Ten allelic apolipoprotein[a] 5′ flanking fragments exhibit comparable promoter activities in HepG2 cells

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Abstract Plasma levels of the atherogenic lipoprotein[a] represent a quantitative genetic trait that is primarily controlled by the polymorphic apolipoprotein[a] locus on chromosome 6q. The more than 1000-fold variation in lipoprotein[a] plasma levels is explained to a large extent by a remarkable size polymorphism of the apolipoprotein[a] gene which is translated into apolipoprotein[a] isoforms and by unidentified sequence variation in apo[a]. In a recent report, sequence variation in a 1.5 kb fragment from the 5′ flanking region of the apolipoprotein[a] gene was associated with different promoter activities, which led to the suggestion that transcriptional control of the apolipoprotein[a] gene might contribute significantly to lipoprotein[a] plasma levels. We have used a reporter gene assay to compare the promoter activities of these 1.5 kb fragments which were cloned from ten well-characterized apolipoprotein[a] alleles. These ten allelic apolipoprotein[a] fragments revealed, despite the same sequence variation as previously reported, comparable and relatively weak promoter activities in HepG2 hepatocarcinoma cells. Promoter activity for the same fragment in non-liver cells and the identification of a liver cell-specific DNaseI hypersensitive site 3 kb upstream from the ATG start codon suggest that longer fragments must be used in order to analyze the transcriptional regulation of the apolipoprotein[a] gene.—**Bopp, S., S. Köchl, F. Acquati, P. Magnaghi, A. Pethö-Schramm, H-G. Kraft, G. Utermann, H-J. Müller, and R. Taramelli.** Ten allelic apolipoprotein[a] 5′ flanking fragments exhibit comparable promoter activities in HepG2 cells. *J. Lipid Res.* 1995. **36:** 1721–1728.

Supplementary key words pulsed field gel electrophoresis · polymerase chain reaction · reporter gene assay · DNaseI hypersensitivity site

Lipoprotein[a] or Lp[a] refers to a low density lipoprotein-like particle containing as protein moieties one molecule each of apolipoprotein B-100 (apoB) and apolipoprotein[a] (apo[a]) (1). The plasma levels of Lp[a] vary to a large extent in human populations, ranging from < 0.1 to > 100 mg/dl (1). Many case control studies have demonstrated an association of atherosclerotic cardiovascular disease and stroke with elevated plasma levels of Lp[a] (2). Genetic studies have revealed that Lp[a] plasma levels are highly heritable and are primarily controlled by the polymorphic apo[a] locus on chromosome 6q (3, 4). Extensive size variation of the apo[a] gene results in the expression of apo[a] isoforms with variable numbers of tandemly repeated kringle domains ranging from 11 to more than 50 (4–6). A large proportion of the observed variation in Lp[a] plasma levels can be attributed to the size polymorphism of the apo[a] gene with the size of the gene being inversely related to the plasma level of Lp[a] (3, 4, 7, 8). However, consistent contributions of the apo[a] gene rely on sequence variations other than size variation (9). For instance, heterogeneity in the poorly defined regulatory sequences of the apo[a] gene could also have an impact on Lp[a] plasma levels. Indeed, one report comparing 1.5 kb apo[a] promoter fragments from two individuals with different Lp[a] plasma levels described different activities for the two cloned promoter fragments which have been explained by minor sequence variations (10). The promoter fragment cloned from the subject with low Lp[a] levels exhibited reduced promoter activity and showed two sequence differences compared to the corresponding fragment from the high expressor subject. One was an additional TTTTA repeat (making nine rather than eight the number of these repeats) at posi-

Abbreviations: Lp[a], lipoprotein[a]; Pg, plasminogen; β-gal, β-galactosidase; PFGE, pulsed field gel electrophoresis; kb, kilobase; PCR, polymerase chain reaction; apo, apolipoprotein.

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tion −1373 from the ATG translational start and the other was a G → A substitution in position −914. Based on these results, it was hypothesized that sequence variation in the 5′ flanking region of the apo[a] gene could contribute significantly to the observed variation in apo[a] plasma levels. Crucial to this question is the assessment, within a given subject, of the contribution of each allelic apo[a] promoter to Lp[a] levels, an issue that was not dealt with in the previous study (10).

To further evaluate genetic variation in the apo[a] 5′ flanking region we amplified ten allelic 1.5 kb apo[a] promoter fragments from five apo[a] heterozygous subjects characterized with respect to the size of their apo[a] alleles and isoforms and with respect to the relative contribution of each apo[a] isoform to Lp[a] plasma levels. Previous studies have revealed liver-specific expression of apo[a] in humans and monkeys (11, 12). Therefore, we used the human hepatocarcinoma cell line HepG2 (13) as a model system to compare the allelic apo[a] fragments in a reporter gene assay. Additional transfection studies with Hela and Caco-2 cells and DNaseI hypersensitivity studies were performed to further characterize the 5′ flanking region of the apo[a] gene.

MATERIALS AND METHODS

Subjects

Five subjects were chosen from an ongoing program studying the apo[a] size polymorphism in the Tyrolean population. Lp[a] concentrations, apo[a] phenotypes, and the sizes of *Ksp*I fragments containing the apo[a] alleles were known for all subjects (Table 1). The following criteria were applied: *i)* representation of a wide range of different sized apo[a] isoforms as well as of high and low plasma concentrations; *ii)* the subjects had to be heterozygous for apo[a]; and *iii)* the size difference between the apo[a] alleles of one individual had to be at least 3 kringles to allow a good separation of the alleles by pulsed field gel electrophoresis (PFGE).

Cell culture

HepG2, Hela, and Caco-2 cells were obtained from American Type Culture Collection and maintained in Eagle's minimal essential medium with Earle's salts, supplemented with 10% FCS (20% for Caco-2 cells), 2 mM L-glutamine, 1% nonessential amino acids, and 1% sodium pyruvate (omitted for Hela cells). Medium and supplements were from Boehringer Mannheim.

Immunoblotting

The contribution of each apo[a] allele to the total Lp[a] plasma concentrations in the five subjects was estimated by immunoblotting. Plasma proteins were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes as described (7). The apo[a] isoforms were visualized by immunodetection with the monoclonal antibody 1A2 (14). The relative amounts of the two isoforms were determined by densitometry.

Reporter gene plasmids

Plasmid pCMVβ (Clontech) comprises the β-galactosidase (β-gal) gene of *Escherichia coli* in an eucaryotic expression vector. To obtain the luciferase reporter gene plasmids pGL3 and pGL3E, the multiple cloning sites at the 5′ end of the luciferase gene in pGL2-Basic and pGL2-Enhancer (Promega) were substituted by a synthetic polylinker comprising *Bgl*II and *Sma*I cloning sites. Plasmid pGL3-TF contains a 658 bp human transferrin promoter fragment (15) cloned into the polylinker of pGL3.

DNA sequence analysis

Double-stranded plasmid DNAs were sequenced (16) using Sequenase version 2.0 (US Biochemical).

PFGE separation of apo[a] alleles

Genomic DNA was isolated from white blood cells as described earlier (17). For each individual, two agarose plugs containing genomic DNA were subjected to digestion with *Ksp*I according to the instructions of the manufacturer (Boehringer Mannheim). *Ksp*I was chosen as restriction enzyme because it generates fragments that comprise the whole apo[a] gene (**Fig. 1,** panel A) including 5′ flanking sequences but excluding the plasminogen gene (4). The plugs were applied on a 1% low melting point agarose gel in two series. After PFGE separation, one half of the gel was subjected to Southern blotting with an apo[a] cDNA probe (17) to identify the positions of each individual apo[a] allele. According to these positions, gel slices containing the separated apo[a] alleles were cut out from the second half of the gel. Treatment of the gel slices with β-agarase (New England Biolabs) and subsequent ethanol precipitation was used to prepare the DNA for PCR amplification.

Amplification and cloning of the apo[a] 5′ flanking region

PFGE fractionated *Ksp*I fragments containing apo[a] alleles were used as templates for the amplification of 1.6 kb fragments from the 5′ region of the apo[a] gene using oligonucleotides 5′-GTC AAG ATC TAC CAC TCT TGC TTT ACT TCA TG-3′ and 5′-GAA TTG CAC ATA AAG CCA TGG C-3′ (Fig. 1B). The 5′ primer contains ten extra nucleotides to introduce a *Bgl*II restriction site at the 5′ end of the PCR product. After restriction of the PCR products with *Bgl*II and *Msc*I, the

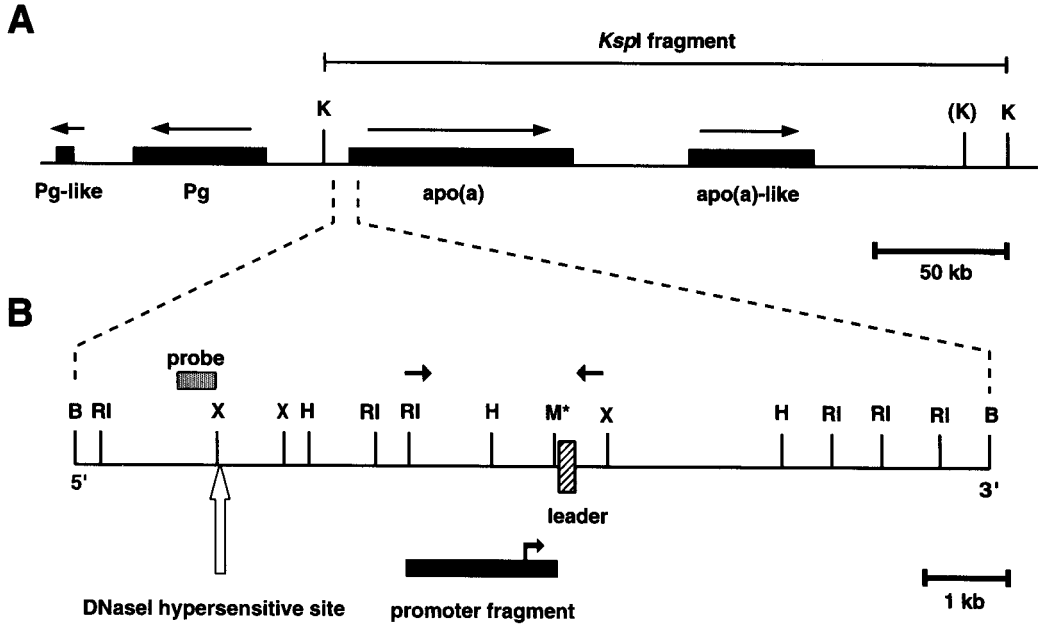

Fig. 1. Long range map of the apo[a]-Pg gene cluster and characterization of the apo[a] 5′ flanking region. A map of the chromosomal region comprising the apo[a]-Pg gene cluster is depicted in panel A. Restriction of human genomic DNA with *Ksp*I results in a fragment comprising the apo[a] gene and an apo[a]-like gene with about 15 kb of the apo[a] 5′ flanking region included (24). Arrows indicate the transcriptional orientation of the genes in the apo[a]-Pg gene cluster. Panel B: the 5′ region of the apo[a] gene is shown enlarged. Priming sites for the oligonucleotides used for the amplification of apo[a] promoter fragments are indicated. The black bar indicates the 1.5 kb promoter fragment that was used for the construction of luciferase reporter gene plasmids. The open arrow indicates the position of a DNaseI hypersensitivity site mainly expressed in liver cells, which was mapped with the indicated probe (shaded bar). K = *Ksp*I; (K) = *Ksp*I, not cut due to methylation; X = *Xba*I; M* = *Msc*I (only one site shown); H = *Hind*III; RI = *Eco*RI; B = *Bam*HI.

resulting 1.5 kb fragments containing nucleotides −1499 to −12 of the apo[a] gene were cloned between the *Bgl*II and *Sma*I restriction sites of pGL3 and or pGL3E.

Transfection and reporter gene assays

DNAs to be used in transfection experiments were purified by Qiagen columns (Qiagen). Lipofection of HepG2 cells was performed as described (18) using a mixture of 5 μg luciferase plasmid and 1 μg pCMV-β. Sixty-four h after transfection, cytoplasmic extracts were prepared as described (19). Ten μl of these extracts were used to determine luciferase activities (19). A 10-sec integral of the relative light units (RLU) was read in a LB950-Luminometer (Berthold). Twenty μl of cytoplasmic extracts was diluted with 30 μl water to measure β-gal activities. After a 5-min preincubation at 37°C, the reaction was started by adding 50 μl prewarmed substrate solution (5 mM *o*-nitrophenol-β-D-galactoside in 100 mM sodium phosphate buffer, 0.1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.3). After another incubation (10–20 min) at 37°C, the yellow reaction products were determined by absorbance reading at 405 nm. Luciferase activities were normalized for β-gal activities to correct for variation in the transfection efficiency.

DNaseI hypersensitivity assays

DNaseI hypersensitive sites were mapped by the indirect end labeling procedure (20). Briefly, 1–5 × 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 centrifuged at 4000 rpm, 10 min at 4°C. Nuclei were resuspended to a final concentration of 10^8/ml. Increasing concentrations of DNaseI (Boehringer Mannheim) were added to 10^7nuclei/100 μl in prepared tubes on ice. The mixtures were incubated for 10 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 100 μg/ml for 30 min at 55°C. DNA was purified by extraction with phenol–chloroform. Portions from the various DNaseI digests, each containing 30 μg of DNA, were further digested with *Bam*HI for 12–16 h at 37°C. South-

ern blots of these experiments with variable concentrations of DNaseI were probed with a 360 bp fragment from the 5′ region of the apo[a] gene (Fig. 1B).

RESULTS

From the long range mapping of the apo[a] gene it was deduced that the entire gene and additional 5′ and 3′ flanking sequences were contained in a large *Ksp*I fragment (17, 21) (Fig. 1A). In order to isolate individual apo[a] genes characterized by a different number of kringle IV units we decided to use this enzyme and to isolate *Ksp*I fragments harboring apo[a] alleles by preparative PFGE. A Southern blot analysis of *Ksp*I digested genomic DNA from five individuals probed with an apo[a]-specific probe is shown in **Fig. 2A.** The presence of two clearly separated alleles in each of the five subjects allowed the enrichment of allelic *Ksp*I fragments by preparative PFGE. The shorter and the longer apo[a] alleles were assigned A and B, respectively. An immunoblot developed with the apo[a]-specific monoclonal antibody 1A2 (14) (Fig. 2B) reveals a major contribution of the apo[a] isoforms expressed from the A alleles to the total apo[a] plasma levels of the five individuals. **Table 1** summarizes the molecular and biochemical values of the ten apo[a] alleles from the five subjects that were studied.

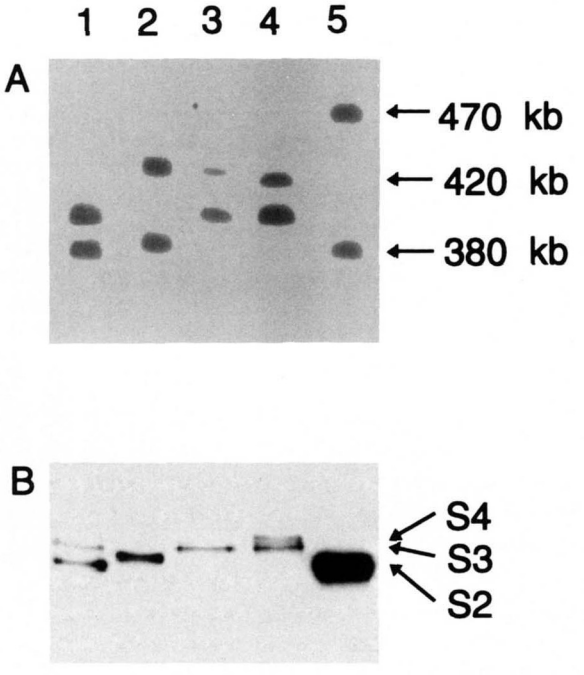

Fig. 2. Characterization of apo[a] alleles and isoforms from five individuals. Panel A shows a Southern blot from a pulsed field gel that was used to isolate *Ksp*I fragments containing the ten apo[a] alleles of five individuals. An apo[a] cDNA fragment was used as hybridization probe (17). The two hybridizing bands per lane reflect the presence of two different-sized apo[a] alleles in all five individuals. Panel B shows an immunoblot of plasma samples from the same individuals. Detection with the apo[a]-specific antibody 1A2 reveals two apo[a] isoforms for individuals 1 and 4 (lanes 1 and 4). Due to the predominant expression of the shorter alleles, individuals 2, 3, and 5 reveal only a single band (lanes 2, 3, and 5).

*Ksp*I fragments containing apo[a] alleles were used for the PCR amplification and subsequent cloning of apo[a] promoter fragments (Fig. 1B) into the luciferase reporter gene plasmids pGL3 and pGL3E, which differ only by the presence of a SV40 enhancer element in the latter plasmid. In a first series of reporter gene assays, we compared the luciferase activities after transfection of pGL3 and pGL3E constructs containing apo[a] promoter fragments which were amplified from the alleles 1B and 2A, respectively. Transfection of the enhancerless apo[a] promoter constructs pGL3-1B and pGL3-2A resulted in luciferase activities similar to the activity obtained after transfection of the promoterless plasmid pGL3 (**Table 2**). The SV40 enhancer in the plasmids pGL3E-1B and pGL3E-2A led to an approximately 5-fold increase of luciferase activities after transfection into HepG2 cells with no corresponding increase for the promoterless plasmid pGL3E (Table 2). In order to achieve a higher sensitivity in the detection of putative differential transcriptional activity of allelic apo[a] promoter fragments, all subsequent experiments were performed with enhancer-containing pGL3E recombinant plasmids. Three clones derived from each of the ten allelic PCR products were characterized by restriction analysis (data not shown) prior to transfection into HepG2 cells. The transfection of three clones per allele was performed to reduce "false negatives" due to misincorporation during the amplification of the promoter fragments. Each of the 30 luciferase plasmids comprising ten allelic apo[a] promoter fragments was transfected at least three times. The promoterless, enhancer-containing plasmid pGL3E served as a negative control. Plasmid pGL3-TF containing a 658 bp human transferrin promoter fragment (15) was included as a reference. **Figure 3** summarizes the promoter activities of the ten allelic apo[a] 5′ region fragments after normalization for transfection efficiency. With the human transferrin promoter activity set to 100%, all ten apo[a] promoters exhibited between 16% and 27% of the transferrin promoter activity with a mean value of 20.4%. The differences are not significant and we observed similar variation of promoter activity within the groups of luciferase plasmids derived from single apo[a] alleles (data not shown).

In order to demonstrate that our result was not biased by an extraordinary high misincorporation rate during the PCR amplification of apo[a] promoter fragments, we sequenced the 1.5 kb promoter fragments from five

TABLE 1. Summary of molecular and biochemical data of ten apo[a] alleles from five heterozygous subjects

Subject Number	Lp[a] Plasma Levels	Size of *Kpn*I Fragments		Contribution to Lp[a] Plasma Levels		Apo[a] Phenotype[a]
		Allele A	Allele B	Allele A	Allele B	
	mg/dl	*kb*		%		
1	80.1	380	400	76	24	S2, S3
2	65.6	390	440	100	ND[b]	S2
3	44.9	400	430	100	ND	S3
4	47.6	400	420	69	31	S3, S4
5	41.3	380	470	100	ND	S2

[a]See reference 14.
[b]ND, not detectable.

independent luciferase plasmids. Three of the sequenced fragments were derived from the A allele, two from the B allele of subject five. We detected five single nucleotide variations in positions –621 (G to A substitution in one B allele fragment), –814 (insertion of one C in all sequenced alleles), –1140 (deletion of an A in one A allele), –1482 (deletion of a T in one A allele), and –1488 (deletion of a T in both B alleles).

Additional sequencing of about 300 bp in the regions –1430 to –1270 and –950 to –800 was undertaken for single cloned copies from each of the ten promoter alleles to look for sequence variation that has previously been suggested to be responsible for a 5-fold difference in promoter activity (10). We could not detect more than the reported sequence variation, namely, a different number of the pentanucleotide repeats TTTTA at position –1374 and a G/A nucleotide substitution at position –914 (Fig. 3). This was not expected as our reporter gene assay indicated comparable promoter activity for the sequenced apo[a] fragments. Three out of four possible combinations of the two previously reported sequence variations occurred in our cohort of ten allelic apo[a] fragments without any correlation to promoter activity.

TABLE 2. Comparison of apo[a] promoter activity in HepG2 cells using enhancerless or enhancer-containing reporter gene plasmids

Transfected Plasmid	% Transferrin Promoter Activity (SD)
pGL3	1.3 (0.3)
pGL3-1B	2.6 (1.0)
pGL3-2A	1.9 (0.1)
pGL3E	1.1 (0.6)
pGL3E-1B	16.2 (2.4)
pGL3E-2A	8.9 (2.0)

Apo[a] promoter constructs based on the enhancerless luciferase plasmid pGL3 or on the SV40 enhancer-containing luciferase plasmid pGL3E were transfected into HepG2 cells. Normalized luciferase activities from three transfections of each reporter construct were used to calculate the mean transcriptional activities as percent transferrin promoter activity (standard deviation).

The presence of consensus sequences for the binding of the liver cell-specific transcription factors HNF-α1, CEBP, and LF-A1 in the 1.5 kb apo[a] promoter fragment led to the hypothesis that this fragment might be sufficient to mediate liver-specific transcription (10). To scrutinize this hypothesis, we performed comparative reporter gene assays with HepG2 cells, CaCo-2 cells (22), and Hela cells, the latter two cell lines being derived from colon and cervix carcinomas, respectively. As shown in **Table 3,** the 1.5 kb fragment from the 5′ flanking region of the apo[a] gene exhibited weak but significant promoter activity in all three cell lines which

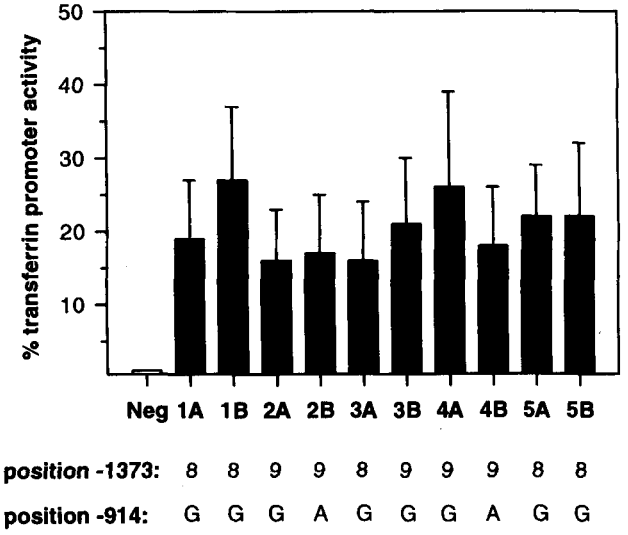

Fig. 3. Promoter activity and sequence variation within the 5′ flanking region of the human apo[a] gene. Ten allelic 1.5 kb apo[a] promoter fragments (1A–5B) from five individuals were analyzed in a luciferase reporter gene assay. The shaded bars represent the mean values (plus SD) of nine transfections, i.e., three clones per allelic promoter fragment and three transfections per clone, expressed as percent of transferrin (TF) promoter activity. The promoterless, enhancer-containing luciferase plasmid pGL3E (Neg) was used as negative control. The number of pentanucleotide repeats (position –1373) and the presence of G or A (position –914) is shown for each allelic promoter fragment.

was detected in each of three independent reporter gene assays.

In order to ascertain control elements either of the constitutive or liver-specific type in the regulation of the apo[a] gene, we started to map DNaseI hypersensitive sites in the 5′ flanking region of the apo[a] gene. A 360 bp restriction fragment from the apo[a] 5′ flanking region (Fig. 1B) was used as probe for DNaseI mapping on nuclei isolated from HepG2 and Hela cells, respectively. After *Bam*HI digestion of genomic DNA this probe detects a major band of 10 kb that belongs to the apo[a] gene. Mild digestion of HepG2 nuclei with DNaseI results in a strong 1.7 kb band, whereas much higher DNaseI concentrations are required for detecting the same site in nonhepatic Hela cells (**Fig. 4**). Several digestions with other enzymes and hybridization with a more upstream probe (data not shown) confirmed that the DNaseI-derived band was located within the apo[a] 5′ flanking region at approximately 3 kb from the ATG start codon. This DNaseI hypersensitive site is located 1.5 kb upstream of the promoter region that was analyzed in the present study.

DISCUSSION

From our data it can be concluded that the 1.5 kb apo[a] 5′ flanking sequences tested comprise a basal, tissue-nonspecific promoter activity. This conclusion is supported by the relatively weak reporter gene expression after transient transfection of apo[a] promoter-containing luciferase plasmids into HepG2, Hela, and Caco-2 cells (Table 3). Luciferase expression in all three cell lines indicates that the analyzed 1.5 kb promoter fragment does not contain all the *cis*-acting elements, which mediate liver-specific apo[a] expression.

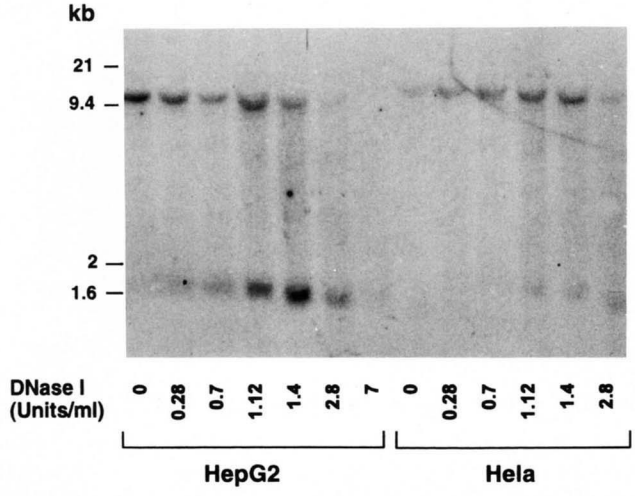

Fig. 4. Analysis of DNase hypersensitivity sites within the 5′ flanking region of the apo[a] gene. Nuclei from HepG2 and Hela cells were digested with DNase at concentrations indicated below each lane in the autoradiogram. The localization of the probe and the DNaseI hypersensitive site is represented in Fig. 1, Panel B. The hypersensitive site as revealed by the 1.7 kb fragment is stronger in HepG2 compared to Hela cells.

Using a sensitive luciferase assay, we did not detect significant variation in the promoter activity of ten allelic apo[a] 5′ flanking fragments cloned from five heterozygous subjects after transient transfection of the reporter gene constructs into HepG2 cells (Fig. 3). Due to the rather low standard deviation in the reporter gene assay (Fig. 3), a 2-fold difference between the apo[a] promoter activities would have been detected. A minor (less than 2-fold) difference in the promoter activity of allelic 1.5 kb fragment from the apo[a] 5′-flanking region is not excluded by our analysis.

If one considers the contribution of single apo[a] alleles to total Lp[a] concentrations, then the results obtained are rather striking. For instance, subjects 3 and 5 have Lp[a] levels of 44.9 and 41.3 mg/dl, respectively, and show a single band on an immunoblot corresponding to an S3 isoform for subject 3 and an S2 isoform for subject 5 (Fig. 2, panel B). At the DNA level, on the other hand, they are both heterozygous for two different *Ksp*I fragments (400 kb and 430 kb fragments encoding S3 and S4 isoforms for individual 3, 380 kb and 470 kb fragments encoding S2 and S4 isoforms for individual 5). In both subjects, the Lp[a] plasma concentrations were determined exclusively by the shorter A alleles despite comparable promoter activity in the 1.5 kb fragments derived from the corresponding B alleles. Sequencing of five cloned promoter fragments from the A and B alleles of one subject revealed five single nucleotide variations (see Results). The extra C at position −814 is likely to reflect either allelic variation or a sequencing error in the published sequence (10) because it occurred in five independently cloned promoter fragments. Another variation (deletion of a T at position −1488) was observed in two promoter fragments cloned from the B allele of subject five and might therefore represent a sequence polymorphism. However, we cannot rule out misincorporation during amplification as causative for the observed deletion of a T at position −1488 as the cloned copies of each allelic promoter fragment were derived from a single PCR. The three other nucleotide variations occurred in one of two or three clones derived from the same allele and hence represent PCR artifacts. Taken together, our finding of five single nucleotide variations in 10 kb of sequenced DNA, two of which might represent polymorphisms rather than PCR artifacts, strongly suggests that our failure to detect heterogeneity in the promoter activity of allelic fragments was not due to PCR-induced sequence variation.

Noteworthy, we could not confirm a previously described correlation between sequence variations in the analyzed 1.5 kb apo[a] flanking fragment and promoter activities. Although we identified the same kind of sequence variation as reported earlier (10) we found almost identical promoter activity for the ten allelic fragments analyzed. This discrepancy might result from the usage of imperfectly matched reporter gene plasmids for the analysis of two allelic apo[a] promoter fragments in the earlier report which was indicated in a more recent report from the same group (23). Due to the larger number of promoter fragments analyzed in identical plasmids and due to the fact that we started our analysis from well-characterized allelic fragments, our data indicate that the reported substitutions and insertions (10) should be regarded as neutral polymorphisms with respect to their influence on the promoter activity in the studied 1.5 kb fragment. Our data do not exclude that the observed sequence variations may modulate the activity of regulatory elements located outside this 1.5 kb fragment.

In our study, we have used SV40 enhancer-containing luciferase plasmids to achieve a higher sensitivity in the detection of variation of the weak transcriptional initiation activity associated with apo[a] promoter fragments, which was barely detectable in the absence of the enhancer (Table 2). We cannot exclude the possibility that the SV40 enhancer present in our plasmids might have masked existing heterogeneity in the transcriptional activity of apo[a] promoter fragments. On the other hand, looking at enhancers as *cis*-regulatory elements that increase rather than initiate transcription from a given promoter, our analysis still appears appropriate to detect variability in the allelic apo[a] promoter fragments. A possible limitation of our analysis comes from the usage of HepG2 cells for the investigation of apo[a] promoter activities. Although HepG2 cells express many liver-specific gene products and are therefore widely used for the in vitro analysis of liver-specific gene expression, they do not express detectable amounts of apo[a] from their endogenous apo[a] alleles. The reason for this remains to be clarified. It might result from the presence of two "null alleles." Alternatively, a relative lack of certain transcription factors that are needed for efficient transcription from the apo[a] promoter might be responsible for the weak promoter activities that we observed in HepG2 cells.

Taken together with the mapping of a DNaseI hypersensitive site located about 3 kb upstream from the ATG start codon (Fig. 4), the weak transcriptional activities in the studied 1.5 kb fragments from the 5′ flanking region of the apo[a] gene suggest to us that the analysis of tissue-specific apo[a] gene regulation and its putative allelic variation requires longer DNA fragments to be analyzed. The apo[a] gene is part of a gene cluster on chromosome 6q containing four homologous genes and the entire cluster spans more than 400 kb of genomic DNA (21, 24). Two of these genes, apo[a] and Pg, are located at a distance of 40 kb from each other in an opposite transcriptional orientation and exhibit tissue-specific expression in the liver. For other clustered genes it has become clear that regulatory elements in so-called locus control regions (LCR) are not restricted to act on any one gene but can be active over long distances and on several genes (25, 26). Likewise, similar sequences might, in fact, be involved in the tissue-specific regulation of the apo[a]–plasminogen gene cluster. Unfortunately, the cloning of large fragments from the apo[a]–plasminogen intergenic region into bacterial vector systems has proved to be extremely difficult (R. Taramelli, unpublished data). This problem might be solved by the use of yeast artificial chromosomes containing large regions of genomic DNA spanning the apo[a]–plasminogen region. Recently, the introduction of recombinant YACs into cultured cells and into the germline of the mouse have been reported to result in authentic tissue-specific and copy number-independent transgene expression (27). Application of this method to the apo[a] gene might be helpful to identify those *cis*-acting regulatory elements that mediate liver-specific apo[a] expression.

In summary, with our in vitro model system we could not detect variability in the promoter activity of the analyzed allelic 1.5 kb apo[a] fragments. However, our data do not exclude that such a variability is encoded outside the fragment that was used in our reporter gene analysis. Alternatively, the remarkable variation in the expression of apo[a] alleles in human plasma might well be explained by sequence variation outside the promoter region which could affect downstream processes like transcriptional elongation, mRNA stability, translocation, post-translational processing, and particle assembly.

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