

# Expression of rainbow trout apolipoprotein A-I genes in liver and hepatocellular carcinoma

Geneviève P. Delcuve, Jian Min Sun, and James R. Davie<sup>1</sup>

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada R3E 0W3

**Abstract** Screening for genes overexpressed in trout aflatoxin B<sub>1</sub>-induced hepatocellular carcinomas resulted in the isolation of cDNA sequences of two apolipoprotein A-I-s, apoA-I-1 and apoA-I-2. The levels of apoA-I-1 and -2 mRNAs of liver and tumor were quite different. ApoA-I-1 mRNA was the major species in liver, while apoA-I-1 and -2 mRNAs were present at similar levels in several tumors. This elevated level of apoA-I-2 mRNA was observed in seven different tumors, suggesting that the overexpression of apoA-I-2 was a general feature of aflatoxin B<sub>1</sub>-induced liver tumors. Hybridization to genomic DNA demonstrated that trout has two different apoA-I genes which is in contrast to other vertebrates which have one gene coding for apoA-I. Liver apoA-I-1 and -2 cDNA clones specified the same amino acid sequence as the tumor apoA-I-1 and -2 cDNA clones. Analysis of the cDNA-derived amino acid sequences showed that trout apoA-I-1 and -2, like human apoA-I, consist largely of multiple 22 amino acid repeats having the potential to generate an amphipathic  $\alpha$ -helix. The similarity of the repeat pattern in trout and human apoA-I-s suggests that all the internal repeats in these sequences arose before the fish-mammal split, some 400 million years ago.—Delcuve, G.P., J.M. Sun, and J.R. Davie. Expression of rainbow trout apolipoprotein A-I genes in liver and hepatocellular carcinoma. *J. Lipid Res.* 1992. 33: 251–262.

**Supplementary key words** apolipoprotein A-I • rainbow trout • liver • hepatocellular carcinoma

Apolipoprotein A-I is the major constituent of high density lipoproteins and participates in the reverse transport of cholesterol from tissues to the liver for excretion. Therefore, apoA-I and HDL are thought to confer protection against coronary heart disease (for reviews, see 1–3). A common feature of apolipoproteins is the presence of multiple repeats of 22 amino acids, each of them having an amphipathic  $\alpha$ -helical structure. Most repeats start with the amino acid proline, an  $\alpha$ -helix breaker. It is thought that the nonpolar face of the amphipathic helices interacts with the hydrophobic lipid core of the lipoprotein particle, while the polar face interacts with the aqueous plasma environment. In mammals and birds, the apoA-I gene is expressed primarily in liver and intestine (for reviews, see 4, 5).

Rainbow trout contains different lipoprotein particles (high, intermediate, low, and very low density lipoproteins) that are similar to those of mammals (6). The major apolipoprotein of rainbow trout HDL appears to be homologous to human apoA-I. It has a comparable molecular weight, a similar amino acid composition, and common antigenic sites with human apoA-I (6–10). This apoA-I-like protein is one of the most abundant proteins in the trout plasma (11). To our knowledge, no sequence data of trout apolipoproteins have been reported previously. However, the amino acid sequence of the amino-terminus of carp apoA-I has been determined (12), and cDNA sequences have been reported for two HDL apolipoproteins from lamprey (13), a cyclostome fish that diverged from other vertebrate lineages before the teleost fish rainbow trout and carp did. The two cDNAs code for two very abundant plasma proteins LAL1 (105 amino acids) and LAL2 (191 amino acids) that have characteristics of typical mammalian apolipoproteins. However, no definitive assignment of the two lamprey proteins to typical mammalian apolipoprotein classes could be made (13).

Neoplastic transformation of a cell is a consequence of the abnormal expression of certain genes. The aberrant expression of these genes may be a result of modification at the gene level and/or changes in chromatin structure. We have been analyzing the chromatin composition of trout liver and hepatocellular carcinoma. These tumors were induced by aflatoxin B<sub>1</sub>, one of the most potent liver toxins and procarcinogens known, and a naturally occurring food contaminant implicated in the etiology of some human hepatic cancers in Africa and China (14, 15). The Shasta strain of rainbow trout (*Oncorhynchus*

Abbreviations: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; SDS, sodium dodecyl sulfate; kDa, kilodalton; nt, nucleotide.

<sup>1</sup>To whom correspondence should be addressed.

*mykiss*) has a near-zero background for any neoplasm and has proved to be the most sensitive experimental animal to the carcinogenic effects of aflatoxin B<sub>1</sub>. Short exposure of fertilized eggs of rainbow trout to aflatoxin B<sub>1</sub> results in 65% of the survivors having at least one liver tumor, 1 year after treatment (16, 17). Aflatoxin B<sub>1</sub>-induced trout liver tumors differ from normal liver tissue in that they have an altered histone H1 subtype composition, more nuclease-sensitive chromatin, and moderately or greatly enlarged hyperchromatic nuclei (18). It is conceivable that these alterations in the composition and organization of tumor chromatin contribute to abnormal hepatocellular gene expression.

The objective of this study was to isolate genes expressed at abnormal levels in rainbow trout hepatocellular carcinomas that were induced by aflatoxin B<sub>1</sub>. Thus, a hepatocellular carcinoma cDNA library was constructed, and differential colony hybridization was used to identify transcripts present at higher levels in tumor than in liver. This resulted in the isolation of two hepatocellular cDNA clones coding for two apolipoprotein A-Is, one of which was overexpressed in hepatocellular carcinoma.

## MATERIALS AND METHODS

### Tissues

Livers and hepatocellular carcinomas, which were induced by aflatoxin B<sub>1</sub>, were obtained from rainbow trout (*Oncorhynchus mykiss*, Shasta strain). To induce hepatocellular carcinomas, the fish were exposed to a 0.5 ppm solution of aflatoxin B<sub>1</sub> for 30 min as 4-week-old fry (28 days post-fertilization) as previously described (16). After 1 year the fish were killed and livers were excised. The tumors were immediately dissected out and frozen in liquid nitrogen. Tissues were kept at -80°C until needed.

### RNA isolation

Total RNA of individual liver or hepatocellular carcinoma was isolated as described (19), and total RNA of testis, gill, heart, intestine, and kidney were isolated as described (20). PolyA<sup>+</sup> mRNA was prepared as described (21).

### cDNA library construction and screening

Double-stranded cDNA was synthesized from an individual hepatocellular carcinoma polyA<sup>+</sup> mRNA using a BRL cDNA synthesis system and cloned into pUC9 by deoxycytidine–deoxyguanosine tailing (21). Screening by differential colony hybridization was performed as described (21). Double-stranded cDNA was syn-

thesized from individual liver polyA<sup>+</sup> mRNA using a Pharmacia cDNA synthesis kit, and cloned into the EcoRI site of λZAPII (Stratagene), using the Promega Riboclone EcoRI adaptor ligation system. Screening for apoA-I clones was performed as previously described (21), using one of the apoA-I-2 hepatocellular carcinoma subclone inserts as a probe.

### Northern and Southern blot analyses

Approximately 10 μg of total RNA was electrophoresed through a 1% agarose gel containing formaldehyde (21), and transferred onto nitrocellulose. Northern blot hybridization was carried out with a <sup>32</sup>P-labeled DNA probe as described (22), except that more stringent washing conditions were applied (68°C instead of 50°C). Isolated cDNA clones pTHC2c and pTHC5c were used as probes. The autoradiograms were scanned with a densitometer. The relation between the signal and the amount of RNA loaded onto the gel was linear, showing that the intensity of the signal was directly proportional to the amount of hybridizable mRNA. Relative steady state levels of THC2c mRNA were determined by first calculating the ratio of signal for THC2c mRNA to that of THC5c mRNA in the different RNA preparations of liver and hepatocellular carcinoma and then calculating the ratio of these values.

After restriction digestions, liver or hepatocellular carcinoma DNA was electrophoresed through a 1% agarose gel and transferred onto MSI Magna nylon 66 membranes (Fisher). Hybridizations to <sup>32</sup>P-labeled probes were done as described (23).

### In vitro translation

Hybridization and recovery of selected polyA<sup>+</sup> mRNA were essentially done according to Ausubel et al. (24). First, plasmid DNA was bound to nitrocellulose filters. To do so, 50 μg DNA in 1.5 ml 10 mM Tris-HCl, pH 8.0, 1 mM EDTA was boiled for 10 min, immediately diluted with 1.5 ml 1 M NaOH, left at room temperature for 10 min, and neutralized by the addition of 9 ml 6 × SSC (20 × SSC = 3 M NaCl, 0.3 M Na citrate). The pH was quickly adjusted to 6.5–7.5 and the solution was slowly passed through a 0.45-μm nitrocellulose filter of 2.5 cm diameter. The filter was washed with 50 ml 6 × SSC, dried at room temperature, and baked for 2 h at 65°C. Disks of 0.5 cm of diameter were used for each hybridization. To remove any DNA loosely bound to the filter, each filter was put in 1 ml water, heated for 1 min in a boiling bath, cooled in ice, and the water was removed by aspiration. PolyA<sup>+</sup> RNA (50–100 μg) in 300 μl hybridization solution (65% deionized formamide, 0.4 M NaCl, 0.2% SDS, 30 mM PIPES, pH 6.5, 50 μg yeast tRNA) was preheated for 10 min at 70°C, and the filters carrying

DNA were hybridized at 50°C for 2 h. Then, the filters were washed (stirred for 30 sec) 10 times in 25 ml each TES/0.5% SDS, prewarmed at 65°C (TES = 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 2 times with 25 ml each TES prewarmed at 65°C. Up to 20 filters were washed together. To recover the mRNAs to be translated, individual filters were added to 0.3 ml water plus 2 µl 10 mg/ml yeast tRNA, boiled for 1 min, and quickly frozen in a dry ice-ethanol bath. After thawing at room temperature and removing the filters, the mRNAs were extracted with phenol-chloroform and precipitated by the addition of 30 µl 2 M KOH and 750 µl ethanol. The selected polyA<sup>+</sup> mRNA or total polyA<sup>+</sup> mRNA from liver or hepatocellular carcinoma was translated by a wheat germ extract (Promega). The <sup>35</sup>S-labeled translation products were analyzed on a 15% polyacrylamide-SDS gel (25) or by two-dimensional gel electrophoresis (26).

### DNA sequencing

pBluescript phagemid containing an apoA-I cDNA insert was excised *in vivo* from the λZAPII vector, according to the Stratagene instruction manual. Single or double stranded DNA template was made from pUC9 and pBluescript vectors containing cDNA inserts. Nucleotide sequence analysis was performed using overlapping templates by the dideoxynucleotide chain-termination method and by primer extension with T7 DNA polymerase (Sequenase 2.0, United States Biochemical Corp.).

## RESULTS

### Characterization of THC2c and THC5c mRNAs

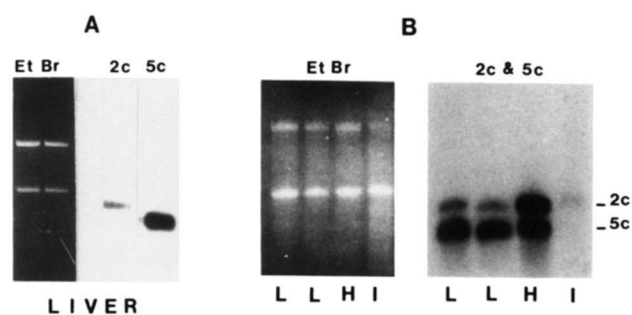
A cDNA library to polyA<sup>+</sup> mRNAs isolated from a single trout hepatocellular carcinoma was constructed in the plasmid pUC9. To isolate sequences that were present at a higher level in hepatocellular carcinoma than in normal trout liver, the cDNA library was screened by differential colony hybridization with <sup>32</sup>P-labeled cDNA of hepatocellular carcinoma or liver (21). The clones that gave a markedly stronger signal with cDNA of hepatocellular carcinoma than with cDNA of liver were submitted to a second differential hybridization, under the same conditions.

After the second screening, 10 clones out of a total of 500 transformants were selected as being overexpressed in hepatocellular carcinoma. One colony, pTHC5c, which gave a similar signal with hepatocellular carcinoma and liver cDNA probes, was isolated as a control. Here, we describe the analysis of eight of the hepatocellular carcinoma overexpressor clones: pTHC5a, pTHC2b, pTHC3b, pTHC4b, pTHC5b,

pTHC7b, pTHC2c, and pTHC3c. When used as probes for hybridization to Northern blots carrying RNA isolated from liver, these eight clones detected a 1.2-kilobase mRNA (Fig. 1A). Further, THC2c cDNA hybridized to the other clones (data not shown), demonstrating that the eight clones had homologous DNA sequences. As pTHC2c was primarily used as a probe in subsequent analysis, we called the hybridizing mRNA THC2c. The control cDNA clone, pTHC5c, hybridized to a 0.9-kilobase mRNA (Fig. 1A).

Since the cDNA clones, pTHC2c and pTHC5c, detected transcripts of different sizes, Northern blot analyses were performed with both labeled cDNA probes (Fig. 1B). The specific activities of pTHC2c and pTHC5c were similar. Thus, the relative signals of THC2c and THC5c mRNAs reflected the relative steady state levels of these two mRNA species. The level of THC5c mRNA in livers was greater than the level of THC2c mRNA. In contrast, the relative levels of THC2c and THC5c mRNA were similar in hepatocellular carcinoma RNA. For the hepatocellular carcinoma RNA sample shown in Fig. 1B, the steady state level of THC2c mRNA was 5.8-fold greater than the level of THC2c mRNA of liver. The relative levels of THC2c and THC5c mRNAs were determined in a total of seven individual tumors. Levels of THC2c mRNA in tumors ranged from 1.5- to 5.8-fold (1.5, 1.5, 2.8, 3.0, 4.0, 4.3, and 5.8) higher than in normal livers. Thus, the Northern blot analysis of RNA isolated from tumors and livers agreed with the results of the differential colony hybridization, that is, levels of THC2c mRNA were higher in the tumors.

The abundance of THC2c and THC5c mRNA in other trout tissues was studied. These mRNA species were not detectable in the RNA prepared from heart, testis, gill, and kidney (not shown). A low level of THC2c, but not THC5c, mRNA was detected in intestine RNA (Fig. 1B).



**Fig. 1.** THC2c and THC5c mRNA levels in liver, hepatocellular carcinoma and intestine. Total RNA from liver (L), hepatocellular carcinoma (H) or intestine (I) was electrophoresed through agarose gels. The gels were stained with ethidium bromide. The RNAs were transferred to nitrocellulose and hybridized to radiolabeled pTHC2c (2c) and/or pTHC5c (5c). The ethidium bromide staining (EtBr) and autoradiograms are shown (2c, 5c, 2c & 5c).

Fig. 2A shows that the liver mRNA selected by pTHC2c codes for a 29-kilodalton protein, while the liver mRNA selected by pTHC5c codes for a 14-kilodalton protein. The same results were obtained when polyA<sup>+</sup> mRNA isolated from hepatocellular carcinoma was used. However, further analysis of these liver and hepatocellular carcinoma THC2c-directed translation products on two-dimensional gels revealed that three proteins were synthesized. The translation products had the same molecular weight of 29,000 but they had different isoelectric points, varying around 5.0 (Fig. 2B). It is important to note that these hybrid-selected transcripts were isolated from the polyA<sup>+</sup> mRNA of an individual liver or tumor. The hybrid select translation results presented in Fig. 2B were identical whether the probe used was pTHC2c, pTHC3c, pTHC5b or pTHC7b. To compare the relative levels of the THC2c-encoded proteins, polyA<sup>+</sup> mRNAs isolated from the liver and hepatocellular carcinoma of individual fish were translated, and the translation products were electrophoretically resolved on two-dimensional gels. The levels of the THC2c-encoded translation products were markedly different for liver and hepatocellular carcinoma. Fig. 3 shows that the most basic of the three THC2c-encoded proteins was the main form in liver, with the central and the most acidic forms being present in very low amounts. Except for two samples out of nine, the central spot was slightly more intense than the most acidic one. The boxes outlined with dashed lines in Fig. 3A and E contained five major translation products that were of similar intensity in the two-dimensional patterns of liver and hepatocel-

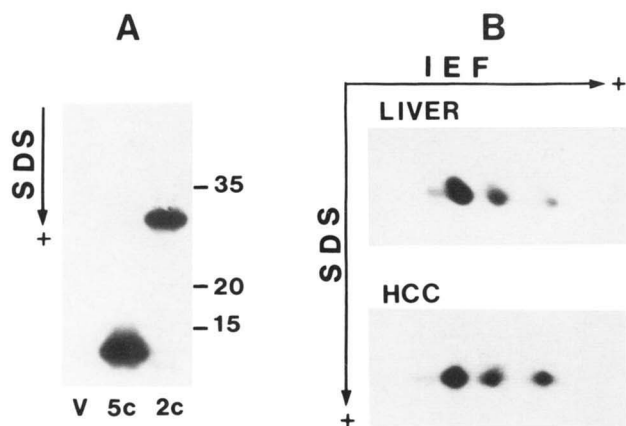


Fig. 2. Identification of proteins encoded by THC2c and THC5c mRNAs. A: The <sup>35</sup>S-labeled translation products of the polyA<sup>+</sup> mRNA hybridizing to pUC9(V), pTHC2c (2c), and pTHC5c (5c) were analyzed on a 15% polyacrylamide-SDS gel. The fluorogram is shown. B: The <sup>35</sup>S-labeled translation products of the polyA<sup>+</sup> mRNA hybridizing to pTHC7b were separated by electrofocusing (IEF) (pH 4.5–7.0) followed by electrophoresis through a 10% polyacrylamide-SDS slab gel. Only the region of the fluorogram with labeled proteins is displayed in the figure. HCC is hepatocellular carcinoma. Markers (in kDa) are the proteins translated with Brome Mosaic Virus as template.

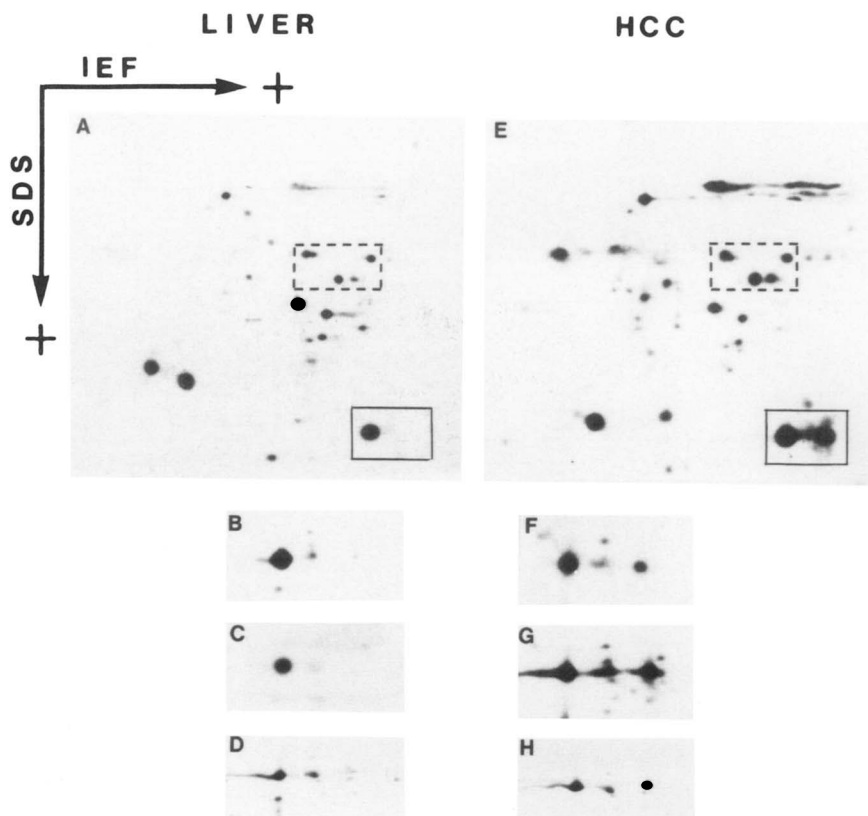
lular carcinoma translation products. Using these translation products as internal standards, we observed that the levels of the basic form of the 29-kDa protein were similar for liver and hepatocellular carcinoma, but clearly the level of the acidic form of the 29-kDa protein was considerably higher in tumor than in liver. In some tumors the acidic form was as abundant as the basic form (Fig. 3). Hepatocellular carcinomas had similar or slightly elevated levels of the central form of the 29-kDa protein than livers.

#### Identification of hepatocellular carcinoma and liver THC2c transcripts as apolipoprotein A-I<sub>s</sub>

DNA sequencing of the eight hepatocellular carcinoma cDNA clones identified two groups of sequences, presenting about 90% homology between each other (Fig. 4 and Fig. 5). The first group contained five clones and the second group had three clones, suggesting that each transcript was about equally represented in the polyA<sup>+</sup> mRNA population of the hepatocellular carcinoma from which the cDNA library was produced. This correlated with the relative abundance of the basic and acidic THC2c-encoded translation products generated from the polyA<sup>+</sup> mRNA of this tumor (Fig. 3E). These observations indicated that the two classes of clones corresponded to the transcripts coding for the most basic and the most acidic 29-kDa proteins.

For the first group of hepatocellular carcinoma cDNA clones, sequence information was obtained from nt – 35 to nt + 1141, showing an open reading frame of 786 nt coding for 262 amino acids (from nt + 1 to nt + 786). For the second group of clones, sequence information encompassed nt – 35 to nt + 775, with an open reading frame starting at + 1. None of the three hepatocellular carcinoma cDNA clones in the second group contained the 3' cDNA sequences required to complete the coding region (Fig. 4). When the amino acid sequences derived from these sequences were searched against an amino acid sequence data base, the greatest homology was found for chicken apolipoprotein A-I in both cases, followed by human, rabbit, and rat apolipoprotein A-I<sub>s</sub>. Thus, we designated these cDNA clones as apolipoprotein A-I<sub>s</sub>.

To compare apoA-I transcripts from liver and hepatocellular carcinoma, a cDNA library from the polyA<sup>+</sup> mRNA of an individual liver was constructed and screened with the 257-bp PvuII-AvaI restriction DNA fragment isolated from clone THC2c (apoA-I-2, see Fig. 4). Fourteen clones were sequenced. Eleven of the clones had sequences homologous to the clones of the first group (apoA-I-1), while the remaining three clones had sequences corresponding to the second group (apoA-I-2). DNA sequence information was obtained from nt – 15 to nt + 1139 for apoA-I-1 and from



**Fig. 3.** Levels of THC2c-encoded proteins in liver and hepatocellular carcinoma. The  $^{35}\text{S}$ -labeled products of *in vitro* translation of four individual livers (A, B, C, and D) and four individual hepatocellular carcinomas (E, F, G, and H) poly(A)<sup>+</sup> mRNAs were separated by electrofocusing (IEF) (pH 3.5–10.0 for A and E; pH 4.5–7.0 for B, C, D, F, G, and H) followed by electrophoresis through a 10% polyacrylamide-SDS slab gel. The fluorograms are shown. Only the region containing the THC2c-encoded proteins is displayed in B, C, D, F, G, and H.

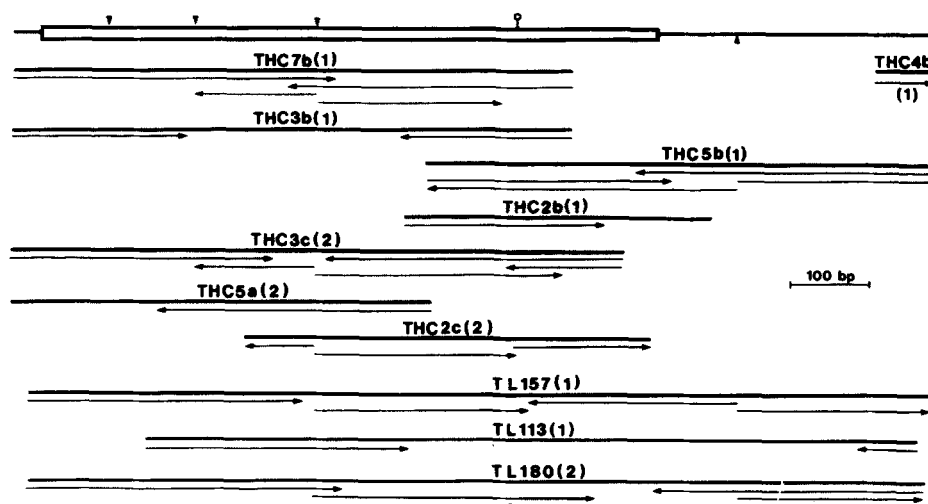
nt -9 to nt +1120 for apoA-I-2. As for apoA-I-1, an open reading frame of 786 nucleotides coding for 262 amino acids was found for apoA-I-2. Only two differences at the nucleotide level were found between liver and hepatocellular carcinoma apoA-I-1 sequences: at positions +366 and +525, Ts in liver were replaced by Cs in tumor. The DNA sequence of apoA-I-1 was found to be heterologous in liver at two other positions: nt +137 (C/T) and nt +973 (A/T). For apoA-I-2 sequences, only one difference was found between liver and hepatocellular carcinoma, where C in liver was replaced by T in tumor (nt +399).

None of the nucleotide differences between liver and hepatocellular carcinoma apoA-I DNA sequences caused a change in amino acid. Thus, the proteins coded for by apoA-I-1 and -2 were identical in liver and tumor. The molecular weights of the proteins apoA-I-1 and -2 were 29,701 and 29,677, respectively. These were compatible with the results of the hybrid-select translation experiments. The isoelectric points of the proteins apoA-I-1 and -2 were, as determined from the amino acid sequences, 5.1 and 4.9, respectively. Thus, the apoA-I-1 protein form was more basic than the

apoA-I-2 and corresponded to the main liver species. This result was consistent with the majority of the liver apoA-I cDNA clones showing the apoA-I-1 DNA sequence, indicating that in liver apoA-I-1 mRNA was more abundant than apoA-I-2 mRNA.

#### Analysis of genomic DNAs

A comparison of apoA-I-1 and -2 cDNA sequences showed that about 10% of the nucleotides were different. These nucleotide differences were scattered throughout the sequences, suggesting that apoA-I-1 and -2 were coded for by different genes. This was confirmed by Southern blot analysis of genomic DNA isolated from an individual liver. The restriction fragments recognized by the clones apoA-I-1 and -2 were different (**Fig. 6**): 5.6 kb and 2.1 kb PstI fragments, 2.9 kb and 4.1 kb EcoRI fragments, 8.6 kb and 9.6 kb HindIII fragments, and 2.9 kb and 3.8 kb BamHI-EcoRI were hybridized by the clones apoA-I-1 and -2, respectively. A genetic variation was observed with the BamHI apoA-I-1 DNA fragment. The apoA-I-1 clone detected an 11 kb BamHI fragment in genomic DNA of a single liver (**Fig. 6**, panel apoA-I-1,



**Fig. 4.** Diagrams representing the sequencing strategies used for each hepatocellular carcinoma or liver cDNA clone. The open box represents the apoA-I translated region from nt + 1 to nt + 786. The restriction sites used for subcloning are (▼), PvuII; (○), AvaI and (▲), PstI. The direction and extent of DNA sequencing are indicated by arrows. THC clones are hepatocellular carcinoma cDNA clones, while TL clones are liver cDNA clones. The classification of the clones in the first (apoA-I-1) or second (apoA-I-2) group is indicated by (1) or (2), respectively.

fourth lane) and 8.6 kb and 11 kb BamHI fragments in genomic DNA isolated from a mixture of several livers (Fig. 6, panel apoA-I-1, second lane). The apoA-I-2 clone detected an 8.6 kb BamHI fragment in the genomic DNA of a single liver (Fig. 6), as well as in the DNA of mixed livers (not shown). The faint bands visualized by the clone apoA-I-2 as a result of cross-hybridization with apoA-I-1 are indicated by arrows. Conversely, cross-hybridization of the clone apoA-I-1 with apoA-I-2 was also faintly visible on the original autoradiogram. Southern blot analysis of genomic DNAs isolated from two individual tumors showed the same restriction patterns as the ones of liver genomic DNA shown in Fig. 6.

#### Analysis of trout apoA-I-1 and apoA-I-2 amino acid sequences

An alignment of the amino acid sequences of trout preproapolipoprotein A-I-s with the chicken and human sequences is shown in Fig. 7. Gaps were introduced for maximum alignment of the four sequences. The two trout apolipoprotein A-I-s presented 84% of homology between each other. Both trout apoA-I-1 and apoA-I-2 had 28% homology with chicken and 24% homology with human apoA-I.

Mammalian and chicken apoA-I-s contain an 18 amino acid long signal peptide and a 6 amino acid long prosegment (Fig. 7). By analogy, a putative 18 amino acid long prepeptide could be identified in both trout apoA-I-s. These putative prepeptides had properties similar to those of signal peptides: they contained a central hydrophobic region flanked by more polar N- and C-terminal regions. The prosegment in

trout apoA-I-s appeared to be only 5 amino acids long as opposed to 6 amino acids for other apoA-I-s. It should be noted that human and rat apoA-II have been reported to contain a 5 amino acid prosegment (5). Fig. 7 shows that both trout apoA-I sequences satisfy the division into different regions as described by Luo, Li, and Chan (29). First, there was a block of 33 residues, which consisted of three 11 amino acid repeats, common to all apolipoproteins. Second, there were repeats of 11 or 22 residues, labeled A-I-4, A-I-5, etc. In the mature apoA-I, 40 residues (about 16.5%) were conserved between human, chicken, and both trout apoA-I proteins, compared to 129 residues (about 53%) conserved among four mammals (human, dog, rat, rabbit) and 80 residues (about 33%) conserved among those four mammals and bird (chicken). Thirty residues (12%) were conserved among the six species mentioned above. When the conservative amino acid substitutions were considered, the extent of homology between trout and human apoA-I-s was 54%. About 70% of the primary sequence of trout apoA-I was predicted by the Chou-Fasman method to have an  $\alpha$ -helical structure.

Fig. 8 illustrates the repetitive nature of the trout apoA-I-1, the main liver form. The repeats have been aligned according to Li et al. (5). The homology between the repeats became striking when the similarities of the biochemical characteristics of the amino acids were considered. For example, columns 2, 6, 10, 14, 17, and 21 contained mainly hydrophobic residues, while columns 4, 5, 15, and 16 contained mainly acidic amino acids and columns 7 and 18 contained mainly basic amino acids. The consensus for

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-35              +1              +20              +40              +60
1 tctcataacgaccaatcaccacccagacctcattc ATG AAA TTC CTG GCT CTC GCA CTA ACC ATC CTG CTG GCC GCA GGT ACC CAG GCT TTT CCT ATG CAG GCT
2 -----g-----C-----T-----C-----C-----G-A-----C-----

              1              10              20
1          met lys phe leu ala leu ala leu thr lle leu leu ala ala gly thr gln ala phe pro met gln ala
2          -  gln  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+80              +100              +120              +140              +160
1 GAT GCT CCC TCT CAG CTG GAG CAT GTG AAG GCA GCC TTG AGC ATG TAC ATA GCT CAG GTG AAG TTG ACC GCA CAG AGG TCC ATT GAC CTT CTG GAT
2 -----T-----A-----TG GA-----G-----GA-----T-----C-----A-----

              30              40              50
1 asp ala pro ser gln leu glu his val lys ala ala leu ser met tyr lle ala gln val lys leu thr ala gln arg ser lle asp leu leu asp
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+180              +200              +220              +240              +260
1 GAC ACA GAG TAC AAA GAG TAC AAG ATG CAG CTG ACC CAG AGC CTT GAC AAC CTC CAG CAG TAT GCT GAT GCC ACC TCC CAG TCC CTG GCC CCC TAC
2 -----G-----T-----A-----C-----C-----G-----C-----G-----A-----T-----G-----

              60              70              80
1 asp thr glu tyr lys glu tyr lys met gln leu thr gln ser leu asp asa leu gln gln tyr ala asp ala thr ser gln ser leu ala pro tyr
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+280              +300              +320              +340
1 AGC GAA GCC TTC GGC ACC CAG TTG ACT GAC GCC ACC GCC GCT GTG CGC GCT GAG GTC ATG AAG GAC GTG GAG GAG CTG CGC TCC CAG CTG GAG CCC
2 ---G---A---GTT-----A-----C-----

              90              100              110
1 ser glu ala phe gly thr gln leu thr asp ala thr ala ala val arg ala glu val met lys asp val glu glu leu arg ser gln leu glu pro
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+360              +380              +400              +420              +440
1 AAG CGC GCT GAA CTC AAG GAA GTC CTG GAC AAG CAC ATA GAT GAG TAC CGC AAG AAG CTG GAG CCC CTG ATC AAG GAG CAC ATC GAA CTG CGC CGC
2 ---A---C---G-----T-----A-----C-----G-----A-----T-----C-----A-----T-----G-----A-----

              120              130              140              150
1 lys arg ala glu leu lys glu val leu asp lys his lle asp glu tyr arg lys lys leu glu pro leu lle lys glu his lle glu leu arg arg
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+460              +480              +500              +520              +540
1 ACC GAG ATG GAG GCC TTC AGG GCT AAG ATG GAG CCC ATT GTG GAG GAG TTG CGC GCC AAG GTG GCC ATC AAT GTG GAG GAG ACC AAG ACC AAG CTC
2 ---C---G-----T-----A-----G-----T-----A-----T-----G-----C-----

              160              170              180
1 thr glu met glu ala phe arg ala lys met glu pro lle val glu glu leu arg ala lys val ala lle asn val glu glu thr lys thr lys leu
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+560              +580              +600              +620              +640
1 ATG CCC ATT GTG GAG ATT GTC CGT GCC AAG CTG ACC GAG CGT CTG GAG GAG CTG AAG ACC CTG GCC GCC CCC TAC GCT GAG GAG TAC AAG GAG CAG
2 ---C-----CC-----T-----

              190              200              210
1 met pro lle val glu lle val arg ala lys leu thr glu arg leu glu glu leu arg thr leu ala ala pro tyr ala glu glu tyr lys glu gln
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+660              +680              +700              +720              +740
1 ATG ATC AAG GCT GTT GGA GAG GTG CGT GAG AAG GTG TCT CCC CTG TCT GAG GAC TTC AAG GGC CAG GTG GGC CCC GCA GCC GAA CAG GCC AAG CAG
2 ---G-----C-----G-----A-----C-----T-----T-----A-----C-----

              220              230              240
1 met lle lys ala val gly glu val arg glu lys val ser pro leu ser glu asp phe lys gly gln val gly pro ala ala glu gln ala lys gln
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+760              +780              +800              +820              +840
1 AAG CTC CTG GCT TTC TAC GAG ACC ATC AGC CAG GCC ATG AAG GCA TAA acacgtctcagcgggccc tcctt cccctcccgctctcactcacactgactcacacac
2 ---A---G-----G-----T-----C-----C-----C-----C-----T-----C-----T-----C-----

              250              260
1 lys leu leu ala phe tyr glu thr lle ser gln ala met lys ala
2 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

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+860              +880              +900              +920              +940              +960
1 catacgtaccacgccaatgccaactgatgcactt ctgcagcggcatggcaggactctgtctctcttaacgaccaccatattgctcaagcagcagcagcagcactaacgcactattg
2 -----a-tg-----cct-----g-----C-----C-----C-----

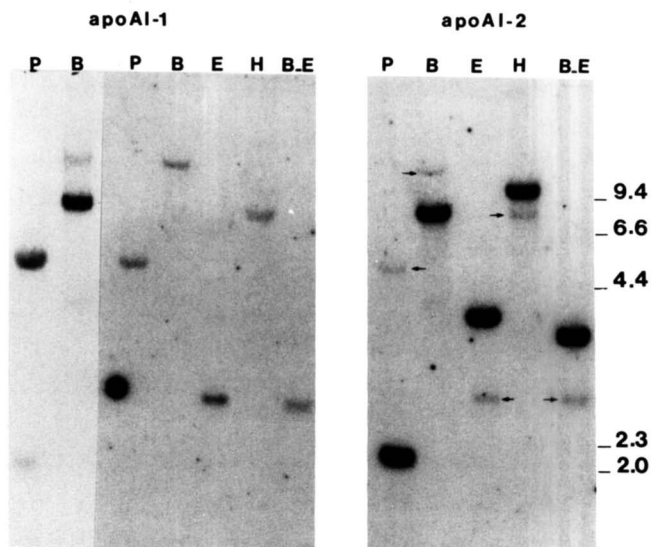
+980              +1000              +1020              +1040              +1060              +1080
1 cttacattcagttttgagttgtgttcaggcctgtgtgcacaactcttgggcctgcataatggttactgtctactatgaacatcaagtgtgstatattctagtgttgccttgttcaagatagctgtgtg
2 -a-----C-----a-----t-----C-----C-----C-----t-----

+1100              +1120              +1140
1 cttcaaacagctcaataacacatcactgctttcatactaaa
2 -g-----

```

Fig. 5. Trout apoA-I-1 and -2 nucleotide sequences and derived amino acid sequences. Each trout apoA-I-2 (2) nucleotide or amino acid identical to the corresponding trout apoA-I-1 (1) nucleotide or amino acid is indicated by (-).

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**Fig. 6.** Southern blot analysis of trout genomic DNA. Trout liver genomic DNA digested with PstI (P), BamHI (B), EcoRI (E), HindIII (H), or BamHI-EcoRI (B-E) was hybridized with TL157 (apoA-I-1) or TL180 (apoA-I-2) cDNA clones. The two first lanes in the apoA-I-1 panel show the hybridization to genomic DNA isolated from a mixture of several livers, while all the other lanes show hybridization to genomic DNA isolated from a single liver. HindIII fragments of phage  $\lambda$  were used as markers and their sizes are indicated in kilobase.

apoA-I-1 and -2 repeats was identical, and it was very similar to the consensus for human apoA-I repeats. Most of these repeats started with the amino acid proline, an  $\alpha$ -helix breaker. When trout apoA-I-1 consensus sequence of the 22 amino acid repeats shown in Fig. 8 was placed in an Edmundsen wheel diagram, it specified an  $\alpha$ -helix with a nonpolar face containing mostly hydrophobic amino acids and a polar face displaying mostly acidic amino acids (data not shown). This is the characteristic feature of an amphipathic  $\alpha$ -helix structure that is common to all apolipoproteins.

#### Comparison of trout apoA-I-s and apolipoproteins of other fish

The amino acid sequences of rainbow trout apoA-I-s were compared with the amino acid sequences of the apolipoproteins LAL1 and LAL2 of the more primitive fish lamprey. LAL1 and LAL2 cDNAs have coding regions of 105 and 191 amino acids, respectively. Fig. 7 shows the alignment of the lamprey LAL1 and LAL2 apolipoproteins with trout apoA-I-s. About 85% of the LAL1 protein, from amino acid 10 to amino acid 99, could be aligned with the amino-terminal region of trout apoA-I-s, from amino acid 5 to amino acid 91, while about 65% of the LAL2 protein, from amino acid 55 to amino acid 178, could be aligned with the carboxy-terminal region of trout apoA-I-s, from amino acid 138 to amino acid 261. The extent of homology

between the aligned portions of trout apoA-I-s and lamprey LAL proteins was about 20%. When the conservative amino acid substitutions were considered, the extent of homology between the aligned portions of the proteins was about 45%. The amino acid sequence of the putative amino-terminus of mature trout apoA-I-1 was compared with the amino acid sequence of carp mature apoA-I which was isolated from plasma (12). Fig. 9 shows there is a high degree of similarity between the amino-terminal regions of trout and carp apoA-I-s. This observation supports our assignment of the first amino acid of the mature trout apoA-I-1.

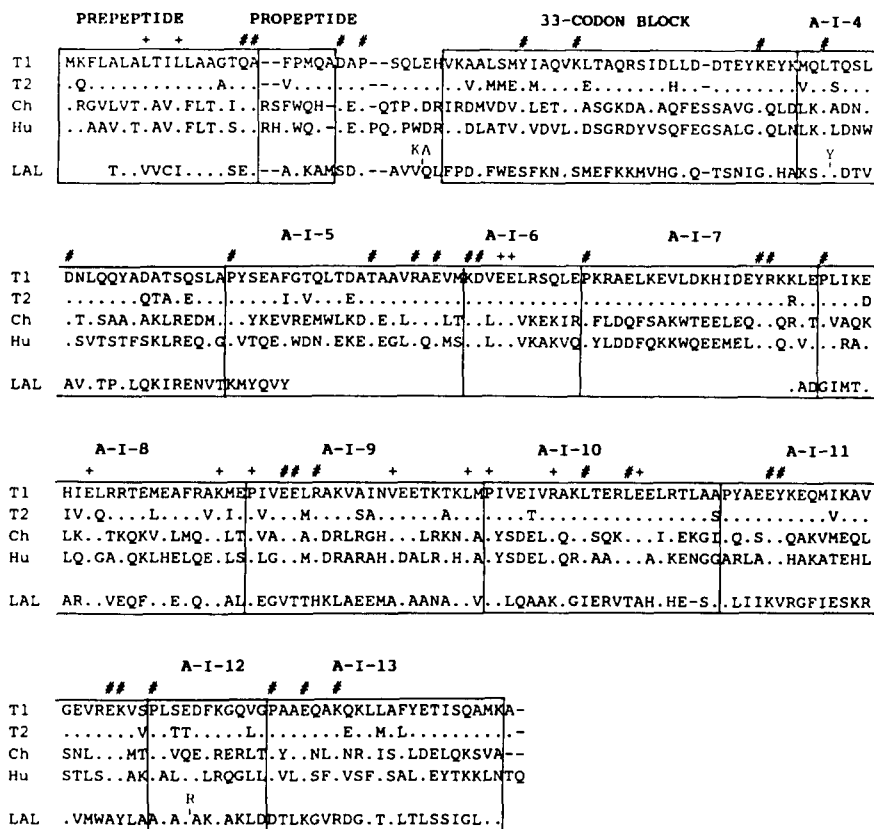
#### DISCUSSION

The principal findings of this study are the following. First, rainbow trout has two apolipoprotein A-I genes, apoA-I-1 and -2. Second, the two apoA-I genes are differentially expressed, with apoA-I-1 mRNA being the major transcript of normal liver. Third, the relative expression of these two transcripts is altered in aflatoxin B<sub>1</sub>-induced hepatocellular carcinomas with the level of apoA-I-2 transcripts being selectively increased. These observations demonstrate that the expression of the two apoA-I transcripts is regulated by different mechanisms that are altered in neoplasia.

We have isolated two trout apolipoprotein A-I cDNA clones whose nucleotide sequences presented about 90% homology with each other. The differences in nucleotide sequence were scattered throughout the sequence. Both of the trout apoA-I cDNA clones had an open reading frame of 786 nucleotides, coding for a 262 amino acid protein. Trout preproapolipoprotein A-I-1 and -2 had predicted molecular weights of 29,702 and 29,678, and predicted isoelectric points of 5.1 and 4.9, respectively. These values are consistent with the results of earlier studies showing that an apoA-I-like protein of trout plasma had a molecular weight of approximately 25,000 (6, 8, 9) and migrated in the pH 5 to 5.8 region of an electrofocusing gel (10). Moreover, the amino acid compositions compiled from the cDNA-derived amino acid sequences of the putative mature apoA-I-1 and -2 were very similar to that of the apoA-I like protein isolated from trout HDL particles (7). The degree of similarity was higher for apoA-I-1 than for apoA-I-2. This is consistent with our observations that suggest that the major synthesized form of apoA-I in liver is apoA-I-1.

Each of the two apoA-I cDNA clones hybridized to a mixed population of mRNAs that directed the in vitro translation of three 29-kDa proteins of different isoelectric points. This suggests that rainbow trout has three apoA-I genes. However, only two apoA-I cDNA clones were isolated, and Southern blot analysis of





**Fig. 7.** Alignment of trout (T1, T2), chicken (Ch), human (Hu) apoA-I protein, and lamprey (LAL) apolipoprotein sequences. The amino acids are represented by the single letter code, and each amino acid identical to the corresponding amino acid of trout apoA-I is indicated by a dot. Chicken and human apoA-I amino acid sequences were obtained from Rajavashisth et al. (27) and Sharpe et al. (28), respectively. Lamprey LAL amino acid sequences were obtained from Pontes et al. (13): LAL1 is the LAL sequence aligned with the amino-terminus of the apoA-I, while LAL2 is the LAL sequence aligned with the carboxy-terminus of the apoA-I. The sequences are divided into regions corresponding to the signal peptide, the propeptide, and the mature protein of human apoA-I. The sequences of the mature protein are sectioned into boxed regions representing the 33-codon block and the 11 or 22 residue repeats which are labeled A-I-4, A-I-5, etc. The apoA-I amino acids that are conserved among the six vertebrate species (human, dog, rabbit, rat, chicken, and trout) are indicated by (#) and those that are conserved among the first four sequences shown in the figure are indicated by (+).

genomic DNA revealed the presence of two different apoA-I genes. We did not detect additional DNA fragments that would indicate the existence of a third gene. Thus, it is possible that the middle 29-kDa spot observed on the two-dimensional gels is not an apoA-I protein. To our knowledge, trout is the first species found to have more than one apoA-I gene. In human (30), rat (31), mouse (32), rabbit (33), and chicken (27), apoA-I is a single copy gene. The duplication of the apoA-I gene in trout might be a result of the possible tetraploid ancestry and ongoing diploidization of salmonid fish. However, a tetraploid origin of rainbow trout has been contested (34).

Trout apoA-I clones were originally isolated as transcripts that were overexpressed in hepatocellular carcinoma. Our results provide evidence that the higher steady state levels of apoA-I transcripts in tumors were due to a selective increase in the level of

the apoA-I-2 transcript. ApoA-I-1 mRNAs appeared to be present at similar levels in tumor and liver. Southern blot analysis of liver and hepatocellular carcinoma genomic DNAs showed that the overexpression of apoA-I-2 was not due to gene amplification or gross gene rearrangement. Furthermore, the elevated expression of apoA-I-2 in hepatocellular carcinoma was not related to increased cell proliferation. An analysis of several different tumor and surrounding liver tissue sections demonstrated that mitotic figures were rare and scattered throughout the tissue sections. Clusters of cells with mitotic activity were not observed (Delcuve, G. P., Hendricks, J. D., Bailey, G., and Davie, J.R., unpublished data). This suggests that the levels of cycling cells were not substantially higher in tumor than in liver. The increased level of apoA-I-2 mRNA was observed in seven different tumors, suggesting that overexpression of apoA-I-2 transcripts is a common

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
64	H	Q	L	T	Q	S	L	D	N	L	Q	Q	Y	A	Δ	A	T	S	Q	S	L	A	A-I-4	
86	P	Y	S	Δ	A	F	G	T	Q	L	T	D	A	T	A	A	V	R	A	E	V	M	A-I-5	
108													K	D	V	Δ	Δ	L	R	S	Q	L	E	A-I-6
119	P	K	R	A	Δ	L	R	E	V	L	D	K	H	I	Δ	Δ	Y	R	K	K	L	E	A-I-7	
141	P	L	I	K	Δ	H	I	E	L	R	R	T	E	M	Δ	A	F	R	A	K	M	E	A-I-8	
163	P	I	V	Δ	Δ	L	R	A	K	V	A	I	N	V	Δ	Δ	T	K	T	K	L	M	A-I-9	
185	P	I	V	Δ	I	V	R	A	K	L	T	E	R	L	Δ	Δ	L	R	T	L	A	A	A-I-10	
207	P	Y	A	Δ	Δ	Y	K	E	Q	M	I	K	A	V	G	Δ	V	R	E	K	V	S	A-I-11	
229	P	L	S	Δ	Δ	F	K	G	Q	V	G												A-I-12	
240	P	A	A	Δ	Q	A	K	Q	K	L	L	A	F	Y	Δ	T	I	S	Q	A	M	K	A-I-13	

## Trout apoAI-1 consensus sequence

P○ x ΔΔΔ○□ x x ○ x x x ○ΔΔ○□ x x ○ x

## Human apoAI consensus sequence

P○○ΔΔ○ x x x ○ x x x ○Δ x ○□ x □○ x

**Fig. 8.** Internal repeats in trout apoA-I-1. Most of the repeats are 22-mers, each of which is made up of two 11-mers, and the other repeats are 11-mers. The numbers on the left of the sequence are the positions in the preproapolipoprotein A-I of the first amino acid in each row. On the right side of the sequences, the repeats are numbered as in Fig. 7. The symbols indicate proline (○; P); aspartic acid or glutamic acid (Δ, D or E); arginine or lysine (□, R or K); and methionine, valine, leucine, isoleucine, phenylalanine, tyrosine, or tryptophan (○, M, V, L, I, F, Y, or W). The remaining amino acids, glycine, alanine, serine, threonine, asparagine, glutamine, histidine, and cysteine (G, A, S, T, N, Q, H, and C) are called indifferent. Any column containing a majority (five or more) of an amino acid of a given class is marked by the corresponding symbol, and that symbol is used for the definition of the consensus sequence of the repeats of both trout apoA-I and human apoA-I.

feature of aflatoxin B<sub>1</sub>-induced hepatocellular carcinomas. It is conceivable that neoplastic transformation of hepatocytes, which results in changes in hepatocellular chromatin structure and composition (18), leads to an increase in the transcriptional activity of the hepatocellular carcinoma apoA-I-2 gene. Thus, it will be of interest to study the mechanisms that govern the levels of the apoA-I-1 and -2 transcripts.

The level of expression of the apoA-I gene was rather high in liver, and when total polyA<sup>+</sup> mRNA isolated from liver was translated in vitro, apoA-I-1 was one of the most abundant proteins synthesized. This is consistent with the high level of HDL in trout plasma (11). As to the overexpression of the apoA-I-2 gene in tumors, the mass of the tumor was small relative to the mass of the liver in 1-year-old fish. Thus, the increased tumor expression of apoA-I-2 would make a minor contribution to the apoA-I plasma levels. However, in older aflatoxin B<sub>1</sub>-treated fish, it is not uncommon to find large tumors that have taken over the whole liver. In these fish, we suspect that the level of apoA-I-2 and HDL containing apoA-I-2 in the trout plasma will be elevated. It should be noted that transgenic mice expressing high amounts of human apoA-I have high plasma apoA-I and HDL levels (35, 36).

Only a few nucleotide differences were found between apoA-I-1 and -2 mRNAs of liver and hepatocellular carcinoma. It is very probable that these nucleotide differences between liver and hepatocellular carcinoma apoA-I-1 or -2 transcripts were due to "usual" genetic variations found in any population. Indeed four of the five differences were CG to TG. It is known that 5-methylcytosine tends to deaminate to thymine, and it is found almost exclusively in the dinucleotide sequence CpG (37). Thus, it is quite possible that four of the five nucleotide differences were due to common deamination events of methylcytosine to thymine. None of the nucleotide differences could be due to aflatoxin B<sub>1</sub> adduction which adducts guanine and induces G to T transversions almost exclusively (38).

Trout apoAI-1	DAP-SQLEHVK-AAL
Carp apoAI	·Q·P·...·L·S··Q

**Fig. 9.** Alignment of trout apoA-I-1 and carp apoA-I amino-terminal amino acid sequences. The amino acids are represented by the single letter code, and each amino acid of carp apoA-I that is identical to the corresponding amino acid of trout apoA-I-1 is indicated by a dot. Gaps (-) have been introduced in the trout amino acid sequence to allow for the maximal alignment of the two sequences.

In mammals and birds, apoA-I is expressed primarily in liver and intestine (27, 29, 31–33, 39–44). One exception is chicken where breast muscle accounts for a major portion of apoA-I mRNA around the time of hatching (27). In trout, liver was found to be the major site of ApoA-I synthesis, while intestine constituted a minor site of synthesis.

It has been proposed that the lamprey LAL1 protein could be the counterpart of mammalian apoA-II and LAL1 was found to be equally similar to apoA-I, A-IV, E, A-II, and C-III (5). Thus the alignment of LAL1 with the amino-terminal portion of trout apoA-Is is not surprising. Although lamprey LAL2 is very different from all known mammalian apolipoproteins and has an uncertain origin, it could be aligned with the carboxyl-terminal portion of trout apoA-Is.

Comparison of trout and human apoA-I amino acid sequences revealed a high degree of analogy, despite the large evolutionary distance between the two species. Trout apoA-Is, like human apoA-I, consist largely of 22-residue repeats that are believed to form an amphipathic  $\alpha$ -helical structure. The similarity of trout and human apoA-I structures is confirmed by the existence of common antigenic sites (10). It has been suggested that the different apolipoprotein genes arose from a common ancestral gene by gene duplications and duplications of 22 or 11 codons (5). The similarity of the repeat pattern in trout and human apoA-Is suggests that all the internal repeats in these sequences arose before the teleost fish-mammal split, some 400 million years ago.

In summary, we demonstrate that rainbow trout has two apoA-I genes, apoA-I-1 and apoA-I-2. ApoA-I-1 was the major transcript of liver, while in aflatoxin B<sub>1</sub>-induced liver tumors the levels of the apoA-I-1 and -2 transcripts were similar. An elevation in expression of apoA-I-2 transcripts was observed in all the tumors analyzed, suggesting that the abnormal expression of the apoA-I-2 gene is a common feature of aflatoxin B<sub>1</sub>-induced hepatocellular carcinoma. Conceivably, fish that bear large liver tumors would have elevated levels of HDL containing apoA-I-2. The ramifications, if any, of such changes in HDL composition will be of interest.

We wish to thank Dr. Leigh Murphy and Helmut Dotzlaw for their help with the DNA sequence identification and Darcy Salo for technical assistance. This project was supported by a grant from the National Cancer Institute of Canada. J. R. Davie is a Scientist of the Medical Research Council of Canada.

Manuscript received 27 August 1991 and in revised form 21 November 1991.

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