Developmental Changes in the Synthesis of α-Fetoprotein and Albumin in the Mouse Liver. Cell-Free Synthesis by Membrane-Bound Polyribosomes†

Katsumi Koga‡ and Taiki Tamaoki*

ABSTRACT: Free and membrane-bound polyribosomes were isolated from the liver of 14-day fetal mice and the ability to produce α-fetoprotein (αFP) and albumin *in vitro* was analyzed. These protein products were characterized by polyacrylamide gel electrophoresis and immunoprecipitation. The results showed that membrane-bound polyribosomes produced αFP and albumin in the amount of 6 and 4%, respectively, of the total protein synthesized *in vitro*. The synthesis of αFP and albumin by free polyribosomes was about one-twentieth of that of membrane-bound polyribosomes, suggesting that αFP and albumin may be synthesized exclusively on membrane-bound polyribosomes *in vivo*. Analysis of αFP and albumin synthesis in relation to the developmental stage of mice (14- and 18-day fetuses and 1.5-day neonate) showed that the percentage of αFP synthesis decreased, whereas that of albumin synthesis remained relatively constant. This suggested a decrease in the population of αFP-synthesizing polyribosomes and an increase in albumin-synthesizing polyribosomes in the growing mice. The ratio of αFP and albumin synthesis by membrane-bound polyribosomes from 14- and 18-day fetal liver and 1.5-day neonatal liver was 1.3, 0.54, and 0.21, respectively. These changes observed *in vitro* appear to reflect the alteration of synthetic activity *in vivo*.

α-fetoprotein (αFP)[1] is a normal constituent α-globulin of fetal and early postnatal sera. In normal adult serum it is absent or present in only small amounts, but can increase to a significant level in certain pathological conditions, particularly primary hepatocarcinomas and teratocarcinomas. The assay of αFP has proved to be of value as an aid in diagnosis of these tumors (see reviews by Abelev, 1971, Uriel, 1969, Alexander, 1972, Masseyeff, 1972, and Anderson and Coggin, 1972).

The study of the control of αFP synthesis may ultimately reveal, at least in part, regulatory mechanisms involved in embryonic development and malignant transformation. In an attempt to understand basic mechanisms which control αFP synthesis we initiated cell-free studies of αFP production in embryonic mouse liver. In our initial approach cell lysates were prepared from fetal and neonatal mouse liver and incubated with radioactive amino acids (Tamaoki *et al.*, 1974). It was found that both αFP and albumin were synthesized in this system and the relative amounts of αFP and albumin synthesized varied depending upon the stage of development of mice from which the liver extract was prepared. As a continuation of cell-free studies of αFP and albumin synthesis in developing liver, we have examined the ability of isolated polyribosomes to produce these proteins *in vitro*. We describe here the results which indicate that membrane-bound, but not free, polyribosomes produce αFP and albumin and their ability to synthesize αFP and albumin changes as a function of the developmental stage of mice.

Materials and Methods

Mice. ICR mice were used throughout the experiment. The gestation period was determined from the day when a vaginal plug was observed.

Preparation of Polyribosomes. Free and membrane-bound polyribosomes were prepared according to Blobel and Potter (1967) with slight modifications. Fetuses were removed by cesarean section and the liver was homogenized manually in a glass homogenizer in a grinding solution which consisted of an equal volume of buffer A (20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 25 mM KCl, 6 mM β-mercaptoethanol, 0.5 mM dithioerythritol (DTE), 0.1 mM EDTA, and 0.25 M sucrose) and postmicrosomal supernatant from adult mouse liver (see below); 1 ml of the grinding solution was used to process ten livers from fetal and neonatal mice. The homogenate was centrifuged at 3000*g* for 15 min in a Sorvall centrifuge. Aliquots of the supernatant (7 ml) were layered over a discontinuous sucrose gradient containing 21 ml of 1.0 M sucrose and 7 ml of 1.8 M sucrose both of which were made in the grinding solution without sucrose. In some experiments, the sucrose cushion was made with 1.45 and 2.0 M sucrose without a significant change in the results. The samples were centrifuged at 25,000 rpm for 16 hr in the SW27 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 4°. Membrane-bound polyribosomes formed a band at the boundary of the two sucrose layers and were recovered from the top by suction. The concentration of membrane-bound polyribosome preparations was 30–50 A_{260}/ml. Free polyribosomes which formed a pellet at the bottom of the centrifuge tube were gently resuspended in buffer A to a final concentration of approximately 50 A_{260}/ml. Figure 1 shows sedimentation patterns of free and membrane-bound polyribosome preparations in a sucrose gradient. The net amount of polyribosomes was determined by measuring the areas of ribosome aggregates larger than monosomes using a planimeter. It ranged from 30 to 60% of the total A_{260} of polyribosome preparations. The total amino acid incorporation measured as described below increased linearly with an increasing amount of polyribosomes at least up to 10 net A_{260} units/ml of reaction mixture.

Postmicrosomal Supernatant from Adult Mouse Liver. The

† From the University of Alberta Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, Edmonton, Alberta, Canada. *Received February 11, 1974.* This work was supported by National Cancer Institute of Canada and Medical Research Council of Canada.

‡ Present address: Agricultural Chemistry Institute, Kyushu University, Fukuoka, Japan.

[1] Abbreviation used is: αFP, α-fetoprotein.

liver from adult mice was homogenized in a Teflon pestle homogenizer in buffer A (3 livers/20 ml). The homogenate was centrifuged at 3000*g* for 15 min and the resulting supernatant was centrifuged at 233,000*g* for 2 hr. The top two-thirds of the high-speed supernatant was dialyzed extensively against buffer A.

Protein Synthesis by Polyribosomes in Vitro. The reaction mixture for cell-free protein synthesis contained the following components per ml: 1 μmol of ATP, 0.3 μmol of GTP, 10 μmol of phosphocreatine, 50 μg of creatine phosphokinase, 50 μg of mouse liver tRNA, 20 mM Tris-HCl (pH 7.8), 4 mM $MgCl_2$, 60 mM KCl, 6 mM β-mercaptoethanol, 0.5 mM DTE, 6 μCi of [^{3}H]leucine (1 Ci/mmol, Schwarz BioResearch, Inc., Orangeburg, N. Y.), 0.025 μmol each of 19 other amino acids, 5 mg of adult mouse liver high-speed supernatant protein, and 5–15 A_{260} units of polyribosome preparations. For a routine assay of polyribosome activity, a reaction mixture of 100 μl was incubated at 30° for 30 min and then given 2 ml of 10% trichloroacetic acid to stop the reaction. The sample was kept in boiling water for 15 min and the insoluble material was collected on a glass fiber filter for measurement of radioactivity in toluene-based scintillation fluid.

For analysis of protein products, a 2–5-ml reaction mixture was incubated at 30° for 60 min. Total protein synthesis was determined using a 100-μl aliquot as described above. The remaining sample was centrifuged at 40,000 rpm for 60 min in a Spinco SW0.1 rotor. The supernatant id not contain newly formed αFP nor albumin (see Results). The pellet was resuspended in 10 mM Tris-HCl (pH 7.4)–0.15 M NaCl of the same volume as the original sample. The suspension was frozen in Dry Ice–acetone, thawed, and sonicated in a Bronson sonifier at setting 5 for 5 min. This process was repeated three times. The sonicated sample was centrifuged as above and the supernatant was assayed for αFP and albumin by polyacrylamide gel electrophoresis and immunoprecipitation.

Polyacrylamide Gel Electrophoresis. Carrier αFP and albumin, approximately 10 μg each, which had been treated with neuraminidase were added to a sample to be analyzed. The treatment with neuraminidase was carried out by incubating a mixture of αFP and albumin, 1 mg each, with 0.2 mg of neuraminidase (*Clostridium perfringens*, 2 units/mg, Sigma Chem. Co., St. Louis, Mo.) at 37° for 30 min. This resulted in a slower electrophoretic mobility of αFP due to the removal of sialic acid residues (Gustine and Zimmerman, 1973) but did not change the migration of albumin.

Polyacrylamide gel electrophoresis was performed in the presence and absence of sodium dodecyl sulfate. Non-sodium dodecyl sulfate gel electrophoresis was carried out in 7% gel (0.5 × 8 cm) at pH 8.9 according to Davis (1964). The gel was stained with Amido Black, scanned at 650 nm, and then sliced into 1-mm fractions. Two consecutive slices were placed in each counting vial and dissolved in 0.4 ml of H_2O_2 at 60° overnight. Aquasol scintillation liquid (NEN Canada, Montreal, Quebec) (10 ml) was added and radioactivity was measured in a liquid scintillation counter.

Sodium dodecyl sulfate gel electrophoresis was performed in 7% gel (0.5 × 7 cm) according to Weber and Osborn (1969) and Dunker and Rueckert (1969). The gel was stained with Coomassie Brilliant Blue, scanned at 600 nm, and then analyzed for radioactivity as described above.

Antiserum against αFP. αFP (2 mg) partially purified from fetal homogenate was mixed with an equal volume of complete Freund's adjuvant and injected into rabbits intramuscularly. This was followed by four additional, weekly injections using incomplete Freund's adjuvant. Two weeks after the last injection, the rabbits were bled. The serum was absorbed several times with adult mouse serum until it showed no reaction with adult serum. In double immunodiffusion the absorbed serum developed a single precipitin line with fetal serum which fused with the precipitin line formed with purified αFP. The specificity of the absorbed serum was further checked by immunoelectrophoresis in which a single precipitin line was observed with fetal serum at a site corresponding to αFP.

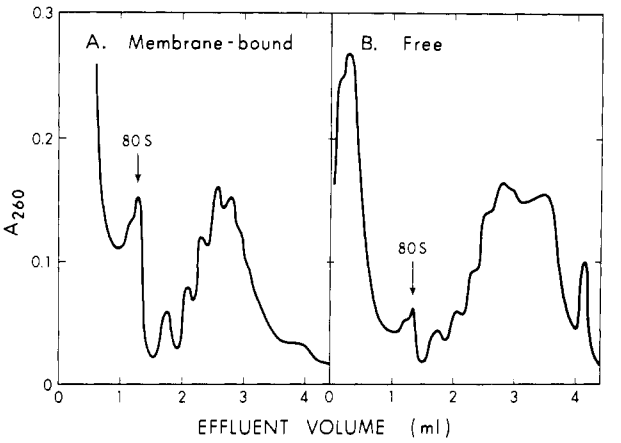

FIGURE 1: Sucrose density gradient centrifugation analysis of membrane-bound and free polyribosomes from 14-day fetal liver. Polyribosomes were prepared as described under Materials and Methods and centrifuged through 10–35% exponential sucrose gradients at 40,000 rpm for 30 min in the Spinco SW50.1 rotor. The gradient was analyzed from the top using a continuous-flow cell attached to a Gilford spectrophotometer. The direction of sedimentation is from left to right: (A) membrane-bound polyribosomes (analyzed without treatment with a detergent); (B) free polyribosomes.

Other Antisera. Rabbit antisera against adult mouse serum and mouse albumin were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Specificity of the anti-albumin serum was established by double immunodiffusion and immunoelectrophoresis.

Immunoprecipitation was carried out by the addition of approximately 10 μg each of carrier αFP and albumin to a sample (50–100 μl) followed by the addition of an excess amount of rabbit antiserum against αFP, mouse albumin, or adult mouse serum. The mixture was incubated at 37° for 60 min and then at 0° for 24–72 hr with occasional stirring. The sample was centrifuged at 5000*g* for 10 min at 4°. The supernatant was analyzed by non-sodium dodecyl sulfate gel electrophoresis. The precipitate was washed three times with 10 mM Tris-HCl (pH 7.4)–150 mM NaCl, dissolved in 1% sodium dodecyl sulfate–1% β-mercaptoethanol–4 M urea at 45° for 60 min, and analyzed by sodium dodecyl sulfate gel electrophoresis.

Results

Cell-Free Synthesis of αFP and Albumin by Membrane-Bound Polyribosomes from 14-Day-Old Fetal Liver. Free and membrane-bound polyribosomes were prepared from 14-day-old fetal liver and incubated with [^{3}H]leucine under the conditions for protein synthesis. The reaction mixture was then centrifuged at high speed to precipitate ribosomes and membraneous material. The supernatant fraction contained about 70% of the total hot-CCl_3COOH precipitable radioactivity for free polyribosomes and about 50% for membrane-bound polyribosomes. This fraction, however, did not contain radioactive αFP and albumin as analyzed by polyacrylamide gel electrophoresis. The pellet was therefore resuspended and subjected to several cycles of freeze-thawing and sonication. This resulted in the release of 30–40% of radioactivity from the pellet. Analysis

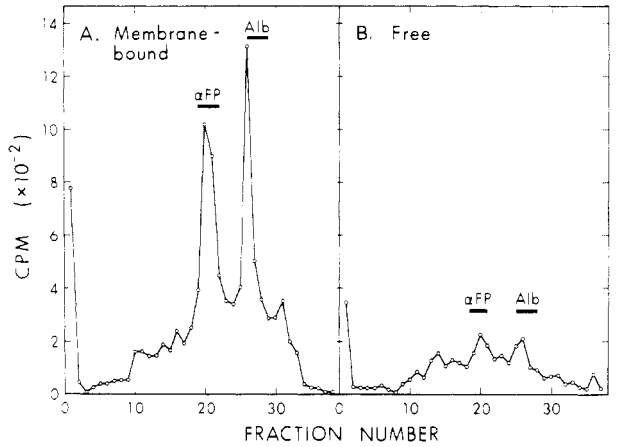

FIGURE 2: Polyacrylamide gel electrophoresis analysis of proteins produced *in vitro* by polyribosomes from 14-day fetal liver. Conditions for protein synthesis *in vitro* and analysis of protein products are described under Materials and Methods. Radioactivity in the samples derived from membrane-bound and free polyribosomes was approximately 12,000 and 6000 cpm, respectively. The bars indicate the positions of the stained bands of carrier αFP and albumin (Alb): (A) membrane-bound polyribosomes; (B) free polyribosomes.

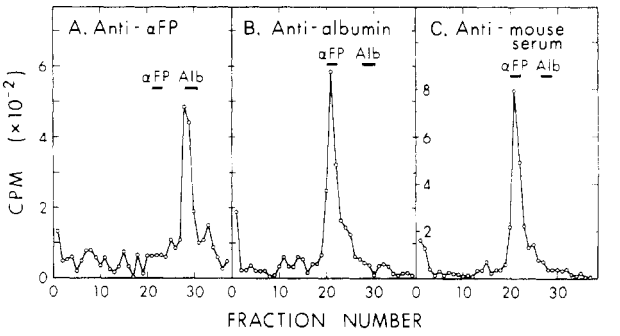

FIGURE 3: Polyacrylamide gel electrophoresis analysis of cell-free protein products not precipitated with various antibodies. The sample shown in Figure 2A containing approximately 12,000 cpm was incubated with antisera against (A) αFP, (B) mouse albumin, and (C) adult mouse serum. Precipitates formed were removed by centrifugation (for subsequent analysis see Figure 4) and the supernatants were analyzed by gel electrophoresis as described under Materials and Methods. The bars indicate the positions of carrier αFP and albumin (Alb).

of the released material on polyacrylamide gel is shown in Figure 2.

In the sample derived from membrane-bound polyribosomes (Figure 2A) two main radioactive fractions were present, one comigrating with carrier neuraminidase-treated αFP and the other migrating somewhat more slowly than carrier albumin. When αFP not treated with neuraminidase was used as carrier, the radioactive peak was found somewhat behind the carrier αFP band. The radioactive protein thus appeared to be αFP devoid of sialic acid residues. The sialation reaction is distinct from the polypeptide synthesis (Gustine and Zimmerman, 1973) and may not occur, at least optimally, under the cell-free conditions employed.

Further characterization of the two main radioactive protein fractions was carried out by immunoprecipitation followed by gel electrophoresis. The sample shown in Figure 2A was divided into two portions; one received antiserum against αFP and the other antiserum against mouse albumin. After incubation the precipitates formed were separated by centrifugation. Analysis of the supernatants which contained proteins not reacted with the antibodies showed that anti-αFP serum removed the slower moving radioactive fraction (Figure 3A), whereas antiserum against mouse albumin eliminated the faster moving radioactive fraction (Figure 3B). This indicated that the faster moving radioactive fraction was albumin and the slower moving fraction αFP. A similar experiment was performed using antiserum against adult mouse serum. Again one major radioactive peak corresponding to the αFP band remained in the supernatant (Figure 3C) confirming the above conclusion.

Analysis of the proteins precipitated with anti-αFP on sodium dodecyl sulfate gel showed that more than 60% of the total radioactivity coelectrophoresed with the carrier αFP of a molecular weight of 70,000 (Figure 4A). At least two additional radioactive fractions were present in the range of mol wt 50,000 and 30,000. Whether they represent incomplete αFP chains or nonspecifically precipitated material is not clear at present.

The sodium dodecyl sulfate gel analysis of immunoprecipitate with antialbumin showed that approximately 50% of the total radioactivity comigrated with carrier albumin (mol wt 68,000) (Figure 4B). The identity of several other fractions is not known at present, but recent evidence has suggested that the fraction with an approximate mol wt of 50,000 may represent nascent albumin peptides (Taylor and Schimke, 1973). If this is the case, the radioactivity of complete and incomplete albumin products combined would amount to 83% of the total radioactivity recovered from the gel.

A very similar gel pattern was obtained with the immunoprecipitate with antiserum against adult mouse serum, in which the sum of complete and incomplete albumin radioactivity accounted for 72% of the total radioactivity (Figure 4C). This suggests that albumin is the major serum protein synthesized under the conditions used and the synthesis of non-albumin serum proteins (except for αFP) is relatively small (~10%).

A slightly slower migration of newly formed albumin than carrier albumin similar to that shown in Figure 2A has also been observed by others (Hill *et al.*, 1972), but the cause of this

TABLE I: Synthesis of αFP and Albumin *in Vitro* by Membrane-Bound and Free Polyribosomes from 14-Day Fetal Mouse Liver.[a]

Polyribosomes		Products		Specific Activity		Ratio of Specific Activity		
Preparation	Net Amount (A_{260})	αFP (cpm)	Albumin (cpm)	αFP (cpm/A_{260})	Albumin (cpm/A_{260})	αFP	Albumin	αFP + Albumin
Membrane bound	1.7	1035	794	609	467	19.7	19.5	19.6
Free	4.0	123	96	30.9	24.0	1	1	1

[a] Free and membrane-bound polyribosomes were incubated with [^{3}H]leucine, and αFP and albumin produced were analyzed in polyacrylamide gel as described under Materials and Methods.

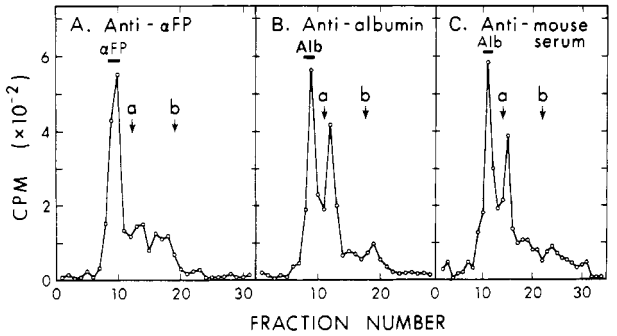

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis analysis of proteins precipitated with various antibodies. The sample shown in Figure 2A containing approximately 12,000 cpm was incubated with antisera against (A) αFP, (B) mouse albumin, and (C) adult mouse serum. Precipitates formed were removed by centrifugation, washed, dissolved in a sodium dodecyl sulfate–urea–β-mercaptoethanol solution and analyzed in sodium dodecyl sulfate gel as described under Materials and Methods. The bars indicate the positions of carrier αFP and albumin (Alb). Arrows indicate the positions of proteins of mol wt 55,000 (a) and 25,000 (b).

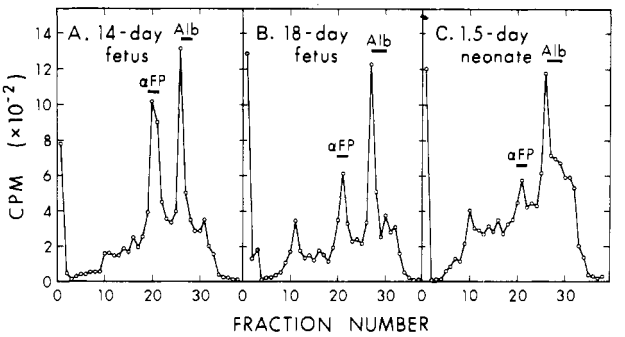

FIGURE 5: Polyacrylamide gel electrophoresis analysis of proteins produced by membrane-bound polyribosomes from pre- and postnatal mouse liver. Membrane-bound polyribosomes from (A) 14-day fetal liver, (B) 18-day fetal liver, and (C) 1.5-day neonatal liver were incubated *in vitro* and protein products containing approximately 12,000 cpm were analyzed by gel electrophoresis as described under Materials and Methods. The bars indicate the positions of carrier αFP and albumin (Alb).

discrepancy is not clear at present. We found that the newly formed albumin was more sensitive than carrier albumin to certain treatments, such as heating. It is therefore possible that the cell-free albumin product differs from the carrier albumin in conformation.

Free polyribosomes appeared to produce some αFP and albumin (Figure 2B), but in amounts that were about one-twentieth of those produced by membrane-bound polyribosomes (Table I). It is possible that this apparent synthesis of αFP and albumin by free polyribosomes may be attributed, at least in part, to a contamination of membrane-bound polyribosomes.

Changes in Synthetic Activity for αFP and Albumin Production in Developing Liver. We have shown previously that the ability of liver lysates to synthesize αFP and albumin depends upon the stage of development of mice (Tamaoki *et al.*, 1974). In order to examine whether or not similar changes are observed at the level of polyribosomes, we analyzed the synthesis of αFP and albumin by membrane-bound polyribosomes isolated from 14-day and 18-day fetal liver and 1.5-day neonatal liver. The results of gel analysis are shown in Figure 5, and the per cent radioactivity in the αFP and albumin fractions is presented in Figure 6. It is seen that the synthesis of αFP decreased rapidly with age (Figure 6A), whereas that of albumin remained relatively unchanged (Figure 6B). It was estimated that the αFP synthesis amounted to about 6% of total protein synthesis obtained with the 14-day fetal liver polyribosomes, 2% with the 18-day fetal liver polyribosomes, and less than 1% with the 1.5-day neonatal liver preparation. Albumin synthesis, on the other hand, represented about 4% of total protein synthesis throughout the growth period studied. The synthesis of αFP and albumin by free polyribosomes remained very small throughout the period of development tested.

The ratio of αFP synthesis to albumin synthesis decreased from 1.3 in 14-day fetus to 0.54 in 18-day fetus and to 0.21 in 1.5-day neonate. These values were in good agreement with those obtained with liver lysates indicating that the translation of endogenous mRNAs by the isolated polyribosomes *in vitro* is quantitatively similar to that in the lysate.

Discussion

It has been shown that the liver and other organs which produce secretory proteins contain two types of polyribosomes, free and membrane bound. Several groups have presented evidence that membrane-bound polyribosomes synthesize proteins which are subsequently transported outside the cell, whereas free polyribosomes produce nonsecretory proteins. Among those secretory proteins shown to be synthesized by membrane-bound polyribosomes are albumin in adult murine liver (Siekevitz and Palade, 1960; Birbeck and Mercer, 1961; Peters, 1962; Redman, 1969; Takagi and Ogata, 1968; Hicks *et al.*, 1969; Ganoza and Williams, 1969; Campbell, 1970), collagen in chick embryo connective tissue (Diegelmann *et al.*, 1973), and immunoglobulin in myeloma cells (Cioli and Lennox, 1973). In the present study, mouse αFP, a serum protein in embryo and neonate, was found to be synthesized predominantly by membrane-bound polyribosomes from the liver providing additional evidence for the production of secretory proteins by membrane-bound polyribosomes.

The mechanism for the specialization of the polyribosomal function is not clear at present. Shafritz (1974) has recently presented evidence that mRNAs for albumin (secretory protein) and ferritin (nonsecretory protein) are not segregated in these two classes of polyribosomes in the rabbit liver. Controls may be exerted at the level of translation through protein factors which are involved in, for instance, initiation of polypeptide chains. It is possible, therefore, that the type of proteins produced by these polyribosome preparations *in vitro* may depend upon experimental conditions.

In analysis of cell-free products on non-sodium dodecyl sul-

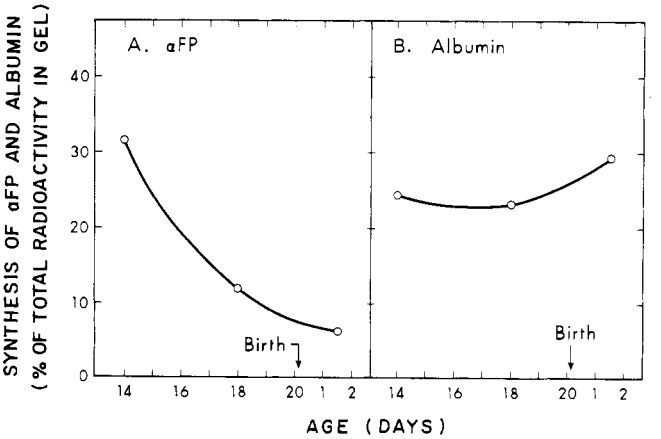

FIGURE 6: Changes in the synthesis of αFP and albumin with the developmental stage of mice. Radioactivity in the αFP and albumin fractions in Figure 5 was calculated as percentage of the total radioactivity recovered from the gel: (A) αFP and (B) albumin.

fate gels, radioactive αFP comigrated with neuraminidase-treated carrier αFP indicating that the cell-free product lacked sialic acid residues and possibly other carbohydrate groups. Alternatively, slower migration of cell-free αFP products than untreated carrier αFP may be attributed to a conformational difference. A similar explanation may be applicable to the electrophoretic behavior of cell-free albumin product.

Sodium dodecyl sulfate gel analysis showed that 60% of the total antibody precipitated material was complete αFP molecules (Figure 4A) and 50% complete albumin molecules (Figure 4B). A second major fraction with an approximate molecular weight of 50,000 (20% for αFP and 33% for albumin) may represent incomplete polypeptides. Conditions are being studied to increase the production of complete polypeptides.

It is to be noted that αFP and albumin, having similar molecular weights, are not clearly distinguished by sodium dodecyl sulfate gel analysis of immunoprecipitates alone. Combined analysis of non-antibody bound proteins in the supernatant fraction is of value in establishing the identity of these proteins.

The production of αFP and albumin by polyribosomes depended upon the stage of development of the mouse liver. The rapid reduction of αFP synthesis with age may reflect a decrease in the polyribosome population synthesizing αFP. On the other hand, a near constant percentage of albumin production suggests a net increase in the albumin-synthesizing polyribosome with the growth of the liver. Further work is necessary to determine whether these changes are due to an alteration of the mRNA production or in the efficiency of the mRNA translation. The similarity of the change in the synthetic ratio of αFP and albumin between polyribosomes and lysates indicates that the isolated polyribosomes retained structural and functional integrity. Isolation of mRNAs for αFP and albumin from these sources and their translation in cell-free systems may provide answers to the above question related to the site and manner of regulation for the αFP and albumin synthesis in the developing liver.

Acknowledgments

We thank Mr. Kelwyn Thomas and Miss Irma Schindler for excellent technical assistance.

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