Regulation of the human apolipoprotein AIV gene expression in transgenic mice

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Abstract The apolipoprotein (Apo) AI-CIII-AIV gene cluster has a complex pattern of gene expression that is modulated by both gene- and cluster-specific *cis*-acting elements. In particular the regulation of Apo AIV expression has been previously studied in vivo and in vitro including several transgenic mouse lines but a complete, consistent picture of the tissue-specific controls is still missing. We have analysed the role of the Apo AIV 3′ flanking sequences in the regulation of gene expression using both in vitro and in vivo systems including three lines of transgenic mice. The transgene consisted of a human fragment containing 7 kb of the 5′ flanking region, the Apo AIV gene itself and 6 kb of the 3′ flanking region (−7+6 Apo AIV). Accurate analysis of the Apo AIV mRNA levels using quantitative PCR and Northern blots showed that the −7+6 kb Apo AIV fragment confers liver-specific regulation in that the human Apo AIV transgene is expressed at approximately the same level as the endogenous mouse Apo AIV gene. In contrast, the intestinal regulation of the transgene did not follow the pattern observed with the endogenous gene although it produced a much higher intestinal expression following the accepted human pattern. Therefore, this animal model provides an excellent substrate to design therapeutic protocols for those metabolic derangements that may benefit from variations in Apo AIV levels and its anti-atherogenic effect.

Key words: Apolipoprotein AI-CIII-AIV; Quantitative polymerase chain reaction; Tissue specificity; Intestine; Liver; Regulation; Transgenic mouse

1. Introduction

The gene encoding apolipoprotein (Apo) AIV can be found with those encoding Apo AI and CIII in a cluster of 17 kb on chromosome 11 [1]. The identification of rearrangements of this cluster in patients suffering from premature atherosclerosis and epidemiological and clinical data have clearly demonstrated the importance of this chromosomal region in the regulation of lipid metabolism [2–4]. The Apo AI and AIV genes are transcribed in the same direction whereas the Apo CIII gene, located between the two, is transcribed in the opposite direction. The Apo AI and CIII genes have been reported to be primarily expressed in liver and intestine, whereas the Apo AIV gene is expressed mainly in the intestine with varying degrees of liver expression in different species [5–8]. Transgenic and knockout mice together with transient expression in tissue culture cells have provided extensive information regarding Apo AI-CIII-AIV gene cluster regulation, identifying a series of *cis*-acting elements which modulate their expression. Fig. 1 shows the organisation of the human Apo AI-CIII-AIV gene cluster. Liver control elements for Apo AI and CIII genes reside in the 5′ flanking region, whereas an intestinal control region thought to regulate the expression of the entire gene cluster at this locus is located in the Apo CIII-AIV intergenic region, close to the Apo CIII promoter [9–12].

The regulation of Apo AIV expression has been previously studied in vivo and in vitro including several transgenic mouse lines [8,9,13–15] but a complete, consistent picture of the tissue-specific controls is still missing. Particularly, the elements that regulate Apo AIV gene expression in both man and mice need further definition. The transgenic mouse experiments and some direct tissue measurements point to minimal expression of Apo AIV mRNA in liver with the main site of expression being the intestine [6]. However, there are reports of variable Apo AIV mRNA intestine/liver ratios even using the same transgene [9,13]. These contradictory results may have their origin not only in the different methodologies used to measure Apo AIV mRNA, which have included dot blot hybridisation [7], RNA excess solution hybridisation [16] and Northern blot quantification [13], but also in the known variability of Apo AIV RNA levels with different physiological conditions [8,13,17] and genetic backgrounds [15]. The latter has been shown in inbred mice to be responsible for up to an 11-fold variation of Apo AIV mRNA levels in the liver. Furthermore, it has been shown that the contribution of the liver towards the Apo AIV pool in plasma is approximately equal to that of the intestine [18] and that hormones that influence Apo AIV levels in plasma have been found to affect Apo AIV mRNA levels in a tissue-selective manner with the liver showing a much tighter regulation than the intestine [19–21]. All these data taken together point to an essential role of the liver in Apo AIV synthesis and metabolism.

In this paper we analyse the role of Apo AIV 3′ flanking sequences in the regulation of gene expression using both in vitro and in vivo systems including three lines of transgenic mice. The transgene consisted of a human fragment containing 7 kb of the 5′ flanking region, the Apo AIV gene itself and 6 kb of the 3′ flanking region (−7+6 kb AIV fragment). Accurate analysis of the Apo AIV RNA levels using quantitative PCR and Northern blots showed that the −7+6 kb AIV fragment confers liver-specific regulation in that the human Apo AIV transgene is expressed at approximately the same level as the endogenous mouse Apo AIV gene. This is not the case for the intestinal expression of the transgene indicating either that there may be important differences between mouse and human Apo AIV gene regulation or that the fragment used for the construction of the transgenic animal is missing essential *cis*-acting regulatory elements.

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Abbreviations: Apo, apolipoprotein; wt, wild type

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2. Materials and methods

2.1. Isolation of transgene genomic fragment

Two partially overlapping clones spanning the Apo AIV gene and flanking regions, clone λ59 and clone λ63, have been previously isolated [22]. A *Bam*HI fragment, containing the Apo AIV −7274/+67 fragment, was excised from clone λ63 and cloned into pBluescript II KS^+. This was in turn cut with *Sph*I (genomic site) and *Hin*dIII (plasmid), into which we introduced the genomic fragment excised, using the same restriction sites, from clone λ59. Finally we cloned a *Hin*dIII (genomic)-*Sal*I (plasmid) fragment obtained again from clone λ59 into the pBluescript II KS^+ clone cut with the same restriction enzymes (Fig. 1), generating the transgene genomic fragment −7+6 Apo AIV.

2.2. Construction of expression vectors

The construction of plasmids p−89/+67 Apo IV/CAT 6, p−3870/+67 Apo IV/CAT 6 and p−7274/+67 Apo IV/CAT 6 has been described in [23]. The addition of 6 kb of the 3′ Apo AIV flanking sequence was carried for the p−89/+67 Apo IV/CAT 6 and p−3870/+67 Apo IV/CAT 6 constructs by the insertion of a *Kpn*-*Kpn* fragment excised from the −7/+6 Apo AIV plasmid into the *Kpn* site in the polylinker of pBLCAT 6. The addition of the 6 kb Apo AIV 3′ flanking region for p−7274/+67 Apo IV/CAT 6 was performed by digestion of the p−3870/+67 Apo IV/CAT 6+3′ with *Nru*I/*Pvu*I and subsequent cloning into and p−7274/+67 Apo IV/CAT 6 cut with the same enzymes.

2.3. Cell culture and transient expression assays

HepG2 and CaCo-2 cells (ATCC) were cultured in DMEM medium containing 4.5 g/l glucose, supplemented with 10% foetal calf serum, 1% non-essential amino acids, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). Cells were plated out at a density of 5×10^5 cells/6-cm dish and the media changed 3 h before transfection. Plasmids were prepared by double banding in caesium chloride or by Qiagen plasmid kit (Qiagen) and introduced into the cells using DOTAP (Boehringer Mannheim) according to the manufacturer's instruction. Each dish was transfected with 1 pmol of CAT plasmid, 2.5 mg of pRSVβ-gal, 0.125 pmol HNF-4 expression vector (pMT2-HNF-4) or control plasmid (pMT2), and carrier plasmid DNA (pBluescript II KS^+) up to 12.5 mg per dish. CAT assays were performed using [^{14}C]chloramphenicol and butyryl coenzyme A. The relative CAT activities were normalised by β-galactosidase activity.

2.4. Human Apo AIV transgenic mice

Transgenic mice were produced by micro-injection of the subgenomic Apo AIV fragment (−7+6 Apo AIV) into the male pronuclei of fertilised mouse oocytes (strain C57/6-CBA), as described [24]. Surviving oocytes were transplanted into the oviducts of pseudopregnant foster mothers. Mice harbouring the transgene were detected by Southern blotting and/or PCR analysis with the human Apo AIV primers using DNA prepared from tail biopsies. Three human Apo AIV transgenic mice lines were established, named 8018, 8021 and 8022. For successive experiments male and female mice were used when they were 3–4 months of age, with a mixed genetic background of 87.5% Swiss and 12.5% C57, and fed at libitum. Litter mates negative for human Apo AIV were used as controls.

2.5. Southern blot analysis

Quantitative Southern blot analysis was performed using equal amounts of *Eco*RI-digested DNA, prepared from tail biopsies of 3-week-old F1 transgenic mice belonging to each pedigree [25]. Filters were exposed overnight to XO-MAT film (Kodak) at −80°C with an enhancer screen. Quantification of the copy number was obtained by densitometric scanning of the film. Hybridisation probes were generated using the *Eco*RI-*Eco*RI fragment of the Apo AIV gene via nick translation of 50 ng of DNA by the random priming method using the oligolabelling kit supplied by Pharmacia. Radiolabelled DNA probes were purified from unincorporated nucleotides using Nick columns (Pharmacia).

2.6. RNA extraction

Total cellular RNA was isolated from liver and the proximal third of the small intestine by the guanidinium thiocyanate procedure [26]. The final pellet was then re-suspended in 200 μl of ddH_20 and treated with RNase-free DNase I (Boehringer Mannheim), according to the manufacturer's instructions. RNA integrity was confirmed by denaturing formaldehyde agarose gel electrophoresis and quantified by measuring absorbance at 260 nm. Using the primer pair for β-actin amplification PCR was performed using 2 μg of RNA to check that no contaminating genomic DNA remained. The two human hepatic and small intestine RNA samples were obtained from Clontech and Invitrogen. Each sample derives from a different individual and is the same used for the commercial poly$(A)^+$ blots obtained from the same suppliers.

2.7. Northern blots

20 μg of total RNA was first denatured by 2.2 M formaldehyde and 50% formamide at 68°C for 15 min and then run on a 1% formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N, Amersham) by capillary blotting and hybridised to probes at 68°C under standard conditions. The mouse and human Apo AIV probes were constructed using the same methodology as described in Section 2.5 using the amplification fragments of the respective primer pairs. The probes were found not to cross-react. A Canberra Packard Instant Imager was used to quantify the mRNA levels. The GAPDH probe was used to normalise for equal loading of RNA between lanes.

2.8. Competitive PCR

The method entailed the addition to the PCR sample of known molar quantities of competitor DNA molecules that share the same sequence as the amplified target (including primer recognition sites) except for an insertion in the middle which allows resolution by gel electrophoresis. It has been shown that in these conditions the amount of competitor DNA yielding equal molar amounts of products gives the initial amount of the target gene [27]. cDNAs were obtained using the Pharmacia cDNA synthesis kit following the manufacturer's instructions. 5 mg of total RNA was used in a 20 μl reaction with oligo dT as primer. The reaction was incubated for 1 h at 37°C after which the volume was brought up to 50 μl. To compensate for variations in the RNA isolation, quantification and tube-to-tube variation in the RT reactions, the cDNAs used for the quantification of the mouse and human Apo AIV mRNA were first normalised by competitive PCR against an endogenous standard, the β-actin gene. All the subsequent PCR reactions measuring the mouse and human hepatic and intestinal Apo AIV mRNA were performed using the amount of cDNA which produced an equivalence point of 5 pg with the β-actin competitor. PCR was performed following the protocols of the Boehringer Taq DNA polymerase. The amplifications were performed on a thermal cycler (Perkin-Elmer) using the following conditions for 30 cycles: (i) denaturation at 94°C for 1 min, (ii) primer annealing at 56°C for 1 min, and (iii) extension at 72°C for 1 min. A slow annealing cycle of −2°C every 10 min from 94 to 56°C was added at the end to prevent cross-hybridisation between target and competitor sequences.

2.9. Primers and competitors

Synthetic DNA oligonucleotide were purchased from Primm s.r.l. (Milan, Italy). Their sequences (5′-3′) and utilisation are as follows. For β-actin competitive PCR: CATGTTTGAGACCTTCAACA (sense), ATCTCCTTCTGCATC-CTGTC (antisense). For mouse Apo AIV competitive PCR: ACAGTTTCAGAA-GACGGATG (sense), GTGGTCTGCATGCGCTGGA (antisense). For human competitive PCR: ACATCTCCAGAAATCTGAAC (sense), GGCGTTCTCCCGCA-GCACTC (antisense). For amplification of GAPDH fragment used in the production of the GAPDH probe: ACATGTTCCAGTATGATTCT (sense), ACGG-AAGGCCATGC-CAGTGA (antisense)

Competitor templates for β-actin, mouse Apo AIV and human Apo AIV were constructed by cloning the PCR cDNA product amplified by the respective oligonucleotide pairs into Puc 18 at the *Sma*I site. A 20-bp oligo in the case of the mouse and human Apo AIV competitor constructs and 200 bp of the intron sequence in the β-actin competitor were inserted into the middle of the cloned sequence of cDNA in order to allow resolution by gel electrophoresis.

2.10. Human Apo AIV protein expression in transgenic mice

5 μl of 1:50 (v/v) plasma in SDS buffer (2% SDS, 6 mM Tris pH 6.8, 10% glycerol, 0.8 M 2-mercaptoethanol, 0.01% bromophenol

blue) was resolved in an SDS-PAGE 7.5% polyacrylamide gel at 150 V for 2 h. The proteins were transferred to nitrocellulose (Schleicher and Schuell) with a semidry transfer unit in the presence of 25 mM Tris pH 6.8, 192 mM glycine, 20% methanol. After 1 h in blocking buffer (PBS, 0.1% Tween 20, 5% non-fat dry milk) the membranes were incubated for 1 h with 0.35 mg/ml of primary monoclonal human Apo AIV antibody (Boehringer Mannheim). The blots were washed for 30 min in blocking buffer, incubated for 1 h with sheep horseradish peroxidase-conjugated secondary antibodies (1:4000) directed against mouse IgG, washed in blocking buffer and then developed using the ECL chemiluminescence system and hyperfilm-ECL (Amersham).

3. Results and discussion

3.1. The 3′ Apo AIV flanking region influences transcription levels

In the course of our studies on the Apo AI-CIII-AIV cluster using intact HepG2 liver nuclei we have noticed that the newly cloned 6-kb Apo AIV showed significant DNase-hypersensitive sites. This preliminary observation was followed up by studying the ability of three different 5′ flanking region fragments to direct reporter gene expression both in the presence and in the absence of the 6-kb Apo AIV 3′ sequence in HepG2 and CaCo-2 cells.

Fig. 2 shows that all three promoter fragments used were able to direct transcription in HepG2 cells although the highest efficiency was found when the regulatory region upstream of the Apo CIII gene and all the intergenic sequence (−7.2 kb fragment) was included. This is consistent with the observation that two enhancers are present in this fragment: the Apo CIII enhancer located 0.8 kb upstream of the cap site of this gene [11] and the Apo CIII-AIV enhancer located 2.5 kb upstream of the Apo CIII gene and 4 kb upstream of the Apo AIV gene [23]. Co-transfection with a plasmid encoding the orphan nuclear receptor HNF-4, which has been demonstrated to activate transcription of the Apo AIV gene in HepG2 and CaCo-2 cells [22], increases the overall activity, except in the −89 construct, but the effect of the 3′ flanking

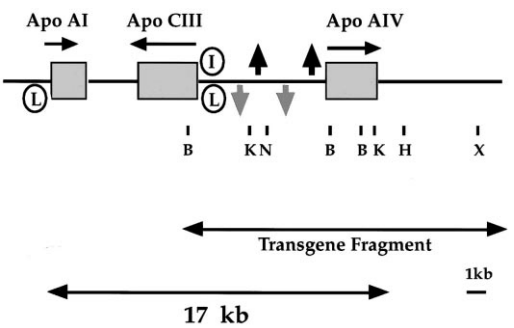


Fig. 1. Scheme representing the Apo AI-CIII-AIV gene cluster localised on the human chromosome 11 (11q23-qter) along with control elements that have been previously characterised. Arrows indicate the direction of transcription of each of the genes. The three promoters, not indicated, can be found at the 5′ end of each gene as can the hepatic enhancers for AI and CIII indicated by L. A single intestinal enhancer, I, located in the intergenic region between Apo CIII and AIV is thought to regulate the expression of the entire gene cluster at this locus. Black and grey arrows correspond respectively to positive and negative regulatory elements found in vitro for Apo CIII and AIV. The positions of all the regulatory elements are indicative. The −7+6 Apo AIV transgene fragment used in the generation of the human Apo AIV mice is also indicated. Abbreviations for restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; N, *Nru*I; K, *Kpn*I; X, *Xho*I.

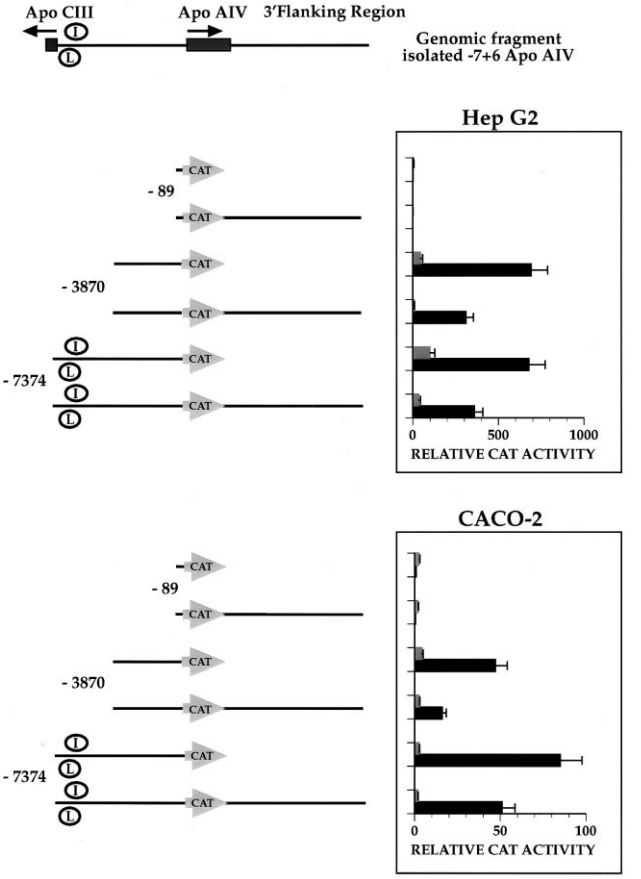


Fig. 2. Histogram showing the CAT activity in HepG2 and CaCo-2 cell lines directed by various lengths of 5′ flanking sequence of the Apo AIV gene in the presence and absence of the 6-kb 3′ flanking sequence. On the left, representations of the constructs used. On the right, the corresponding CAT activity obtained. Grey columns indicate basal levels. Black columns indicate the level of expression obtained upon co-transfection with the HNF-4 expression vector. Each CAT activity represents the mean of at least three separate transfections.

region was still clearly visible. The results on CaCo-2 cells were not as clear due to the fact that the three promoter constructs showed a very weak activity, unless the orphan receptor HNF-4 was co-transfected. In this case the pattern was similar to that observed in HepG2, only for the two largest promoter fragments. The promoter efficiency is clearly dependent on the 3′ flanking region which down-regulates transcription both in HepG2 (over 60% decrease in transcription, and 50% decrease in co-transfections with HNF-4) and CaCo-2 cells co-transfected with HNF-4 (40–60% decrease). Furthermore, it is also clear that the regulatory effect of the 3′ flanking region is independent of the control exerted by HNF-4 whose *cis*-acting elements are present in the intergenic region [22]. These results indicate that Apo AIV expression could be regulated by both 5′ and 3′ flanking sequences, a phenomenon already observed in this cluster with the Apo AI intestinal enhancer [12]. It was particularly interesting to take this study beyond the in vitro stage as hepatic and intestinal cell models are rather far from the tissue conditions. The only system able to give more accurate information was the use of transgenic animals including the 6 kb of the 3′ flanking region. This element was absent in previous studies with Apo AIV transgenic mice which had identical 5′ fragments up-

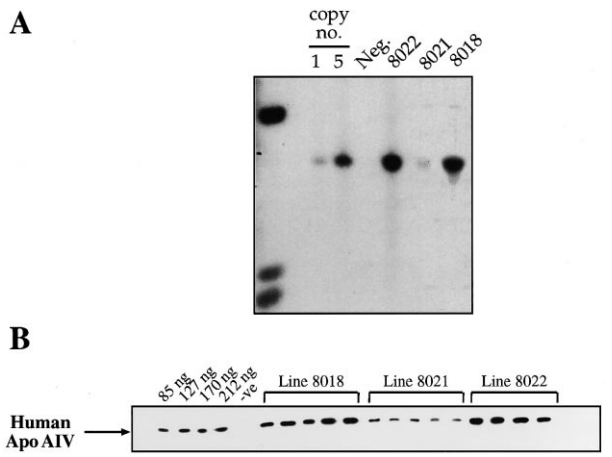


Fig. 3. A: Quantitative Southern blot analysis of the copy number of the human Apo AIV transgene present in the three lines. Dilutions of the plasmid corresponding to one and five copies of the transgene can be seen on the left. Copies of the transgene in the transgenic mice were estimated by densitometric scanning and are eight copies for line 8022, one for line 8021 and nine for line 8018. B: Human Apo AIV levels in mouse plasma was quantified by Western blot. Left, four different dilutions of a human plasma containing 17 mg/dl of Apo AIV. This is followed by the plasma of a non-transgenic littermate (−ve) and then four to five samples for each transgenic line. The human Apo AIV in the plasma of these mice was found to be for line 8018 181 ± 30 mg/dl, for line 8021 65 ± 11 mg/dl and for line 8022 202 ± 32 mg/dl.

stream of the Apo AIV gene but only 1 kb of 3′ flanking sequence [9,13]. We then undertook the study of the presence of putative long-distance control regions that can influence Apo AIV expression in vivo by introducing the −7+6 Apo AIV fragment in transgenic mice.

3.2. Human Apo AIV transgenic mice

Three transgenic lines were produced by microinjection of the −7+6 fragment into fertilised mouse oocytes. The transgene copies were estimated by densitometric measurements of Southern blot signals (Fig. 3A) and are: line 8021, one copy; line 8018, nine copies; line 8022, eight copies. All three lines were found to express human Apo AIV protein in plasma which was found to be at levels about three times higher than human levels for the mouse carrying the single copy and about 11 times for the other two lines (Fig. 3B). Thus, although not directly related to the gene copy number, an increase in protein level is observed. A strict correlation of Apo AIV mRNA and plasma protein levels has not been observed either in inbred species of mice [8,15] or in all previous studies of Apo AIV transgenic mice reported. In fact, DNA, RNA and protein concentrations were shown not to have a linear correlation perhaps suggesting some form of post-transcriptional control that modulates secretion and degradation of Apo AIV [13,14].

3.3. Tissue-specific expression of human Apo AIV mRNA in transgenic mice

A first analysis of the tissue distribution of Apo AIV mRNA in wild type (wt) mice, transgenic mouse lines and human tissues was carried out by Northern blot. Fig. 4A shows the analysis of mouse wt tissues on a poly(A)$^+$ commercial membrane (Origene), using a mouse Apo AIV probe. As expected, liver and small intestine gave a significant signal, the small intestine approximately 6-fold higher than in the liver. A faint but reproducible band was seen in RNA derived from stomach. No signal was observed by Northern analysis on total RNA carried out on kidney, heart and lung (data not shown). Given the levels of expression observed in the wt tissues the transgenic mouse analysis was carried out using total RNA derived from the tissues of the three lines and hybridising with both human and mouse Apo AIV probes in conditions that only the species-specific signal was detected (see Section 2). Fig. 4B shows the levels of the endogenous mouse Apo AIV mRNA for 8018 (similar results were obtained from the other two lines). The normalisation with the GAPDH and InstantImager quantification indicates that the levels in liver and intestine of the mouse Apo AIV mRNA levels reflect the ratio observed in wt mice, which is on average an 8-fold higher expression in intestine than in the liver. A parallel membrane was hybridised with the human probe (Fig. 4C) and after normalisation with GAPDH the expression of the human Apo AIV was on average 60-fold higher in intestine than in liver.

It was desirable for a comparative regulatory study to also have a clear idea of expression of Apo AIV in normal human tissues. Previous studies [7] had indicated the intestine to liver ratio of Apo AIV mRNA to be 100:1. In order to confirm this observation we used two pairs of commercial poly(A)$^+$ blots that included samples of intestine and liver from different individuals. Our results showed that, after normalisation with GAPDH, whilst in one blot the intestinal/hepatic ratio of Apo AIV mRNA levels was 1:1 (Fig. 4D), in the second (Fig. 4E) the expression was exclusively intestinal. Therefore we could only partially confirm the previous studies on human Apo AIV expression in intestine and liver, making a conclusion about the basal human Apo AIV mRNA levels difficult to draw. Obviously, these differences in highly standardised commercial samples are indicative of different physiological conditions at the time of sample collection, not to mention the potential differences in the genetic background. It is interesting to note in Fig. 4E that in the human gastrointestinal tract expression of Apo AIV mRNA is not detected in the stomach (as was the case in wt mice, Fig. 4A) and that there are similar levels in jejunum and ileum. The quantitative PCR studies in the mouse intestinal tract have shown that the levels of mouse Apo AIV mRNA detected along the whole small intestine were comparable (data not shown).

At this point it is clear that in vitro assays or direct tissue measurements are only indicative. Furthermore, it is well known that there are considerable variations in Apo AIV levels due to genetic background and physiological conditions. It follows that comparison of control elements can only be achieved using tissues from the same animal expressing both human and mouse Apo AIV. Therefore the measurement of human and mouse Apo AIV mRNA levels in the same sample obtained from a transgenic animal will allow direct comparison since the regulation of the transgene and the endogenous gene is subjected to the same genetic background and physiological conditions.

3.4. Quantitative PCR measurements

To this end, a detailed study of the tissue-specific expression of human and mouse Apo AIV was carried out using a purpose-developed competitive quantitative PCR method to

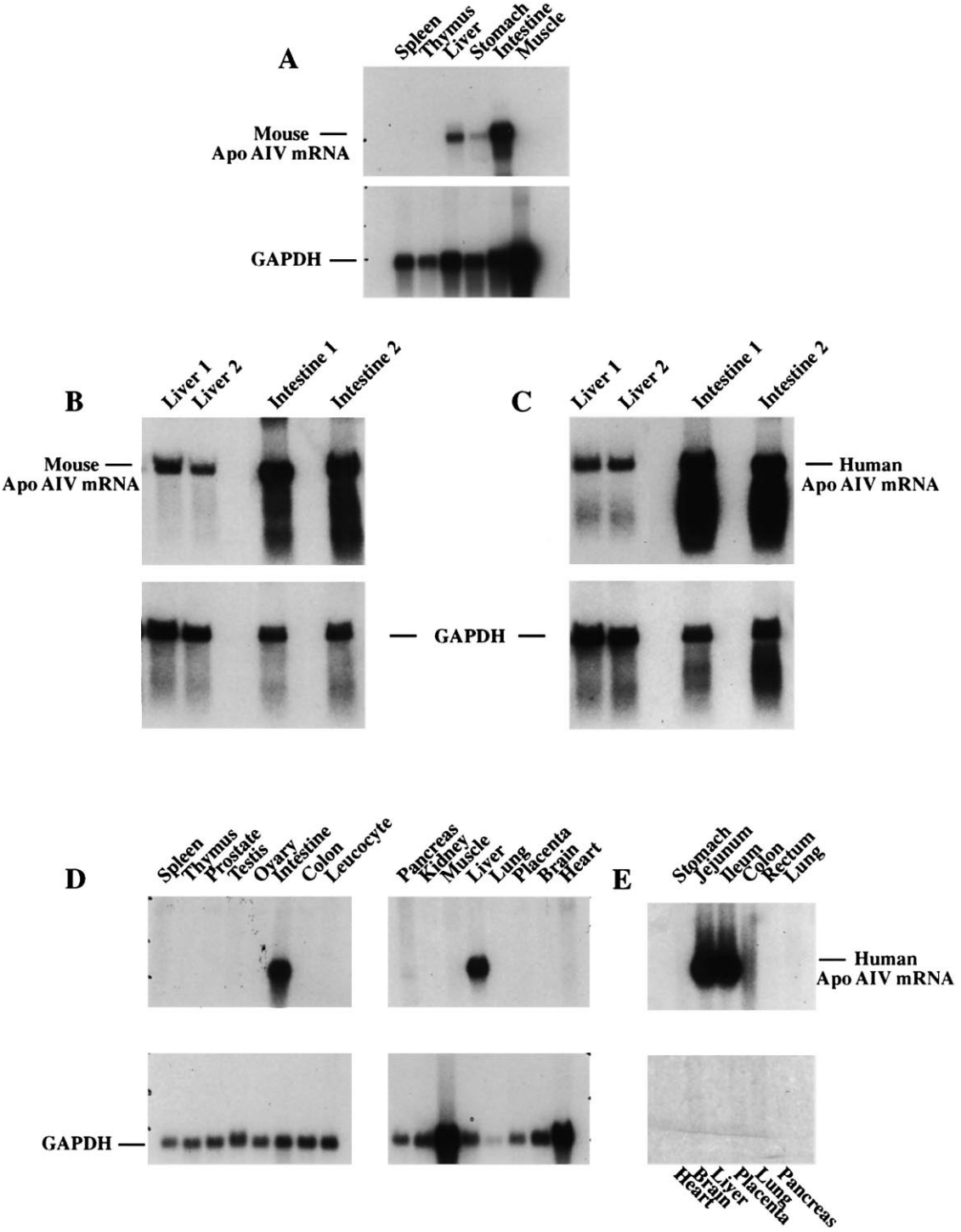
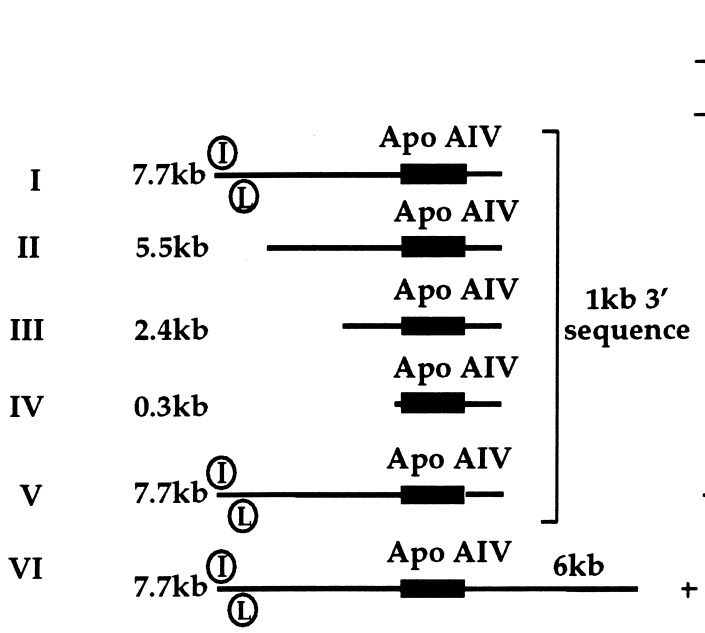


Fig. 4. Characterisation of the expression of the Apo AIV gene was carried out by quantitative Northern blot analysis with both human and mouse probes that did not cross-react, using the GAPDH mRNA for normalisation. A: Commercially available (Origene) mouse poly(A)$^+$ blot, probed with mouse Apo AIV. B: 8018 transgenic mouse RNA probed with mouse Apo AIV probe. C: Identical mRNA used in B, probed with human Apo AIV probe. D: Commercially available (Clontech) human poly(A)$^+$ RNA blots, probed with human Apo AIV probe. E: Commercially available (Invitrogen) human poly(A)$^+$ blots probed with human Apo AIV probe. Normalisation with GAPDH already performed by the suppliers.

measure accurately human and mouse Apo AIV mRNA and the β-actin mRNA as internal reference. The same Clontech mRNA sample of the poly(A)$^+$ blot was used to covalidate the quantitative PCR method. In fact, the level of Apo AIV mRNA by the quantitative PCR normalised for 5 pg of β-actin was 0.6 pg in the intestine and 0.55 pg for the liver, a 1:1 ratio, identical to the one obtained by the Northern blot normalised for equal amounts of GAPDH.

Fig. 5 shows an example of such determinations carried out in liver and intestine of the 8018 line. The data from all three

Table 1
Scheme of previous constructs used in the production of human Apo AIV transgenic mice with an estimate of the expression of the transgene



	Human Apo AIV transgene expression		Mouse Apo AIV endogenous expression	
	Intestine	Liver	Intestine	Liver
I	+	−	nd	nd
II	−	−	nd	nd
III	−	−	nd	nd
IV	−	−	nd	nd
V	+ + +	+	+ + +	−
VI	+ + + + +	+	+ + +	+

Constructs I–IV [9]; construct V [13]; construct VI, this paper.

transgenic lines normalised for 5 pg of β-actin mRNA are shown in Fig. 5B. In all cases, there are comparable levels of mouse and human Apo AIV mRNA in liver. In this tissue the values range from 0.3 to 0.8 pg for the mouse Apo AIV (the wt being 1.5 pg) and from 0.6 to 1.2 pg for the human Apo AIV. On the other hand, intestinal Apo AIV mRNA derived from the endogenous mouse gene was on average 1.18 (range 1–2 pg, wt 3.8 pg). These values were on average 18-fold lower than the human Apo AIV mRNA derived from the transgene (average 21.6 pg, range 15–27 pg). These results indicate a different intestine to liver ratio in the transgene compared to the endogenous gene, since human Apo AIV mRNA levels in the intestine are on average 25 times higher than those observed in the liver. The intestine to liver ratio of the mouse Apo AIV mRNA levels showed that the intestine expressed Apo AIV mRNA only three times more than the liver. These ratios reflect the results obtained from the Northern blot analysis (Fig. 4A,B). Table 1 shows a scheme of the different fragments used for producing Apo AIV transgenic mice with an estimation of the liver and intestinal expression. Our transgenic differs from those previously reported in the fact that the hepatic expression of endogenous mouse Apo AIV mRNA and the human transgene were at comparable levels. Table 2 shows that in all three transgenic lines the ratio between human and mouse AIV messenger in liver is 1.5–2. This indicates an essentially identical hepatic regulation of the human and mouse Apo AIV expression. In contrast, Table 2 shows that the human intestinal expression is 11–23-fold higher than that of the endogenous gene.

In previous human Apo AIV transgenic mouse studies (Table 1, construct V) it was reported that no endogenous hepatic expression was observed. However, the same mouse strains have been shown to express Apo AIV in the liver [8,28]. These differences can be explained by the techniques used for mRNA detection. In fact, in the analysis of the transgenics derived from construct V, the detection of Apo AIV mRNA was performed by Northern blot using very high stringency of hybridisation and short riboprobes to differentiate the human and mouse RNAs. This procedure obviously lowers the sensitivity of the method and only substantial levels of Apo AIV mRNA are detected. Construct V gave a significant human Apo AIV mRNA signal in the transgenic mouse liver but no endogenous mouse Apo AIV mRNA was detected. This indicates that the human/mouse liver Apo AIV mRNA ratio was very high. In the transgenic mice described in this paper (Table 1, construct VI) we see a human/mouse liver Apo AIV ratio close to 1:1. Our interpretation is that the absolute levels of hepatic human Apo AIV mRNA we are seeing are lower than those seen with transgene derived from construct V and are then comparable to the endogenous levels in this strain. This result is then consistent with the inhibitory effect of the 6-kb 3′ flanking region observed in vitro in hepatic-derived cells (Fig. 2A).

In contrast, the intestinal regulation of the transgene did not follow the pattern observed with the endogenous gene in the same tissue, possibly requiring regulatory elements in addition to those present in the 6-kb 3′ flanking region that may be present elsewhere in the 17-kb locus spanning the AI-CIII-AIV gene cluster. Alternatively, our results could indicate that the transgene includes a set of *cis*-acting elements that pro-

Table 2
Ratios of human intestine/mouse intestine and human liver/mouse liver using the mean values obtained from the quantitative PCR

	8018	8021	8022
H.In/M.In	23.00	18.30	11.00
H.Li/M.Li	2.00	1.50	2.00

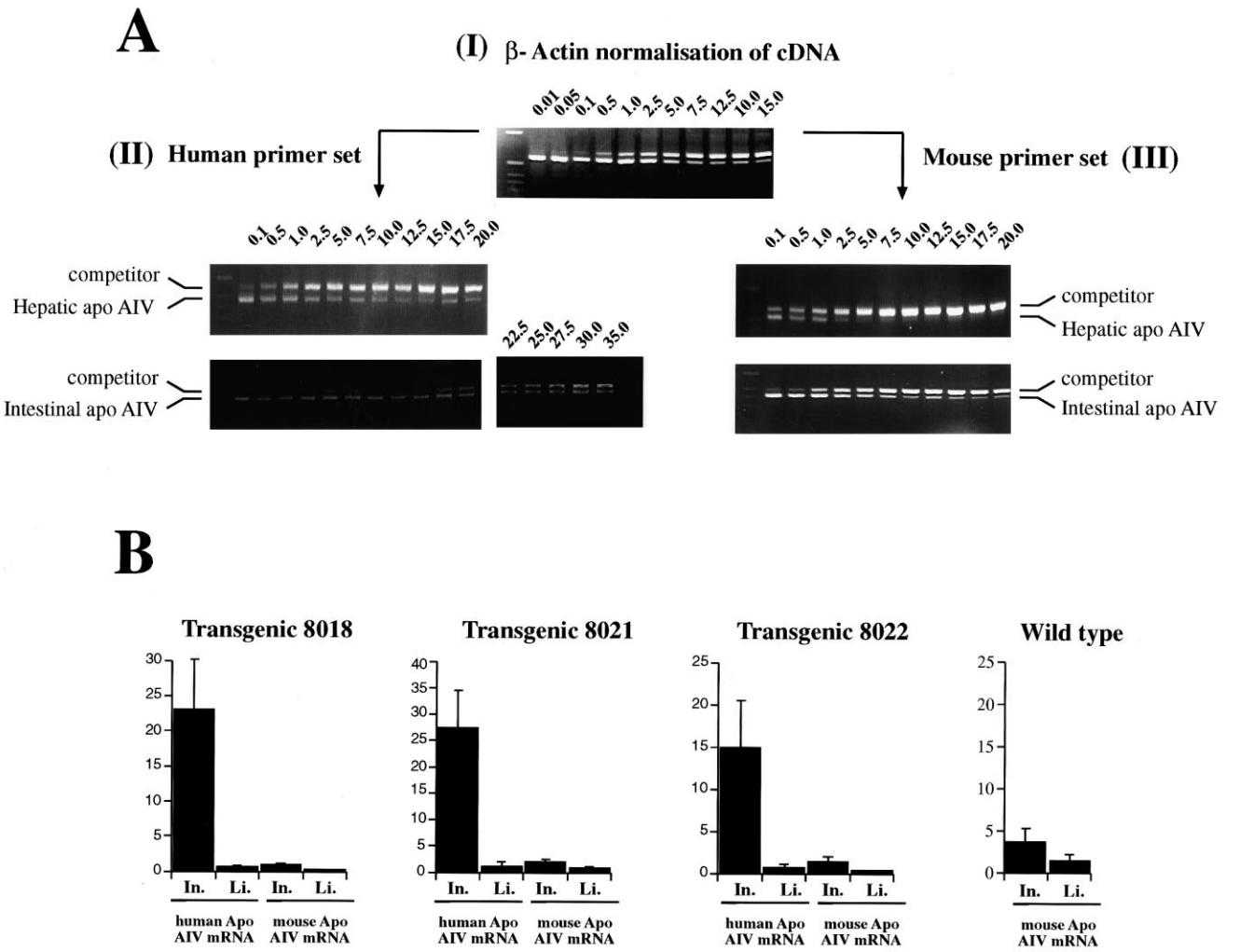


Fig. 5. A: cDNA made from RNA isolated from the proximal third of the intestine and the liver of a transgenic mouse are first normalised by competitive PCR against the β-actin gene. The upper band is given by the internal standard, the lower band by the β-actin cDNA present in the sample (I). Subsequently, the amount of cDNA which gives an equivalence point of 5 pg is used in the competitive PCR reaction using the human Apo AIV primer set (II) and the mouse Apo AIV primer set (III) for both the intestine and liver. B: Mean results obtained from five mice of each transgenic line and for the control mice with this methodology.

duce the accepted human pattern of expression to be dominant in mice, or that the human *cis*-acting elements are improperly recognised by the mouse *trans*-acting factors. The distribution of human Apo AIV mRNA in our mouse model is qualitatively similar to that of the accepted pattern in human with only very slight alteration of the endogenous mouse Apo AIV mRNA levels. We are currently using these transgenic lines to elucidate the mechanism by which the elements dispersed throughout the cluster are activated or inhibited by the action of natural or therapeutic effectors in vivo, a subject of great interest considering the anti-atherogenic action of the Apo AIV protein. Our mouse model thus provides an excellent substrate to design therapeutic protocols for those metabolic derangements that benefit from variations in Apo AIV levels.

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