Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5031–5035, August 1984
Biochemistry

Differential DNase I sensitivity of the albumin and α-fetoprotein genes in chromatin from rat tissues and cell lines

(transcriptional control/chromatin structure/hepatocyte differentiation/hepatocarcinogenesis)

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Communicated by James Bonner, April 9, 1984

ABSTRACT **We have examined the DNase I sensitivity of the albumin and α-fetoprotein (AFP) genes in different rat tissues (adult liver and kidney) and cloned cell lines (hepatoma 7777-C8, JF1 fibroblasts), which show drastic differences in the level of expression of these two genes. This was done by studying the disappearance of defined restriction endonuclease fragments of these genes as a function of limited DNase I digestion. The sensitivity of these genes was compared to that of a gene not expressed in the hepatic cells and to that of a ubiquitously expressed gene. In nuclei from adult rat liver the albumin and AFP genes were preferentially degraded by the nucleolytic action of DNase I, whereas they were not in rat kidney nuclei. In the hepatoma cells the AFP gene was much more sensitive to DNase I digestion than the albumin gene; both genes were very resistant to DNase I action in fibroblastic nuclei. When analyzed in relation to the level of gene expression our results indicate that (*i*) alterations in the chromatin structure of the albumin and AFP genes might be involved in the early establishment of the tissue-specific potential of overt gene expression; (*ii*) such alterations reflected in an altered DNase I sensitivity do not appear to be responsible for the changes in gene activity occurring during the terminal differentiation of the hepatocyte; and (*iii*) modifications in the chromatin structure of these genes might occur during oncogenic events; these structural modifications could be related to the changes in gene expression observed in hepatocarcinogenic processes.**

The control of albumin and α-fetoprotein (AFP) synthesis in the mammalian liver provides a powerful model system to investigate the molecular mechanisms responsible for changes in gene expression during developmental and oncogenic processes. These two serum proteins have similar physicochemical properties but their serum concentrations show a reciprocal relationship during normal ontogenic development and in some carcinogenic events (1, 2). Thus AFP is the dominant protein species in the plasma of the mammalian fetus, where it is synthesized by the yolk sac and the liver (3). Its concentration is drastically decreased in the serum of adult animals except in cases of liver neoplasia and germinal tumors (1, 4). On the contrary, albumin is the major plasma protein during adult life, and its serum level increases during early fetal development to reach high, approximately constant, values in postnatal life (1). A reduced rate of albumin synthesis in relation to that of total protein is observed in most of the transplantable rat hepatomas (5).

Different studies from our own and other laboratories have indicated that the production of albumin and AFP during rodent liver development and in different hepatomas is regulated mainly at the transcriptional level (6–9). The molecular mechanisms responsible for the changes in transcriptional activity are not known but could involve modifications at the gene level, changes in chromatin structure, or both. We have previously reported that changes in albumin and AFP synthesis during liver development and in different rat hepatomas do not appear to result from alterations in gene number or gross rearrangements of the corresponding genes (ref. 10; unpublished data). Further, we have recently shown that methylation changes at specific DNA sequences within the albumin and AFP genes are not responsible for the changes in gene occurring during rat liver development (11).

In this paper, we describe the use of DNase I as a probe to investigate the chromatin structure of the albumin and AFP genes in different rat tissues (adult liver and kidney) and cell lines (hepatoma 7777-C8, JF1 rat fibroblasts). The choice of the tissues and cell lines analyzed was directed by the interest in investigating the functional relationship between chromatin structure and the oncodevelopmental activation (or inactivation) of these genes. We have found tissue-specific differences in the DNase I sensitivity of these two genes; these differences suggest that alterations in their chromatin structure might occur during the early events of cell differentiation and during oncogenic processes. Further, our results indicate that the structural modifications can be related to the pattern of changes in gene expression. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

Animals and Cells Lines. Male rats of the Sprague–Dawley strain (Iffa, Credo, St. Germain sur l'Abresle, France) were used as sources of whole cell RNA and nuclei from the different tissues. The hepatoma 7777-C8 cell line is a clonal line derived from the Morris transplantable hepatoma 7777 originally induced in Buffalo rats (11). The JF1 line was cloned by C. Szpirer from rat fibroblast cells.

Isolation of Nuclei and Digestion with DNase I. Two different procedures have been used to isolate purified nuclei. Method I was a modification of the Hewish and Burgoyne procedure (13), which uses polyamines as stabilizing agents in the homogenization buffer. Method II, which was developed in our laboratory, combines the use of 3 mM Mg^{2+} as a nuclear stabilizer and of 0.5 mM EGTA as an inhibitor of endogenous nucleases (14). The nuclei were resuspended at a concentration of about 1 mg of DNA per ml in digestion buffer (15 mM Tris·HCl, pH 7.4/60 mM KCl/15 mM NaCl/3 mM $MgCl_2$/0.5 mM dithiothreitol/0.25 M sucrose for method I; 10 mM Tris·HCl, pH 7.4/10 mM NaCl/3 mM $MgCl_2$/0.25 M sucrose for method II). Nuclei were digested to a limited extent with several concentrations of DNase I (Worthington; 0.85–17 units per mg of DNA) for 2 min (method I) or 1 min (method II) at 37°C.

Preparation of DNA and Whole Cell RNA. DNA was isolated from all samples as previously described (15). Total RNA

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Abbreviations: AFP, α-fetoprotein; kb, kilobase pair(s).

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from the different cells and tissues was isolated by using the guanidinium thiocyanate procedure (16).

Restriction Endonuclease Digestions and Gel Electrophoresis. DNA samples were digested with a 5-fold excess of the restriction endonucleases *Eco*RI (Boehringer Mannheim) or *Hin*dIII (Bethesda Research Laboratories) in the recommended reaction buffers, and 10- to 15-μg samples of DNA were loaded on 0.8% agarose gels as described (15).

Southern DNA Transfer and Filter Hybridization. The separated DNA fragments were transferred onto nitrocellulose or diazobenzyloxymethyl filters and hybridized to the albumin (17) and AFP (18) cDNA probes or to the albumin genomic probes subA and subC (17, 19), labeled by nick-translation as described previously (8). The dihydrofolate reductase (20) and tyrosine hydroxylase (21) cDNA probes were kindly provided by R. T. Shimke and J. Mallet, respectively. Filter autoradiograms were quantitatively scanned with a Joyce–Loebl double-beam recording microdensitometer.

RESULTS

Expression of Albumin and AFP Genes in Different Rat Tissues and Cell Lines. We first measured the levels of albumin and AFP mRNA sequences present in total RNA preparations from adult liver and kidney and from the cloned hepatoma 7777-C8 and JF1 fibroblastic cell lines by RNA-excess hybridization in solution (Fig. 1 *A* and *B*) and by dot blot analysis (Fig. 1 *C* and *D*). The results show that the albumin gene is expressed at high levels in adult liver but it is transcribed at a very low rate in adult kidney (≈5–10 albumin mRNA molecules per cell). No significant amounts of albumin gene transcripts were detected in the RNA preparations from the C8 or JF1 cell lines. In contrast, the AFP gene is highly transcribed in the hepatoma cells, whereas only a residual level of transcription is detected in the adult liver and kidney (≈10–20 and 5–10 AFP mRNA molecules per cell, respectively). The RNA from the fibroblast line JF1 contains no measurable amounts of AFP mRNA sequences. These results are in agreement with those previously obtained in our laboratory except for the low levels of transcription of the albumin gene observed in the cloned C8 line by reference to the original hepatoma (6, 8). Blot hybridization analysis of these RNA preparations indicated that the mRNA sequences detected by solution and dot hybridization correspond to mature albumin and AFP mRNA molecules (refs. 8 and 23; unpublished data).

Analysis of the DNase I Sensitivity of the Albumin and AFP Gene Regions in Nuclei from Different Rat Tissues and Cell Lines. Because of the growing body of evidence suggesting that transcriptionally active genes are in an "open" chromatin conformation that renders them more accessible to DNase I digestion than transcriptionally inactive genes (24–27), we have compared the DNase I sensitivities of the albumin and AFP genes in the different rat tissues and cell lines mentioned in the previous section. As a first approach we examined the sensitivities of these genes to extensive DNase I digestion by liquid hybridization using uncloned ^{32}P-labeled cDNA probes. As shown previously, these studies demonstrated that the albumin and AFP genes are preferentially digested to nonhybridizable fragments in liver nuclei, while DNase I treatment of kidney nuclei resulted in no selective degradation of these genes (12, 14).

To extend these results of solution hybridization we have characterized the DNase I sensitivities of the different regions of the albumin and AFP genes by analyzing the disappearance of defined restriction fragments after limited digestion of nuclei with DNase I. Nuclei from rat liver and kidney were incubated with increasing concentrations of DNase I (up to 17 units per mg of DNA) and the extent of DNA degradation was monitored by measuring the degree of trichloroacetic acid solubility (which never exceeded 2% even for the higher DNase I concentrations) and by electrophoretic analysis in agarose gels followed by ethidium bromide staining. The DNA was isolated, digested with *Eco*RI or *Hin*dIII, and analyzed by the method of Southern (28) with specific cloned cDNA probes, a procedure that allowed visualization of the complete albumin and AFP transcription units.

The autoradiogram obtained after hybridization with the albumin cDNA probes is shown in Fig. 2*A*. It can be observed that *Eco*RI digestion of DNA isolated from untreated rat liver (lane 1) and kidney (lane 7) nuclei generated six fragments, approximately 3.6, 2.7, 2.5, 1.5, 1.3, and 1.0 kilobase pairs (kb), similar in size to the *Eco*RI fragments obtained from the albumin genomic clone λRSA 40 (lane 5), as expect-

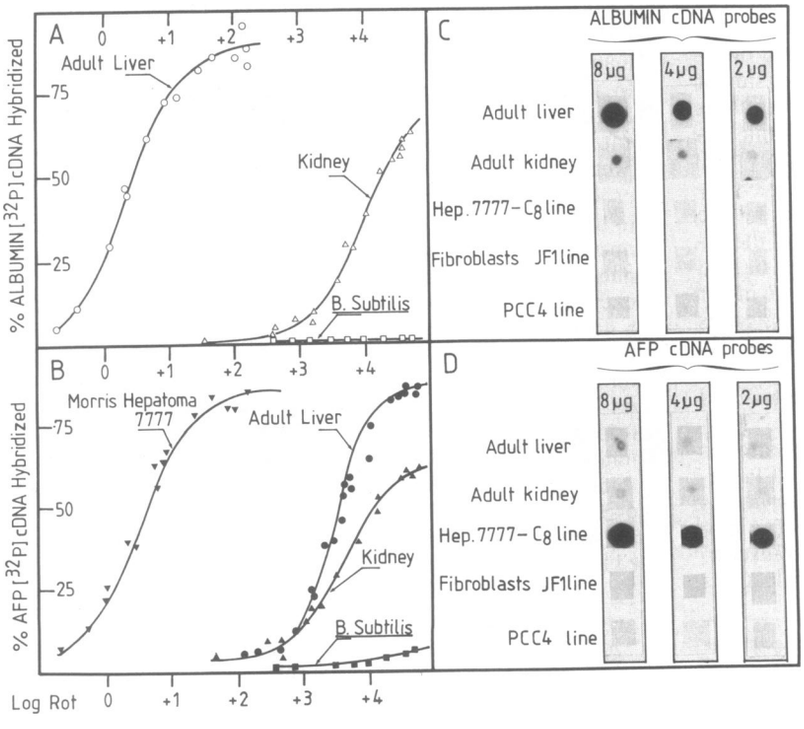

FIG. 1. Hybridization kinetic (*A* and *B*) and dot blot (*C* and *D*) analysis of albumin and AFP mRNA levels in adult rat liver and kidney, and in two cell lines. RNA-excess hybridizations with single-stranded albumin (*A*) or AFP (*B*) cDNA probes were carried out as described elsewhere (6, 8). Serial dilutions of total cellular RNA were dotted onto nitrocellulose paper and hybridized to cloned ^{32}P-labeled albumin (*C*) or AFP (*D*) cDNA probes as previously reported (11, 22). Whole cell RNA samples from *Bacillus subtilis* and from the mouse embryonal carcinoma cell line PCC4 were included as controls for background hybridization. The units for R_0t are mol of RNA nucleotide per liter times incubation time in sec.

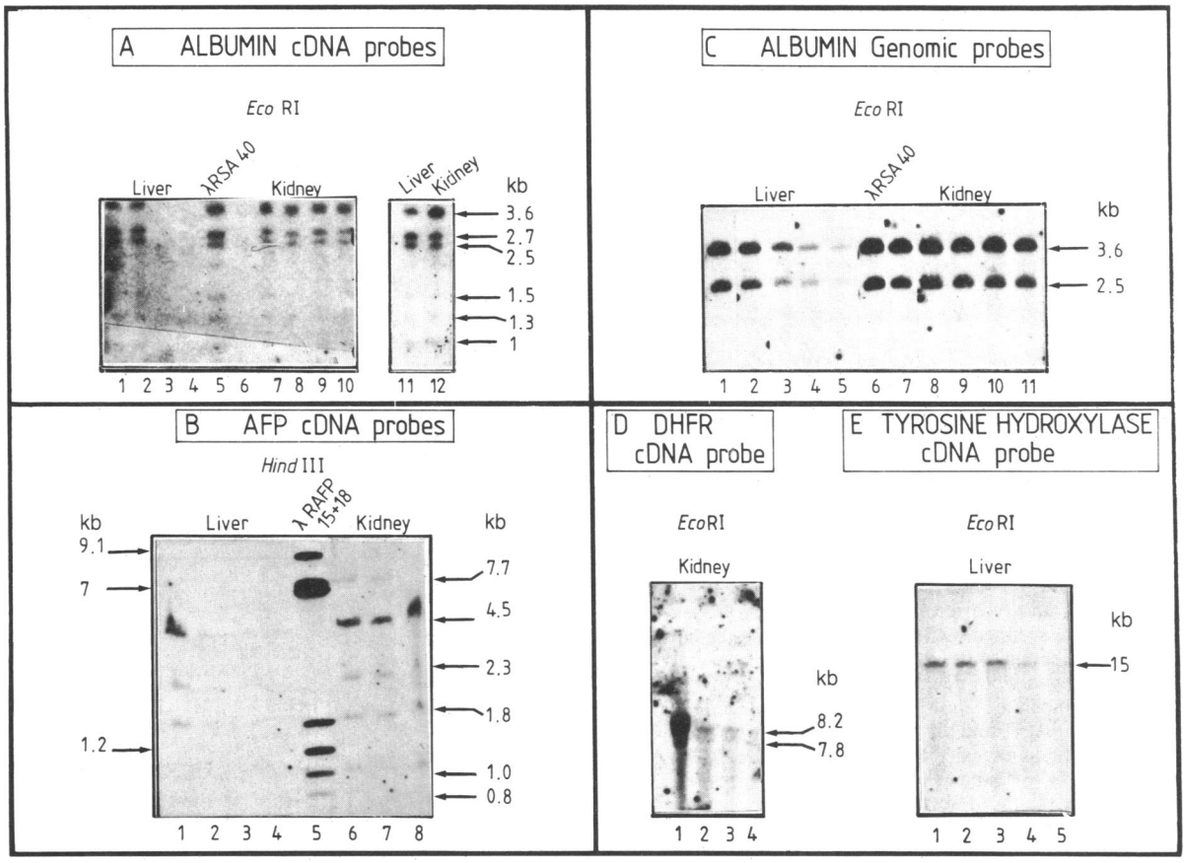

FIG. 2. Preferential DNase I sensitivity of the albumin and AFP genes in liver nuclei. Nuclei from adult rat liver and kidney were isolated by method I (*A*, *C*, and *E*) or method II (*B* and *D*) and digested with increasing amounts of DNase I. DNA was purified, digested with *Eco*RI or *Hin*dIII, and analyzed by the method of Southern with albumin (*A*), AFP (*B*), dihydrofolate reductase (*D*), or tyrosine hydroxylase (*E*) cDNA probes or with the albumin genomic probes subC and subA (*C*). Undigested DNA samples from liver and kidney are shown in lanes 1 (*A*, *B*, *C*, and *E*) and lanes 7 (*A*), 6 (*B*), 7 (*C*) and 1 (*D*), respectively. DNase I-treated samples were as follows: (*A*) Lanes 2–4, liver nuclei with 1.7, 8.5, and 17 units of DNase I per mg of DNA. Lanes 8–10, kidney nuclei with 3.4, 6.8, and 10.4 units per mg of DNA. DNA from nuclei incubated in the absence of added DNase is shown in lanes 11 and 12. (*B*) Lanes 2–4, liver nuclei with 3.4, 5.1, and 6.8 units per mg of DNA. Lanes 7–8, kidney nuclei with 3.4 and 6.8 units per mg of DNA. (*C*) Lanes 2–5 and 8–11, liver and kidney nuclei with 1.7, 3.4, 5.1, and 6.8 units per mg of DNA, respectively. *Eco*RI-digested DNA from the albumin genomic clone λRSA 40 (17) is shown in lanes 5 (*A*) and 6 (*C*). *Hin*dIII-digested DNA from two rat AFP genomic clones (29) was loaded in lanes 6 (*A*) and 5 (*B*). (*D*) Lanes 2–4, kidney nuclei with 3.4, 5.1, and 6.8 units per mg of DNA. (*E*) Lanes 2–5, liver nuclei with 3.4, 5.1, 6.8, and 10.4 units per mg of DNA. The size of the genomic DNA fragments containing specific albumin, AFP, dihydrofolate reductase, and tyrosine hydroxylase gene sequences is given on the right. Sizes indicated on the left correspond to the λRAFP 15 and λRAFP 18 fragments containing additional λ Charon 4A sequences.

ed from the physical map of the rat albumin gene (17, 19). In rat liver nuclei all these fragments readily disappear after DNase I digestion, while they are not sensitive to DNase I treatment in kidney nuclei. The *Eco*RI digestion patterns presented in lanes 11 and 12 clearly show that the sensitivity of the albumin gene region is directly due to the DNase I action and not to the endogenous nuclease activity. Intermediate DNase I digestions (Fig. 2*C*) allow one to follow the gradual disappearance of the albumin gene fragments in liver nuclei (the 3.6- and 2.5-kb bands correspond to the gene regions homologous to the genomic subclones subA and subC used as hybridization probes in this blot; see ref. 19).

The autoradiogram shown in Fig. 2*B* illustrates that the different *Hin*dIII fragments of the rat AFP gene are very sensitive to DNase I digestion in rat liver nuclei but they are not accessible to the nuclease action in kidney nuclei.

Although we have monitored DNase I digestion in both liver and kidney nuclei, and we have compared similar degrees of DNA digestion in both tissues, it seemed important to demonstrate that the differential sensitivity of the albumin and AFP genes was a characteristic of these two genes and not a random feature of the genome in the two tissues. Therefore we used cDNA probes for tyrosine hydroxylase and dihydrofolate reductase as an internal control to measure DNase I sensitivity of a gene that is not expressed in the liver tissue and of another gene that is constitutively expressed in the kidney cells (unpublished data). As shown in Fig. 2*D*, the dihydrofolate reductase gene is sensitive to DNase I in kidney nuclei, contrary to the refractoriness of the albumin and AFP genes in the same nuclei. Although the tyrosine hydroxylase gene appears sensitive to DNase I in rat liver nuclei (Fig. 2*E*), a densitometric analysis of the results presented in Fig. 2 (including results obtained after washing and rehybridization of the nitrocellulose filters used in Fig. 2*E* with albumin and AFP gene probes) indicates that after DNase I digestion of these nuclei the 3.6- and 4.5-kb fragments of the albumin and AFP genes, respectively, disappear to a 2- to 3-fold greater extent than the 15-kb tyrosine hydroxylase DNA fragment. When the differences in fragment length (30) are taken into account, these results imply that the albumin and AFP genes in hepatic cells are about 6 to 10 times more accessible to the nucleolytic action than is the tyrosine hydroxylase gene (Table 1).

We next compared the effects of DNase I digestion on nuclei prepared from the hepatoma 7777-C8 cell line and the fibroblast JF1 line. The DNA was purified, digested with *Eco*RI or *Hin*dIII, and covalently blotted onto diazobenzyloxymethyl paper; the paper was hybridized to the albumin

Table 1. Quantitative comparison of the accessibility to DNase I digestion of specific regions of the albumin, AFP, and dihydrofolate reductase genes relative to the reference "inactive" tyrosine hydroxylase gene in nuclei from rat tissues and cell lines

Gene region (fragments)	Corrected relative intensity			
	Adult liver	Adult kidney	Hepatoma 7777-C8	Fibroblasts JF1
Albumin				
4.0 kb *Eco*RI	—	—	2.2	—
3.6 kb *Eco*RI	7.6	1.03	—	—
2.5 kb *Eco*RI	12.8	1.1	—	—
4.9 kb *Hin*dIII	—	—	2.0	1.06
AFP				
6.0 kb *Eco*RI	—	—	9.7	—
4.5–4.7 kb *Hin*dIII	5.6	1.02	5.2	1.08
1.8–2.3 kb *Hin*dIII	7.9	1.05	8.8	1.03
Dihydrofolate reductase				
8.2 kb *Eco*RI	—	4.8	—	—
3.7 kb *Hin*dIII	5.4	6.3	3.2	4.24

The blots shown in Figs. 2 and 3, as well as similar additional blots, were successively hybridized with the albumin, AFP, dihydrofolate reductase, and tyrosine hydroxylase cDNA probes. The autoradiograms were scanned with a densitometer and the integrated areas under the albumin, AFP, dihydrofolate reductase, and tyrosine hydroxylase bands were determined in the same lane, corresponding to an intermediate DNase I digestion. The integrated intensities of the albumin, AFP, and dihydrofolate reductase bands were divided by the integrated intensity of the 15-kb *Eco*RI (or 17-kb *Hin*dIII) tyrosine hydroxylase band. These values were then normalized to 1.0 by reference to the ratios obtained for DNA from the undigested nuclei. The data were finally corrected by taking into account the difference in fragment length (30).

cDNA probes (Fig. 3*A*), washed, and rehybridized to the AFP cDNA recombinant plasmids (Fig. 3*B*). Visual inspection of the autoradiograms directly shows that the albumin and AFP genes are not cut by DNase I in JF1 nuclei even at the highest concentration of DNase I used. The ubiquitously expressed dihydrofolate reductase gene proved to be sensitive to DNase I in these cells (autoradiogram not shown; see Table 1). In the hepatoma nuclei the albumin gene is hardly affected by DNase I digestion. The different *Eco*RI restriction patterns observed for the λRSA 40 genomic clone and the hepatoma DNA is due to the albumin gene allelic polymorphism in the Sprague–Dawley and Buffalo strains of rats (ref. 15; unpublished data). The AFP gene in the hepatoma cells appears relatively more sensitive to digestion by DNase I than the albumin gene (Fig. 3). A quantitative analysis of the densitometric data indicates that in the hepatoma cells the AFP gene is 4 to 5 times more sensitive to DNase I than is the albumin gene (Table 1). The autoradiogram of Fig. 3*B* further shows the sequential appearance of three subfragments of the AFP gene after DNase I digestions (see lanes 3 and 4 in reference to lane 2). The sum of the size of the two first fragments could correspond to the size of the 5.0-kb fragment. Since the 6.0- and 5.0-kb fragments disappear much faster than the 4.3-kb fragment, it is tempting to conclude that DNase I hypersensitive sites exist at the 5′ end of the AFP gene in the hepatoma cells (see refs. 11 and 24 for restriction maps of the rat AFP gene).

DISCUSSION

Our studies on the chromatin structure of the albumin and AFP genes in adult rat liver and kidney allow us to draw several conclusions about the tissue-specific expression of these genes. The results obtained unambigously demonstrate that in nuclei from adult liver the albumin and AFP genes are preferentially degraded by the nucleolytic action of DNase I, whereas they are not in rat kidney nuclei. This conclusion is based, on one hand, on results of solution hybridization ex-

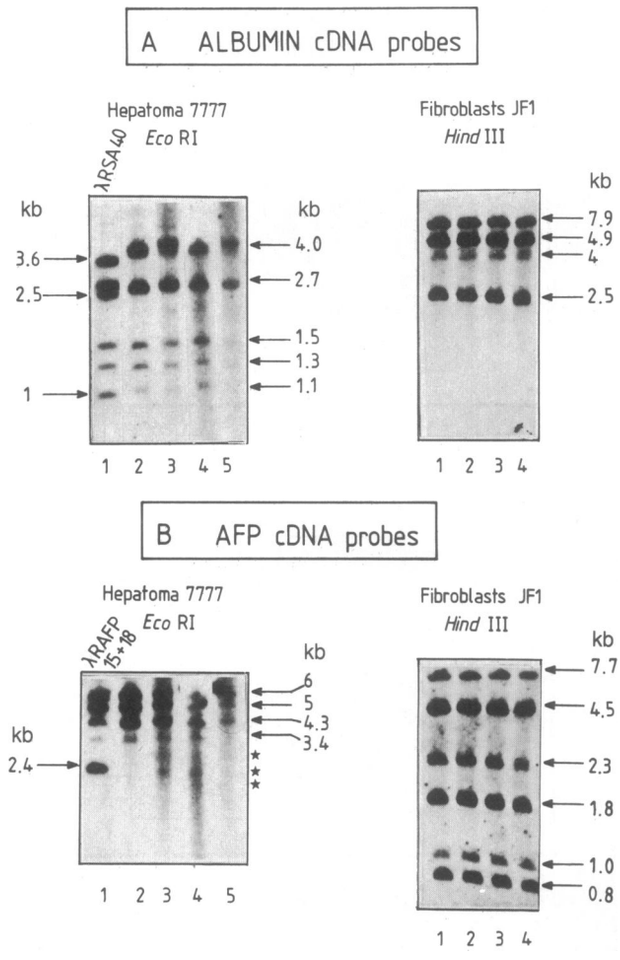

FIG. 3. DNase I sensitivity of the albumin (*A*) and AFP (*B*) gene regions in chromatin from the hepatoma 7777-C8 and the rat fibroblast JF1 cell lines. Nuclei from the corresponding cells were isolated according to method I and incubated with increasing amounts of DNase I (3.4 units per mg of DNA for lanes 3 and 2 for the hepatoma and fibroblasts cells respectively; 6.8 units per mg for lanes 4 and 3; 9.9 units per mg for lanes 5 and 4), and the isolated DNA was digested with *Eco*RI (for the hepatoma cells) or with *Hin*dIII (for the fibroblast cells). After electrophoresis on a 0.8% agarose gel the DNA fragments were transferred to diazobenzyloxymethyl paper and hybridized to the nick-translated albumin (*A*) or AFP (*B*) cDNA probes. Undigested DNA samples from the hepatoma and fibroblast cells are shown in lanes 2 and lanes 1, respectively. *Eco*RI-digested DNA samples from the albumin genomic clone λRSA 40 and the AFP genomic clones λRAFP 15 and 18 are also shown in lanes 1 of the hepatoma 7777 autoradiograms. The sizes of the genomic DNA fragments containing specific albumin and AFP gene sequences are given on the right of the autoradiograms. The stars indicate the positions of subfragments (2.7, 2.35, and 2.1 kb) generated by the DNase I cleavage, which are clearly visible in the original autoradiograms.

periments that allowed us to relate the sensitivity of the albumin and AFP genes to that of the total genome in extensive nuclease digestions (12, 14). On the other hand, the limited DNase I digestion experiments permitted us to compare the degree of sensitivity of these genes to that of other specific loci such as the tyrosine hydroxylase in liver nuclei and the dihydrofolate reductase in kidney nuclei. Furthermore, the Southern blot experiments indicated that, in adult liver, the albumin and AFP genes are equally sensitive to DNase I digestion throughout the transcribed sequences. These studies clearly show that although the AFP gene is expressed at a similar, extremely low, level in both adult liver and kidney, its chromatin structure is completely different in the two tissues. In adult liver the high sensitivity of the AFP gene to nucleolytic digestion may reflect its potential for reexpres-

sion in oncogenic processes. In several developmentally regulated systems it has been demonstrated that the DNase I sensitivity is maintained after transcription has ceased, indicating that an irreversible alteration in chromatin structure has occurred (see ref. 26 for a review).

The finding that, in spite of the low but detectable levels of transcription, the albumin and AFP genes are insensitive to DNase I digestion in adult kidney is difficult to conciliate with current hypothesis on the relationship between chromatin structure and gene expression (26). The low transcriptional activity of these genes in kidney tissues could result from a residual level of transcription in all cells or, alternatively, from a relatively high level of expression in a small subpopulation of albumin- and AFP-producing cells. This last situation cannot be excluded but seems unlikely due to the different ontogenic origins of the hepatocytes and kidney tissues. A residual level of transcription of these genes in all kidney cells could mirror the "transcriptional leakage" observed for most genes in bacterial cells (31). If this was the case, our results would indicate that the "compact" chromatin structure of certain genes is compatible with a residual nonspecific level of transcription. In this context it should be noted that the results of Bellard *et al.* (25) on the chromatin structure of the ovalbumin and globin genes in hen oviduct and erythrocytes indicate the existence of several types of nuclease sensitivities, all characterized by relatively increased rates of digestion but to different levels, and the highest corresponding to the very actively transcribed genes. The dilemma presented by our results will be solved only by appropriate *in situ* hybridization experiments.

The results with the rat cell lines support a close relationship between DNase I sensitivity and gene expression. Indeed, both genes are totally resistant to DNase I digestion in the fibroblast cells, which do not show detectable transcription of these genes. The higher sensitivity of the AFP gene than the albumin gene to DNase digestion in nuclei from the hepatoma cells is in fair correlation with the transcriptional activities of these genes. The comparative analysis of the degree of DNase I sensitivity of these genes in adult hepatocytes and in the hepatoma cells (Table 1) suggests that alterations in the chromatin structure of the albumin gene occur during the process of carcinogenesis. These alterations might occur in parallel with changes in the methylation pattern of the albumin gene, which becomes highly hypermethylated in the hepatoma cells (11).

Although we have not analyzed the chromatin structure of the albumin and AFP genes in fetal hepatocytes, it is expected that in these cells both genes should be accessible to the nucleolytic action of DNase I, since high levels of albumin and AFP mRNA sequences are already found in hepatocytes from 15-day-old rat fetuses (11, 22). The results presented here thus provide no evidence to sustain the idea that alterations in the chromatin structure of the albumin and AFP genes are responsible for the changes in gene activity occurring during the terminal differentiation of the hepatocyte. However, the different chromatin conformations of the albumin and AFP genes in adult rat liver and kidney suggest that modifications in the chromatin structure of these genes might be necessary for establishing the tissue-specific potential of overt expression. Further, our studies do not rule out the possibility that other differences not manifested in the DNase I sensitivity do exist for the albumin and AFP genes in hepatocytes. For instance, it remains to be determined whether the AFP gene is more sensitive to the action of micrococcal nuclease in fetal than in adult hepatocytes. In addition, the preparation of different albumin and AFP genomic subclones allows us to search for stage- and cell-specific DNase I-hypersensitive sites at the 5′ flanking sequences of these genes. DNase I-hypersensitive regions have been found at the 5′ end of a number of active genes and are generally hypothesized to be associated with the transcriptional promoter (32–34).

We are grateful to Dr. A. Poliard for help in the culture of the cell lines. We thank Dr. J. Mallet and Dr. N. Faucon-Biguet for providing us with the nick-translated tyrosine hydroxylase cDNA probe. This work was supported by an Action Thématique Programée from the Centre National de la Recherche Scientifique (Biologie du Développement) and by grants from the Institut National de la Santé et de la Recherche Médicale (Contract 7940 222) and the Association pour le Développement de la Recherche sur le Cancer (Contract 6109). A.G. was supported by a fellowship from the Institut National de la Santé et de la Recherche Médicale.

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