A Liver-specific Factor and Nuclear Factor I Bind to the Rat \( \alpha \)-Fetoprotein Promoter*

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Proteins putatively involved in the transcriptional control of rat \( \alpha \)-fetoprotein (AFP) gene expression in the liver were identified by analyzing the in vitro binding of proteins from nuclear extracts of fetal and adult rat livers to the cloned rat AFP promoter region \((-197\text{ to }+48)\) using a combination of gel shift and DNsI footprinting assays. Three stable and specific high affinity complexes (I, II, and III) were detected by gel shift analysis of fetal rat liver extracts. Complex II was specific to extracts from fetal liver while the two others (I and III) were also formed with extracts from adult liver. Complex I was highly liver-specific since it was not detected with extracts of kidney, spleen, and brain. DNsI I and gel shift competition experiments using a synthetic oligonucleotide indicated that it is formed upon binding of a liver-specific factor to region \(-65\text{ to }-46\) of the rat AFP promoter. This region, perfectly conserved in the rat, mouse, and man, had been previously shown to be crucial for liver-specific expression of the mouse AFP gene. The binding of this liver-specific factor to this DNA region may thus represent a key step in the specificity of expression of AFP gene in liver.

Gel shift and DNsI I footprinting competition experiments showed that complex III was formed by binding of the widely distributed nuclear factor I to the rat AFP promoter in the region \(-125\text{ to }-100\). It is potentially significant that, in extracts from fetal liver, nuclear factor I is also involved with another binding factor in the formation of the stage-specific complex II.

\( \alpha \)-Fetoprotein (AFP), mainly synthesized by the liver and yolk sac, is the major plasma protein during fetal life. Its level decreases abruptly after birth to reach almost undetectable levels during normal adult life (1). However, reexpression of the gene can be observed during hepato-carcinogenesis and chemical or physical injuries to the liver (1-3). This gene thus provides a particularly interesting model for studying the molecular mechanisms which govern the tissue-specific expression of a gene at a given period of development or during carcinogenesis (see Ref. 4 for review).

The regulation of the AFP gene expression takes place mainly at the transcriptional level (5-7). It is now well accepted that regulation of transcription involves interactions between cis- and trans-regulatory elements (see Refs. 8-10 for reviews). Very often, these DNA-protein interactions contribute to chromatin changes which can be revealed by the reactivity of the corresponding DNA regions toward the action of DNsI I (see Ref. 11 for review).

Recent results from transient expression experiments and from transgenic mice have clearly indicated that several cis-regulatory elements located within a large region in the 5’ end upstream region of the AFP gene are involved in the control of its expression in the rat (12, 13)° mouse (14-16), and man (17).

In particular, the region covering the promoter of the AFP gene is able to direct the liver-specific expression of the AFP gene. The marked conservation of DNA sequences in this region between the three species (7) strongly suggests that similar mechanisms, involving factors conserved during evolution, may account for the specificity of the AFP gene expression in the liver.

The rat AFP promoter has recently been characterized in our laboratory (7), and a DNsI I hypersensitive site whose presence is directly correlated with the actual transcription of the gene has been mapped in this region of the rat genome (18).

These elements prompted us to search for DNA-binding proteins present in nuclei from fetal and adult rat liver which possess a high affinity for the rat AFP promoter region and which, consequently could be trans-acting regulatory factors involved in the liver-specific and or stage-specific expression of the AFP gene.

MATERIALS AND METHODS

Plasmids and DNA Probes—Recombinant plasmid pPO4 was constructed by inserting the BbvI-BbvI fragment of rat AFP genomic subclone PO4 (7) at the HincII site of the polynuker region of pUC9. It contains the promoter region of the rat AFP gene (from \(-197\text{ to }+48\)).

Recombinant plasmid PE\( \alpha \)EAP (kindly provided by Dr. M. Sawadogo, Rockefeller University, New York) contains the origin of replication (region 1-451) of the adenovirus 2 (Ad2) cloned in a pUC13 vector.

The DNA fragments were labeled using \( ^{32} \text{P} \)ATP (6000 Ci/mmol) and T4 polynucleotide kinase after opening and dephosphorylation of the plasmids at either the HindIII or BamHI sites on the polynuker. After recutting with either BamHI or HindIII, respectively, the labeled fragment was isolated by 5% polyacrylamide gel electrophoresis followed by diffusion from the gel or electroreoluation.

Preparation of the Nuclear Extracts—Adult livers, kidneys, spleens, and brains were obtained from normal adult Sprague-Dawley rats (IFFA CREDO, France). Fetal livers and yolk sac were obtained from
rats fetuses taken at day 18 of gestation. Nuclei were purified from the freshly removed organs as previously described (19, 20). Protease inhibitors, phenylmethylsulfonyl fluoride at a final concentration of 1 mM, leupeptin, antipain, and chymostatin, all at a final concentration of 1 µg/ml were added to all buffers. Nuclei were resuspended at a concentration of 1 mg/mL DNA/mL in 5 mM Na-EDTA, 15 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4 (buffer A), plus 0.35 M NaCl, and proteins were extracted by gentle shaking for 30 min at 4 °C (21). The suspension was centrifuged at 40,000 × g for 20 min and the supernatant collected. Glycerol (0.2 volume) was added, and the crude nuclear extract was stored frozen at −80 °C or fractionated immediately by addition of solid (NH₄)₂SO₄ (to 45% saturation) and incubation at 4 °C for 30 min with gentle shaking. The precipitated proteins were recovered after centrifugation at 10,000 × g for 10 min, and the protein pellet was dissolved in one-half or ⅓ of the original volume in buffer A plus 70 mM NaCl for gel shift or DNase I footprinting experiments, respectively. After dialysis for 4 h, and centrifugation at 40,000 × g for 20 min, 0.2 volume of glycerol was added to the clear supernatant, and the protein extract was aliquoted and kept frozen at −80 °C. The protein concentration of the extracts were about 1 mg/mL for gel shift assays and 3–6 mg/mL for footprinting experiments (Bradford method (22) using immunoglobulin as a standard).

Non-specific Competitor DNA—Unless otherwise stated DNA from Micrococcus lysodeikticus sonicated to an average chain length of 400 base pairs was used as an unspecific competitor in gel shift and DNase I footprinting experiments.

Gel Shift Assay—Binding experiments were performed by incubating first 1 µg of nuclear protein extract with 0.125–4 µg of the unspecific DNA competitor for 10 min at room temperature in a binding buffer containing 70 mM NaCl, 4% glycerol, 1 mM Na-EDTA, 10 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4 (final concentration) (21). The labeled probe (0.5–1 ng, 5,000–10,000 cpm Cerenkov) was then added, and incubation was continued for a further 25 min in a final volume of 25 µl.

After addition of 5 µl of a 30% glycerol solution, the reaction mixture was loaded onto a 4% acrylamide gel (30:1 acrylamide/Bis acrylamide), which has been cast and pre-electrophoresed for 2 h at 12 V/cm in 1 mM Na-EDTA, 3.3 mM sodium acetate, 6.7 mM Tris-HCl, pH 7.5 buffer. Electrophoresis was performed for 3 h at 4°C in a Protean I1 cell (Bio-Rad) with a continuous buffer recirculation. Gels were then dried and autoradiographed at −80 °C using an intensifying screen.

DNase I Footprinting Experiments—Similarly, nuclear protein extract (3–50 µg) was incubated with unspecific DNA (50–250 ng) for 10 min at 0°C in a buffer containing 70 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 7.5. The labeled probe (0.5 ng, 5,000 cpm Cerenkov) was then added and incubation continued for a further 15 min at room temperature. DNase I (10–60 ng), MgCl₂, and CaCl₂ were added (final concentrations 5 and 2.5 mM, respectively) in a final volume of 10 µl. Digestion by DNase I was performed for 30 s in a 25 °C water bath and stopped by addition of 80 µl of a solution containing 12.5 mM EDTA, 0.1% sodium dodecyl sulfate, 25 µg/ml Proteinase K, and 50 µg/ml yeast tRNA. After incubation for 15 min at 37°C and extraction once with 100 µl of phenol then twice with 200 µl of diethyl ether, DNA was ethanol-precipitated, dissolved in 2.5 µl of a 90% formamide solution in water, heated at 100°C for 3 min, and electrophoresed on an 8% acrylamide–7 M urea gel. The labeled probe which had been submitted to the G + A chemical cleavage of the Maxam and Gilbert method (23) was used as molecular weight markers. The gel was fixed, dried, and autoradiographed at −80 °C using an intensifying screen.

Heparin-Ultrogel Chromatography—A 2-ml aliquot of the ammonium acetate-deprecipitated protein extract from adult liver nuclei (4 mg of proteins) was loaded onto a 1-ml heparin-Ultrogel column (IBF, France) equilibrated in 70 mM NaCl, 5 mM Na-EDTA, 15 mM 2-mercaptoethanol, 10% glycerol, 10 mM Tris-HCl, pH 7.4. The column was eluted with a step gradient of the same buffer containing 70, 200, 400, 600 mM, and 1 M NaCl at a flow rate of 2 ml/h. 0.5-ml fractions were collected and individually dialyzed against the buffer containing 70 mM NaCl for 3 h at 4°C and stored frozen at −80°C.

RESULTS

Nuclear Proteins from Fetal and Adult Rat Liver Bind to the Rat AFP Promoter Region—Binding of liver nuclear proteins to the rat AFP promoter was assessed by gel shift and DNase I footprinting experiments.

The gel shift assay (21) was first used to test the nuclear extracts obtained from fetal or adult liver for their ability to bind the 32P-labeled AFP probe which includes the promoter region (−197 to +48).

As shown in Fig. 1, three stable high affinity complexes (I–III) were formed between the 32P-AFP probe and nuclear proteins from fetal rat liver.

Only the two complexes I and III were detected when the AFP probe was incubated with extracts from adult rat liver indicating that complex II is specific of the fetal stage. Complex specificities were assessed by gel shift competition which showed that formation of the radioactive complexes was readily inhibited when unlabeled rat AFP promoter region was added as a competitor while similar amounts of pBR322 did not affect the formation of any of the complexes (not shown).

The region of DNA involved in the binding was roughly localized by gel shift experiments using probes obtained by enzymatic cleavage at sites MboI (−141) or MspI (−65) of the 32P-AFP probe labeled on one or the other strand. They indicated that the observed complexes were formed by binding of factors to the −141 to +5 region of the AFP promoter. As shown in the next paragraph, this is in perfect agreement with the results given by DNase I footprinting experiments.

Proteins having a high affinity for the promoter region of the rat AFP gene were also detected and their target on DNA located by direct DNase I footprinting experiments using nuclear extracts from fetal and adult rat liver. The results are shown in Fig. 2. Two main regions were consistently protected from the action of DNase I by extracts from fetal or adult liver nuclei. The first protected region between −61 and −45 in the upper strand (coding strand) and −65 and −48 in the lower strand (noncoding strand), will be subsequently referred to as region A. Hypersensitive sites were detected between −70 and −67. The second protected region between −123 and −99 in the upper strand and between −128 and −100 in the
lower strand will be referred to as region B. Hypersensitive sites were detected at -127 and -98/96.

This indicated that proteins from liver nuclear extracts can bind to the rat AFP promoter region A and B.

No obvious differences could be observed in the protection by nuclear extracts from adult or fetal liver of either regions A and B or other regions of the rat AFP promoter.

**Tissue Specificity of the Complexes**—The tissue specificity of the complexes was tested to detect any liver-specific factor interacting with the rat AFP promoter region since this region is involved in liver-specific expression of the AFP gene (12, 13). The gel shift assay was used to compare extracts from fetal or adult rat liver with extracts from adult rat kidney, brain, and spleen, for their ability to bind the 32P-AFP probe (Fig. 3). The results clearly showed that complexes I and II were only formed with nuclear extracts of liver origin. This indicated that fetal and adult rat liver contain specific factors which can bind to the rat AFP promoter region, giving rise to complexes I and II in the fetal period and complex I at the adult stage.

By opposition complex III was detected with the extracts from several organs, indicating that the factor responsible for formation of complex III is ubiquitous.

A Liver-specific Factor Binds to Region A of the Rat AFP Promoter—The liver-specific activity which led to formation of complex I in the gel retention assay, while the 0.6 M NaCl fraction of the heparin-Ultrogel column was assayed for its ability to bind the 32P-AFP probe using the DNase I footprinting assay, the region -65 to -44 appeared to be strongly protected. In addition hypersensitive sites at -70 and -67 could be observed (Fig. 4B). Interestingly, the location of the hypersensitive sites and limits of the region protected with the 0.4 M NaCl fraction of the heparin-Ultrogel column fitted very well with those observed in region A by direct footprinting experiments with nuclear extracts from fetal and adult rat liver (Fig. 2). This experiment strongly suggested that the liver-specific complex I is formed by fixation of a liver-specific factor to region A of the probe. This hypothesis was confirmed using a synthetic double-stranded oligonucleotide (AFP1) corresponding to the protected region A (from -68 to -45) in gel shift competition experiments. As shown in Fig. 4C, the oligonucleotide AFP1...
FIG. 4. The liver-specific factor binds region A of the rat AFP promoter. A and B, analysis of the binding of proteins present in the 0.4 M NaCl fraction of the heparin-Ultrogel column to the $^{32}$P-rat AFP promoter. A, gel shift analysis. 5 $\mu$l of the 0.4 M NaCl fraction of the heparin-Ultrogel column (lane 1) or 5 $\mu$l of buffer (lane 2) were tested for their ability to bind the $^{32}$P-AFP probe in the presence of 500 ng of unspecific DNA. B, DNase I footprinting analysis. A $^{32}$P-AFP probe labeled at the 5¢ end of the lower strand was incubated with 7 $\mu$l (lane 1), 3.5 $\mu$l (lane 2) of the 0.4 M fraction of the heparin-Ultrogel column, or with buffer alone (lane 3) and submitted to the action of DNase I. G + A reactions of the probe were run as markers (lane 4). C, gel shift competition experiment. 1 $\mu$g of nuclear extracts from adult liver (lanes 1–4) or from fetal liver (lanes 5–8) were incubated with 0.7 ng of the $^{32}$P-AFP probe in the presence of 0.25 ng of unspecific DNA, 0.6, 2.5, and 10 ng of a synthetic double-stranded oligonucleotide covering the region A of the rat AFP promoter from −98 to −45 (AFPI) were present as a competitor in lanes 2 and 6, 3 and 7, 4 and 8, respectively.

competed quite readily with the $^{32}$P-AFP probe for formation of complex I with nuclear extracts from both fetal and adult livers. Oligonucleotide AFPI did not compete at all for the formation of complexes II and III.

Taken together these results indicate that complex I is formed by fixation of a liver-specific factor to the region A of the rat AFP promoter. It is of interest to note that this region of the AFP gene promoter is perfectly conserved in the rat (7), mouse (24), and human (24) species (Table I). It possesses some peculiar features such as a 4-base pair repeat GTTA (twice in the upper strand and once in the lower strand) and a palindromic sequence GTTAAC (−54 to −49).

As we have observed that the rat albumin promoter region (−175 to +16) competes in the formation of the liver-specific complex I with the $^{32}$P-AFP probe (not shown), the nucleotide sequence of the rat albumin promoter. It is of interest to note that this region of the AFP gene promoter is perfectly conserved in the rat (7), mouse (24), and human (24) species (Table I). It possesses some peculiar features such as a 4-base pair repeat GTTA (twice in the upper strand and once in the lower strand) and a palindromic sequence GTTAAC (−54 to −49).

As we have observed that the rat albumin promoter region (−175 to +16) competes in the formation of the liver-specific complex I with the $^{32}$P-AFP probe (not shown), the nucleotide sequence of the rat albumin promoter (RAFP) was examined for a region which might be homologous to region A of the rat AFP promoter. Region −60 to −44 of the rat albumin promoter exhibits a high degree of homology (69%) with region −62 to −47 of the rat AFP promoter. In addition the DNA sequences in these regions of both genes are completely conserved in the rat (25), mouse (26), and man (27) (Table I). Consequently, gel shift competition experiments were performed with a double-stranded oligonucleotide covering region −60 to −50 of the rat albumin promoter. They showed that this oligonucleotide competed very efficiently and specifically for formation of complex I upon incubation of nuclear extracts from fetal and adult rat liver with the $^{32}$P-labeled AFP probe since the patterns were absolutely identical to those shown for oligonucleotide AFPI in Fig. 4. This suggests that the liver-specific factor which binds to region A of the

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<th>Table I</th>
<th>Similarities between the nucleotide sequence of some hepatic gene promoters</th>
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<td>Region A of the rat AFP promoter, the proximal element of the rat albumin promoter (25), and regions of the rat α-fibrinogen and human α₁-antitrypsin promoters recognized by HNF (37).</td>
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Nuclear Factor I (NF-I) Binds the Rat AFP Promoter Region—Extracts from both fetal and adult liver nuclei strongly protected the region B from the action of DNase I in footprinting experiments (Fig. 2). The region which is involved in the binding encompasses the sequence 5'ATTGGCACAATTGGCTAAG3'/TAACCGTTITTACGGATT from -120 to -104 of the rat AFP promoter. This observation led us to test whether NF-I could be involved in the binding of the promoter region of rat AFP at region B. Footprinting and gel shift competition experiments were therefore performed using the plasmid pEIAEP, which carries the cloned NF-I binding site of the Ad5 origin of replication, and a synthetic double-stranded oligonucleotide which represents a consensus sequence for the binding of NF-I.

As shown in Fig. 5A, plasmid PEIAEP readily prevented the binding of the factor from adult liver nuclei which recognized region B of the 32P rat AFP promoter. In gel shift analysis of adult liver extracts the Ad5 DNA from plasmid PEIAEP competed in the formation of complex III with the labeled probe, while formation of complex I was not affected (Fig. 5B). These two results strongly suggested that NF-I is the factor involved in the formation of complex III by binding region B of the probe. This was confirmed using a synthetic double-stranded oligonucleotide containing a consensus NF-I-binding site GATCCATTGCACTTGCCAAATAT/GTAAACCGTACAACGGTTATACTAG (oligonucleotide NF-I).

Indeed, this oligonucleotide was able to specifically compete with the 32P-AFP probe for formation of complex III with extracts from fetal and adult rat liver nuclei (Fig. 5). Further evidence for binding of NF-I to region B of the rat AFP promoter was provided by the facts that complex III formation (Fig. 3) and protection of region B from DNase I digestion (not shown) both occurred upon incubation of the 32P-AFP probe with nuclear extracts from several organs. Indeed, NF-I is known to be a widely distributed factor.

Analysis of fetal liver nuclear extracts must take into account the fact that Ad5 DNA from plasmid pEIAEP and oligonucleotide NF-I competed for formation of complex III but also for the formation of complex II (Fig. 5). This indicated that NF-I or a closely related factor is also involved in the formation of complex II and strongly suggested that complex II is the result of the simultaneous binding of NF-I and another factor to the rat AFP promoter region. It is impossible as yet to precisely determine whether complex II is formed by the simultaneous binding of NF-I and another factor to two distinct DNA regions of the AFP promoter or whether it results from protein-protein interaction between NF-I, fixed on region B and an additional factor. However, the second possibility is supported by the observation that formation of complex II is totally inhibited by raising the ionic strength of the incubation medium to 150 mM while complexes I and III are much less affected (not shown). The exact nature of the additional factor, which would be specific to fetal liver, remains to be determined. However, the liver-
specific factor which leads to formation of complex I can be excluded since the oligonucleotide AFP1 did not compete for the formation of complex II.

**DISCUSSION**

A combination of gel shift and DNase I footprinting experiments have been used to show that the rat AFP promoter, which controls the liver specificity of AFP expression (12, 13) can bind nuclear proteins within at least two different regions.

Several lines of evidence indicated that one of these proteins is NF-I or a very closely related factor. This protein, which is also known as TGGCA-binding protein (29, 30) and CTF (31), recognizes DNA sequences which have been mapped upstream of many viral or cellular genes. Most of these finding sites correspond to DNase I-hypersensitive sites concerned with gene expression. This suggests that NF-I, which is a ubiquitous factor, has a general role in the regulation of transcription (31). It may also play a role in the mode of action of the glucocorticoid receptor (32). However, the precise function of NF-I in these operations is not yet definitely established.

NF-I belongs to the group of CAAT box-binding proteins which has been recently characterized (33-35). In this respect it is interesting to note that no CAAT box is present at the expected place (−80) in the promoter regions of AFP genes in rat, mouse, and man but that a perfect 5’CCAAT box exists in the lower strand around position −120 of these 5’ end extragenic regions (7). In the present paper we have shown that this CCAAT box is part of region B (−125 to −100) recognized by NF-I. Thus NF-I, upon binding to its target in the promoter region of the AFP gene, would play a role (although not yet clearly elucidated) similar to that played by other CCAAT box-binding proteins fixed to their recognition sequence.

Our data indicate that in extracts from fetal liver, NF-I and another factor are simultaneously involved in formation of a fetal liver-specific complex with the AFP promoter. This might play a role in the change of the chromatin structure revealed by the presence of a DNase I-hypersensitive site (18) in that region in fetal liver nuclei and might be in relation with the specific expression of the AFP gene at that stage of the liver development.

The other nuclear factor which interacts with the rat AFP promoter region is liver-specific. It binds to region A (−65 to −46) which is absolutely conserved in the promoter regions of AFP genes in the rat, mouse, and man (7). This region belongs to the region (−85 to −52) which has been shown to be of crucial importance for the liver-specific expression of the mouse AFP gene (15). In views of the perfect conservation of the sequences between region A of the AFP promoter in the mouse, rat, and man (7), it is not unreasonable to presume that this DNA sequence is of particular significance in directing the liver-specific expression of AFP also in the rat and in man.

It is thus logical that binding of the liver-specific factor to region A should represent an important step in the control of the liver-specific expression of the AFP gene.

The competition experiments indicated that this factor, which binds region A of the AFP promoter or a closely related one, may also recognize a proximal element of the rat albumin promoter (from −63 to −41) which is perfectly conserved in the rat, mouse, and man (25-27) and which shares a high degree of homology with region A of the AFP promoter. These results fit well with a recent report which indicates that only liver extracts can protect this region of rat albumin promoter from the action of DNase I in direct DNase I footprinting experiments (36). In addition this region of the albumin promoter has recently been shown to be important for liver-specific in vitro transcription (26).

The regulation of expression of some other liver-specific genes has very recently been reported. A liver-specific factor (HNF4) was found to bind to a region (from −102 to −75) involved in the specificity of expression of the β-chain of rat fibrinogen in liver (37). HNF4 also binds to a regulatory region located at about −70 in the human α-antitripsin promoter (37, 38). These regions show a high degree of homology with region A of the AFP promoter. The electrophoretic properties of the complex formed between HNF4 and the fibrinogen regulatory element are similar to those observed in this study for complex I in the case of the rat AFP promoter. Lastly, we have found that an oligonucleotide covering the regulatory region for the β-fibrinogen gene recognized by HNF4 (from −101 to −74) was as potent as oligonucleotide AFP1 in competing with the 32P-AFP probe for the formation of complex I. Altogether these observations strongly suggested that the liver-specific factor which binds to region A of the rat AFP promoter and to the proximal element of the rat albumin promoter may be HNF4 or closely related to HNF4.

These results all point to liver specificity of expression being conferred, at least in part, by binding of a liver factor (or a class of closely related factors) to a relatively well-conserved DNA element within several 5’ end extragenic regions of genes expressed in liver. The regulation of the hepatic expression of the AFP gene probably follows this scheme. However, in contrast to the other cited liver genes for which, most if not all, the DNA sequences important for high level of expression in liver cells are located within a limited part of their 5’ end extragenic region, full AFP expression requires regulatory elements located far upstream from the cap site.

This renders the study of the regulation of the expression of the AFP gene even more promising as a model for obtaining insight into the mechanism of the cis-acting long distance effects. The next step, presently in progress in our laboratory, requires the precise mapping of these regulatory elements and the characterization of the proteins which interact with them.

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