *Cancer Res. 34*, 865–871.
Sell, S., Becker, F., Leffert, H., & Watabe, H. (1976) *Cancer Res. 36*, 4239–4249.
Shapiro, D. J., & Schimke, R. T. (1975) *J. Biol. Chem. 250*, 1759–1964.
Shapiro, D. J., Taylor, J. M., McKnight, G. S., Palacios, R., Gonzalez, C., Kiely, M., & Schimke, R. T. (1974) *J. Biol. Chem. 249*, 3665–3671.
Sheiness, D., & Darnell, J. E. (1973) *Nature (London), New Biol. 241*, 265–268.
Strair, R. K., Yap, S. H., & Shafritz, D. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4346–4350.
Strauss, A. W., Bennett, C. D., Donohue, A. M., Rodkey, J. A., & Alberts, A. W. (1977) *J. Biol. Chem. 252*, 6846–6855.
Studier, W. F. (1965) *J. Mol. Biol. 11*, 373–390.
Taylor, J. M., & Shimke, R. T. (1973) *J. Biol. Chem. 248*, 7664–7668.
Taylor, J. M., & Tse, T. P. H. (1976) *J. Biol. Chem. 251*, 7461–7467.
Tse, T. P. H., Morris, H. P., & Taylor, J. M. (1978) *Biochemistry 17*, 2121–2128.
Van Furth, R., & Adinolfi, M. (1969) *Nature (London) 222*, 1296–1299.
Watabe, H. (1971) *Cancer Res. 31*, 1192–1994.
Watanabe, A., Taketa, K., & Kosaka, K. (1975) *Ann. N.Y. Acad. Sci. 295*, 95–108.

Ribosomal Ribonucleic Acid Synthesis and Processing in Embryos of the Hawaiian Sea Urchin *Tripneustes gratilla*†

Jeffrey K. Griffith and Tom D. Humphreys*

ABSTRACT: Pulse-labeled RNA from plutei of the sea urchin *Tripneustes gratilla* was fractionated in sucrose gradients and sodium dodecyl sulfate–polyacrylamide gels and hybridized to recombinant plasmid DNA containing a portion of the *T. gratilla* ribosomal gene to identify a large, ribosomal RNA (rRNA) precursor of 2.5×10^6 daltons. Both pulse-chase and incorporation experiments indicate that the 2.5×10^6 dalton moiety is sequentially processed to 1.6×10^6 and 1.0×10^6 dalton intermediates and then to mature 1.45×10^6 (26 S) and 0.7×10^6 (18 S) dalton species. As judged by the respective molecular weights of the precursor and mature rRNA species, processing is nonconservative with approximately 15% of the precursor mass degraded and 85% contributing mature 26S and 18S rRNA. The absolute rate of synthesis of the rRNA precursor was measured by quantitating the flow of radioactivity through the embryos' GTP pool and into each of the rRNA species identified above. The precursor is synthesized at a rate of 3100 molecules h^{-1} $nucleus^{-1}$ and is present at a steady-state concentration of 1450 molecules/nucleus. Its half-life is 19 min. Comparison of the rate of synthesis of the precursor with the rate of accumulation of mature 26S and 18S rRNA shows that most precursor molecules yield mature rRNA. The number of copies of the ribosomal genes in the *T. gratilla* genome was measured by comparing the rates of reassociation of *T. gratilla* ribosomal DNA (rDNA) and single copy DNA with total *T. gratilla* DNA. These results indicate that the ribosomal genes are repeated 50 times per haploid genome. The ribosomal genes in pluteus cells are thus transcribed at an average rate of about 31 copies $sequence^{-1}$ h^{-1}.

The quantitative aspects of RNA metabolism and its developmental regulation have been extensively studied in sea urchin embryos (Emerson & Humphreys, 1970; Brandhorst & Humphreys, 1971, 1972; Dolecki et al., 1977; Galau et al., 1977; Brandhorst & Bannet, 1978; Dworkin & Infante, 1978). However, no unified picture of the synthesis, processing, and regulation of sea urchin embryo rRNA has emerged.

The paucity of information concerning sea urchin rRNA metabolism stems from its low rate of transcription during

† From the University of Hawaii, Pacific Biomedical Research Center, Kewalo Marine Laboratory, Honolulu, Hawaii 96813. *Received December 21, 1978.* This work was supported by grants from the National Institutes of Health. Jeffrey K. Griffith was a National Institutes of Health Postdoctoral Fellow.

0006-2960/79/0418-2178$01.00/0