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Isolation of α-Fetoprotein Messenger Ribonucleic Acid from AH-66 Ascites Hepatoma Cells

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α-Fetoprotein (AFP) produced by rat AH-66 hepatoma cells was purified and specific antibody was obtained from the serum of an immunized horse. AFP messenger ribonucleic acid (mRNA) (approximately 2700 nucleotides) was isolated by affinity chromatography with oligo(dT)-cellulose of RNA extracted from AFP-synthesizing polysomes of the hepatoma cells.

Keywords——α-fetoprotein; AH-66 hepatoma cells; AFP mRNA; carcinoembrionic protein; cancer

α-Fetoprotein (AFP) is one of the carcinoembryonic proteins. It is produced by the yolk sac in the early embryo and by the liver at the later stage, appearing in the circulation. The synthesis of AFP stops after birth and its concentration in the blood decreases to trace. However, since many yolk sac tumors and hepatoma cells produce AFP, the blood level of AFP increases when animals bear such tumors. This phenomenon provides a valuable means for identifying patients bearing hepatoma or yolk sac tumors, and a radioimmunoassay procedure for AFP has been developed for routine use.

On the other hand, it is of much interest that the AFP gene is transcriptionally active in such tumor cells as well as in fetal cells, but is silent in the cells of adult animals. Undoubtedly, this is an attractive and convenient model for studying gene expression. In order to investigate the mechanism of transcriptional control of the AFP gene, it is requisite to synthesize a deoxyribonucleic acid (DNA) sequence complementary to AFP mRNA.

The present paper describes the isolation of the AFP mRNA from AH-66 rat hepatoma cells and the characterization of the purified preparation.

Materials and Methods

Transplantation of AH-66 Hepatoma Cells——AH-66 hepatoma cells producing large amounts of AFP were kindly given by Professor H. Hirai of Hokkaido University. Approximately 1×10^8 cells were injected intraperitoneally into a Donryu rat weighing 100—120 g. Nine days later about 30 ml of ascites fluid was drawn off from the rat. One ml of the ascites fluid containing approximately 1×10^8 hepatoma cells was transplanted successively.

Compositions of the Solutions used in the Present Study——Sol. A: 0.3 M KCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 12 mM 2-mercaptoethanol and 10% glycerol. Sol. B: 1% SDS, 1% 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl (pH 8), 10% sucrose and 0.001% pyronin G. Sol. C: 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$ and 200 μg/ml heparin. Sol. D: 1 M sucrose, 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$ and 200 μg/ml heparin. Sol. E: 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$ and 200 μg/ml heparin. Sol F: 0.1 M Tris-HCl (pH 9) containing 0.5% SDS and 0.025 M EDTA. Sol. G: mixture of phenol, chloroform and isoamyl alcohol (25: 25: 1).

Purification of Rat AFP——Rat AFP was purified by affinity chromatography by making use of its binding property to estradiol.[1] The purification was performed according to the method of Innis *et al.*[2] Ascites fluid from Donryu rats bearing AH-66 hepatoma cells was centrifuged to sediment the cells and the supernatant was fractionated by ammonium sulfate precipitation. The fraction of 40—65% saturation was adsorbed batchwise onto estradiol-Sepharose at 4°C overnight using Sol. A. AFP was eluted twice with Sol. A containing 10% ethanol and 0.2 mM estradiol for more than 3 h. Ten to fifteen mg of purified AFP was obtained using 1.65 mg of estradiol bound to 8 g of Sepharose.

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SDS-Polyacrylamide Gel (PAG) Electrophoresis——SDS-PAG electrophoresis was performed by the procedure of Fairbanks *et al.*[3] Lyophilized protein samples were dissolved in Sol. B, boiled for 3 min, and subjected to electrophoresis in 5.6% polyacrylamide containing 0.1% SDS.

Analysis of Carbohydrates, Protein and Amino Acids——Hexose was determined by means of the phenol–sulfuric acid reaction[4] using D-glucose as a standard, hexosamine by means of the Elson–Morgan reaction[5] using D-glucosamine as a standard and sialic acid by means of the periodate–resorcinol reaction[6] using N-acetylneuraminic acid as a standard. Protein was determined by the Lowry method[7] using bovine serum albumin as a standard. AFP was hydrolyzed with 6 N HCl at 110°C for 24 h and amino acid analysis was carried out with a JEOL JLC-5AH amino acid analyzer.

Purification of Anti-AFP Antibody——Specific anti-AFP antibody was purified from anti-rat AFP horse antiserum (also a gift from Dr. Hirai). A 25 to 40% saturation ammonium sulfate fraction of antiserum was passed through columns of CM-cellulose and DEAE-cellulose to remove ribonuclease.[8] This ribonuclease-free IgG fraction (215 mg of protein) was then incubated with AFP (45 mg) conjugated to Sepharose-4B (8 g) at 4°C overnight. The specific anti-AFP antibody (50 mg) was eluted with 4.5 M $MgCl_2$ (pH 4) and dialyzed against the phosphate buffer (pH 7.0).

Agar Diffusion of Anti-AFP Antibody——Agar diffusion was performed as described by Ouchterlony.[9] The agar plate was kept standing at 4°C for a few days.

Quantitative Precipitin Reaction of AFP——Immunoprecipitation of AFP was performed by the procedure of Shibata *et al.*[10] The amount of immunoprecipitates was determined by using bromsulfophthalein.

Preparation of Anti-Horse IgG Rabbit Antibody——Horse IgG was prepared from normal horse serum by ammonium sulfate precipitation and DEAE-cellulose chromatography according to Levy *et al.*[11] Rabbits were immunized with horse IgG as described by Higashi *et al.*[12] Anti-horse IgG rabbit antibody was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography, as was anti-AFP antibody.

Purification of AFP mRNA——Polysomes synthesizing AFP were isolated immunochemically according to Shapiro *et al.*[13] AH-66 cells collected from ascites fluid were homogenized with 2 volumes of Sol. C and the homogenate was centrifuged at $15000 \times g$ for 10 min. The supernatant was mixed with 0.1 volume of 20% Triton X-100 and overlayed on Sol. D then centrifuged at $100000 \times g$ for 2 h to precipitate polysomes. The polysomes (1000 A_{260}) suspended in Sol. E were incubated with 1 mg of specific anti-AFP horse antibody for 1 h at 0°C, then anti-horse IgG rabbit antibody (100 mg) was added and the whole was incubated for an additional 2 h. The mixture was overlayed on Sol. E+50% sucrose and 2% Triton X-100, and centrifuged at $15000 \times g$ for 10 min. The precipitates were suspended in Sol. E and centrifuged at $8000 \times g$ for 10 min to sediment the immunoprecipitates.

RNA was extracted from the immunoprecipitates described above by a modification of the procedure of Jost *et al.*[14] The immunoprecipitates were suspended in Sol. F and extracted with 2 volumes of Sol. G for 5 min. The phenol phase was reextracted with 1 volume of Sol. F and the combined water phase was treated twice with 1 volume of cold chloroform. RNA was precipitated with 2 volumes of ethanol in the presence of 1% sodium acetate (pH 5) at −20°C overnight and separated by centrifugation.

The RNA, 30—50 A_{260}/ml of 10 mM Tris-HCl (pH 7.4) containing 0.5 M KCl, was applied to a column of oligo(dT)-cellulose previously equilibrated with the same buffer, and the AFP mRNA was eluted with water.[15]

Electrophoresis of RNA——Electrophoresis of RNA was performed at 4°C on a 0.6×9 cm gel composed of 3% agarose in 6 M urea and 0.025 M citrate buffer (pH 3.5) as described by Rosen *et al.*[16]

Translation of AFP mRNA by Wheat Germ Cell-free System——Wheat germ S-30 was prepared according to Roberts *et al.*[17] The composition of the reaction mixture for translating AFP mRNA is as follows: 5 mg protein/ml of wheat germ S-30, 0.5 A_{260}/ml of AFP mRNA, 20 mM Tris-HCl (pH 7.4), 120 mM KCl, 5 mM $(CH_3COO)_2Mg$, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 50 μg/ml of creatine kinase, 2 mM 2-mercaptoethanol, 40 μM/ml of amino acids mixture and 1 μCi/ml of [^{14}C]-amino acids mixture (55 mCi/milliatom of carbon, Radiochemical Centre, Amersham). The mixture was incubated at 30°C for 1 h.

Results

Properties of Purified AFP

Purified AFP was analyzed by SDS-PAG electrophoresis. As shown in Fig. 1 the preparation gave two peaks (m.w. 74000 and 72000) having lower mobilities than that of bovine serum albumin (m.w. 68000).

The carbohydrate content and the amino acid composition of AFP are given in Table I.

Properties of Specific Anti-AFP Antibody

The specificity of purified anti-AFP antibody was examined by the Ouchterlony method (Fig. 2). A single distinct precipitin line was observed with ascites fluid and the purified AFP, no spur being detectable. The anti-AFP antibody gave no visible precipitin line against adult rat serum. These results indicate that the antibody was monospecific to AFP. On

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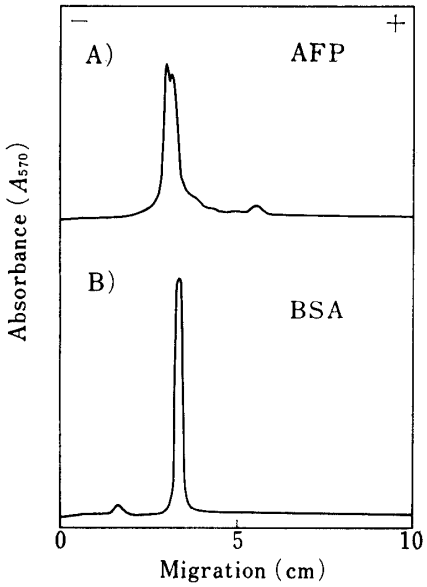

Fig. 1. SDS-PAG Electrophoresis of Purified AFP

A) 8 μg of AFP purified by estradiol-Sepharose chromatography and B) 8 μg of bovine serum albumin were electrophoresed for 4 h at 5 mA/gel (0.5×10 cm). The gels were stained with Coomassie brilliant blue R-250 and scanned at 570 nm in a densitometer (Fuji Riken, FD-AIV).

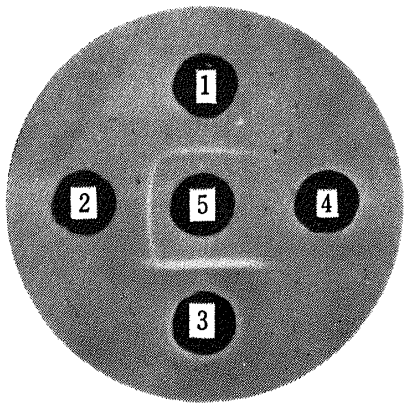

Fig. 2. Agar Diffusion of Anti-AFP Antibody

The wells contained: 1, AH-66 ascites fluid; 2, purified rat AFP; 3, anti-horse IgG rabbit antibody; 4, adult rat serum; 5, purified anti-AFP horse antibody.

TABLE I. Carbohydrate Content and Amino Acid Composition of AFP

Carbohydrate	Content (%)
Hexose[a)]	1.5
Hexosamine[b)]	1.5
Sialic acid[c)]	0.9

Amino acid[d)] (mol/1000 mol)			
Asp	89	Met	5
Thr	73	Ile	41
Ser	111	Leu	83
Glu	126	Tyr	25
Pro	50	Phe	36
Gly	66	Lys	60
Ala	73	His	25
Cys	41	Arg	31
Val	61	Trp	n.d.

a) determined by means of the phenol–sulfuric acid reaction.
b) determined by means of the Elson–Morgan reaction after hydrolysis with 2 N HCl (12 h, 100°C).
c) determined by means of the periodate–resorcinol reaction.
d) determined after hydrolysis with 6 N HCl (24 h, 110°C).
n.d.: not determined.

the other hand, anti-horse IgG rabbit antibody used for the isolation of AFP-synthesizing polysomes clearly reacted with the anti-AFP antibody.

The precipitin reaction between the purified anti-AFP antibody and the purified AFP

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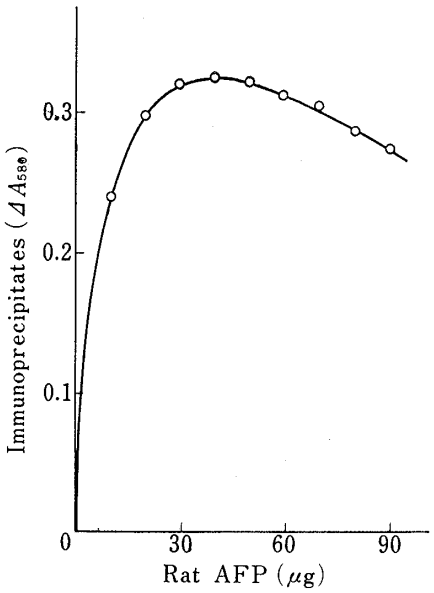

Fig. 3. Precipitin Reaction with Purified Anti-AFP Antibody

Increasing amounts of AFP were added to a fixed quantity (100 μg) of anti-AFP antibody and the resulting immunoprecipitates were assayed by the BSP method.

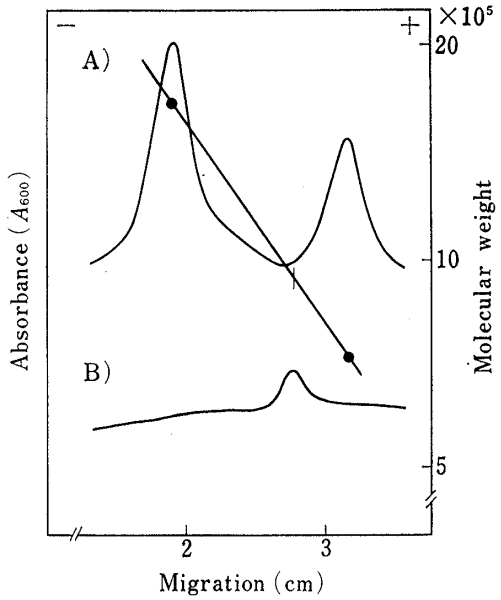

Fig. 4. Acid-Urea Agarose Gel Electrophosis of AFP mRNA

A) 40 μg of ribosomal RNAs and B) 5 μg of AFP mRNA were electrophoresed for 4 h at 2 mA/gel (0.6×9 cm). The gels were stained with methylene blue after electrophoresis and scanned at 600 nm in a densitometer (Fuji Riken, FD-AIV). Ribosomal RNAs were assumed to have S values of 18 and 28 and molecular weights of 700000 and 1580000.

TABLE II. *In Vitro* Translation of AFP mRNA

Exp, No.	^{14}C in AFP (cpm/A_{260}mRNA)	^{14}C in Total protein (cpm/A_{260} mRNA)	Purity (%)
1	1160	1440	81
2	1020	1380	74
3	1090	1363	80

After incubation, the reaction mixture was centrifuged at 100000×g for 1 h and the supernatant was divided into two fractions. One was treated with 1 volume of 10% TCA, and the other with 100 μg of specific anti-AFP antibody and 30 μg of carrier AFP. Each precipitate was centrifuged, washed and dissolved in a small amount of formic acid. Ten ml of toluene–Triton scintillation cocktail (toluene 667 ml, Triton X-100 337 ml, DPO 5.5 g and POPOP 0.1 g) was added and the radioactivity was measured.

was investigated quantitatively (Fig. 3). One hundred μg of the specific antibody completely precipitated up to 40 μg of AFP. The result indicates that the molar ratio of antibody to antigen is approximately one at the equivalent point.

Properties of AFP mRNA

1) **Electrophoresis of AFP mRNA**——Isolated AFP mRNA was analyzed on an acid-urea agarose gel (Fig. 4). AFP mRNA (B) showed a single peak at a molecular weight of approximately 9×10^5, based on the mobilities of 28-s (m.w. 15.8×10^5) and 18-s (m.w. 7×10^5) rRNA as standards. The number of nucleotide residues calculated for the molecular size of AFP mRNA would be approximately 2700.

2) **Translation of AFP mRNA in cell-free System**——Purified AFP mRNA was translated in wheat germ S-30 cell-free system (Table II). Radioactivities incorporated in the immunoprecipitates isolated by anti-AFP antibody were approximately 80% of those in TCA pre-

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cipitates (total protein). These results suggest that AFP mRNA was obtained in at least 75% purity in terms of messenger activity.

Discussion

As shown in Fig. 1, purified AFP appeared to consist of two molecules which were slightly different in size. Kerckaert *et al.*[18] also reported two types of rat AFP which were the same in carbohydrate content and in amino acid composition, but differed slightly in carbohydrate composition and in the sequence of amino acids.

The present preparation of rat AFP contained 1.5% hexose, 1.5% hexosamine and 0.9% sialic acid. These values are similar to the data reported by Watabe,[19] but the content of hexosamine is somewhat lower. The amino acid composition of AFP is also substantially identical with that reported previously,[18,19] except that the content of methionine is somewhat lower.

Messenger RNA coding for AFP was isolated from mouse yolk sac by Miura *et al.*[20] AFP-synthesizing polysomes were precipitated by indirect immunoprecipitation as employed by the present authors, and poly(A)-containing mRNA was separated by poly(U)-Sepharose affinity chromatography, whereas oligo(dT)-cellulose was used in the present study.

Miura *et al.*[20] reported the size of the isolated AFP mRNA to be about 2700 nucleotides, in good agreement with that of AFP mRNA from rat ascites hepatoma cells described in the present paper. Since 1700 nucleotides are needed to code for AFP protein, purified AFP mRNA has about 1000 extra nucleotides which are apparently not translated.

In wheat germ extracts, the mouse AFP mRNA directed the synthesis of proteins, 98% of which reacted with specific anti-AFP. Rat AFP mRNA preparations obtained by the present authors coded for AFP synthesis, the ratio of radioactivities incorporated into the immunoprecipitates with anti-AFP antibody to those into TCA-insoluble materials being 80%.

Using this purified AFP mRNA as a template, the authors have synthesized cDNA by the use of reverse transcriptase and studied the transcriptional activity of AFP gene, the results will be reported in the following paper.[21]

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