

Evaluation of the function of the human apolipoprotein B gene nuclear matrix association regions in transgenic mice

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Abstract The human apolipoprotein B (apoB) gene resides in a 47.5 kb DNaseI-sensitive chromosomal domain in hepatic and intestinal cells, flanked by the 5' distal matrix association region (MAR) and the 3' proximal MAR. A third MAR, the 5' proximal MAR, is found only in transcriptionally active hepatic (HepG2) cells. Hepatic expression of the apoB gene requires a tissue-specific promoter (-898 to +121) and an enhancer from the second intron of the gene (+360 to +1064). A vector containing this portion of the gene linked to the β -galactosidase reporter is sufficient for low level expression in the livers of transgenic mice. Expression in transgenic mice was increased when the promoter-enhancer β -gal vector was flanked by MARs. The results were similar whether the 5' distal, the 5' proximal or the 3' proximal MARs were placed at both ends of the construct, or whether the construct was flanked by the 5' distal and the 3' MAR, suggesting that the apoB MARs play a role in gene expression in vivo. When the MAR-containing constructs were transiently transfected into HepG2 cells, the resulting β -gal activities were similar to that of the construct lacking MARs, thus demonstrating that the MARs do not exhibit any enhancer activity. ■ Recent experiments (Kalos, M., and R. E. K. Fournier. 1995. *Mol. Cell. Biol.* 15: 198–207) examining stable integration of some of our constructs into human and rat hepatoma transfectants suggest that in single and double copy transfectants, the apoB MARs behave as boundary "insulators", protecting the integrated transgenes against position effects regardless of their site of integration. However, multicopy transfectants are transcriptionally inactive and when the MARs are absent, expression of the transgenes drops to background levels. Our results to date with single and low-copy number transgenes do not support an insulator function for the apoB MARs, although they appear to be required to increase the levels of expression.—Wang, D-M., S. Taylor, and B. Levy-Wilson. Evaluation of the function of the human apolipoprotein B gene nuclear matrix association regions in transgenic mice. *J. Lipid Res.* 1996. 37: 2117–2124.

Supplementary key words chromatin structure and in vivo transcription • gene expression in transgenic mice • apolipoproteins • atherosclerosis

Apolipoprotein B (apoB) is the sole protein present in low density lipoproteins (LDL) and plays a pivotal role

in lipid metabolism and transport (for reviews see 1, 2). ApoB is encoded by a single copy gene that is primarily expressed in the liver and intestine in humans (3), thus it provides a model for the study of tissue-specific gene regulation. Our goal has been to identify the key regulatory elements responsible for apoB expression in hepatic and intestinal cells using both cell culture models and transgenic mice (for review see 4). During these studies we determined that expression in the livers of transgenic mice of a reporter gene linked to apoB regulatory sequences occurs when the regulatory region contains the tissue-specific promoter (-898 to +121) and a segment from the second intron enhancer (+360 to +1064) (5).

These two control regions represent the minimum requirement for liver specific expression of a reporter gene driven by the apoB promoter in transgenic mice. However, these two regions do not suffice for expression of the transgenes in intestinal cells and the levels of expression in liver are very low. Higher levels of expression are observed when additional sequences from both the 5' and the 3' regions are included in the constructs (5), with the highest levels deriving from a construct that includes the segment from -899 to -5265 upstream of the promoter-enhancer β -gal region plus the segment from +43104 to +44329 of the apoB gene at the 3' end of the construct.

The 3' segment contains the apoB gene 3' matrix association region (MAR) and the segment from -899 to -5265 includes the 5' distal and the 5' proximal MARs (6) plus a region that displays a repressor effect upon

Abbreviations: CAT, chloramphenicol acetyltransferase; β -gal, β -galactosidase; apoB, apolipoprotein B; MAR, matrix attachment site or matrix association region; SAR, scaffold association region.

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transcription in transient assays in liver-derived HepG2 and intestine-derived CaCo-2 cells (7). The question arises as to whether the MARs behave as enhancers in transgenic mice or whether the higher levels of expression seen in the constructs harboring MARs are due to a boundary effect of the MARs, thus protecting the associated control elements against negative influences from nearby repressor sequences at the site of integration (position effects). Recent work by Kalos and Fournier (8), using stably transfected human and rat hepatoma cell lines and some of our apoB constructs, have demonstrated that the apoB promoter-enhancer β -gal construct is not expressed above background levels. However, when this construct is flanked by the 5' distal MAR at its 5' end and the 3' MAR at its 3' end, position effects are eliminated because every stable cell line containing one or two copies of the integrated transgene expresses it at high levels. Cells harboring multiple-copy integrations of the transgene are transcriptionally inactive (8). To better understand the role of the apoB MARs in transgenic mice, we devised a series of constructs in which the apoB promoter-enhancer β -gal region was flanked by different combinations of the MARs and analyzed their effects upon transgene expression.

MATERIALS AND METHODS

Plasmid construction

Plasmid p β -gal.PE was previously described (5) and contains a 1020 bp *Pvu*II fragment representing the apoB promoter upstream of the β -gal gene. A 704 bp *Pst*I fragment containing the apoB second intron enhancer is inserted immediately upstream of the apoB promoter, in the reverse orientation. Plasmid p β -gal.EP was similar to the previously described p β -gal.PE, except that in p β -gal.EP, the second intron enhancer was in the forward orientation. The 3' MAR fragment (used in the construction of p β -gal.3EP3 and p β -gal.5EP3) was a 1.2 kb *Xba*I fragment spanning nucleotides +43104 to +44329 of the human apoB gene; the 5' distal MAR was a 1214 bp *Xba*I-*Nco*I fragment previously described (6) and the 5' proximal MAR was a 329 bp *Sty*I-*Hind*III fragment isolated from plasmid p β -gal.2.7 (7). p β -gal.3EP3 was made as follows: first, p β -gal.EP was linearized by digestion with *Nhe*I followed by dephosphorylation and ligation of the 3' MAR fragment to the 3' end to generate plasmid p β -gal.EP3. Clones in which the MAR was in the forward orientation were selected by digestion with *Bam*HI. To add the 3' MAR to the 5' end, the construct was linearized with *Xba*I, dephosphorylated, and ligated to the 3' MAR. The orientation of the MAR was confirmed by digestion with *Xho*I. Plasmid

p β -gal.5EP3 was made from p β -gal.EP3, by linearization with *Xba*I followed by dephosphorylation and ligation to the 5' distal MAR. The construct was cut with *Bam*HI to select for the forward orientation of the MAR. Plasmid p β -gal.5EP5 was made as follows: p β -gal.EP was linearized with *Xba*I and the 5' MAR was ligated to the 5' end of the construct to make p β -gal.5EP. This plasmid was then linearized with *Nhe*I and ligated to the 5' MAR to make p β -gal.5EP5. Plasmid p β -gal.5PEP5P was made from plasmid p β -gal.EP. First, the construct was linearized with *Xba*I and the 5' proximal (5'P)MAR was ligated to the 5' end. The resulting plasmid was linearized with *Nhe*I and ligated to insert the (5'P) MAR at the 3' end.

Transient transfections

Transfections into HepG2 cells were performed as described before (5). In brief, cotransfections were made with 10 μ g of the apoB- β -gal constructs and 5 μ g of plasmid Pvu6-6, in which expression of the chloramphenicol acetyl transferase (CAT) gene is driven by the apoB promoter and the 443 bp *Sma*I-*Pvu*II enhancer fragment from the second intron of the gene. The CAT plasmid was included as an internal control for transfection efficiency.

Generation of transgenic mice and Southern blot analysis

Preparation of DNA from the various constructs for transgenic mice generation was as previously described (5). Transgenic mice were generated as described by Simonet et al. (9). Genomic DNA was prepared from the tail tips of the offspring at 3–4 weeks of age as previously described (5). Ten μ g of DNA from each sample was digested with *Bam*HI, fractionated in 0.8% agarose gels, and then transferred to Gene Screen membranes (DuPont), by the method of Southern (10). Founder mice were identified by hybridization with a labeled *Cla*I fragment from the β -gal gene, and copy number was determined by comparison of the intensity of the band in each sample with the intensity of copy number controls representing 8 and 40 copies of the transgenes, respectively, that were run in parallel with the samples. DNA was also prepared from the livers, intestines, and spleens of these animals at the time of killing to verify that the transgene was present in these tissues. Quantitation of the radioactivity associated with each band was performed with a phosphorimager.

Preparation and analysis of RNA

To determine the expression levels of the transgenes, total cellular RNA was isolated from the tissues of transgenic mice as described before. Transcripts from

the human transgenes and the endogenous mouse apoB gene were detected with a quantitative RNase protection assay as previously described (5). The probe used to detect transgene RNA was from plasmid pBSSmaK/X (linearized with *Xba*I) by transcription with T7 RNA polymerase in the presence of [³²P]UTP, and the probe used to detect mouse apoB RNA was prepared from plasmid pBSSKmaB+138 in a similar manner. In all cases, 10 µg of RNA from each tissue or from HepG2 cells was used per assay and half of each sample was fractionated on an 8% polyacrylamide-urea sequencing gel. The relative levels of transgene RNA and endogenous mouse apoB mRNA were quantitated on a phosphorimager, with correction for differences in the specific activities of the two probes.

RESULTS

The apoB promoter (-898 to +121) together with the second intron enhancer (+346 to +1064) represent the minimal number of regulatory elements required for low-level expression of the associated β-gal reporter gene in the livers of transgenic mice. High levels of transgene mRNA expression, comparable to the levels of expression of the endogenous mouse apoB mRNA, can be obtained with constructs that include the segment from -899 to -5265 upstream of the promoter containing both the 5' distal MAR (-5265 to -4051), the 5' proximal MAR (-2131 to -1802) and the region in between them (-4050 to -2132), together with the 3' MAR (+43104 to +44329) at the 3' end of the construct (5). To determine whether the high expression levels are conferred by the MARs or by the sequences between them, we made several constructs in which the minimal expression vector was flanked by various combinations of the apoB MARs. A summary of the most relevant constructs is shown in Fig. 1.

Expression of the transgenic constructs in cell culture

As a test for structural integrity, all constructs to be tested in transgenic animals (Fig. 1) were transiently transfected into the human hepatoma cell line HepG2. All constructs contained the apoB promoter fused to a β-galactosidase expressing cassette (5) containing the polyadenylation and splicing signals from simian virus 40. Transcription from the apoB promoter was quantitated by assaying β-gal activity in extracts made from the transfected cells and the results are presented in Fig. 2. As shown earlier (5), the construct containing only the apoB promoter and the second intron enhancer (EP) was expressed at high levels in HepG2 cells. The addition of either the 3' MAR at both ends of the construct

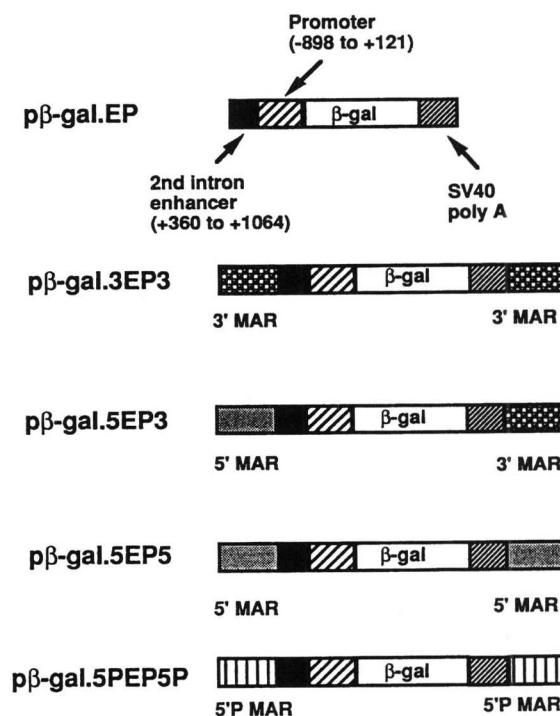


Fig. 1. Schematic representation of constructs used to evaluate the role of the apoB MARs. In all constructs, the reporter gene is β-galactosidase. pβ-gal.EP contains the apoB promoter and the second intron enhancer; this construct is flanked by the 5' distal MAR at both its 5' and 3' ends in pβ-gal.5EP5; by the 3' proximal MAR at both ends in pβ-gal.3EP3; by the 5' distal MAR and the 3' proximal MAR in pβ-gal.5EP3, and by the 5' proximal MAR at both ends in pβ-gal.5PEP5P.

(3EP3), or the 5' MAR and the 3' MAR (5EP3), resulted in a slight reduction in the β-gal activity, while addition of either the 5' distal or the 5' proximal MAR at both ends of the EP construct (5EP5 and 5PEP5P, respectively), caused a slight increase in β-gal activity. Nevertheless, these variations are not statistically significant. Therefore, we conclude that the apoB MARs do not exhibit either a stimulatory (enhancer) or an inhibitory (repressor) activity when flanking the apoB promoter-enhancer constructs. Furthermore, the MAR-containing constructs are functional in transiently transfected HepG2 cells.

Expression of the constructs in transgenic mice

To minimize the use of animals, we performed studies with founder mice and selected only those founders harboring one or a few copies of the transgenes for analysis of expression levels, thus minimizing the chances of mosaicism. We were particularly interested in the expression levels of the single-copy and low-copy founders, as comparisons between them might shed

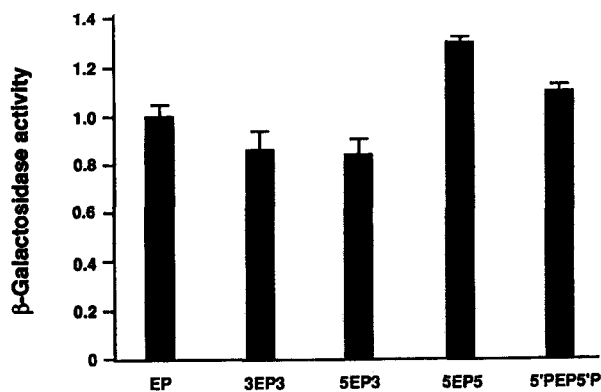


Fig. 2. Transient transfections into HepG2 cells. The names of the constructs are shown in the abscissa and the relative β -gal activities are shown on the ordinate. The vertical bars represent an average of four independent transfections for each construct. A value of 1.0 was assigned to the β -gal activity of the EP construct. Standard deviations varied between 2 and 10% (shown on top of each construct).

light on a possible insulator function of the MARs. High-copy number founders, although most abundant, were not included in the analysis because they did not express the transgenes.

Construct p β -gal. PE, including the apoB promoter and the second intron enhancer, is expressed at low levels in the livers of transgenic mice. In this construct, the enhancer is situated upstream of the promoter in the reverse orientation (5). For the purposes of this study, we made a new construct, p β -gal. EP, where the enhancer was placed upstream of the promoter in the forward orientation and was used to generate transgenic mice. Four founder animals were generated with this construct, varying in copy number from 3 to 40 (Table 1). Copy number of the transgenes was determined by Southern blot analysis as described in Materials and Methods.

Expression of the transgenes was determined with a sensitive RNase protection assay that measured the steady state level of transgene RNA in the livers of expressing mice (Fig. 3). None of the transgenes were expressed in intestine or spleen. To control for RNA degradation and to correct for differences in the amount of RNA analyzed, the results were normalized to the levels of endogenous mouse apoB mRNA. In Fig. 3, we illustrate the RNase protection assay. Panel A shows a control non-transgenic mouse, together with transgenic mice #1 and #12, harboring the p β -gal. 5EP5 construct. Only mouse #1 expresses the transgene in the liver (Table 1). Panel B shows RNase protection assays with mice #148 and #151, harboring the p β -gal. 3EP3 construct. Similarly, panel D shows results with the p β -gal. EP construct and panel C, with the p β -gal. 5EP3 construct. Three of the four founder animals carrying

p β -gal. EP transgenes expressed it in liver, but not in intestine or spleen, at levels varying from less than 0.1% to 0.7% of the value for the endogenous mouse apoB mRNA levels (Table 1).

Four founder animals were generated with the p β -gal. 3EP3 construct, containing one or three copies of the transgene (Table 1 and Fig. 3). Of these, only the single-copy animals expressed the transgene in the liver, at levels varying from 3.8% to 9% of those of the endogenous mouse apoB mRNA. Similarly, eight founder animals were generated with the p β -gal. 5EP3 construct, varying in copy number between 1 and 28 (Table 1). All but one of them expressed the transgene in the liver, at levels between 0.3 and 8.2% of the endogenous mouse apoB mRNA levels (Table 1). Five founder animals were obtained with the p β -gal. 5EP5 construct, harboring between 2 and 12 copies of the transgene (Table 1). Expression levels in the livers varied between 1.0% and 6.7%. One founder was generated with construct p β -gal. 5PEP5P and another one with a construct containing the segment from -4050 to -2132 upstream of the promoter. Their expression levels were 2% and 0.2% as compared to the mouse apoB mRNA levels, respectively (data not shown).

The overall data from these experiments suggest that the MAR-containing constructs express the transgenes at higher levels than does the p β -gal. EP construct. There appear to be no significant differences between the 3' MAR or the 5' distal MAR in their ability to stimulate transcription of the transgenes.

DISCUSSION

Most eukaryotic genes are thought to reside in chromosomal looped domains that vary in length between 20 and 100 kb (11, 12). These domains may contain one or several genes (6, 13). For some genes, such as the chicken lysozyme gene (14) and the human apolipoprotein B gene (6), the structural domain coincides with the functional domain and contains all of the regulatory (*cis*-acting) elements required for its expression in the appropriate tissues or cell-types. Such a concept of domains implies the existence of boundaries or borders at the 5' and 3' ends of these domains. The function of these border or boundary elements is to organize genes or gene clusters into separate domains and to protect the gene(s) within the domain from adverse regulatory effects from nearby enhancers or repressors situated just outside the domain borders (insulator function). For some genes (6, 14), the boundaries of the DNaseI sensitive chromatin domain coincide with the locations of nuclear matrix association regions (MARs), also called scaffold attachment regions (SARs) (15). How-

TABLE 1. Quantitation of transgene copy number and liver expression levels for transgenic mice harboring the p β -gal.EP, p β -gal.3EP3, p β -gal.5EP3, p β -gal.5EP5 constructs

Transgene	Animal Designation ^a (F0)	Transgene Copy No. ^b	% of Endogenous Mouse Liver ApoB Expression ^c
p β -gal.EP	41 (F)	6	—
	42 (F)	3	0.7
	76 (M)	40	0.1
	123 (F)	16	0.1
p β -gal.3EP3	19 (F)	1	3.8
	148 (M)	3	—
	151 (F)	1	9
	157 (M)	3	—
p β -gal.5EP3	22 (M)	28	8.2
	111 (F)	1	0.3
	125 (M)	14	0.8
	126 (M)	1	—
	132 (M)	11	2.5
	136 (F)	1	2.1
	139 (F)	1	2.7
p β -gal.5EP5	142 (F)	3	1.9
	1 (M)	2	6.7
	10 (M)	5	—
	25 (F)	4	1.0
	26 (F)	4	—
	29 (M)	12	1.7

^aMice are identified individually by number, and the sex of each mouse that was analyzed for expression is indicated in brackets after the mouse number.

^bQuantitation of transgene copy number was achieved by radio analytical imaging of the Southern blot filters on a phosphorimager.

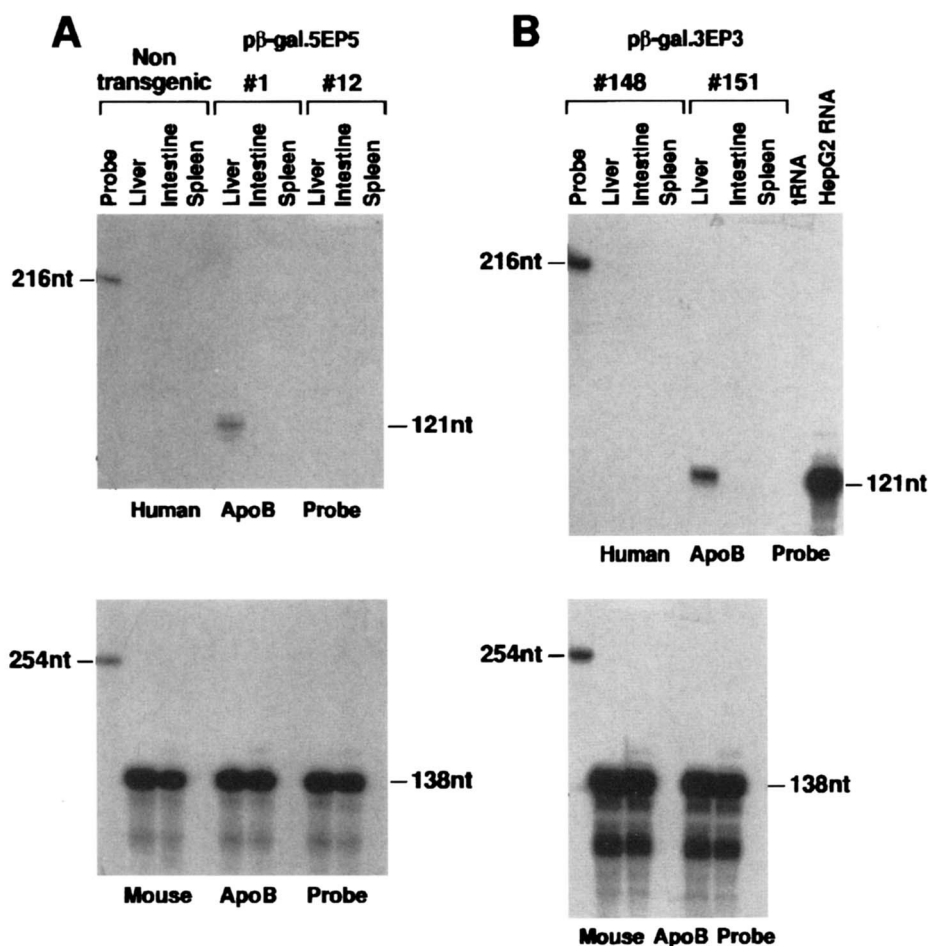
^cIn every case, RNA from the liver, intestine, and spleen was analyzed for expression. Transgene expression was not found in intestine or spleen. The expression levels are presented as hepatic transgene RNA levels relative to that of the endogenous mouse liver apoB mRNA, and take into account the differences in the specific activities of the two ribonuclease protection probes. —: no detectable expression.

ever, not all MAR sequences represent border elements and some are found within genes, in introns (16).

Sequences whose function resembles that of a boundary insulator have been identified in *Drosophila*. Thus, the specialized chromatin structure (scs) elements that flank the 87A7 heat shock gene confer position-independent expression of transgenes at many chromosomal locations (17). The scs elements themselves do not exhibit enhancer or repressor effects but they block enhancer activity when placed between an enhancer and its promoter (18). In higher eukaryotes, the interferon β SAR (13), the chicken lysozyme MAR (19), the chicken β -globulin insulator (20), and the human apoB gene MARs (8) confer insulating properties as they promote high-level, position-independent transgene expression in stably transfected cells. None of these sequences display enhancer activity in transient transfection assays; therefore, the elimination of position effects observed

in their presence may be attributed to a boundary/insulating role.

Boundary elements could become essential when designing vectors for expression of heterologous genes in transgenic animals because, in the majority of cases, founder animals harboring transgenes are transcriptionally inactive due to position effects at the site of integration, but also to possible heterochromatinization of multicopy tandem arrays of transgenes (21). Flanking a transgene and its required regulatory elements with insulator sequences may ensure expression. To date no clear function has been established for these potential boundary insulator sequences in transgenic mice. Original claims of insulator activity with the A-elements of the chicken lysozyme gene (MARs or SARs) have been put into question because the A-elements reside next to a strong tissue-specific enhancer in a segment of DNA that behaves like a locus control region (LCR) (22). Locus



control regions are defined as elements that confer copy number-dependent expression to an associated transgene (23). However, LCRs do not function by an insulating mechanism; instead, they override position effects and activate transcription directly, in a dominant positive manner, by a yet unknown mechanism probably involving the modification of chromatin structure (24).

Recently, Thompson et al. (25) reported that the 5' and 3' SARs from the human β -interferon locus stimulate transgene expression in a copy number-dependent manner, thus providing some degree of insulation against position effects. This effect is observed only in pre-implantation mouse embryos but not in differentiated tissues of newborn and adult mice and may be due to a role of SARs in chromatin remodeling for transcription early in development.

We tested the function of the apoB MARs in the expression of transgenes harboring the apoB promoter-enhancer- β -gal cassette in mice and showed that flanking the promoter-enhancer- β -gal vector with either the 5' distal MAR or with the 3' proximal MAR at both ends of the construct or with the 5' MAR at the 5' end and

the 3' MAR at the 3' end of the construct yields similar results. Mice carrying transgenes flanked by MARs express them at higher levels than mice carrying the construct lacking MARs. However, transgene expression was low in all the mice, about 1–10% of endogenous levels, indicating that all of the transgenes carrying this minimal promoter-enhancer cassette were inefficiently expressed. This is attributable, at least in part, to the absence in all constructs of segments from the apoB gene that are required for high level expression in the livers of transgenic mice; namely, the segment from -899 to -5265 in the p β -gal.3EP3 construct, the segment from -4051 and -899 in p β -gal.5EP3, and the segments from -899 to -4051 plus that from +43104 to +44329 in p β -gal.5EP5. Additionally, because founder mice rather than established lines were examined, cell mosaicism in the liver may contribute to variability in expression levels.

In contrast to what has been reported for transgenes containing LCRs, we did not observe copy number-dependent expression. Indeed, we have consistently observed that low copy number transgenes are expressed

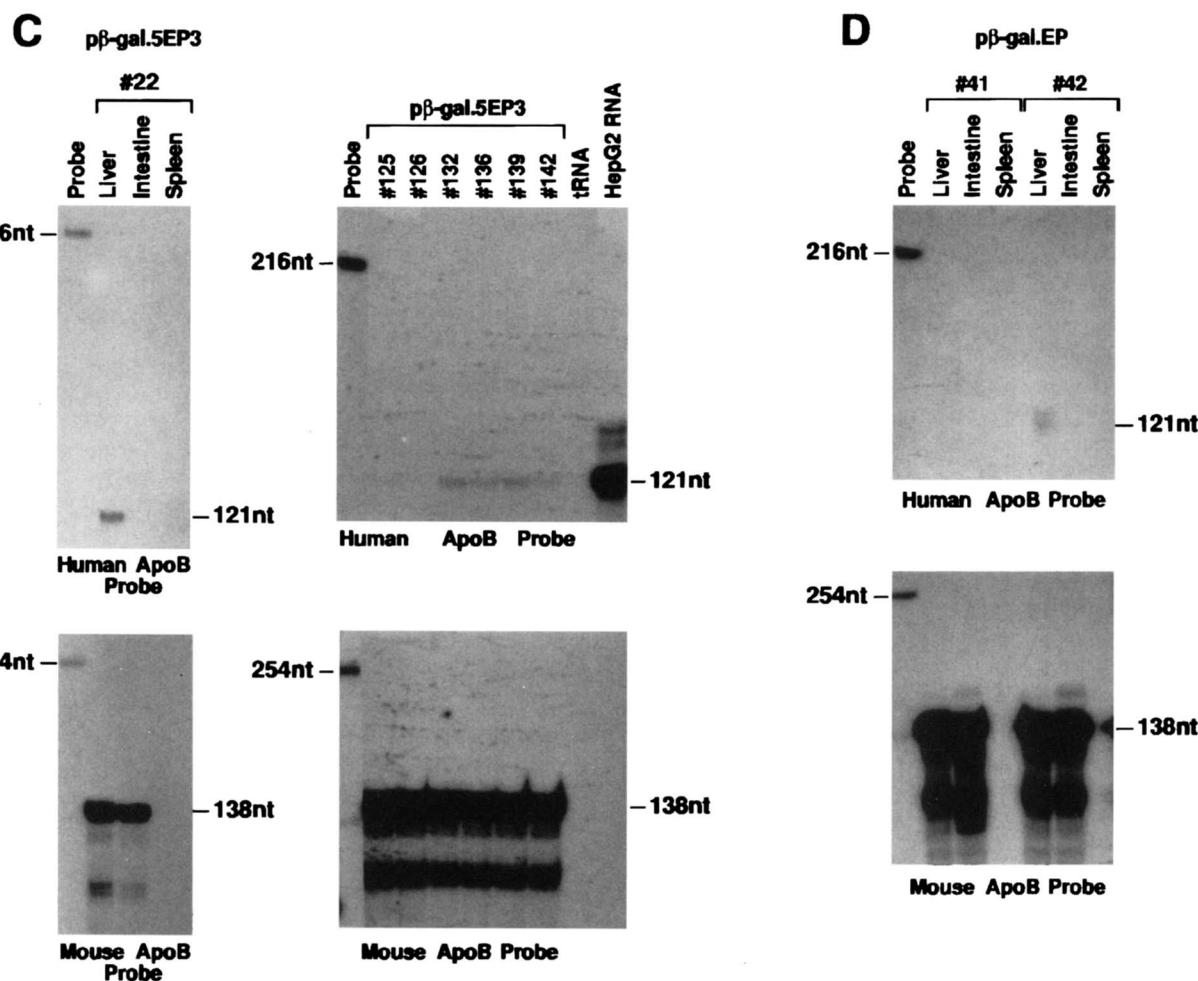


Fig. 3. Analysis of transgene RNA expression. Representative results of RNase protection experiments with transgenic mice carrying constructs p β -gal.5EP5 (panel A), p β -gal.3EP3 (panel B), p β -gal.5EP3 (panel C), and p β -gal.EP (panel D) are shown. In each instance, 10 μ g of total cellular RNA from the tissues indicated above each lane was analyzed in parallel with a human apoB probe and a mouse apoB probe in separate assays. RNA from HepG2 cells and tRNA were used as positive and negative controls, respectively. The mouse numbers are shown on top of the gels; the size of the human and mouse probes as well as the sizes of the protected fragments from the transgenes are shown at either side of the gels in nucleotides (nt). The middle part of panel C shows RNA from the livers of various founder mice injected with the p β -gal.5EP3 construct.

at higher levels than high copy number transgenes, an observation consistent with the heterochromatinization theory (21). Our results suggest that the apoB MARs may not function as boundary insulators, as some of the low copy number constructs are not expressed, suggesting that they are subject to position effects. However, because only one single-copy insertion was transcriptionally inactive, the possibility that the apoB MARs confer position-independent expression cannot be entirely ruled out.

Another difference between the results obtained in cell culture models, using either transient or stable transfectants and our data with transgenic mice, is that the DNA segment situated in between the two 5' MARs (-4051 to -2132), represses transcription from the apoB promoter in cultured cells. In contrast, in transgenic mice that segment is required together with the remain-

der of the 5' region extending to -5265 for full-level expression of the transgenes in the liver (5). Although at present we do not understand the molecular basis for these differences, it is clear that experiments such as those presented in this report are necessary to fully understand the intricacies of gene expression in vivo.

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