Nuclease-hypersensitive Sites Define a Region with Enhancer Activity in the Third Intron of the Human Apolipoprotein B Gene*

(Received for publication, May 4, 1992)

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The positions of several DNase I-hypersensitive (DH) sites have been mapped in the second and third introns of the human apolipoprotein B gene. Two such DH sites, I and V, are present both in human hepatoma (HepG2) and colon carcinoma (CaCo-2) cells that express the gene but absent from HeLa cells that do not express the gene. These DH sites map near sequence elements that have been highly conserved between the human and mouse genes. A PvuII-EcoRI fragment (+1064 to +2977) from the hypersensitive region exhibited enhancer activity, which was further localized by means of deletion experiments to a 155-base pair segment located entirely within the third intron and flanked by two DH sites. Three DNase I footprints were observed within this core enhancer, one of which contains putative binding sites for three liver specific nuclear proteins. Experiments are presented that suggest that this enhancer operates by a similar mechanism as that described previously for the strong second intron enhancer, involving an interaction with the basal transcriptional machinery. Digestions with low levels of micrococcal nuclease were performed to ascertain whether nucleosomes were present in the DNase I sensitive enhancer region. Nine different micrococcal nuclease-hypersensitive (MH) sites were detected in HepG2 cells but not in HeLa cells; one MH site was common to both cell types, and HeLa cells exhibited three unique MH sites. The first six MH sites (I–VI) are spaced approximately 200 base pairs apart, suggesting the presence of positioned nucleosomes in that region. MH sites VI–X are more closely spaced, suggesting either additional cutting sites within the core particle or the absence of one or two nucleosomes in this segment of the third intron enhancer.

A useful tool in studies designed to identify tissue-specific developmentally regulated or hormonally induced regulatory regions in eukaryotic genes is the determination of the locations of nuclease-hypersensitive sites, in particular, of DNase I-hypersensitive sites (DH sites) for reviews see Eissenberg et al., 1985; Elgin, 1988. Thus, as a first step in trying to identify a regulatory region such as a tissue-specific segment of DNA, it is desirable to map the positions of DH sites in the vicinity of the region of interest. Once one or more tissue-specific sites have been identified, one can proceed with functional assays using either transient or stable transfections into cell types that express or do not express the gene, to localize specific DNA sequence elements required for promoter (Paulweber et al., 1991b), enhancer (Brooks et al., 1991), or repressor activity (Paulweber et al., 1991a). For genes that are regulated in a tissue-specific manner, these manipulations performed in cultured cells can yield valuable information comparable with that obtained using transgenic mouse models to address questions regarding sequences involved in tissue-specific control of gene expression (Simonet et al., 1991).

In the case of the human apolipoprotein B (apoB) gene, which is expressed exclusively in the liver and intestine (Knott et al., 1986), the combination of approaches using DNase I hypersensitivity first, to delineate a regulatory region of interest, followed by transient transfections, DNase I footprinting, and gel retention experiments, has yielded a large body of information regarding its regulation in hepatic and intestinal cells in culture together with some insight into why the gene fails to be expressed in cells such as HeLa cells (Levy-Wilson et al., 1988; Levy-Wilson and Portier, 1989). Thus, important elements have been characterized in the +1 to −898 region of the gene (the promoter region) (Paulweber et al., 1991b) as well as in the 5′ upstream region extending from −898 to −3200 (the repressor region) (Paulweber et al., 1991a) and in a segment of the second intron enhancer (from +621 to +1064) (Brooks et al., 1991; Brooks and Levy-Wilson, 1992).

Earlier studies examining the nuclease sensitivity of the apoB promoter region revealed several DH and MH sites in the region from −898 to +121 (Levy-Wilson et al., 1988). Furthermore, a strong DH site was localized to approximately position +760, in the second intron of the gene. Subsequent work in our laboratory revealed a tissue-specific enhancer in the region surrounding this DH site (Brooks et al., 1991). Most of the enhancer activity was recovered as a 147-bp fragment “the core enhancer,” containing a 98-bp DNase I footprint. Detailed studies of the core enhancer further revealed that three liver-enriched transcription factors act syn-
ergistically to promote enhancement of transcription (Brooks and Levy-Wilson, 1992).

One of our goals is to understand how the transcription factors that bind to the enhancer interact with the enhancer DNA in native chromatin. We wish to learn whether binding of regulatory proteins prevents or interferes with the association of nucleosomes in that region. During the course of these studies, we asked whether tissue-specific DH and MH sites (that may be indicative of a nucleosome-free region) may lie 3' to the second intron enhancer. Our studies revealed several new DH and MH sites in this region of the gene encompassing the second and third introns. These findings prompted us to search for additional regulatory elements. Our data demonstrate that the segment of the apoB gene from +1064 (PvuII) to +2977 (EcoRI) harbors an enhancer activity, distinct from that present in the 5' end of the second intron. Deletion experiments have localized the enhancer activity to a small segment in the third intron of the gene. The chromatin structure close to the intron 3 enhancer differs between transcriptionally active HepG2 cells as compared with transcriptionally inactive HeLa cells.

**MATERIALS AND METHODS**

**Tissue Culture, Isolation of Nuclei, Nuclease Digestion, Gel Electrophoresis, and Southern Blotting**—HepG2, CaCo-2, and HeLa cells were cultured as described previously (Levy-Wilson et al., 1988). Preparations of nuclei, digests with DNase I and micrococcal nuclease (Mnase), gel electrophoresis, and Southern blotting were as described in Levy-Wilson et al. (1988).

**Plasmid Constructions and Transfections**—The PvuII-EcoRI fragment spanning nucleotides +1064 to +2977 of the human apoB gene was inserted at the HindIII site of the plasmid pPVUCAT (Brooks et al., 1991) that contains sequences from -898 to +121 of the apoB gene fused to the chloramphenicol acetyltransferase (CAT) gene. A 479-bp StyI-HaeIII fragment spanning nucleotides +1542 to +2021 of the human apoB gene was cloned using XbaI linkers into the XbaI sites of the plasmids pPVUCAT (Brooks et al., 1991) and -85CAT (Paulweber et al., 1991). To create a deletion of the StyI-HaeIII fragment, the DNA fragment from +1803 to +2977 was amplified by polymerase chain reaction using the oligonucleotides XCORE3' and XCORE5' as primers. The resulting DNA was digested with XbaI and inserted at the XbaI site of the plasmids pPVUCAT and -85CAT. The PvuII-EcoRI fragment (+1064 to +2977) to which HindIII linkers had been added was also cloned into the HindIII site of the plasmid containing the Mus-B1-XbaI fragment from the apoB reducer (+5627 to -2730) inserted at the XbaI site of -139CAT (Paulweber et al., 1991b).

Sequences of oligonucleotides listed 5' to 3' are as follows.

**XCORE3':** AAATCGTCTAGAGTACTGAGCAAACGCAGC

**XCORE5':** AAATCGTCTAGAGCATCTGGGGGAAGTTAACG

**RESULTS**

**DNase I Hypersensitivity in Introns 2 and 3 of the Human apoB Gene**—To identify possible DH sites in the second and third introns of the apoB gene, several sets of experiments were performed, a subset of which is described below. Employing small DNA fragment probes within a single-copy human gene in Southern blots produced signal intensities that varied from blot to blot. For this reason, we used three different sets of restriction digests and two different probes for each cell type in every blot. Therefore, our assignment of a particular DH or MH site is based on its localization in at least four independent blots.

Nuclei from transcriptionally active HepG2 and CaCo-2 cells and transcriptionally inactive HeLa cells were incubated with increasing amounts of DNase I followed by digestion of the DNA with StuI and EcoRI. In Fig. 1, we illustrate results obtained using the 3' probe (BglII-EcoRI). As we augmented the DNase I concentration we observed that the original 3615-bp fragment was progressively digested, giving rise to several new bands designated 1-V. By comparing the mobilities of the bands relative with that of DNA size markers, we localized the position of the DH sites along introns 2 and 3 as illustrated in the lower panel of Fig. 1. In HepG2 cells, five DH sites were clearly apparent, namely I, II, III, IV, and V. In CaCo-2 cells, only DH sites I, II, and V were evident. Instead of DH sites III and IV, CaCo-2 cells exhibited bands designated II', III', and IV'. HeLa cells, that do not transcribe the gene, revealed two DH sites resembling sites III' and IV' seen in CaCo-2 cells, in addition to DH site II, which was evident in all three cell lines.

DH site I, present in HepG2 and CaCo-2 cells but not in HeLa cells, fell within the well characterized 443-bp Smal-PvuII enhancer segment (Brooks et al., 1991). It is located in the vicinity of position +940 at the 3' boundary of the 98-bp footprint E described previously by us (Brooks et al., 1991) in a segment whose DNA sequence is highly conserved between the human and the mouse apoB genes (Ludwig et al., 1991, Fig. 2). Recently, we have shown that this sequence is the binding site for a nuclear protein designated protein I1 that is essential for the activity of the second intron core enhancer (Brooks and Levy-Wilson, 1992). DH site II, situated near position +1120, is a strong DH site in all three cell lines, suggesting that its presence is unrelated to transcriptional activity. However, DH II does not appear to be open and available to all nucleases, since micrococcal nuclease does not cut preferentially at this site, at least in HepG2 and CaCo-2 cells (Fig. 6). In CaCo-2 cells, two additional sites not apparent in HepG2 cells are seen within the second intron, at positions +1180 (DH II') and +1580 (III').

Within the third intron lie DH sites III (+1730), IV (+1780), IV' (+1890), and V (+1860). DH site IV' appears to be present in both CaCo-2 and HeLa cells and is therefore not transcriptionally significant. On the other hand, DH site V is observable in both expressing cell lines, although not in HeLa cells, suggesting that the region in which it resides may be important for the transcriptional activity of the gene in HepG2 and CaCo-2 cells. This proposition is supported by the high degree of homology in the vicinity of DH V, between the human and the mouse apoB genes (Fig. 2). The localization of all DH sites shown above was confirmed in all three cell lines by using a 339-bp 5' probe (StuI-NcoI) in StuI-EcoRI blots and in separate EcoRI blots probed with the BglII-EcoRI probe of Fig. 1 (data not shown).

**The Third Intron of the Human apoB Gene Contains a Transcriptional Enhancer**—The finding of tissue-specific DH sites in intron 3 suggested the presence of additional important regulatory elements in that region. To test this hypothesis, we first made a construct including sequences from the PvuII site at +1064 (immediately following the intron 2 enhancer characterized previously) to the EcoRI site at +2977.
Fig. 1. DH sites in introns 2 and 3 of the human apoB gene. Nuclei from HepG2, CaCo-2, 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 Stul and EcoRI, electrophoresed on 1.2% agarose gels, blotted onto nitrocellulose paper, and probed with a $^{32}$P-labeled BglI-EcoRI fragment shown at the bottom right-hand side of the figure. The numbers on the left of the first autoradiogram indicate the positions of $^{32}$P-labeled $\phi$x174 + $\lambda$HindIII restriction fragments, electrophoresed with the samples, to be used as size markers. 3615 is the size, in base pairs, of the Stul-EcoRI band. The roman numerals on the right of the autoradiograms indicate the sites cut by DNase I. Below the autoradiograms is a restriction map of the region of interest. Exons 2 and 3 are shown as black boxes. Numbers below the restriction enzyme sites indicate their positions, in base pairs, relative to the transcriptional start site (+1). Arrows show the positions of the DH sites in each of the three cell lines.
that the StyI-HaeIII fragment contains most, if not all, of the tested for enhancer activity. The core enhancer is a 155-bp fragment containing within it three DNase enhancer being consistently stronger than the forward one. This observation proves promoter by % fold in the forward orientation in HepG2 and the -898 promoter and upon a weak apoB promoter (-85 to -898 to +121). Data from four independent transfections of HepG2 cells (Fig. 3) revealed a 5-11-fold enhancement of the -85 promoter (right side) which were set at 100%, in HepG2 and CaCo-2 cells. F, forward orientation of the enhancer segment; R, reverse orientation of the enhancer segment. CAT activities are expressed as the mean value ± the standard deviation. The numbers in parentheses represent the number of independent transfections performed for each construct.

In CaCo-2 cells, the StyI-HaeIII fragment enhanced transcription of the -85 construct by 10-fold, but only in the reverse orientation.

We then performed DNase I footprinting analysis of a segment (from +1692 to +2021) of the StyI-HaeIII fragment that excludes exon 3. When this fragment was incubated with varying amounts of nuclear extract from mouse liver, three footprints were observed (Fig. 4) and designated A, B, and C. The results of the footprinting experiments suggested that the segment containing the three footprints may be crucial for the enhancer activity. To test this hypothesis, a DNA fragment (+1803 to +2021) designated the “core” enhancer, containing the three footprints was tested for its ability to enhance transcription from the -898 promoter and the -85 promoter in HepG2 and CaCo-2 cells (bottom of Fig. 3). In the context of the strong apoB promoter (-898), the core enhancer was weaker than the StyI-HaeIII fragment in HepG2 cells and failed to enhance at all in CaCo-2 cells. However, in the context of the -85 promoter, the core enhancer was stronger than the StyI-HaeIII fragment in both orientations, whereas in CaCo-2 cells it was about the same in the reverse orientation even though it did enhance 5-fold in the forward orientation.
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According to data presented above we conclude that the third intron contains an enhancer whose activity is strongest upon a weak apoB promoter (−85 to +121). Most of this activity can be localized to a region (the core enhancer) that includes three DNase I footprints. A computer search for similarities between footprint B and the binding sites for known transcription factors revealed some similarities between footprint B and the binding sites for the liver enriched factors: C/EBP (Costa et al., 1988) (a 7/10 match), TGT3 (Petropoulos et al., 1991) (a 6/8 match), and HNF-2 (LF-A1) (Monaci et al., 1988) (a 8/15 match), and HNF-2 (LF-A1) (Monaci et al., 1988) (a 7/10 match), TGT3 (Petropoulos et al., 1991) (a 6/8 match), and HNF-2 (LF-A1) (Monaci et al., 1988) (a 8/15 match) (Table I). A 6/8 match with the AP-1 recognition sequence (Lee et al., 1987) occurs within footprint C. No significant similarities were found with footprint A.

The Mechanism of Action of the Intron 3 Enhancer Probably Involves an Interaction with the Basal Transcriptional Machinery—Recently we showed that the mechanism of action of the intron 2 enhancer involves an interaction between the enhancer-bound proteins and components of the basal transcriptional machinery, rather than an interaction with proximal promoter sequences (Paulweber and Levy-Wilson, 1991). To determine whether the intron 3 enhancer acts by a similar mechanism we performed experiments illustrated in Fig. 5. In CaCo-2 cells, a construct containing the sequence from −139 to +121 of the apoB promoter (−139CAT) exhibits the highest transcriptional activity (Paulweber and Levy-Wilson, 1991). Indeed, it is transcribed so efficiently in CaCo-2 cells that insertion of intron 2 enhancer sequences immediately upstream of it actually decreases transcription presumably by disrupting the optimal interaction between the proximal promoter-bound factors and the initiation complex. Insertion of the PvuII-EcoRI fragment harboring the intron 3 enhancer upstream of the −139CAT construct yielded a similar result, that is, transcriptional activity of the −139CAT construct was reduced to 37% (Fig. 5). When an MboI-XbaI fragment, harboring a “reducer region” that down-regulates transcription of the −139CAT construct was inserted upstream of the −139CAT construct, an expected reduction of promoter activity was seen (Paulweber and Levy-Wilson, 1991).

We have proposed previously that the reducer exerts its negative effect on transcription by blocking the action of an activator that binds to the −111 to −88 segment of the apoB promoter (Paulweber and Levy-Wilson, 1991) and recently confirmed this hypothesis by identifying the reducer sequence in the 5’ upstream region of the gene and its target sequence in the proximal promoter region.2 This reducer effect leaves the basal transcriptional machinery accessible to other regulatory proteins, such as, for instance, those bound to an enhancer, as we showed was the case for the intron 2 enhancer (Paulweber and Levy-Wilson, 1991). An obvious corollary was whether the intron 3 enhancer would be capable of reversing the negative effect of the reducer. Indeed, results in Fig. 5 show that the PvuII-EcoRI segment containing this new enhancer activity does increase transcription (7-fold) of the −139CAT promoter that had been depressed by the reducer. This enhancer, however, is weaker than the intron 2 enhancer, since it cannot restore completely the activity of the original −139CAT construct, as does the second intron enhancer (Paulweber and Levy-Wilson, 1991). In summary, these data suggest that the intron 3 enhancer acts by a mechanism similar to the intron 2 enhancer, involving a direct interaction with the basal transcriptional complex.

Intron 3 of the Human apoB Gene Is Especially Sensitive to MNase—To elucidate whether nucleosomes may be present within the enhancers in introns 2 and 3 of the human apoB gene, we first performed an analysis of the hypersensitivity of these regions to MNase. To this end, nuclei from HepG2 and HeLa cells were incubated for 5 min at 37°C with increasing amounts of MNase (Fig. 6). The purified DNA was then cleaved with NotI and EcoRI or StuI and EcoRI. As a control, free DNA from HepG2 cells was digested with MNase in parallel with the nuclei, followed by restriction enzyme digestion and analysis by Southern blotting in the same manner (A). In this series of experiments, the 3’ BglII-EcoRI probe shown below the restriction map of Fig. 6 was used. In the case of HepG2 cells (A, Fig. 6), the original NotI-EcoRI fragment was digested progressively with MNase to yield fragments I–X. None of the same MH sites was observed when free DNA was cut with MNase, suggesting that they represent regions in HepG2 chromatin open and accessible to MNase. The positions of the MH sites are indicated in the bottom panel. MH sites I–III are approximately 200 bp distant from one another, as are MH sites IV–VI, suggesting that

2 B. Paulweber et al., submitted for publication.
MNase cuts in the accessible linker regions once every 200 bp, implying that nucleosomes may be "positioned" or "phased" (Eisenberg et al., 1985; Gross and Garrard, 1987; Grunstein, 1990). However, MH sites VI–X are much closer to one another, some 50–100 bp apart, implying the presence of some additional cutting sites accessible to MNase within the core particle or perhaps an absence of nucleosomes along the segment from MH sites VI–X. Indeed, no typical nucleosomal repeat (monomer to pentamer) is observable even at the higher MNase levels (A Fig. 6).

In contrast, MNase digests of nonexpressing HeLa nuclei contrast with those from HepG2 cells (A, Fig. 6). First, with the exception of MH site II, none of the other MH sites are present, but are replaced by three new bands, namely x, y, and z. Because x and y are sites open in free DNA, they do not reflect chromatin structure. If nucleosomes are phased in this region in HeLa cells, their phase is likely to differ from that in HepG2 cells. Moreover, in HeLa cells a clear nucleosomal repeat is seen in the HepG2 nuclear digests, even at the higher MNase levels (A Fig. 6).

The fascinating array of MH sites within intron 3, highly suggestive of a nonrandom nucleosome positioning in that region, prompted us to examine whether a similar situation applies to the intron 2 enhancer. Several sets of experiments were performed, one of which involved probing of the same blots shown in Fig. 6 with a 5′ probe. The results are shown Fig. 6B. The contrast between these data and that in Fig. 6A is evident; we see clear evidence of a nucleosomal repeat in both the HepG2 and the HeLa samples, suggesting that the segment beginning at the 5′ end at the NotI site and extending for at least 1 kilobase pair 3′ of that position (and therefore including the Smal-PvuII intron 2 enhancer) contains a normal array of nucleosomes in both HepG2 and HeLa cells. From these data we cannot discern whether the nucleosomes are phased equally, differently, or not at all in either cell line. However it appears that the nucleosomal organization of the gene differs in intron 2 as compared with intron 3. The reasons for these differences are not apparent at the moment but will be studied further.

**Discussion**

We recently characterized in detail a tissue-specific enhancer in the 5′-half of intron 2 of the human apoB gene (Brooks et al., 1991; Brooks and Levy-Wilson, 1992). Discovery of a tissue-specific DH site (DH4 (Levy-Wilson et al., 1988)) that mapped to an intron 2 segment that had been highly conserved between the human and mouse apoB genes alerted us to the presence of this important regulatory region.

In attempting to study in more detail the chromatin structure in the vicinity of the intron 2 enhancer, the sensitivity of the adjacent region to DNase I and MNase was examined. A number of new DH and MH sites were defined in HepG2 and CaCo-2 cells, where the gene is transcriptionally active. A few hypersensitive sites were also detected in HeLa cells, that do not express the gene. A summary of these data is presented in Fig. 7.

In addition to the previously identified tissue-specific DH site 4 at position +760 (Levy-Wilson et al., 1988), two new DH sites were found within intron 2 in HepG2 cells, namely I and II, four in CaCo-2 cells, namely I, II, II', and III', and two in HeLa cells. Within intron 3, HepG2 cells exhibited 3 DH sites, namely III, IV, and V; CaCo-2 cells displayed DH sites IV' and V and HeLa cells, DH site IV'. The finding of DH sites II, III', and IV' in HeLa cells indicates that formation of these sites is unrelated to the transcriptional activity of the gene. On the other hand, DH sites I, IV, and V
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**Fig. 6.** MH sites in introns 2 and 3 of the human apoB 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 NodI and EcoRI or Stul and EcoRI, electrophoresed on 1.2% agarose gels, blotted onto nitrocellulose paper, and probed with the BglII-EcoRI fragment shown as a black line at the bottom on the right-side of the figure (Panel A) or with the NodI-Apal fragment (B). Numbers on the left of the HepG2 blot indicate the positions of the size markers run on the same gel with the samples. The roman numerals on the right of the HepG2 blot indicate the MH sites, as do the letters and numbers on the left of the HeLa blot. The positions of the monomer to pentamer nuclease bands, visible in the HeLa blot are shown on the right side of the autoradiogram. Below the autoradiograms is a restriction map of the area of interest, similar to that described in Fig. 1. The arrows show the positions of the MH sites.
map to the immediate vicinity of footprints in highly conserved segments of DNA (Fig. 2), suggesting that their occurrence is related to the transcription process.

Indeed, the finding of DH IV and V in HepG2 cells prompted us to ask whether any regulatory elements of the gene may lie in the second half of intron 2 and in intron 3. Our results in Fig. 3 clearly show that intron 3 contains an enhancer activity localized within a 155-bp fragment harboring three DNase I footprints that is most active upon a weaker apoB promoter construct. At present we are testing the function of the intron 2 and intron 3 enhancers upon the tissue-specific expression of the apoB gene in transgenic mice. We wish to ascertain whether both enhancers are required for correct tissue expression in the liver and intestine of transgenic mice or whether one enhancer suffices for expression in the liver and the other one for intestinal expression. Preliminary results indicate that constructs lacking both enhancers are not expressed in transgenic mice.

We were also interested in the relative strength and mechanism of action of the intron 3 enhancer, as compared with the previously studied intron 2 enhancer. Data in Figs. 3 and 5, when compared with similar data for the intron 2 enhancer already reported (Paulweber and Levy-Wilson, 1991), suggest that the intron 3 enhancer is less active than the intron 2 enhancer and that both enhancers operate by a mechanism in which the enhancer-bound factors or their mediators (Flanagan et al., 1991; Lin et al., 1990; Pugh and Tjian, 1990) interact with the basal transcriptional machinery (Mitchell and Tjian, 1989).

Micrococcal nuclease digestion revealed a number of sites hypersensitive to this nuclease in introns 2 and 3 (Fig. 6). Indeed, the location of some of the MH sites coincided with those of some of the DH sites (Fig. 7). For instance, MH site II coincided with DH site II', whereas MH sites IV and V coincided with DH sites IV and V in HepG2 cells. This latter observation is interesting: the MH data suggest that sites IV and V, sensitive to both types of nucleases, must be positioned in the internucleosomal linker regions, and the DH data imply a proximal binding site for a nuclear protein. That this is indeed the case is demonstrated in Fig. 4, where footprints A and C lie very near DH (and MH) sites IV and V, respectively.

The MH data also indicate a difference in the distance between cutting sites along the region shown in Fig. 7. For example, MH sites I–III and IV–VI are positioned at approximately 200-bp intervals, suggesting the presence of precisely positioned or phased nucleosomes (Eissenberg et al., 1985; Gross and Garrard, 1987; Grunstein, 1990) in HepG2 cells. MH sites VI–X, on the other hand, are much more closely spaced, suggesting either the absence of one or two nucleosomes in this region or alternatively a more “open” nucleosome structure with additional cutting sites accessible within the core particles of the two nucleosomes that may reside in this area. The apparent absence of these MH sites in HeLa cells suggests that, at least within this segment in intron 3, the phasing frame may differ in a transcriptionally active cell type such as HepG2 cells versus an inactive cell type such as HeLa cells. Should our parallel studies in transgenic animals validate the importance of the enhancers in introns 2 and 3 in the expression of the apoB gene in liver and intestine in vivo, we will continue our chromatin studies to ascertain how the various transcription factors that bind to these enhancers interact with their cognate sites in a chromatin template. Alternative phasing configurations may offer an option to the removal of one or more nucleosomes as a mechanism for allowing transcription of a chromatin template; such a mechanism may only apply to regulatory elements that augment or decrease basal expression levels, such as enhancers and silencers and not be possible for promoters. It is not unreasonable to propose, based on our micrococcal nuclease data for the third intron, that in HepG2 cells, nucleosomes must occupy defined positions along the DNA with important regulatory binding sites left available in linker regions for interaction with the corresponding protein factors. In some small segments, a nucleosome or two may also be absent or removed if necessary to accommodate additional factors. In HeLa cells, however, the phasing would differ, either a second frame would be chosen in which the key DNA sequences would be placed within the core particles or positioning could be random, with multiple phases allowed, some of which would be sufficient to block binding of transcription factors. Such a mechanism may be appropriate for a gene like the apoB gene that is thought to be expressed in a constitutive manner in hepatic cells (Pullinger et al., 1989). The existence of a few MH sites in HeLa cells, albeit in different locations as those in HepG2 cells, argues against random phasing.

The observation that DH and MH sites IV and V coincide suggests that these important DNA-binding regions are positioned in internucleosomal linker regions. This fact alone may be sufficient to account for their functional interaction via DNA looping with the transcription initiation complex to enhance transcription of the gene. Unlike a gene whose expression is stimulated by a hormone, such as the glucocorticoid induction of the mouse mammary tumor virus promoter, and accomplished by selective and temporal removal of nucleosomes from the proximal promoter region (Cordingley et al., 1987), the apoB gene is transcribed constitutively in adult liver, and removal of nucleosomes would also have to be a constant process, perhaps energetically unfavorable. Studies aimed at detecting the presence of nucleosomes in the promoter and in the intron 2 enhancer of the apoB gene would yield data consistent with phased nucleosomes in HepG2 cells.

Although the detection of signals in these experiments with the single-copy apoB gene is not optimal, we consistently observe the presence of nucleosomes in these regulatory regions. Of course this does not preclude temporal nucleosome displacement from these regions during transcription (Felsenfeld, 1992) that we just cannot detect. In any event, it is likely that some structure with additional cutting sites accessible within the core particles of the two nucleosomes that may reside in this area.

Fig. 7. Summary of the locations of MH and DH sites in HepG2, CaCo-2, and HeLa cells. The upper panel shows the positions of the MH sites; the lower panel shows the locations of the DH sites. The central horizontal line shows a restriction map of the area of interest, with exons 1, 2, and 3 as black boxes and footprints A, B, and C as white boxes. A few relevant restriction sites are indicated below the line. The numbers on top of the central line represent a scale, in base pairs. The arrows represent the MH (upper panel) and DH (lower panel) sites, with the roman numerals corresponding to those in Figs. 1 and 6.

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3 A. Brooks, unpublished results.

4 B. Levy-Wilson, unpublished data.
that nucleosomes are positioned in expressing and nonexpressing cell lines in ways that facilitate or preclude reversible interactions of important regulatory sequences with transacting factors.

Acknowledgments—We are indebted to Dr. Brian J. McCarthy for "lending us" the talent of Erwin H. Ludwig in DNA sequencing and for his encouragement and comments on this manuscript. We also thank Kurt Haubold for his help with the database searches and William Doolittle for typing this manuscript.

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