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α_1- Fetoprotein mRNA of rat yolk sac and hepatoma

Jen-Fu Chiu, Warapam Decha-Umphai and Patricia Commer

Department of Biochemistry, University of Vermont, College of Medicine, Burlington, VT 05405, USA

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ABSTRACT

Rat α_1-fetoprotein mRNA was isolated and purified to apparent homogeneity by means of immunoadsorption and oligo (dT) cellulose affinity chromatography. Purified AFP mRNA migrated as a 21S peak in 2.5% SDS-polyacrylamide gels. The translation product of this mRNA in micrococcal nuclease treated reticulocyte lysate was identified as AFP by specific immunoprecipitation, SDS-gel electrophoresis and tryptic digestion analysis. DNA complimentary to AFP mRNA was synthesized with avian meyloblastosis virus RNA-dependent DNA polymerase. This AFP cDNA was used as a probe to quantitate AFP mRNA in the developing rat liver and to compare the complexity and diversity of AFP mRNA derived from the normal rat liver and Morris hepatoma 7777. We found that the amount of functional AFP mRNA is decreasing during liver development. There is very little, if any, AFP mRNA in the adult rat liver. A high degree of homology between the AFP mRNA sequences of yok sac and hepatoma was also found.

INTRODUCTION

Alpha$_1$-fetoprotein (AFP) is a glycoprotein which is normally present in fetal serum and in amniotic fluid of many species. This protein is synthesized mainly by the yolk sac and the fetal liver, and to a small extent by the gastrointestinal tract during fetal life (1-5) Although AFP is present in trace amounts (< 0.06 μg/ml) in the serum of the adult rat (6) and human (7,8) it is present in very high concentrations (up to over 10,000 μg/ml) in the serum of fetal and hepatoma-bearing animals (9-11). Disappearance of AFP in the postnatal life and appearance in the malignancy reflects sequential gene activation and repression. The control of AFP expression in the normal liver and hepatomas provides a powerful model for study on gene regulation during normal development and neoplastic transformation.

By examining the similarities and differences in the model of AFP synthesis between the normal and the cancer tissues, it may be possible to elucidate the molecular mechanism involved in the control of AFP gene expression. In this paper, AFP mRNA was purified and characterized from rat yolk sac. Com-

plementary DNA to AFP mRNA was synthesized and used as a probe to quantitate AFP mRNA in the normal developing rat liver and the Morris hepatoma 7777. During this study we also compared the complexity and diversity of AFP mRNA derived from the normal rat liver and hepatoma 7777.

MATERIALS AND METHODS

Animals and Hepatoma 7777. Sixteen day gestation of pregnant albino Sprague Dawley rats were used for yolk sac RNA preparations. Adult and newborn rat liver RNAs were also isolated from the same strain of rats. Morris hepatoma 7777 was maintained and transplanted in the hind legs of adult female Buffalo rats. All rats were fed water and rat chow ad libitum.

Preparation of antibodies. Antibodies against homogeneous rat AFP were prepared in rabbits as described in our previous paper (12). Anti-AFP was immunopurified by affinity chromatography on columns of AFP-sepharose, then made RNase-free by ion exchange chromatography on DEAE-cellulose and CM-cellulose columns (13). Rabbit γ-globulin was paritally purified by ammonium sulfate fractionation and QAE-Sephadex A-50 chromatography and used as antigen to make sheep anti-rabbit γ-globulin (12). Sheep anti-rabbit γ-globulin was purified by ammonium sulfate fractionation and made RNase-free as above. An insoluble matrix of the sheep anti-rabbit γ-globulin was prepared by crosslinking the antibody with glutaraldehyde as described by Schimke et al (14).

Isolation of AFP specific polysomes. The procedure for the isolation of total polysomes has been described in a previous paper (15). Yolk sac polysomes (15 A260 units/ml) were reacted in solution with monospecific antibodies against AFP (10 μg of anti-AFP/A260 unit of polysomes). After 1 h at 2°C and gentle shaking, the unreacted free antibodies were separated from the polysomes by centrifugating the polysomes over a step gradient made of 4 ml of 2.5 M sucrose and 7 ml of 1 M sucrose in polysomal buffer (16). Centrifugation was carried out for 4.25 h at 25,000 rpm in a SW 27 Beckman rotor. The polysomes were collected from the 2.5 M sucrose layer and diluted to 0.4 M sucrose with 140 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes pH 7.5 and 500 μg/ml of heparin. Then the matrix of sheep IgG against rabbit IgG was added at the ratio of 250 mg/1000 A260 units of polysome. The mixture was placed on ice for 3 h. The mixture was then spun down at 5,000 x g and washed three times in 10 vol of polysome buffer containing 1% Triton X-100 and 1% sodium deoxycholate, 150 mM NaCl and 0.5 M sucrose to reduce nonspecific binding. The matrix was then finally washed with polysome buffer alone. Adsorbed polysomes and mRNA were released by two extractions with 4 volumes of 50 mM EDTA and incubated for

15 min with gentle stirring on ice (14).

Isolation of AFP mRNA. Immunopurified polysomes were dissociated in 1% sodium dodecyl sulfate and 1% of sarkosyl. The RNA was sedimented away from protein and heparin on 5 to 20% linear sucrose gradients. Sixteen to twenty-four S RNAs were collected, adjusted to 0.2 M NaCl and precipitated with 2.5 volumes of ethanol at -20°C poly (A)-containing mRNA was isolated by oligo (dT) cellulose chromatography (15).

Synthesis of complementary DNA to AFP mRNA. [^{3}H] cDNA to purified AFP mRNA was synthesized with avian myeloblastosis virus RNA-dependent DNA polymerase using the procedure of Myers et al. (17) with some modifications. The cDNA synthesis reaction mixture contained 200 µM each of dTTP, dGTP and dATP, 50 µM [^{3}H] dCTP (23.8 Ci/mmol, New England Nuclear), 8 mM $MgCl_2$, 20 mM KCl, 50 mM Tris-HCl buffer (pH 8.2), 0.4 mM dithiotreitol, 4 mM sodium pyrophosphate, 20 µg/ml oligo (dT) 12-18, actinomycin 50 µg/ml, 20 µg/ml mRNA, and 180 untis/ml reverse transcriptase. The reaction was performed at 45°C for 30 min. The reaction was terminated by adding sodium dodecyl sulfate and EDTA, pH 7.0 to final concentrations of 1% and 10 mM respectively. The cDNA was separated from the unreacted deoxynucleoside by passing the reaction mixture over a column (0.7 X 40 cm) of sephadex G-50 equilibrated and run in 100 mM NaCl, 10 mM EDTA, and 20 mM HEPES at pH 7.5.

The peak fractions were pooled, adjusted to 0.2 M NaOH, incubated at 75°C for 15 min, then cooled quickly to 0°C. After neutralizing the solution with 1 M HEPES at 7.0, the cDNA was adjusted to 50 µg/ml with yeast tRNA and 0.2 M NaCl, and precipitated with 2.5 volumes of ethanol at -20°C overnight. The specific activity was 4 X 10^7 dpm/µg with a range of size approximately 800-1,600 nucleotides.

Isolation of total polysomal RNA and mRNA fractions. Liver, yolk sac, or hepatoma tissue was homogenized in 5 volumes of polysomal buffer. After centrifuging at 10,000 x g for 10 min to remove nuclei and mitochondria, total polysomal magnesium-precipitable RNA was isolated by the procedure of palmiter (18). Poly (A)-containing RNA was prepared from the magnesium-precipitable ribonucleoprotein complexes by phenol chloroform isoamylalcohol (49:49:2) extraction and oligo (dT) column chromatography (19).

Hybridization of cDNA to RNA. The hybridization reaction was performed in a final volume of 20 µl of 50% formamide, 0.5 M NaCl, 25 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA containing 1500 cpm of cDNA and various amounts of RNAs. The components were sealed in glass capillary tubes presoaked in polyvinyl sulfate (5 µg/ml). After heating at 95°C for 5 min to denature the

nucleic acid. The reaction mixtures were incubated at 41°C for 72 hours. The formed hybrids were assayed utilizing nuclease S_1 from Aspergillus Oryzae (20).

Polyacrylamide gel electrophoresis. RNA electrophoresis was carried out on 2.5% polyacrylamide gels as described previously (15). RNA gels were scanned at 260 nM in a Gilford spectrophotometer.

Protein samples were dissolved in 1% sodium dodecyl sulfate, 1% dithiothreitol and 50 mM sodium phosphate at pH 7.2, heated at 90°C for 3 min and run on 10% polyacrylamide gels (0.6 X 10 cm). The electrophoresis was performed in 50 mM sodium phosphate buffer at pH 7.2 containing 0.1% sodium dodecyl sulfate for 7-8 h at 8 MA/gel. Gels were sliced and radioactivity was determined as previously described (15).

Cell free protein synthesis. Cell free protein synthesis was performed in nuclease-treated rabbit reticulocyte lysate system as described by Pelham and Jackson (21). The translation was dependent on the addition of exogenous mRNA, resulting in about a 5-7 fold stimulation above endogenous background. The AFP translation product labeled with [^{3}H] leucine (60 Ci/mmol, New England Nuclear) or [^{3}H]tyrosine (60 Ci/mmol, New England Nuclear) was measured immunologically with rabbit anti-AFP. AFP immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis. Total protein synthetic activity was determined by examining the trichloroacetic acid precipitable material.

Tryptic peptide analysis. Protein samples (1 to 2 mg/ml) were dissolved in 7 M guanidine - 0.3 M Tris HCl pH 8.2 - 1% EDTA and dithiothreitol was added to a final concentration of 100 mM. After incubating for 1 h at 37°C, freshly prepared sodium iodoacetate was added to 0.2 M and the incubation continued at 37°C for an additional 45 min. The iodoacetate-treated samples were collected by precipitation with 10 volumes of acetone:1N HCl (40 = 1) at 0°C. After washing with ether, the samples were dissolved and digested with TPCK-trypsin in buffer of 0.1 M ammonium bicarbonate pH 8.0 for 8 h with gentle shaking at a substrate enzyme ratio of 50. Digested samples were lyophilized and dissolved in 1 ml pyridine acetate buffer A (278 ml acetic acid, 16.1 ml pyridine, water 705.9 ml pH 3.1) and applied to a 0.9 X 55 cm column containing Bio-Rad AG 50 W-2X resin previously equilibrated in pyridine acetate buffer A. The peptides were eluted with a gradient consisting of 200 ml pyridine acetate buffer A (pH 3.1) and 250 ml pyridine acetate buffer B (139 ml acetic acid, 161 ml pyridine, water 700 ml, pH 5.0) (22). Samples of about 2.5 ml were collected. Radioactivity was determined using a liquid scintillation counter.

RESULTS

The method designed for the isolation of AFP mRNA from rat yolk sac is based on the selection of polysomes synthesizing a single specific protein. Monospecific antibodies which were raised against AFP can recognize the nascent polypeptide chain on the polysomes and provide the specificity of the isolation proceudres (23,24). Polysomes which formed complexes with the specific rabbit antibody could be separated from the rest of the polysome population by adsorption to a sheep anti-rabbit antibody matrix (14). The matrix was then collected by centrifugation and extensively washed to remove unspecifically bound polysomes. The specifically bound polysomes were then dissociated in 1% sodium dodecyl sulfate and 1% sarkosyl. Deproteinized polysomal RNA was fractionated by sucrose gradient centrifugation. 16 to 24S RNAs were collected and poly(A) containing RNA was purified by oligo (dT)-cellulose chromatography.

Upon examination of the translational activity of AFP mRNA in the cell free micrococcal nuclease-treated reticulocyte lysate system, AFP mRNA was found to be enriched 5.1 fold in the immunoadsorbed fraction compared to the initial yolk sac polysomal preparation (Table 1). Immunoadsorbed polysomes were dissociated and fractionated by oligo (dT) cellulose column chromatography Approximately 72% of the initial AFP mRNA was recovered at this step. It was enriched 78 fold over the starting material (data not shown).

The profile of purified AFP mRNA on polyacrylamide gels is shown in Fig

Table 1 Purification of AFP synthesizing polysomes from rat yolk sac by immunoadsorption.

Fraction	RNA A260 unit	specific activity cmpx10^3/ A260	Enrich fold	Total AFP mRNA activity cpm x 10^6	% Total Protein Synthesis	yield
Original polysome	55	546	1	30.0	19.1	100
Immuno-adsorbed polysome	9.2	2785	5.1	25.6	95.5	85
Non-adsorbed polysome	40	82	0.15	3.0	2.9	10.1

The assay for AFP mRNA translation activity was perforemd in the cell-free micrococcal nuclease treated rabbit reticulocyte protein synthesizing system as described in the Methods.

1. No significant contamination by molecules of different length can be observed. The molecular weight of AFP mRNA was estimated from its electrophoretic mobility on polyacrylamide gels under denaturing conditions in comparison to 28S and 18S rRNA markers. Figure 1 shows the molecular weight for AFP mRNA in 9.1 X 10^5 dalton. This gives an approximate size of 2527 bases.

To ascertain the identity and purity of the isolated mRNA, the protein directed by the mRNA in a cell-free protein-synthesizing system was examined. We have used the micrococcal-treated rabbit reticulocyte lysate system (21) for this purpose. Residual protein synthesis represents globin synthesis almost exclusively (Fig 2a). Endogenous globin synthesis was represented by a discrete peak when

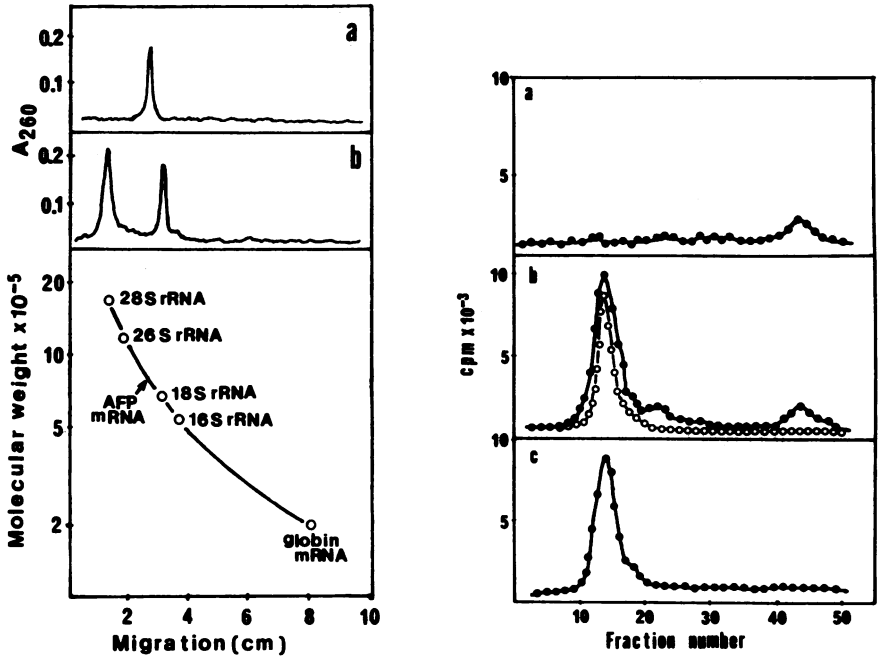

FIG. 1.

Molecular weight determination of AFP mRNA by polyacrylamide gel electrophoresis (13). The patterns of gel electrophoresis of pure AFP mRNA (a) and polysomal RNAs (b) are shown in the top and middle panel.

FIG. 2

SDS-polyacrylamide gel electrophoresis of translational product which was primed with purified AFP mRNA (a) the products synthesized in reticulocyte lysate in the absence of exogenous mRNA (b) the total (^{3}H) labeled reaction product (●) and authentic ^{125}I-AFP (O) were subjected to the electrophoresis. (c) Immunoprecipitated (^{3}H) labeled reaction product.

translation products were analyzed by SDS-gel electrophoresis. Fig 2b shows that the majority of radioactivity (over 90%) migrates as a single sharp peak. This material co-migrates with a ^{125}I-labeled AFP standard prepared from amniotic fluid. This peak was missing if no mRNA was added. The synthesis of the polypeptide corresponding to AFP was further characterized. A γ-globulin fraction containing anti-AFP activity was used to specifically precipitate AFP. The immunoprecipitates from the reticulocyte lysate consisted predominantly of one polypeptide which co-migrates with authentic rat AFP (Fig 2c).

Evidence that the immunoprecipitated translation product was indeed AFP is presented in Fig 3. Pure AFP was labeled with ^{125}I. AFP synthesized in the reticulocyte lysate system was labeled with (^{3}H) tyrosine in the reaction mixture. The protein was then immunoprecipitated. Both the ^{125}I-AFP standard and the *in vitro* translated ^{3}H-labeled product were subjected to tryptic digestion. The ^{125}I-labeled tryptic peptide patterns of the authentic AFP and the *in vitro* synthesized AFP are identical. The excellent agreement between the pure AFP standard and the immunoprecipitated translation product is very strong evidence that only AFP is translated from our purified mRNA.

The purity of the isolated AFP mRNA is also demonstrated by the hybridization kinetics of AFP mRNA and cDNA. Complementary DNA copies of AFP mRNA isolated from yolk sac were synthesized by means of a viral reverse transcriptase and labeled labeled with (^{3}H) dCTP as described in Methods. As shown in Fig 4, the

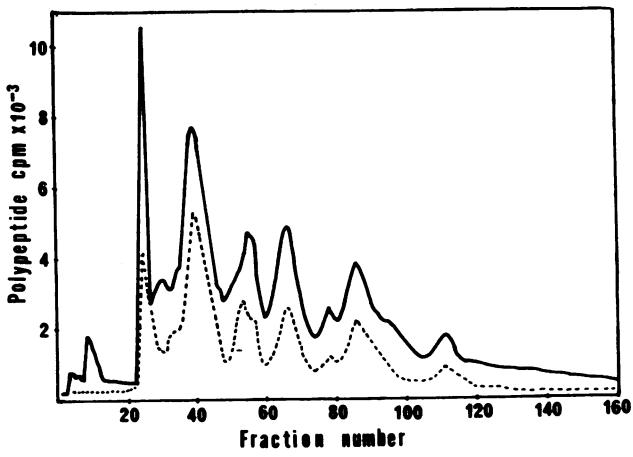

FIG 3.

Column chromatographic pattern of peptides from the tryptic digestion of ^{3}H-AFP translation product and ^{125}I-AFP standard (- - - - -).

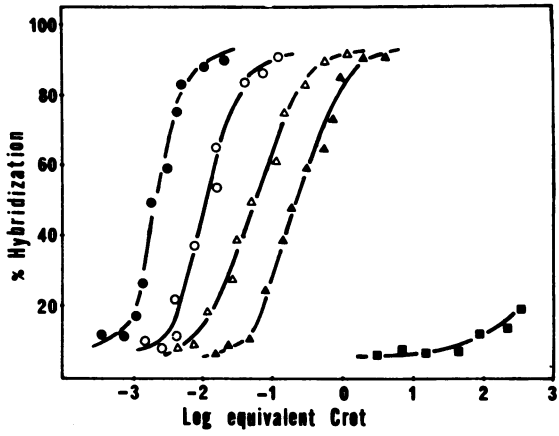

FIG 4.

Kinetics of AFP cDNA hybridization to RNAs of various origins. ●—●, purified AFP mRNA; ○—○, total yolk sac mRNAs; △—△, total hepatoma 7777 mRNAs; ▲—▲, total mRNAs isolated from 10 day old rat liver; ■—■, total mRNA isolated from adult rat liver.

absence of additional hybridization when reassociated reactions of AFP mRNA and cDNA are carried out at high C_rot values and the failure of AFP cDNA to hybridize to adult rat liver mRNAs suggest that AFP mRNA is a single reactive species and is not extensively contaminated with other species of mRNA. The yolk sac and Morris hepatoma 7777 total mRNAs hybridized to AFP cDNA with a $C_rot_{1/2}$ of 9×10^{-3} and 4.9×10^{-2}. The $C_rot_{1/2}$ ratio of AFP cDNA for purified AFP mRNA to total mRNA isolated from hepatoma 7777 is comparable to those reported by Innis and Miller (25). A change of AFP mRNA concentration in developing rat liver was also observed. This data is in correlation to the AFP synthesis by developing liver as described by Koga and Tamaoki (26) using an *in vitro* cell free translation system. mRNA from adult rat liver does not undergo significant hybridization with AFP cDNA even at C_rot value greater than 100.

In order to examine the sequence hormology between AFP mRNA isolated from rat yolk sac and hepatoma 7777, heterologous RNA-cDNA hybridization reactions were perforemd to allow a determination of the degree to which the AFP mRNA sequence form normal rat yolk sac and hepatoma 7777 were shared. Hepatoma 7777 AFP mRNA was isolated as described in a previous paper (15). Fig 5 shows the hybridization kinetics of yolk sac AFP cDNA hybridized with hepatoma AFP mRNA. The kinetics is essentially similar to the homologous hybridization, i.e., the

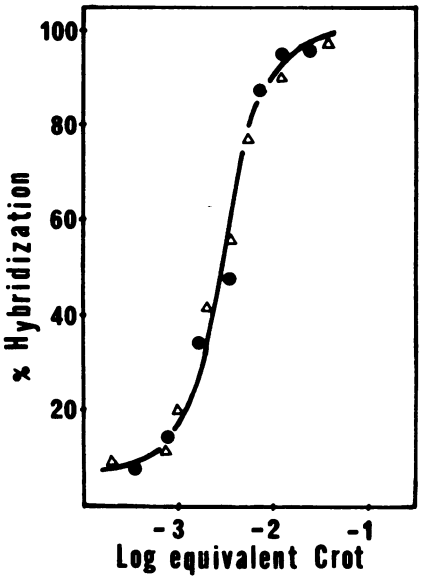

FIG 5.

Kinetic of yolk sac AFP cDNA hybridization to AFP mRNA which isolated from yolk sac (●—●) and hepatoma 7777 (△—△).

yolk sac AFP cDNA hybridization with yolk sac AFP mRNA. These findings suggest that there has been little nucleotide sequence change in the hepatoma AFP mRNA. These data can also be confirmed with the results from thermal stability of the hybrids. The Tm of the hybrids formed between yolk sac AFP cDNA and AFP mRNA derived from the normal rat yolk sac and hepatoma 7777 was 87°C accompanying a sharp transition (our published data). This indicates that the structure of AFP mRNA is similar in both normal rat tissue and hepatoma.

DISCUSSION

The immunoadsorption method used here to isolate AFP mRNA from yolk sac was originally developed by Schimke *et al.* (14). Through the specificity of binding of monospecific rabbit antibodies against rat AFP to the nascent polypeptide chains of polysomes synthesizing this protein, we have isolated AFP specific polysome fractions from total rat yolk sac polysomes by immunoadsorption onto sheep antirabbit IgG antibodies matrix. The extent of contamination of purified AFP mRNA by rRNAs or other species of mRNAs was examined by analytical gel electrophoresis and *in vitro* translation.

A single sharp symmetrical peak of mRNA is observed on the gel (Fig 1). The ribosomal RNA is undetectable. The absence of contamination by other mRNAs was demonstrated by examination of the translation products coded for by the purified mRNA in micrococcal nuclease treated reticulocyte lysate. The polyacrylamide gel electrophoretic profiles of the total translation products and of the material immunoprecipitated with monospecific AFP antibody are identical with authentic AFP (Fig 2). The tryptic digestion product of AFP and of the translation product are virtually identical. Taken together, these two findings indicate that the purified AFP mRNA is free of significant contamination by other species of translationable RNA.

The purity of the isolated AFP mRNA was also demonstrated by examination of the kinetics of its hybridization to cDNA synthesized form this purified mRNA template. If purified mRNA is contaminated with heterogenous mRNAs, the hybridization kinetics will reflect this heterogeneity with a gradual increase in the extent of hybridization over a wide range of C_rot value (27). Fig 4 shows that the hybridization curve of AFP mRNA-cDNA covers only 2 magnitudes of C_rot values. These data demonstrate that the purified AFP mRNA used as a template for cDNA synthesis is not extensively contaminated with other species of mRNAs. AFP cDNA does not undergo significant hybridization with adult liver RNAs even at high C_rot value (Fig 4), demonstrating that the purified AFP mRNA used as a template for cDNA synthesis is free of significant contamination by albumin mRNA which is present at a level of about 11% total mRNAs in rat liver (28).

A comparison of the physical and chemical properties of AFP which is synthesized in normal tissues and in hepatoma is helpful in clarifying the physiological roles of this protein. Cross-hybridization of cDNA to mRNA permits us to study the sequence homogeneity of AFP mRNA in the normal rat liver and Morris hepatoma 7777. Results in Fig 5 show a high degree of homology between AFP messenger RNA sequences of the rat liver and the hepatoma. These results are in agreement with the data that similar amino acid compositions were found in AFP of fetal umbilical cord serum or hepatoma ascite fluid (29-31). There is only a small difference in the carbohydrate composition between fetal and hepatoma AFP (29). These results suggest that there is no change in the gene in normal tissue and the hepatoma. The mechanisms causing the disappearance of AFP mRNA in adult rat liver and reappiearing in hepatoma are under investigation.

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