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The apolipoprotein (a) gene: a transcribed hypervariable locus controlling plasma lipoprotein (a) concentration

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Abstract. Lipoprotein(a) [Lp(a)] is a quantitative trait in human plasma. Lp(a) consists of a low-density lipoprotein and the plasminogen-related apolipoprotein(a) [apo(a)]. The apo(a) gene determines a size polymorphism of the protein, which is related to Lp(a) levels in plasma. In an attempt to gain a deeper insight into the genetic architecture of this risk factor for coronary heart disease, we have investigated the basis of the apo(a) size polymorphism by pulsed field gel electrophoresis of genomic DNA employing various restriction enzymes (*Swa*I, *Kpn*I, *Ksp*I, *Sfi*I, *Not*I) and an apo(a) kringle-IV-specific probe. All enzymes detected the same size polymorphism in the kringle IV repeat domain of apo(a). With *Kpn*I, 26 different alleles were identified among 156 unrelated subjects; these alleles ranged in size from 32 kb to 189 kb and differed by increments of 5.6 kb, corresponding to one kringle IV unit. There was a perfect match between the size of the apo(a) DNA phenotypes and the size of apo(a) isoforms in plasma. The apo(a) DNA polymorphism was further used to estimate the magnitude of the apo(a) gene effect on Lp(a) levels by a sib-pair comparison approach based on 253 sib-pairs from 64 families. Intra-class correlation of log-transformed Lp(a) levels was high in sib-pairs sharing both parental alleles ($r = 0.91$), significant in those with one common allele ($r = 0.31$), and absent in those with no parental allele in common ($r = 0.12$). The data show that the intra-individual variability in Lp(a) levels is almost entirely explained by variation at the apo(a) locus but that only a fraction (46%) is explained by the DNA size polymorphism. This suggests further heterogeneity relating to Lp(a) levels in the apo(a) gene.

Introduction

The level of lipoprotein(a) [Lp(a)] is a quantitative genetic trait in human plasma (Utermann 1989). Lp(a) concentrations may vary 1000-fold among individuals but are a stable characteristic in most healthy subjects. High concentrations of Lp(a) are associated with premature coronary heart disease (CHD) and stroke (Scanu and Fless 1990). Recent studies have established that Lp(a) is a genetically controlled risk factor for atherosclerotic vascular disease (Sandholzer et al. 1992). Understanding the genetic regulation of Lp(a) concentrations is of theoretical interest but may also have considerable practical implications.

Lp(a) is composed of a low-density lipoprotein and a high molecular weight glycoprotein called apolipoprotein(a) [apo(a)] (Utermann and Weber 1983; Gaubatz et al. 1983). The latter exhibits a genetic size polymorphism with apparent molecular weights of isoforms ranging from about 300 kDa to more than 800 kDa (Utermann et al. 1987). The size of the apo(a) isoforms is negatively correlated with the concentration of Lp(a) in plasma (Utermann et al. 1987, 1988), and both apo(a) isoform size and Lp(a) concentration are controlled by the apo(a) gene locus on chromosome 6q2.6–q2.7 (Weitkamp et al. 1988; Lindahl et al. 1989; Drayna et al. 1988). It is presently not known how the structural variation at the apo(a) locus (=apo(a) isoform size polymorphism) affects Lp(a) levels in plasma and how much of the intra-individual variability of Lp(a) levels is explained by variation at the apo(a) locus. Using a measured genotype approach, we have previously shown that the fraction of the variability in Lp(a) levels that is explained by the apo(a) size polymorphism varies among populations, ranging from about 30%–40% in Caucasians to almost 70% in Asian populations (Sandholzer et al. 1991). These figures are minimal estimates of the contribution of the apo(a) locus to Lp(a) level variability, since only the size polymorphism of apo(a) and no other possible variation at the apo(a) locus has been considered.

The basis for the understanding of the apo(a) size polymorphism was established when Lawn and coworkers demonstrated that the apo(a) gene resembles a plasminogen gene "gone awry", and that it probably arose from a plasminogen gene during primate evolution (McLean et al. 1987). Like plasminogen, apo(a) contains a 5′ signal peptide and a 3′ protease domain. However, instead of the five different copies of the so-called kringle

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motifs (kringles I, II, III, IV, and V), human apo(a) contains a single kringle-V-like structure and multiple repeats of a kringle-IV-like motif, about 20 of which are completely identical in the sequenced cDNA (McLean et al. 1987). This repeat structure immediately suggested that the genetic variation in the size of the apo(a) protein is a result of variation in the number of kringle IV repeats in the apo(a) gene among subjects. Evidence for this proposal has been provided by quantitative Southern blotting using kringle-IV-specific probes (Lindahl et al. 1990; Gavish et al. 1989), Northern blotting of apo(a) mRNA, which has demonstrated a size polymorphism correlated with the protein size polymorphism in humans and baboons (Koschinsky et al. 1990; Hixson et al. 1989), and a recent study using pulsed field gel electrophoresis (PFGE) of *Kpn*I-digested genomic DNA (Lackner et al. 1991).

In order to obtain a deeper insight into the genetic regulation of plasma Lp(a) levels, we have further investigated the structural basis of the apo(a) size polymorphism and have related the genetic variation at the apo(a) locus to apo(a) isoform size in Lp(a) and to Lp(a) concentrations in plasma. By using a sib-pair comparison approach, we demonstrate that in the general population, Lp(a) concentrations are almost entirely controlled by the apo(a) gene locus.

Materials and methods

Subjects

EDTA-treated blood was collected from 156 unrelated healthy Tyrolean subjects (laboratory personal and students of the medical school of the University of Innsbruck). Analysis of *Kpn*I fragments was performed on all samples and a determination of apo(a) protein isoforms was carried out on 134. For the sib-pair study, all available relatives of the above individuals were invited for blood sampling. A total of 234 subjects from 64 families accepted this invitation and were analysed for Lp(a) concentration by enzyme-linked immunosorbent assay (ELISA). The apo(a) DNA phenotypes were determined by PFGE. Using all possible combinations, 253 sib-pairs were deduced from these families.

PFGE

Lymphocytes were prepared from 10 ml EDTA-treated blood by Ficoll-Paque (Pharmacia, Sweden) centrifugation. The cell number was determined using a Coulter Counter (Coulter Electronics, UK) and adjusted to 2×10^7 cells/ml. Equal volumes of cell suspension and 1% LMP (low melting point) agarose were mixed carefully and poured into plug-forming moulds. After proteinase K (Sigma, FRG) treatment (2 mg/ml) for at least 48 h at 55°C, the DNA-containing plugs were washed extensively in TE buffer, followed by phenylmethane sulfonyl-fluoride (PMSF, 0.04 mg/ml) in TE buffer, and then TE buffer alone. The plugs were either digested directly or stored in 0.5 *M* EDTA (pH, 8.0) for several months. Among several restriction endonucleases that were tested, digestion with *Kpn*I, *Swa*I, *Not*I, *Sfi*I and *Ksp*I resulted in apo(a) gene fragments of ~50–1000 kb. The digestion was performed employing 2×40 U enzyme per plug following the instructions of the supplier (Boehringer-Mannheim, FRG).

The DNA restriction fragments were size-separated in the Chef mapper system (Biorad, USA) using its autoalgorithm to determine the best parameters. Electrophoresis was performed in 1% LE agarose (Seakem, USA) in 0.5% TAE buffer at 14°C. To separate the small *Kpn*I or *Swa*I fragments, the chosen fragment size limits were 50–400 kb. The separation of the large *Not*I and *Sfi*I fragments were undertaken with the same parameters, except that 100 kb and 1000 kb were selected as the lower and upper limits, respectively.

After electrophoresis, the gel was stained with ethidium bromide. The DNA fragments were nicked with 60 mJ UV light in the Gene Linker (Biorad, USA), photographed and transferred to Hybond N membrane (Amersham, UK) using the alkaline buffer method. Following baking, the nylon filter was prehybridized and hybridized using the DIG luminescent detection kit (Boehringer-Mannheim, FRG). As the probe, a digoxigenin-labelled 340-bp *Pst*I fragment derived from the apo(a) cDNA clone Lamda a41 (a gift from R. Lawn) (McLean et al. 1987) was employed. Bound probe was visualized by chemoluminescence using AMPPD 3-(2′-spiro-adamantane)-4-methoxy-4-(3′′-phosphoryloxy)-phenyl-1,2-dioxetane (Boehringer-Mannheim, FRG) as substrate.

Polymerase chain reaction and restriction site mapping

Two different sets of primers were chosen, following consideration of the published sequence of apo(a) (McLean et al. 1987) and the plasminogen gene (Petersen et al. 1990), to obtain specific amplification of the apo(a) kringle IV (= kringle 2–22) structure. The first pair (E1, E2) was a 30-mer (E1, codogen) 5′-GTTCCAAGCCTAGAGGCTCCTTCCGAACAA corresponding to bases 748–777, and a 45-mer (E2, complement) 5′-CATTTGGGTAGTATTCTGGGGTCCGACTATGCGAGTGTGGTGTCA corresponding to bases 595–551, and was used for the amplification of an intron situated between two kringle IV repeats.

The second pair of primers was used for the amplification of a putative intron within the kringle IV structure. The first primer (E3, codogen) 5′-TCTATGACACCACACTCGCATAGTCGG was a 27-mer corresponding to base 547–573 and the second primer (E4, complement) 5′-TTGTTCGGAAGGAGCCTCTAGGCTTGGAAC was a 30-mer corresponding to bases 777–748. The polymerase chain reaction (PCR) was performed according to Saiki et al. (1988). The reaction conditions were: 1st step, 1.5 min at 96°C; 2nd step, 2 min at 60°C; 3rd step, 10 min (primers E1 and E2) and 15 min (primers E3 and E4) at 72°C for 40 cycles. Perfect Match (1 µl, Stratagene, LaJolla, CA) was added to the reaction mixture. The PCR products were isolated using the Gene Clean method (Bio 101, LaJolla, CA).

Restriction size mapping of the PCR products was performed using the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Bgl*II, *Pst*I, and *Pvu*II. The digests of the PCR products were subjected to Southern blot analysis. Blots were hybridized with a radioactively labelled probe corresponding to the kringle IV exon of apo(a), base 436–778 and a 45 mer (base 551–595) (McLean et al. 1987) to elucidate the orientation of the fragments.

Apo(a) phenotyping by immunoblotting (Sandholzer et al. 1991) and determination od Lp(a) plasma concentration by ELISA (Menzel et al. 1990) were performed as described previously.

Statistical methods

Gene counting was used to estimate allele frequencies. Because of the highly polymorphic apo(a) system, most potential apo(a) genotypes could not be observed and the standard chi-square statistic to test the hypothesis that the observed allele frequencies are in Hardy-Weinberg equilibrium was not applicable. We therefore calculated a standard chi-square statistic to compare the observed numbers of homozygotes and heterozygotes with the expected numbers assuming Hardy-Weinberg equilibrium (Chakraborty cited in Lackner et al. 1991).

Because the skewness coefficient for the distribution of Lp(a) concentration was generally high, Lp(a) levels were either natural-log-transformed prior to analyses or non-parametric tests were applied. For sib-pair comparison, the sibs were grouped into pairs

with two identical *Kpn*I apo(a) alleles, one identical apo(a) allele, or no apo(a) allele in common. Comparisons of mean Lp(a) plasma concentrations and mean differences of Lp(a) values in these groups were made using the Kruskal-Wallis non-parametric analysis of variance by ranks. For pairwise comparisons, the Mann-Whitney U-test was performed (Siegel and Castellan 1988).

Intra-class correlations were used to compare Lp(a) plasma concentrations among siblings by group and for the pooled data set of all sib-pairs (Cavalli-Sforza and Bodner 1971). As an estimate of the heritability of Lp(a) concentrations, twice the intra-class correlation coefficient among all sib-pairs was used (Falconer 1964).

The contribution of apo(a) genotypes to the variance in the various groups was estimated by the analysis of variance (Scheffe 1959). Because of the extensive polymorphism of the *Kpn*I alleles, most theoretically possible apo(a) phenotypes could not be observed in our sample, resulting in very small cells. In order to keep the conditions comparable with our earlier investigations of apo(a) protein phenotypes, the *Kpn*I isoforms were therefore binned (Budowle et al. 1991), according to the closest respective apo(a) isoform. Thus, each bin contained all *Kpn*I fragments that corresponded to a particular apo(a) protein isoform (see Table 1). Furthermore, the Spearman rank correlation coefficient was calculated as a measure of association between apo(a) DNA fragment size and Lp(a) levels. As an index of apo(a) kringle IV repeats in the genome of an individual, the allele numbers of the two alleles in each subject were added. Starting with the smallest *Kpn*I fragment (32 kb = allele number 1), each *Kpn*I fragment was designated by an allele number ("empty" places in the 5.6-kb ladder were also given a number; see Results).

The statistical analyses were performed using the SPSS 4.0 statistical package (SPSS 1990).

Results

Intron-exon structure of the apo(a) kringle IV repeat domain

Genomic DNA was digested with different restriction enzymes (*Eco*RI, *Rsa*I, *Pvu*II, *Sac*I, *Bam*HI, *Kpn*I, *Swa*I), subjected to Southern blotting and probed with an apo(a)-specific oligonucleotide (Lindahl et al. 1990). Only one major restriction fragment was obtained with each enzyme, suggesting that a conserved consensus sequence exists in the intron(s) of the apo(a) kringle IV repeat. The largest fragment of a defined size obtained with the frequently cutting enzymes was 5.6 kb in length. With some enzymes (*Kpn*I, *Swa*I), large fragments (>20 kb) were generated that only poorly entered the gel (Fig. 1).

In order to determine the number, size, and restriction map of introns in the highly repeated kringle IV domain of the apo(a) gene, two pairs of primers designated E1, E2 and E3, E4 (see Methods) were used to amplify the intronic sequences of this region by PCR. These primers were designed to start the reaction from the putative intron-exon boundaries in the apo(a) gene as deduced from the respective sequence in the plasminogen gene (Petersen et al. 1990). Thus, any intron(s) within a kringle IV coding unit and between the kringle IV repeats should be amplified. With the primer pair E1, E2, one fragment of 1.4 kb was obtained, whereas amplification with the primer pair E3, E4 resulted in a major fragment of 4.2 kb. Together, these fragments add up to 5.6 kb, which corresponds to the size of kringle IV-containing fragments obtained by digestion of genomic DNA with *Bam*HI, *Eco*RI, and *Pvu*II (Fig. 1). A partial restriction map of the PCR fragments and of the derived exon-intron structure of the kringle IV domain is shown in Fig. 2. The data suggest a repeat structure where the coding region for one kringle IV is split into two exons of 162 bp (exon 1) and 180 bp (exon 2) by the 4.2-kb intron 1, and that the 1.4-kb intron 2 is located between the 3′ end of exon 2 and the 5′ end of exon 1.

Fig. 1. Southern blot of genomic DNA from 2 subjects. DNA was digested with different restriction enzymes as indicated at the *top*. The fragments were hybridized with a 45-nucleotide oligomer corresponding to a sequence (nucleotide 551 to 595) in the repeated kringle IV domain of apo(a). Sizes of the major fragments obtained with each enzyme are indicated. Note that *Kpn*I fragments are retained at the *top* of the gel

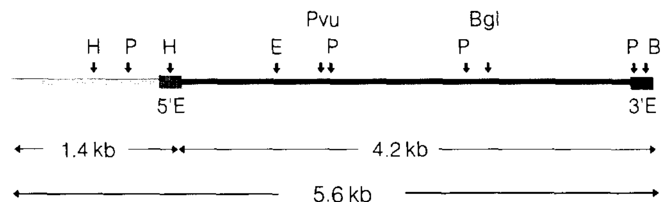

Fig. 2. Consensus restriction site map of the apo(a) kringle IV intron-exon motifs. The map starts with the intron that separates two kringle IV repeats. The intron is followed by the 5′ exon (5′E), the intron within the kringle IV, and the 3′ exon *(3′ E)*. The positions of the restriction sites are indicated. Abbreviation of the restriction enzymes: *B Bam*HI; *E Eco*RI; *H Hin*dIII; *Bgl Bgl*II; *P Pst*I; *Pvu Pvu*II

Digestion of genomic DNA with rare cutting restriction enzymes and PFGE

Genomic DNA from 7 different subjects was digested with the restriction enzymes *Not*I, *Swa*I, *Sfi*I, *Ksp*I and *Kpn*I. The subjects were either heterozygotes ($n = 4$) or homozygotes ($n = 3$) for apo(a) isoforms (defined by immunoblotting and family analysis). Digests were subjected to PFGE, and apo(a) Kringle IV-containing fragments were demonstrated by Southern blotting using a digoxigenin-labelled apo(a) kringle-IV-specific probe. With all enzymes, either one or two restriction fragments were generated depending on the subject. The

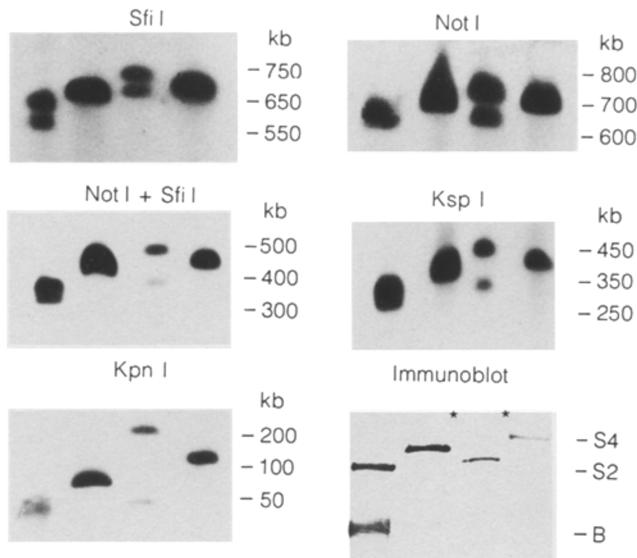

Fig. 3. PFGE of genomic DNA from 4 subjects of defined heterozygous (*lanes 1, 3* of each blot) and homozygous (*lanes 2, 4* of each blot) apo(a) type. DNA was digested with different restriction enzymes as indicated at the *top*. The fragments were hybridyzed with a cDNA-derived probe specific for the repeated kringle IV domain of apo(a). The conditions of PFGE for this experiment were optimized for DNA fragments of a size from 100–1000 kb. Therefore, separation of small *Kpn*I fragments (*left lane* of the *lower* blot) is suboptimal (compare Figs. 4, 6–8). Plasma from the same subjects was subjected to SDS-PAGE/immunoblotting using the apo(a) monoclonal antibody (MAB) 1A[2] *(lower right panel)*. The apo(a) isoform corresponding to the largest DNA fragment (*lane 3* on each blot) is not seen on the immunoblot because of its low concentration. The position is indicated by *asterisks*

three subjects defined as apo(a) homozygous had only one apo(a) fragment irrespective of the enzyme used. Likewise, the apo(a) heterozygous subjects exhibited two restriction fragments with each of the enzymes. Although the sizes of fragments were different depending on the enzyme used, there was a complete correspondence of fragment sizes generated by the different restriction enzyme. There was also complete correspondence with the protein isoforms. This suggests that all enzymes detect the same size polymorphism in the apo(a) gene. The kringle IV-containing fragments generated with *Not*I were large (range ~620 kb to 760 kb), those generated with Sfi ranged from 550 kb to 710 kb, and the *Ksp*I fragments ranged from 340 kb to 430 kb. *Swa*I and *Kpn*I digestion resulted in smaller fragments (Fig. 3). The fragments generated with *Swa*I were each approximately 5 kb smaller than those generated with *Kpn*I. Double digests with *Not*I and *Ksp*I, and *Sfi*I and *Ksp*I resulted in fragments of the same size as *Ksp*I alone, suggesting that both *Ksp*I restriction sites are contained in the *Not*I and *Sfi*I fragments, respectively. Double digests with *Not*I and *Sfi*I generated fragments of 340 kb to 480 kb. Hence, these fragments overlap. Double digests with *Kpn*I and *Not*I, or *Sfi*I and *Ksp*I demonstrated that the *Not*I, *Sfi*I, and *Ksp*I sites are all downstream of the 3′ *Kpn*I site. This site has been localized in the intron between kringle V and the protease domain region of the apo(a) gene (Lackner et al. 1991).

DNA from 156 independent subjects were then digested with *Kpn*I. Fragment sizes ranged from 32 kb to 189 kb. They differed in size by increments of 5.6 kb, which correspond to one kringle IV intron/exon repeat unit. Beginning with the smallest *Kpn*I fragment (32 kb), which was designated allele number 1, fragments were numbered consecutively towards the larger fragments. The largest detected so far was number 29 (189 kb). No more than two *Kpn*I fragments were detected, in any of the 156 subjects, by the apo(a) cDNA probe. *Kpn*I fragments from selected subjects with apo(a) alleles of different size are arranged as a "step-ladder" in Fig. 4.

Correlation of apo(a) isoform size with KpnI fragment size and co-segregation in families

Apo(a) isoforms and *Kpn*I fragments were determined by immunoblotting and PFGE in 134 selected subjects. A striking correlation of isoform size with *Kpn*I fragment length was also seen in this sample (Fig. 5). Subjects with small *Kpn*I fragments had small apo(a) isoforms in plasma, and those with large DNA fragments had high molecular weight isoforms in plasma. All subjects with two protein isoforms also had two *Kpn*I fragments. Subjects with a single *Kpn*I fragment had only a single isoform in plasma (Fig. 6). Apo(a) isoforms (by immunoblotting) and *Kpn*I fragments (by PFGE) were further determined in 87 members of 17 families. This

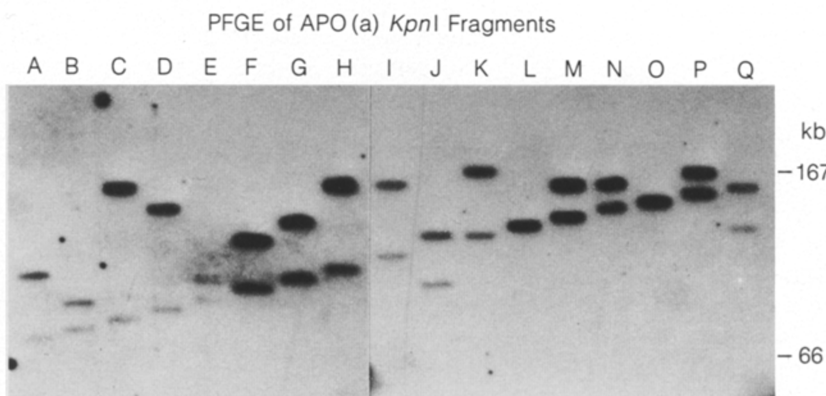

Fig. 4. Southern blot of fragments from genomic DNA separated by PFGE. DNA fragments were detected by hybridization with the cDNA-derived kringle-IV-specific probe. DNA from 17 selected subjects was digested with *Kpn*I and samples were arranged in the form of a step-ladder demonstrating the increment in the size of the DNA fragments. The sample in *lane J* was initially misclassified. The size difference between two adjacent fragments in the ladder is ~5.6 kb

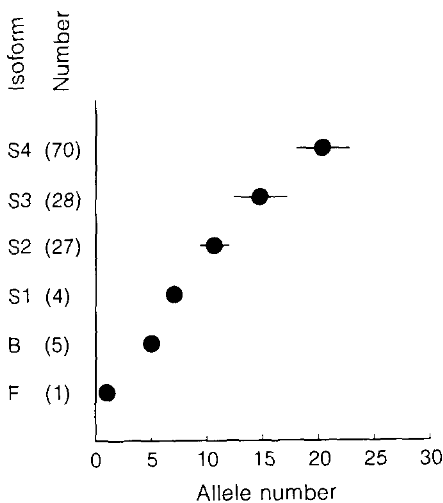

Fig. 5. Graphic representation of the correlation between apo(a) protein isoforms and apo(a) *Kpn*I fragments (given as *allele number*). Numbers of subjects in each isoform class are given *left*

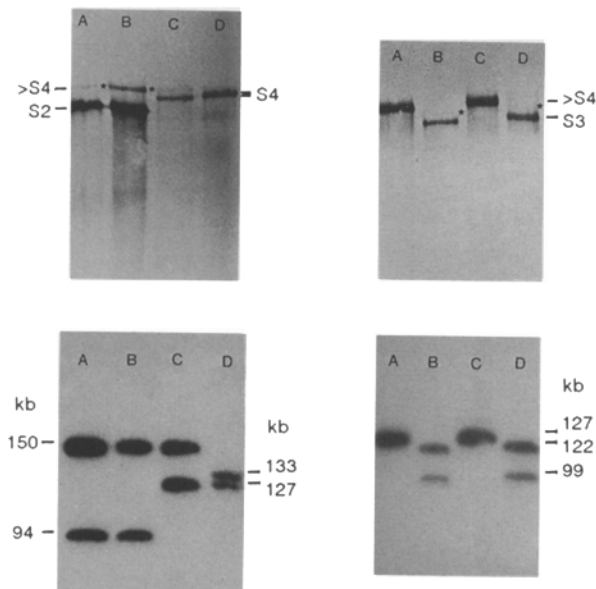

Fig. 6. Immunoblot of apo(a). Plasma from 8 subjects was subjected to SDS-PAGE and immunoblotted with the MAB 1A[2] against apo(a). *Lower part* PFGE of *Kpn*I fragments. DNA from the same 8 subjects was digested with *Kpn*I and fragments were hybridized with the apo(a) kringle-IV-specific cDNA probe. In the *right panels,* two homozygous subjects are shown in *lanes A, C.* Note also the low expression of the 122-kb allele. The protein isoform corresponding to this DNA fragment was recognized only in retrospect. *Left* The phenomenon of pseudohomozygosity is demonstrated in *lane D*

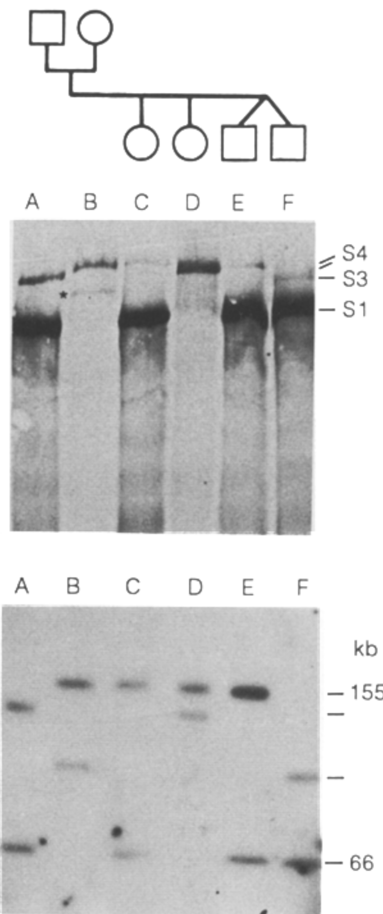

Fig. 7. Immunoblot *(upper part)* and Southern blot *(lower blot)* analysis of apo(a) isoforms and apo(a) *Kpn*I fragments in a family whose pedigree is shown at the *top.* Immunoblot and PFGE/Southern blot analysis was performed as described. Note the inheritance and correspondence of apo(a) isoforms and DNA fragments. The S3 isoform in *lane B* occurs at a lower concentration than the S4 isoform from the same subject

was particularly informative with respect to the relationship between apo(a) isoforms in plasma and *Kpn*I restriction fragments. In all families investigated, apo(a) fragment size was inherited as an autosomal co-dominant trait (Fig. 7). In all family members, the number and size of apo(a) isoforms detected by Western blotting corresponded to the number and size of apo(a) *Kpn*I alleles (Figs. 7, 8). Thus, *Kpn*I fragments and apo(a) isoforms co-segregated in all families. All family members with two different *Kpn*I fragments also had two protein isoforms in plasma, although they were not always easily detected by immunoblotting (Figs. 6–8). There were two reasons for this. First, separation was generally better at the DNA level than at the protein level for large isoforms. In some subjects, *Kpn*I fragments were well separated but resolution of isoforms by SDS-polyacrylamide gel electrophoresis (PAGE) immunoblotting was poor, resulting in pseudo-homozygosity (Budowle et al. 1991) (Figs. 6–8). Some samples initially typed as "single-band" apo(a) phenotypes were only retrospectively recognized as double-band types (Fig. 8). Secondly, some isoforms were present in low concentrations and therefore were difficult to detect by immunoblotting (Figs. 6, 7). The correspondence of previously described protein isoforms to *Kpn*I fragments is shown in Fig. 5 and Table 1.

There were only a few subjects with only one *Kpn*I fragment. They had only one apo(a) isoform in plasma (Fig. 6). In heterozygotes, the intensity of the larger apo(a) DNA fragment was always stronger on the blots

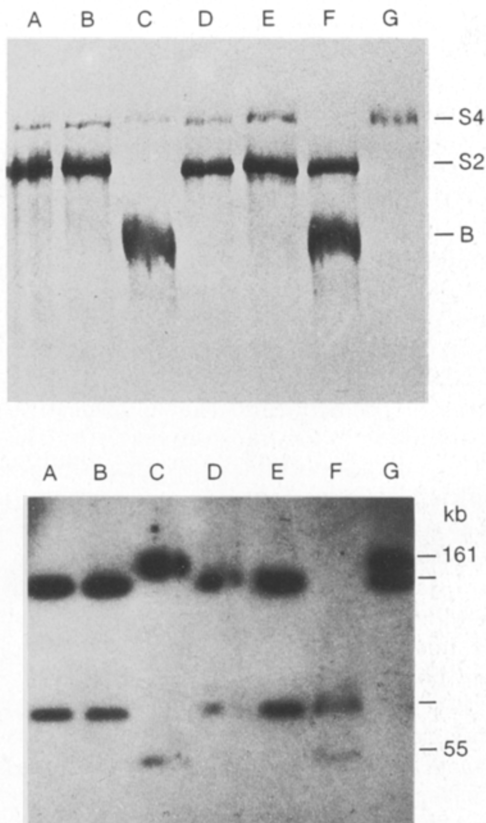

Fig. 8. Immunoblot *(upper panel)* and Southern blot *(lower panel)* analysis of apo(a). Plasma from 7 sibs was subjected to SDS-PAGE/immunoblotting using the MAB 1A[2] against apo(a) *(upper panel)*. DNA from the same sibs was digested with *Kpn*I and the fragments were hybridized with the apo(a)-specific cDNA-derived kringle IV probe *(lower part)*. Note that, on the immunoblots, the lower molecular weight bands give the stronger signal, and that on the Southern blots, the larger fragments exhibit a stronger signal

Table 1. Relationship between apo(a) protein isoforms and *Kpn*I alleles

Apo(a) protein isoform	Apo(a) isoform size [kD]	Apo(a) allele number	*Kpn*I fragment size [kb]
F	<400	1	32
B	460	4– 5	49– 54
S1	520	7– 9	66– 77
S2	580	10–12	82– 94
S3	640	13–15	99–110
S4	>700	16–29	116–189

than that of the smaller fragment (Figs. 4, 6–8). This most probably reflects the higher number of kringle IV repeats that are detected by hybridization with the kringle IV probe in the larger fragments. This situation was generally reversed for the apo(a) protein isoforms. As a rule, immunostaining of the lower molecular weight isoforms was stronger on the Western blots than that of higher molecular weight isoforms (Figs. 6–8). This reflects the well-known inverse association of apo(a) isoform size with Lp(a) plasma levels (Utermann et al. 1987, 1988; Sandholzer et al. 1991; Gaubatz et al. 1990). There were however some notable exceptions from this rule. In some heterozygous subjects, isoforms of higher molecular weight were significantly more intense on the blots than the respective lower molecular weight isoforms (Fig. 7). Again, this is consistent with the broad range of Lp(a) concentrations seen in a given isoform class (Boerwinkle et al. 1989).

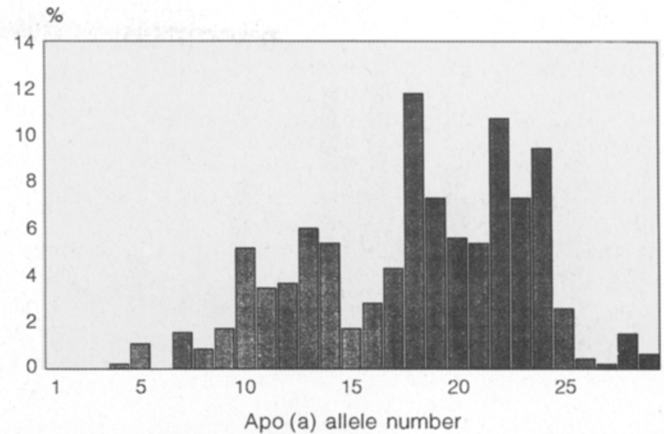

Fig. 9. Apo(a) allele frequency. Frequency distribution of *Kpn*I apo(a) fragments in a sample of $n = 156$ unrelated subjects

Frequencies of KpnI alleles in the population

A total of 26 different *Kpn*I fragments were detected in the 234 subjects investigated. However, some of these subjects were related and some had been included in the study because they were known to have certain protein isoforms (e.g. the F-type). These subjects were not included in the further analysis. The frequencies of *Kpn*I fragments (= DNA size alleles) in the remaining 156 unrelated unselected subjects are represented graphically in Fig. 9. The frequency distribution was unimodal but skewed towards larger fragments. This distribution is opposite to the distribution of Lp(a) levels. The most frequent *Kpn*I fragments in the sample were alleles 18, 22, and 24. These correspond to isoforms S4 (and larger) on the protein level (Table 1). The frequencies of *Kpn*I phenotypes in the sample were not significantly different from those expected assuming Hardy-Weinberg equilibrium. Only 6.4% of subjects were homozygous for one apo(a) size fragment, whereas 93.6% were heterozygotes. The apo(a) system therefore has a very high polymorphism information content (PIC) value (0.93).

Effects of the apo(a) KpnI polymorphism on Lp(a) levels in plasma

Apo(a) isoform size in plasma is negatively associated with Lp(a) levels (Utermann et al. 1987, 1988; Sandholzer et al. 1991; Gaubatz et al. 1990). Since the apo(a) size polymorphism is a reflection of the DNA size polymorphism, the same kind of relationship between *Kpn*I fragment size and Lp(a) level could be expected. Because of the more precise definition of restriction frag-

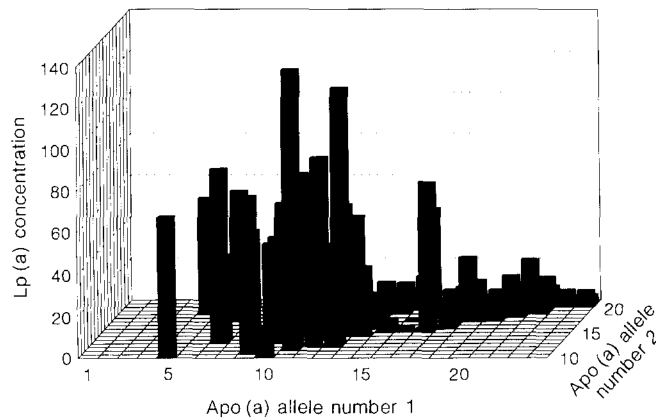

Fig. 10. Relation between Apo(a) alleles and Lp(a) concentration. Graphical representation of the relationship between apo(a) *Kpn*I fragments and Lp(a) concentration in plasma. The two *Kpn*I fragments in a subject are denoted as *allele number 1* and *allele number 2*. Note the decrease in Lp(a) concentration with the increase in allele number (= apo(a) *Kpn*I fragment size)

ments, the recognition of their absence or presence as opposed to protein isoforms and the higher resolution at the DNA level (see above), we anticipated a more accurate determination of the magnitude of the effect of the apo(a) size polymorphism on Lp(a) levels.

Indeed, the size of *Kpn*I fragments is negatively correlated with Lp(a) levels in plasma. When the sum of the two alleles in an individual was taken as a measure [assuming an additive effect of apo(a) alleles on Lp(a) levels (Kraft et al. 1992)], the Spearman rank correlation coefficient was $r = 0.36$. A graphic representation of the relationship between apo(a) fragment size and Lp(a) levels is shown in Fig. 10. There are 351 theoretical apo(a) phenotypes, 107 of which were observed in the sample resulting in very small cells. For the calculation of the effects of apo(a) size on Lp(a) levels, types were therefore binned as described under Methods. The *Kpn*I polymorphism explained 46% of the variability of Lp(a) levels in the sample.

Sib-pair comparison

Lp(a) concentrations and the apo(a) *Kpn*I polymorphism were determined in 64 families with a total of 234 family members. From this material, 253 sib-pairs were obtained. For most sib-pairs, the parents were also analysed assuring the identity by descent or the non-identity of *Kpn*I fragments among sibs. The sibs were then grouped into pairs with two identical apo(a) alleles ($n = 71$), one identical apo(a) allele ($n = 139$), and no apo(a) allele in common ($n = 43$). Mean Lp(a) levels between these groups were not significantly different (Table 2). Lp(a) levels were significantly more similar in allele-identical sibs than in sibs sharing only one parental allele. Lp(a) levels were most dissimilar in sib-pairs with no apo(a) allele in common. Mean sib-pair differences were 5.9 ± 8.9 mg/dl, 20.6 ± 20.3 mg/dl, and 21.8 ± 21.6 mg/dl in the sib-pairs with two, one, or no identical allele, respectively. Intra-class correlation of log-transformed Lp(a) concentrations was high in the apo(a) *Kpn*I identical siblings ($r = 0.91$), significant in siblings with one common apo(a) *Kpn*I fragment ($r = 0.31$), and absent in those sib-pairs that shared no apo(a) fragment ($r = 0.12$) (Table 2).

The high intra-class correlation in the apo(a) identical sibs compared with the other sib-groups suggests that Lp(a) levels are almost entirely controlled by the apo(a) gene locus in this population of healthy sibs. The lack of any significant correlation in sib-pairs that had no apo(a) allele in common is particularly striking and suggests that no other genes contribute significantly to Lp(a) level variation in the general population. The variability of Lp(a) levels in the total group of sibs was not significantly different from the variability in the group of unrelated subjects. A heritability of Lp(a) levels of $h^2 = 0.92$ was calculated from the intra-class correlation (0.46) in all sib-pairs (Falconer 1964). Together, the heritability estimate of 0.92 from the total group, the high intra-class correlation of Lp(a) levels in the apo(a) identical sib-pairs, and the lack of a significant intra-class correlation in sib-pairs that differ for both apo(a) alleles strongly suggest that Lp(a) concentrations in plasma are almost completely heritable and that the apo(a) locus is the only gene with a significant effect in the population at large. Nevertheless, only about 46% of the variability of Lp(a) levels is explained by the apo(a) size polymorphism. Hence, there must be other unrecognized differences between apo(a) alleles identical in size, i.e. differences that contribute to Lp(a) level variability among subjects.

Table 2. Intra-class correlation and sib-pair differences in Lp(a) plasma levels (mg/dl)

	Number of shared alleles in sib-pairs			
	Two	One	Zero	Pooled
Number of pairs	142	278	86	506
Lp(a)-concentration (mean ± SD)	17.7 ± 20.3	21.1 ± 23.7	19.1 ± 20.3[1]	19.8 ± 22.3
Lp(a) difference between pairs (mean ± SD)	5.9 ± 8.9	20.6 ± 20.3	21.8 ± 21.6[2]	16.7 ± 19.3
Intra-class correlation	0.91[3]	0.31[4]	0.12[5]	0.46[3]

[1] Kruskal-Wallis test statistic: 1.1; 2 *df*; $P = 0.58$
[2] Kruskal-Wallis test statistic: 41.53; 2 *df*; $P < 0.001$
[3] $P < 0.001$
[4] $P < 0.005$
[5] Not significant

Discussion

In the present study, we have further investigated the genetic basis of the Lp(a) polymorphism and have demonstrated that most of the variation in Lp(a) concentrations in a Caucasian population sample is explained by variation at the apo(a) gene locus on chromosome 6q2.6–q2.7. Using a panel of rare cutting restriction enzymes (*Not*I, *Swa*I, *Sfi*I, *Kpn*I, *Ksp*I), separation by PFGE, and detection by a cDNA probe specific for the kringle IV repeat in apo(a), we show that all these enzymes detect a size polymorphism in the apo(a) gene. The correspondence of patterns obtained by the different enzymes in a panel of defined subjects suggests that the same polymorphism is detected by these enzymes. Secondly, we have related the apo(a) DNA polymorphism to the previously described protein polymorphism of apo(a). Thirdly, the contribution of the DNA polymorphism to the variability of Lp(a) levels was estimated in unrelated subjects. Furthermore, we have used a sib-pair comparison approach to estimate the contribution of the apo(a) locus to the total variability of Lp(a) levels in the sample. Our results confirm and extend those of Lackner et al. (1991) who have previously demonstrated a size polymorphism in the apo(a) gene using *Kpn*I restriction fragments. They observed 19 different alleles in a sample of 102 subjects. In our study, 26 different apo(a) size alleles were detected by *Kpn*I digestion of genomic DNA and separation of fragments by PFGE. This heterogeneity is larger than the size heterogeneity of apo(a) previously detected at the protein level by SDS-PAGE and Western blotting (Utermann et al. 1987; Sandholzer et al. 1991; Gaubatz et al. 1990). However, in a recent study employing agarose gel electrophoresis, Kamboh et al. (1991) were able to distinguish 26 apo(a) isoforms. These authors estimated a mean heterozygosity of 94% in their sample; this is identical to the one observed here (94%) and by Lackner et al. (1991). Although no relationship to DNA heterogeneity or Lp(a) levels was investigated in that study, it is likely that the same polymorphism is detected by agarose gel electrophoresis. As previously concluded by Lackner et al. (1991), the DNA size polymorphism that was detected with an intronic apo(a) probe in their work and by an apo(a) cDNA probe here resides in the apo(a) structural gene and is attributable to a variable number of tandemly arranged kringle IV repeats in the apo(a) gene. This variable number of repeats is likely to be responsible for the protein size polymorphism. This conclusion is based on three lines of evidence. (1) The same DNA polymorphism was detected by different restriction enzymes all of which had no restriction site in the exon or intron sequences of the apo(a) kringle IV repeat. The fragments obtained were different in size and varied from less than 50 kb to 190 kb (*Kpn*I, *Swa*I), approximately 340–480 kb (*Ksp*I, and *Not*I plus *Sfi*I), up to approximately 600–800 kb (*Sfi*I, *Not*I). Nevertheless, the same fragment patterns were obtained. (2) The differences between neighbouring *Kpn*I and *Swa*I fragments were approximately 5.6 kb. This corresponds to the size of one kringle IV unit as determined by PCR analysis and Southern blotting (for the large fragments generated with *Not*I and *Sfi*I, no precise measurement of the distance of neighbouring fragments could be made but the identity of patterns suggests that the same size difference is revealed). The precise locations of the *Kpn*I sites flanking the kringle IV repeat have been determined. The 3′ site is in the kringle V-protease domain region (Lackner et al. 1991) and the 5′ site is in a large 14-kb intron between the leader sequence and the first kringle in apo(a) (Ichinose 1992). Double digests with *Kpn*I and *Not*I or *Sfi*I showed that the *Not*I and *Sfi*I sites are upstream of the 5′ *Kpn*I site and downstream of the 3′ *Kpn*I site (data not shown), and that the *Not*I and *Sfi*I fragments, but not the *Kpn*I and *Swa*I, fragments are detected by a 5′ signal sequence probe near the apo(a) promoter; this suggests that these fragments contain most or all of the apo(a) gene (H. G. Kraft, R. Taramelli, G. Utermann, unpublished data). (3) There was a perfect correspondence of apo(a) isoform size with the *Kpn*I (and *Swa*I, data not shown) fragments. Notably, none of the enzymes *Not*I, *Swa*I, *Sfi*I, *Ksp*I or *Kpn*I, has a single restriction site in any of the (at least) 30 intron 1 and intron 2 repeats in the kringle IV motif that are present in the larger apo(a) genes; there is only one restriction site in each of the kringle IV repeats for *Eco*RI, *Pvu*II and *Bam*HI. Together, the data suggest that a 5.6-kb kringle IV repeat exists in the apo(a) gene; this repeat is variable in number but amazingly conserved in structure, even in the introns. This explains the size polymorphism at the DNA (Lackner et al. 1991), mRNA (Koschinsky et al. 1990) and protein level (Utermann et al. 1987).

The principal strategy used for the demonstration of the DNA size polymorphism in the apo(a) gene is the same as that widely used for variable number of tandem repeats (VNTRs), and micro- and minisatellites in non-transcribed regions of the genome (Nakamura et al. 1987). However, in contrast to most other size variation in the genome, this variation is in a transcribed gene. Moreover, the repeats are large, necessitating the separation of fragments by PFGE. The apo(a) locus may thus be envisaged as a transcribed VNTR. There are only a few examples of variation in the number of modules in transcribed genes in the literature. These include the size polymorphism of the human keratin 10 chain caused by variable numbers of glycine loops (Korge et al. 1992), and the length variation in the PUM genes (Swallow et al. 1987) and in human proline-rich protein (Lyons et al. 1988).

The relationship between apo(a) alleles (DNA fragments), apo(a) isoforms and Lp(a) levels is both simple and complex. The simultaneous determination of apo(a) DNA fragments (= apo(a) alleles) and apo(a) isoforms in plasma has provided new insights into this relationship. First, subjects considered to have a single isoform in plasma are rarely homozygous with respect to kringle IV repeat number (Figs. 4, 6–8). More frequently, they have different numbers of kringle IV repeats on both alleles, but the product of one allele is too low in concentration to be detected by conventional SDS-PAGE/immunoblotting. Alternatively, such subjects may be heterozygotes for two apo(a) DNA fragments of very

similar size. The resulting isoforms may then simply not be separated by SDS-PAGE/immunoblotting, a phenomenon that has been termed pseudo-homozygosity (Budowle et al. 1991). Sometimes, they may be recognized as separate bands only in retrospect (Fig. 6).

As a rule, isoforms of lower molecular weight are present at higher concentration in heterozygotes than those of higher molecular weight, but this is not always the case (Fig. 7). However, sometimes it is difficult to differentiate between a situation where a low molecular weight band occurs in considerably lower concentration or where a degradation product of apo(a) forms a spurious band. Knowledge of the DNA phenotype will ensure the correct interpretation of the protein phenotype in such a situation. Thus, DNA phenotyping helps to overcome some problems related to protein phenotyping, e.g. problems that are caused by the different concentrations of the two apo(a) species that may be present in a heterozygote. However, DNA typing is not generally superior to protein phenotyping but gives a different, though complementary, type of information. No information on the expression of an allele is obtained from DNA fragment size. Thus, it cannot be concluded from this type of DNA analysis alone whether or not there exist null alleles in the apo(a) system, as has been done by Lackner et al. (1991). The combination of both methods, DNA and protein typing together with the determination of Lp(a) levels presently provides the most complete information on the structure and function of apo(a) alleles in an individual.

The detailed characterization of the apo(a) DNA size polymorphism and its relationship to the protein polymorphism was essential to the major questions addressed here. How much of the inter-individual variability in Lp(a) levels is explained by the apo(a) locus and how much is exlained by the apo(a) DNA size polymorphism? In other words, is the contribution of the apo(a) locus to Lp(a) level variability explained solely by the effects of apo(a) size alleles? The DNA size polymorphism of apo(a) explains about 46% of the variation in Lp(a) levels in the Tyrolean population sample studied here. This is slightly more than the 35%–42% explained by the protein size polymorphism in the same population (Boerwinkle et al. 1989; Sandholzer et al. 1991). Most probably, this is caused by a loss of information when protein isoforms, instead of DNA phenotypes, are determined. The variability in Lp(a) levels that is explained by the DNA or protein size polymorphism is considerably smaller than the variability that can be attributed to variation at the apo(a) locus.

The contribution of the apo(a) locus to the total variability of Lp(a) levels was estimated by a sib-pair comparison approach based on a total of 253 sib-pairs. The underlying idea of the sib-pair comparison is that sibs with identical alleles at a given locus show less variation and a higher intra-class correlation for a quantitative trait than sibs that share only one or no allele at that locus, if the locus under investigation contributes to the variability of the trait. For a quantitative trait that is under complete control by the locus under investigation, the intra-class correlation for allele-identical sibs theoretically becomes one. Thus, unlike a classical twin study, the sib-pair approach allows us to estimate the contribution of a single locus to a quantitative trait and, as performed here, may be envisaged as a special case of the measured genotype approach. By comparing sib-pairs with identical and non-identical apo(a) DNA types, we have demonstrated that the intra-class correlation of Lp(a) levels is much higher in the allele-identical sibs ($r = 0.91$) than in those with only one allele in common, and that no significant correlation exists in sibs that are non-identical for both apo(a) alleles. This suggests that a large fraction of the variation in Lp(a) plasma levels is explained by the apo(a) locus. The finding that the 1000-fold variation in Lp(a) levels in the population is almost exclusively explained by the apo(a) locus agrees with the results of a twin study that demonstrated that Lp(a) levels in twins are almost completely under genetic control (M. Austin, C. Sandholzer, J. V. Selby, B. Newman, R. M. Krauss and G. Utermann, unpublished data). The heritability of 0.92 calculated here from the sib-pair analysis is remarkably close to the heritability of Lp(a) in the twin study (0.94). Moreover, the mean difference in Lp(a) concentrations between apo(a)-identical sibs (5.9 mg/dl) is close to the mean difference in monozygotic twins (3.6 mg/dl). Combined with the results from this study, we conclude that the variation in Lp(a) levels in the general population is entirely explained by genetic variation and that this genetic variation is to a large extent the result of variation at the apo(a) locus. These findings also suggest that there is variation in the apo(a) gene beyond the size polymorphism of apo(a), because the latter explains only a fraction (46%) of the variability. The search for this variation may provide further insights into the genetic regulation of Lp(a) levels. Azrolan et al. (1991) have shown that, in the cynomolgus macaque, the apo(a) size variation and mRNA levels have major independent effects on plasma Lp(a) concentration. These data are consistent with our observation that the length variation in apo(a) explains only a fraction of the variability in Lp(a) levels that is caused by the apo(a) locus, and suggest that a similar situation may exist in humans.

The finding that Lp(a) levels in the general population are largely controlled by the apo(a) locus does not contradict previous reports that other genetic and non-genetic factors may also affect Lp(a) concentration. Examples are familial hypercholesterolemia (Utermann et al. 1989) and end-stage renal disease (Parra et al. 1987; Takegoshi et al. 1990). In both conditions, Lp(a) levels are significantly elevated over controls matched for apo(a) phenotype (H. Dieplinger, F. Kronenberg, G. Utermann, unpublished data). The mechanisms underlying these elevations are presently unknown. However, familial hypercholesterolemia effects only about 1/500 subjects in the population and hence the contribution of this condition to the total variability of Lp(a) in the population is neglegibly small. It should however be pointed out that the sample studied here represented healthy subjects with no obvious disease. We cannot therefore conclude, from the results of this study and a recent twin study (M. Austin, C. Sandholzer, J. V. Selby, B. Newman, R. M. Krauss and G. Utermann, unpublished

data), that the Lp(a) level is an unchangeable genetic marker. Lp(a) levels may well be affected by certain diseases and metabolic disturbances. Although this may be important for the respective disease group, it will not be reflected in a healthy population sample.

Our data may also explain the initial paradox of why a continuously quantitative trait was originally described as a qualitative autosomal dominant marker (Berg 1963). Apo(a) alleles appear to affect Lp(a) levels in an additive fashion (Utermann et al. 1987; Boerwinkle et al. 1989; Kraft et al. 1992). The Lp(a) concentration in heterozygotes is the sum rather than the mean of the Lp(a) concentrations determined by each allele singly, but the effects of apo(a) alleles on Lp(a) levels are extremely different, resulting in up to 1000-fold differences in concentration among individuals. Thus, in a given subject or family, one allele may "dominate" over the other(s), if the contribution of one allele to Lp(a) levels is much larger than that of the other(s). Moreover, alleles that are associated with high Lp(a) levels are much less frequent than those associated with low levels. In a simple non-sensitive test with a threshold, this may result in an apparent dominant transmission of the trait. Taken together, the molecular and population genetic analysis of the apo(a) protein and DNA polymorphism, in relation to plasma Lp(a) levels and the sib-pair analysis performed here, have resulted in a detailed dissection of the genetic architecture of a quantitative trait in humans that is associated with coronary heart disease.

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