

## Modification of the Apolipoprotein B Gene in HepG2 Cells by Gene Targeting

Robert V. Farese, Jr., Laura M. Flynn, and Stephen G. Young

Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute, Department of Medicine, University of California, San Francisco, California 94141-9100

### Abstract

The HepG2 cell line has been used extensively to study the synthesis and secretion of apolipoprotein (apo) B. In this study, we tested whether gene-targeting techniques can be used to inactivate one of the apo B alleles in HepG2 cells by homologous recombination using a transfected gene-targeting vector. Our vector contained exons 1–7 of the apo B gene, in which exon 2 was interrupted by a promoterless neomycin resistance (*neo<sup>r</sup>*) gene. The recombination of this vector with the cognate gene would inactivate an apo B allele and enable the apo B promoter to activate the transcription of the *neo<sup>r</sup>* gene. To detect the rare homologous recombinant clone, we developed a novel solid phase RIA that uses the apo B-specific monoclonal antibody MB19 to analyze the apo B secreted by G418-resistant (G418<sup>r</sup>) clones. Antibody MB19 detects a two-allele genetic polymorphism in apo B by binding to the apo B allotypes MB19<sub>1</sub> and MB19<sub>2</sub> with high and low affinity, respectively. HepG2 cells normally secrete both of the apo B MB19 allotypes. Using the MB19 immunoassay, we identified a G418<sup>r</sup> HepG2 clone that had lost the ability to secrete the MB19<sub>1</sub> allotype. The inactivation of an apo B allele of this clone was confirmed by the polymerase chain reaction amplification of an 865-bp fragment unique to the targeted apo B allele and by Southern blotting of genomic DNA. This study demonstrates that gene-targeting techniques can be used to modify the apo B gene in HepG2 cells and demonstrates the usefulness of a novel solid phase RIA system for detecting apo B gene targeting events in this cell line. (*J. Clin. Invest.* 1992. 90:256–261.) **Key words:** cholesterol • radioimmunoassay • homologous recombination • hypobetalipoproteinemia • lipoproteins

### Introduction

Apo B100 is secreted by the liver as a component of VLDL (1), a portion of which are then metabolized in the circulation to the denser, cholesterol-enriched LDL. Apo B100 is virtually the sole protein component of LDL, and plasma apo B100 levels correlate directly with the risk of developing atherosclerotic coronary heart disease (2). Plasma apo B100 levels are determined by a steady state resulting from the rate of synthesis

and secretion of apo B100 from the liver and from the rate of apo B100 clearance from the plasma. The human hepatoma cell line HepG2 has been used extensively as a cell culture model to study the synthesis and secretion of apo B100 from liver cells (3, 4).

To further understand apo B synthesis and secretion, it would be useful to be able to introduce specific mutations into the apo B gene in HepG2 cells. This is now possible in principle, using the recently developed techniques of gene targeting. These techniques have made it possible to inactivate genes or to introduce subtle mutations into genes in embryonic stem cells or in permanent cell lines (5). To inactivate a gene, the DNA vector used contains a fragment of the gene of interest, in which one of the exons is interrupted by a selection marker coding for drug resistance. The homologous recombination of the vector with the cognate gene in a cultured cell inactivates one allele of the gene and results in a drug-resistant phenotype. In this study, we tested whether gene-targeting techniques can be used to inactivate an apo B allele in HepG2 cells. The targeting strategy that we chose used a targeting vector containing a promoterless neomycin resistance (*neo<sup>r</sup>*) gene as a selection marker. The use of a promoterless *neo<sup>r</sup>* gene in the vector limits viable cell clones to those in which the transfected vector recombines near an active cellular promoter; this strategy is applicable when the gene of interest is expressed in the cell line, as apo B is in HepG2 cells, and has been reported to enrich greatly the population of selected cell clones for homologous recombination (6, 7).

Despite the use of various enrichment strategies, identifying a targeted clone is often laborious, requiring genomic DNA analysis of hundreds or thousands of individual clones by Southern blotting or the polymerase chain reaction (PCR). To detect the rare homologous recombinant clone in this study, we developed a novel solid phase RIA using the apo B-specific monoclonal antibody MB19. Antibody MB19 detects a two-allele genetic polymorphism in apo B by binding to the apo B allotypes MB19<sub>1</sub> and MB19<sub>2</sub> with high and low affinity, respectively. HepG2 cells normally secrete both of the MB19 allotypes. The inactivation of an apo B allele in HepG2 cells would therefore be expected to alter the MB19 phenotype of the secreted apo B, which can be rapidly assessed in a double label solid phase RIA. We used the MB19 immunoassay to identify a targeted HepG2 clone that had lost the ability to secrete the MB19<sub>1</sub> allotype. This MB19 screening system will be useful for future gene-targeting studies designed to introduce subtle mutations into the apo B gene, such as those that occur in familial hypobetalipoproteinemia.

1. *Abbreviations used in this paper:* *neo<sup>r</sup>*, neomycin resistance; PCR, polymerase chain reaction.

Address correspondence and reprint requests to Robert V. Farese, Jr., M.D., Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100

Received for publication 11 February 1992.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/92/07/0256/06 \$2.00

Volume 90, July 1992, 256–261

## Methods

**Gene-targeting vector construction.** The strategy for the construction of our targeting vector was similar to that used for the inactivation of an *N-myc* allele in cultured lymphocytes (6). Our vector, ~ 10 kb long, contained three segments: a 5' 564-bp segment of the human apo B gene containing exon 1, intron 1, and a portion of exon 2; a middle segment containing a promoterless *neo<sup>r</sup>* gene either with or without a poly(A) signal; and an 8.3-kb 3' apo B segment extending from intron 2 to intron 7 (Fig. 1). To construct the poly(A)<sup>-</sup> vector, four DNA fragments were inserted into the polylinker of pBIISK (Stratagene Inc., La Jolla, CA). First, a BglII-NruI (1083-bp) fragment [*neo<sup>r</sup>*/poly(A)<sup>-</sup>] from pSV2Neo was inserted into the BamHI and SmaI sites of pBIISK. Second, a 169-bp fragment of the apo B gene extending from just 3' of the apo B promoter (-15 relative to the transcriptional start site) to the middle of exon 1 was enzymatically amplified from the human apo B clone  $\lambda$ 5c (8) and inserted into the XbaI and SpeI sites of pBIISK. Third, a 410-bp fragment of the apo B gene extending from the middle of exon 1 to exon 2 (apo B cDNA nucleotide 245) was amplified from  $\lambda$ 5c and inserted into the SpeI site of pBIISK. The amplification oligonucleotides that we used for the 169-bp and 410-bp fragments were designed to eliminate the ATG translation initiation codon in exon 1 and two ATGs in exon 2. The ATGs were eliminated to prevent translation from beginning at these sites in a targeted cell. Finally, an 8.3-kb AsuII-KpnI fragment of  $\lambda$ 5c extending from intron 2 to intron 7 was inserted into the ClaI and KpnI sites of the vector. Before this final ligation, a lone SacII site within the AsuII-KpnI fragment was eliminated so that it would be possible to linearize the vector at the polylinker SacII site. A similar vector in which the *neo<sup>r</sup>* gene had a polyadenylation signal [*neo<sup>r</sup>*/poly(A)<sup>+</sup>] was also constructed. All cloning steps for this vector were the same as those described above except that the BglII/BamHI fragment of pSV2Neo was inserted into the BamHI site of pBIISK.

**Tissue cultures and electroporations.** HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% sodium pyruvate. For the electroporations, ~ 20  $\mu$ g of DNA vector were digested with SacII and KpnI (releasing the plasmid sequences), ethanol precipitated, and resuspended in 20  $\mu$ l of Tris-EDTA. Next, HepG2 cells were harvested by trypsinization from 80% confluent T-150 flasks, centrifuged at 200 *g* for 5 min, and resuspended in calcium- and magnesium-free PBS, pH 7. The DNA was then transfected into  $2 \times 10^7$  HepG2 cells (0.8 ml) by electroporation using a 250-V, 500- $\mu$ F pulse from a Gene Pulser (Bio-Rad Labs.-Chem.-Div., Richmond, CA). After 5 min, the cells were plated onto two 150-mm dishes. Selection with G418 (800  $\mu$ g/ml) was begun 24–48 h after the electroporation. After 3–4 wks, G418<sup>r</sup> colonies were transferred to 96-well culture plates for RIA screening.

**RIA screening.** A double label, solid phase RIA was developed to detect G418-resistant (G418<sup>r</sup>) HepG2 clones that contained an inactivated apo B allele. The RIA was based on the use of the apo B-specific monoclonal antibody MB19, which detects a two-allele genetic polymorphism in human apo B (9); antibody MB19 binds to the apo B allotypes MB19<sub>1</sub> and MB19<sub>2</sub> with high and low affinity, respectively. The MB19 immunochemical polymorphism is associated with a C  $\rightarrow$  T transition in exon 4 of the apo B gene (apo B cDNA nucleotide 421), which results in a Thr  $\rightarrow$  Ile substitution at apo B amino acid 71 (10). Previous studies have shown that HepG2 cells are trisomic for chromosome 2 (11), where the apo B gene is located (12). We confirmed this finding by karyotyping several HepG2 clones. We next examined the MB19 phenotype of the apo B secreted by HepG2 cells in competitive assays using antibody MB19 (9) and determined that HepG2 cells have three functioning apo B alleles: one MB19<sub>1</sub> allele and two MB19<sub>2</sub> alleles (see Fig. 2B). The successful inactivation of an apo B allele would therefore result in a change in the MB19 phenotype of the secreted apo B from 1/2/2 to either 1/2 or 2/2.

For the RIA, Dynatech Immulon 1 96-well plates (Fisher Scientific Co., Pittsburgh, PA) were coated for 2 h with 50  $\mu$ l of PBS containing 1

$\mu$ g of the apo B-specific monoclonal antibody MB47/ml (epitope between apo B100 amino acids 3429 and 3523 [13]). The plates were then washed as described (9). Nonspecific binding sites were blocked with PBS containing 3% BSA, and the plates were washed again. After G418<sup>r</sup> clones were grown in fresh cell culture medium for 24 h, 50  $\mu$ l of medium from each of the 96 wells in the cell culture plates was transferred to the RIA plates and allowed to incubate for 16 h at 4°C. The plates were washed, and 50  $\mu$ l of PBS containing 3% BSA, 0.2% Tween 20, <sup>125</sup>I-MB19 (8,000 cpm/ $\mu$ l), and <sup>131</sup>I-MB3 (8,000 cpm/ $\mu$ l) was added. Antibody MB19 binds within the amino-terminal 100 amino acids of apo B100 (14), and antibody MB3 (which does not detect the MB19 polymorphism) binds between apo B100 amino acids 995 and 1082 (15). MB19 and MB3 were radioiodinated to specific activities of 5,000–15,000 cpm/ng by the lactoperoxidase method (Enzymobeads; Bio-Rad), then added to the plates for incubation for 4–8 h at 4°C. The plates were then washed, and the individual wells were counted. In this assay, the <sup>131</sup>I-MB3 cpm reflects the amount of apo B captured by antibody MB47, and the ratio of <sup>125</sup>I-MB19 cpm/<sup>131</sup>I-MB3 cpm is a measure of the MB19 phenotype of the clone. For controls, we used medium from nontransfected HepG2 cells (MB19 phenotype 1/2/2), medium from Hep3B cells (MB19 phenotype 2/2), and plasma samples from laboratory personnel having MB19 1/1, 1/2, or 2/2 phenotypes. G418<sup>r</sup> clones whose <sup>125</sup>I/<sup>131</sup>I ratio was significantly different from the standard HepG2 ratio were identified as potentially targeted and were grown in larger culture dishes for genomic DNA analysis.

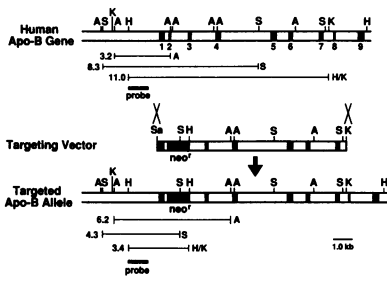
**DNA analysis by PCR and Southern blotting.** Genomic DNA from G418<sup>r</sup> clones was prepared using standard methods (16) and was analyzed by two PCRs. In a control reaction, an 869-bp fragment of the apo B gene extending from the promoter region (sequences not contained in the targeting vector) to intron 2 was amplified using oligonucleotide primer 1 (5' AGAAGCCAGTGTAGAAAAGCAAACAGG 3') and primer 2 (5' AGGCCAGGTAGAAGAGAGTTGGCATCC 3') (see Fig. 3). To detect a targeted clone, a target reaction was performed using primer 1 and primer 3 (5' GGTCGGTCTTGACAAAAGAACCG 3'), which is located within the coding segment of the *neo<sup>r</sup>* gene. This reaction amplifies an 865-bp fragment that is present only in a targeted allele. Both amplifications were carried out for 40 cycles using denaturing, annealing, and extension temperatures of 98° (15 s), 62° (45 s), and 72°C (3 min), respectively. For each reaction, 2.5 U of *Taq* polymerase (Cetus Corp., Emeryville, CA) was added at the beginning of the reaction and after 15–20 cycles.

For Southern blotting, ~ 10  $\mu$ g of genomic DNA was digested with SphI, ApaLI, or HindIII-KpnI, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Zetaprobe; Bio-Rad). The membrane was probed with a <sup>32</sup>P-labeled HindIII-StuI apo B fragment located in the apo B promoter region outside the vector sequences (apo B nucleotides -1800 to -639) (Fig. 1).

**Isolation and characterization of lipoproteins.** Serum-free medium was placed on HepG2 cells for 24 h, and samples were collected and concentrated on Centriflo membrane cones (Amicon Corp., Danvers, MA). The size of the secreted apo B-containing lipoprotein particles was assessed by nondenaturing polyacrylamide gradient gel electrophoresis and Western blotting as described previously (17). For electron microscopy, aliquots of the concentrated cell culture media were ultracentrifuged to obtain the *d* < 1.063 g/ml fraction. These lipoproteins were dialyzed against 150 mM NaCl and 1 mM EDTA, negatively stained, and examined by electron microscopy as described (18). Lipoprotein sizes determined by electron microscopy were compared using an unpaired *t* test.

## Results

Electroporations of  $2 \times 10^7$  HepG2 cells with the promoterless *neo<sup>r</sup>*/poly(A)<sup>-</sup> apo B gene-targeting vector typically yielded 10–150 G418<sup>r</sup> clones. In one experiment, in which we analyzed 87 G418<sup>r</sup> clones with the double-label screening RIA, we identified one clone that had a <sup>125</sup>I-MB19/<sup>131</sup>I-MB3 ratio that was significantly lower than that of HepG2 cells and nearly

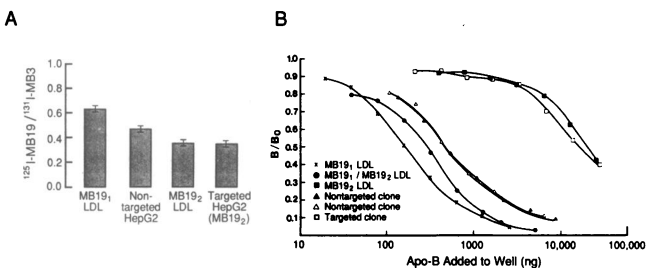


**Figure 1.** Scheme for the inactivation of an apo B allele in HepG2 cells. Restriction maps for the apo B gene (through exon 9), the apo B gene-targeting vector, and a targeted apo B allele resulting from a double crossover homologous recombination (sequence replacement) event are shown. The targeting vector contained exons 1–7 of the apo B gene in which a portion of exon 2 and intron 2 were replaced with a promoterless *neo<sup>r</sup>*/poly(A)<sup>-</sup> gene. The homologous recombination of the targeting vector with an apo B allele enables the apo B promoter to activate the expression of the *neo<sup>r</sup>* gene and inactivates the apo B allele. A hybridization probe located outside the vector sequences [a HindIII-StuI fragment (–1799 to –638)] detects changes in the restriction pattern for several enzymes as indicated. A, ApaLI; S, SphI; H, HindIII; K, KpnI; Sa, SacI.

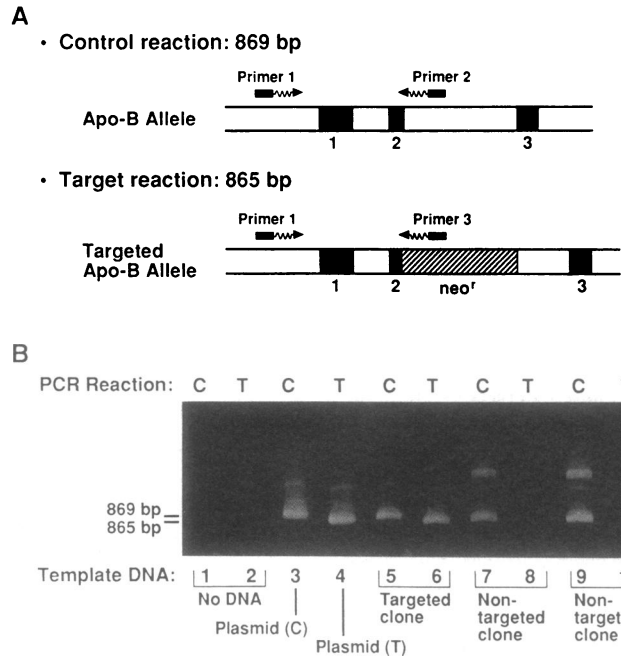
identical to that of LDL isolated from an MB19<sub>2</sub> (low-affinity) homozygote (Fig. 2 A). The <sup>125</sup>I-MB19/<sup>131</sup>I-MB3 ratios for the remainder of the clones in this experiment varied by only 5–10%, demonstrating the reliability of the screening assay; no other clones had ratios approaching the MB19 2/2 (Fig. 2 A) or 1/2 LDL standards (data not shown). To confirm that the clone having the altered <sup>125</sup>I-MB19/<sup>131</sup>I-MB3 ratio had an altered MB19 phenotype, we then tested the ability of the apo B-containing lipoproteins from this clone and other G418<sup>r</sup> clones to compete with an LDL standard for binding to antibody MB19 in a competitive RIA. This RIA demonstrated that the clone identified in the screening RIA had lost the ability to produce apo B of the MB19<sub>1</sub> (high-affinity) allotype (Fig. 2 B).

The PCR analysis of genomic DNA demonstrated an 869-bp control reaction amplification product that was detectable in each clone tested and an 865-bp target reaction amplification product that was detectable only in the potentially targeted clone and in a plasmid control (Fig. 3), suggesting that this

clone was indeed targeted. Further supporting this conclusion, a more slowly migrating doublet band was present in the control reaction for each of the nontargeted clones, but was absent from the control reaction for the targeted clone. This doublet band is created by the formation of heteroduplex DNA during the amplification that is due to a 9-bp insertion/deletion polymorphism in the apo B signal peptide region (19). For the targeted clone, this heteroduplex DNA was absent because the recombination of the targeting vector with the MB19<sub>1</sub> apo B allele (which is a signal peptide deletion allele) resulted in the loss of the portion of intron 2 where primer 2 binds (Fig. 3 A). Thus, the control PCR amplified only DNA from the two remaining nontargeted apo B alleles, which are both signal peptide insertion alleles, and no heteroduplex DNA was formed.

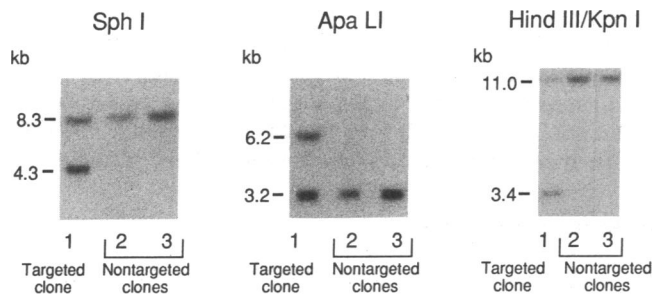


**Figure 2.** Alteration in the MB19 phenotype as shown by MB19 RIAs of the targeted and nontargeted HepG2 clones. (A) Representative MB19 screening assay. Microtiter plates were coated with antibody MB47 and incubated overnight with 50  $\mu$ l of cell culture medium from each G418<sup>r</sup> clone or with LDL standards. After the plates were washed, <sup>125</sup>I-MB19 and <sup>131</sup>I-MB3 were added and allowed to incubate for 4–6 h. The wells were then washed and counted to determine the <sup>125</sup>I-MB19/<sup>131</sup>I-MB3 ratio. (B) MB19 competitive RIA. The ability of the apo B secreted by G418<sup>r</sup> clones to compete with an apo B standard (MB19<sub>1</sub> LDL) for binding to antibody MB19 was compared (9). Dilutions of HepG2 apo B samples and of several LDL standards were added to the MB19 assay on the basis of their apo B content, which was determined in a competitive RIA using antibody MB3 as described (17).



**Figure 3.** PCR analysis of G418<sup>r</sup> clones. (A) Scheme for PCRs. A control reaction used a 5' oligonucleotide (primer 1) located in the apo B promoter region, upstream of the targeting vector sequences, and a 3' oligonucleotide (primer 2) in intron 2; this reaction was predicted to be positive in each G418<sup>r</sup> clone. A target reaction used the same 5' oligonucleotide but a 3' oligonucleotide (primer 3) in the *neo<sup>r</sup>* gene sequence. (B) PCR analysis of two nontargeted clones and the targeted clone. A more slowly migrating heteroduplex DNA band was seen in the control reactions for the two nontargeted clones, but was absent in the control reaction for the targeted clone (explanation in text).

The successful inactivation of an apo B allele was confirmed by Southern analysis of genomic DNA from the targeted clone (Fig. 4). Using three different restriction enzyme digests and a probe located outside the vector sequences, we demonstrated that the targeted clone had unique restriction fragments that resulted from the homologous recombination of the vector with an apo B allele. Because the gene-targeting vector contained two SpeI sites (one in exon 1 and one at the exon 2/*neo<sup>r</sup>* gene junction) that were introduced during the construction of the vector and that are not present in the apo B gene, we were able to assess whether these sites existed in the targeted HepG2 apo B allele in order to localize the region of crossover. Only the SpeI site at the exon 2/*neo<sup>r</sup>* gene junction was present in the targeted allele, indicating that the crossover



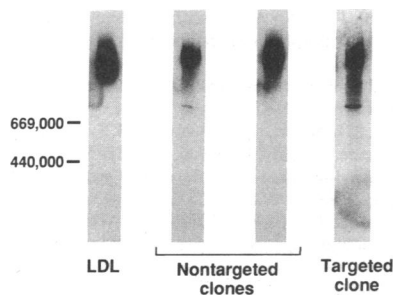
**Figure 4.** Southern analysis of G418<sup>r</sup> clones. The analysis for two nontargeted clones and the targeted clone with three different restriction enzyme digests is shown. 10 μg of genomic DNA was digested with SphI, ApaLI, or HindIII-KpnI, separated on an agarose gel, and transferred to a nylon membrane. The membrane was probed with a HindIII-StuI fragment from the apo B promoter sequences (see Fig. 1). All clones contained restriction fragments (SphI, 8.3 kb; ApaLI, 3.2 kb; HindIII-KpnI, 11.0 kb) from normal apo B alleles. The targeted clone also contained restriction fragments (SphI, 4.3 kb; ApaLI, 6.2 kb; HindIII-KpnI, 3.4 kb) from the targeted apo B allele.

must have occurred within the 410-bp apo B segment immediately 5' of the *neo*<sup>r</sup> gene (data not shown). We screened several hundred G418<sup>r</sup> clones obtained from the *neo*<sup>r</sup>/poly(A)<sup>-</sup> vector and the *neo*<sup>r</sup>/poly(A)<sup>+</sup> vector electroporations, but did not identify any other targeted clones.

HepG2 cells have been shown previously to produce apo B-containing particles that are similar in size and density to human LDL (20). To determine whether inactivating an apo B allele altered lipoprotein size, we measured the size of the apo B-containing lipoproteins within the *d* < 1.063 g/ml fraction of the culture medium from the targeted clone and from nontargeted clones. No significant difference in the size of the apo B-containing lipoproteins was observed by nondenaturing polyacrylamide gels (Fig. 5) or by electron microscopy (mean particle diameters for the targeted and nontargeted clones were 16.7±0.6 nm and 15.0±0.6 nm, respectively; *P* < 0.10). Karyotyping of the targeted clone revealed that, like the other HepG2 clones examined, it had trisomy of chromosome 2.

## Discussion

In this study, we demonstrate that it is possible to modify the apo B gene in HepG2 cells by gene targeting. Using an apo



**Figure 5.** A nondenaturing 2.5–16% gradient polyacrylamide gel demonstrating the size of the apo B-containing lipoproteins that were secreted from targeted and nontargeted HepG2 clones. An Isophore 2.5–16% gel was used as described (17), in which each lane contained ~ 40 ng of apo

B. Antibody MB44 [epitope between apo B amino acids 2488 and 2658 (38)] was used for Western blotting. Human LDL (lane 1) was prepared from the plasma of a normolipidemic donor. The migrations of two molecular weight standards (thyroglobulin, 669,000; ferritin, 440,000) are indicated.

B-targeting vector containing a promoterless *neo*<sup>r</sup> gene, we inactivated one of the three apo B alleles normally found in HepG2 cells. The successful inactivation of an apo B allele was demonstrated by a change in the MB19 phenotype of the apo B secreted by the targeted clone (Fig. 2) and by PCR and Southern analysis of the genomic DNA from the targeted clone (Figs. 3 and 4). This is one of only several examples of the successful inactivation of a gene in human cells (21–23), and the first example, to our knowledge, of successful gene targeting in a hepatoma cell line.

The identification of a targeted clone was greatly facilitated in this study by the development of a solid phase RIA capable of rapidly screening many clones to detect a change in the secreted apo B phenotype. A major difficulty with gene targeting in mammalian cells is that low rates of homologous recombination (~ 1 in 10<sup>6</sup> transfected cells) make it arduous to identify a targeted clone (5). The use of positive and negative drug selection markers has improved targeting frequencies to manageable rates (5), yet it still may be necessary to screen hundreds or thousands of clones to identify a targeted clone. The PCR has greatly facilitated this task by allowing one to screen the DNA from pools of clones in an attempt to identify a unique amplification product resulting from homologous recombination (24). The use of the PCR, however, has been complicated by the occurrence of both false positive (24, 25) and false negative reactions (26). Additionally, in our study, the GC-rich promoter region of the apo B gene proved to be difficult to amplify consistently from genomic DNA, making the PCR difficult to use as a screening method (data not shown). The fact that HepG2 cells normally secrete two distinct MB19 protein allotypes enabled us to develop a simple immunoassay screening system capable of screening hundreds of clones in just 2 d. Using this double label immunoassay, we successfully identified a targeted clone (Fig. 2A) despite a very low targeting frequency.

The targeting frequency that we observed, ~ 1 in 450 for the *neo*<sup>r</sup>/poly(A)<sup>-</sup> vector, was much lower than the 20% frequency described for a similar promoterless *neo*<sup>r</sup> gene strategy used to inactivate an *N-myc* allele in lymphocytes (6). Several factors may account for this. First, gene targeting frequencies are highly variable among different genes and different cell lines (5). Second, our targeting vector contained less sequence homology on the short end of the vector (572 bp) than did the vector used to target the *N-myc* gene. It is possible that the short 5' homologous segment in our vector decreased the targeting frequency, although Hasty et al. (27) have recently demonstrated that high targeting frequencies can be maintained with as little as 472 bp of sequence homology included on the short end of the targeting vector, provided there is an adequate overall length of homology. Third, our gene-targeting vector lacked a portion of intron 2 that has recently been shown to contain an apo B enhancer element (28). In a targeted cell, the deletion of this element may have resulted in diminished apo B promoter activity and, therefore, diminished expression of the *neo*<sup>r</sup> gene. Finally, the expression of the *neo*<sup>r</sup> gene in a targeted clone was also dependent on translation termination-reinitiation. Although this phenomenon has been shown to occur in eukaryotic cells (29), it may have occurred inefficiently in a targeted clone and resulted in a relatively low level of G418 resistance.

It is possible that a different apo B gene-targeting strategy may have resulted in a higher targeting frequency. For example, the inclusion of a thymidine kinase gene in the targeting

vector as a negative selection marker, a strategy that has been successful for a number of other genes (5), may have improved the targeting efficiency. Alternatively, an apo B sequence insertion-type vector may have worked better than the sequence replacement vector that we used. Sequence insertion vectors have been shown to result in increased frequencies of homologous recombination at the *hprt* gene locus (26), and, in preliminary studies, we have found a sequence insertion vector to be more efficient than a sequence replacement vector for targeting the mouse apo B gene in embryonic stem cells (Farese, R., Jr., and S. Young, unpublished data).

Despite the low targeting frequency that we observed with the vector used in this study, the combination of better apo B gene-targeting vectors and the powerful MB19 screening system that we developed should provide a versatile system for introducing mutations into the apo B gene in HepG2 cells. For example, the MB19 RIA that we used to detect an inactivated apo B allele should be applicable to future gene-targeting studies designed to introduce nonsense mutations into the more 3' exons (exons 26–29) of the apo B gene in HepG2 cells. For this type of study, a targeting vector could be designed in which a *neo<sup>r</sup>* gene interrupts the apo B gene at the unique SalI site in exon 26. In this case, a successfully targeted allele would be expected to produce a truncated apo B, apo B37. Because apo B37 would not be detected by the capture antibody (MB47) in the MB19 screening RIA, a change in the MB19 phenotype would be observed.

A cell line producing such a truncated apo B would be useful in understanding the syndrome of hypobetalipoproteinemia. In this syndrome, apo B nonsense mutations result in the production of truncated apo Bs, which are invariably found in very low concentrations in the plasma. In many cases, the explanation for the low plasma apo B levels is unknown. For example, apo B31 is found at less than 5% of normal apo B100 levels (30), even though apo B31 lacks the region of the apo B molecule necessary for binding to LDL receptors. Transfection studies using apo B cDNA vectors containing strong viral promoters suggest that apo B31 and several other truncated apo Bs are secreted rapidly from cells (31). These transfection studies do not address, however, whether nonsense mutations at the apo B gene locus result in diminished apo B synthesis rates; nonsense mutations in other genes are associated with low mRNA levels and low protein synthesis rates (32–34). By introducing nonsense mutations into exon 26 of the apo B gene in HepG2 cells, the mRNA level and synthesis rate of a truncated apo B could be compared directly with those of apo B100.

The MB19 immunoassay could also be used to screen for the introduction of missense mutations into the apo B gene in HepG2 cells. Missense mutations could be introduced using the recently described “hit and run” gene-targeting strategy that has made it possible to introduce subtle mutations into a gene (35, 36). Using gene-targeting methodology to express altered forms of apo B to study apo B structure and function might be easier than using apo B cDNA expression strategies. Introducing missense mutations into apo B100 cDNA expression vectors (37) involves many cloning steps, and the development of stable cell lines expressing apo B isoforms longer than apo B50 has proven to be difficult.

In summary, we have demonstrated that the techniques of gene targeting can be used to modify the apo B gene in HepG2 cells. In addition to successfully inactivating an apo B allele in

the HepG2 cell line, we have developed a novel double label RIA that greatly facilitates the detection of apo B gene targeting events in these cells. The use of this powerful detection assay together with targeting vectors designed to introduce subtle mutations into the apo B gene should create valuable new cell culture models for studies of apo B synthesis and secretion.

## Acknowledgments

We thank Linda Curtiss for providing the monoclonal antibody, MB19; Susan Hubl for technical assistance; Nigel Killeen for helpful advice; Steve Schoenberg and the UCSF cytogenetics laboratory for the karyotyping of HepG2 clones; Dale Newland and David Sanan for the electron microscopy; Rosalind Levin for manuscript preparation; Tom Rolain and Charles Benedict for graphics assistance; and Al Averbach and Mimi Zeiger for editorial assistance.

This work was supported by a Grant-in-Aid from the American Heart Association, California Affiliate. Dr. Farese was supported by a National Institutes of Health—Diabetes, Endocrinology, and Metabolism training grant (DK07418) and a National Institutes of Health Clinical Associate Physician Award.

## References

- Hamilton, R. L., A. Moorehouse, and R. J. Havel. 1991. Isolation and properties of nascent lipoproteins from highly purified rat hepatocytic Golgi fractions. *J. Lipid Res.* 32:529–543.
- Sniderman, A., S. Shapiro, D. Marpole, B. Skinner, B. Teng, and P. O. Kwiterovich, Jr. 1980. Association of coronary atherosclerosis with hyperapobetalipoproteinemia [increased protein but normal cholesterol levels in human plasma low density ( $\beta$ ) lipoproteins]. *Proc. Natl. Acad. Sci. USA.* 77:604–608.
- Pullinger, C. R., J. D. North, B. B. Teng, V. A. Rifci, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* 30:1065–1077.
- Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* 266:5080–5086.
- Capecchi, M. R. 1989. Altering the genome by homologous recombination. *Science (Wash. DC).* 244:1288–1292.
- Charron, J., B. A. Malynn, E. J. Robertson, S. P. Goff, and F. W. Alt. 1990. High-frequency disruption of the N-myc gene in embryonic stem and pre-B cell lines by homologous recombination. *Mol. Cell. Biol.* 10:1799–1804.
- te Riele, H., E. R. Maandag, A. Clarke, M. Hooper, and A. Berns. 1990. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. *Nature (Lond.)* 348:649–651.
- Blackhart, B. D., E. H. Ludwig, V. R. Pierotti, L. Caiati, M. A. Onasch, S. C. Wallis, L. Powell, R. Pease, T. J. Knott, M. L. Chu, et al. 1986. Structure of the human apolipoprotein B gene. *J. Biol. Chem.* 261:15364–15367.
- Young, S. G., S. J. Bertics, L. K. Curtiss, D. C. Casal, and J. L. Witztum. 1986. Monoclonal antibody MB19 detects genetic polymorphism in human apolipoprotein B. *Proc. Natl. Acad. Sci. USA.* 83:1101–1105.
- Young, S. G., and S. T. Hubl. 1989. An ApaLI restriction site polymorphism is associated with the MB19 polymorphism in apolipoprotein B. *J. Lipid Res.* 30:443–449.
- Simon, D., D. P. Aden, and B. B. Knowles. 1982. Chromosomes of human hepatoma cell lines. *Int. J. Cancer.* 30:27–33.
- Knott, T. J., S. C. Rall, Jr., T. L. Innerarity, S. F. Jacobson, M. S. Urdea, B. Levy-Wilson, L. M. Powell, R. J. Pease, R. Eddy, H. Nakai, et al. 1985. Human apolipoprotein B: structure of carboxyl-terminal domains, sites of gene expression, and chromosomal localization. *Science (Wash. DC).* 230:37–43.
- Doduri, R. K., R. K. Austin, L. K. Curtiss, S. G. Young, J. L. Witztum, and R. S. Smith. 1991. Definition of a nonlinear epitope on apoprotein B for MB47, a monoclonal antibody that completely blocks the binding of LDL to cellular LDL receptors. *Circulation.* 84(Suppl. II):227. (Abstr.)
- Xiong, W., E. Zsigmond, A. M. Gotto, Jr., K. Y. Lei, and L. Chan. 1991. Locating a low density lipoprotein-targeting domain of human apolipoprotein B-100 by expressing a minigene construct in transgenic mice. *J. Biol. Chem.* 266:20893–20898.
- Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J. C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B100. *J. Biol. Chem.* 265:553–568.

16. Frohman, M. A., and G. R. Martin. 1990. PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego. 228–236.
17. Young, S. G., S. J. Bertics, L. K. Curtiss, and J. L. Witztum. 1987. Characterization of an abnormal species of apolipoprotein B, apolipoprotein B-37, associated with familial hypobetalipoproteinemia. *J. Clin. Invest.* 79:1831–1841.
18. Young, S. G., F. P. Peralta, B. W. Dubois, L. K. Curtiss, J. K. Boyles, and J. L. Witztum. 1987. Lipoprotein B37, a naturally occurring lipoprotein containing the amino-terminal portion of apolipoprotein B100, does not bind to the apolipoprotein B,E (low density lipoprotein) receptor. *J. Biol. Chem.* 262:16604–16611.
19. Boerwinkle, E., and L. Chan. 1989. A three codon insertion/deletion polymorphism in the signal peptide region of the human apolipoprotein B (APOB) gene directly typed by the polymerase chain reaction. *Nucleic Acids Res.* 17:4003.
20. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J. Lipid Res.* 27:236–250.
21. Smithies, O., R. G. Gregg, S. S. Boggs, M. A. Koralewski, and R. S. Kucherlapati. 1985. Insertion of DNA sequences into the human chromosomal  $\beta$ -globin locus by homologous recombination. *Nature (Lond.)* 317:230–234.
22. Zheng, H., P. Hasty, M. A. Brenneman, M. Grompe, R. A. Gibbs, J. H. Wilson, and A. Bradley. 1991. Fidelity of targeted recombination in human fibroblasts and murine embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* 88:8067–8071.
23. Itzhaki, J. E., and A. C. G. Porter. 1991. Targeted disruption of a human interferon-inducible gene detected by secretion of human growth hormone. *Nucleic Acids Res.* 19:3835–3842.
24. Kim, H. S., B. W. Popovich, W. R. Shehee, E. G. Shesely, and O. Smithies. 1991. Problems encountered in detecting a targeted gene by the polymerase chain reaction. *Gene (Amst.)* 103:227–233.
25. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Hasty, P., J. Rivera-Pérez, C. Chang, and A. Bradley. 1991. Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol. Cell. Biol.* 11:4509–4517.
27. Hasty, P., J. Rivera-Pérez, and A. Bradley. 1991. The length of homology required for gene targeting in embryonic stem cells. *Mol. Cell. Biol.* 11:5586–5591.
28. Levy-Wilson, B., L. Soria, E. H. Ludwig, M. Argyres, A. R. Brooks, B. D. Blackhart, W. Friedl, and B. J. McCarthy. 1991. A polymorphism in a region with enhancer activity in the second intron of the human apolipoprotein B gene. *J. Lipid Res.* 32:137–145.
29. Peabody, D. S., and P. Berg. 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. *Mol. Cell. Biol.* 6:2695–2703.
30. Young, S. G., S. T. Hubl, R. S. Smith, S. M. Snyder, and J. F. Terdiman. 1990. Familial hypobetalipoproteinemia caused by a mutation in the apolipoprotein B gene that results in a truncated species of apolipoprotein B (B-31). A unique mutation that helps to define the portion of the apolipoprotein B molecule required for the formation of buoyant, triglyceride-rich lipoproteins. *J. Clin. Invest.* 85:933–942.
31. Yao, Z., B. D. Blackhart, M. F. Linton, S. M. Taylor, S. G. Young, and B. J. McCarthy. 1991. Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. Evidence that the length of apolipoprotein B has a major effect on the buoyant density of the secreted lipoproteins. *J. Biol. Chem.* 266:3300–3308.
32. Baserga, S. J., and E. J. Benz, Jr. 1988. Nonsense mutations in the human  $\beta$ -globin gene affect mRNA metabolism. *Proc. Natl. Acad. Sci. USA.* 85:2056–2060.
33. Chelly, J., H. Gilgenkrantz, M. Lambert, G. Hamard, P. Chafey, D. Récan, P. Katz, A. de la Chapelle, M. Koenig, I. B. Ginjaar, et al. 1990. Effect of dystrophin gene deletions on mRNA levels and processing in Duchenne and Becker muscular dystrophies. *Cell.* 63:1239–1248.
34. Ritchie, H. H., M. R. Hughes, E. T. Thompson, P. J. Malloy, Z. Hochberg, D. Feldman, J. W. Pike, and B. W. O'Malley. 1989. An ochre mutation in the vitamin D receptor gene causes hereditary 1,25-dihydroxyvitamin D<sub>3</sub>-resistant rickets in three families. *Proc. Natl. Acad. Sci. USA.* 86:9783–9787.
35. Hasty, P., R. Ramirez-Solis, R. Krumlauf, and A. Bradley. 1991. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature (Lond.)* 350:243–246.
36. Valancius, V., and O. Smithies. 1991. Testing an “in-out” targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. *Mol. Cell. Biol.* 11:1402–1408.
37. Blackhart, B. D., Z. Yao, and B. J. McCarthy. 1990. An expression system for human apolipoprotein B100 in a rat hepatoma cell line. *J. Biol. Chem.* 265:8358–8360.
38. Milne, R., R. Théolis, Jr., R. Maurice, R. J. Pease, P. K. Weech, E. Rassart, J. C. Fruchart, J. Scott, and Y. L. Marcel. 1989. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J. Biol. Chem.* 264:19754–19760.