Transgenic mice expressing a human apolipoprotein[a] allele

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Abstract The most important determinant of plasma levels of Lp[a] are sequence differences at the highly polymorphic apolipoprotein[a] (apo[a]) locus. To define the sequences that mediate the regulation of apo[a] expression, we cloned a 370 kb DNA fragment that included a 130 kb apo[a] gene, and 40 kb 5′- and 200 kb 3′-flanking region from an individual with high plasma levels of Lp[a] using a YAC vector. This genomic clone was used to generate transgenic mice. In the YAC-apo[a] transgenic mouse, apo[a] was only expressed in the liver, as it is in humans. The mean serum level of apo[a] in 4-week-old YAC-apo[a] transgenic mice was 20 mg/dl. In the female mice the levels of apo[a] varied over a 1.5-fold range during the 4-day estrus cycle and the levels correlated directly with serum progesterone levels. The serum levels of apo[a] decreased to almost undetectable level in male mice after puberty and this decrease was reversed by castration. Ingestion of a high-fat diet resulted in a ~100-fold fall in hepatic apo[a] mRNA levels and >60-fold decrease in serum apo[a] levels. To delimit the control elements that mediate tissue-specific and sex hormone-responsive apo[a] transcription, we derived a reporter YAC in which 40 kb of 5′ flanking sequences from the cloned apo[a] allele were linked to a luciferase reporter gene. Analysis of four independent transgenic lines revealed no hepatic luciferase expression, suggesting that important *cis*-acting elements located outside the apo[a] 5′-flanking region are necessary for in vivo expression of apo[a].—Acquati, F., R. Hammer, B. Ercoli, V. Mooser, R. Tao, V. Rönicke, A. Michalich, G. Chiesa, R. Taramelli, H. H. Hobbs, and H-J. Müller. **Transgenic mice expressing a human apolipoprotein[a] allele. *J. Lipid Res.* 1999.** 40: **994–1006.**

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Plasma lipoprotein[a] (Lp[a]) is distinguished from low density lipoprotein (LDL) by the presence of an additional glycoprotein component called apolipoprotein[a] (apo[a]), which is covalently linked to the apolipoprotein B-100 (apoB-100) of LDL. Plasma levels of Lp[a] vary more than 1000-fold among individuals of a population but remain relatively constant within individuals (1). In vivo turnover studies demonstrated that interindividual variation of plasma levels of Lp[a] are due to differences in Lp[a] biosynthesis and not Lp[a] degradation (2, 3). Relative plasma levels of Lp[a] are determined almost exclusively by the highly polymorphic human apo[a] gene, which is located 40 kb from the plasminogen gene (4) on human chromosome 6q2.6–q2.7 (5, 6). The highly polymorphic apo[a] component of Lp[a] exhibits homology to the fibrinolytic proenzyme plasminogen. Based on the nomenclature for plasminogen, apo[a] comprises ten distinct kringle IV (K-IV) units (named K-IV type 1 to K-IV type 10), a single kringle V domain, and a catalytically inactive protease domain (7). Nine of the K-IV units (K-IV type 1, 3–10) are unique in sequence; apo[a] alleles differ by the number of identical K-IV type 2 units (8), which are each encoded in a 5.5 kb fragment (9). The numbers of K-IV type 2 repeats in the apo[a] gene vary from 3 to 31 and are inversely correlated to Lp[a] plasma levels (8). About 60% of the total variation of Lp[a] plasma levels can be attributed to the length polymorphism in the apo[a] gene and protein (10). However, plasma levels of Lp[a] vary significantly in individuals with similar-sized apo[a] alleles (5, 11) and much of these differences are due to variations in or tightly linked to the apo[a] gene.

Variation in the sequences controlling apo[a] gene ex-

Abbreviations: apo[a], apolipoprotein[a]; apoB-100, apolipoprotein B-100; kb, kilobase pair(s); LDL, low density lipoprotein; Lp[a], lipoprotein[a]; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PFGE, pulse-field gel electrophoresis; SDS, sodium dodecyl sulfate; YAC, yeast artificial chromosome.

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pression may influence plasma levels of apo[a]. In cynomolgus monkeys, hepatic mRNA abundance correlates with Lp[a] plasma levels (12). What regulates the synthesis of apo[a] in vivo has been difficult to analyze because of the restricted tissue pattern of apo[a] expression in mammals (7). The present knowledge about transcriptional regulation of apo[a] expression is mainly based on in vitro assays. In reporter gene assays, 1.5 kilobase fragments from the immediate 5′ flanking sequences of apo[a] are associated with only weak promoter activity (13, 14). A series of polymorphisms in the 5′-flanking region of the apo[a] gene (15–17) has been associated with specific plasma levels of Lp[a]. However, none of them has been shown to influence the transcription of the apo[a] gene in vitro suggesting that these polymorphisms are in linkage disequilibrium with yet-to-be identified sequences which participate in the determination of Lp[a] plasma levels.

Recently, Frazer et al. (18) derived yeast artificial chromosome (YAC) transgenic mice from an apo[a] allele cloned from a YAC library which was derived from an anonymous individual. The YAC transgene was 250 kb in length and contained >60 kb of 5′- and 3′-flanking sequences. The transgenic animal expressed an apo[a] transcript only in the liver and this was associated with the presence of a 370 kDa apo[a] isoform (12 K-IV repeats) in the plasma. Thus, the 250-kb genomic fragment contained the *cis*-acting elements required to mediate tissue-specific expression of apo[a] in vivo. However, the unknown 'descent' of the apo[a] transgene precludes a comparison of the expression of apo[a] in the human donor and transgenic mice. Therefore, maintenance of the integrity of the sequences in the apo[a] gene after the YAC cloning and generation of the transgenic mice could not be verified. The apo[a] gene is highly recombinogenic, as evidenced by the impressive length polymorphism of the gene in the population. Moreover, recently apo[a] transgenic rabbits were generated in which there was a gene rearrangement; these animals expressed a 200 kDa apo[a] isoform which was significantly smaller as compared to the apo[a] isoform in YAC transgenic mice which were obtained with the same YAC (18, 19).

Most apo[a] alleles in the population are associated with a low plasma level of Lp[a]. For this reason, we have cloned an apo[a] allele from an individual with a high plasma level of Lp[a] (72 mg/dl). A 370 kb genomic fragment containing an apo[a] allele with 14 kringle IV repeats and 40 kb and 200 kb of apo[a] 5′- and 3′-flanking sequences, respectively, was cloned and used to produce transgenic mice referred to as YAC33. These mice have liver-specific expression of the apo[a] transgene. We show that the apo[a] gene is regulated at the level of transcription by sex steroids and by increasing the fat content of the diet. As a first step to define the control elements which mediate tissue specificity and sex hormone responsiveness of apo[a] transcription in vivo, we performed a homologous recombination experiment in YAC33 containing yeast cells to derive YAC33-LUC, in which the apo[a] coding and 3′-flanking regions were replaced by a luciferase reporter gene, retaining 40 kb of apo[a] 5′-flanking region from the original human genomic DNA insertion in YAC33. A total of four independent transgenic mice were developed and none expressed luciferase in the liver or spleen. These results strongly suggest that the sequences in the 5′-flanking region of the apo[a] gene are not sufficient for apo[a] transcription which demonstrated that if a recently identified enhancer (20, 21) that resides within this sequence participates in apo[a] transcription, other sequences are also required.

EXPERIMENTAL PROCEDURES

Chemicals

Chemicals were obtained from Sigma (Deisenhofen, Germany). Yeast growth media were from Difco (Augsburg, Germany). Restriction enzymes, Proteinase K, and MP agarose were from Boehringer Mannheim (Mannheim, Germany). T4 DNA ligase (400,000 U/ml) and β-Agarase were purchased from New England Biolabs (Schwalbach, Germany). Yeast Lytic Enzyme was obtained from ICN (Meckenheim, Germany). SeaPlaque and NuSieve GTG agarose were from FMC Bioproducts (Hessisch Oldendorf, Germany).

Yeast strains

Saccharomyces cerevisiae strain AB1380 (*MATa*, *ura3–52*, *trp1*, *ade2–1*, *lys2–1*, *his5*, *can1–100*) was kindly provided by Dr. Zachau.

Immortalization of lymphocytes

Epstein-Barr virus transformation (22) of lymphocytes from a donor with elevated levels of plasma Lp[a] was used to establish the lymphoblastoid cell line L413.

Preparation of YAC vector arms

The plasmids pCGS969 and pCGS966 were kindly provided by Dr. D. Smith. To make the left arm (pCGS969-L1), 3 μg of pCGS969 was cut to completion with *Bam*HI and *Not*I and loaded onto a preparative agarose gel. A 9 kb fragment corresponding to the CEN4-ARS1-TRP1-TK-TEL left vector arm was purified from the gel and ligated with a synthetic linker generated by annealing the following primers: 5′-GAT CCG TCG ACG AAT TCA AAT GC-3′ and 5′-GGC CGC ATT TGA ATT CGT CGA CG-3′. To make the right arm (pCGS966-R3) 3 μg of pCGS966 was digested with *Bam*HI and *Eco*RI and a 7.5 kb fragment corresponding to the TEL-URA-NEO-ARS1 right vector arm was gel-purified and ligated with a synthetic linker derived from the annealing of the following primers: 5′-GAT CCG TCG ACA TAG AGC GGC CGC AAT AG-3′ and 5′-AAT TCT ATT GCG GCC GCT CTA TGT CGA CG-3′; the resulting construct (pCGS966-R1) was then digested with *Sfi*I and the DNA fragment was purified. The DNA was treated with T4 DNA polymerase and religated; the resulting plasmid (pCGS966-R2), which lacked the *Sfi*I site localized 5′ to the Neo cassette, was cut with *Sna*BI and *Not*I, purified and ligated in the presence of a linker derived from the annealing of the following primers: 5′-GGC CGC GGC CCC CTT GGC CTA C-3′ and 5′-GTA GGC CAA GGG GGC CGC-3′ to introduce a *Sfi*I site. The sequence of this *Sfi*I site was obtained by direct sequencing of a PCR product from genomic DNA of immortalized donor lymphocytes.

YAC library construction

Thirty-four plugs containing 12 μg each of high molecular weight DNA from the L413 cell line were digested to completion

with *Sfi*I and *Not*I. The individual from which the L413 cell line was derived has two apo[a] alleles with 14 and 37 KIV repeats. The DNA from the plugs was size-fractionated on a 1% SeaPlaque agarose gel using pulsed-field gel-electrophoresis in a CHEF III apparatus (Bio-Rad Laboratories, Munich, Germany). The switch time was gradually increased from 10 to 30 s, with a 7 volt/cm field for 19 h at a 106° angle. When the electrophoresis was terminated, the right and left lanes of the gel, which contained a yeast chromosome marker, were stained in 1 μg/ml ethidium bromide at 4°C for 1 h. Under UV light examination, the marker lanes were notched in a position corresponding to a molecular weight range of 360–420 kb. The central part of the gel was cut, using the notches as reference for the lower and upper cutting line. The gel slice was equilibrated four times (30 min) at 4°C in ligase buffer (50 mm Tris-HCl, pH 7.5, 10 mm $MgCl_2$, 30 mm NaCl, 0.75 mm spermidine trihydrochloride, 0.3 mm spermine tetrahydrochloride), then cut with a sterile scalpel into pieces of 1 ml to be processed separately. Each piece was placed in an Eppendorf tube and melted at 68°C in a water bath. After 5 min, 10 μg of both vector arms (pCGS966-R3 *Sfi*I-*Bam*HI digested and pCGS969-L1 *Not*I-*Bam*HI digested, both dephosphorylated only at the *Bam*HI termini) were added in a minimal volume (20 μl) and melting was allowed to proceed for another 5 min. The tubes were incubated for 1 h at 37°C before adding 1/10 of the volume of 10× ligase buffer (1× ligase buffer from New England Biolabs (Germany) supplemented with 10 mm ATP, pH 7.5, and 10 mm DTT) and 5 μl T4 DNA ligase followed by successive incubations at 37°C (30 min) and at room temperature (overnight). The ligation reactions were then melted at 68°C for 10 min in a water bath, cooled for few minutes at 37°C, and loaded with a tip into a long well of a 1% SeaPlaque agarose gel. The ligated vector–human DNA material was separated from the unligated vector and vector dimers by pulsed-field gel electrophoresis for 8.5 h with a switch time gradually increasing from 13 to 15 s, a 5.5 V/Cm field and a 106° angle. Fragments larger than 350 kb were excised from the gel and incubated four times (30 min each) at 4°C in 10 mm Tris-HCl, pH 7.5, 30 mm NaCl, 1 mm EDTA, 0.75 mm spermidine trihydrochloride, 0.3 mm spermine tetrahydrochloride. The gel slice was then cut with a sterile scalpel into blocks of 1 cm^3; these were placed into Eppendorf tubes, melted at 68°C in a water bath for 10 min, and cooled for 5 min at 40°C; 1 unit of β-agarase was then added for each 100 μl of molten agarose and the reaction incubated at 40°C for 2 h. A second aliquot of enzyme was then added after a further melting step, and the reaction was allowed to continue for another 2 h. Finally, 200 μl aliquots of the agarose-free DNA were microdialyzed at 4°C for 4 h on 0.2 μm Millipore filters (Millipore, Eschborn, Germany) against 20 ml of STC (yeast transformation buffer) containing 0.75 m spermidine trihydrochloride and 0.3 mm spermine tetrahydrochloride; the DNA was immediately used for yeast transformation.

Yeast transformation

Transformation was carried out according to the procedure developed by Burgers and Percival (23). Spheroplasts were prepared with Yeast Lytic Enzyme using 0.1–1 units/10^7 cells according to the yeast strain used. Optimal conditions for transformation were defined in preliminary experiments using Ycp50 as a standard plasmid; transformation efficiencies were consistently in the range of 0.5–1 × 10^6 colonies/μg DNA. One hundred μl aliquots of spheroplasts resuspended in STC buffer at a concentration of 2 × 10^9/ml were mixed with 100 μl DNA from the ligation reaction. The transformation was performed at 22°C in 8-ml polystyrene tubes. Cells from each single tube were added to 8 ml of top agar without uracil and plated onto prewarmed plates also lacking uracil. Colonies grew up in about 5–6 days and were replicated onto plates lacking uracil and tryptophan to check for the presence of both YAC vector arms; the colonies that grew on the plates were subjected to PCR screening to identify apo[a]-containing YACs. Genomic DNA from AB1380 transformants carrying apo[a] YAC33 was analyzed by PCR with primer pairs spanning different regions of the apo[a] coding as well as the 5′- and 3′- flanking regions of the apo[a] gene. Primer sequences were as follows: apo[a] promoter–pentanucleotide repeat: 5′-ATT TGC GGA AAG ATT GAT ACT ATG C-3′ and 5′-CCA AAT CAC GTC AGT GCA CTT CAA-3′, 30 cycles with 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C; apo[a] kringle IV-type 2: 5′-TGT CAG GTG GGA GTA CTG CAA C-3′ and 5′-GCC CAT CCT AAA GAC AAA GCC C-3′, 30 cycles with 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C; apo[a] 3′ untranslated region: 5′-TCA ACC TAC TTA GAA GCT GAA C-3′ and 5′-TTT GTC AGA CCT TAA AAG C-3′, 30 cycles with 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; apo[a]-like gene: 5°-CAG GAA TCC GGA TTG TTC-3′ and 5′-TTG TAT GAA TGA ATC CTC-3′, 30 cycles with 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C. To check for the integrity of the YAC insert, high molecular weight DNA in agarose plugs was prepared from both L413 cell line and YAC33-bearing yeast cells as described (24); several restriction enzyme digestions were performed on both DNA samples followed by PFGE blotting and hybridization with probes spanning the apo[a] promoter, kringle IV and protease-domain regions.

Retrofitting of YAC33 to produce YAC33-LUC

PFGE-purified YAC33 was used as a template for the amplification of a 462 bp fragment linking 5′ sequences from −421 to −1 (with respect to the ATG start codon) of the apo[a] gene to the first 41 bases of the firefly luciferase coding region. The primers used for PCR were 5′-ATC GGA CGT CAC TTG GCA GAG GGT CTG G-3′, which contains an *Aat*II site at its 5′ end and 5′-AAT GGC GCC GGG CCT TTC TTT ATG TTT TTG GCG TCT TCC ATT TTG GGA CTG GCC AGC AGT-3′, which contains a natural *Nar*I site at its 5′ end. The amplified product was gel-purified, digested with *Nar*I, and ligated to a 2,635 bp *Nar*I/*Sal*I fragment from pGL2-Basic (Promega, Heidelberg, Germany) containing the luciferase coding sequences from the *Nar*I site at +42 and an SV40 polyadenylation signal. The ligation product was restricted with *Aat*II and *Sal*I and cloned into pBL-ASS, a derivative of pBlueScriptII-SK(+) (Stratagene, Heidelberg, Germany) that contains restriction sites for *Aat*II, *Sal*I, and *Swa*I placed between the vector's *Sac*I and *Apa*I sites to make pApoLUC. The gene encoding yeast alfa-aminoadipate reductase, an enzyme essential in lysine biosynthesis, was isolated as an *Xba*I-*Hind*III fragment from pLNA-1 (25) and cloned into *Xba*I- and *Hind*III-digested pBlueScriptII-SK(+); the gene was then re-isolated as a *Not*I-*Sal*I fragment and ligated to a *Not*I-*Swa*I fragment spanning the CEN4 sequence amplified from pCGS966. The ligated fragment was finally restricted with *Swa*I and *Sal*I and ligated to *Sal*I- and *Swa*I-digested pApoLUC to obtain the retrofitting vector pApoLLC. pApoLLC was digested with *Aat*II and *Swa*I and a 6.4 kb linear fragment was gel purified and used to transform AB1380 yeast spheroplasts containing YAC33. Transformed cells that had undergone homologous recombination were selected on plates lacking uracil, tryptophan, and lysine. The clones were analyzed by PFGE and those containing the 70 kb retrofitted YAC33-LUC were grown up.

Preparation of purified YAC DNA for microinjection

Agarose plugs containing high molecular weight DNA from AB1380 cells containing recombinant YACs were prepared as previously described (24). Ten such plugs were loaded on a preparative, 0.5% agarose gel and YAC33 was separated from the endogenous yeast chromosomes by PFGE in 0.5× TBE buffer at 5

V/cm for 25 h with pulses of 17.5 s. The YAC 33 position on the gel was determined as described above and an unstained gel slice containing the YAC was isolated and immediately embedded in a 4% Nusieve GTG agarose gel for a second run at 50 V for 12 h with a 90° angle in order to concentrate the DNA. A small gel block containing the YAC was equilibrated for 1 h at 4°C in TE, 100 mm NaCl, 0.75 mm spermidine trihydrochloride, 0.3 mm spermine tetrahydrochloride with three buffer changes, melted at 68°C for 10 min, and digested with 10 units of β-agarase/500 μl DNA at 45°C for 2 h. Finally, the DNA was dialyzed at 4°C for 4 h on 0.05 mm microdialysis membranes (Millipore, Germany) floating on microinjection buffer (10 mm Tris-HCl, pH 7.5, 0.1 mm EDTA, 100 mm NaCl, 0.75 mm spermidine trihydrochloride, 0.3 mm spermine tetrahydrocloride). A small DNA aliquot was dialyzed against TE and subjected to PFGE analysis to verify the integrity of the YAC.

Microinjections

A total of ~2 picoliter of purified YAC-DNA at a concentration of ~1 ng/μl (corresponding to ~5 YAC copies) was microinjected into each fertilized C57BL/6 × SJL F2 hybrid mouse egg using standard procedures (26). Tail DNA samples from 17 newborn mice were PCR amplified with primer pairs specific for the apo[a] kringle IV-type 2 region (see Yeast transformation section); the animals found positive for such PCR were further analyzed by PCR with primer pairs specific for the apo[a] promoter and 3′ untranslated region and for the apo[a]-like gene (see Yeast transformation section). Further PCR analysis was done to check for the presence of the junction between the right YAC vector arm and the genomic insert and the CEN4 gene on the left YAC vector arm with the following primer pairs: right YAC arm-genomic junction: 5′-TCT GGA AGA GCC TCA GGA C-3′ and 5′-AGC TTA GTA TAT AAA TAC AC-3′, 30 cycles with 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C; left YAC arm (CEN4 gene): 5′-AAG TCG ATA TGG ACT TAG TC-3′ and 5′-GTT ATC TAT GCT GTC TCA CC-3′; 30 cycles with 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C.

Animals and diet

Mice were weaned at 21 d and housed in a non-barrier animal facility on a 12-h light–dark cycle, with free access to a normal chow diet (Ssniff GmbH, Soest, Germany) and water. Blood samples were collected from the retroorbital sinus and transferred into Microvette CB1000 tubes (Sarstedt, Nürnbrecht, Germany). After a 20 min incubation at 37°C, the samples were centrifuged for 5 min at 1,500 *g*. Apo[a] and apoB serum levels were determined by enzyme linked immunoabsorbent assay as described previously (27). Serum levels of progesterone were determined by radioimmunoassay (28). Four 18-week-old female YAC apo[a] transgenic animals were placed on a high-fat diet (purchased from ICN Biomedicals GmbH, Eschwege, Germany) containing 17.84% butter, 0.98% corn oil, 48.33% sucrose, 19.33% casein, 1.23% cholesterol, 0.48% sodium cholate, 4.67% alphacel nonnutrive bulk, 4.79% American Institute of Nutrition mineral mixture, 0.97% American Institute of Nutrition vitamin mixture, 0.97% choline chloride, 0.29% dl-methionine, and 0.12% vitamin E (250 U/g). After 2 weeks on a high-fat diet, the animals were placed back on normal chow diet. Serum samples were obtained prior to initiation of the high-fat diet, after 2 weeks on the high-fat diet, and 14 and 28 days after the animals were placed on normal chow diet.

Northern blot

Total RNA was prepared from various tissues using RNA STAT-60 (TelTest B Inc., Friendswood, TX) following the instructions of the manufacturer. A total of 15 μg of RNA from each tissue was size-fractionated on a 1% (w/v) agarose, 0.6 m formaldehyde gel and then transferred to a nylon membrane (Hybond N+, Amersham, Arlington Heights, IL). A probe was generated from apo[a] KIV type 2 repeat using reverse transcriptase-PCR (Promega RT-PCR kit, Promega, Madison, WI) and two oppositely oriented oligonucleotides (5′-TGA CAC CAC ACT CGC ATA GTC GGA C-3′ and 5′-GAT GAC CAA GCT TGG CAG GTT CTT CC-3′) to amplify a 315 bp fragment from hepatic YAC33 apo[a] poly(A)+ RNA. The fragment was gel-purified and radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol) using Megaprime DNA Labeling System (Amersham, IL). A radiolabeled 1141 bp fragment from the rat glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (29) was used as a loading control. The ^{32}P-labeled probes (~1×10^6 cpm/ml) were allowed to hybridize with the filter for 2 h at 65°C in Rapid-hyb buffer (Amersham, IL). The filter was washed with 0.1% (w/v) SDS, 0.1 × SSC (1 × SSC contains 150 mm sodium chloride and 15 mm sodium citrate) at 70°C for 30 min prior to being exposed to Reflection™ NEF 496 film (DuPont-NEN, Boston, MA) with intensifying screens at −80°C for the indicated times.

SDS-PAGE and immunoblotting

Fractionation of serum samples (0.25 μl) by reducing and non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting was performed as described previously (27) using POD-conjugated fab fragments of the monoclonal antibody 1A2 (30) and the Enhanced Chemiluminescence Western Blot Detection Kit (Amersham Buchler GmbH, Braunschweig, Germany).

Density gradient centrifugation

Density gradient centrifugation of mouse plasma (100 μl per tube) was performed as described (31). Tubes were unloaded from the bottom into 15 fractions.

Castration and sex steroid treatment

Eight-week-old male and female YAC apo[a] transgenic mice (n = 5 per group) were castrated under anesthesia (0.04 ml/g of the mixture: 1 ml normal saline, 0.8 ml Ketavet®, 0.2 ml Rompun®). Plasma samples were collected 15, 29, 76, 97, and 114 days after surgery. Sex steroid treatments were performed with castrated female and male animals (n = 3 per group) 14 days after surgical castration. Animals were treated for 12 days with single subcutaneous applications per day of vehicle (1 volume benzylbenzoate, 9 volumes castor oil), testosterone (50 μg/ml) or estradiol (5 μg/ml). Plasma samples were collected before the treatment, and 0, 14, and 62 days after the treatment, respectively.

Luciferase reporter gene assays

Transient transfection of luciferase plasmids into HepG2 hepatocarcinoma cells and subsequent determination of cytosolic luciferase activity was performed as previously described (14).

RT/PCR analysis

DNA-free total liver RNA was prepared from 5-week-old male and female YAC33-LUC transgenic mice. The product of a first strand cDNA synthesis with random primers and AMV reverse transcriptase (Boehringer Mannheim, Germany) was taken as a template for PCR amplification using Taq DNA polymerase (Boehringer Mannheim, Germany) and the following primer pairs specific for GAPDH and luciferase, respectively: GAPDH-5′: 5′-CCT GGC CAA GGT CAT CCA TGA CAA C-3′, GAPDH-3′: 5′-GTC CAC CAC CCT GTT GCT GTA GCC-3′, Luciferase-5′: 5′-GAT TCT AAA ACG GAT TAC CAG G-3′, Luciferase-3′: 5′-GCG TTT TGC

AAC CGC TTC C-3′. A standard PCR with 25 cycles for GAPDH and 40 cycles for luciferase cDNA detection was performed.

RESULTS

YAC cloning of a human apo[a] allele

Most apo[a] alleles in the population are associated with low plasma levels of Lp[a]. Therefore, to develop mice that expressed high plasma apo[a] levels from a human gene in its own genomic context, we cloned a specific apo[a] allele from an individual with high plasma levels of Lp[a]. The individual whose apo[a] allele was cloned had a plasma level of Lp[a] of 72 mg/dl and had two apo[a] alleles; one allele contained 14 KIV repeats and the other contained 37 repeats. Immunoblot analysis of plasma from this individual revealed that only the apo[a] allele with 14 KIV repeats was associated with circulating apo[a] protein; the apo[a] allele with 37 repeats was a null allele. Genomic DNA from EBV-transformed lymphocytes from this individual was used to clone a 370 kb fragment that contained the apo[a] allele with 14 KIV repeats as well as ~40 kb and ~200 kb of 5′ and 3′ flanking regions, respectively (**Fig. 1A**). A genomic YAC library was made using size-enriched *Not*I/*Sfi*I-fragments of ~370 kb in length. A total of 504 recombinant yeast clones were arranged in 42 pools of 12 clones. These pools were screened for the presence of apo[a] gene sequences using PCR and apo[a] specific primers. One pool gave a positive PCR product leading to the isolation of YAC33 which had the expected size of 390 kb and gave amplification products with PCR primer pairs specific for different regions of the human apo[a] gene (data not shown). Southern blot analysis of the cloned apo[a] gene in YAC33 cells revealed no differences with the donor allele in EBV-transformed lymphocytes. Size heterogeneity of the *Hpa*I restriction fragments, which include the highly repetitive KIV type 2 repeats of the apo[a] gene, was seen (data not shown) but >80% of the *Hpa*I restriction fragments had an identical size as the corresponding fragment in EBV-transformed lymphocytes from the donor individual.

Development of YAC33 transgenic mice

Purified YAC33 DNA was microinjected into fertilized C57BL/6 × SJL F_2 hybrid mouse eggs. DNA from 17 animals was screened by PCR for the presence of apo[a] kringle sequences leading to the identification of five PCR positive animals which were further analysed by PCR for the presence of the right YAC arm/human genomic DNA junction, the apo[a] promoter, the apo[a] KIV type 2 repeats, the apo[a] 3′ untranslated region, the apo[a]-like gene, and the left YAC arm (CEN4). Three animals gave specific amplification products with all primer pairs and had apo[a] plasma levels between 5 and 20 mg/dl (data not shown). The founder with the highest apo[a] plasma level was bred with C57BL/6 mice to establish a YAC33 transgenic line, which was maintained as an apo[a]-hemizygous line by repeated backcrosses with C57BL/6 mice. No apo[a]-homozygous animals were obtained after >150 matings of hemizygous animals, probably due to embryonic lethality caused by the interruption of an essential gene by the apo[a] transgene. The integrity of the YAC transgene in the mice was assessed using both PCR and PFGE Southern blot analysis. The sizes of the restriction fragments from the apo[a] gene of the transgenic

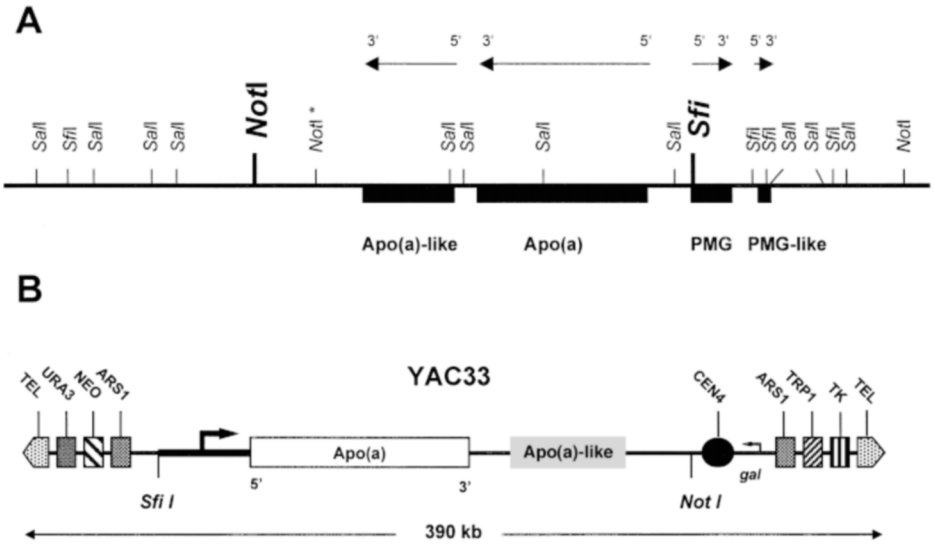

Fig. 1. Chromosomal and YAC localization of human apo[a] gene. Panel A: long-range restriction map of the apo[a]–plasminogen gene family on human chromosome 6q2.6–2.7, indicating in bold the *Not*I and *Sfi*I restriction sites, which have been used to clone a defined apo[a] allele. The asterisk labeled *Not*I site is not cleaved in human genomic DNA, probably due to methylation. The arrows above the map indicate the transcriptional orientation of the four genes. Panel B: schematic organization of YAC33 comprising a 370 kb insert of human genomic DNA cloned into the YAC vector (56). TEL, *Tetrahymena* telomere; CEN4, yeast centromere; ARS1, yeast autonomously replicating sequence; URA3, TRP1, genes for selection of YAC transformants; NEO, neomycin resistance cassette; TK, *gal*, *Escherichia coli* gal1 promoter.

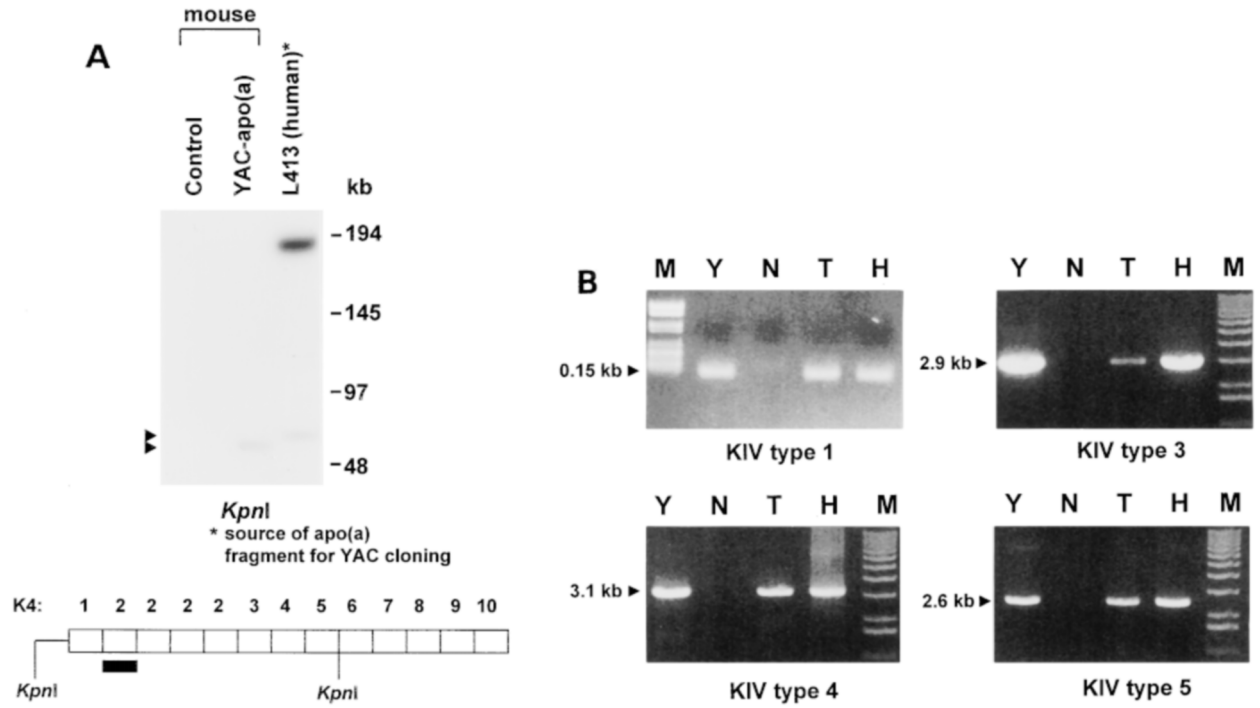

Fig. 2. Characterization of YAC33 integrate in transgenic mice. Panel A: PFGE blot of *Kpn*I restricted genomic DNA from non-transgenic and YAC33 transgenic mice and from L413 lymphocytes. The localization of *Kpn*I sites within the human apo[a] gene are shown below the blot. The blot was hybridized to a KIV type 2 probe (black bar). The arrows indicate the position of *Kpn*I fragments in the lanes that contain YAC-apo[a] and L413 DNA, respectively. Panel B: PCR analysis of YAC33 yeast DNA (**Y**), non-transgenic mouse DNA (**N**), YAC33 transgenic mouse DNA (**T**), and human DNA (**H**) using kringle IV type 1, 3, 4, and 5 specific primers (32).

mouse were identical to the apo[a] allele from which it was cloned with one exception, which is shown in **Fig. 2A**. Genomic DNA from the transformed cell line and the transgenic mice was digested with *Kpn*I and Southern blot analysis was performed using a genomic probe from the intron separating the two exons of apo[a] kringle IV type 2 (Fig. 2A). The *Kpn*I fragment was ~5.5 kb shorter in the transgenic mouse. A single KIV type 2 repeat is encoded by 5.5 kb. To ensure that all the other unique KIV repeats were present in the mice PCR reactions were performed with primers specific for apo[a] kringle IV type 1, type 3, type 4 and type 5 (32); products of the expected size were seen using DNA from the transformed cell line and from the YAC33 transgenic mouse (Fig. 2B). Taken together, our data are consistent with the smaller-sized *Kpn*I fragment being due to the loss of a single KIV type 2 repeat in the apo[a] transgene of our mice. Thus, our mouse has four rather than five copies of the KIV type 2 repeat.

Apo[a] mRNA in YAC33 transgenic mice

To investigate apo[a] mRNA expresssion in YAC33 transgenic mice, we performed a Northern blot analysis using total liver RNA. Non-transgenic mice and transferrin–apo[a] mice expressing an apo[a] minigene with 18 kringles under the control of the mouse transferrin promoter (33) were included as controls. Apo[a] transcripts of 7.5 kb and 9.5 kb were detected in the liver samples from YAC33 and transferrin–apo[a] mice, respectively (**Fig. 3A**). As human plasma apo[a] is derived almost exclusively from the liver (7, 34) we addressed the tissue-specificity of apo[a] expression in YAC33 transgenic mice. Northern blot analyses of total RNA from different tissues (liver, heart, brain, muscle, kidney, intestine, and spleen) showed liver-specific expression of the 7.5 kb apo[a] transcript (Fig. 3B).

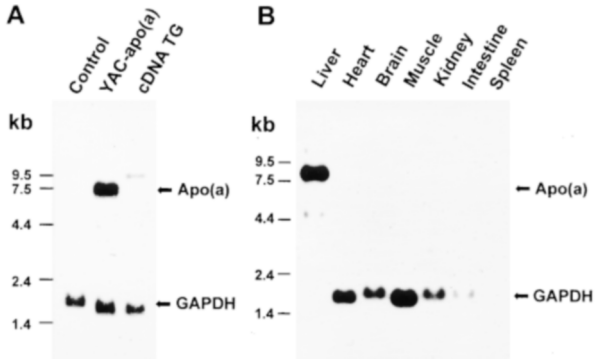

Fig. 3. Northern blot analysis of apo[a] mRNA in YAC33 transgenic mice. Fifteen μg of total RNA from indicated tissues was fractionated on formaldehyde agarose gels. The positions of size markers are indicated in each panel. The filters were hybridized simultaneously with an apo[a] kringle IV probe and a GAPDH probe. Panel A: liver RNA from YAC33 transgenic mice contained a 7.5 kb apo[a] transcript as compared to 9.5 kb apo[a] transcripts in the liver of minigene transgenic mice which were obtained by microinjection of a 18 kringle construct. Panel B: tissue-specific expression of apo[a] mRNA in the liver of YAC33 transgenic mice.

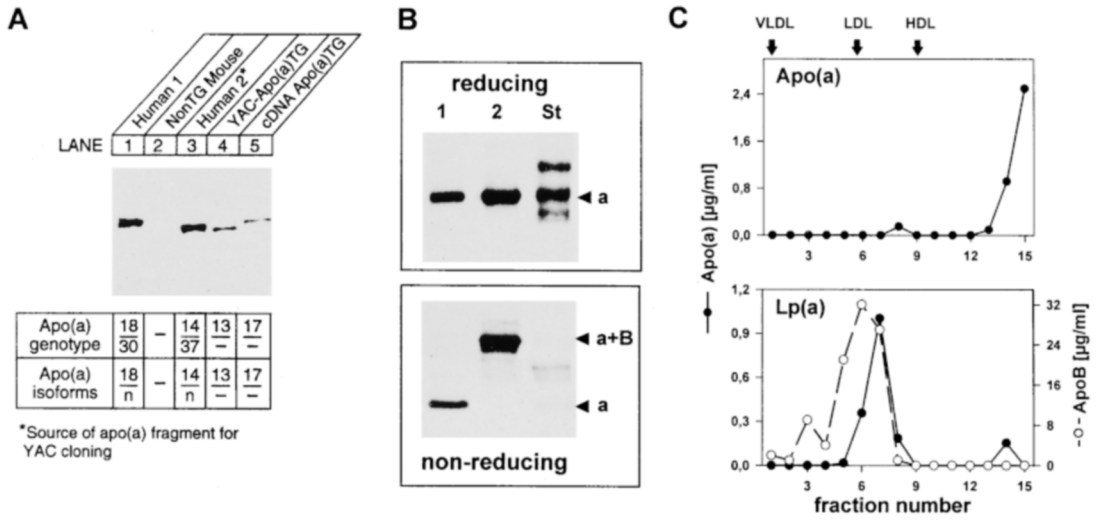

Fig. 4. Lp[a] assembly in transgenic mice. Panel A: immunoblot comparing apo[a] isoforms in human plasma (lanes 1 and 3) and in mouse plasma (lane 2: nontransgenic, lane 4: YAC33 transgenic, lane 5: apo[a] cDNA transgenic). The band in lane 3 represents the gene product from the human apo[a] allele which was cloned into YAC33. Panel B: immunoblot analysis of transgenic apo[a] after reducing and non-reducing SDS-PAGE. Plasma samples (0.25 μl) from YAC33 transgenic (lane 1) and YAC33 and apoB transgenic mice (lane 2) were fractionated on a 4.5% polyacrylamide gel. A recombinant apo[a] standard (*ST*) with 10, 14, and 18 kringle domains was included as a size marker. Panel C: density gradient fractionation of plasma from YAC33 transgenic mice (upper panel) and YAC33 and apoB transgenic mice (lower panel). The fractions were tested by ELISA for their apo[a] and apoB content as indicated. The positions of VLDL, LDL, and HDL are indicated.

Apo[a] in the plasma of YAC33 transgenic mice

Immunoblot analysis with a monoclonal antibody directed against human apo[a] was performed to compare the apo[a] glycoproteins in YAC33 transgenic mice and in the human subject (human 2), whose transformed lymphocytes were used as a source for the cloning of YAC33 (**Fig. 4A**). Also included in this immunoblot analysis was plasma from the transferrin–apo[a] cDNA mouse. The slightly reduced size of the apo[a] in the plasma of YAC33 transgenic mouse (lane 4) as compared to the donor individual (lane 3) is compatible with the YAC-apo[a] mice having one less kringle IV repeat.

Previous studies with transferrin–apo[a] mice revealed that apo[a] does not form a covalent complex with mouse apoB-100 (35). However, in transgenic mice expressing both human apo[a] and human apoB-100, the apo[a] is covalently linked with apoB-100 (36). To determine whether the apo[a] circulating in the plasma of the YAC-apo[a] mice could assemble into Lp[a] particles, we bred the YAC33 transgenic mice with human apoB-100 transgenic mice (36). Immunoblot analysis with an anti-apo[a] monoclonal antibody was performed under reducing (Fig. 4B, upper panel) and non-reducing (Fig. 4B, lower panel) conditions. Formation of a covalent complex between apo[a] and apoB-100 results in a retardation in the migration of apo[a] under non-reducing conditions. Apo[a] migration was significantly retarded in the double transgenic mice (Fig. 4B, lower panel), which is consistent with apo[a] forming a covalent complex with human apoB-100. Plasma samples from single and double transgenic mice were also subjected to density gradient centrifugation. Almost all the apo[a] in YAC33 apo[a] transgenic mice was present in the lipoprotein-free infranatant fractions of the gradient (Fig. 4C, upper panel). In contrast, the vast majority of apo[a] in mice expressing both the YAC-apo[a] and human apoB-100 transgenes was associated with lipoproteins (Fig. 4C, lower panel) that had a density between LDL and HDL, which is characteristic of Lp[a].

Effect of sex hormones on apo[a] serum levels

We measured apo[a] serum levels in five female and five male YAC33 transgenic mice every 2 weeks. At each time point, serum levels of apo[a] varied markedly among the different female mice; there also was significant intra-individual variation in apo[a] levels. No consistent age-associated trends were seen (**Fig. 5A**, left panel). In contrast, in male mice, serum levels of apo[a] decreased dramatically to almost undetectable levels between 4 and 8 weeks of age in all the male mice (Fig. 5A, right panel). A similar age-dependent decrease in plasma apo[a] levels was seen in another strain of YAC-apo[a] transgenic mice (18). Variation in serum levels of apo[a] was apparent in the males at the 28 and 42 day point.

To determine why the serum levels of apo[a] varied so much in our female mice, we measured serum levels of apo[a] and progesterone on 5 consecutive days. In mice, the estrus cycle is 4–5 days and is associated with significant variation in the serum progesterone concentrations. The serum levels of apo[a] appeared to vary in synchrony with the progesterone levels (Fig. 5B). The maximal intra-individual variation in serum levels of apo[a] during the estrus cycle was 1.5-, 1.6-, and 2.4-fold.

Next, we compared the effect of castration of female and male YAC33 transgenic mice on serum levels of apo[a]. Eight-week-old male animals have an almost undetectable

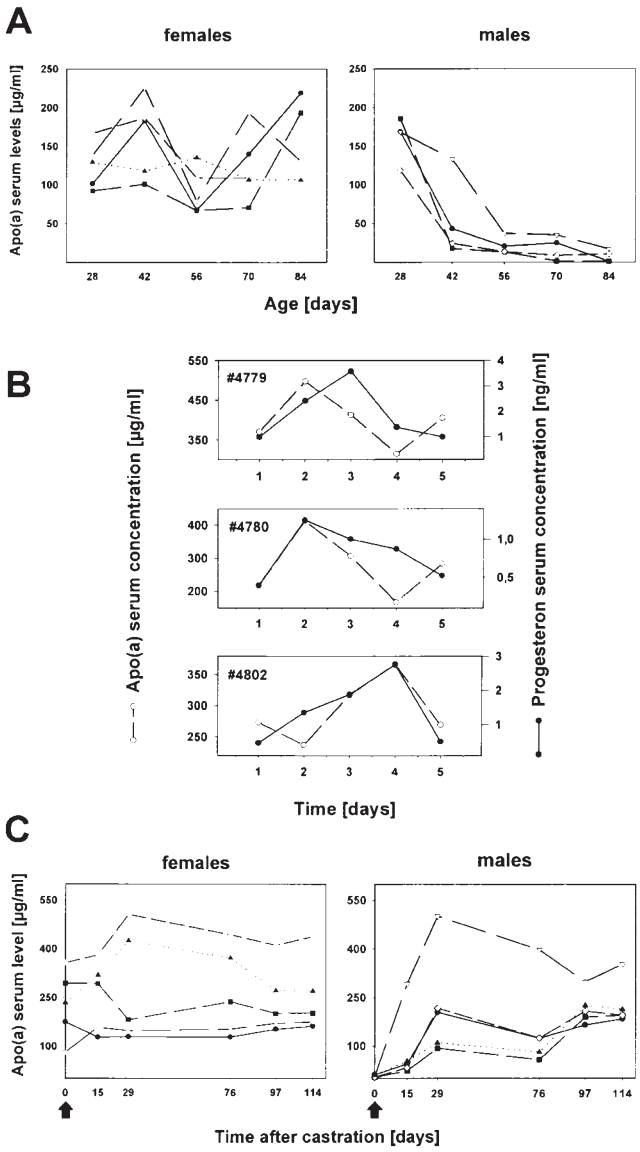

Fig. 5. Variation of apo[a] serum level with age, sex, estrus cycle, and castration. Panel A: female animals exhibited significant intra-individual variation of their apo[a] serum levels. At the age of 6 to 8 weeks (sexual maturity), the apo[a] serum level of male animals dropped to almost undetectable levels. Panel B: apo[a] and progesterone serum levels were determined in three 8-week-old animals on 5 subsequent days. In each animal, the day with the lowest progesterone level was defined as day 1. Note the parallelism in the course of apo[a] and progesterone serum levels. Panel C: after castration of 10-week-old female and male YAC33 transgenic mice, apo[a] serum levels were monitored for up to 114 days. Note the dramatic increase of serum apo[a] concentration after castration of male animals.

serum level of apo[a]; after castration there was a dramatic increase in their apo[a] serum levels (Fig. 5C), as was previously observed in another YAC–apo[a] transgenic mouse model (18). In contrast, no consistent effect of castration was seen on the serum levels of apo[a] in female mice (Fig. 5C, right panel), although castrated female mice appeared to have less intra-individual variation in their apo[a] serum levels (compare Fig. 5A and Fig. 5C).

Effect of high-fat diet on apo[a] serum levels in YAC33 transgenic mice

Female YAC33 transgenic mice were fed a synthetic high-fat diet. Within 2 weeks of initiating the diet, a dramatic (>60-fold) decrease of serum levels of apo[a] was seen despite an associated increase in serum cholesterol levels (**Fig. 6A**). Serum levels of apo[a] and cholesterol returned to pre-diet levels on a chow diet (Fig. 6A). To identify the component(s) in the high-fat diet responsible for the decrease of apo[a] serum levels in the YAC33 transgenic animals, the mice were fed high-fat diets lacking either cholesterol or sodium cholate. A much smaller effect on apo[a] and cholesterol serum levels was seen when sodium cholate or cholesterol was omitted from the high-fat diet (Fig. 6B). A Northern blot analysis of liver RNA revealed that compared to the normal chow diet, the high-fat diet and the high-fat diet minus cholesterol were associated with a 99% and 87% decrease, respectively, in the levels of apo[a] mRNA (Fig. 6C) whereas the high-fat diet minus cholate led only to a 52% fall of hepatic apo[a] mRNA levels (Fig. 6C).

Transcriptional control of apo[a] in transgenic mice

Previous in vitro studies in HepG2 hepatocarcinoma cells with reporter gene plasmids containing up to 1.5 kb apo[a] 5′ flanking sequences have indicated the presence of important *cis*-acting elements in the proximal apo[a] promoter (13) and in the far upstream region (20). However, the in vivo relevance of these elements remains to be shown. To address the question whether the plasminogen–apo[a] intergenic region contains the transcriptional control elements that mediate tissue-specific, hormone-responsive expression of apo[a] in vivo, we used a 'gene-targeted YAC transgene approach', which has been previously used to delineate the structural requirements in human apoB-100 for Lp[a] assembly (37). We performed a homologous recombination experiment in YAC33-containing yeast cells to obtain the reporter YAC33-LUC, in which the apo[a] coding region and the human genomic sequences at the 3′ end of the apo[a] gene in YAC33 were replaced by a luciferase reporter gene. The strategy for this gene targeting experiment is outlined in **Fig. 7A**. By detailed Southern blot analyses, PCR analyses, and sequence analysis of the junction between the apo[a] promoter region and the luciferase coding sequence in the retrofitting vector, we could not detect any differences in the 40 kb plasminogen apo[a] intergenic region between YAC33 and YAC33-LUC (data not shown). We microinjected the YAC33-LUC DNA into fertilized mouse eggs and obtained four independent lines of transgenic mice. The integrity of the transgene was assessed by Southern PFGE and long-range PCR analysis in three of the strains. With these methods we could not detect any rearrangements in the integrated YAC33-LUC DNA of the four transgenic lines (Fig. 7B and data not shown). Furthermore, we addressed the integrity of the luciferase gene in YAC33-LUC transgenic mice. Cloning of PCR-amplified luciferase coding sequences from two lines of YAC33-LUC transgenic mice into a CMV promoter expression vector and subsequent transient transfection of

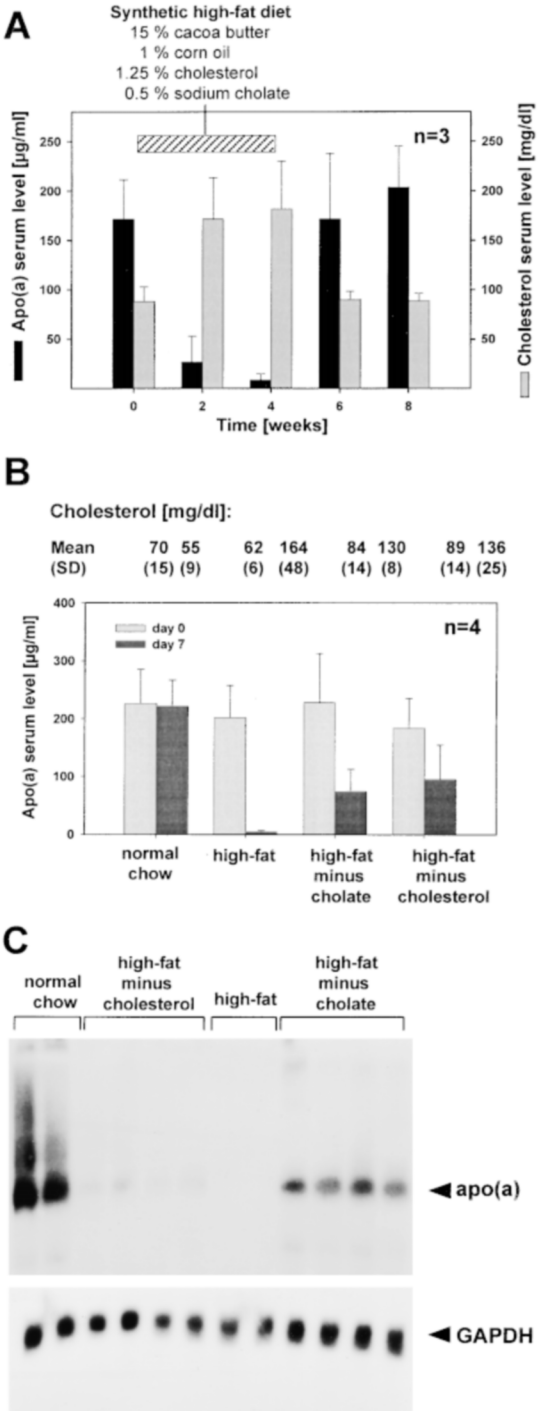

Fig. 6. Effect of high-fat diet on apo[a] and cholesterol plasma levels in YAC33 transgenic mice. Panel A: 8-week-old female YAC33 transgenic mice (n = 3) were placed for 4 weeks on a high-fat diet (hatched bar). Thereafter, the animals were placed back on normal chow diet. Apo[a] and cholesterol serum levels were determined every 2 weeks. Panel B: 8-to-12 week old female YAC33 transgenic mice (n = 4) were fed for 1 week with the indicated diets. Apo[a] serum levels (mean/SD) and cholesterol serum levels (mean day 0/mean day 7) at the beginning and at the end of the diet are indicated within and on top of the graph, respectively. Panel C: Northern blot analysis of total liver RNA (10 µg per lane) from 12 different YAC33 transgenic mice after 9 day feeding with the indicated diets. The blot was hybridized with probes for apo[a] kringle IV (upper part) and GAPDH (lower part), respectively.

the resulting reporter plasmids into HepG2 cells yielded high levels of cytosolic luciferase activity, as compared to mock-transfected cells (**Fig. 8A**). However, by RT-PCR analysis of total RNA from liver and spleen we could not detect any luciferase transcripts even after 40 PCR cycles, whereas 25 cycles yielded a plentiful fragment from the GAPDH RNA (Fig. 8B).

DISCUSSION

In this paper we describe the development of the first apo[a] transgenic mouse in which a well-characterized human apo[a] allele associated with high plasma levels of Lp[a] has been successfully expressed. A 370 kb human genomic DNA fragment that contains the entire apo[a] gene plus ~40 kb and ~200 kb of 5′- and 3′-flanking sequences was cloned into a YAC vector and subsequently transferred into the germline of transgenic mice. The resultant YAC33 transgenic mice revealed a liver-specific apo[a] expression pattern. The serum levels of apo[a] ranged from 5 to 25 mg/dl and showed significant sex- and age-specific effects. In female YAC33 transgenic mice, the serum level of apo[a] varied over a 3-fold range and paralleled the variations of progesterone levels over the 4-day estrus cycle of the animals. Male mice experienced a dramatic decrease of apo[a] levels after puberty, which was reversed by castration. A pronounced decrease of hepatic apo[a] mRNA and apo[a] serum levels was observed in female YAC33 transgenic mice after feeding a synthetic high-fat, high-cholesterol, cholate-rich diet. Four independent lines of transgenic mice containing 40 kb of 5′ flanking sequences from the apo[a] gene linked to a luciferase reporter gene did not express detectable levels of luciferase, suggesting that additional sequences are required for expression of apo[a] in vivo.

This is not the first YAC-apo[a] mouse that has been made. Previously, a transgenic mouse model expressing a human apo[a] allele from a YAC library was developed (18). The source of the genomic DNA used to construct the library was not known. Thus, this is the first apo[a] transgenic mouse that expresses apo[a] in its own genomic context, in which the construct and the apo[a] expression can be compared between the mouse and the human from which the apo[a] allele was cloned. We show that no recombination occurred outside the KIV type 2 repeat region. This allele, which is associated with high levels of Lp[a] (72 mg/dl) is associated with a lower level in mice (<8 mg/dl). It is highly unlikely that the >9-fold lower level of Lp[a] in the mice is due to the lack of a single K-IV repeat in the apo[a]; this would be expected to be associated with higher rather than lower levels of plasma Lp[a] given the inverse relationship between apo[a] isoform size and plasma levels of Lp[a]. It is more likely that the lower serum levels of apo[a] in YAC33 transgenic mice reflect differences in the metabolism of apo[a] in mice and men. We cannot rule out a mutation in the apo[a] gene construct in the YAC-apo[a] mice that contributes to the lower expression level of apo[a]. White, Guerra, and Lan-

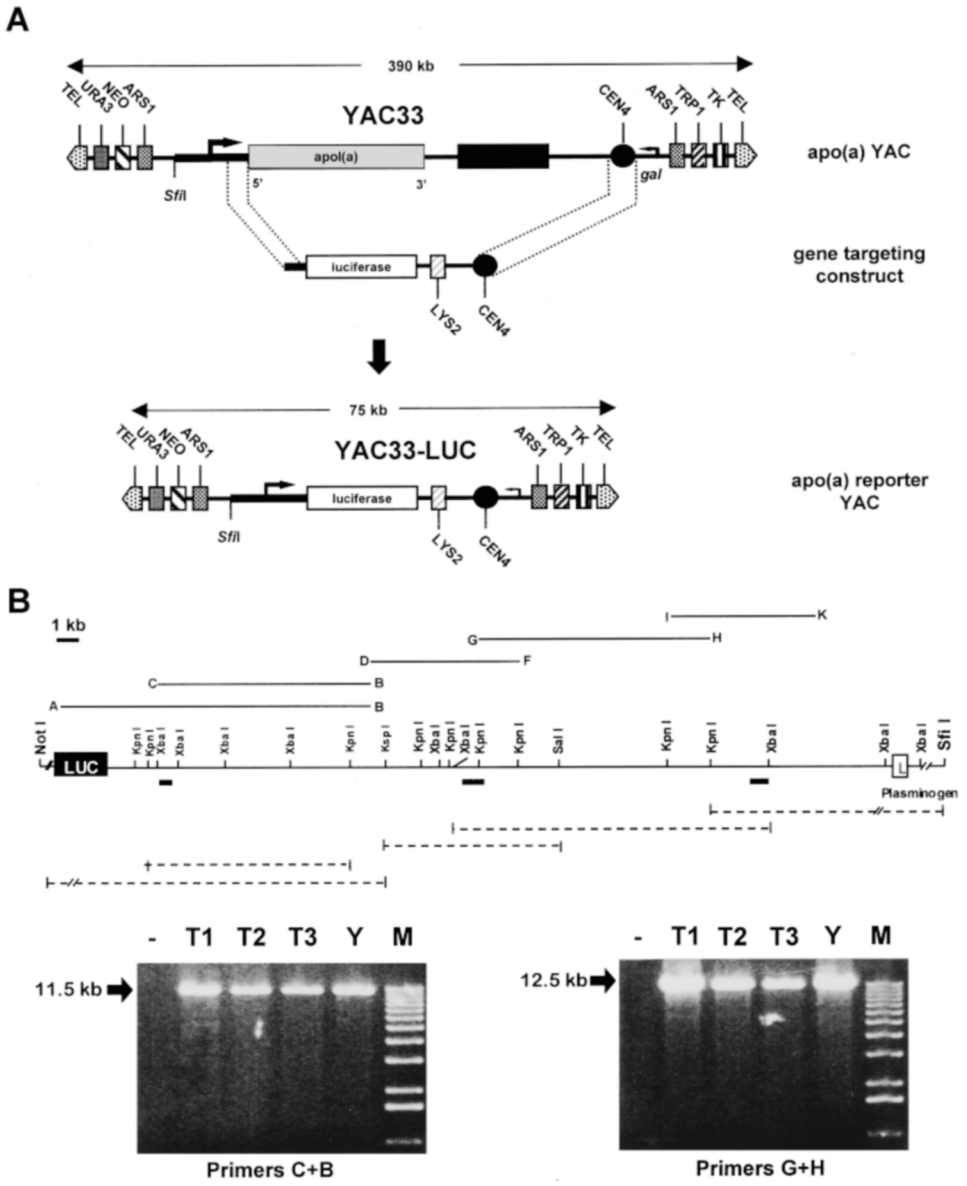

Fig. 7. YAC33-LUC transgenic mice. Panel A: the gene targeting construct LUC-LYS2-CEN4 was transformed into YAC33 containing yeast cells to obtain YAC33-LUC, in which 40 kb 5′ flanking sequences of the human apo[a] gene are linked to the luciferase reporter gene. Panel B: PCR and Southern blot analysis of YAC33-LUC transgenic mice. Fragments that have been detected in YAC33-LUC transgenic mice by PCR and by Southern blotting are shown on top and below a long-range map of the *Not*I/*Sfi*I fragment of YAC33-LUC, respectively. The PCR primers A to K are given in Experimental Procedures. Black bars below the map indicate the probes that were used for the Southern blot analysis. Two examples of long-range PCR analysis with indicated primer pairs and template DNAs from nontransgenic mice (−), from three independent lines of YAC33-LUC transgenic mice (**T1**, **T2**, and **T3**) and from YAC33-LUC containing yeast cells (**Y**).

ford (38) have shown that some apo[a] alleles associated with low plasma levels of Lp[a] are retained in the ER in a not fully glycosylated store. Immunoblot analysis of liver proteins from YAC-apo[a] transgenic mice failed to show any accumulation of the precursor form of apo[a] (data not shown), suggesting that it at least is folded and secreted properly.

Expression of large human genomic fragments in mice is often associated with an expression pattern that is similar to that seen in humans, due to conservation of proximal and distal *cis*-acting elements between the species (39). The expression pattern of apo[a] in the YAC33 transgenic mice resembles that seen in humans. Plasma apo[a] in humans is almost completely liver-derived (34) and the apo[a] mRNA in our transgenic model was detected only in the liver. Thus, the sequences necessary for hepatic expression of apo[a] are contained in the 370 kb transgene.

The serum levels of apo[a] fall dramatically in male YAC33 transgenic mice in association with puberty. This fall in serum levels of apo[a] is presumably due to the Lp[a]-lowering effect of testosterone which is supported by the re-

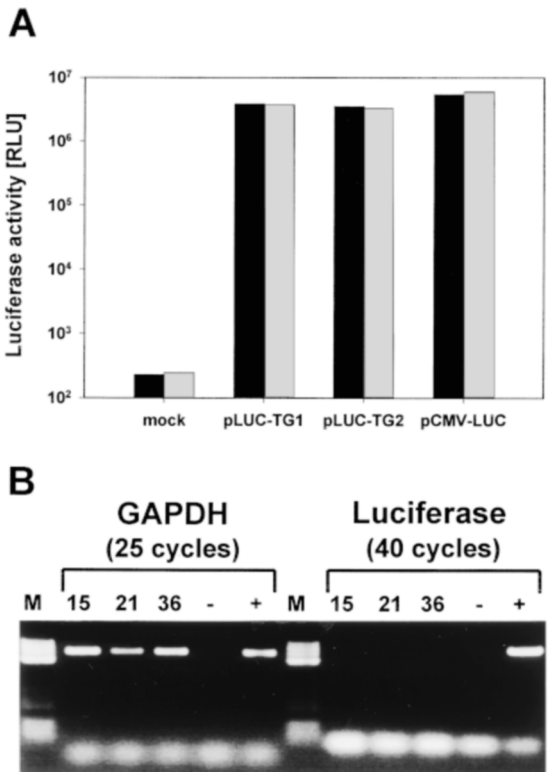

Fig. 8. Functional luciferase gene in YAC33-LUC transgenic mice is not expressed in 5-week-old animals. Panel A: the luciferase genes from two YAC33-LUC transgenic lines were PCR amplified and subsequently cloned into a CMV promoter plasmid. Transient transfection of the resulting reporter gene plasmids into HepG2 cells revealed high-level luciferase expression similar to the level obtained after transfection of the pCMV-LUC control plasmid in two independent transfection experiments (light and dark bars). Panel B: DNA-free total liver RNA from three independent transgenic lines was subjected to reverse transcription and PCR amplification using GAPDH primers or luciferase primers. Amplifications without template and with control templates (human genomic DNA and luciferase plasmid) were included as negative (−) and positive (+) controls, respectively.

versal of the effect by castration (Fig. 5B and C). Subsequent treatment of castrated male YAC33 transgenic mice with pharmacological doses of testosterone or estradiol leads to almost undetectable apo[a] serum levels (data not shown), as has also been shown in another strain of YAC-apo[a] transgenic mice (18). In humans, no corresponding decrease in plasma apo[a] levels is seen at puberty, but administration of testosterone to adult males is associated with a fall in plasma levels of Lp[a] (40) and orchidectomy with a ~20% increase in plasma levels of Lp[a] (41). At this stage, we can only speculate about the basis of the differences between humans and transgenic mice in their response to male sex hormones. Human and murine testosterone receptors might differ with respect to their intracellular concentration and/or in their affinity to response elements in or around the apo[a] gene.

Plasma levels of Lp[a] are reduced by estrogen in humans (42–45). However, the level of reduction of plasma Lp[a] by endogenous hormones is much more pronounced in YAC transgenic mice. Recently, plasma levels of Lp[a] were shown to be increased in the luteal phase of unstimulated and stimulated menstrual cycles (46). Similarly, YAC33 transgenic mice exhibited a parallel time-course of apo[a] and corpus luteum-derived progesterone serum levels during the estrus cycle. The 1.5-fold to 2.4-fold variation of apo[a] serum levels along the estrus cycle in three analyzed animals (Fig. 5B) was comparable to that recently reported in another strain of YAC-apo[a] transgenic mice (47). The reduced intra-individual variation of apo[a] serum levels after castration (Fig. 5A and 5C) is probably due to the elimination of the effects of estrus cycle on apo[a] serum levels.

Human Lp[a] levels do not change in response to cholesterol (48) and most other dietary pertubations (49). In YAC33 transgenic mice, a synthetic high-fat, high-cholesterol diet led to a 2-fold increase in serum cholesterol but a pronounced decrease in serum apo[a] levels. The fall of serum apo[a] levels was associated with a similar fall in the hepatic apo[a] mRNA levels. Thus, the high-fat diet affects either the rate of apo[a] transcription or apo[a] mRNA stability. The same high-fat diet, which lacked either synthetic cholesterol or cholate, had similar, but less pronounced, effects on serum levels of apo[a]. In rodents, unlike humans, cholesterol feeding results in an up-regulation of 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis. In this way, the rodent is able to efficiently excrete much of the excess dietary cholesterol as bile acids. Cholate feeding inhibits 7α-hydroxylase and thus leads to a reduction in bile acid production. As a consequence there is an increase in hepatic cholesterol levels. Cholesterol is known to mediate transcriptional repression of some genes involved in lipid metabolism by means of a sterol responsive element (SRE) (50) localized in the proximal 5′ flanking region of the regulated genes. We have found the sequence 'AAAATCACGTCAGTGC' about 1300 bp upstream of the apo[a] ATG start codon which shares 13 of the 16 bp of the SRE-containing sequence 'AAAATCACCCCACTGC' in the human LDL receptor gene, suggesting that a similar mechanism of transcriptional control could occur for the apo[a] gene. We must, however, caution that such putative mechanism, whether existing, seems not to operate in humans but could nevertheless account for the observed apo[a] down-regulation observed in the mouse by means of the presence of species-specific transcription factors or to a particularly favorable chromatin environment in the transgene integration site.

Feeding YAC-apo[a] transgenic mice with high-fat diet or with the same diet lacking either cholesterol or cholate resulted in a reduction of hepatic apo[a] mRNA to 2%, 10%, and 35% of the level in animals fed with a normal chow diet, indicating an additive effect of cholate and cholesterol on apo[a] mRNA levels (Fig. 6C). The 3.5-fold difference in apo[a] mRNA levels after feeding the high-fat diets lacking cholate or cholesterol was not seen at the level of serum apo[a] (Fig. 6B), suggesting that high cholesterol in the cholate-deficient diet in addition to its effect on apo[a] mRNA levels also inhibits a posttranscriptional step in apo[a] synthesis.

In an attempt to delimit the *cis*-acting elements that govern the expression of apo[a], we developed four lines

of transgenic mice containing the YAC33-LUC construct in which 40 kb of 5′ flanking sequences from the apo[a] gene were linked to a luciferase reporter gene. As judged by PCR and Southern blot analysis of genomic DNA from three independent lines, we could not detect any rearrangement in the YAC33-LUC integrates. In addition, we observed strong cytosolic luciferase activity in HepG2 cells transfected with plasmids containing a CMV promoter linked to PCR-cloned luciferase genes from YAC33-LUC transgenic mice indicating that the luciferase gene in YAC33-LUC transgenic mice is intact. The absence of detectable luciferase mRNA and/or enzyme activity in liver and spleen of four independent YAC33-LUC transgenic lines suggests that important *cis*-acting elements for liver-specific apo[a] expression in vivo are located outside the apo[a]-5′ flanking sequences used. Apo[a] is a member of a gene family on human chromosome 6q2.6–q2.7 which includes three other members, one of which being plasminogen (4, 51). All four members show a striking homology with each other and seem to be expressed mainly in the liver, thus raising the possibility of a common *cis*-acting element being responsible for their co-ordinated expression in liver. Such a hepatic control region (HCR) has been identified in another apolipoprotein gene cluster on human chromosome 19, 18 kb downstream of the apoE promoter and 9 kb downstream of the apoC-I promoter (52). Other downstream locus control regions (LCR) have been identified in the 3′ flanking region of human T-cell receptor alpha/delta locus (53) and of the CD2 gene (54), respectively.

The comparison of apo[a] and luciferase expression in YAC33 and YAC33-LUC transgenic mice, respectively, points to the apo[a] structural gene and/or downstream sequences to harbor the *cis*-acting elements that are necessary for liver-specific expression of apo[a]. Our data are compatible with the extremely weak basal apo[a] promoter activity as observed by several authors (13, 14, 55). Recently, two enhancer regions located 20 kb and 28 kb upstream from the apo[a] transcriptional start point have been described, which mediate a 10- to 15-fold transcriptional stimulation from apo[a] and other promoters in HepG2 and in HeLa cells (20). The absence of detectable luciferase expression in YAC33-LUC transgenic mice suggests that these enhancer regions, which are contained in the 40 kb apo[a] 5′ flanking sequences in the YAC33-LUC construct, are not sufficient to trigger liver-specific apo[a] expression in vivo. Alternatively, the lack of luciferase expression in YAC33-LUC transgenic mice might be caused by undetected minor rearrangements in the YAC integrates or by positional effects due to the YAC integration sites. Although we cannot exclude these possibilities, they appear improbable from a comparison of transgene expression in the obtained lines of YAC33 and YAC33-LUC transgenic mice with a complete integrate: each of three independent YAC33 transgenic lines containing an intact YAC33 construct showed a high level of apo[a] expression. In contrast, we could not detect luciferase expression in the liver and in the spleen of three independent transgenic lines carrying a complete YAC33-LUC construct. Our present work is aimed at the generation of additional YAC33 variants by homologous recombination in yeast cells. The characterization of transgenic mice obtained by microinjection of such YAC33 variants should lead to a better understanding of the transcriptional regulation of apo[a] expression and could lead to the identification of attractive molecular targets for the development of Lp[a]-lowering therapeutics that inhibit apo[a] transcription.

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