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DNase I- and Micrococcal Nuclease-Hypersensitive Sites in the Human Apolipoprotein B Gene Are Tissue Specific

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We have mapped the DNase I- and micrococcal nuclease-hypersensitive sites present in the 5′ end of the human apolipoprotein B (apo-B) gene in nuclei from cells expressing or not expressing the gene. Four DNase I-hypersensitive sites were found in nuclei from liver-derived HepG2 cells and intestine-derived CaCo-2 cells, which express the apo-B gene, but not in HeLa cells, which do not. These sites are located near positions −120, −440, −700, and +760 base pairs relative to the transcriptional start site. Undifferentiated CaCo-2 cells exhibited another site, near position −540. Six micrococcal nuclease-hypersensitive sites were found in nuclei from HepG2 and CaCo-2 cells, but not in HeLa cells or free DNA. These sites are located near positions −120, −390, −530, −700, −850, and +210. HepG2 cells exhibited another site, near position +460. Comparison of the DNA sequence of the 5′ flanking regions of the human and mouse apo-B genes revealed a high degree of evolutionary conservation of short stretches of sequences in the immediate vicinity of each of the DNase I- and most of the micrococcal nuclease-hypersensitive sites.

Apolipoprotein B (apo-B) is the major protein component of low-density lipoproteins, which play a central role in the metabolism and transport of cholesterol. Apo-B is the ligand responsible for the uptake and clearance of low-density lipoproteins from the circulation via the apo-B,E(LDL) receptor pathway (13, 20, 29, 30). We and others have recently determined the primary structure of apo-B by sequencing its cDNA (5, 7, 24, 26), and we have elucidated the complete structure of the 43-kilobase (kb) human apo-B gene (3). The gene comprises 29 exons and 28 introns, with most introns appearing in the 5′-terminal one-third of the gene. Apo-B mRNA has been detected only in the liver and intestines of several mammals, including humans (23), demonstrating that transcription of the apo-B gene is regulated in a tissue-specific manner.

To understand the molecular mechanisms involved in the tissue-specific expression of the human apo-B gene, we have begun to map *cis*-acting regulatory sequences in the 5′ flanking region of the gene. We also have examined this region for the presence of DNase I-hypersensitive (DH) sites in cells expressing or not expressing the gene. These sites are located near the 5′ ends of the genes that are active or inducible in the cell under study, and their presence often correlates with binding sites for regulatory proteins (for a review, see reference 9). We have also examined the susceptibility of the 5′ end of the apo-B gene to another nuclease, micrococcal nuclease (MNase). This enzyme differs from DNase I in its mode of nucleolytic cleavage (22) and thus provides complementary structural information.

In the experiments in this study, we show that a 1-kb *Pvu*II fragment from the 5′ end of the gene exhibits cell-specific promoter activity. Furthermore, several DH and MNase-hypersensitive (MH) sites were detected in the 5′ proximal region of the human apo-B gene in human hepatoma HepG2 cells and colon carcinoma CaCo-2 cells. These sites are absent from cell types that do not express the gene, such as HeLa cells. Most of these sites map to regions in which highly conserved base sequence motifs occur.

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MATERIALS AND METHODS

Tissue culture. HepG2 cells were grown in monolayer in T150 flasks (Corning) in a CO_2 incubator at 37°C in minimum essential medium supplemented with 10% fetal bovine serum. HeLa cells were grown in Dulbecco modified Eagle medium in the presence of 10% fetal calf serum. All cultures were supplemented with 1% penicillin-streptomycin.

Undifferentiated CaCo-2 cells were grown as described above, but with 15% serum, and were harvested at confluency. Differentiated CaCo-2 cells were maintained in culture by being fed every other day after confluency for periods of 13 to 15 days.

Isolation of cellular RNA and Northern blots. Total RNA was isolated from HepG2, CaCo-2, and HeLa cells by the procedure of Chirgwin et al. (6). Northern (RNA) blots were performed as described by Thomas (36).

Transfection of the apo-B promoter fragment into mammalian cells in culture. A *Pvu*II fragment (−899 to +121) was isolated from a human apo-B gene clone and inserted into the *Sma*I site of the Bluescribe plasmid (Stratagene). The construct was then digested with *Xba*I and *Sst*I, and the fragment containing the apo-B promoter region was ligated into pLS1 (pLS1 is modified pTE without the thymidine kinase promoter). The constructions were introduced into various cell lines and later examined for chloramphenicol acetyltransferase (CAT) activity (14). A calcium phosphate coprecipitate containing 15 μg of DNA was added to the cells and shocked with 15% glycerol after a 4-h incubation. Cells were collected 48 h after addition of DNA, and extracts were prepared by freezing the cells and then subjecting them to heat shock at 65°C. The reaction mixture for the enzymatic assays contained 140 mM Tris (pH 7.8), 0.44 mM acetyl coenzyme A, 0.2 mCi of [^{14}C]chloramphenicol (40 to 60 mCi/mmol; New England Nuclear Corp., Boston, Mass.), and cell extract (100 μg of total protein). The reactions were allowed to proceed for 3 h at 37°C. Samples were extracted

with 1 ml of ethyl acetate, and the organic phase was dried under nitrogen. The residue was suspended in 15 μl of ethyl acetate and analyzed by ascending thin-layer chromatography with chloroform-methanol (95:5, vol/vol). The chromatograms were subjected to autoradiography. All CAT activity measurements were normalized for the differences in the transfection efficiency between various cell lines by cotransfection with a plasmid containing the β-galactosidase gene under the control of the Rous sarcoma virus promoter and measurement of the total β-galactosidase activity in the same cell protein extracts.

Isolation of nuclei and DNase I digestion. Nuclei were isolated essentially as described previously (27). Confluent monolayers from the various cell types were harvested by centrifugation at 2,000 rpm in an HB-4 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). The cell pellet was washed once in reticulocyte standard buffer (RSB)-sucrose (10 mM Tris [pH 7.5], 10 mM NaCl, 1 mM $MgCl_2$, 0.25 M sucrose) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) by gentle homogenization with a Dounce homogenizer, followed by a low-speed spin as described above. Cells were suspended in 3 volumes of RSB-sucrose-PMSF and lysed by the addition of Nonidet P-40 at a final concentration of 0.5% for 5 min at 0°C. The nuclear pellet was recovered by centrifugation at 4,000 rpm in the Sorvall HB-4 rotor. Nuclei were washed once with RSB-sucrose-PMSF, and their integrity and purity were checked by light microscopy after they had been stained with methyl green.

Intact nuclei were suspended at a DNA concentration of 1 mg/ml in RSB-sucrose-PMSF. DNase I (1 mg/ml, 2,000 U/ml; Worthington Diagnostics, Freehold, N.J.) was added to various final concentrations, and the mixtures were incubated for 10 min at 37°C. The reactions were terminated by the addition of an equal volume of 2× lysis buffer (0.6 M NaCl, 20 mM EDTA, 20 mM Tris hydrochloride [pH 7.5], 1% sodium dodecyl sulfate). For MNase digestions, nuclei were suspended at a DNA concentration of 0.5 mg/ml in RSB-sucrose-PMSF containing 1 mM $CaCl_2$. MNase (30 U/μl; Worthington) was added to various final concentrations, and the mixtures were incubated for 5 min at 37°C. The reactions were terminated by the addition of an equal volume of 2× lysis buffer.

RNase, previously boiled for 10 min to remove traces of DNase, was added at 40 μg/ml for 30 min at 37°C, and then proteinase K was added at 100 μg/ml for 4 to 16 h at 37°C. DNA was purified by extraction with an equal volume of phenol-chloroform (1:1, vol/vol), followed by precipitation with ethanol for 16 h at −20°C. The DNA was recovered by centrifugation, washed once with 70% ethanol, and suspended in a small volume of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). Portions from the various DNase I and MNase digests, containing 30 μg of DNA each, were further digested with appropriate restriction enzymes for 12 to 16 h at 37°C. The DNA was purified by phenol extraction and ethanol precipitation.

Gel electrophoresis and Southern blotting. Aliquots containing 30 μg of DNA digested with both DNase I and restriction enzymes were electrophoresed in agarose gels, transferred by Southern blotting onto nitrocellulose filters, and hybridized with various probes derived from the 5′ end of the human apo-B gene (3). All hybridizations were done in 50% formamide–5× SSPE (0.75 M NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA [pH 7.4])–5× Denhardt solution (1% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], polyvinylpyrrolidone, and bovine serum albumin in 1.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate])–100 μg of yeast tRNA per ml at 42°C. All probes were ^{32}P labeled by random priming.

Isolation and sequencing of the mouse apo-B gene 5′ end. Mouse genomic clones derived from the 5′ end of the apo-B gene were obtained by screening a mouse genomic library with a 1.1-kb *Pvu*II fragment from the 5′ end of the human apo-B gene. Subcloning and sequencing were performed by standard procedures.

RESULTS

Apo-B gene expression. As an initial attempt to map promoter elements in the 5′ region of the human apo-B gene accurately, a 1-kb *Pvu*II fragment immediately preceding the translational start site (−899 to +121) was inserted in front of the bacterial CAT gene and transfected into three human cell lines. The results (Fig. 1) show that CAT activity was observed only in HepG2 and CaCo-2 cells, but not in HeLa cells, suggesting that the *Pvu*II fragment has promoter activity specific for liver-derived and intestine-derived cell lines. Because these activity measurements were normalized to the β-galactosidase activity of a coinfected plasmid, it appears that the apo-B promoter displays a twofold-greater activity in HepG2 cells than in CaCo-2 cells. This result is in good agreement with measurements of apo-B mRNA in the same cell lines. For instance, apo-B100 mRNA is present in HepG2 and CaCo-2 cells, but absent from HeLa cells (Fig.

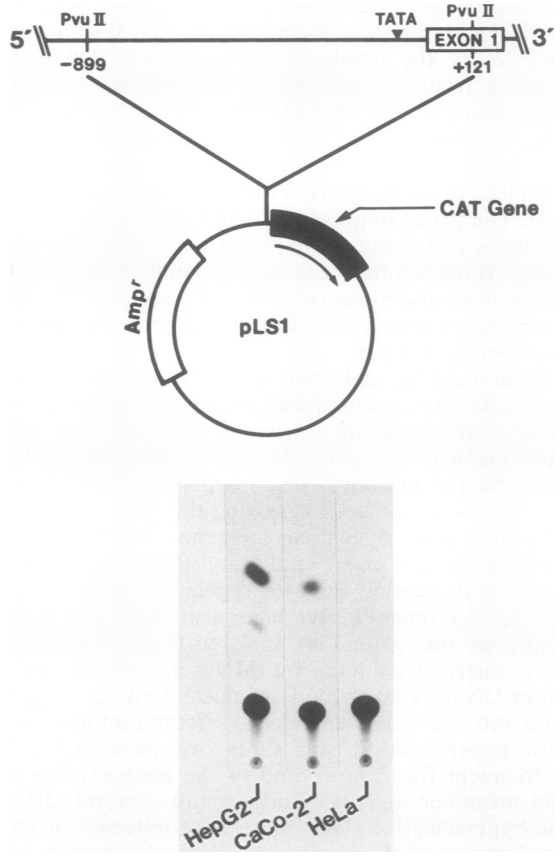

FIG. 1. Cell specificity of apo-B promoter expression. A 1,020-base-pair fragment whose 3′ end is just prior to the translational start site was inserted into pLS1 upstream of a promoterless CAT gene. The plasmid was transfected into the three human cell lines shown above, and the level of CAT activity in each cell line was determined.

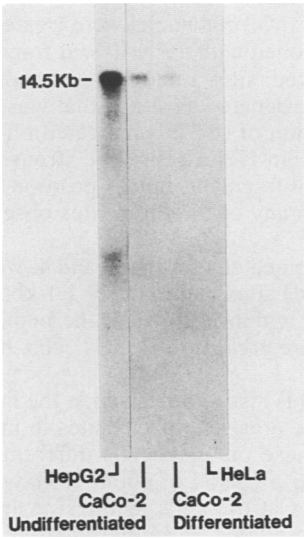

FIG. 2. Northern blot hybridization of apo-B sequences to RNA from three human cell lines. Thirty micrograms of RNA was used per lane. The probe was a 3.5-kb cDNA insert corresponding to the amino terminus of the protein. Appropriate size markers were run in parallel with the samples.

2). Furthermore, higher levels of mRNA are apparent in HepG2 than in CaCo-2 cells.

The combined results in Fig. 1 and 2 demonstrate that HepG2 and CaCo-2 cells express the apo-B gene in vivo and recognize apo-B promoter sequences and are therefore suitable cells with which to study the structural features of chromatin that correlate with expression of the human apo-B gene. HeLa cells, on the other hand, do not transcribe the apo-B gene, nor do they recognize exogenously added apo-B promoter sequences, and are therefore an appropriate representative of the inactive state of the apo-B gene.

DNase I hypersensitivity in the 5′ end of the human apo-B gene. To determine whether there are any DH sites in the promoter region of the human apo-B gene, several sets of experiments were performed, a subset of which is described below. Because of the inherent limitations of the detection system, i.e., difficulties in visualizing small DNA fragments from a unique gene in the human genome on Southern blots, the intensity of the signal of some of the hypersensitive bands varied from experiment to experiment. For this reason, three different sets of restriction digests were used for each cell type and two or more probes were used for every blot. Agreement among the various sets of experiments supports our assignment of each of the hypersensitive sites. Nuclei from either HepG2 cells or HeLa cells (control) were treated with increasing amounts of DNase I, and then the purified DNA was digested with *Hin*dIII and *Pst*I (Fig. 3, panel 1) or *Hin*dIII and *Bgl*II (Fig. 3, panel 2). The blots were probed with probe A (*Hin*dIII to *Stu*I) or B (*Pvu*II to *Pvu*II).

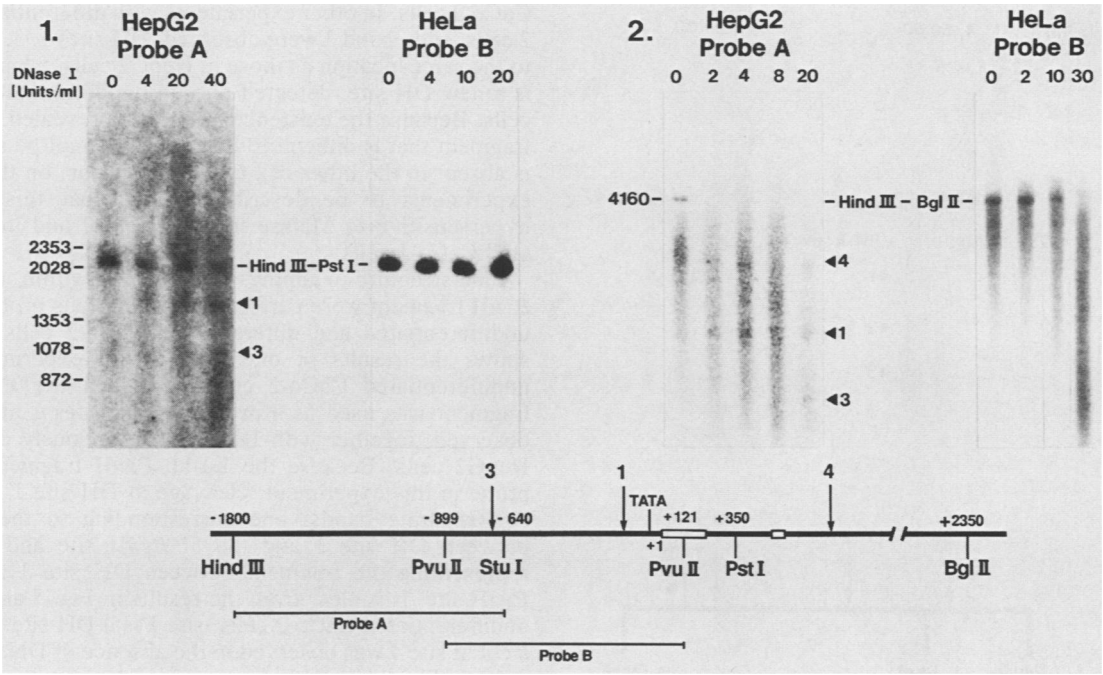

FIG. 3. DH sites in the 5′ end of the human apo-B gene. Nuclei from HepG2 or HeLa cells were digested with DNase I at the concentration indicated above each lane in the autoradiograms. After digestion, DNA was purified from each sample, secondarily cleaved with either *Hin*dIII and *Pst*I (panel 1) or *Hin*dIII and *Bgl*II (panel 2), electrophoresed on 1.2% agarose gels, blotted onto nitrocellulose paper, and probed with ^{32}P-labeled probe A or B. The numbers on the left of the first autoradiogram show the positions of ^{32}P-labeled ϕX174 + λ *Hin*dIII restriction fragments, electrophoresed with the samples, to be used as size markers. The number on the left of the HepG2 *Hin*dIII-*Bgl*II digest indicates the size of the original fragment. The numbers on the right of the HepG2 autoradiograms indicate the sites cut by DNase I. Below the autoradiograms is a restriction map of the *Hin*dIII-to-*Bgl*II region of interest. The two small open boxes in the linear map represent exons 1 and 2 of the gene. The numbers above the line represent the distance in base pairs relative to the transcriptional start site (+1). Key restriction sites are indicated below the line. TATA denotes the TATA box. The arrows show the positions of the DH sites. Below the map are shown the segments of DNA used as probes.

Figure 3 shows some representative results. A genomic map of the 5′ region of the human apo-B gene, from −1800 (*Hin*dIII site) to +2350 (*Bgl*II site) from the transcriptional start site, is shown at the bottom of Fig. 3.

When the *Hin*dIII-*Pst*I fragment was analyzed with probe A or B in HepG2 cells, it was observed that the original 2.1-kb fragment was progressively digested with increasing amounts of DNase I. Concomitantly, two new bands appeared, designated as 1 and 3. From the sizes of bands 1 and 3, determined by their gel mobilities relative to known restriction fragments, two DH sites were localized on the map at the positions indicated by arrows (Fig. 3, bottom). When nuclei from HeLa cells were treated in an identical manner with DNase I, the *Hin*dIII-*Pst*I fragment was resistant to degradation and no DH sites were detected.

To verify these results, the region from *Hin*dIII at −1800 to *Bgl*II at +2350 was examined by using the same techniques. Again, we observed in HepG2 cells a rapid degradation of the original 4.1-kb fragment by DNase I and the appearance of a new DH site localized in intron 2, designated as site 4, in addition to sites 1 and 3 already described. The positions of sites 1, 3, and 4 were confirmed by using probe B (data not shown). These sites were absent from HeLa cell nuclei, although some degradation of their DNA by DNase I did occur, generating a smear below the parental band. Next, we examined in more detail the region from −899 to +121 (*Pvu*II-*Pvu*II) that exhibits tissue-specific promoter activity. When HepG2 cell nuclei were treated with DNase I and *Pvu*II and probed with a *Pvu*II-*Stu*I fragment, three DH sites were observed: sites 1 and 3, described above, plus an additional site, designated as site 2, that was not detected in Fig. 3. The location of site 2 is indicated on the map at the bottom of Fig. 4. In HeLa cells, one strong band appeared below the original fragment, but its position on the map did not coincide with any of the three sites observed in HepG2 cells.

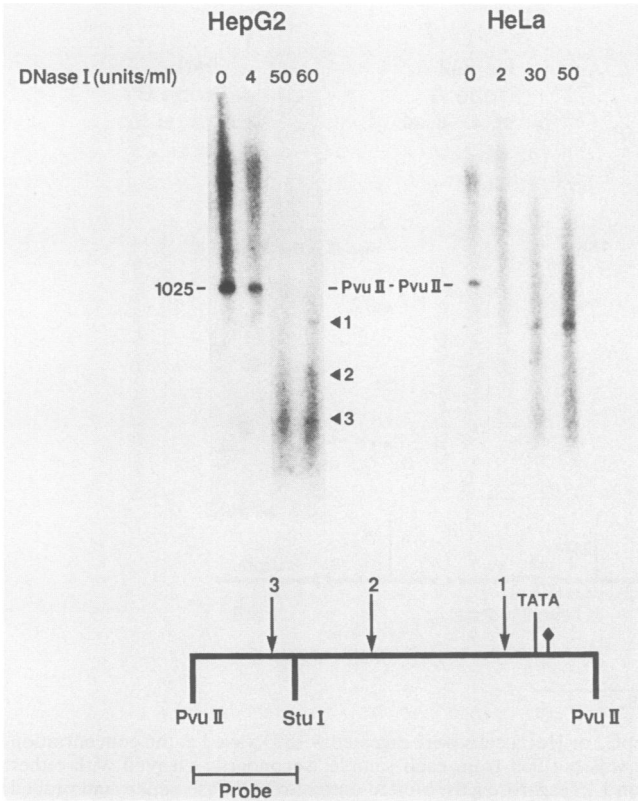

FIG. 4. DH sites in the *Pvu*II-*Pvu*II promoter fragment of the apo-B gene. Nuclei from HepG2 or HeLa cells were isolated and processed as described in the legend to Fig. 3, except that the secondary digestion was with *Pvu*II. On top are the autoradiograms in which the *Pvu*II-*Stu*I fragment was used as a probe. Arrows above the restriction map denote DH sites. ↟, Transcriptional start site.

From the combined data in Fig. 3 and 4, we conclude that there are three DH sites within the 5′ 1.1-kb *Pvu*II fragment and one DH site within intron 2 of the human apo-B gene. These DH sites are present in HepG2 cells, but not in HeLa cells.

Because apo-B is also synthesized in the human intestine, we looked for the presence of DH sites in intestine-derived CaCo-2 cells. These cultured cells differentiate into intestinelike cells over a period of about 13 days after reaching confluency (18). CaCo-2 cells synthesize apo-B mRNA in both the undifferentiated and differentiated states (Fig. 2). However, because it is not known whether there are structural rearrangements in chromatin during the differentiation process, the DH sites in CaCo-2 cells before and after differentiation were compared.

Figure 5 illustrates two sets of experiments. In the experiment shown in panel 1, the *Hin*dIII-to-*Pst*I region was examined; in the experiment shown in panel 2, the *Hin*dIII-to-*Bgl*II region was analyzed for the presence of DH sites in both undifferentiated and differentiated CaCo-2 cells. DH sites 1, 3, 3′, and 4 were observed in undifferentiated CaCo-2 cells, and DH sites 1 and 4 were observed in differentiated CaCo-2 cells. In other experiments with differentiated CaCo-2 cells, sites 2 and 3 were observed. DH sites 1, 3, and 4 map to the same location as those in HepG2 cells, whereas site 3′ is a new DH site, detected only in undifferentiated CaCo-2 cells. Because the existence of site 3′ is revealed by a DNA fragment that is difficult to detect, we cannot be sure that it is absent in the other cell types. It is clear, on the basis of experiments to be described below, that this region is hypersensitive to MNase in both HepG2 and undifferentiated CaCo-2 cells.

Fine-structure mapping experiments within the *Pvu*II-*Pvu*II fragment were performed with various probes in both undifferentiated and differentiated CaCo-2 cells. Figure 6 shows the results of one such set of experiments with undifferentiated CaCo-2 cells, in which the *Pvu*II-*Pvu*II fragment was used as a probe. Again, sites 1 and 3′ were observed, together with DH site 2, previously detected in HepG2 cells. Because the 1.1-kb *Pvu*II fragment was the probe in this experiment, cleavage at DH site 1 resulted in two separate bands: one corresponding to the fragment between DH site 1 and the 5′ *Pvu*II site and the other representing the fragment between DH site 1 and the 3′ *Pvu*II site. It is clear from the results in Fig. 5 and 6 that in undifferentiated CaCo-2 cells, site 1 is a DH site. However, a cut at site 4 was observed in the absence of DNase I and is presumably attributable to an endogenous nuclease activity. Similarly, sites 1 and 4 in differentiated CaCo-2 cells were sensitive to an endogenous nucleolytic activity. Thus, from these data alone, we cannot be certain that these two sites are hypersensitive to DNase I in CaCo-2 cells.

When the results of all the experiments with DNase I are summarized (Fig. 7, bottom), several conclusions emerge. First, HepG2, undifferentiated CaCo-2, and differentiated CaCo-2 cells all exhibit sensitivity at sites 1, 2, 3, and 4. The first three sites are localized in the 5′ flanking region of the

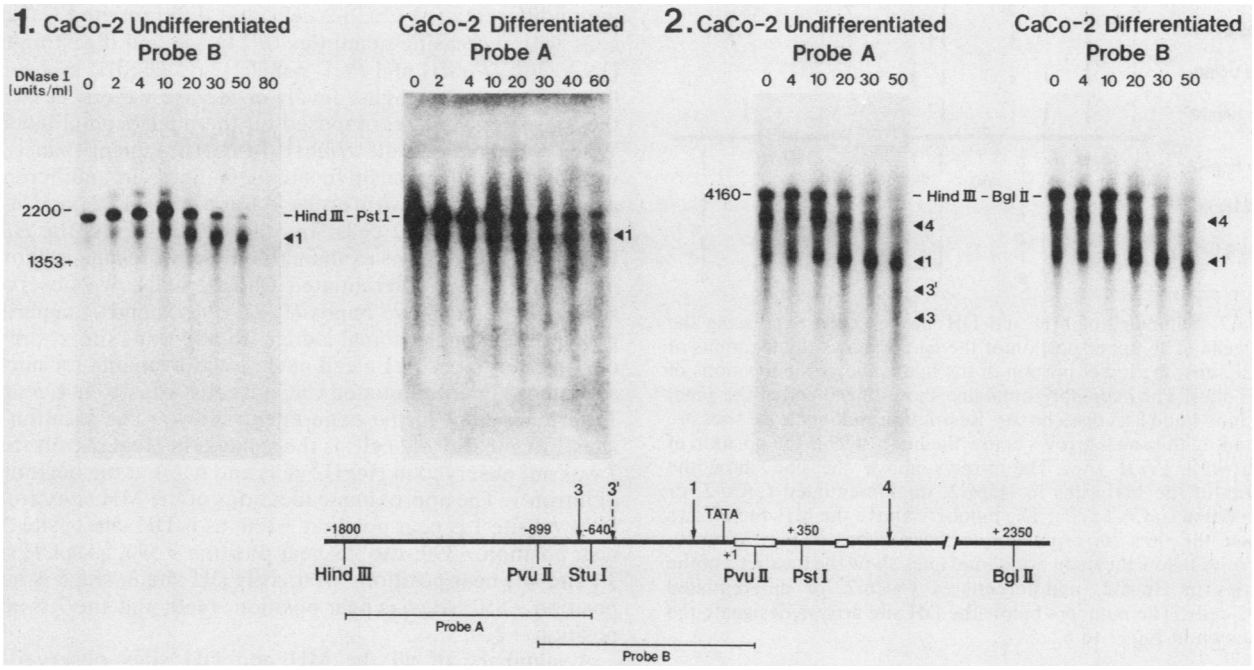

FIG. 5. DH sites in the 5′ end of the human apo-B gene in intestine-derived cells. Nuclei from either undifferentiated or differentiated CaCo-2 cells were prepared, treated with DNase I, and secondarily digested with restriction enzymes and processed as described in the legend to Fig. 3.

gene, while site 4 is localized in intron 2. Undifferentiated CaCo-2 cells exhibit an additional DH site (designated as 3′) not evident in the other cell types. None of these DH sites are present in HeLa cells. Because of the many different experiments performed with each cell type, the locations of the DH sites could be mapped accurately, with a resolution of ± 10 to 40 nucleotides, depending on the nature of the band in question. Thus, DH site 1 maps near position −120, DH site 2 maps near position −440, DH site 3′ maps near position −540, DH site 3 maps near position −700, and DH site 4 maps near position +760 relative to the transcriptional start site.

In addition to the complete sequence of the human apo-B promoter region (28), we have determined the sequence of the corresponding portion of the mouse gene (B. J. McCarthy, E. H. Ludwig, unpublished results). Therefore, we searched for homologies between the genes of the two species in regions proximal to all nuclease-sensitive sites. Surprisingly, relatively long stretches of sequence similarities were found in the vicinity of every DH site: not only in the 5′ flanking region, but also in intron 2, near DH site 4. These sequences are compared in Fig. 8. DH site 1 maps some 12 bases 5′ to the region of sequence conservation. The same is true for DH sites 2 and 3′. Site 3 falls within the region of sequence homology, while site 4 is localized some 2 to 3 bases 3′ to the segment of sequence homology.

MNase hypersensitivity in the 5′ end of the human apo-B gene. To determine whether there are MH sites in the 5′ end of the human apo-B gene, we incubated nuclei from either HepG2 cells or from HeLa cells for 5 min at 37°C with low levels of MNase (Fig. 9). The purified DNA was then digested with *Hin*dIII and *Pst*I (Fig. 9, panel 1) or *Hin*dIII and *Bgl*II (Fig. 9, panel 2). As an additional control experiment, purified HepG2 DNA was digested with MNase in parallel with the nuclei and then cut with restriction enzymes in a similar manner. The resulting blots were probed with probe A (*Hin*dIII to *Stu*I) or B (*Pvu*II to *Pvu*II) or both. Figure 9 depicts some of these results. When HepG2 nuclei were digested with MNase, a progressive degradation of the original *Hin*dIII-to-*Pst*I 2.1-kb fragment was observed with increasing amounts of MNase. As MNase digestion proceeded, a series of broad bands appeared. These correspond to nucleosome polymers derived from MNase cuts in linker regions between nucleosomes. Superimposed on this pat-

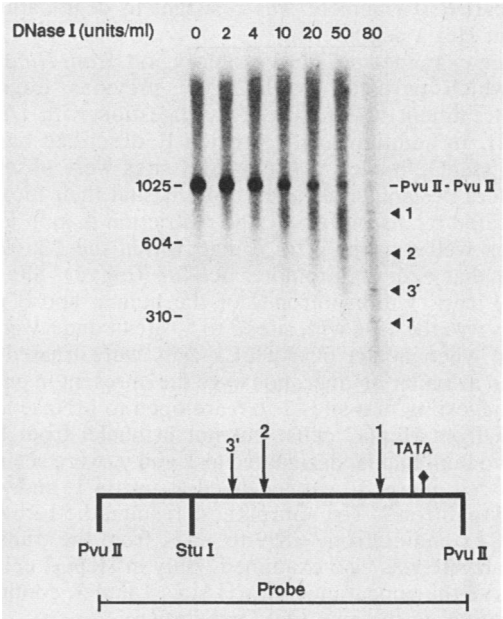

FIG. 6. DH sites in the promoter region of the apo-B gene in undifferentiated CaCo-2 cells. The experimental design and data layout are as described in the legend to Fig. 4.

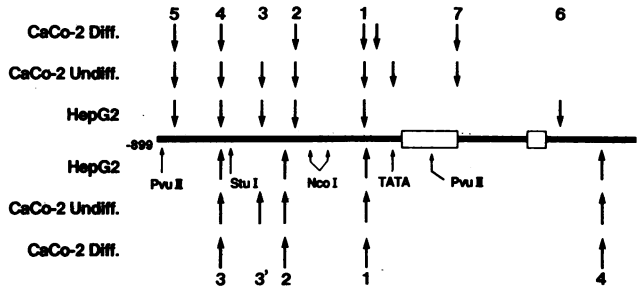

FIG. 7. Summary of MH and DH sites in cells expressing the apo-B gene. The upper portion of the figure shows the locations of the MH sites; the lower portion of the figure shows the locations of the DH sites. The main horizontal line shows the 5′ end of the gene, with exons 1 and 2 as open boxes. Restriction endonuclease sites are indicated by the small arrows below the line. −899 is the position of the leftward *Pvu*II site. The arrows above the line show the locations of the MH sites in HepG2, undifferentiated CaCo-2, or differentiated CaCo-2 cells. The numbers above the MH site arrows designate the sites corresponding to those shown in Fig. 9 and 10. The arrows below the main horizontal lines show the locations of the DH sites in HepG2, undifferentiated CaCo-2, or differentiated CaCo-2 cells. The numbers below the DH site arrows designate the sites shown in Fig. 3 to 6.

tern, four sharper bands, representing MH sites, appeared below the original band and have been designated as bands 2, 3, 4, and 5. The arrows above the restriction map in Fig. 9 show the positions of these MH sites. Their location was determined from the size of the DNA bands, deduced from their relative mobilities with respect to markers of known length. These sites were not found when HeLa cell nuclei were treated with MNase, nor were they evident in isolated HepG2 cell DNA, suggesting that the chromatin structure of the human apo-B gene in HepG2 cells selectively exposes these sites to MNase; therefore, MNase does not merely cut DNA in a sequence-specific manner (8, 17). Furthermore, the *Hin*dIII-*Pst*I fragment was resistant to degradation by MNase in HeLa cell nuclei.

We then examined a slightly larger region, from *Hin*dIII to *Bgl*II, which partially overlaps the previous fragment. MNase treatment was followed by digestion with *Hin*dIII and *Bgl*II. In addition to the previously described bands 4 and 5 in HepG2 nuclei, two new MH sites were observed. These were designated as sites 1 and 6, and their locations are indicated by arrows above the restriction map in Fig. 9. Site 1, as well as sites 2 to 5, map within the 1-kb *Pvu*II fragment that exhibits promoter activity (Fig. 1). Site 6, in contrast, maps within intron 2 of the human apo-B gene. Again, as was the case with sites 2 to 5, sites 1 and 6 were not observed when nuclei from HeLa cells were treated with MNase in a similar manner, nor were they present in purified DNA, suggesting that sites 1 to 6 are open to MNase attack in nuclei from HepG2 cells, but not in nuclei from HeLa cells. Two faint bands, designated as x and y, were seen only in HeLa cell nuclei; y was localized to intron 1, and x was localized to intron 2. To confirm these results, the 1-kb *Pvu*II fragment extending from −899 to +121 from the transcriptional start site was also examined. Only in HepG2 cells did we observe the appearance of MH sites 1 and 3, confirming their location on the map (data not shown).

The 5′ end of the apo-B gene was then examined for MH sites in CaCo-2 cells at confluency and 15 days after confluency. Figure 10 illustrates the results obtained from treating undifferentiated CaCo-2 cells and differentiated CaCo-2 cells with increasing quantities of MNase and digesting the DNA with *Hin*dIII and *Pst*I (panel 1) and *Hin*dIII and *Bgl*II (panel 2). Because higher levels of MNase were necessary, the MH sites are superimposed on the nucleosomal ladder. When we examined the *Hin*dIII-to-*Pst*I fragment (panel 1), we observed hypersensitive sites 1, 4, and 5 in undifferentiated CaCo-2 cells (with probe A) and sites 1, 2, and 4 in differentiated CaCo-2 cells. In other experiments, the *Hin*d III-to-*Bgl*II region was examined; one set of results is shown in panel 2. In undifferentiated CaCo-2 cells, we observed several hypersensitive bands (1, 2, 3, 4, 5, and 7) superimposed on the nucleosomal ladder. In addition, site x, previously observed in HeLa cell nuclei, was available for nuclease attack. In differentiated CaCo-2 cells, sites 1, 2, 4, 5, and 7 were apparent in the experiment shown. The location of sites 1 to 5 in CaCo-2 cells is the same as in HepG2 cells. Site 7 was not observed in HepG2 cells and maps at the beginning of intron 1. The approximate locations of the MH sites are as follows: site 1 is near position −120, as is DH site 1; site 2 is near position −390; site 3 is near position −530, as is DH site 3′; site 4 is near position −700, as is DH site 3; site 5 is near position −850; site 6 is near position +460; and site 7 is near position +210.

A summary of all the MH and DH sites observed is provided in Fig. 7. MH sites 1, 2, 4, and 5 were present in HepG2 cells, as well as in undifferentiated and differentiated CaCo-2 cells. Site 6 was observed in HepG2 cells, and site 7 was observed in undifferentiated and differentiated CaCo-2 cells. Two additional sites, indicated in Fig. 4 by arrows between site 1 and the TATA box, were sometimes observed in CaCo-2 cells. MH sites 2, 5, 6, and 7 appear to have no counterpart DH sites, because the specificities of the two enzymes do not always overlap.

Because we had observed a high degree of evolutionary conservation of the DNA sequences surrounding DH sites, we compared the DNA sequences in the vicinity of the MH sites in the mouse and human apo-B genes. The results are shown in Fig. 11. As with all of the DH sites, we found

FIG. 8. Base sequence conservation of DH sites in the human and mouse apo-B gene. The boxes indicate stretches of sequences from the human and mouse apo-B genes located near the five DH sites, with divergent flanking sequences outside the boxes. Lowercase letters indicate mismatched bases.

stretches of sequence similarity between the mouse and human genes in the vicinity of MH sites 1 to 6. Although MH site 7 did not show any significant degree of sequence conservation, these sequences are listed for comparison in Fig. 11.

Presence of nucleosomes in the apo-B promoter region. Within the 1-kb *Pvu*II fragment exhibiting tissue-specific promoter activity (Fig. 1), there are at least three DH sites and five MH sites. To determine whether any nucleosomes were present along this DNA fragment, we incubated nuclei from HepG2 cells, undifferentiated CaCo-2 cells, and HeLa cells with sufficient MNase to generate nucleosome ladders that were evident on ethidium bromide-stained gels. Southern blot analysis of these DNA samples, with the 1-kb *Pvu*II fragment as a probe, revealed a regular array of nucleosomes in all three cell types (Fig. 12). Furthermore, the spacing between individual nucleosomes appeared to be slightly different among the various cells, suggesting that the positioning of nucleosomes along the promoter region of the human apo-B gene may vary depending on the cell line.

DISCUSSION

It is now well established that sites within the promoter region of active or potentially active genes are hypersensitive to various nucleases (9). Although it is not clear in detail what feature of chromatin structure is recognized, evidence points toward stretches of DNA free of nucleosomes, binding sites for other proteins, and regions of torsional stress. Enzymes used to demonstrate hypersensitivity include DNase I (10, 39), MNase (21, 22, 31), undefined endogenous nucleases (11), S1 nuclease (25), and restriction endonucleases (33).

Of the two enzymes used in our experiments, DNase I has been used most often as a probe for hypersensitivity. Nevertheless, despite its marked preference for A+T-rich regions and its preferential affinity for nucleosome linker regions, MNase has also proven to be a useful probe of chromatin structure (22). Because MNase has a strong preference for internucleosomal linker regions, additional information regarding nucleosome positioning often can be derived from these types of studies (1). In many cases, DH and MH regions are coincident (19, 21).

The 5′ flanking region of the human apo-B gene is DH and MH in nuclei from two eell lines that express the gene, HepG2 and CaCo-2 cells, but not in HeLa cells. The hypersensitive region extends over 2 kb. Most sensitive sites are common to HepG2 and CaCo-2 cells, although site 3′ appears only in undifferentiated CaCo-2 cells. Five MH sites within the promoter region are common to HepG2 and CaCo-2 cells. Two of these sites are hypersensitive to both nucleases, demonstrating that they reflect highly accessible chromatin regions. The occurrence of such sites in the region of the gene immediately 5′ to the CAAT and TATA elements characteristic of many promoters is common to many genes (19). Likewise, interspecies conservation of base sequences of the region from the transcriptional start site to base −200

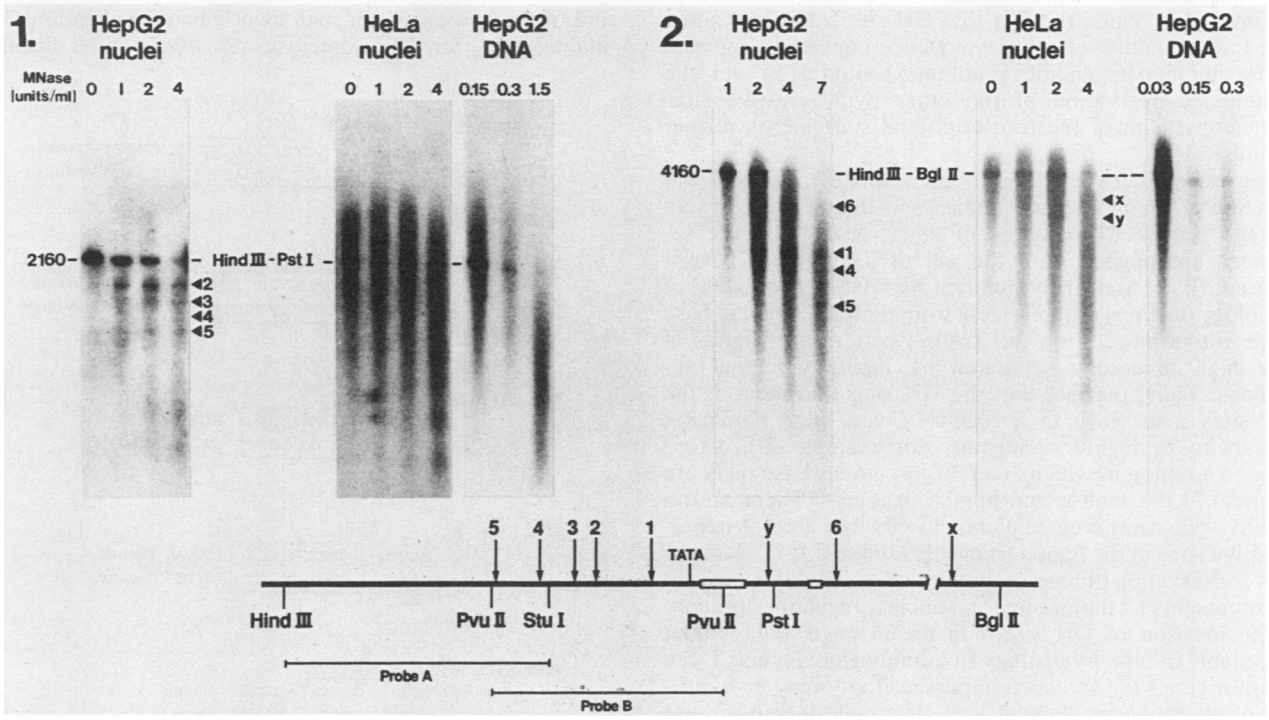

FIG. 9. MH sites in the 5′ end of the human apo-B gene. Nuclei from HepG2 or HeLa cells and purified DNA from HepG2 cells were digested with MNase at the concentration indicated above each lane in the autoradiograms. After digestion, DNA was purified from each sample, secondarily cleaved with either *Hin*dIII and *Pst*I (panel 1) or *Hin*dIII and *Bgl*II (panel 2), electrophoresed on 1.2% agarose gels, blotted onto nitrocellulose paper, and probed with ^{32}P-labeled probe A or B. The number on the left of the HepG2 *Hin*dIII-*Pst*I and *Hin*dIII-*Bgl*II digest indicates the size of the original fragment. The numbers on the right side of the HepG2 autoradiograms indicate the sites cut by MNase. Below the autoradiograms is a restriction map of the *Hin*dIII-to-*Bgl*II region of interest. The two small open boxes in the linear map represent exons 1 and 2 of the gene. Key restriction sites are indicated below the line. TATA denotes the TATA box. The arrows show the positions of the MH sites. Below the map are shown the segments of DNA used as probes in the above experiments.

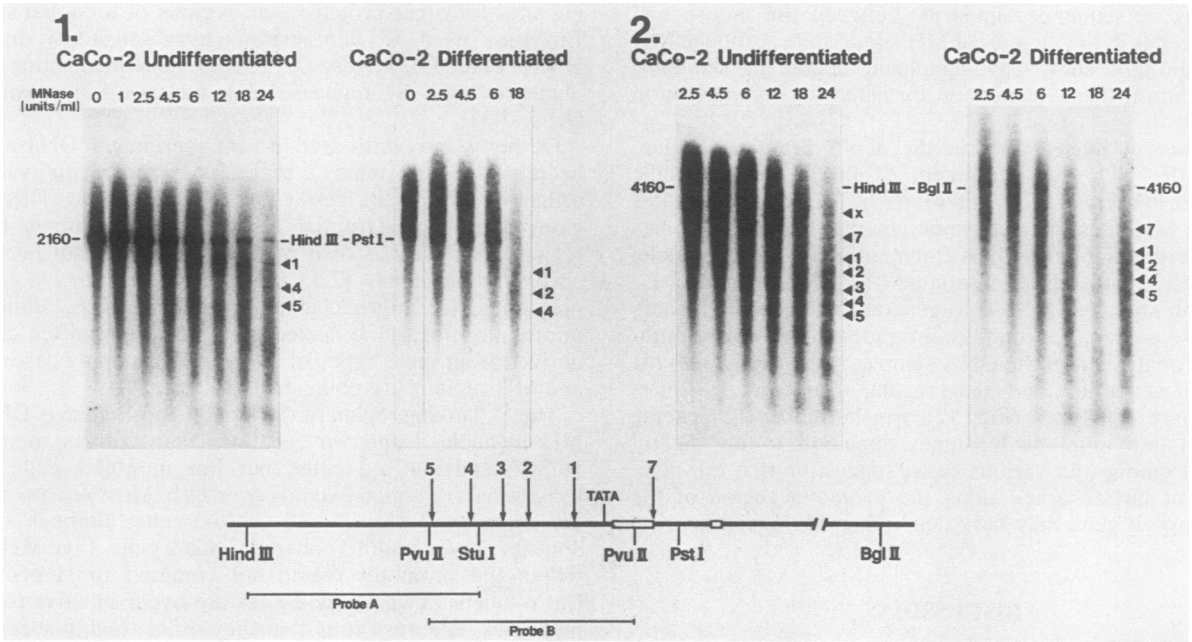

FIG. 10. MH sites in the 5′ end of the human apo-B gene in intestine-derived cells. Nuclei from either undifferentiated or differentiated CaCo-2 cells were treated with MNase, digested with restriction enzymes, and processed as described in the legend to Fig. 9.

has been observed in several mammalian genes. For example, five sequence blocks between bases −130 and +54 are highly conserved in the promoter region of the hepatocyte serum albumin gene in the human, rat, mouse, and chicken genomes (16). Thus, the fact that DH site 1 and MH site 1 map to the vicinity of a base sequence conserved between mouse and human genomes is not unexpected. However, the sequence conservation of the other hypersensitive sites farther upstream of the transcriptional start merits further comment.

The region between base −900 and the transcriptional start site of the mouse and human apo-B genes displays an average sequence homology of about 50% when the sequences are aligned with the aid of a computer best-fit program (B. J. McCarthy, unpublished data). The extent of homology declines progressively from the TATA box to base −900. Superimposed on this overall pattern are islands of very high homology separated by highly divergent sequences. Thus, the fact that the DH sites and most of the MH sites map close to a region of very high homology appears to be highly significant. For example, DH site 2 maps to a region in which 26 of 30 contiguous base pairs are identical in the mouse and human genomes. There are no equally well-conserved sequence motifs that are not hypersensitive sites in the region from −399 to the CAAT element. This conservation of base sequence is most readily explained if it represents a binding site for a nuclear regulatory protein.

The location of DH site 4 in an intron is not without precedent. In fact, by analogy to immunoglobulin and T-cell receptor genes (2, 38), such hypersensitivity may be indicative of an enhancer element (12). The high degree of base sequence conservation in the region of DH site 4 in intron 2 is even more striking, because intronic sequences in general display a high rate of evolutionary sequence divergence. Beginning at base +727, 19 of 24 base pairs in mouse and human genes are identical.

How might these DH and MH sites be related to transcriptional control? DH sites are often diagnostic of regions important in transcription, although not all regions in which transcription factors bind are DH. Furthermore, some DH sites reflecting deformations in chromatin structure may correlate with other important elements in the overall structure of the chromosome, such as origins of replication (32), binding sites for topoisomerases (4, 40), or cell lineage-

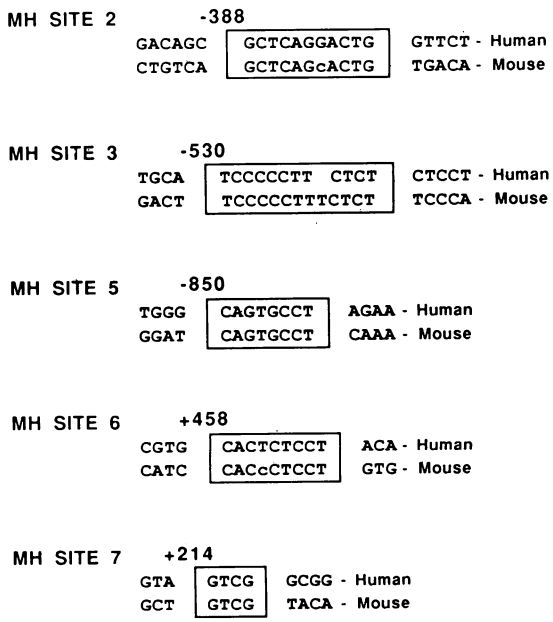

FIG. 11. Base sequence conservation of MH sites in the human and mouse apo-B genes. The boxes indicate stretches of sequences from the human and mouse apo-B genes located near the MH sites, with divergent flanking sequences outside the boxes. MH sites 1 and 4 correspond to DH sites 1 and 3, respectively, and their corresponding sequences are shown in Fig. 8. Lowercase letters indicate mismatched bases.

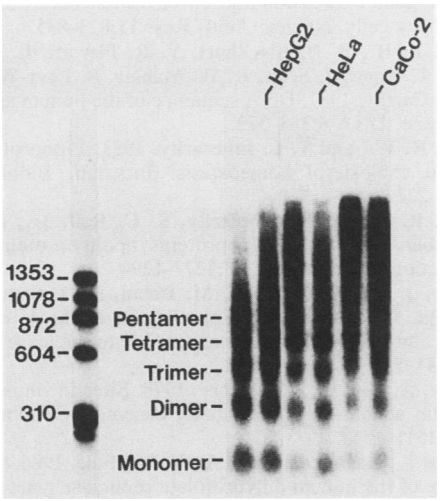

FIG. 12. Presence of nucleosomes in the human apo-B promoter region. Nuclei from HepG2, undifferentiated CaCo-2, and HeLa cells were digested with MNase. The purified DNA was electrophoresed on a 1.8% agarose gel and then subjected to Southern blot transfer onto nitrocellulose paper and hybridization with the ^{32}P-labeled 1-kb *Pvu*II fragment used in Fig. 1. The left-hand column represents radioactively labeled DNA fragment markers. For each cell line, two adjacent lanes representing two levels of MNase are shown.

specific signals for differentiation (15, 37). Among the DH sites that do correspond to binding sites for transcription factors, some may be for factors common to many genes (34), some may be tissue specific, i.e., liver or intestine specific, and still others may be gene-specific regulatory elements. Moreover, a number of proteins may interact with even a limited part of the 5′ flanking region, as is the case for the 75-base-pair repeat of the Moloney murine leukemia virus enhancer (35).

We have determined, through the use of fusions of the bacterial CAT gene with a set of deletions of the apo-B promoter, that tissue-specific negative and positive regulatory elements in the human apo-B gene both reside in the region between −250 and −80 bp relative to the transcriptional start site (B. D. Blackhart et al., unpublished results). Furthermore, gel retention assays have shown that several different nuclear protein-binding sites occur in the region between −899 and the transcriptional start and that the pattern of binding differs among nuclear proteins from different cells. To date, two of these sites have been localized by footprinting: one corresponding to DH site 1 and MH site 1, and the other corresponding to DH site 3 and MH site 4. In both cases the footprint corresponds to a linker region as revealed by MNase digestion and maps within the evolutionary conserved sequences listed in Fig. 8 (M. A. Onasch et al., unpublished data). By analogy with these results, we expect that the other regions of conserved sequences will also bind regulatory proteins. These experiments are in progress.

In many instances, DH sites occur in a region free of one or more nucleosomes. This does not appear to be the case in the human apo-B promoter region, since in the vicinity of all DH sites there are short MNase-sensitive stretches at about 200-base-pair intervals that may correspond to internucleosomal linker regions. Furthermore, the data in Fig. 12 show that nucleosomes are present along the promoter region (*Pvu*II fragment) of the apo-B gene in all three cell lines examined. The nucleosomes appear to be regularly spaced, with a periodicity of about 190 base pairs for HepG2 cells and slightly longer for CaCo-2 cells and HeLa cells. This result, together with the data on the tissue-specific localization of the MH sites, suggests that nucleosomes may occupy unique positions along the apo-B promoter region in all three cell types examined. In HepG2 cells and CaCo-2 cells, the nucleosomes would expose MH sites in the linker regions, while in HeLa cells, the same sequences would be within the core particle.

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