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A SPECIFIC α-FETOPROTEIN GENE BINDING PROTEIN IN α-FETOPROTEIN PRODUCING RAT HEPATOMAS

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A specific α-fetoprotein (AFP) gene binding nuclear protein (Mol. Wt. 149,000) was determined in Morris hepatoma 7777 cells by the protein blotting technique. This protein is not present in normal adult rat liver and non-AFP producing Morris hepatoma 5123tc. Neoplasia induced in rats fed the hepatocarcinogen 3'-methyl-4-dimethylaminoazobenzine enhanced AFP gene activity and re-expressed specific AFP gene binding nuclear protein. The precise role of this protein in AFP gene regulation remains to be determined. © 1987 Academic Press, Inc.

There are many examples in both eukaryotes and prokaryotes demonstrating that site-specific interaction of protein with DNA is required for transcriptional activity (1-5). Purified RNA polymerase II alone is known not to initiate specific transcription from eukaryotic promoters. However, in the presence of crude cell extracts, initiation of transcription by RNA polymerase II comes under promoter control and occurs at same sites as are used in vivo. (6,7)

Transcription systems containing crude extracts have been used extensively in an attempt to determine the basis of transcriptional selectivity, and in particular to determine what sequences and proteins are needed to constitute a functional promoter (8-11). Recently, several proteins have been isolated and characterized These proteins are demonstrated to be the factors required for transcription of specific genes (1, 12-14).

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Abbreviations: AFP, α-fetoprotein; 3'-MDAB, 3'-methyl-4-Recently, several proteins have been isolated and characterized. dimethylaminoazobenzene, SDS, sodium dodecyl sulfate

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A variety of methods have been described for the detection, isolation, and characterization of nonhistone DNA-binding proteins (12-24). Recently the protein blotting technique has been successfully used in the identification of sequence-specific binding proteins by the Drosophilia heat shock gene (24), and of the rat α-fetoprotein (AFP) gene (25). The present study demonstrates AFP gene binding proteins in rat hepatomas. These proteins are only present in AFP-producing hepatomas, but not in normal adult rat liver and non-AFP producing hepatomas. The specific AFP-binding proteins appear in rat liver after transformation to an AFP producing hepatoma.

MATERIALS AND METHODS

Animals and Tumors. Morris hepatomas 7777 and 5123tc were maintained as transplantable tumors in the hind legs of Buffalo rats. Normal liver tissue was obtained from Buffalo rats. Hepatocarcinogenesis experiments were performed as described in our previous paper (26). Male Fischer rats were fed with diet containing 10% corn oil and 0.06% 3'-methyl-4-dimethylaminoazobenzene (3'-MDAB). The rats in control groups were fed diet containing 10% corn oil.

Preparation of Nuclear Extract. Nuclei were prepared from livers and hepatoma tissues as described (25). Nuclei were treated with DNase I for 1 - 2 hrs on ice and then dissolved in equal volume of 0.125 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, and 10% β-mercaptoethanol. Samples prepared in this way can be used directly for electrophoresis or can be stored at -70°C for extended periods until use.

Gel Electrophoresis and Transfer. SDS-polyacrylamide gel electrophoresis was performed as previously described (25). Following electrophoresis, proteins were transferred to nitrocellulose paper in 24 mM Tris, pH 8.3, 192 mM glycine, 20% methanol for 3-5 hr at 60 V.

DNA-binding and Detection Following electrophoretic transfer, the nitrocellulose filters were preincubated in binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.1% Ficoll, 0.1% bovine serum albumin and 0.1% polyvinyl pyrrolidone) at room temperature with gentle agitation for 1 hr. The nitrocellulose filters were then incubated with fresh binding buffer containing 150 μg/ml sheared E. coli DNA and 0.1 μg/ml 32P-labeled p-λRAF 6/R6.2 for one more hour at room temperature. Following binding, the filters were washed 3x in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 02. M NaCl and 1x Denhardt's solution (0.02% Ficoll, 0.22% bovine serum albumin and 0.02% polyvinyl pyrrolidone) at room temperature. DNA binding was determined by autoradiography using Kodak XRP X-ray film.

AFP Gene Probe and End-labeling. The DNA probe is a 6.2 Kb EcoR1 fragment containing the 5'-end AFP gene originating from the bacteriophage λRAF6 and has been subcloned into EcoR1 site of the Vector pUC8 (25). This subclone was designated pλRAF6/R6.2. The plasmid pλRAF 6/R6.2 was restricted with EcoR1 to create insert fragments of rat AFP and vector DNA that exist in equimolar amounts. The restricted pλRAF 6/R6.2 DNA was end-labeled as previously described with 32p-dATP (27).

Determination of DNA-binding Specificity. Following autoradiography the radiolabeled bands were localized to their corresponding positions on the

nitrocellulose filters. The bands were cut from the filter and the strip was shaken in 0.75 ml of 0.1% SDS, 1 mM EDTA for 1 hr. The filter was removed and the DNA was concentrated by lyophilization. Lyophilized DNA was dissolved in 3% Ficoll containing bromphenol blue as a tracking dye, and was electrophoretically separated on 1% agarose gels. Agarose gels were dried and autoradiographed to detect the presence of DNA.

RESULTS AND DISCUSSION

Initial experiments were performed to compare the AFP gene-binding proteins in normal rat liver in which the AFP gene is transcriptionally inactive, and in the Morris hepatoma 7777 in which the AFP gene is active. Total chromosomal proteins of these two tissues were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper by the Western method. The chromosomal proteins blotted onto the nitrocellulose filter were then exposed to a mixture of ^{32}P-labeled DNA fragments containing an equimolar mixture of AFP insert and pUC 8 vector DNA, plus 1500 folds excess of cold *E. coli* DNA. The ^{32}P-labeled pUC 8 vector DNA was used as an internal control for nonspecific-binding. *E. coli* DNA was used to compete out most of the nonspecific binding. Figure 1A shows the DNA binding pattern of the fractionated normal rat liver (lane a) and Morris hepatoma 7777 (lane b) chromosomal proteins. To determine the specificity of this binding, DNA fragments were eluted from each radioactive band and electrophoresed on agarose gels to determine the molecular weights of the bound DNA's. As shown in Figure 1B electrophoresis of DNA eluted from histone proteins reveals two DNA bands of 6.2 Kb (AFP gene insert) and 2.7 Kb (pUC 8 vector DNA). The autoradiographic intensity is equal for the two bands indicating that histones do not show preferential or specific binding. Whereas, the nonhistone proteins vary in their ability to bind DNA. Three nonhistone proteins isolated from Morris hepatoma 7777 show preferential binding to AFP gene DNA. The molecular weights of these proteins are 149,000 (protein band #1) 98,000 (protein band #4) and 83,000 (protein band #5). Among these, the nonhistone protein of 149 Kd binds specifically to AFP DNA. Proteins of comparable molecular weight in normal liver do not exhibit specific DNA binding ability. Two nonhistone proteins of molecular weights of 62,000 (protein band #7) and 73,000 (protein band #6) isolated from normal liver show specific binding to AFP gene DNA.

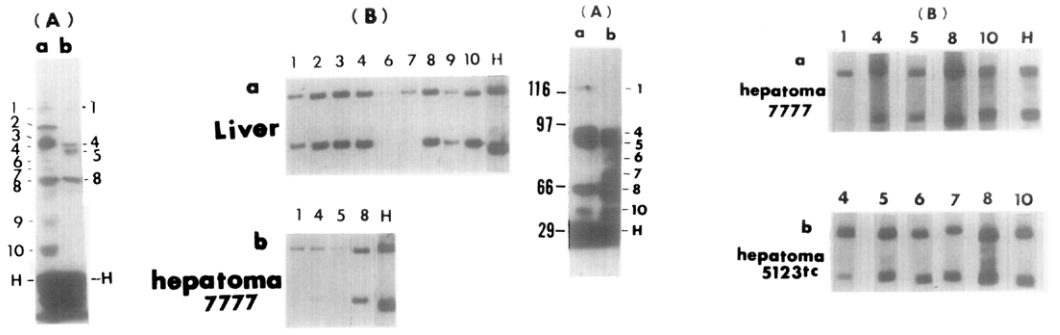

Figure 1. The specificity of DNA binding proteins in nuclear chromosomal proteins isolated from adult rat liver (lane a) and Morris hepatoma 7777 (lane b). (A) Autoradiographs of filters. (B) Autoradiographs of agarose gel containing DNA eluted from proteins in (A). Numbers correspond to bands in (A). H, histone protein bands.

Figure 2. The AFP gene binding protein in AFP producing and non-producing Morris hepatoma nuclear proteins. (A) Autoradiographs of DNA binding proteins isolated from Morris hepatoma 7777 (lane a) and 5123tc (lane b). (B) Autoradiographs of agarose gel containing DNA eluted from proteins in (A). Numbers correspond to bands in (A). H, histone protein bands.

Figure 2 compares the AFP gene binding proteins found in the non AFP-producing Morris hepatoma 5123tc with Morris hepatoma 7777. As expected the AFP gene binding proteins in the Morris hepatoma 7777 (lane a) are the same as shown in Figure 1. The Morris hepatoma 5123tc (lane b) has only one nonhistone protein (protein band #4, Mr 83,000) exhibiting preferential AFP gene binding activity. The highly specific AFP-binding protein of molecular weight 149,000 (protein band #1) does not exist in Morris hepatoma 5123tc.

To see if the pattern of AFP gene binding proteins change during induction of primary hepatocellular carcinoma, rats were fed the hepatocarcinogen 3'-MDAB and chromosomal proteins assayed at various times after initiation of the carcinogenic diet (Figure 3). We found the Mr 149,000 AFP gene specific binding protein was induced after 150 days on the diet (Figure 3, lane C); AFP is produced in large amounts by this hepatoma as indicated by the greatly elevated AFP serum levels and AFP mRNA concentration in liver (28). The specific AFP gene binding protein (Mr 149,000) was not detectable in other two samples isolated from liver of rats treated with 3'-MDAB for 70 and 100 days. Neoplastic nodules were clearly visible in rat livers at these stages, however, AFP synthesis in these livers is very low.

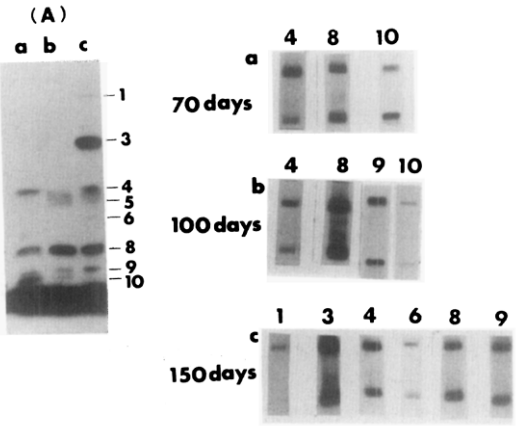

Figure 3. The appearance of AFP gene binding proteins in liver nuclear proteins from rats fed 3'-MDAB. (A) Autoradiographs of DNA binding proteins from rats fed 3'-MDAB for (a) 70 days, (b) 100 days and (c) 150 days. (B) Autoradiographs of agarose gel containing DNA eluted from proteins in (A). Numbers correspond to bands in (A). H, histone protein bands.

The results presented here correlated the existence of Mr 149,000 AFP gene binding protein with AFP production in primary and cultured hepatomas. This protein is not tumor-specific as we have also identified it in fetal and regenerating rat liver (date not shown). Therefore, it is likely that the Mr 149,000 protein plays an important role in the expression of the AFP gene.

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