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# α-Fetoprotein and albumin genes of rats: No evidence for amplification–deletion or rearrangement in rat liver carcinogenesis

(gene frequency/gene regulation/cDNA/DNA hybridization/"blot" hybridization)

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ABSTRACT **Full-length radiolabeled albumin and α-fetoprotein (AFP) cDNAs were synthesized from pure albumin and AFP mRNA preparations by using avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase). The cDNAs have been used to quantitate the number of albumin and AFP genes in different rat tissues by two independent methods, both of which yielded similar results. First, the kinetics of the association of these cDNAs with nuclear DNA from rat liver, rat kidney, and Morris hepatoma 7777 under conditions of vast DNA excess indicated that the albumin and AFP mRNAs are transcribed from "nonrepetitive DNA." Second, saturation hybridization experiments in which a constant amount of rat liver DNA or Morris hepatoma 7777 was hybridized with increasing amounts of cDNA to albumin mRNA have shown the presence of 1–2 albumin genes per rat haploid genome. The number of AFP genes obtained in similar titration experiments was approximately 2–3. This was true whether rat liver DNA or hepatoma 7777 DNA was used in the reassociation experiments. When high molecular weight DNA preparations from both these tissues were digested with the restriction endonuclease *Eco*RI and the fragments were transferred to a nitrocellulose filter, the albumin and AFP [$^{32}$P]cDNA probes hybridized to different sets of DNA fragments. However, each probe gave the same hybridization pattern whether Buffalo rat liver DNA or hepatoma 7777 DNA was utilized.**

α-Fetoprotein (AFP) is a plasma glycoprotein whose synthesis is associated with developmental and neoplastic processes (1, 2). This "oncodevelopmental" protein is the dominant protein species in the plasma of the mammalian fetus, where it is synthesized by the yolk sac and the liver. Its concentration is drastically decreased in the serum of adult animals except in cases of liver neoplasia and germinal tumors (3–5). The serum levels of albumin, the major plasma protein during adult life, show a reciprocal relationship with those of AFP. They increase from low levels early in fetal development up to high, approximately constant levels in postnatal life (1, 6). A reduced rate of albumin synthesis in relation to that of total proteins is, however, observed in adult rats bearing hepatomas (7, 8,§). We have recently shown that the changes in the serum levels of these two proteins, and in the albumin and AFP synthetic capacities of the liver and hepatoma tissues, are closely correlated with the steady-state concentration of the corresponding functional mRNAs.¶ These studies provided no information as to whether the regulation of the levels of polysomal albumin and AFP mRNA molecules is due to transcriptional control, to modulation at the level of processing or transport, or to gene amplification–deletion mechanisms. In this paper, we show that the processes responsible for changes in albumin and AFP phenotypic expression associated with liver tumorigenesis do not involve permanent changes in gene number. Furthermore, experiments involving hybridizations of specific albumin and AFP cDNA probes to *Eco*RI restriction endonuclease fragments of rat liver and Morris hepatoma 7777 cellular DNA have provided no evidence for permanent rearrangement of albumin and AFP genes during liver neoplasia.

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## METHODS

**Tumor Source and Animals.** Male rats of either the Sprague–Dawley or the Buffalo strain were used as sources of nuclear DNA or high molecular weight whole cell DNA. Rats bearing hepatoma 7777 tumors were kindly supplied by Harold P. Morris of the Department of Biochemistry, Howard University College of Medicine, Washington, DC; the tumors were maintained and transplanted in S. Sell's laboratory as described (9).

**Isolation and Purification of Albumin and AFP mRNAs.** Albumin and AFP mRNAs were isolated in a pure homogeneous state by a combination of immunoprecipitation of polysomes synthesizing these proteins, poly(U)-Sepharose 4B chromatography, and sucrose gradient centrifugation as described elsewhere.¶

**Synthesis of cDNA Probes.** cDNA probes complementary to albumin mRNA and AFP mRNA were synthesized as reported (ref. 10,¶), using 15–20 μg of the mRNA templates per ml and either [$^{3}$H]dCTP (20 Ci/mmol) or [$^{32}$P]dCTP (130 Ci/mmol or 28 Ci/mmol) as labeled substrate (1 Ci = $3.7 \times 10^{10}$ becquerels). The [$^{3}$H]cDNA preparations were calculated to have a specific activity of $1.4 \times 10^{7}$ cpm/μg, and the [$^{32}$P]-cDNAs to be $2.2 \times 10^{8}$ cpm/μg (used for association kinetics analysis) and $5 \times 10^{7}$ cpm/μg (used for saturation hybridization experiments).

**Preparations of Nuclear DNA.** Nuclei were prepared from rat liver, rat kidney, and Morris hepatoma 7777 tissue by a modification of the citric acid procedure of Higashi *et al.* (11). The nuclear pellets were dissolved in 100 mM NaCl/1 mM EDTA/1% sodium dodecyl sulfate ($NaDodSO_4$)/10 mM Tris·HCl buffer, pH 8.0, and brought to pH 8.0 with 1 M Tris base. Proteinase K was then added to a concentration of 100 μg/ml and the samples were incubated at 37°C for 2 hr. The solutions were then extracted with 1 vol of phenol/chloro-

Abbreviations: AFP, α-fetoprotein; $NaDodSO_4$, sodium dodecyl sulfate; Nt, nucleotides; $R_0t$ ($C_0t$), concentration of RNA (DNA) in moles of nucleotide per liter times incubation time in seconds; kb, kilobase pairs.

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§ S. Sell, unpublished.

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form/isoamyl alcohol (49:49:2, vol/vol), and the DNA was spooled from the aqueous phase after the addition of 2 vol of cold ethanol. Spooled DNA was lyophilized, dissolved in 50 mM Tris·HCl buffer, pH 7.5, containing 100 mM NaCl and 2 mM EDTA and then treated with 50 μg of pancreatic ribonuclease A per ml for 90 min at 37°C. $NaDodSO_4$ and proteinase K were then added to final concentrations of 0.5% and 50 μg/ml, respectively, and the incubation was continued for another 60 min at 37°C. The solution was then extracted with phenol/chloroform and the aqueous phase was precipitated overnight at −20°C with 2 vol of ethanol. Precipitated DNA was pelleted by centrifugation for 15 min at 7000 × *g*, lyophilized, and dissolved in 10 mM Na acetate, pH 6.6. The DNA solutions were sheared in a VirTis model 60 homogenizer under conditions that give DNA lengths of 300–400 nucleotides (12). Fragment sizes of the final preparations were routinely measured on 5–20% alkaline sucrose gradients.

**mRNA/cDNA Hybridization Reactions.** mRNA in excess was hybridized with the [$^{32}$P]cDNAs as described.¶ Data were analyzed by using a computer program (13) designed to fit the data according to the equation

$$c/C_0 = P[1 - \exp(-\ln 2 \cdot R_0t/R_0t_{1/2})],$$

in which $c/C_0$ represents the fraction of [$^{32}$P]cDNA in hybrid form at time $t$, $R_0t$ is the product of molar concentration of RNA nucleotides and incubation time in seconds, $R_0t_{1/2}$ is the $R_0t$ value of 50% hybridization, and $P$ is the fraction of cDNA hybridized at completion of the reaction.

**cDNA/DNA Reassociation Reactions under Conditions of DNA Excess.** cDNA/DNA reactions took place in 100-μl silanized capillary tubes containing 0.5 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, 20 mM Tris·HCl buffer at pH 7.5, 1000–2000 cpm of [$^{32}$P]cDNA, and nuclear DNA as indicated in the figure legends. The reaction mixtures were heated at 105°C for 4 min to denature DNA and incubated at 69°C for various times up to 72 hr. The extent of hybridization was assayed by S1 nuclease digestion as described (10). $C_0t$ values (DNA concentration in moles of nucleotide per liter times sec) were corrected for acceleration of renaturation rate due to higher cation concentration (12). The rate constants and curves through the data were obtained by using a computer program (13) designed to fit the data according to the equation

$$d/D_0 = P\{1 - [1 + (C_0t/C_0t_{1/2})]^{-0.44}\},$$

in which $d/D_0$ represents the fraction of cDNA in duplex form at time $t$, and $P$ is the fraction of the cDNA probe hybridized at completion of the reaction.

**Saturation Hybridization Experiments.** Fifty micrograms of nuclear DNA was mixed with increasing amounts of the albumin and AFP [$^{32}$P]cDNA ($5 \times 10^7$ cpm/μg) probes in 10 μl of 0.5 M NaCl/1 mM EDTA/0.1% $NaDodSO_4$/20 mM Tris·HCl buffer, pH 7.5. The reaction mixtures were sealed in silanized capillary tubes, heated at 105°C for 4 min, and incubated for 65 hr at 69°C. Duplex formation was assayed by S1 nuclease treatment. Background S1 resistance was measured by incubating parallel samples under the same conditions except for the use of micrococcal DNA instead of rat DNA. The amount of [$^{32}$P]cDNA hybridized was corrected for the background S1 nuclease resistance and converted to pg of cDNA.

**Preparation of Restriction Fragments and Agarose Gel Electrophoresis.** High molecular weight cellular DNA was extracted from rat livers and Morris hepatoma 7777 by a modification of the method described by Blin and Stafford (14). The DNA preparations obtained were digested three times by a 3-fold excess of the restriction endonuclease *Eco*RI in 50 mM NaCl/10 mM $MgCl_2$/100 μg of gelatin per ml/100 mM Tris·HCl buffer, pH 7.5, for 60 min at 37°C. The resulting fragments were separated by electrophoresis on 0.7% agarose gels containing 0.1 μg of ethidium bromide per ml, and the DNA bands were visualized under UV light (15).

**Filter Hybridizations.** DNA fragments were transferred to nitrocellulose filters by a modification of the technique of Southern (16). DNA fragments on filter strips were hybridized to $^{32}$P-labeled albumin cDNA or AFP cDNA (20 ng/ml; $2.2 \times 10^8$ cpm/μg) at 65°C for 20 hr in 10 ml of hybridization solution consisting of 1 M NaCl, 10-fold concentrated Denhardt's solution (17), 6 mM EDTA, 0.1% $NaDodSO_4$, 25 μg of denatured *Escherichia coli* DNA per ml, and 90 mM Tris·HCl at pH 7.2. After hybridization, the filters were washed at 63°C in 75 mM NaCl/15 mM Tris, pH 7.2/1 mM EDTA/0.1% $NaDodSO_4$/0.1% sodium pyrophosphate and exposed to preflashed Kodak XR-5 x-ray film (18) with a Cronex x-ray intensifying screen for various times at −70°C.

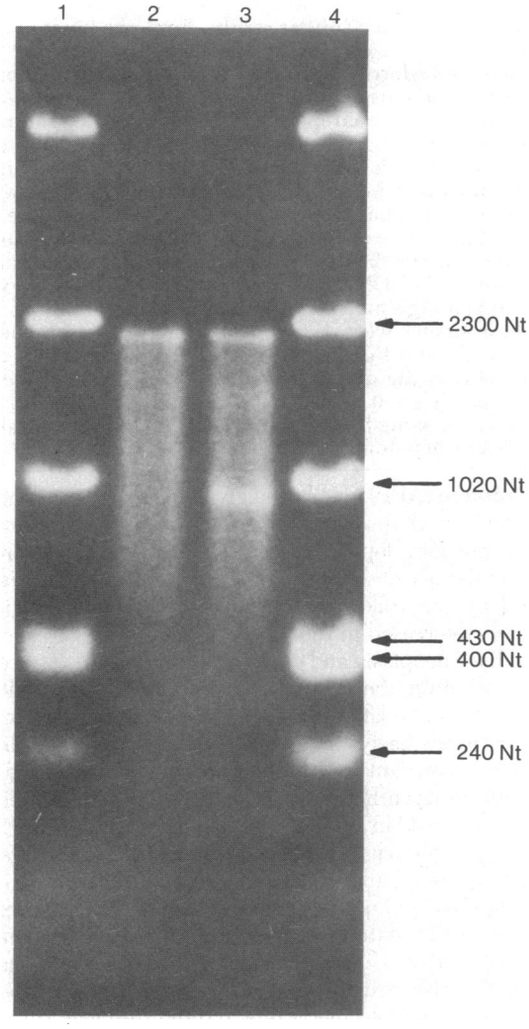


FIG. 1. Autoradiogram of albumin and AFP [$^{32}$P]cDNAs after electrophoresis on an alkaline agarose slab gel. Albumin and AFP [$^{32}$P]cDNA samples were electrophoresed on a 1% agarose gel containing 30 mM NaOH/2 mM EDTA for 10 hr at 30 V as described (19). After electrophoresis, the gels were neutralized, dried, and exposed to Kodak XR-5 x-ray film. Lanes 1 and 4: PM2 DNA markers. *Hin*dIII digests of PM2 DNA (gift of Horace B. Gray, Jr., University of Houston), labeled *in vitro* with [$\gamma$-$^{32}$P]ATP and phage T4 polynucleotide kinase. Lane 2: AFP [$^{32}$P]cDNA. Lane 3: albumin [$^{32}$P]-cDNA.

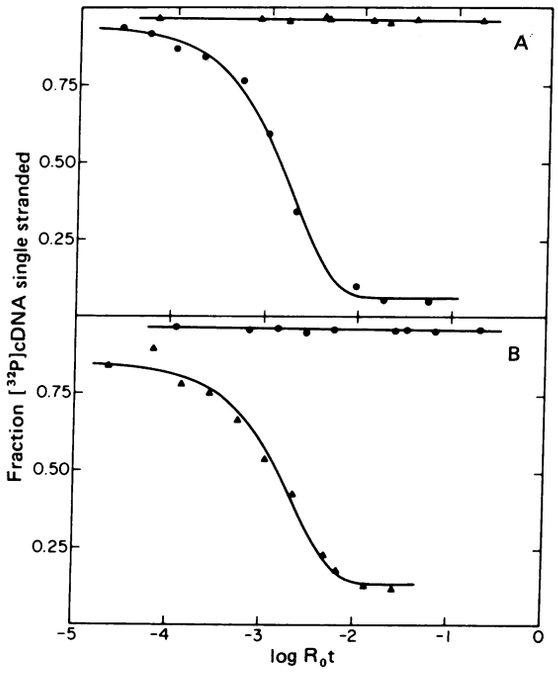


FIG. 2. Kinetics of hybridization of albumin and AFP [$^{32}$P]-cDNAs to their homologous and heterologous templates. Excess RNA hybridized with highly purified albumin in mRNA (●) and AFP mRNA (▲) samples. (*A*) Hybridizations to albumin [$^{32}$P]cDNA. (*B*) Hybridizations to AFP [$^{32}$P]cDNA.

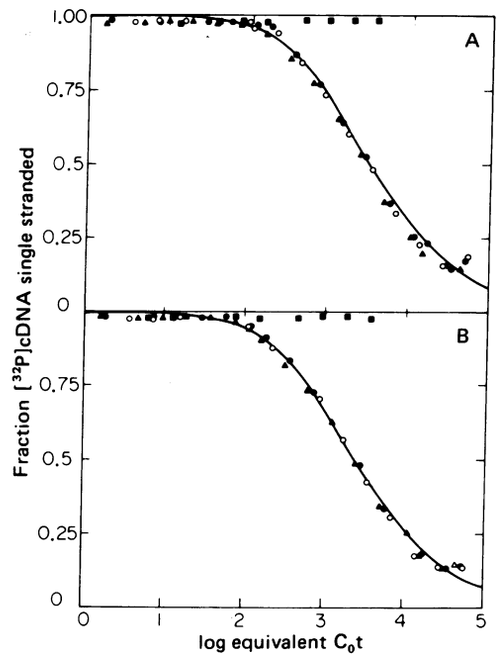


FIG. 3. Association kinetics of albumin [$^{32}$P]cDNA (*A*) and of AFP [$^{32}$P]cDNA (*B*) with DNA prepared from rat kidney (○), rat liver (●), and Morris hepatoma 7777 (▲). Approximately 1–1.5 mg of each DNA preparation was mixed separately with 1000–2000 cpm of the $^{32}$P-labeled cDNAs (5–10 pg), heat denatured, and annealed at 69°C in the 0.5 M NaCl hybridization buffer. The extent of reaction was measured by S1 nuclease resistance. The amount of duplex formed at each $C_0t$ value was normalized by taking the reactivity of each probe (determined from Fig. 2) as 100%. The curve drawn in *A* represents the best fit for the rat liver DNA data, and that shown in *B* is the best fit for the hepatoma DNA points. Reactions of the albumin and AFP [$^{32}$P]cDNA probes with *Micrococcus lysodeikticus* DNA are also shown (■).

## RESULTS

**Characterization of the cDNA Probes; Hybridization with Their Template mRNAs.** The isolation of albumin and AFP mRNA in a pure homogeneous state is described elsewhere.¶ The mRNA preparations were used as templates to generate highly radiolabeled cDNAs with avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase). The size of the $^{32}$P-labeled transcripts was analyzed on alkaline agarose slab gels with *Hin*dIII fragments of phage PM2 DNA as markers (Fig. 1). The autoradiogram shows that, from both templates, a certain proportion of the $^{32}$P-labeled products migrate as a discrete band, slightly faster than the 2300 nucleotides (Nt) of the PM2 marker. The largest transcripts were 2180 Nt for albumin [$^{32}$P]cDNA and 2120 Nt for the AFP cDNA (average determination of three independent electrophoretic runs). These values are very close to the size of the albumin mRNA [≈2265 Nt, including a poly(A) tract of 102 adenosine residues] and AFP mRNA [≈2235 Nt including a poly(A) tail of 190 bases] templates.¶ These results suggest that our [$^{32}$P]cDNA preparations contain all the complexity represented in the albumin and AFP mRNA sequences. Another discrete very intense band, migrating near the 1020-Nt band of the PM2 markers, can be seen in the albumin cDNA transcripts. Such a band has no parallel in the AFP cDNA preparation. This might indicate the existence of a region of extensive secondary structure within the albumin mRNA that could block further enzyme action.

In Fig. 2 is shown the kinetics of hybridization of the [$^{32}$P]-cDNA probes with the homologous and heterologous templates. Under our experimental conditions, the albumin cDNA hybridizes to the albumin mRNA with a $R_0t_{1/2}$ of $1.38 \times 10^{-3}$ mol liter$^{-1}$ sec and the AFP cDNA reacts with its mRNA template with a $R_0t_{1/2}$ of $1.53 \times 10^{-3}$ mol liter$^{-1}$ sec. These values are similar to those previously found for our $^{3}$H-labeled cDNA probes, and the kinetics are consistent with those expected for single mRNA species 2200 Nt in size. Under our reaction conditions, the albumin and AFP [$^{32}$P]cDNAs do not crossreact with the heterologous mRNA templates (Figs. 1*A* and 2*B*). Furthermore, when the cDNAs were hybridized to rat kidney polysomal RNA, no reaction was observed up to very high $R_0t$ values (not shown). Thus, our cDNA probes appear to be highly specific for albumin and AFP mRNA sequences, respectively.

**Kinetics of Association of Albumin and AFP cDNAs with Rat DNA.** Measurement of gene reiteration frequency by reassociation kinetics requires that there be a large excess of complementary sequences in the "driver" DNA (20). We have used high specific activity $^{32}$P-labeled cDNA probes ($2.2 \times 10^8$ cpm/$\mu$g) and milligram quantities of nuclear DNA to achieve a driver/tracer sequence ratio of at least 40. The data in Fig. 3 show that the reactions of both $^{32}$P-labeled albumin and AFP cDNAs with different rat DNAs achieve completion at nearly 90% saturation of the probes. The kinetics of the reactions were very similar for DNA extracted from rat liver, rat kidney, and Morris hepatoma 7777. Computer analysis of the data provided the following second-order rate constants in liter mol$^{-1}$ sec$^{-1}$ for the albumin cDNA reactions: $1.11 \times 10^{-3}$, $1.17 \times 10^{-3}$, and $1.12 \times 10^{-3}$ for rat liver, rat kidney, and Morris hepatoma 7777 DNA, respectively. Reactions with the AFP cDNA probe proceeded with rate constants of $1.34 \times 10^{-3}$, $1.32 \times 10^{-3}$, and $1.42 \times 10^{-3}$ for the same DNA samples. These values are consistent with those expected for the reassociation of sequences present approximately one to four times in the rat genome (10, 21). While those results indicate that the albumin and AFP mRNAs

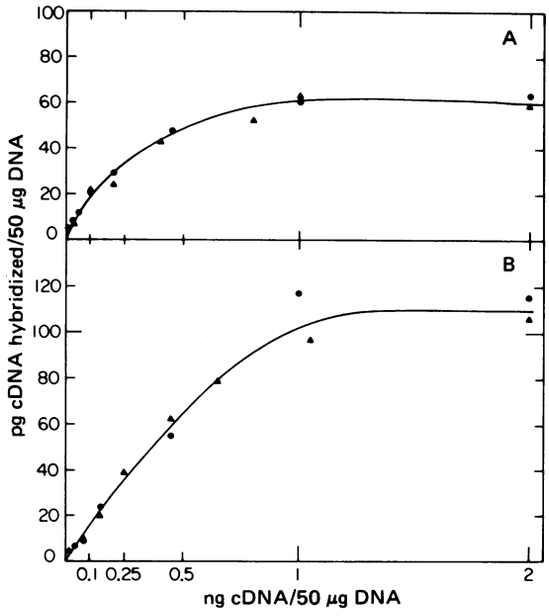


FIG. 4. Saturation analysis of the hybridization reactions of albumin [$^{32}$P]cDNA (*A*) and AFP [$^{32}$P]cDNA (*B*) with rat liver DNA (●) and Morris hepatoma DNA (▲).

are transcribed from the "nonrepetitive" fraction of the rat genome in the different tissues studied, the limited accuracy of the kinetic approach does not allow us to differentiate between one and a few copies of each gene.

**Saturation Hybridization Analysis of Albumin and AFP Gene Numbers.** A reliable measurement of the number of genes coding for a particular protein can be obtained by a saturation experiment in which fixed amounts of genomic DNA are reannealed in the presence of increasing amounts of a pure cDNA probe containing all the sequence complexity encoding for that protein. We have carried out such experiments, using our full-length albumin and AFP cDNA probes. The reactions are shown in Fig. 4. Confirming the kinetic studies, the saturation data show that there are no significant differences between the plateau levels achieved when either rat liver or Morris hepatoma 7777 DNA was used in the hybridization reactions. At saturation, approximately 60 pg of albumin cDNA was able to hybridize per 50 μg of nuclear DNA. When the AFP cDNA probe was used, saturation occurred at a ratio of 100 pg of cDNA per 50 μg of DNA. Therefore, rat DNA sequences homologous to albumin and AFP mRNA comprise fractions of $1.2 \times 10^{-6}$ and $2.2 \times 10^{-6}$ of the total genomic DNA, respectively. For a rat haploid genome size of 2.9 pg or $2.8 \times 10^{9}$ base pairs (21, 22), we can calculate that 3360 base pairs are albumin-encoding sequences and that 6160 base pairs correspond to AFP-encoding sequences. We have recently determined the size of the albumin and AFP mRNA molecules by their electrophoretic migration on agarose gels under fully denaturing conditions (methylmercury hydroxide/agarose gels).¶ Taking these values (albumin mRNA, 2265 Nt; and AFP mRNA, 2235 Nt), we can estimate there are about 1.5 albumin genes and 2.8 AFP genes per haploid genome.

**Albumin and AFP Gene Sequences in Rat Liver and Morris Hepatoma 7777 DNA Fragments Generated with *Eco*RI Endonuclease.** High molecular weight rat liver DNA and Morris hepatoma 7777 DNA were digested with *Eco*RI endonuclease, and the resulting DNA fragments were fractionated according to size by agarose gel electrophoresis. The

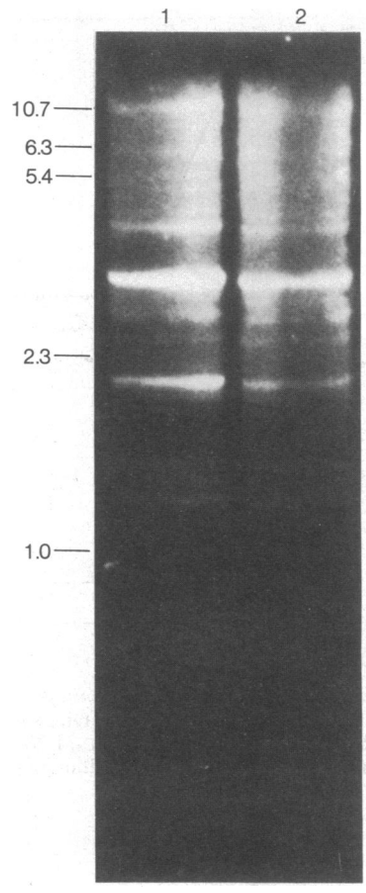


FIG. 5. Filter hybridization of the AFP [$^{32}$P]cDNA probe to *Eco*RI fragments of rat liver and Morris hepatoma 7777 DNA. Lane 1: Buffalo rat DNA. Lane 2: Hepatoma DNA. The numbers indicate the size in kilobases of fragments estimated from *Hin*dIII fragments of PM2 DNA and *Eco*RI digests of λ Charon 4A used as markers.

fragments were then transferred to nitrocellulose filters (16) and hybridized to the albumin and AFP [$^{32}$P]cDNA probes.

The hybridizing fragments were located by autoradiography and their sizes were determined by comparison with appropriate molecular weight standards (Figs. 5 and 6). No significant differences are observed between the hybridization patterns obtained with the Buffalo rat DNA and the hepatoma DNA. Several bands hybridized to the AFP cDNA with different relative intensities (Fig. 5). Two bands with sizes of 3.3 and 2.0 kilobases (kb) ± 5% can be identified that hybridized intensely. Other bands probably containing AFP gene material migrated at positions corresponding to 10.7, 4.2, 2.8, 1.3, and 1.1 kb. Less intense bands are also present but their significance cannot yet be assessed.

Hybridizations with the albumin [$^{32}$P]cDNA probe yielded a completely different set of bands (Fig. 6). In Buffalo rat DNA, the following DNA fragments containing albumin gene sequences can be identified in order of decreasing size (in kb): 8.7, 7.6, 4.3, 3.1, 2.9, 2.4, 1.9, 1.6, 1.3, and 1.0. The Morris hepatoma DNA displays a similar banding pattern. The 3.1-kb fragment was consistently seen as the most intense band. In Fig. 6 is also shown the albumin hybridization pattern generated by *Eco*RI fragments of Sprague–Dawley rat liver DNA. While most of the bands displayed by the Buffalo rat DNA were also present, the 4.3- and 1.9-kb bands seen in Buffalo DNA were absent here. In addition, two new bands, 3.9 and 2.7 kb in size, were found in *Eco*RI-digested DNA from the Sprague–Dawley strain.

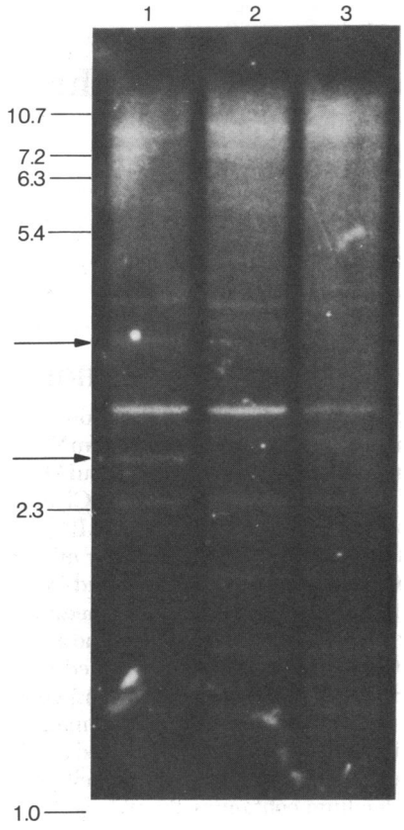


FIG. 6. Autoradiogram of Sprague–Dawley rat liver DNA (lane 1), Buffalo rat DNA (lane 2), and Morris hepatoma 7777 DNA (lane 3) *Eco*RI fragments after filter hybridization to albumin [$^{32}$P]cDNA. The arrows point to the 3.9- and 2.7-kb fragments present in Sprague–Dawley DNA and absent in the other DNA samples.

## DISCUSSION

In the present study we have first used albumin and AFP cDNA probes of high specific activity to estimate the reiteration frequency of albumin and AFP genes in different rat tissues and Morris hepatoma 7777. Two different, though complementary, approaches have been utilized. The kinetic technique under conditions of vast DNA excess allowed us to conclude that both albumin and AFP mRNAs are transcribed from approximately single-copy DNA in the different tissues. The saturation hybridization experiments afforded more accurate estimates of the number of albumin and AFP genes present in the rat genome. Again, no significant differences were found in the final plateau values in experiments with rat liver DNA or Morris hepatoma 7777 DNA. Values obtained for the number of albumin and AFP sequences per rat haploid genome were 1–2 and 2–3, respectively. These results indicate that the highly enhanced levels of stable AFP mRNA sequences observed during hepatic carcinogenesis appear to result from a transcriptional activation or a post-transcriptional stabilization of polysomal AFP coding sequences rather than to a specific amplification of the AFP genes.

We have taken those studies one step further by hybridizing the cDNA probes to *Eco*RI-digested, filter-bound rat DNA and Morris hepatoma 7777 DNA. The similarity in the hybridization patterns displayed by the DNA from normal and neoplastic tissue suggests that the gross organization of the albumin and AFP genes in the rat genome is not permanently altered by the processes involved in changing the phenotypic expression of albumin and AFP that occurs during liver carcinogenesis. An additional outcome of these studies is the evidence obtained for the existence of different patterns of sequence organization of the albumin genes in rats of different, though related, genetic origins. Thus, it would appear that the structural organization of the albumin genes is not highly conserved in related rat strains.

A more detailed analysis of the structural organization of the albumin and AFP genes in the genomic DNA will require further restriction fragment studies. We have recently isolated albumin and AFP cDNA clones in the bacterial strain *E. coli* $\chi$1776 with the plasmid pBR322. These clones can be used as probes to obtain a physical map of the albumin and AFP chromosomal genes.

We thank Dr. Harold Morris, who graciously furnished the tumor-bearing rats. We are indebted to Dr. J. Beard for providing us with reverse transcriptase of avian myeloblastosis virus as part of the joint National Institutes of Health–National Cancer Institute program. We also thank Dr. J. Dever and K. Thomas for help in the early part of this work, R. F. Murphy for help in the computer analysis of the hybridization and reassociation data, and James Posakony for helpful discussion. The skillful technical assistance of John Rising is gratefully acknowledged. This work was supported by U.S. Public Health Service Grant GM-13762, by National Cancer Institute Grant CA-22227, and by the French Research Council (Centre National de la Recherche Scientifique).

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