Cloning of Rat α-Fetoprotein 3′-Terminal Complementary Deoxyribonucleic Acid Sequences and Preparation of Radioactively Labeled Hybridization Probes from Cloned Deoxyribonucleic Acid Inserts†

Warren S. L. Liao,* George A. Ricca, and John M. Taylor‡

ABSTRACT: Double-stranded complementary deoxyribonucleic acid (cDNA) was synthesized from rat yolk sac α-fetoprotein (AFP) mRNA, inserted into the *Pst*I site of plasmid pBR322 by an oligo(deoxyguanylic acid)·oligo(deoxycytidylic acid) joining technique, and cloned in *Escherichia coli* χ1776. A plasmid containing an inserted AFP double-stranded cDNA with a contiguous poly(adenylic acid) [poly(A)] segment was identified and subsequently employed in a new method for preparing AFP-specific hybridization probe. Following an initial digestion of the AFP plasmid with *Hin*dIII to create an open, recessed 3′ end, λ exonuclease III was employed to remove the DNA strand opposite the coding strand of the cDNA insert. Oligo(thymidylic acid) was then annealed to the poly(A) segment and employed as primer for *E. coli* DNA polymerase I to synthesize a ^{32}P-labeled cDNA copy of the AFP coding strand. The single-stranded cDNA product was easily isolated by sedmentation through isokinetic alkaline sucrose gradients. Hybridization with this AFP-specific cDNA probe showed that the yolk sac contained a 6-fold greater concentration of AFP mRNA than that of the fetal liver. AFP mRNA was also found in the normal adult liver, but at a much lower level than in the fetal liver. The concentrations of AFP mRNA in Morris hepatomas 7777 and 8994, however, were significantly elevated to a 2- to 3-fold higher concentration than in the fetal liver.

AFP[1] (α-fetoprotein) is a major constituent of fetal plasma and amniotic fluid and, in late gestation, is the major protein product of the liver and the yolk sac (Abelev, 1974; Ruoslahti et al., 1974; Ruoslahti & Seppälä, 1979). AFP production decreases markedly just prior to birth, and it becomes a trace component of normal adult plasma. However, highly elevated plasma levels of AFP can be detected in adult animals during liver regeneration (Abelev, 1974; Taketa et al., 1975) and in animals bearing hepatomas (Abelev, 1974; Sell et al., 1976). In addition, abnormally high levels of AFP in human amniotic fluid and in the serum of pregnant women have been associated with fetal neural tube defects, fetal distress, and threatened abortions (Monk & Goldie, 1976; Brock, 1977; Kimball et al., 1977). Rat AFP is a single-chain glycoprotein with a molecular weight of 72 000, containing about 4.0% carbohydrate (Smith & Kelleher, 1973; Watanabe, 1975). It is encoded by a 17S mRNA of about 2200 nucleotides (Sala-Trepat et al., 1979a,b). Hybridization assays with a specific cDNA indicate that AFP mRNA is produced from the class of unique sequence genes (Sala-Trepat et al., 1979a). Further studies of rat AFP gene expression would be greatly facilitated by the availability of a large amount of AFP-specific cDNA from cloned recombinant DNA.

Several different procedures for labeling cloned DNA inserts for hybridization assays have been described. The most commonly used procedure is nick translation (Rigby et al., 1977; Tsai et al., 1978; Dworkin & Dawid, 1980) which uses DNase digestion followed by DNA polymerase I extension with labeled deoxyribonucleoside triphosphates. Alternatively, DNA inserts have been labeled with ^{125}I (Lev et al., 1980) or made radioactive at their 5′ termini with [γ-^{32}P]ATP (Lev et al., 1980). While labeled cDNA probes prepared by using these procedures have been employed successfully in hybridization to nucleic acids immobilized on filters, there are several modest disadvantages for them to be used as probes for quantitative measurements of DNA–RNA hybridization in solution. Since these procedures label both complementary strands of the DNA where only one strand can form a hybrid to its corresponding mRNA, this results in a maximum of only 50% hybridization of the input probe. Reassociation of complementary strands of the probe may present a problem in some assays. Various efforts have been undertaken to overcome these problems. Tsai et al. (1978) prepared single-stranded probes from labeled double-stranded cDNA (ds-cDNA) inserts by limited hybridization with excess mRNA, hybrid isolation, and alkaline digestion of the mRNA. However, the preparative requirement for relatively large amounts of mRNA is a disadvantage for low abundance species. Lev et al. (1980) separated labeled probes into complementary strands by electrophoresis in polyacrylamide gels. But it would be difficult to use this method with nick-translated material because a heterogeneous reaction product is obtained initially. Recloning cDNA inserts into the single-stranded bacteriophage M13 may be offered as an alternative procedure (Messing et al., 1977; Schreier & Cortese, 1979). However, this procedure requires significant additional preparative effort.

In this communication, we report the cloning of rat AFP ds-cDNA, corresponding to the complete 3′-terminal region of the mRNA with a contiguous poly(A) segment, and the development of an easy method for synthesis of a single-

† From the Department of Microbiology and the Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received August 28, 1980.* This investigation was supported by Grants CA 16746 and CA 18450 from the National Institutes of Health, U.S. Public Health Service, Bethesda, MD.

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[1] Abbreviations used: AFP, α_1-fetoprotein; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; cDNA, complementary deoxyribonucleic acid; ss-cDNA, single-stranded cDNA; ds-cDNA, double-stranded cDNA; 1 × SSC, 0.15 M NaCl and 0.015 M trisodium citrate; bp, base pairs; kb, kilobases; R_0t, the product of initial RNA concentration in moles of nucleotides per liter and the time in seconds; $R_0t_{1/2}$, the R_0t value at 50% hybridization; oligo(dG), oligo(deoxyguanidylic acid); oligo(dC), oligo(deoxycytidylic acid); oligo(dT), oligo(thymidylic acid); poly(A), poly(adenylic acid).

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stranded cDNA (ss-cDNA) hybridization probe from the cloned ds-cDNA inserts. The probe synthesized is the functional equivalent of a cDNA copy of AFP mRNA, which then can be used for quantitative measurements of AFP mRNA.

Experimental Procedures

Preparation of AFP and Antibodies. AFP was purified from the amniotic fluid of 19-day pregnant Sprague–Dawley rats by ammonium sulfate precipitation, affinity chromatography, gel filtration, ion-exchange chromatography, and isoelectric focusing.[2] Antibodies were raised in rabbits by serial injection with Freund's complete and incomplete adjuvant. Antibodies were evaluated by crossed immunoelectrophoresis (Weeke, 1973) which showed a single immunoprecipitation line with amniotic fluid and no cross-reactivity with normal adult rat serum.

Preparation of mRNA. Yolk sacs, pooled from 19-day pregnant Sprague–Dawley rats, were homogenized and then extracted with phenol–chloroform as previously described (Keller & Taylor, 1976). Poly(A)-containing RNA was prepared by two successive affinity chromatography column steps with poly(U)–Sepharose (Taylor & Tse, 1976). The RNA was sedimented through 5%–29.9% isokinetic sucrose gradients (McCarty et al., 1974) containing 1% $NaDodSO_4$, 25 mM Hepes, and 5 mM EDTA at pH 7.4 in a Beckman SW41 rotor at 30000 rpm for 16 h at 20 °C. The 17S poly(A)-containing RNA fraction was collected and resedimented, and the center portion of the 17S peak was collected. Immunoprecipitation and gel electrophoresis of cell-free translation products identified this RNA material as primarily AFP mRNA (Figure 1).

AFP mRNA was also isolated from Morris hepatoma 7777 (maintained in buffalo rats) by polysome immunoprecipitation by using a double antibody technique essentially as described (Taylor & Tse, 1976). The poly(A)-containing RNA was further purified by poly(U)–Sepharose affinity chromatography and size fractionation as described above.

Synthesis of cDNA. Single-stranded cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (kindly provided by Dr. Joseph Beard, Life Sciences, Inc., St. Petersburg, FL), with a 20-fold weight excess of oligo(dT) primer to mRNA, essentially as described (Tse et al., 1978), but with the omission of actinomycin D. Double-stranded cDNA was synthesized from a ss-cDNA template with *E. coli* DNA polymerase I (Boehringer Mannheim) according to Wickens et al. (1978). Separate assays indicated that 80% of the first strand and 95% of the second strand reaction products were resistant to S1 nuclease digestion.

Construction of Recombinant Plasmids. The ds-cDNA was incubated with S1 nuclease (Miles) to create blunt ends (Kioussis et al., 1979). Terminal transferase (P-L Biochemicals) was employed to add approximately 30 dCMP residues to the ds-cDNA 3′-OH termini with reaction conditions similar to those of Wahl et al. (1979). About 15 residues of dGMP were added in a similar reaction to the plasmid pBR322 that had been previously cleaved by *Pst*I restriction endonuclease (Wahl et al., 1979). Equimolar amounts of dC-tailed ds-cDNA and dG-tailed plasmid were annealed in 200 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl at pH 7.6 for 16 h at 45 °C. Transformation of *E. coli* χ1776 was carried out essentially as described by Norgard et al. (1978) in accordance with the *National Institutes of Health Guidelines for Research involving Recombinant DNA Molecules* (1978). Colonies containing recombinant plasmids were selected by their resistance to tetracycline and sensitivity to ampicillin.

Identification of Transformants Containing AFP Sequences. Transformants were replica plated, grown on nitrocellulose filters (Millipore), and prepared for hybridization according to Grunstein & Hogness (1975). Replicate filters were hybridized with ^{32}P-labeled ss-cDNAs synthesized from AFP mRNA prepared by polysome immunoprecipitation, 19-day yolk sac mRNA, and adult liver mRNA (Kioussis et al., 1979). The filters were washed, dried, and autoradiographed. Colonies containing AFP sequences were then grown for plasmid preparation (Kioussis et al., 1979). The presence of AFP sequences in recombinant plasmids was demonstrated by the hybrid arrest of translation technique of Paterson et al. (1977) and further confirmed by nucleotide sequence analysis of cloned inserts (Liao et al., 1980).

Identification of Plasmids Containing Poly(A) Sequences. Isolated recombinant plasmids were digested with *Pst*I, extracted with phenol and ether, and precipitated with ethanol. The DNA cleavage fragments were resolved by electrophoresis in 1% agarose gels. The DNA was transferred to nitrocellulose filters (5 × 11 cm) by the Southern blotting technique (Southern, 1975). Filters were then treated with 10 mL of hybridization buffer (5 × SSC, 50% formamide, 0.5 mg/mL of sonicated and boiled calf thymus DNA, and 50 mM sodium phosphate at pH 6.5) containing 5 × Denhardt's reagent (Denhardt, 1966) and 1% glycine for 4 h at 42 °C. Hybridization was then carried out with 7.5×10^6 cpm of ^{32}P-labeled poly(dT) in 10 mL of hybridization buffer containing 1 × Denhardt's reagent and 10% sodium dextran sulfate (Pharmacia) for 15 h at 42 °C. After the filters were washed 4 times for 5 min each with 2 × SSC containing 0.1% $NaDocSO_4$ at 25 °C and 4 times for 15 min each with 0.1 × SSC containing 0.1% $NaDocSO_4$ at 50 °C, they were dried and autoradiographed. The poly(dT) hybridization probe was synthesized by elongating 2 μg of oligo$(dT)_{12-18}$ (Collaborative Research) with 50 units of terminal transferase in a 50-μL reaction volume containing 140 mM cacodylic acid, pH 6.9, 1 mM $CoCl_2$, 2 mM dithiothreitol, and 200 μM [α-^{32}P]dTTP (specific activity adjusted to 12.2 Ci/mmol). The elongated probe was purified by gel filtration through Sephadex G-100 equilibrated with 100 mM NaCl, 10 mM EDTA, and 20 mM Hepes at pH 7.5.

Restriction Endonuclease Analysis. Digestions with various restriction endonucleases (Bethesda Research Laboratories, New England Biolabs, Boehringer Mannheim) were carried out according to the manufactuer's descriptive literature. Cleavage fragments were separated by electrophoresis in horizontal 1–2% agarose gels or in vertical 5% polyacrylamide gels (Maniatis et al., 1975). Unlabeled DNA fragments were visualized by ethidium bromide staining (25 μg/mL), and ^{32}P-labeled fragments were identified by autoradiography (Maniatis et al., 1975). All gels included a separate lane of DNA standards prepared by digesting pBR322 or SV40 DNA with appropriate restriction endonucleases.

Synthesis of Plasmid-Derived AFP cDNA. Superhelical AFP plasmid DNA was prepared as a cleared lysate by the method of Clewell & Helinski (1970) and further purified by two successive bandings in ethidium bromide–CsCl gradients as described by Radloff et al. (1967). Plasmid DNA was digested with *Hin*dIII and precipitated with ethanol after extraction with phenol and chloroform. One microgram of the linear plasmid was then digested in 20 μL with 12 units of lambda exonuclease III (Bethesda Research Laboratories) for 20 min at 37 °C in a buffer containing 6 mM $MgCl_2$, 100 mM NaCl, 5 mM dithiothreitol, and 50 mM Tris-HCl at pH

[2] W. S. L. Liao and J. M. Taylor, unpublished results.

8.3. The reaction was terminated by cooling on ice; the digested plasmid DNA was denatured by heating at 100 °C for 5 min and plunged immediately into an ice–water bath. Two micrograms of oligo(dT) primer (Collaborative Research) was then added to the reaction mixture and allowed to anneal to the poly(A) sequence of the plasmid DNA for 10 min at 4 °C. The exonuclease III reaction mixture and the annealed oligo(dT) primer were added to an equal volume of cold DNA polymerase I reaction buffer containing 200 mM Hepes, pH 6.9, 70 mM KCl, 40 μM each of unlabeled deoxyribonucleoside triphosphates (dATP, dGTP, and dTTP), 7 μM of [α-^{32}P]dCTP (750 Ci/mmol, New England Nuclear), and 5 units of the Klenow fragment of *E. coli* DNA polymerase I (Bethesda Research Laboratories). The reaction mixture was incubated for 2 h at 25 °C, followed by phenol and chloroform extractions. The DNA was then denatured in 0.2 M NaOH by heating at 75 °C for 15 min and sedimented through 5–27.8% isokinetic sucrose gradients (McCarty et al., 1974) containing 0.1 M NaOH, 0.9 M NaCl, and 1 mM EDTA for 24 h in a Beckman SW41 rotor at 41 000 rpm at 4 °C. Fractions containing radioactive DNA were pooled, and the DNA was collected by ethanol precipitation. This DNA with a specific radioactivity of 9×10^7 cpm μg^{-1} was then employed for RNA-excess hybridization in solution according to the standard reaction conditions (Tse et al., 1978).

Electrophoresis of Denatured DNA. Electrophoresis of denatured DNA in agarose slab gels was performed by the method of McMaster & Carmichael (1977) with some modifications. DNA molecules were initially denatured in a buffer containing 70% (v/v) Me_2SO and 10 mM sodium phosphate buffer (pH 7.0) at 90 °C for 15 min. The reaction mixture was then adjusted to 1 M glyoxal, 50% Me_2SO, and 10 mM sodium phosphate buffer (pH 7.0) by adding 5 M glyoxal. The denaturation of DNA with glyoxal and Me_2SO was continued for 1 h at 50 °C (McMaster & Carmichael, 1977). Following electrophoresis in agarose gels, the ^{32}P-labeled DNA fragments were visualized by autoradiography by using Kodak XR-5 film at −70 °C.

Results and Discussion

Isolation of AFP mRNA. AFP mRNA was found to be the major poly(A)-containing RNA species of the rat yolk sac (Figure 1A), in agreement with the observations of other investigators who examined the mouse yolk sac (Tilghman et al., 1979; Miura et al., 1979). Immunoprecipitation and gel electrophoresis of the cell-free translation products (Figure 1A) indicated that our size-fractionated AFP mRNA was greater than 70% pure and was enriched about 3.5-fold over the starting material. This mRNA preparation was used for ds-cDNA synthesis which was then employed for cloning in pBR322. AFP mRNA prepared by polysome immunoprecipitation and size fractionation from Morris hepatoma 7777 (Taylor & Tse, 1976) was employed to synthesize ^{32}P-labeled cDNA for screening AFP-containing recombinant plasmids.

Cloning of AFP cDNA. A ds-cDNA synthesized from yolk sac AFP mRNA was prepared and examined by gel electrophoresis (Figure 1B). A major band of 2250 base pairs (bp) in length was detected which corresponded to the length of AFP mRNA (Sala-Trepat et al., 1979b). Several minor discrete bands were consistently detected which may have resulted from premature termination of transcription, possibly due to mRNA secondary structure (Challberg & Englund, 1979). The ds-cDNA was digested by S1 nuclease and inserted into the *Pst*I site of the ampicillin resistance gene of pBR322 by the oligo(dG)-oligo(dC) joining technique. *E. coli* χ1776 was transformed by the recombinant DNA, and tetracycline-resistant, ampicillin-sensitive transformants were selected.

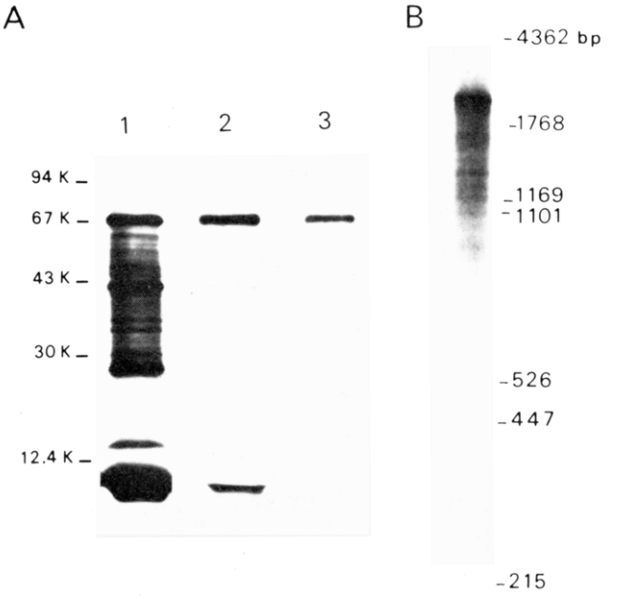

FIGURE 1: Translation of yolk sac mRNA. (A) Total poly(A)-containing RNA from 19-day yolk sac (lane 1) and 17S size-fractionated AFP mRNA (lane 2) were translated in a micrococcal nuclease treated rabbit reticulocyte cell-free system (Pelham & Jackson, 1976) containing [^{3}H]leucine. Lane 3 shows the translation produce which was immunoprecipitated with specific AFP antibodies and treated as described (Taylor & Tse, 1976). Electrophoresis of the translation products was performed in $NaDodSO_4$-containing 7.5–20% linear gradient polyacrylamide gels (Laemmli, 1970) which were then analyzed by fluorography (Laskey & Mills, 1975). (B) Total ^{32}P-labeled ds-cDNA prepared from AFP mRNA was examined by electrophoresis in 2% agarose gels followed by autoradiography (Kioussis et al., 1979). Avian myeloblastosis virus reverse transcriptase was employed to synthesize single-stranded cDNA, and the cDNA was then used as a template for second-strand synthesis by *E. coli* DNA polymerase I. The numbers indicate the lengths of DNA standards which were run in an adjacent land and identified by ethidium bromide staining.

Characterization of Recombinant AFP Plasmids. Colonies were selected by the replica filter screening technique (Grunstein & Hogness, 1975) for plasmids containing AFP sequences. Two colonies, pAFP-2 and pAFP-3, which showed intense hybridization to ^{32}P-labeled ss-cDNAs synthesized from two different preparations of AFP mRNA (size-fractionated yolk sac mRNA and polysome immunoprecipitated mRNA) and no hybridization to probes synthesized from normal adult liver mRNA were selected for further study (Figure 2). Recombinant plasmid, pAFP-3, was further demonstrated to contain an AFP sequence corresponding to the 3′-terminal portion of the mRNA by nucleotide sequence analysis (Liao et al., 1980).

The presence of poly(A) in the cloned inserts was demonstrated by its hybridization with ^{32}P-labeled poly(dT). Plasmid pAFP-3 was digested with *Pst*I, and the cleavage fragments were separated by electrophoresis in 1% agarose gels. The DNA was transferred to a nitrocellulose filter sheet and hybridized with ^{32}P-labeled poly(dT). The probe hybridized with fragments corresponding to the AFP ds-cDNA insert but did not hybridize with pBR322 (Figure 3). These results indicated that pAFP-3 contained at least a portion of the mRNA poly(A) segment and, probably, the complete putative 3′-terminal noncoding sequence of AFP mRNA. The doublet observed at the 530–580 bp position was due to incomplete digestion of the *Pst*I site within the AFP ds-cDNA insert. The close proximity of this site to the GC-tail sequence may have

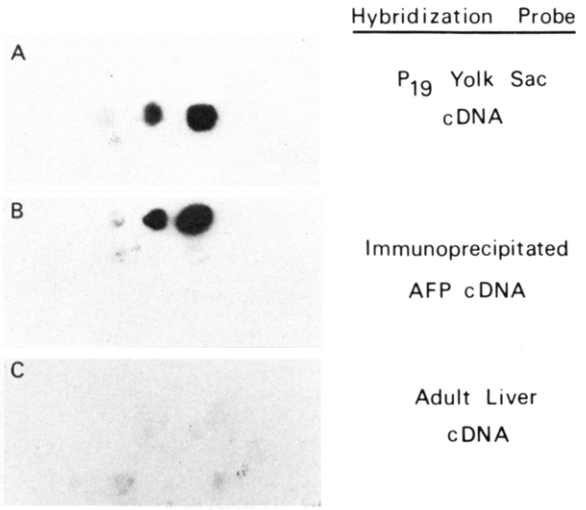

FIGURE 2: Screening of filter-bound recombinant plasmid DNA. Autoradiograms of replicate filters are shown on which transformant colonies had been grown, adsorbed, and hybridized to ^{32}P-labeled cDNA probes prepared from (A) 17S yolk sac mRNA, (B) hepatoma polysome immunoprecipitated AFP mRNA, and (C) normal adult liver mRNA. A typical experiment is shown in which each filter contains two rows of four different transformant colonies in each row.

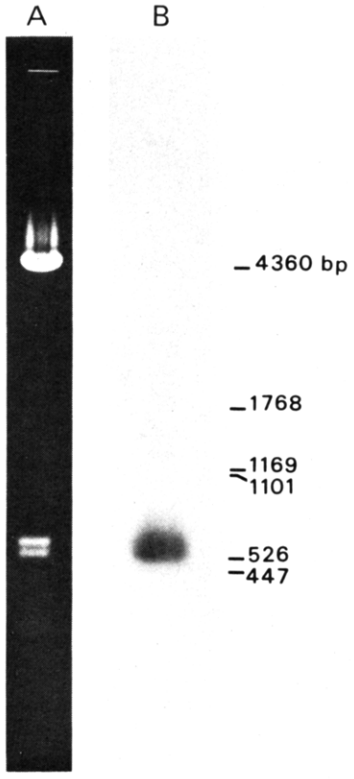

FIGURE 3: Identification of poly(A) in a recombinant plasmid. The cleavage fragments of *Pst*I-digested pAFP-3 were separated by agarose gel electrophoresis, transferred to nitrocellulose filter sheets, hybridized with ^{32}P-labeled poly(dT), and autoradiographed as indicated. The probe hybridized to the ds-cDNA insert but not to the pBR322 DNA fragment (lane B). Lane A shows the same DNA stained with ethidium bromide. The positions of DNA standards are indicated.

been responsible for this problem. This procedure, therefore, provides a convenient way to select for recombinant clones containing inserts with continguous poly(A) segment which may subsequently be employed to generate highly specific cDNA hybridization probes.

Preparation of AFP-Specific Hybridization Probe. The pAFP-3 plasmid was employed to develop a highly specific, easily prepared hybridization probe for use in mRNA quantitation in an RNA-driven reaction in solution. The procedure takes advantage of the availability of a stretch of homopolymer in the ds-cDNA insert and relies upon the sequential enzymatic activities of exonuclease III and DNA polymerase I. Thus, the method is termed the exo-pol procedure.

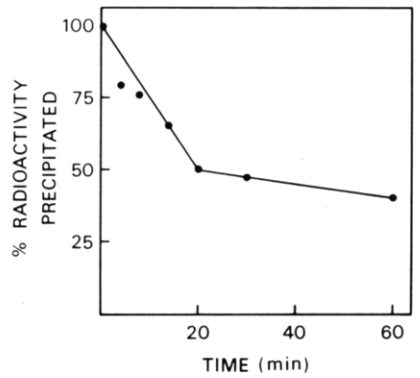

FIGURE 4: Time course of exonuclease III digestion of linear SV40 DNA. Uniformly labeled SV40 DNA (1 μg) was digested with *Eco*RI restriction endonuclease and was subsequently incubated with 12 units of exonuclease III. At the indicated time period of incubation, the reaction was stopped by cooling on ice. The labeled DNA was immediately precipitated with 10% trichloroacetic acid, and the radioactivity in the precipitate was determined (Tse et al., 1978).

The specificity of exonuclease III has been well characterized (Richardson & Kornberg, 1964; Richardson et al., 1964; Weiss, 1976). The enzyme releases 5′-mononucleotides from the 3′ termini of DNA, and it is specific for double-stranded DNA. For this reason, in preparing the hybrid plasmid for exonuclease III digestion, the covalently closed circular plasmid DNA was digested with a restriction endonuclease which produced an end with a recessed 3′ terminus on the appropriate complementary strand (although a blunt end also would have been suitable). Ideally, the restriction enzyme should not cleave within the ds-cDNA insert and should cleave at only one site in the plasmid, near the insert. For the pBR322 cloning vector, the preferred restriction endonucleases are *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*I.

As an initial characterization of the reaction conditions and the specificity of exonuclease III reaction, uniformly labeled SV40 DNA, previously digested with *Eco*RI restriction endonuclease, was incubated with an excess amount of exonuclease III. The extent of exonuclease III digestion was followed by the amount of radioactivity precipitated with 10% trichloroacetic acid. As Figure 4 shows, the initial exonuclease III digestion reaction occurred at a fast rate and was linear with time. The rate of hydrolysis, however, was greatly decreased after approximately 50% of the radioactive material had been rendered trichloroacetic acid soluble. There was no significant further digestion even after prolonged incubation. These results indicated that about 50% of the SV40 DNA was sensitive to exonuclease III digestion while the residual DNA was largely single stranded and resistant to further exonuclease III digestion. These results are similar to those reported by Richardson et al. (1964). Since pAFP-3 has essentially the same size DNA as SV40, the exonuclease III reaction conditions for SV40 were directly applied to the reactons for pAFP-3.

Since knowledge of the orientation of cloned AFP ds-cDNA insert within the cloning vector was a prerequisite for the exo-pol procedure to label the appropriate complementary strand of DNA, a physical map based on the cleavage reactions of several restriction endonucleases was determined. The orientation of the AFP sequence in pAFP-3 is indicated in Figure 5. According to this map, the poly(A) segment is

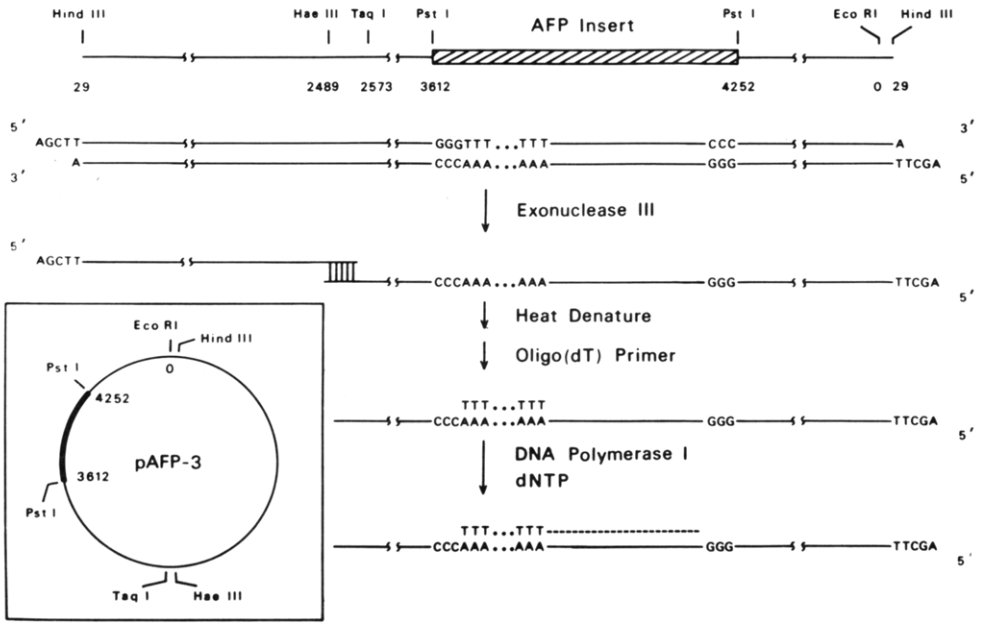

FIGURE 5: Protocol for the synthesis of plasmid-derived AFP cDNA hybridization probe. The positions, in nucleotides, of restriction endonuclease sites from the single *Eco*RI position in pBR322 are taken from Sutcliffe (1978). The schematic insert shows the orientation of AFP ds-cDNA within the cloning vector, pBR322.

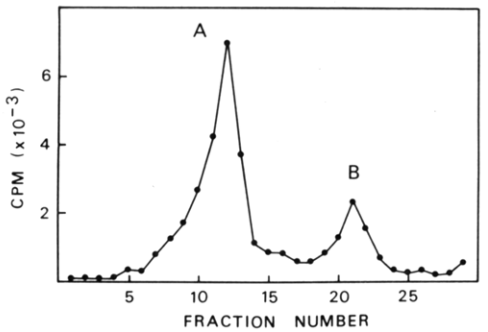

FIGURE 6: Alkaline isokinetic sucrose gradient. Plasmid-derived ^{32}P-labeled cDNA was denatured by heating in 0.2 M NaOH and subjected to sedimentation through an alkaline isokinetic sucrose gradient as described under Experimental Procedures. Fractions (0.4 mL) were collected, and 10-μL aliquots were removed to determine the radioactivity. The direction of sedimentation was from left to right. Fractions 11–13 (Peak A) and 20–22 (Peak B) were taken for further study.

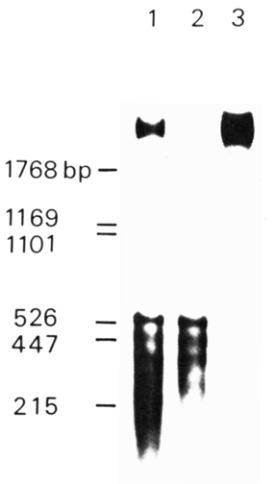

FIGURE 7: Agarose gel electrophoresis of denatured cDNA. ^{32}P-Labeled DNA samples were denatured with Me_2SO and glyoxal and electrophoresed 4 h at 100 V in 1.5% agarose slab gel. Lane 1 shows the total cDNA products before sucrose gradients. Lanes 2 and 3 show DNAs from peaks A and B, respectively, from the alkaline isokinetic sucrose gradients (Figure 6). The numbers indicate the lengths of DNA standards which were run in adjacent lanes. In lane 2, the majority of the DNA was concentrated in a 550-nucleotide band.

located close to the first *Pst*I restriction endonuclease site (position 3612) of the recombinant plasmid pAFP-3. Following digestion of pAFP-3 with *Hin*dIII, the linear plasmid was treated with an excess amount of exonuclease III to increase the probability of synchronous digestion at the 3′ end of the duplex DNA molecule. After heat denaturation, oligo(dT) was annealed to the poly(A) segment of the AFP coding strand. Subsequently, the only material synthesized by DNA polymerase I appeared to be that which was primed by oligo(dT). A schematic diagram of these reactions is shown in Figure 5.

Characterization of AFP-Specific Hybridization Probe. Following the synthesis reaction, the radioactively labeled DNA material was isolated by an alkaline isokinetic sucrose gradient sedimentation procedure that readily dissociated the noncovalently attached probe from its template. Radioactive DNAs with two different sedimentation values were obtained, having sharp sedimentation profiles which suggested DNAs of uniform lengths (Figure 6). The lengths of these DNAs were estimated to be approximately 500 and 2200 nucleotides for peaks A and B, respectively. It can be estimated from the gradient profile that approximately 70% of the DNA synthesized was in the shorter species.

The labeled DNAs were also analyzed in agarose gels following rigorous denaturation with glyoxal and Me_2SO. Under these conditions, the electrophoretic mobility of DNA becomes a function of its molecular weight (McMaster & Carmichael, 1977). Figure 7 shows the analysis of denatured radioactive DNA products on 1.5% agarose slab gels before and after alkaline sucrose gradient sedimentation. Two major species of DNA were clearly evident, each with electrophoretic mobility corresponding to approximately 2300 and 550 nucleotides. These length estimations were in close agreement with those obtained from isokinetic gradients. Since these denaturing gels included internal DNA standards of known length, 2300 and 550 nucleotides for slow and fast migrating DNA species, respectively, were taken as a more reliable size estimation.

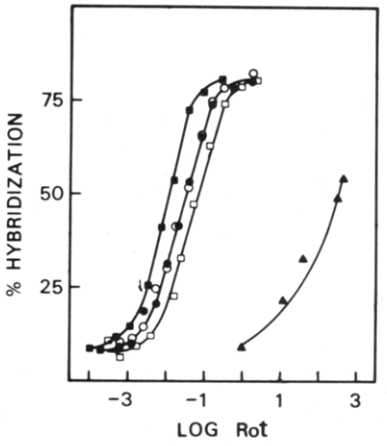

FIGURE 8: Hybridization of plasmid-derived AFP cDNA probe to poly(A)-containing RNA. Poly(A)-containing RNA from Morris hepatomas 7777, 72 ng (●), Morris hepatomas 8994, 72 ng (○), 19-day yolk sac, 12 ng (■), 19-day fetal liver, 36 ng (□), and adult liver, 9080 ng (▲), were hybridized in excess to 22 pg of plasmid-derived AFP cDNA probe (550-nucleotide DNA), and the extent of hybridization was determined by S1 nuclease (Tse et al., 1978). The S1 nuclease resistant background of the AFP cDNA probe was about 4%.

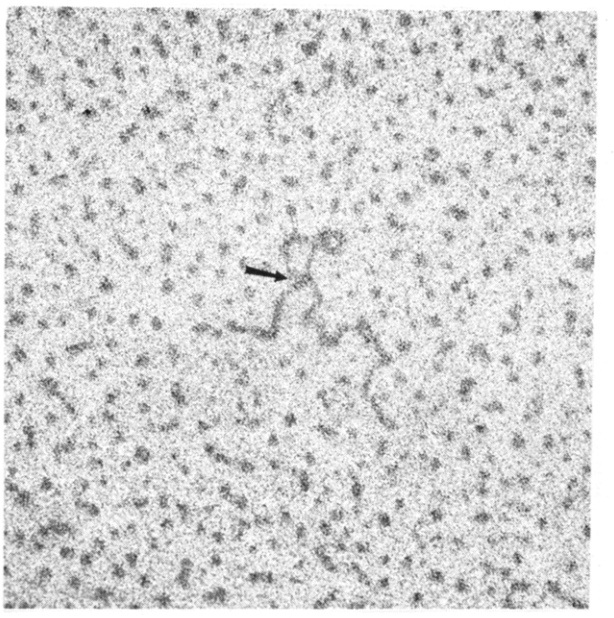

FIGURE 9: Electron micrograph of foldback structure of pAFP-3 DNA. *Hin*dIII-digested pAFP-3 DNA was treated with exonuclease III which exposed stretches of oligo(dC) and oligo(dG) homopolymers as shown in Figure 5. The pAFP-3 DNA was then denatured, allowed to reassociate briefly, and immediately mounted for electron microscopic observation, as described by Miller & Hyman (1978). An example is given of the foldback structure observed which is composed of two single-stranded arms and a single-stranded loop with a short double-stranded stem structure. The arrowhead points to the stem structure.

Further characterization of the reaction products indicated that the 550-nucleotide DNA is a ss-cDNA copy of the coding strand of the AFP insert whereas the 2300-nucleotide DNA is a copy of both the coding strand of the AFP insert and the plasmid strand adjacent to the AFP insert. These conclusions were derived from the following hybridization studies. When the 550-nucleotide DNA was hybridized to poly(A)-containing RNA from fetal liver, yolk sac, and AFP-producing Morris hepatomas 7777 and 8994, the hybridization went to at least 80% of completion with pseudo-first-order kinetics, occurring over a log R_0t range of approximately 2 (Figure 8). The S1 nuclease resistant background of the 550-nucleotide DNA in the absence of RNA was about 4%. These results suggest that the 550-nucleotide DNA preparation consisted mainly of single-stranded sequences complementary to a mRNA species. Control hybridizations showed that the probe did not hybridize to pBR322 plasmid DNA. In contrast, the 2300-nucleotide DNA hybridized to both the poly(A)-containing RNA from Morris hepatomas as well as the pBR322 plasmid DNA.

The hybridization data and the uniformity of the DNA lengths strongly suggest that the transcription with the DNA polymerase I appeared to terminate at the oligo(dG) segment at the 5′ region of the AFP coding strand (site adjacent to position 4252 in Figure 5) or to transcribe the oligo(dG) segment at low frequencies. Two different mechanisms may be used to explain these observations. First, it is possible that after the exonuclease III digestion which exposes stretches of oligo(dC) and oligo(dG) segments (Figure 5) the single-stranded DNA could fold back and allow the oligo(dG) segment to anneal with the oligo(dC) segment and thus form a stable hybrid with a single-stranded loop of approximately 500 nucleotides long. This reasoning is strongly supported by the electron microscopy studies in which pAFP-3 plasmid DNA tends to form a looping structure following exonuclease III digestion (Figure 9). The observation of numerous DNA molecules with the same folding conformation in the same relative position in the molecule argues against random crossing of the DNA molecule. Since this folding event is an intramolecular reaction, it is independent of the DNA concentration and occurs rapidly with zero-order kinetics.

Alternatively, Travaglini et al. (1975) have reported that the nature of the base in the polynucleotide template alters the maximal rate of polynucleotide synthesis by DNA polymerase I. By use of homopolymers as templates, DNA polymerase I transcribed poly(dG) 31 times slower than poly(dC). It is quite possible that both mechanisms are involved to produce cDNA of relatively uniform length.

Measurement of AFP mRNA Using Plasmid-Derived Hybridization Probe. The plasmid-derived AFP cDNA was employed in RNA-driven hybridization reactions to determine the relative concentrations of AFP mRNA in yolk sac, fetal liver, and adult liver as well as in hepatomas. Figure 8 shows that in late gestation the yolk sac ($R_0t_{1/2}$ = 8.5 × 10^{-3} mol·s·L^{-1}) contains about 6-fold more AFP mRNA than the fetal liver ($R_0t_{1/2}$ = 5.0 × 10^{-2} mol·s·L^{-1}). Since the yield of total poly(A)-containing RNA from the yolk sac (24 μg/g of tissue) is about the same as that from the fetal liver (28 μg/g of tissue), the yolk sac appears to be the major source of AFP in both amniotic fluid and fetal serum during rat fetal development. Hybridization of the AFP-specific cDNA to total liver RNA from normal adult (200 g) rats (Figure 8) shows the presence of a very low level of AFP mRNA. This degree of AFP gene expression in the adult liver may be due to a limited amount of transcription which remains from fetal gene activity. Alternatively, a continuous low level of liver regeneration may be characteristic of the adult liver, resulting in the previously reported low basal level of AFP production (Masseyeff et al., 1974). AFP and albumin cDNAs have previously been shown not to cross-hybridize to the heterologous mRNAs (Sala-Trepat et al., 1979b), so that the results shown in Figure 8 represent true AFP mRNA sequences. Since these hybridization results were obtained with a cloned AFP cDNA, possible problems due to non-AFP cDNA contaminants were avoided.

It is especially noteworthy that both Morris hepatomas 7777 and 8994 ($R_0t_{1/2}$ = 2.5 × 10^{-2} mol·s·L^{-1}), originally derived from adult livers (Morris & Wagner, 1968; Sell & Morris, 1974), produced AFP mRNA at a level not only significantly

higher than that of the normal adult liver but also attained an AFP mRNA level 2–3 times higher than the 19-day fetal liver. This reappearance of high levels of AFP mRNA in the hepatomas 7777 and 8994 may represent an increased AFP gene activity as the reult of carcinogenesis. However, the possibility that the relative abundance of other mRNA species may be affected is also likely.

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