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SEVERAL LIVER SPECIFIC DNASE HYPERSENSITIVE SITES ARE PRESENT IN THE INTERGENIC REGION SEPARATING HUMAN PLASMINOGEN AND APOPROTEIN(A) GENES

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Abstract: The genes coding for plasminogen and apoprotein(a) are clustered, together with two related genes, on the telomeric region of chromosome 6 at band 6q26-27. Moreover, the two genes are 40 Kb apart and transcriptionally pointing to opposite directions. Plasminogen and apoprotein(a) share a high degree of homology in the promoter and 5' flanking regions and their expression is mainly confined to liver. To assess whether common controlling elements mediate the tissue specific expression of these two genes, the region between them was investigated for DNase hypersensitive sites in hepatic cell nuclei.

YAC cloning has recently allowed to discover that the genes coding for plasminogen and apoprotein(a) are physically linked and separated by 40 Kb of genomic DNA(1). Furthermore, chromosome walking disclosed the presence of two additional genes (apoprotein(a)-like and plasminogen-like) sharing with the previous two a leader and sequences highly homologous to apoprotein(a)-plasminogen kringleIV, kringleV and protease modules (2). The entire plasminogen-apoprotein(a) gene cluster spans almost 400 Kb of genomic DNA and is located on the telomeric region of chromosome 6 at band 6q26 (2,3).

The inter and intragenic duplication events that gave rise to the four members of this family are also reflected in the promoter and 5' flanking sequences which share a high degree of homology. In particular, analysis of both apoprotein(a) and plasminogen promoters and 5' flanking regions revealed consistent homology for several hundred base pairs from the first ATG (1,4).

Interestingly the expression pattern of both genes seems to be mainly confined to liver although reduced amounts of apoprotein(a) RNA were found in testes and brain (5). On the other hand plasminogen RNA is also detected in kidney and very low amounts in testes (5).

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In order to ascertain whether common transcriptional control elements mediate the liver specificity of those two genes we started studying the cromatin conformation encompassing the 40 Kb plasminogen-apoprotein(a) intergenic region. Indeed experiments with DNase established that several liver specific hyperensitive sites (DH) were detected in nuclei prepared from the liver derived HepG2 cell line; however these sites were absent in the non hepatic HeLa. cell line.

MATERIALS AND METHODS

YAC cloning and lambda subcloning were as previously described (1).
Probes for DNase hypersensitive mapping were obtained as follow (see also Fig1B):
probe A is a 170 bp PCR amplified product from the plasminogen 5' flanking region (R.T. manuscript in preparation). Oligonucleotides used for PCR amplification were 5'GGTTGCTTCCATTTCAGACA3' and 5'TTTGTATGTTATATGTATGA3'. PCR conditions were denaturation for 1 min at 94°C, annealing for 1 min at 42°C and extention for 1 min at 72°C.The sequences corresponding to this region of the plasminogen 5' flanking region were submitted to GenBank data base with the accession number U07744. Probe B is a HindIII-BamHI fragment situated 4.7 Kb from the plasminogen start of transcription (12).
Probe C is a EcoRI-SacI restriction fragment within the plasminogen 5'flanking region and 6.7 Kb further upstream from probe B. Probe D is a XbaI-EcoRI fragment located 4 Kb from the apoprotein(a) start of transcription (4). The EcoRI site is derived from the plasmid used for subcloning and therefore is not represented in genomic DNA.
DNaseI hypersensitive sites were mapped by the indirect end labelling method procedure (13). Briefly, 1-5 x 10^8 cells were washed twice in PBS and resuspended in 25 ml of RSB (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$). The cells were lysed in 0.1% Nonidet P-40 in RSB and nuclei were visualized by staining with trypan blue and spun down at 1200 rpm, 5 min at 4°C. Nuclei were resuspended to a final concentration of 10^8/ml. Increasing concentrations of DNaseI (Boehringer Mannheim) were added to 10^7 nuclei/100ul in prepared tubes on ice. The mixtures were incubated for 5 min at 37°C. the reactions were terminated by adding 100 µl of a solution containing 0.6 M NaCl, 20 mM tris pH 7.5, 20 mM EDTA, 1%SDS. Proteinase K was added at 250 µg/ml for overnight at 55°C. DNA was purified by extraction with phenol-chloroform. Portions from the various DNaseI digests, each containing 10 µg of DNA, were further digested with HindIII, XbaI, EcoRI BamHI for 12 to 16 h at 37 °C. Southern blots of these time course experiments were probed with the probes described above.

RESULTS AND DISCUSSION

The plasminogen-apoprotein(a) gene family is clustered on the telomeric region of chromosome 6 (6q26-27) and the restriction map of the four members of this family is depicted in Fig1A. As can be assessed from both Fig1A and B plasminogen and apoprotein(a) genes are transcriptionally pointing to opposite directions (1,2)

The plasminogen-apolipoprotein(a) intergenic region was obtained from YAC 219E11 by cosmid and lambda subcloning (1). Some sequences belonging to this region were refractory to cloning and several attempts were made before obtaining overlapping clones. The restriction map of two of them (clone cos2B and λA1) is presented in Fig1B.

As depicted in Fig 2 A-D several fragments that were employed as probes revealed more than one band on genomic southerns due to the presence of related sequences in the 5' flanking regions of the apo(a)-like and plasminogen-like genes .

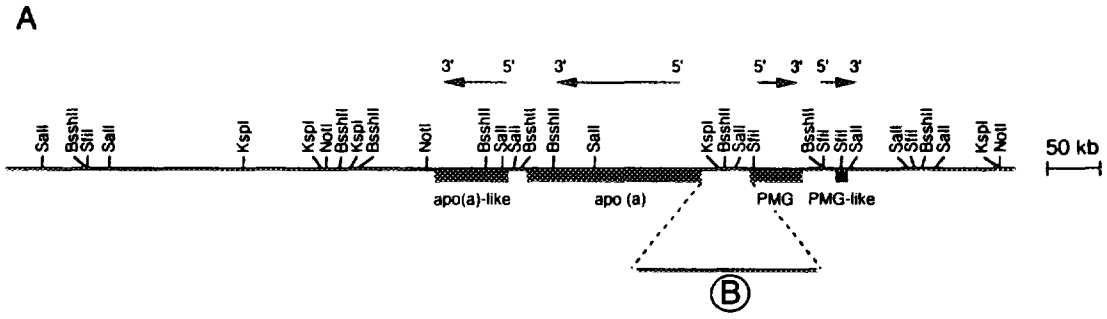

Fig1A. Long range map of the plasminogen-apoprotein(a) gene family. Close rectangles represent the four related genes. The transcriptional orientation of these genes is indicated by arrows.

In order to unambigously assign DH sites to the intergenic region, several restriction enzyme digestions and different probes had to be tested. Fig.2 A-D shows only one combination of restriction digestion and genomic probe which is specific for the intergenic region.

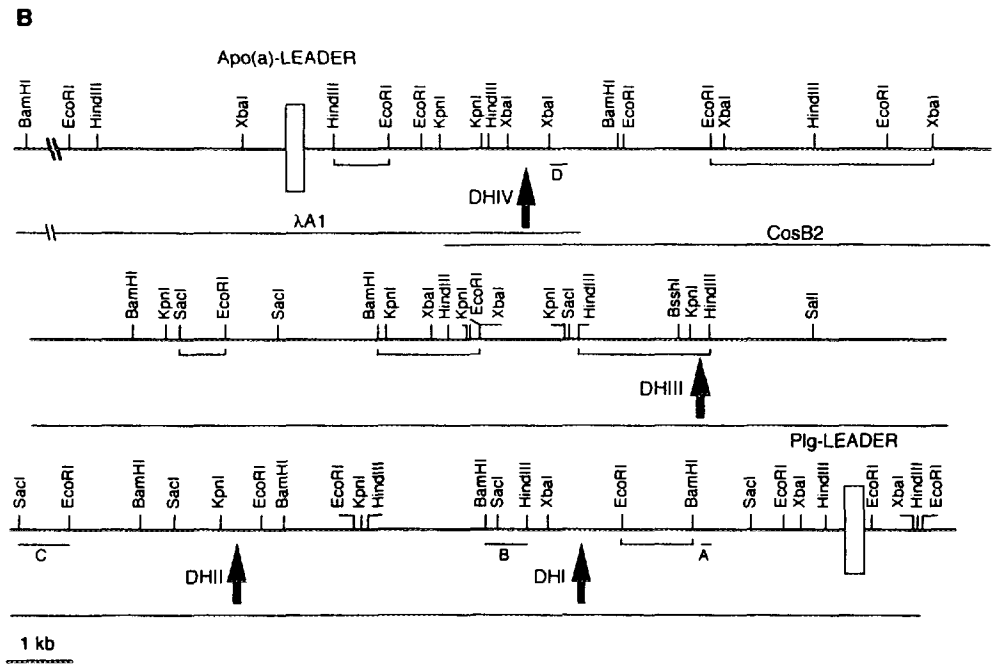

Fig1B. Restriction map of clones cos2B and λ1A comprising the plasminogen-apoprotein(a) intergenic region. White rectangles are the plasminogen and apoprotein(a) leader-5'UT exons respectively. Probes used for DNase hypersensitive mapping experiments are underlined and named A,B,C and D (see Materials and Methods). Brackets define restriction fragments harboring repetitive sequences. Black vertical arrows depict DNase hypersensitive sites.

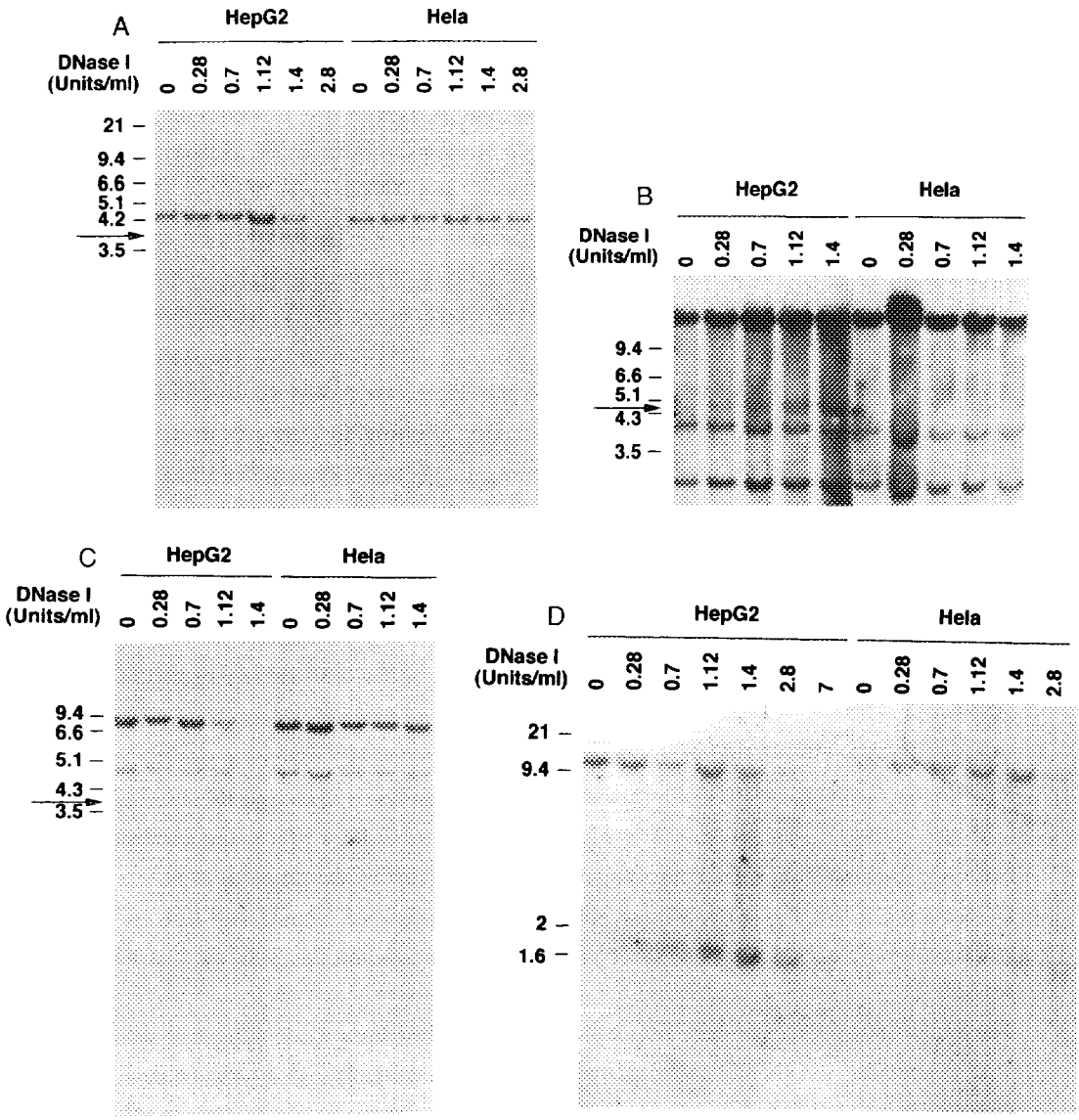

Fig 2. DNase hyperensitive site (DH) mapping.
HepG2 and HeLa nuclei were treated with serial dilutions of DNase as indicated above each blot. DNA was extracted, restricted with a suitable restriction enzyme and after southern transfer hybridized with different probes. A (DHI) HindIII digestion and hybridisation with probe A.
B.(DHII) XbaI digestion and hybridisation with probe B.
C (DHIII) EcoRI digestion and hybridisation with probe C. D (DHIV) BamHI digestion and hybridisation with probe D.

As pointed out in the same figure two liver specific DH sites (DHI, DHII) were mapped -4 and -9 Kb respectively from the plasminogen start of transcription (Fig. 2 A-B and Fig1B). An additional DH site (DHIII), exclusively present in liver, was localized at position -16 Kb (Fig.2 C and Fig1B) while another one (DHIV), which is also present in HeLa cell nuclei (although at

much higher DNase concentrations), is positioned -4 Kb from the apoprotein(a) cap site (Fig 2 D and Fig1B).

Sensitivity to various nucleases, in particular DNaseI, is a useful tool to assess the chromatin confomation of certain gene regions engaged in transcription (6,7). Active genes are preferentially sensitive to mild digestion by DNaseI and DNase hypersensitive sites reflect discrete regions of open chromatin accessible to various regulatory proteins.

We begun to study the chromatin structure of the genomic region encompassing the apoprotein(a) and plasminogen genes which are clustered, together with two pseudogenes, on the peritelomeric region of chromosome 6 (6q26) (1,2). The entire cluster has been shown to span approximately 400 Kb of genomic DNA and the genes coding for apoprotein(a) and plasminogen are 40 kb apart and transcritionally pointing to opposite directions(1,2). Additionally, the two genes share a great deal of homology not only in their coding sequences but also in their promoters and 5' flanking regions (1,4).

Such a structural organisation and the fact that both genes are mainly expressed in the hepatocyte prompted us to search, in the intergenic region between them, for common controlling sequences (LCR-like) that could mediate their liver preferential expression.

Precedents for such coordinated and tissue specific control has been already described in the globin gene clusters(8,9) and in other gene clusters such as those of the apoprotein CI-E (10) and AI-CIII-AIV (11) Common regulatory sequences responsible for the liver specificity were found in the first cluster (10) and for intestinal specific expression in the second one (11).

The apoprotein(a)-plasminogen intergenic region seems to be a promising candidate for harboring liver specific regulatory sequences as evidentiated by the presence of at least three DNase hypersensitivity sites present only in the liver derived HepG2 cell line. A fourth additional site is also present in the non hepatic HeLa cell line that was used as negative control.

The functional relevance of these sites will be tested in mouse lines made transgenic with YACs containing the apoprotein(a), plasminogen and the intergenic region between them. Deletion mutagenesis of each individual site will follow in order to assess the contribution of each of them in mediating the liver specific expression of these two genes

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