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Genetics

Generation of targeted retroviral vectors by using single-chain variable fragment: An approach to *in vivo* gene delivery

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ABSTRACT **We report the generation of a retroviral vector that infects human cells specifically through recognition of the low density lipoprotein receptor. The rationale for this targeted infection is to add onto the ecotropic envelope protein of Moloney murine leukemia virus, normally trophic for murine cells, a single-chain variable fragment derived from a monoclonal antibody recognizing the human low density lipoprotein receptor. This chimeric envelope protein was used to construct a packaging cell line producing a retroviral vector capable of high-efficiency transfer of the *Escherichia coli* β-galactosidase gene to human cells expressing low density lipoprotein receptor. This approach offers a generalized plan to generate cell and tissue-specific retroviral vectors, an essential step toward *in vivo* gene therapy strategies.**

Retroviral vectors are widely used as tools for the efficient transfer of genes into various cell types (1). This utility has been exploited toward genetic therapies where a number of clinical protocols utilize retroviral vectors for the transfer of therapeutic genes. Mostly these protocols involve *ex vivo* approaches, where cells are explanted from the patient, infected with the retroviral vector, and implanted back into the patient. These strategies provide the framework for establishing the efficacy of gene therapy. However, the widespread use of gene therapy will require the development of *in vivo* delivery systems. The long term goal is to inject a patient with a vector capable of specifically delivering the therapeutic gene to a target tissue.

Transducing retroviruses are typically constructed using a packaging cell line and a vector (2). The former provides all the proteins needed to assemble a retrovirus (the products of the *gag, pol,* and *env* genes); the latter consists of the gene of interest and a retroviral packaging signal bounded by the long terminal repeats. Hence, when the vector is introduced into the packaging cell line, the mRNA generated from the long terminal repeat is packaged, and a transducing retrovirus is produced. The tropism of the retrovirus is dictated by the product of the *env* gene, and the commonly used packaging cell lines provide either an ecotropic envelope, which restricts infection to rodent cells, or an amphotropic envelope, which permits infection of most mammalian cells. However, neither of these types of packaging cell lines is desirable as *in vivo* delivery systems for gene therapy, which ideally require a specific tissue tropism. This can be approached either by incorporating targeting molecules into the virion membranes (3) or by altering the envelope protein. For the latter case the possibilities include the following: (*i*) to engineer onto the envelope a ligand with a cognate receptor expressed on the target tissue (4, 5), (*ii*) to engineer onto the envelope an antibody binding site that recognizes a cell-specific antigen on the target tissue, and (*iii*) to exploit a recognition protein from a virus that has evolved a tropism for the target tissue (pseudotyping).

We investigated the second of the possibilities listed above, by grafting a single-chain variable fragment (scvf) (6) directed against the low density lipoprotein receptor (LDLR), onto the ecotropic envelope of Moloney murine leukemia virus. We report that the recombinant retrovirus containing the chimeric envelope infects human cells specifically through recognition of the LDLR.

MATERIALS AND METHODS

Assembly of Chimeric Envelope. Primers for PCR amplification of the variable regions were designed from the sequence of the C7 hybridoma κ and γ cDNA. For the γ variable region, GGGCCCAGGCTCGAGACCATGCAGGTTCAGCTGCAGCAGTCTGGGGCT and GCCTTTAATTAATGAGGAGACTGTGAGAGTGGTGCCTTG; for the κ region, TCGGGCGCGCCAGATGTTTTGATGACCCAAACTCCA and TGAGGAGACGGTGACCGTTTTGATTTCCAGCTTGGTGCCTCC. The primers incorporate unique restriction enzyme sites that facilitated cloning into pBS $(Gly_4\text{-}Ser)_3$; hence, κ and γ regions were linked by the $(Gly_4\text{-}Ser)_3$ linker, ATAAAAACGTTAATTAAAGGCGGTGGCGGATCGGGCGGTGGCGGATCGGGCGGTGGCGGATCGGGCGCGCCACTGCAGGAGACT. The resulting 759-bp cassette was cloned as a *Xho* I–*Bst*EII fragment into pEnv20.22, an ecotropic envelope expression vector modified to give unique *Xho* I and *Bst*EII sites, to generate pC7env. Nucleic acid manipulations were done by using standard techniques (7).

Immunoprecipitation. Cells (10^6) were labeled overnight in 2 ml of Dulbecco's modified Eagle's medium (minus methionine and cysteine) and [^{35}S]methionine at 0.4 mCi/ml (14.8 GBq/ml; DuPont). Nine hundred microliters was immunoprecipitated with a goat anti-envelope polyclonal serum G230 (from J. Elder, Scripps Research Institute). The complex was bound to rabbit anti-goat antiserum and protein A-Sepharose. This complex was washed once in 1 ml of 100 mM Tris·HCl, pH 8.0/0.5% Nonidet P-40/0.5 M LiCl and then washed with phosphate-buffered saline. The remaining protein was size-separated on a 7.5% polyacrylamide gel and visualized by autoradiography after fluorescence enhancement.

Viral Infections. Near-confluent cell cultures were grown for 24 hr in serum-free medium (HB GRO, Irvine Scientific); the supernatant was harvested, filtered, and kept at −70°C. Cells (5×10^4) were infected overnight with 2 ml of the supernatant containing Polybrene at 4 μg/ml (Sigma). The medium was then replaced, and the cells were stained for β-galactosidase 48 hr later (8). Note that the HeLa cells used in this experiment did not overexpress the LDLR.

Immunoblot Analysis. Eighty-five micrograms of cell extract from a HeLa cell line overexpressing the human LDLR was fractionated on a 7.5% polyacrylamide gel in the absence of

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Abbreviations: LDLR, low density lipoprotein receptor; scvf, single-chain variable fragment.

reducing agents. The proteins were blotted onto nitrocellulose, and the filters were incubated overnight with either C7 antibody or cell culture supernatants at 4°C. The filters were then washed in either Tris saline buffer/0.05% Nonidet P-40, for antibody probing, or Tris saline buffer, for "far" immunoblots. Primary binding was visualized by probing with a rabbit anti-mouse antibody conjugated to horseradish peroxidase or with anti-env antibody G230, followed by rabbit anti-goat horseradish peroxidase antibody for the far immunoblot (2 hr at room temperature), and visualized by using the enhanced chemiluminescence system (Amersham). For the competition assay, 20 μg and 10 μg of total cell extract were prepared for immunoblot as above. The filter was then incubated overnight with cell culture supernatant and subsequently probed with the C7 antibody.

Cell Transfections. Five micrograms of Bluescript KS+ (Stratagene) or an expression vector pCMV-LDLR was transfected into 5×10^4 QT6 cells using calcium phosphate precipitation (9). Twenty-four hours later the cells were infected with cell culture supernatant of Ψ2.C7env.lacZ. Forty-eight hours later the cells were stained for β-galactosidase (8).

RESULTS

Construction of the Chimeric Envelope and Packaging Cell Lines. A scvf was generated from the variable region of the γ and κ chains from hybridoma C7 (American Type Culture Collection no. 1691-CRL) (10). This hybridoma produces an IgG_{2b} monoclonal antibody that recognizes human and bovine LDLR and is internalized with kinetics similar to low density lipoprotein (10). The sequence of the κ and γ variable region was obtained through cDNA isolated from a C7 mRNA-derived library. Primers were designed for PCR amplification of the variable regions to include amino acid residues 1–112 and 1–120 of the κ and γ mature chains, respectively. The primers included convenient restriction enzyme sites to engineer a $(Gly_4\text{-}Ser)_3$ linker (11) between the κ and γ variable regions and to assemble this scvf module (253 amino acids) into the coding sequence of the ecotropic envelope (see Fig. 1*A*). The resultant expression plasmid, pC7Env, was transfected into 293 cells together with plasmids pGag-polGpt (12) and the retroviral vector LNL-SLX CMV β-gal (13). Virus produced in this transient system did not result in any observable transduction of β-galactosidase to human HeLa cells or to murine 3T3 cells. This result is probably due to the observation that some functions of the envelope are not tolerant to insertions (14). Consequently, we reasoned that because the viral coat is assembled by multimerization, the presence of a normal ecotropic envelope may trans-complement the chimeric envelope protein. Hence, viral particles were produced by transient transfection of pC7Env, LNL-SLX CMV β-gal (13), and SV-Ψ-E-MLV (15) vectors into 293 cells. Virus produced in this experiment did transduce β-galactosidase to HeLa cells at low efficiency (data not shown). To increase viral titer we then established permanent clones of Ψ2 packaging cells (16) containing the chimeric envelope, by cotransfection of plasmid pC7Env and a hygromycin B phosphotransferase expression vector. This procedure results in a cell line producing the *gag, pol, env* gene products (from the Ψ2 packaging cell), as well as the chimeric envelope protein. Twenty-eight hygromycin B-resistant clones were screened for expression of the chimeric envelope transcript using the scvf as a probe (data not shown). The clone with the highest expression, Ψ2.C7env, was infected with an amphotropic viral vector transducing LNL-SLX CMV β-gal (13). This transduction gives a pool of cells producing a recombinant retrovirus containing neomycin resistance and β-galactosidase. The resultant G418-resistant cells were cloned by limiting dilution, and 24 lines were analyzed for β-galactosidase expression by staining (8). The criteria used for selecting the highest expression clone, Ψ2.C7env.lacZ, were (*i*) rapidity of blue color development and (*ii*) 100% of the cells should be blue (17). The clone does not produce any detectable amphotropic helper virus (data not shown). This clone was compared with a packaging clone, Ψ2.lacZ, that produces an ecotropic virus transducing the same vector, LNL-SLX CMV β-gal. Fig. 1*B* illustrates the result of an immunoprecipitation, with anti-envelope antibodies, from the supernatants of the Ψ2.C7env.lacZ clone, the Ψ2.lacZ clone, and mouse 3T3 cells incubated overnight with [^{35}S]methionine. As expected, the Ψ2.C7env.lacZ clone produces a chimeric envelope protein (lane a), larger than the normal envelope (lane b) due to the insertion of the scvf. The size of the chimeric envelope is commensurate with it being ≈250 amino acids larger.

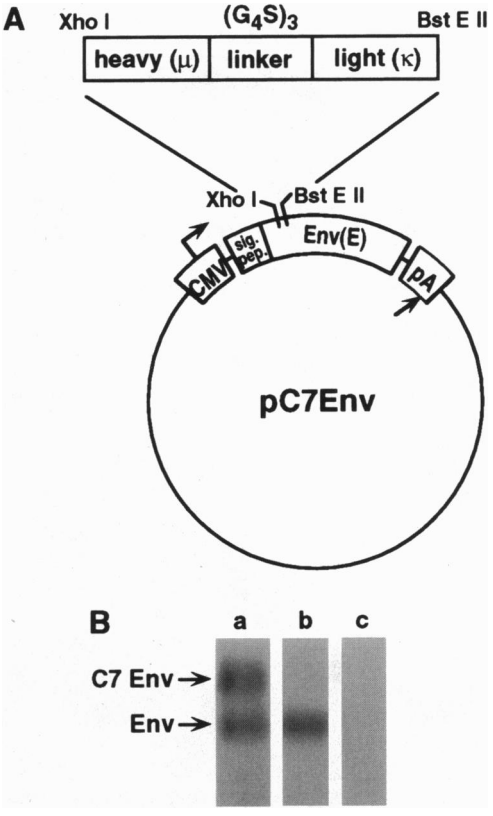

FIG. 1. (*A*) Schematic of the chimeric plasmid. A scvf to LDLR was assembled and cloned into an envelope expression vector, to generate C7env. $(G_4S)_3$, $(Gly_4\text{-}Ser)_3$; CMV, cytomegalovirus early promoter. (*B*) Characterization of chimeric envelope protein. Immunoprecipitation from the culture supernatant of Ψ2.C7env.lacZ (*a*), Ψ2.lacZ (*b*), and 3T3 (*c*) cells.

Virus was also harvested over a 24-hr period from nearly confluent Ψ2.C7env.lacZ and Ψ2.lacZ cells in serum-free medium. The rationale for omission of serum was that the presence of low-density lipoprotein in serum would down-regulate the LDLR and hence decrease the effective titer on the target cells. The virus was used to infect a number of cell types (Fig. 2). As expected, murine 3T3 cells were infected by the virus produced from both packaging cells (Fig. 2 *A* and *E*), due to the presence of ecotropic envelope. However, cells of human origin only were transduced to β-galactosidase positive when the chimeric envelope was present in the virus (Fig. 2 *F–H*). The titer of the virus from the Ψ2.lacZ clone was 3×10^5/ml on 3T3 cells and was not detectable on HeLa cells ($<10^1$ per ml), whereas the titer from the Ψ2.C7env.lacZ clone was 1.8×10^5/ml on 3T3 cells and an average of 1×10^4/ml on HeLa cells.

Specificity of Binding and Infection. The binding of the chimeric virus to its cognate receptor was examined on a far

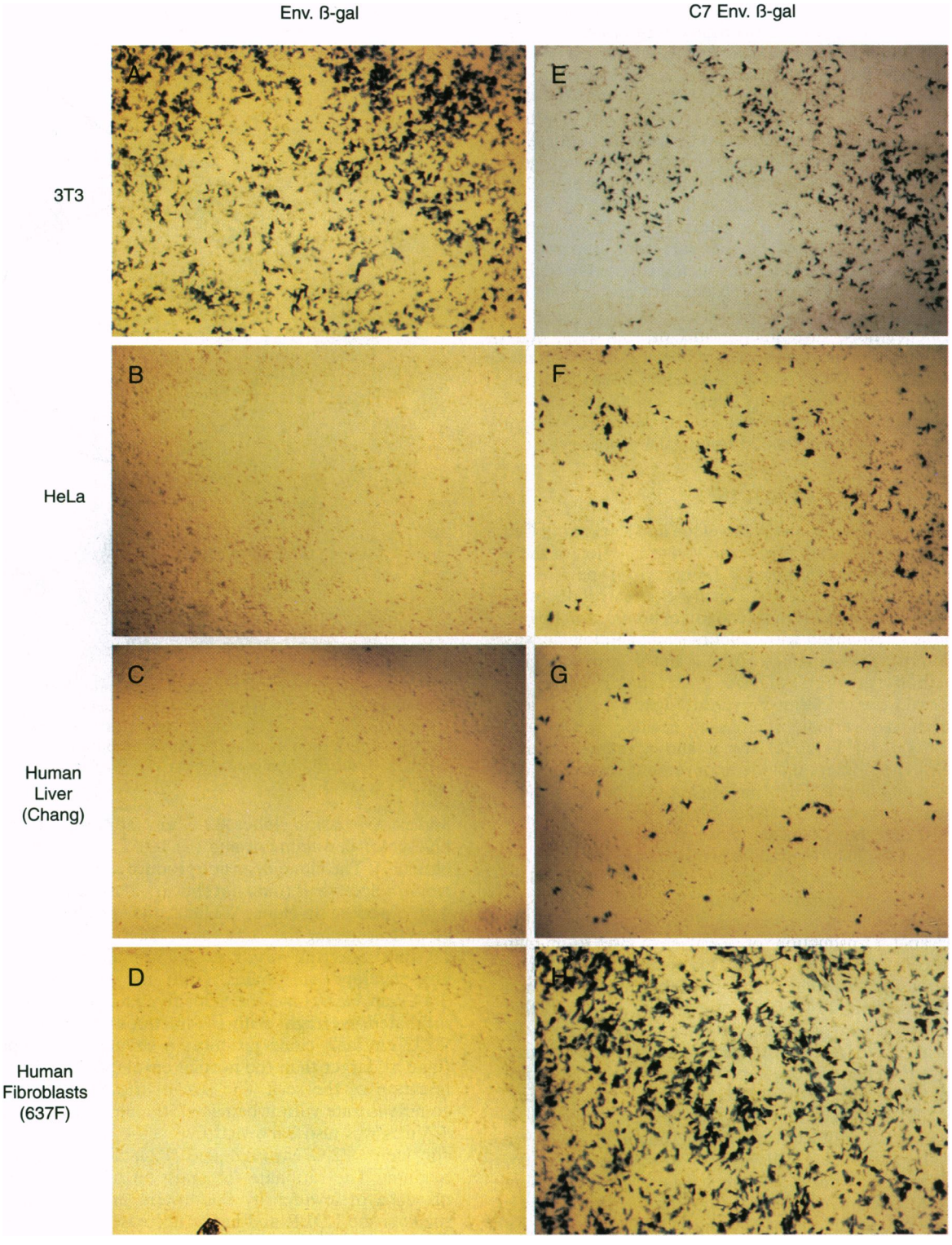

FIG. 2. Infectivity of chimeric scvf–envelope virus. Infection of various cell types from the culture supernatant of Ψ2.lacZ cells (*A–D*) and Ψ2.C7env.lacZ cells (*E–H*).

immunoblot. Extract from a HeLa cell line overexpressing the human LDLR was gel-fractionated and transferred onto nitrocellulose membrane. The presence of LDLR was confirmed by using C7 monoclonal antibody as a probe (Fig. 3*A*, lane 1). Duplicate filters were then incubated with supernatant from the Ψ2.lacZ clone (Fig. 3*B*, lane 1) or the Ψ2.C7env.lacZ clone (Fig. 3*B*, lane 2). The filter was then incubated with a polyclonal antibody against the Moloney murine leukemia virus envelope protein. A signal is apparent at 100 kDa (Fig. 3*B*, lane 2), the size expected for the human LDLR, which migrates above a background band, apparent in the control (Fig. 3*B*, lane 1). The identity of the cross-reacting band migrating at ≈180 kDa (Fig. 3*B*, lane 2) is not known. Fig. 3*C* illustrates that the 100-kDa signal is the LDLR in a competition assay. Duplicate immunoblots were preincubated with supernatant either from the Ψ2.lacZ clone (Fig. 3*C*, lanes 1 and 2) or from the Ψ2.C7env.lacZ clone (Fig. 3*C*, lanes 3 and 4) and then incubated with the C7 anti-LDLR monoclonal antibody. For

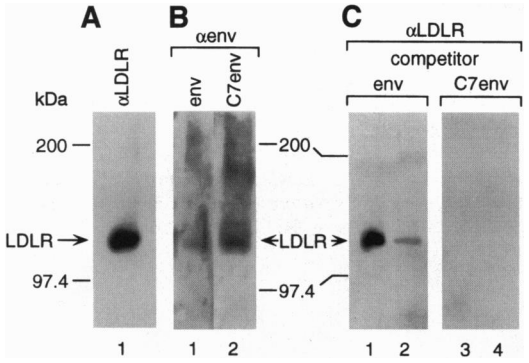

FIG. 3. Chimeric virus binding. (*A*) Immunoblot probed with C7 antibody. (*B*) Far immunoblot with culture supernatants of Ψ2.lacZ cells (*B*, lane 1) or Ψ2.C7env.lacZ cells (*B*, lane 2). (*C*) Competition for LDLR binding with culture supernatant of Ψ2.lacZ cells (lanes 1 and 2) or Ψ2.C7env.lacZ cells (lanes 3 and 4), and C7 antibody. Twenty micrograms (lanes 1 and 3) or 10 μg (lanes 2 and 4) of HeLa cell extract were prepared for immunoblot. Note that the enhanced chemiluminescence system gives a nonlinear response for the exposure conditions used.

equivalent loading of proteins the signal is effectively competed against by the Ψ2.C7env.lacZ virus. Taken together, these data demonstrate that the chimeric envelope mediates binding to a 100-kDa protein and that this protein is the LDLR.

To prove that the infection occurs through the LDLR, we used a quail cell line, QT6 (18), known to resist ecotropic virus infection. QT6 cells were transfected with either Bluescript KS(+) or an expression vector for human LDLR and infected 48 hr later with virus from the Ψ2.C7env.lacZ clone. Fig. 4 shows that only those cells transfected with the LDLR can be transduced with the β-galactosidase gene. The figure illustrates independent infections, and the effective titer in this experiment is low, probably due to the transient nature of LDLR expression and a toxicity associated with its overexpression. However, this experiment provides genetic proof that the LDLR is required for infection of nonmurine cells by the chimeric ecotropic virus.

DISCUSSION

We have altered the tropism of a retroviral vector so that it will now target infection through the LDLR. Other reports of altered retroviral tropisms have focused on the erythropoietin receptor (4) and the integrin receptor (5). The use of a ligand (4, 5) may be restrictive because a ligand has to be identified, and the size of ligands vary considerably. It has not been established whether large ligands can be tolerated, and also the titer using this strategy was low (4). Targeting through the specificity of antigen–antibody interactions has been demonstrated, although with very low efficiency (19), by means of biotinylated monoclonal antibodies to ecotropic envelope, biotinylated monoclonal antibodies to either human major histocompatibility complex I or II and a streptavidin bridge to drive infection of human cells through this specific interaction. However, the present results show that high-titer chimeric virus ($>10^4$/ml) can be produced by the use of scvf–envelope fusions. Previously, Russell *et al.* (20) reported use of this approach, but the antibody was directed against the unphysiological hapten 4-hydroxy-5-iodo-3-nitrophenacetyl caproate, and hence no functional infection was demonstrated. The use of antibody–antigen interactions as the basis for targeting has a number of attractions. This approach provides more scope due to a reagent base of monoclonal antibodies that have been developed to exquisitely define cell types, to define/target tumor antigens, or to investigate receptor functions. Furthermore the advantage of an antibody-based approach is that a monoclonal can be developed that acts as an antagonist for a given receptor, whereas its ligand will inevitably elicit a biological response on binding. This result also suggests an obvious extension to retroviral targeting experiments, where the scvf acts as an agonist that on binding the targeting molecule stimulates the cell into one round of division. This strategy would overcome a restriction of Moloney-based vectors being able to infect only dividing cell populations. In the long term we envisage this approach will be used in concert with lentivirus-based retroviral vectors, which can infect nondividing cells (21). Furthermore, in principle, a similar approach may be used with DNA viral vectors, such as adenovirus and adeno-associated viruses. This demonstration of targeting using a retroviral vector is a beginning toward directed *in vivo* gene therapy.

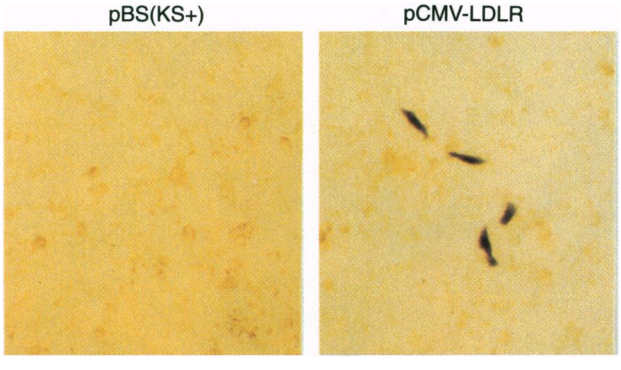

FIG. 4. Receptor specificity. Infection of quail cells transfected with either Bluescript plasmid or pCMV-LDLR and infected 24 hr later with Ψ2.C7env.lacZ vector. Infection was only apparent in cells transfected with the LDLR expression vector. Note that the QT6 cells were transiently transfected with an LDLR expression construct—hence, the resulting titer was low.

Note Added in Proof. A similar approach, using an avian retrovirus, was recently reported (22).

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1. Miller, A. D. (1992) *Nature (London)* **357,** 455–460.
2. Miller, A. D., Miller, D. G., Garcia, J. V. & Lynch, C. M. (1993) *Methods Enzymol.* **217,** 581–599.
3. Young, J. A. T., Bates, P., Willert, K. & Varmus, H. E. (1990) *Science* **250,** 1421–1423.
4. Kasahara, N., Dozy, A. M. & Kan, Y. W. (1994) *Science* **266,** 1373–1376.
5. Valsesia-Wittmann, S., Drynda, A., Deleage, G., Aumailley, M., Heard, J. M., Danos, O., Verdier, G. & Cosset, F. L. (1994) *J. Virol.* **68,** 4609–4619.
6. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S. & Whitlow, M. (1989) *Science* **242,** 423–426.
7. Sambrook, J., Fritsch, E. F. & Maniatis, T., eds. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
8. Sanes, J. R., Rubenstein, J. L. & Nicolas, J. F. (1986) *EMBO J.* **5,** 3133–3142.
9. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
10. Beisiegel, U., Schneider, W. J., Goldstein, J. L., Anderson, R. G. & Brown, M. S. (1981) *J. Biol. Chem.* **256,** 11923–11931.
11. Batra, J. K., FitzGerald, D., Gately, M., Chaudhary, V. K. & Pastan, I. (1990) *J. Biol. Chem.* **265,** 15198–15202.

12. Markowitz, D., Goff, S. & Bank, A. (1988) *J. Virol.* **62,** 1120–1124.
13. Scharfmann, R., Axelrod, J. H. & Verma, I. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4626–4630.
14. Gray, K. D. & Roth, M. J. (1993) *J. Virol.* **67,** 3489–3496.
15. Landau, N. R. & Littman, D. A. (1992) *J. Virol.* **66,** 5110–5113.
16. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33,** 153–159.
17. Hoeben, R. C., Migchielsen, A. A., van der Jagt, R. C., van Ormondt, H. & van der Eb, A. J. (1991) *J. Virol.* **65,** 904–912.
18. Moscovici, C., Moscovici, M. G., Jimenez, H., Lai, M. M., Hayman, M. J. & Vogt, P. K. (1977) *Cell* **11,** 95–103.
19. Roux, P., Jeanteur, P. & Piechaczyk, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9079–9083.
20. Russell, S. J., Hawkins, R. E. & Winter, G. (1993) *Nucleic Acids Res.* **21,** 1081–1085.
21. Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., Sharova, N., Adzhubel, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M. & Stevenson, M. (1993) *Nature (London)* **365,** 666–669.
22. Chu, T. H. & Dornburg, R. (1995) *J. Virol.* **69,** 2659–2663.