Sustained somatic gene inactivation by viral transfer of Cre recombinase

Astrid Rohlmann, Michael Gotthardt[2], Thomas E. Willnow[2], Robert E. Hammer[1], and Joachim Herz*

*Departments of Molecular Genetics and [1]Biochemistry, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235; [2]Max-Delbrück-Center, Berlin, Germany. *Corresponding author (e-mail: herz@utsw.swmed.edu).*

Received 2 July 1996; accepted 21 August 1996

Transgenic and knockout mice have proven invaluable tools for analyzing physiologically relevant functions of numerous genes. In some cases, however, pleiotropic effects that result from a variable requirement for a particular gene in different tissues, cell types, or stages of embryonic development may complicate the analysis due to a complex phenotype or embryonic lethality. The loxP/Cre-mediated recombination system, which allows tissue-specific gene targeting in the mouse, can be used to overcome these problems. A limitation of current methods is that a mouse carrying a loxP-tagged gene must be crossed with a transgenic mouse expressing the Cre recombinase in an appropriate tissue to obtain the desired gene rearrangement. We have used recombinant adenovirus carrying the Cre recombinase to induce virtually quantitative somatic cell gene disruption in the liver. The targeted gene was the multifunctional low-density lipoprotein receptor-related protein (LRP), a cell surface receptor for α_2-macroglobulin and other ligands. Transient expression of Cre following adenoviral infection produced the predicted gene rearrangement, functionally inactivating LRP in the liver. Rearrangement occurred within 6 days after infection and remained stable for at least 28 days. The results demonstrate the suitability of adenoviral Cre gene transfer to induce long-term, quantitative, and temporally controlled gene disruption in the mouse.

Keywords: homologous recombination, LRP, protease, adenovirus

The 38-kD Cre protein mediates the site-specific insertion and excision of bacteriophage P1 into the host genome[1]. This simple binary recombination system has since been adapted for activating or inactivating various genes in mammalian cells[2] and in transgenic animals[3–6]. LoxP sites consist of a palindromic 34-bp sequence with an 8-bp core sequence that determines the directionality of the site. Two unidirectional loxP sites located on the same linear DNA molecule (e.g., a chromosome) are recognized by the Cre recombinase, which then efficiently excises and circularizes the intervening DNA sequence. It is thus possible to introduce precise, predetermined deletions of any desired size into the mammalian genome, e.g., a transgenic mouse, by first inserting loxP sites by homologous recombination into the targeted gene locus and then expressing Cre recombinase in all cells or only in specific tissues of the animal[5–8]. This technology is particularly useful to study the function of developmentally essential genes[9] in various tissues and at various times of development by inserting loxP sites into "silent" locations within the target gene (i.e., insertions sites that do not interfere with normal transcription, splicing, and protein translation) and subsequently expressing the Cre recombinase in a temporally and spatially controlled fashion.

We have generated a mouse strain in which loxP sites have been inserted into the gene encoding the low-density lipoprotein (LDL) receptor-related protein (LRP). Mice in which this multifunctional receptor[10] has been knocked out die early during gestation for reasons that are largely unclear[11]. In contrast, animals in which both LRP alleles have been modified by the insertion of loxP sites ($LRP^{flox/flox}$) are viable and phenotypically normal. By injecting a recombinant adenovirus carrying the Cre-recombinase gene under the cytomegalovirus promoter[12] intravenously into homozygous $LRP^{flox/flox}$ mice we were able to achieve essentially complete recombination and functional inactivation of the LRP gene in the liver. Adenovirus-mediated overexpression of an exogenous gene typically lasts for only a short period of time due to attack by the immune system[13–15]. In contrast, the Cre-induced genomic rearrangement and functional inactivation of the LRP gene occurred rapidly and remained stable for at least 4 weeks. These results demonstrate the usefulness of adenovirus-mediated transfer of Cre recombinase to study the function of essential genes in the liver by inactivating the "floxed" target gene in a temporally and completely controlled manner without the need for crossbreeding the loxP gene-altered mice with a transgenic mouse line expressing the Cre recombinase.

Results

Generation of $LRP^{flox/flox}$ mice. Mice carrying an LRP allele into which loxP sites had been integrated were generated by gene targeting in embryonic stem cells. The structure of the recombinant ("floxed") allele is shown in Figure 1A. Cre-mediated recombination is expected to result in deletion of part of the promoter including the transcription start site, exon I encoding the signal peptide, and exon II, thus rendering the allele nonfunctional. Wild-type, homozygous (flox/flox), and heterozygous animals were identified by Southern blot analysis of EcoRI digested genomic DNA using the indicated hybridization probe (Fig. 1B). We and others have previously shown that, following intravenous injection, recombinant adenovirus can infect almost 100% of the hepatic parenchymal cells[16,17]. We intravenously injected a purified recombinant adenovirus carrying the Cre recombinase (AdCre1) into the livers of homozygous $LRP^{flox/flox}$ mice to determine the extent to which it was capable of converting the floxed LRP allele to the desired knockout allele. Southern blot analysis of liver genomic DNA, 6 days after virus injection into homozygous $LRP^{flox/flox}$ mice, revealed virtually complete conversion of the floxed allele to the knockout allele (Fig. 2A). As expected, no detectable

recombination was seen in tail DNA isolated from the same animal. A similar result was obtained when the filter was hybridized with a pol2neo probe[18] (Fig. 2B). In the tail DNA, this probe detected the 9.7-kb floxed allele and also a 3.7-kb band indicative of the endogenous murine RNA polymerase 2 (pol2) promoter. In contrast, the floxed allele was almost completely absent in the liver DNA. Instead, the neo probe hybridized to a band of approximately 1.7 kb, which most likely represents the excised loxP-flanked neo cassette. The presence of this expected episomal circular DNA fragment could also be demonstrated by a polymerase chain reaction (PCR) assay that specifically detected only the circularized neo-containing fragment (not shown). Preliminary observations suggest that the episome was lost from the Cre virus-infected livers at a rate of approximately 50% per month.

Adenovirus-mediated LRP gene inactivation in vivo. We next determined whether AdCre1-mediated recombination of the floxed LRP allele resulted in the functional inactivation of the gene and the expected loss of protein expression. Wild-type and homozygous $LRP^{flox/flox}$ mice were intravenously injected with AdCre1 or with a control virus carrying the *Escherichia coli* β-galactosidase gene (Fig. 2C). Liver and brain were removed from each animal 6 days later and membrane extracts were subjected to immunoblot analysis with antibodies directed against LRP or the LDL receptor (LDLR, a control protein). LRP expression levels in liver and brain were equivalent in wild-type mice injected with either virus and in $LRP^{flox/flox}$ animals injected with AdCMV-βgal. This result demonstrates that (1) Cre expression does not affect the wild-type LRP allele, and (2) LRP expression is unaffected by the integration of the loxP sites and the neo cassette. In contrast, LRP expression was virtually eliminated in the livers of AdCre1-injected $LRP^{flox/flox}$ animals, but not in the brain, which is not a target for intravenously injected adenovirus[17]. Expression of the LDLR in either tissue was unaffected by the genotype of the animal or the virus used.

Loss of LRP protein in the liver correlated with the loss of LRP receptor activity (Fig. 3) as shown by measuring the clearance rates of ^{125}I-labeled α_2-macroglobulin in uninjected and virus-injected mice. α_2-macroglobulin is a specific ligand for the multifunctional LRP (refs. 19, 20). $LRP^{flox/flox}$ animals that had been injected with Cre virus were significantly impaired in their ability to clear ^{125}I-labeled α_2-macroglobulin from their plasma. In contrast, the ligand was efficiently cleared from the circulation of uninjected animals and wild-type and $LRP^{flox/flox}$ mice that had been injected with control virus.

Long-term effectiveness of Cre-mediated gene disruption. To determine whether this somatic gene disruption was transient or permanent we injected wild-type or $LRP^{flox/flox}$ animals with AdCre1 of AdCMV-βGal (Fig. 4). Partial hepatectomies were performed on the same animals 6 and 28 days after virus injection, respectively, and liver tissues were processed for immunoblotting with anti-LRP and anti-LDLR antibodies as well as for Southern blot analysis. Again, LRP expression was virtually abolished 6 days after injection of AdCre1 into $LRP^{flox/flox}$ mice and remained almost undetectable 28 days after virus administration. LDLR expression was not affected by virus injection regardless of the genotype of the animal or time point examined. Southern blot analysis of liver genomic DNA revealed that the floxed LRP allele had been largely converted to the knockout allele in the Cre-virus–injected $LRP^{flox/flox}$ animals. A variable level of nonrecombined, floxed LRP allele was detectable in some animals 6 days (between 7% and 28%) and, slightly more pronounced, 28 days (between 26% and 34%) after virus injection (Figs. 2 and 4). In contrast, LRP was almost completely absent after 6 days (between 0% and 4% of control) and 4 weeks (between 4% and 10% of control). The presence of nonrecombined DNA was likely due to the inflammatory response characteristic of adenoviral infection and the subsequent lymphocytic

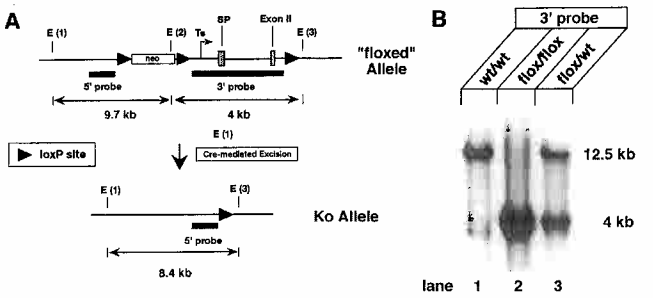

Figure 1. Introduction of loxP sites into the LRP gene. (A) Gene structure. A PGK-neo cassette flanked by loxP sites was introduced into a HindIII site approximately 240 bp upstream of the transcription initiation site. A third loxP site was introduced into a KpnI site downstream of exon II. Digestion of the "floxed" LRP allele with EcoRI produces a 9.7-kb fragment (detected with the 5′ probe) and a 4-kb fragment (detected with the 3′ probe). The wild-type allele (not shown) is detected by either probe as a 12.5-kb EcoRI fragment. Following Cre-mediated recombination the recombined allele is detected by the 5′ probe as a 8.4-kb EcoRI fragment. EcoRI sites, E(1–3), are indicated. (B) Southern blot analysis of mice wild-type (lane 1), homozygous (lane 2), or heterozygous (lane 3) for the floxed LRP allele. Approximately 10 μg of genomic DNA were digested with EcoRI and analyzed using the 3′ probe. Wt allele, 12.5 kb band; floxed allele, 4 kb band.

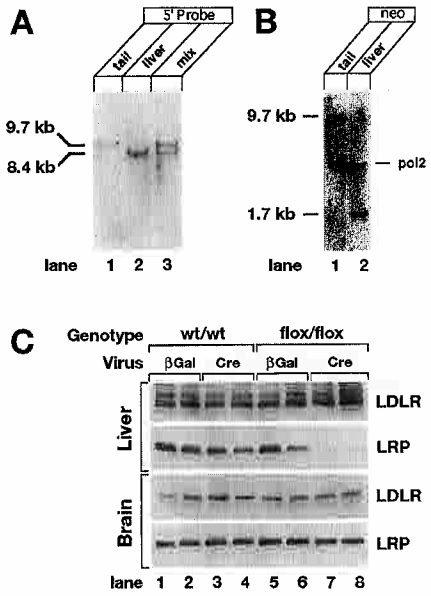

Figure 2. Southern and Western blot analysis of mouse tissues. (A) Ten micrograms of tail (lane 1) or liver (lane 2) DNA from a mouse homozygous for the floxed LRP allele were subjected to digestion with EcoRI and Southern blot analysis with the 5′ probe 6 days after AdCre1 injection. The positions of the floxed allele (9.7 kb) and the recombined allele (8.4 kb) are indicated. (Lane 3) Equal amounts of tail and liver DNA were mixed to illustrate the different migration of the floxed and recombined gene fragments. (B) Ten micrograms of tail (lane 1) or liver (lane 2) DNA from a mouse homozygous for the floxed LRP allele were subjected to digestion with EcoRI and Southern blot analysis with a neo probe 6 days after AdCre1 injection. The 9.7 kb band indicative of the floxed allele (lane 1) is absent in the liver (lane 2). Instead, the probe hybridizes to a 1.7 kb fragment that most likely represents the episomal excised neo cassette. The endogenous murine RNA polymerase II gene (pol2) is detected by pol2 promoter sequences present in the neo probe. (C) Immunoblot analysis of liver and brain membrane proteins. Mice wild-type (wt/wt; lanes 1–4) or homozygous for the floxed LRP allele (flox/flox; lanes 5–8) were either injected with AdCMV-βGal (lanes 1, 2, 5, and 6) or AdCre1 (lanes 3, 4, 7, and 8). Six days later, liver and brain were removed from the animals and membrane proteins were subjected to immunoblot analysis using polyclonal antibodies directed against the LDL receptor or LRP.

recombination was seen in tail DNA isolated from the same animal. A similar result was obtained when the filter was hybridized with a pol2neo probe[18] (Fig. 2B). In the tail DNA, this probe detected the 9.7-kb floxed allele and also a 3.7-kb band indicative of the endogenous murine RNA polymerase 2 (pol2) promoter. In contrast, the floxed allele was almost completely absent in the liver DNA. Instead, the neo probe hybridized to a band of approximately 1.7 kb, which most likely represents the excised loxP-flanked neo cassette. The presence of this expected episomal circular DNA fragment could also be demonstrated by a polymerase chain reaction (PCR) assay that specifically detected only the circularized neo-containing fragment (not shown). Preliminary observations suggest that the episome was lost from the Cre virus-infected livers at a rate of approximately 50% per month.

Adenovirus-mediated LRP gene inactivation in vivo. We next determined whether AdCre1-mediated recombination of the floxed LRP allele resulted in the functional inactivation of the gene and the expected loss of protein expression. Wild-type and homozygous $LRP^{flox/flox}$ mice were intravenously injected with AdCre1 or with a control virus carrying the *Escherichia coli* β-galactosidase gene (Fig. 2C). Liver and brain were removed from each animal 6 days later and membrane extracts were subjected to immunoblot analysis with antibodies directed against LRP or the LDL receptor (LDLR, a control protein). LRP expression levels in liver and brain were equivalent in wild-type mice injected with either virus and in $LRP^{flox/flox}$ animals injected with AdCMV-βgal. This result demonstrates that (1) Cre expression does not affect the wild-type LRP allele, and (2) LRP expression is unaffected by the integration of the loxP sites and the neo cassette. In contrast, LRP expression was virtually eliminated in the livers of AdCre1-injected $LRP^{flox/flox}$ animals, but not in the brain, which is not a target for intravenously injected adenovirus[17]. Expression of the LDLR in either tissue was unaffected by the genotype of the animal or the virus used.

Loss of LRP protein in the liver correlated with the loss of LRP receptor activity (Fig. 3) as shown by measuring the clearance rates of ^{125}I-labeled α_2-macroglobulin in uninjected and virus-injected mice. α_2-macroglobulin is a specific ligand for the multifunctional LRP (refs. 19, 20). $LRP^{flox/flox}$ animals that had been injected with Cre virus were significantly impaired in their ability to clear ^{125}I-labeled α_2-macroglobulin from their plasma. In contrast, the ligand was efficiently cleared from the circulation of uninjected animals and wild-type and $LRP^{flox/flox}$ mice that had been injected with control virus.

Long-term effectiveness of Cre-mediated gene disruption. To determine whether this somatic gene disruption was transient or permanent we injected wild-type or $LRP^{flox/flox}$ animals with AdCre1 of AdCMV-βGal (Fig. 4). Partial hepatectomies were performed on the same animals 6 and 28 days after virus injection, respectively, and liver tissues were processed for immunoblotting with anti-LRP and anti-LDLR antibodies as well as for Southern blot analysis. Again, LRP expression was virtually abolished 6 days after injection of AdCre1 into $LRP^{flox/flox}$ mice and remained almost undetectable 28 days after virus administration. LDLR expression was not affected by virus injection regardless of the genotype of the animal or time point examined. Southern blot analysis of liver genomic DNA revealed that the floxed LRP allele had been largely converted to the knockout allele in the Cre-virus–injected $LRP^{flox/flox}$ animals. A variable level of nonrecombined, floxed LRP allele was detectable in some animals 6 days (between 7% and 28%) and, slightly more pronounced, 28 days (between 26% and 34%) after virus injection (Figs. 2 and 4). In contrast, LRP was almost completely absent after 6 days (between 0% and 4% of control) and 4 weeks (between 4% and 10% of control). The presence of nonrecombined DNA was likely due to the inflammatory response characteristic of adenoviral infection and the subsequent lymphocytic

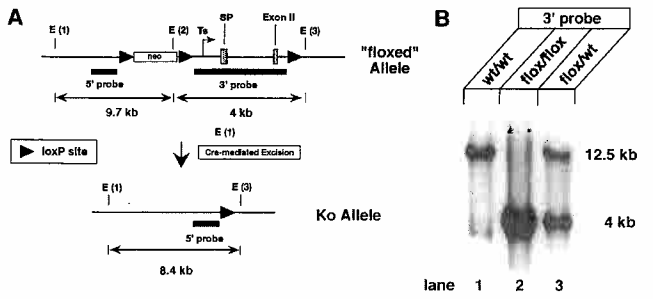

Figure 1. Introduction of loxP sites into the LRP gene. (A) Gene structure. A PGK-neo cassette flanked by loxP sites was introduced into a HindIII site approximately 240 bp upstream of the transcription initiation site. A third loxP site was introduced into a KpnI site downstream of exon II. Digestion of the "floxed" LRP allele with EcoRI produces a 9.7-kb fragment (detected with the 5′ probe) and a 4-kb fragment (detected with the 3′ probe). The wild-type allele (not shown) is detected by either probe as a 12.5-kb EcoRI fragment. Following Cre-mediated recombination the recombined allele is detected by the 5′ probe as a 8.4-kb EcoRI fragment. EcoRI sites, E(1–3), are indicated. (B) Southern blot analysis of mice wild-type (lane 1), homozygous (lane 2), or heterozygous (lane 3) for the floxed LRP allele. Approximately 10 μg of genomic DNA were digested with EcoRI and analyzed using the 3′ probe. Wt allele, 12.5 kb band; floxed allele, 4 kb band.

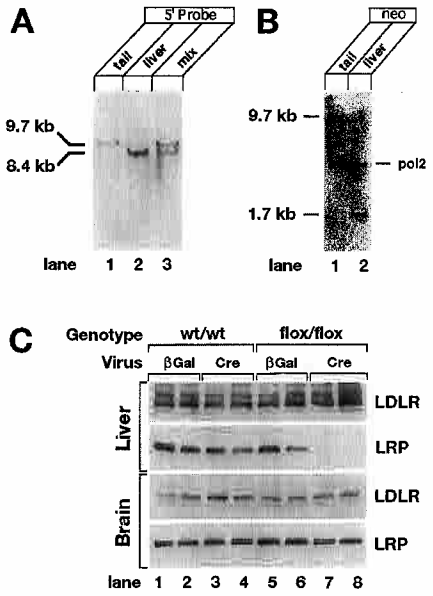

Figure 2. Southern and Western blot analysis of mouse tissues. (A) Ten micrograms of tail (lane 1) or liver (lane 2) DNA from a mouse homozygous for the floxed LRP allele were subjected to digestion with EcoRI and Southern blot analysis with the 5′ probe 6 days after AdCre1 injection. The positions of the floxed allele (9.7 kb) and the recombined allele (8.4 kb) are indicated. (Lane 3) Equal amounts of tail and liver DNA were mixed to illustrate the different migration of the floxed and recombined gene fragments. (B) Ten micrograms of tail (lane 1) or liver (lane 2) DNA from a mouse homozygous for the floxed LRP allele were subjected to digestion with EcoRI and Southern blot analysis with a neo probe 6 days after AdCre1 injection. The 9.7 kb band indicative of the floxed allele (lane 1) is absent in the liver (lane 2). Instead, the probe hybridizes to a 1.7 kb fragment that most likely represents the episomal excised neo cassette. The endogenous murine RNA polymerase II gene (pol2) is detected by pol2 promoter sequences present in the neo probe. (C) Immunoblot analysis of liver and brain membrane proteins. Mice wild-type (wt/wt; lanes 1–4) or homozygous for the floxed LRP allele (flox/flox; lanes 5–8) were either injected with AdCMV-βGal (lanes 1, 2, 5, and 6) or AdCre1 (lanes 3, 4, 7, and 8). Six days later, liver and brain were removed from the animals and membrane proteins were subjected to immunoblot analysis using polyclonal antibodies directed against the LDL receptor or LRP.

collected by retro-orbital puncture and the amount of trichloroacetic acid-precipitable radioactivity in plasma was measured[25]. Values are expressed as the percent of radioactivity present in plasma at 1 min after injection of the label.

Acknowledgments

We thank Jamey Marth for providing the loxP/PGK-neo cassette, Dudley Strickland for activated α_2-macroglobulin, Jay Horton for helpful suggestions, and David Russell for critical reading of the manuscript. We are indebted to Martina Anton and Frank Graham for sharing the Cre-expressing adenovirus and to Wen-Ling Niu and Laura Quinlevin for outstanding technical assistance. This work was supported by grants from the NIH (HL20948), the Keck Foundation, the Perot Family Foundation, and the Lucille P. Markey Charitable Trust. A.R. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and M.G. by the Boehringer Ingelheim Fonds. J.H. is an Established Investigator of the American Heart Association.

1. Sternberg, N., Hamilton, D., Austin, S., Yarmolinsky, M., and Hoess, R. 1981. Site-specific recombination and its role in the life cylce of bacteriophage P1. *Cold Spring Harbor. Symp.* **45:**297–309.
2. Sauer, B. 1993. Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol.* **225:**890–900.
3. Orban, P.C., Chui, D., and Marth, J.D. 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89:**6861–6865.
4. Lakso, M., Sauer, B., Mosinger, Jr., B., Lee, E.J., Manning, R.W., Yu, S.-H., et al. 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89:**6232–6236.
5. Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewski, K. 1994. Deletion of the DNA polymerase ß gene in T cells using tissue-specific gene targeting. *Science* **265:**103–106.
6. Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. 1995. Inducible gene targeting in mice. *Science* **269:**1427–1429.
7. Ramirez-Solis, R., Liu, P., and Bradley, A. 1995. Chromosome engineering in mice. *Nature* **378:**720–724.
8. Van Deursen, J., Fornerod, M., Van Rees, B., and Grosveld, G. 1995. Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc. Natl. Acad. Sci. USA* **92:**7376–7380.
9. Copp, A.J. 1995. Death before birth: clues from gene knockouts and mutations. *TIG* **11:**87–93.
10. Krieger, M. and Herz, J. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Ann. Rev. Biochem.* **63:**601–637.
11. Herz, J., Clouthier, D.E., and Hammer, R.E. 1992. LDL receptor-related protein internalizes and degrades uPA/PAI-1 complexes and is essential for embryo implantation. *Cell* **71:**411–421.
12. Anton, M. and Graham, F.L. 1995. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* **69:**4600–4606.
13. Pereira, D.S., Rosenthal, K.L., and Graham, F.L. 1995. Identification of adenovirus E1A regions which affect MHC class I expression and susceptibility to cytotoxic T lymphocytes. *Virol.* **211:**268–277.
14. Yang, Y. and Wilson, J.M. 1995. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J. Immunol.* **155:**2564–2570.
15. Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., and Wilson, J.M. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **91:**4407–4411.
16. Stratford-Perricaudet, L.D., Makeh, I., Perricaudet, M., and Briand, P. 1992. Widespread long-term gene transfer to mouse skeletal muscle and heart. *J. Clin. Invest.* **90:**626–630.
17. Herz, J. and Gerard, R.D. 1993. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc. Natl. Acad. Sci. USA* **90:**2812–2816.
18. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64:**693–702.
19. Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M., and Argraves, W.S. 1990. Sequence identity between the α_2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.* **265:**17401–17404.
20. Kristensen, T., Moestrup, S.K., Gliemann, J., Bendtsen, L., Sand, O., and Sottrup-Jensen, L. 1990. Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the α_2-macroglobulin receptor. *FEBS Lett.* **276:**151–155.
21. Wang, Y., Krushel, L.A., and Edelman, G.M. 1996. Targeted DNA recombination in vivo using an adenovirus carrying the *cre* recombinase. *Proc. Natl. Acad. Sci. USA* **93:**3932–3936.
22. Kütt, H., Herz, J., and Stanley, K.K. 1989. Structure of the low-density lipoprotein receptor-related protein (LRP) promoter. *Biochim. Biophys. Acta* **1009:**229–236.
23. Willnow, T.E. and Herz, J. 1994. Homologous recombination for gene replacement in mouse cell lines, pp. 305–334 in *Methods in cell biology 43*. Roth, M. (ed.). Academic Press, San Diego.
24. Willnow, T.E., Armstrong, S.A., Hammer, R.E., and Herz, J. 1995. Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proc. Natl. Acad. Sci. USA* **92:**4537–4541.
25. Willnow, T.E., Sheng, Z., Ishibashi, S., and Herz, J. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science* **264:**1471–1474