Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2135–2139, April 1987
Biochemistry

Specific sets of DNase I-hypersensitive sites are associated with the potential and overt expression of the rat albumin and α-fetoprotein genes

(transcriptional control/chromatin structure/hepatocyte differentiation/hepatoma cell lines)

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Communicated by James Bonner, November 17, 1986

ABSTRACT **We have examined the chromatin structure of the 5′-flanking region of the albumin and α-fetoprotein (*Afp*) genes in different developing rat tissues and cloned cell lines that display various functional states of these genes. Nuclease-hypersensitive sites were probed with DNase I, using an indirect end-labeling technique. In albumin-producing rat cells two major DNase I-hypersensitive sites were found near the promoter region and one additional site was located ≈3 kilobases (kb) upstream. Similarly, in Afp-producing rat tissues and cell lines we mapped one DNase I-hypersensitive region close to the promoter region and two cleavage sites further upstream at ≈2.2 and ≈3.8 kb from the cap site. The DNase I-hypersensitive sites of both genes were absent in nonhepatic rat cells and therefore appear to be tissue specific. Loss of specific sets of DNase I-hypersensitive sites accompanies the cessation of transcription for the *Afp* gene in adult rat liver and in a "dedifferentiated" hepatoma cell line. Likewise, specific sets of DNase I-hypersensitive sites disappear during the inactivation of the albumin gene in hepatoma cells. The distal upstream sites of the *Afp* and albumin genes display the same DNase I sensitivity in expressing and potentially expressible states. These findings suggest that reversible changes in short chromatin regions may be involved in the actual transcription of the albumin and *Afp* genes, while more permanent tissue-specific changes at other sites correlate with the capacity of these genes to be expressed during hepatic differentiation and neoplasia.**

A growing body of evidence suggests that chromatin structure plays an essential part in the control of gene transcription during cellular differentiation (1–3). For instance, short stretches of nucleosomal-free DNA highly sensitive to DNase I occur generally at or near the 5′ region of active or potentially active genes (3–6). While it was originally postulated that such DNase I-hypersensitive sites are necessary for efficient transcription of a gene (6), the formation and function of these chromatin structures now appear much more complex than was first thought (3, 7). Moreover, relatively little information is available on the analysis of chromatin changes in situations in which a gene ceases to be transcribed during development (8, 9) or neoplasia (10, 11).

In this context, the regulation of albumin and α-fetoprotein (*Afp*) genes provides a powerful model system to examine the molecular events implicated in the activation and inactivation of specific genes during developmental and oncogenic processes (12–14). Albumin and *Afp* gene expression is mainly regulated at the transcriptional level during normal rodent development and neoplasia (15–17). Previous studies have demonstrated that modifications in chromatin structure in the region of the albumin and *Afp* genes appear to be involved in the establishment of the tissue-specific potential of gene expression during rat liver development (14, 18, 19).

In this article, we have examined the chromatin structure upstream from the rat albumin and *Afp* genes in different tissues during rat development and in various cell lines that exhibit different patterns of albumin and *Afp* gene expression. Our study suggests the existence of two distinct classes of DNase I-hypersensitive sites; one appears to be associated with overt gene expression, while the other is more related to the potential for albumin and *Afp* gene transcription during hepatic differentiation and neoplasia. A preliminary report of this work has been presented (20).

MATERIALS AND METHODS

Animals and Cell Lines. Male Sprague–Dawley rats (Iffa Credo, St. Germain sur L'Abresle, France) were used as a source of nuclei from developing rat liver and other tissues. Fetal tissues (yolk sac and liver) were obtained from pregnant rats at day 19 of gestation. Newborn and adult tissues were isolated when the animals were 7 days, 14 days, and 3 months old.

The hepatoma 7777-C8 cell line was cloned from the transplantable Morris hepatoma 7777 originally induced in Buffalo rats (21). The JF1 cell line was a kind gift of C. Szpirer (Université Libre de Bruxelles, Belgium). It is derived from Sprague–Dawley rat fibroblast cells.

Clone 2 was isolated from the dexamethasone-resistant Faza 967 cell line, which is derived from the Reuber H35 rat clone Faza 967. The isolation procedure and properties of this cell line have been described (22). Using an indirect fluorescence staining assay "dedifferentiated" clone 2 cells were uniformly negative for both albumin and Afp (22).

Isolation of Nuclei. *Rat tissues.* Nuclei were prepared from fresh rat liver, yolk sac, and kidney by a modification of the Hewish and Burgoyne procedure (23) using polyamine-containing buffers in the presence of EDTA and EGTA as described (18).

Rat cell lines. Cells from 10 plates were pooled for preparation of nuclei. They were scraped or trypsinized and collected by centrifugation for 5 min at 2000 rpm. Subsequent steps were carried out in a cold room and nuclei were kept at 0°C–4°C. The pellet was resuspended in 10 ml of homogenization buffer (15 mM Tris·HCl, pH 7.4/0.5 mM EGTA/0.5 mM spermidine/0.15 mM spermine/60 mM KCl/15 mM NaCl/2 mM EDTA/15 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/0.34 M sucrose). Then 0.2% Nonidet P-40 and/or 0.1% Triton X-100 was added. Nuclei

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Abbreviation: Afp, α-fetoprotein.

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were homogenized with a Dounce apparatus (clone 2 cells) or a motor-driven Potter homogenizer (hepatoma 7777-C8 and fibroblastic JF1 cell lines). Nuclei were pelleted and washed twice in homogenization buffer without detergent.

DNase I digestion. Nuclei were resuspended at ≈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). Nuclei were digested with 0.5–5 μg (rat tissues and 7777-C8 and JF1 cell clones) or 10–16 μg (clone 2) of DNase I per ml (Worthington; 1684 units per mg of enzyme). Sets of nuclear suspension were incubated with or without DNase I at 37°C for 2 min. The reaction was stopped and DNA was purified as described (18, 24).

Southern blotting and DNA probes. After DNase I digestion and restriction enzyme cleavage (*Eco*RI; Boehringer Mannheim), DNA samples (5–10 μg per lane) were run on a 0.8% or a 2% agarose gel in Tris borate buffer (pH 8.2) and then transferred onto nitrocellulose, diazobenzyloxymethyl, or nylon (Biodyne Electronics, Santa Monica, CA) filters as described (24). Filter prehybridization, hybridization, and washing conditions and autoradiography were as reported (24).

All the probes used for analysis of the two genes were pBR325 subclones derived from the genomic albumin clone λRSA30 (25, 26) or the genomic *Afp* clone λRAFP15 (27). The albumin and *Afp* genomic probes are shown, respectively, in Figs. 1*C* and 4*D*. The albumin SubJB probe is 1457 base pairs (bp) long and the Afp pO4 probe is 1390 bp long. The probes were labeled with [^{32}P]deoxyribonucleotides by nick-translation as described (16). The specific activity of the probes was 2–4 × 10^8 cpm/μg.

RESULTS

To examine the chromatin structure of the 5′-flanking sequences of the rat albumin and *Afp* genes, nuclei were isolated from developing rat tissues and cell lines and mildly digested with DNase I. Purified DNA was restricted and analyzed by Southern blotting. The positions of DNase I cleavage sites were determined by indirect end-labeling as described (4). In this study, we distinguished minor and major DNase I-hypersensitive sites. The so-called "major sites" were present in Afp- and albumin-expressing and potentially expressing tissues and cells, while no such correlation was found for the so-called "minor sites." The bands corresponding to the major sites were usually but not always more intense than the bands corresponding to the minor sites.

The Presence of Tissue-Specific DNase I-Hypersensitive Sites Is Correlated with Albumin Gene Expression. The genomic subclone subJB (Fig. 1*C*) was used first to probe DNase I-hypersensitive sites in adult rat liver chromatin. As shown in Figs. 1*A* and 2*B*, the 8.3-kilobase (kb) *Eco*RI fragment containing the DNA sequences upstream from the promoter region and the first two exons was rapidly degraded by mild DNase I digestion. This cut generated three major subfragments, which migrated as 4.0-, 1.2- to 1.0-, and 0.95-kb DNA populations. These subfragments indicate the presence of three major DNase I-hypersensitive sites named, respectively, AlbIII, AlbII, and AlbI. A thorough examination of the broad fragment corresponding to the AlbII site revealed the presence of at least three distinct sites (Figs. 1*A* and 2*A*). Additional subbands of very low intensity (Figs. 1*A* and 2*A*) were also detected with the probe subJB and correspond to the so-called "minor" A, B, C, D, and E sites (★ in Fig. 1*A*). None of these major and minor sites was detected after DNase I digestion of naked DNA isolated from adult rat liver (data not shown). To ascertain the location of the hypersensitive sites, we hybridized the same blot with the genomic probe subJC (Fig. 1*B*). Two strong subfragments appear as the original 8.3-kb band is digested. The band at 4.0 kb results

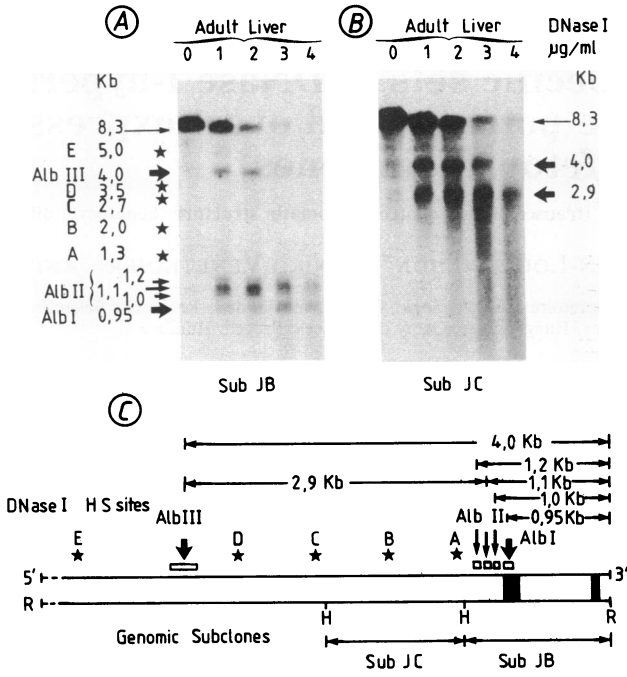

FIG. 1. Mapping of DNase I-hypersensitive sites in the 5′-flanking region of the rat albumin gene. Nuclei were isolated from adult rat liver and then treated with DNase I (0–4 μg/ml). The purified DNA was digested with *Eco*RI, electrophoresed (10 μg per lane), and transferred to diazobenzyloxymethyl-paper. The same filter was hybridized with ^{32}P-labeled subJB probe (*A*) and, after washing, with ^{32}P-labeled subJC probe (*B*) (see map in *C*). Thin arrow corresponds to the initial *Eco*RI fragment. Thick arrows and stars indicate, respectively, the position of fragments resulting from cleavage at the major and minor hypersensitive sites. Locations of major DNase I-hypersensitive sites AlbI, AlbII, and AlbIII and the minor DNase I-hypersensitive sites A, B, C, D, and E, in the 5′ region of the albumin gene are shown in a partial restriction map (*C*). Horizontal open bars show regions of DNA covered by the DNase I-hypersensitive sites. Solid boxes represent exon regions. R, *Eco*RI sites; H, *Hin*dIII sites.

from localized cleavage at the hypersensitive site designated AlbIII, and broader band at 2.9 kb indicates the presence of the AlbII site. These results confirm our mapping and demonstrate that no major sites exist between the AlbIII and AlbII sites.

The positions of the major and minor DNase I-hypersensitive sites are shown in the partial restriction map of the albumin 5′-flanking gene region (Fig. 1*C*). The AlbI site appears located inside the first exon at about +70 bp from the cap site. The region of the AlbII site covers the putative promoter region of the rat albumin gene at about −240, −140, and −70 bp from the cap site. The most distant site (AlbIII) is located ≈3 kb upstream from the cap site.

These DNase I-hypersensitive sites were next examined in yolk sac and developing rat liver and in nonhepatic adult rat tissues (kidney, spleen). Using the smallest amount of DNase I necessary to generate bands on the resulting autoradiograms (1 and 2 μg/ml), one subfragment of 4.0 kb and a broad region of 1.0–1.2 kb corresponding to the AlbIII and AlbII sites, respectively, were clearly visible in the samples isolated from newborn and adult liver that synthesized high levels of albumin mRNA sequences (Fig. 2*A*); the AlbI site was also detected with a longer exposure time (not shown). The band corresponding to the AlbII site was just distinguishable in fetal liver chromatin. As shown in Fig. 2*C*, the 4.0-kb band (AlbIII site) was found at a low DNase I concentration in adult and newborn liver and at a reduced level in fetal liver. In fetal liver, the low intensity of the major

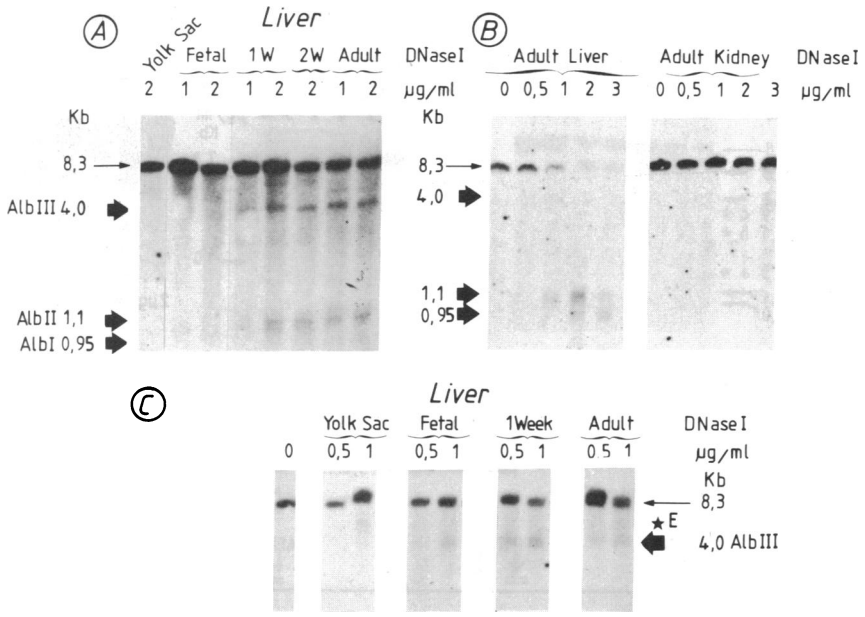

FIG. 2. Detection of DNase I-hypersensitive sites 5′ to the albumin gene in chromatin from rat tissues. Nuclei from yolk sac, fetal, newborn, and adult liver and adult kidney were isolated and incubated with increasing amounts of DNase I. After purification, the DNA was digested with *Eco*RI. Aliquots of DNA (10 µg) were electrophoresed on a 0.8% agarose gel and transferred to Biodyne filter (*A*) or diazobenzyloxymethyl-paper (*B* and *C*). The DNase I-hypersensitive sites were mapped with the probe subJB (see Fig. 1*C*). Thin and thick arrows and stars are the same as in Fig. 1.

DNase I-hypersensitive sites upstream from the albumin gene (Fig. 2*A*) may be due to the low content of albumin-producing hepatocytes at this developmental stage. The minor bands A–E were visible in both fetal and adult liver. An additional minor band at 6.0 kb was detected in the newborn liver. The significance of this observation is not clear. None of the major sites was detected in the chromatin of the albumin nonproducer yolk sac, while a faint signal corresponding to the minor site E was visible (Fig. 2 *A* and *C*). As shown in Fig. 2*B*, the 8.3-kb band is hardly digested in adult kidney chromatin (and spleen; not shown) and the digestion products migrate as a uniform smear, indicating the absence of hypersensitive sites upstream from the albumin gene in these nonhepatic tissues.

DNase I-hypersensitive site mapping was also carried out in hepatoma and fibroblast cell lines, as reported for the rat tissues, using the genomic subclone subJB.

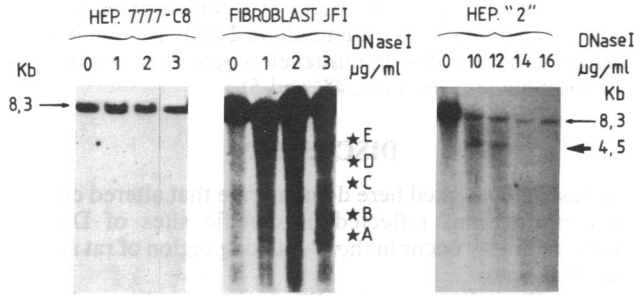

FIG. 3. Mapping of DNase I-hypersensitive sites in the 5′-flanking region of the albumin gene in cell lines. Nuclei from hepatoma 7777-C8, fibroblast JF1, and hepatoma clone 2 cell lines were digested with various DNase I concentrations, as indicated. Due to an unusual resistance to DNase I digestion of the nuclei isolated from the clone 2 cell line, we were obliged to use high concentrations of DNase I to detect DNase I-hypersensitive sites. After purification, the *Eco*RI-digested DNA was electrophoresed and transferred to a diazobenzyloxymethyl filter. The DNase I-hypersensitive sites were mapped with the nick-translated probe subJB (see Fig. 1*C*).

As shown in Fig. 3, the 5′-flanking sequence of the albumin gene appears almost and fully resistant to DNase I digestion in 7777-C8 and JF1 cell lines, respectively. In addition, none of the major DNase I-hypersensitive sites (AlbI, AlbII, AlbIII) was detected in the chromatin of these cell lines, which do not transcribe the albumin gene. Only the bands corresponding to the minor sites (A, B, C, D, and E) were distinguishable upstream from the albumin gene, which is highly amplified in the JF1 cell line (18) (Fig. 3 *Center*).

Interestingly, we found that a 4.5-kb band was fairly detectable in the clone 2 cells, which do not synthesize albumin mRNA sequences (Fig. 3). This band was also detected in albumin-expressing hepatoma cell lines, which derived from the clone Faza 967 (ref. 22; I. Tratner, J.-L.N., J.M.S.-T., and A.V., unpublished data). An additional band at ≈6.0 kb, which appears similar to that detected in the newborn liver (Fig. 2*A*), and the minor bands A–E were also visible on longer exposures (results not shown) in this cell line.

Three DNase I-Hypersensitive Sites Occur in Afp-Expressing Rat Cells, and Two of Them Disappear After Cessation of Transcription. DNase I-hypersensitive sites were mapped upstream from the *Afp* gene using the indirect end-labeling procedure (4) with the cloned genomic probe pO4 (see map in Fig. 4*D*).

In the yolk sac, which synthesize a high level of Afp mRNAs (15), the initial 7.8-kb *Eco*RI fragment was cleaved to yield 2 subfragments of 4.8 and 3.2 kb and a doublet 1.1- and 1.0-kb band corresponding, respectively, to the AfpIII, AfpII, and AfpI hypersensitive sites (Fig. 4*A*). The 4.8- and 3.2-kb bands and a strong 1.0-kb band were also detected in Afp-expressing fetal liver (Fig. 4*C*; data not shown). In 1-week-old liver, the 4.8- and 3.2-kb subfragments were clearly visible, while the 1.1- and 1.0-kb band intensities were much lower than in yolk sac or fetal liver (Fig. 4 *A–C*). The bands corresponding to the AfpI site were barely visible in the 2-week-old liver, which synthesizes 1/10th as much Afp mRNA as fetal liver (15, 16) (Fig. 4*C*; data not shown). Interestingly, only the 4.8-kb band (AfpIII site) was detected in the adult liver, which does not produce Afp, even at high

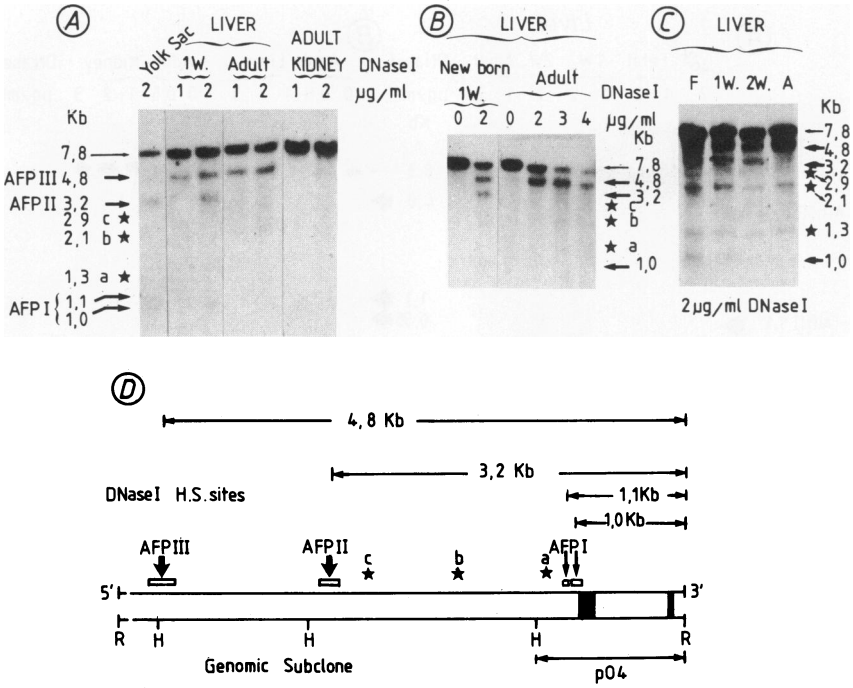

FIG. 4. Detection and mapping of DNase I-hypersensitive sites in the 5'-flanking region of the *Afp* gene in rat tissues. DNA was isolated from nuclei of rat tissues after treatment with DNase I as described in the legend of Fig. 2. After *Eco*RI cleavage, the purified DNA was electrophoresed on 0.8% (*A* and *B*) or 2% (*C*) agarose gel, transferred to Biodyne paper (*A* and *C*) or nitrocellulose paper (*B*), and probed with nick-translated genomic probe pO4 (see map in *D*). Thin arrow denotes the original DNA fragment of 7.8 kb generated by *Eco*RI cleavage. Thick arrows and stars mark the position of DNA fragments resulting from cleavage at, respectively, the major and minor hypersensitive sites. The location of the DNase I-hypersensitive sites—namely, AfpI, AfpII, AfpIII, and a, b, c—is shown in the partial restriction map (*D*). Vertical open bars delimit the DNase I-hypersensitive regions. Solid vertical boxes represent exon regions. R, *Eco*RI sites; H, *Hin*dIII sites.

DNase I concentrations (Fig. 4 *A–C*). These three specific sites were not found in the chromatin of adult kidney and spleen (Fig. 4*A*; data not shown), demonstrating the tissue specificity of these hypersensitive sites.

In addition, on the 0.8% agarose gel in Fig. 4*A*, very weak bands of 1.3, 2.1, and 2.9 kb were found corresponding to the so-called minor sites a, b, and c. The bands corresponding to sites a and b are much more visible on a 2% agarose gel (Fig. 4*C*), suggesting that the weak signal of hybridization of these components may result in part from the high target size for DNase I cleavage.

Mapping of the major DNase I-hypersensitive sites onto the partial map of the 5' region of the *Afp* gene shows that (Fig. 4*D*) (*i*) the two hypersensitive sites corresponding to AfpI site regions are located near the promoter region at about −140 and −30 bp from the putative cap site (J.-L.N., J. L. Danan, and M. Poiret, unpublished data). (*ii*) The AfpII site appears to be ≈2.2 kb from the cap site. (*iii*) The most distal AfpIII site is located far upstream of the promoter region, ≈3.8 kb from the cap site.

As shown in Fig. 5 (*Left*) the same cleavage sites as those detected in fetal liver (AfpI, AfpII, and AfpIII) are observed after DNase I digestion of hepatoma 7777-C8, which actively synthesizes Afp. None of these sites was detected in fibroblast JF1 cells, and only the minor sites were visible upstream from the *Afp* gene, which appears highly amplified in this cell line (Fig. 5 *Center*).

Of interest is that only the AfpIII site was identified in the chromatin of the Afp-negative cell line 2 (Fig. 5 *Right*). In this respect, the difference in pattern of DNase I hypersensitive sites in hepatoma 7777-C8 and clone 2 cells is similar to the changes observed during rat development between the fetal and adult stages (see Figs. 4*C* and 5).

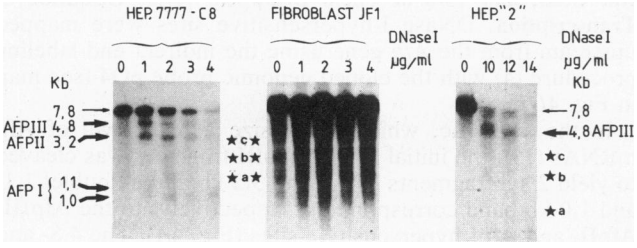

FIG. 5. DNase I-hypersensitive sites mapping upstream of the *Afp* gene in cell lines. Nuclei from hepatoma 7777-C8, fibroblast JF1, and clone 2 cell lines were digested with increasing amounts of DNase I. After purification, the DNA was cut with *Eco*RI and 5 μg of each sample was separated on a 0.8% agarose gel and then blotted onto nitrocellulose (7777-C8 and JF1 clones) or Biodyne (clone 2) filters. As a probe for the resulting autoradiogram, the genomic probe pO4 was used (see map in Fig. 4*D*). The original *Eco*RI fragment and the DNase I-hypersensitive sites are marked with arrows and stars as described in Fig. 4 legend.

DISCUSSION

The results presented here demonstrate that altered chromatin conformations reflected in specific sites of DNase I hypersensitivity occur in the 5'-flanking region of rat albumin and *Afp* genes.

Using the indirect end-labeling technique (4), we detected the presence of three major DNase I-hypersensitive sites upstream (AlbIII) and close to the promoter region (AlbII, AlbI) of the albumin gene in the chromatin of developing liver, which actively synthesizes albumin mRNA sequences. In agreement with many reports (for review, see ref. 3), these sites appear to be tissue specific, since none of them is observed in adult kidney or in a fibroblast cell line. Thus, these sites may be involved in both tissue-specific activation and actual transcription of the albumin gene during rat liver development.

The absence of DNase I-hypersensitive sites in the chromatin of the albumin gene in the hepatoma 7777-C8 cells corroborates our former results (18) and suggests strongly that alterations in the chromatin structure of this gene were correlated to the changes in gene expression observed during carcinogenesis. In addition, it is notable that one distal hypersensitive site remains unaltered after cessation of the albumin gene activity in the dedifferentiated clone 2 cells. In this context, partial reexpression of albumin synthesis was found when these dedifferentiated hepatoma cells grew in the form of solid tumors in nude mice (28).

We have identified three tissue-specific DNase I-hypersensitive sites upstream (AfpIII, AfpII) and in the promoter region (AfpI) of the *Afp* gene in rat tissues and cell lines that actively transcribe this gene (yolk sac, fetal liver, hepatoma 7777-C8). In nonhepatic tissues (kidney, spleen) and in the fibroblast JF1 cell line, these regions are in an inaccessible chromatin structure. In adult liver and clone 2 cells, where the transcription of the *Afp* gene was shut off, only the AfpIII cleavage site was detected. The disappearance of the AfpI and AfpII sites in the adult liver provides a clear indication that changes in chromatin structure of the *Afp* gene correlate with differential gene activity during the terminal differentiation of the hepatocyte. In this context, we propose that the distal AfpIII site upstream from the *Afp* gene was induced during commitment and subsequently maintained by a self-propagation mechanism (29). It would act as a differentiator regulatory element. The other DNase I-hypersensitive sites might mark the position of putative activator (AfpI site) or modulator (AfpII site) elements of transcription.

A causal relationship between DNase I-hypersensitive sites, unmasking regulatory DNA sequences (3, 7), and albumin or *Afp* gene expression still remains to be demonstrated. Ott *et al.* (30) reported that the sequence of 400 bp close to the cap site of the rat albumin gene (corresponding to the AlbII hypersensitive site region described in our report) is necessary to direct highly efficient chloramphenicol acetyltransferase expression only in albumin-producing hepatoma cells. Furthermore, expression of an *Afp* minigene and 5′-flanking sequence construct was investigated after introduction into teratocarcinoma cell lines (31), human cell lines (32), or in pronucleus leading to transgenic mice (33). The results of these studies lead to the conclusion that ≈7 kb of the 5′-flanking sequence of the mouse *Afp* gene is sufficient to direct both tissue-specific expression and developmental regulation. This region includes the three DNase I-hypersensitive sites detected upstream from the rat *Afp* gene. Interestingly, the AfpII site would be located in a region with enhancer properties (32, 34), while the AfpIII site would cover a cell-type negative element (34). Such identification of sequences essential for the tissue-specific expression and modulation of albumin and *Afp* gene activity might reveal whether distinct roles might be attributed to distinct chromatin structures in gene activation and transcription, as suggested by our results. Furthermore, in chromatin of various eukaryotic genes the DNase I-hypersensitive sites were found to mark sequences to which regulatory proteins bind (for review, see ref. 35). It is now of obvious interest to search for specific proteins, which will preferentially bind to the DNase I-hypersensitive regions of the rat albumin and *Afp* genes.

In memory of J.M.S.-T., who died while this manuscript was in preparation. We thank I. Tratner and M. Poiret for the samples of cloned genomic DNA and Drs. M. Buckingham, A. Poliard, J. L. Danan, and T. Erdos for careful reading of the manuscript. This work was supported by an Action Thématique Programmée from the Centre National de la Recherche Scientifique ("Organisation et expression du génome") and by grants from the Institut National de la Santé et de la Recherche Médicale and the Association pour le Développement de la Recherche sur le Cancer. A.V. was recipient of a grant from the Institut National de la Santé et de la Recherche Médicale.

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