The Presence of Intervening Sequences in the α-Fetoprotein Gene of the Mouse*

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Messenger RNA for the oncosfetal protein α-fetoprotein was isolated from yolk sacs of 16- to 18-day-old mouse embryos. This RNA was used as a template to synthesize full-length double-stranded DNA containing predominantly α-fetoprotein coding sequences. Chimeric plasmids containing the double-stranded DNA were constructed in two ways. First, molecular linkers containing the recognition site for the restriction endonuclease HindIII were ligated to the blunt ends of the DNA. After following cleavage with HindIII, the recombinant molecules were ligated to the single HindIII site in the plasmid vehicle pBR322. Second, internal Pst I fragments of the double-stranded DNA were ligated to the single Pst I site of pBR322. Following transformation of the host Escherichia coli χ1776, colonies containing those chimeric plasmids which had incorporated α-fetoprotein sequences were identified by selective hybridization and arrest translation procedures. Two cloned sequences derived from the 5' and 3' regions of the mRNA were used as hybridization probes to detect the presence of the α-fetoprotein gene within a two-dimensional fingerprint of mouse genomic DNA cleaved with EcoRI, an enzyme which does not cleave within the messenger RNA sequence. The detection of 3 EcoRI fragments demonstrates that the single α-fetoprotein gene must be represented discontinuously in the mouse genome, with at least two intervening sequences.

α-fetoprotein is a major constituent of fetal serum in all mammalian species which have been examined (1–4). During development in the mouse, AFP is synthesized by the yolk sac and liver and secreted into the blood and amniotic fluid. After birth, the amount of AFP in serum decreases markedly in most strains of mice to a basal level which represents less than 0.01% of that in fetal serum (3, 5). This decline has recently been shown by Olsson et al. (5) to be inherited as an autosomal recessive trait, which they termed regulation of α-fetoprotein (raf).

Reinitiation of α-fetoprotein synthesis in the adult occurs under a select number of pathological conditions: following liver injury (6, 7), in hepatomas (1, 2), and in teratocarcinomas (8, 9). In addition, a number of congenital birth defects, including congenital nephrosis, severe Rh-hemolytic disease, and several open neural tube defects are associated with abnormally elevated concentrations of AFP in the maternal and fetal circulation and amniotic fluid (10–12). These striking and reproducible increases in the production of AFP in fetal abnormalities, in liver regenerative diseases, and in carcinomas of the liver and germ cells in humans has generated interest in the mechanism of control of the expression of AFP.

Recent studies by Tamaoki and his co-workers (13, 14) have used cell-free translation assays to demonstrate that the rate of synthesis of α-fetoprotein in fetal and neonatal mouse liver is related to the concentration of translatable AFP mRNA. Taking this analysis one step further, Innis and Miller (15) reported the isolation of rat AFP mRNA. Complementary DNA to the AFP mRNA was used in hybridization kinetic experiments to show that the actual amount of AFP mRNA in adult liver mRNA was less than 1% of that in hepatoma cells. These studies imply that the control of AFP synthesis occurs at the level of mRNA transcription or processing, or both.

Distinguishing between these possibilities will require large amounts of specific hybridization probes to the α-fetoprotein gene, as well as intimate knowledge about its structural organization in the genome. Toward this end, we have constructed chimeric plasmids containing portions of the mouse AFP mRNA sequence, and used them to identify three EcoRI fragments of mouse genomic DNA which encode in a discontinuous manner the single copy AFP gene.

EXPERIMENTAL PROCEDURES

Purification of α-Fetoprotein—Amniotic fluid, pooled from 16- to 18-day mouse fetuses, was subjected to 35 to 70% ammonium sulfate precipitation. The 70% pellet was dialyzed against 50 mM Tris-HCl (pH 7.5) and electrophoresed through a nondenaturing 7% polyacrylamide gel (16). The AFP band was identified by a comparison to fetal and adult serum protein markers, extracted and concentrated by ethanol precipitation (16). This material was used to raise antiserum to AFP by bimonthly injections into a rabbit. The rabbit antisera were regularly tested for anti-AFP activity by Ouchterlony analysis (17) against purified AFP, amniotic fluid, and adult mouse serum.

Preparation of RNA—Yolk sac and livers, obtained from 16- to 18-day-old Swiss mouse fetuses were frozen in liquid N2. Poly(A) RNA was prepared by phenol extraction of total cellular RNA (18) followed by oligo(dT)-cellulose chromatography (19). The RNA was fractionated on an 8 to 25% sucrose gradient in 2 mM EDTA, 100 mM NaCl, 10 mM Hepes (pH 7.5), which was centrifuged at 25,000 rpm for 22 h in a Beckman SW40 rotor. Individual fractions were precipitated by the addition of 2 volumes of ethanol, resuspended in H2O and tested for AFP mRNA activity using a cell-free synthesizing system derived from wheat germ (20, 21). After treatment with 200 μg/ml of

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RNase A for 30 min the [35S]methionine-containing products of translation were analyzed on sodium dodecyl sulfate polyacrylamide gels (22). To identify *de novo* AFP, 25-μl portions of each translation were incubated at 37°C for 1 h in the presence of an excess of anti-AFP serum (23). AFP was added for several hours, and the chilled precipitate was washed by centrifugation through a discontinuous sucrose gradient (23). The pellets were resuspended in 1% SDS, 1% β-mercaptoethanol, 20% glycerol, 60 mM Tris-HCl (pH 6.8) and subjected to electrophoresis as before. In most experiments, RNA obtained from adult mouse liver was treated identically, and used as a negative control for the presence of RNA from RNA using Sepharose 2B chromatography in 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA. Twenty micrograms of DNA was cleaved with EcoRI (20), extracted with phenol and ether, and concentrated by ethanol precipitation. The arrest translation procedure of Paterson *et al.* (30) was followed. The DNA was dissolved in 20 μl of deionized formamide and heated at 90°C for 5 min. Five microliters containing 5 μg of poly(A) RNA, 2 μM NaCl, and 50 mM 1,4-piperazinediethanesulfonic acid (pH 6.8) was added, and the mixture was incubated at 50°C for 2 h. After the addition of 200 μl of H2O, the solution was divided into two equal portions, one of which was heated to 90°C for 1 min before precipitation with 2 volumes of ethanol and one which was precipitated directly. The two pellets after centrifugation were dissolved in 10 μl of H2O and added to wheat germ lysates as before.

Restriction Endonuclease Mapping of DNA—Double-stranded DNA or plasmid DNA was cleaved with EcoRI, Pst I, HindIII, or BamHI, under conditions previously described (31). The fragments were analyzed on 1 to 2% agarose gels or 5% acrylamide gels (32) by staining with 25 μg/ml of ethidium bromide. When fragments were isolated for the preparation of radiolabeled probes, the bands were cut out, placed in a dialysis membrane with electrophoresis buffer, and submerged in a horizontal slab gel electrophoresis apparatus. After 4 to 6 h at 150 mA, the buffer was removed, filtered, and concentrated with ethanol and tRNA carrier. The DNA was labeled to specific activities of 10 to 50 × 10⁶ cpm/μg by nick translation in the presence of [α-³²P]dCTP (53).

**RESULTS**

Isolation of α-Fetoprotein mRNA—In order to identify a suitable source of murine AFP mRNA, total poly(A)-containing RNA was extracted separately from 16 to 18-day-old fetal livers and yolk sacs. These were subjected to sucrose gradient centrifugation, and individual fractions were tested for AFP template activity using a wheat germ in vitro protein synthesis system. The products of translation were analyzed by SDS-polyacrylamide gel electrophoresis, and AFP and albumin were identified using monospecific antibodies to each protein. The AFP mRNA template activity migrated as an 18 S RNA species, which agrees with the size determinations of previous investigators for rat AFP (15) and mouse (14) AFP mRNA, and with the molecular weight of the protein being 70,000 (38).

The α-Fetoprotein mRNA translations are shown in Fig. 1. In Lane 1, two predominant bands which co-migrate with mouse albumin (upper dot) and AFP (lower dot) were synthesized by 18 S fetal liver RNA. The lower band is completely immunoprecipitable by anti-AFP sera (Fig. 1, Lane 2). When the total translation products derived from 18

**Note:** NIH Guidelines for Research Involving Recombinant DNA (1976) National Institutes of Health, Bethesda, Md.
FIG. 1. In vitro translation of RNA and identification of AFP. An 18 S fraction from adult liver 16- to 18-day-old fetal liver or yolk sac RNA was translated in a 50-μl wheat germ lysate in the presence of [35S]methionine. Either the total translation products (Lanes 1, 3, and 5) or the translation product precipitated with anti-AFP (Lanes 2, 4, and 6) were analyzed by electrophoresis in slab 9% SDS-polyacrylamide gels, and autoradiographed. The RNAs were: 1 and 2, fetal liver RNA; 3 and 4, yolk sac RNA; and 5 and 6, adult liver RNA. The upper and lower dots indicate the migration of authentic mouse albumin and AFP, respectively.

S yolk sac mRNA were analyzed (Fig. 1, Lane 3), there was a single predominant protein band which was completely immunoprecipitable by anti-AFP sera (Lane 4) and could be competed for by authentic AFP (data not shown).

The striking predominance of α-fetoprotein, the only major protein encoded by the 18 S yolk sac RNA, supports the previous observations of Wilson and Zimmerman (39), who concluded that AFP represented 40 to 60% of the protein synthesis of yolk sac, while only 20% of that in fetal liver. As a control, poly(A) RNA from adult mouse liver which should contain no AFP template activity was used to direct protein synthesis in the wheat germ system. As shown in Fig. 1, Lanes 5 and 6, no labeled protein which could be immunoprecipitated with AFP antibody was synthesized.

The relative migration of albumin and α-fetoprotein in the gel buffer system used (Fig. 1) is contrary to their molecular weights of 68,000 and 70,000, respectively (38). By using a different buffer system (40), we could reverse the order of migration of both the in vitro products and the mature proteins, such that albumin exhibited a faster migration. We conclude that the aberrant migration observed is a result of the primary amino acid sequences of these proteins and not to any post-translational modification.

Insertion of α-Fetoprotein mRNA Sequences into a Plasmid Vehicle—The 18 S poly(A) mRNA preparation from yolk sac should contain greater than 50% AFP mRNA, ensuring that at least one in two hybrid plasmids contain AFP sequences. The heterogeneous mRNA’s were transcribed by avian myeloblastosis virus reverse transcriptase using the conditions of Wickens et al. (24). The second strand was synthesized using DNA polymerase and the resulting hairpin loop and single stranded tails removed by treatment with S1 nuclease from Aspergillus oryzae. Agarose gel electrophoresis of the double-stranded cDNA’s (Fig. 2) demonstrated the presence of a predominant band migrating as 2,150 base pairs of DNA. This is the size one would predict for a full-length transcript of an 18 S mRNA.

To choose a suitable strategy for cloning the α-fetoprotein cDNA, the 2,150 bp dsDNA was cleaved with PsI, HindIII, and BamHI (Fig. 2). These enzymes were chosen because they each cleave the genome of the plasmid vector pBR322 DNA.
Once, in either the ampicillin resistance gene (Pst I) or the tetracycline resistance gene (BamHI and HindIII) (41), and thus insertion into any one of these sites allows for discrimination between colonies that contain recombinant plasmids and those that contain parental plasmids. All three enzymes cut the AFP sequence, generating unique fragments detectable by autoradiography. The presence of clearly identified bands which correspond to a single species supports the previous estimate by translation that AFP is the only predominant species in the 18S yolk sac mRNA. Both HindIII and BamHI cleave the sequence once (Fig. 2, Lanes 6 and 7) and Pst I makes 3 cuts (Lane 9). In contrast, EcoRI (Lane 5) does not cleave within the AFP sequence. By performing a series of double digests such as the BamHI-HindIII cleavage illustrated in Fig. 2, Lane 8, it was possible to construct a restriction map of the cDNA (Fig. 3).

The orientation of the restriction map relative to the 5' and 3' ends of the α-fetoprotein mRNA, illustrated in Fig. 3, was determined using ds cDNA which had not been treated with S1 nuclease. Following cleavage of the dsDNA with Hha I, which recognizes one asymmetric site in the cDNA, the fragments were denatured by glyoxal and electrophoresed in a 1.5% agarose gel (34). The presence of a 700-bp denatured fragment demonstrates that Hha I must cleave closer to the open 3'-derived end of the dsDNA (compare Fig. 4, Lanes 2 and 3). On the other hand, denaturation of BamHI fragments, derived from a cleavage at the other end of the dsDNA, produces a 1.4-kb denatured fragment (Fig. 4, Lanes 5 and 6). The fragments representing the 5' end of the mRNA are not clearly resolved in this system, due to the difficulty in completely denaturing a snap-back sequence.

The cleavage of α-fetoprotein cDNA with Pst I generated two internal fragments, 520 and 900 bp in length (Fig. 3). These fragments were ligated directly to pBR322, which had been digested with Pst I and treated with bacterial alkaline phosphatase (25) to reduce the number of parental colonies. After the ligated mixture was used to transform CaCl2-treated χ776 cells, hybrid colonies were identified and screened by the method of Grunstein and Hogness (26). Replica filters were hybridized separately to [32P]cDNA's prepared from 18S mRNA's of yolk sac, 18-day fetal liver and adult liver (Fig. 5). Thus, colonies which hybridized strongly to the cDNA from yolk sac and fetal liver mRNA's, but not to adult liver cDNA's, would be expected to contain α-fetoprotein sequences and were selected for analysis. The plasmids were isolated by the procedure of Meager et al. (28) and digested with Pst I. Those containing the 520- and 900-bp (or both) Pst I fragments were thus identified.

To clone those portions of the α-fetoprotein cDNA sequence which were outside the two Pst I internal fragments, we used the method of Ulrich et al. (25) which required attaching 10-bp molecular linkers to the ends of ds cDNA. HindIII linkers were ligated to the 2,150-kb AFP cDNA and the resulting molecules cleaved with a vast excess of HindIII to destroy excess linkers and create ligatable ends. In this way, the dsDNA would also be cleaved internally at the HindIII site. The dsDNA fragments were ligated to pBR322 cut by HindIII and transformants were generated and selected by hybridization. Several clones, all derived from the 5' end of the AFP mRNA were identified.

**Characterization of pBR322-AFP1 and pBR322-AFP2—**
Two recombinant plasmids, termed pBR322-AFP1 and pBR322-AFP2, were selected for further characterization.
Cleavage of pBR322-AFP1 with HindIII and electrophoresis in an acrylamide gel yielded the parental 4.3-kb fragment and a 960-bp HindIII insert (Fig. 6, Lane 2). On the basis of digests with combinations of HindIII and Pst I or BamHI (Fig. 6, Lanes 3 and 4), it was possible to map the insert to the 5' end of the α-fetoprotein dsDNA sequence, as shown in Fig. 3. Likewise, pBR322- AFP2 was shown by a series of digests with Pst I (Fig. 6, Lane 5) and HindIII (Lane 6) to contain the 900-bp Pst I fragment of AFP dsDNA derived from the 3' end of AFP mRNA. The two inserts overlapped each other by approximately 200 bp, within a common Pst I-HindIII fragment (Fig. 6, arrow).

As further evidence that the two cloned DNA's contained AFP sequences, the hybrid arrest procedure of Paterson et al. (30) was used. Total pBR322- AFP1 DNA was hybridized to poly(A)-containing yolk sac RNA under conditions favoring RNA-DNA hybrids (30), and the mixture was translated in a wheat germ extract. The translation products from the control RNA are shown in Fig. 7, Lane 1, and the migration of authentic α-fetoprotein is indicated by the arrow. When the pre-hybridized RNA is translated, the AFP band preferentially disappears (Lane 2). If the hybrids are dissociated by heating, the AFP template activity is recovered (Lane 3). When parental pBR322 DNA is used to form hybrids instead of pBR322- AFP1 DNA, there is no preferential loss of AFP mRNA activity (Lanes 4 and 5). Similar results were obtained with pBR322- AFP2 (data not shown). The specific arrest of AFP translation from a yolk sac RNA population demonstrates that these clones contain AFP mRNA sequences.

Detection of α-Fetoprotein mRNA Sequences within Mouse Genomic DNA—With the availability of monoclonal hybridization probes to the AFP mRNA sequence, it was possible to visualize the genomic sequences in mouse DNA which encode the!AFP gene(s). Thus, a two-dimensional “fingerprint” of EcoRI-digested genomic DNA was prepared by chromatography on an RPC-5 ion exchange column followed by agarose gel electrophoresis of individual column fractions. The DNA was transferred to Millipore filters (37), and hybridized to labeled DNA probes derived from specific regions of the AFP mRNA sequence. The ethidium bromide stain of a gel is shown at the top of Fig. 8. The insert from pBR322- AFP1, which contains sequences derived from the 5' end of the AFP mRNA, hybridizes to two EcoRI fragments, 6.0 and 3.0 kb in size. On the other hand, the pBR322- AFP2 insert, derived from the 3' end of the AFP mRNA, and overlapping the pBR322- AFP1 by approximately 200 bp (see Fig. 3), detected the 3.0 kb fragment, along with a different 5.2 kb fragment.
but not the 6.0 kb fragment. That the two probes recognized nonidentical fragments argues that the number of EcoRI fragments which hybridize to AFP sequences cannot be explained on the basis of multiple AFP gene copies. Rather, the differential hybridization pattern supports the conclusion that there is a single copy of the gene, represented in at least three EcoRI fragments. The absence of any EcoRI cleavage site in the entire AFP cDNA sequence (Fig. 2), further suggests that the two detectable EcoRI sites in the genomic sequence are contained within intervening sequences in the gene. The Southern blots also establish the orientation of the three fragments with respect to the AFP mRNA, with the 6.0-kb fragment representing the 3' end of the mRNA, the 3.0-kb fragment representing a portion of the mRNA which includes the region overlapped by the two probes, and the 5.2-kb fragment representing the 5' end of the mRNA.

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