

## A system for tissue-specific gene targeting: Transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors

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Contributed by Harold E. Varmus, August 3, 1994

**ABSTRACT** Avian leukosis viruses (ALVs) have been used extensively as genetic vectors in avian systems, but their utility in mammals or mammalian cell lines is compromised by inefficient viral entry. We have overcome this limitation by generating transgenic mice that express the receptor for the subgroup A ALV under the control of the chicken  $\alpha_{sk}$ -actin promoter. The skeletal muscles of these transgenic animals are susceptible to efficient infection by subgroup A ALV. Because infection is restricted to cell lineages that express the transgene, the method has utility for studies of development and oncogenesis and will provide models for tissue-specific gene therapy.

Retroviruses can be used as vectors to deliver and express genes in cultured cells and animals (1). Several mammalian retroviruses, most notably murine leukemia viruses, have been used extensively as vectors in experiments with mammalian cells and, more recently, as vectors in gene therapy trials in humans (2–4). In general, such vectors suffer from two problems. (i) Because they are inherently replication defective, they must be propagated in helper cells that are programmed to provide viral proteins not encoded by the vector; as a result, viral titers are often relatively poor. (ii) The target cells can produce infectious virus vectors if they become infected with any of a variety of helper viruses of either endogenous or exogenous origin. This means that both vector stocks and the target cells must be carefully monitored for contaminating viruses to minimize the risk of unintended spread of the vector.

Avian retroviral vectors, especially those derived from avian leukosis viruses (ALVs), offer potential advantages over their mammalian counterparts. First, ALV-based vectors can be constructed to encode all of the proteins required for assembly of infectious particles in addition to the transferred gene of interest (5–8). Second, these vectors replicate to high titers in avian cells including cells, such as line 0 chicken embryo fibroblasts (CEFs), that lack endogenous viruses with which the vectors are likely to recombine. Third, ALV RNAs and proteins are inefficiently produced in mammalian cells so that infectious ALVs are rarely, if ever, produced in these cells; as a consequence, the vectors cannot spread in the target animals (9, 10). Despite these virtues, however, ALV vectors have not been used in mammalian hosts because the cells lack suitable cell surface receptors for virus entry and infection is extremely inefficient (11).

Five major subgroups (A–E) of ALV have been ascribed to differences in the surface envelope proteins encoded by various strains. Susceptibility of avian cells to these subgroups is governed by polymorphisms at three genetic loci,

known as *tv-a*, *tv-b*, and *tv-c* (11, 12). We have recently obtained cDNA clones representing two alternatively spliced versions of mRNA from the quail *tv-a* locus, the genetically defined locus for the subgroup A ALV [ALV(A)] receptor (refs. 13 and 14; L. Crittenden, P.B., J.A.T.Y., and H.E.V., unpublished data). These cDNAs encode small, cell surface-associated proteins that contain sequences homologous to the low density lipoprotein receptor (14). Expression of these proteins in mammalian cells renders the cells susceptible to efficient infection by ALV(A). A *tv-a* cDNA was linked to a promoter that is expressed specifically in muscle cells and used to create transgenic mice. When ALV(A) vectors are delivered to the skeletal muscles of these mice early in life, the muscle becomes infected and expresses foreign genes carried by the vector. By expressing a viral receptor in a tissue-specific manner, we can obtain restricted infection with vectors that replicate in avian but not in mammalian cells.

### MATERIALS AND METHODS

**Transgene Construction.** A 218-bp *Sal* I/*Sma* I fragment from the promoter region of the chicken  $\alpha_{sk}$ -actin gene was isolated from CLA12-191ACTCAT-1 (15) and linked to the pg800 quail cDNA (13), which encodes the ALV(A) receptor (*tv-a*). The plasmid pSPKE0.8, which consists of the *tv-a* cDNA inserted in the *Kpn* I/*Eco*RI sites of the pSP73 vector (Promega), was digested with *Sal* I and *Sma* I and ligated to the  $\alpha_{sk}$ -actin promoter fragment. The  $\alpha_{sk}$ -actin–receptor cassette was isolated as a *Cla* I fragment and used for microinjection.

**RNase Protection Assay.** Total RNA was isolated from homogenized leg muscle of 8- to 10-wk-old animals by the RNazol method (Cinna/Biotecx Laboratories, Friendswood, TX). Two <sup>32</sup>P-labeled antisense RNA probes were synthesized with an RNA transcription kit (Stratagene): a fragment from *tv-a*, which encodes the final 12 amino acids and the 3' untranslated region of the pg800 sequence (see Fig. 1), and a control fragment of the mouse glyceraldehyde-3-phosphate dehydrogenase gene, which was used as a control for the quality and quantity of the RNA. These probes were hybridized with 20  $\mu$ g of total RNA from four  $\alpha$ AKE transgenic lines (lines 1391, 1552, 1494, and 1506) and to RNA from a negative control animal (16). The RNA samples were then digested

Abbreviations: ALV, avian leukosis virus; ALV(A), ALV subgroup A; AP, human placental alkaline phosphatase; CEF, chicken embryo fibroblast; CAT, chloramphenicol acetyltransferase.

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with an RNase A/T1 mixture (Ambion, Austin, TX). The protected RNA probe fragments were separated on a 6% acrylamide/7.6 M urea gel and exposed to Kodak X-Omat film.

**Western Analysis.** Protein extracts were prepared by homogenizing leg muscle from 8- to 10-wk-old animals in protein gel loading buffer (14). The protein extracts (20  $\mu$ g) were separated on a SDS/12% polyacrylamide gel and transferred to nitrocellulose. After blocking, the filter was incubated with a 1:2500 dilution of a rabbit polyclonal antiserum raised against a purified glutathione *S*-transferase–pg800 fusion protein produced in *Escherichia coli*, followed by incubation with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit second antibody. The protein–antibody complexes were visualized by enhanced chemiluminescence (Amersham).

**Virus Propagation.** CEFs derived from 10-day line 0 embryos were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with 10% tryptose phosphate broth (GIBCO/BRL), 5% fetal bovine serum (HyClone), 5% newborn bovine serum (Advanced Biotechnologies, Silver Spring, MD), 100 units of penicillin per ml, and 100  $\mu$ g of streptomycin per ml. CEFs were passaged 1:3 at confluence. Virus propagation was initiated by transfection of plasmid DNA that contained the retroviral vector in proviral form. In standard transfections, 5  $\mu$ g of purified plasmid DNA was introduced into early passage CEFs by the calcium phosphate precipitation method (15). The course of virus infection was monitored by assaying confluent cells for chloramphenicol acetyltransferase (CAT) activity or by staining for cells producing human alkaline phosphatase (AP) (see below).

**AP Assay.** The AP assay was modified from procedures of Cepko and co-workers (17, 18). Frozen muscle sections or leg whole mounts were first washed in phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 30 min at 25°C (sections) or overnight at 4°C (whole mounts). Tissues were washed twice in PBS for 10 min each (sections) or 1 h each (whole mounts). Tissues in PBS were then heat treated at 65°C for 45 min in PBS to inactivate the endogenous AP, washed twice with AP detection buffer (100 mM Tris-HCl, pH 9.5/100 mM NaCl/50 mM MgCl<sub>2</sub>) for 10 min, and then exposed to the AP chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (GIBCO/BRL). Enzymatically active AP produced an insoluble purple precipitate.

**CAT Assay.** Protein extracts were prepared by homogenizing leg tissue in 0.25 M Tris-HCl (pH 7.8). CAT activity was determined by using an equivalent amount of protein from each sample as described (15). Reaction mixtures were incubated for 24 h at 37°C with two additions of fresh acetyl-CoA substrate. Acetylated and nonacetylated forms of chloramphenicol were separated on thin-layer silica gels, and the separated products were quantitated by radioanalytic imaging (Ambis Systems).

## RESULTS AND DISCUSSION

Previous work with transgenic mice carrying a 200-bp fragment of the chicken  $\alpha_{sk}$ -actin promoter linked to the bacterial CAT gene resulted in CAT expression that was restricted predominantly to skeletal muscle and heart (15). To target expression of *tv-a* to these tissues, the same promoter fragment was linked to the *tv-a* cDNA clone pg800 (Fig. 1). The DNA was microinjected into fertilized mouse eggs from C57BL/6C3F<sub>2</sub> matings to produce four lines of mice— $\alpha$ AKE 1391, 1494, 1506, and 1552. Receptor RNA and protein were detected in each of the four transgenic lines (Fig. 2). Lines 1391 and 1552 express relatively high levels of *tv-a*, while line 1494 produces a moderate level, and line 1506 produces a relatively low level.

The avian retroviral vector RCASBP/AP(A) (5000–7500 infectious units in a vol of 20–30  $\mu$ l) was injected into the leg muscles of 5-day-old mice from each of the four transgenic lines. The RCASBP/AP(A) virus contains the subgroup A *env* and carries the AP gene, which expresses a heat stable protein that can be easily visualized by a simple histochemical procedure (17, 18). This assay was performed 7 days after infection to allow sufficient time for proviruses to be established and for AP to be expressed. In contrast to their nontransgenic siblings, which cannot be infected by RCASBP/AP(A), animals from all four transgenic lines were susceptible to RCASBP/AP(A). The highest number of AP-stained muscle fibers was found in line 1494 (Fig. 3A). Infection was confined to the injected leg and did not spread to the contralateral leg. In individual infected legs, there were regions with high concentrations of AP-positive fibers, while other regions were not infected (Fig. 3B). Infected fibers in individual animals expressed different levels of AP, presumably an effect of the various integration sites on expression of different proviruses (Fig. 3C).

To measure the susceptibility of each transgenic line to ALV(A) infection more quantitatively, the experiment was repeated with RCASBP/CAT(A), a vector carrying the CAT gene (19). Extracts of infected leg muscle from lines 1494 and 1506 contained  $\approx$ 5-fold more CAT activity than did muscle extracts from lines 1552 and 1391 (Table 1), a result that correlated with the more qualitative AP assay. The average CAT activity for each transgenic line was determined by measurements on tissue from at least four animals. The wide range of CAT activities among mice from each line probably reflects variation in the amount of virus that remained in the leg after injection, since, in some cases, part of the injected viral stock leaked from the injection site.

Unexpectedly, the relative susceptibility of the four lines was inversely correlated with the relative levels of TV-A protein produced by each line (Fig. 2). The TV-A protein encoded by the pg800 clone appears to associate with the plasma membrane through a glycosphatidylinositol linkage (H. Wang, H.E.V., and P.B., unpublished data). Recent work has shown that this version of the receptor protein is

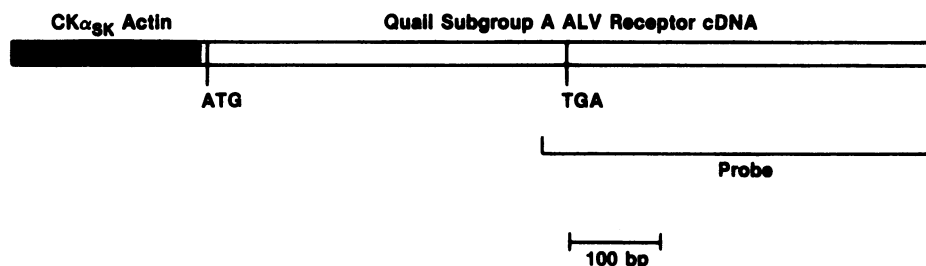


FIG. 1. Schematic representation of the  $\alpha_{sk}$ -actin promoter–ALV receptor transgene. The promoter region of the chicken  $\alpha_{sk}$ -actin gene (solid bar), contained on a 218-bp fragment, was linked to the gp800 quail cDNA, which encodes the ALV(A) receptor (*tv-a*). The region that was used as an RNase protection probe is marked and contains the last 12 codons and the 3' untranslated region from pg800.

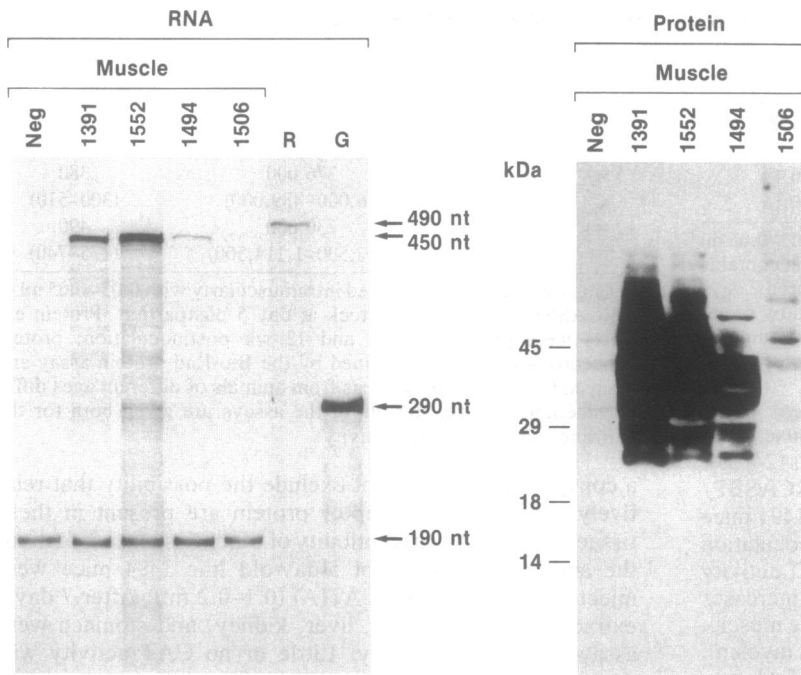


FIG. 2. Analysis of *tv-a* RNA and protein levels of the four  $\alpha$ AKE transgenic lines. The RNA panel shows an autoradiogram of a 6% polyacrylamide/7.6 M urea gel used to separate the protected RNA probe fragments produced in an RNase protection assay with RNA from the transgenic lines and a negative control (NEG). When the appropriate RNA is present, a 450-nt receptor RNA fragment is protected by the 490-nt full-length probe (R), and a 190-nt glyceraldehyde-3-phosphate dehydrogenase RNA fragment is protected by the 290-nt probe (G). The protein panel shows an autoradiogram of a Western transfer analysis of the TV-A protein levels in leg muscles of the transgenic lines and a negative control separated on a SDS/12% polyacrylamide gel. The TV-A protein undergoes post-translational modification (14) and consequently runs as a broad band on protein gels.

inefficiently secreted from NIH 3T3 cells (J. Gilbert and J. White, personal communication; H. Wang and H.E.V., unpublished data). Recently, Connolly *et al.* (20) engineered a soluble form of *tv-a* that efficiently blocked the infection of avian cells by ALV(A) by binding to the virus. A possible explanation for the lower susceptibility of the lines that express high levels of protein is that the version of receptor protein expressed in the

transgenic mice may be shed from the cell surface in sufficient quantity to interfere with productive infection.

ALVs appear to require mitotically active cells to complete their life cycle in cultured cells (21–23). We have found that the ability to infect line 1494 animals with RCASBP/CAT(A) declines rapidly after 5 days of age (Table 2). Extrapolating from data on skeletal muscle development in rats, the infect-

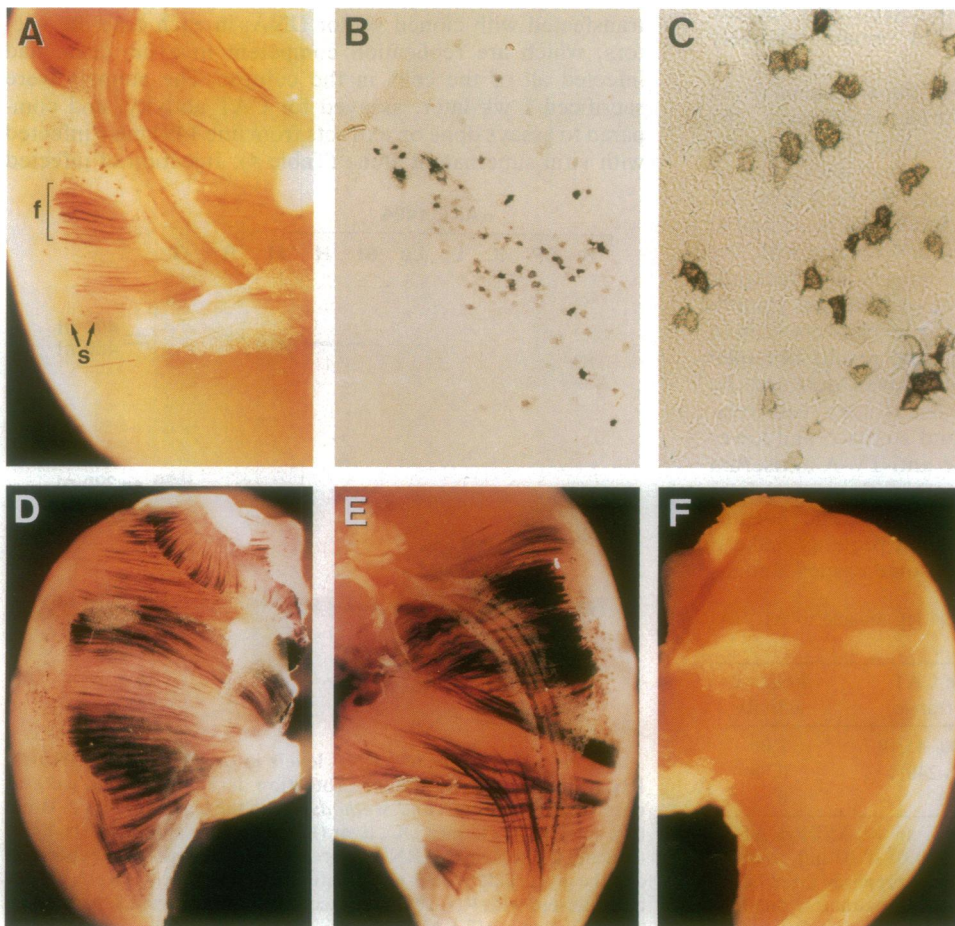


FIG. 3.  $\alpha$ AKE transgenic mice infected with RCASBP/AP(A). Between 5000 and 7500 infectious units (0.02–0.03 ml) of RCASBP/AP(A), the RCASBP(A) retroviral vector carrying the AP gene (17, 18), was injected intramuscularly into the legs of 5-day-old mice. Seven days later, the animals were sacrificed and leg whole mounts (A) or 10- $\mu$ m frozen sections from the leg muscle (B and C) were prepared. (A) Infected leg from line 1494. AP-positive muscle fibers can be seen as purple streaks and satellite cells as purple dots. Representative AP-stained muscle fibers (f) and satellite cells (s) are indicated in A. (B) Transverse section of an RCASBP/AP(A)-infected leg through the lateral and medial gastrocnemius. (C) Higher magnification of B. The experiment was repeated with RCASBP/AP(A)-infected CEFs (D–F). (D and E) AP-positive fibers in both sides of a whole mount of a leg from a transgenic animal infected by this procedure. (F) Leg from a nontransgenic animal that was injected in parallel with infected CEFs.

Table 1. Susceptibility of the  $\alpha$ AKE transgenic lines to ALV(A)

Line	Animals tested	Average CAT activity (range)	Relative activity
1494	6	2500 (1400–4330)	1.00
1506	7	2400 (1290–4260)	0.96
1552	4	480 (310–640)	0.19
1391	4	410 (180–720)	0.16

Mice (5 days old) were injected intramuscularly with 0.03–0.05 ml of viral stock of RCASBP/CAT(A), a retroviral vector that contains and expresses the CAT gene. Protein extracts were prepared 7 days after injection, assayed for CAT activity, and quantitated by radioanalytic imaging (Ambis Systems). Each assay mixture contained approximately one-fifth of the protein from the muscles of the hind leg.

ability of skeletal muscle in the transgenic mouse lines correlates with the mitotic potential of the myoblasts (24, 25).

To determine the ability of muscle infected with RCASBP/CAT(A) to maintain the expression of CAT, line 1494 mice were assayed for CAT activity at 5 and 12 wk postinoculation and the results were compared with the level of CAT activity at 1 wk (Table 3). The total CAT activity in the leg increases during the first 12 wk, increasing with the size of the muscle, in parallel with muscle-specific proteins (e.g., actin, myosin). The increase in CAT specific activity between 1 and 5 wk may reflect increasing levels of CAT expression, reaching maximum sometime after 1 wk postinfection. Although the ability to infect myoblasts declines rapidly after 5 days of age (Table 2), some myoblasts are mitotically active up to day 15 postpartum, when the majority of the myoblasts have already fused into the muscle fibers. Consequently, the number of cells producing CAT protein may increase over a span of at least 10 days, as infected myoblasts divide and/or new myoblasts are infected. Once the myoblasts have stopped dividing and CAT expression has stabilized, the specific activity of CAT in the muscle should remain relatively constant as seen at 5 and 12 wk.

The  $\alpha_{sk}$ -actin promoter was used in the transgene in an attempt to restrict expression of *tv-a* to skeletal muscle. The receptor RNA and protein levels were determined in a variety of tissues from each of the transgenic mouse lines, and, as expected, all four lines expressed relatively high levels of receptor RNA in skeletal muscle and in heart (Figs. 2 and 4; data not shown), consistent with earlier results with the  $\alpha_{sk}$ -actin promoter (15). However, moderately high receptor RNA levels were also found in tissues containing smooth muscle (stomach and lung). Much lower levels were seen in the other tissues tested, illustrated by the results obtained with line 1494 (Fig. 4). TV-A protein was detected by Western transfer analysis only in muscle tissues, with relatively high levels in skeletal muscle and stomach and lower levels in heart (data not shown). The anti-TV-A antiserum used in these experiments cross-reacts with several host proteins that are approximately the same size as the receptor protein (see Fig. 2). These cross-reacting proteins make it difficult to detect low levels of TV-A in some tissues, and, as

Table 2. Susceptibility of  $\alpha$ AKE line 1494 leg muscle to ALV(A) infection as a function of age

Injected	Animals tested	Average CAT activity (range)	Relative activity
Day 5	6	2500 (1400–4330)	1.00
Day 10	4	200 (100–240)	0.08
Day 15	4	<10	0

Mice were injected intramuscularly with an RCASBP/CAT(A) virus stock at day 5 (0.03–0.05 ml), day 10 (0.05–0.07 ml), and day 15 (0.07 ml) postpartum. Protein extracts were prepared after 7 days and assayed for CAT activity. Protein from approximately one-fifth of the total leg muscle was used in each of these assays.

Table 3. CAT protein expression by infected muscle fibers

	Animals tested	Total CAT activity per leg (range)	Specific activity (range)
1 wk	6	12,500 (7000–21,600)	100 (60–170)
5 wk	4	376,000 (286,000–489,000)	380 (300–510)
12 wk	3	730,000 (262,500–1,114,500)	490 (175–740)

Line 1494 mice were injected intramuscularly with 0.03–0.05 ml of a RCASBP/CAT(A) virus stock at day 5 postpartum. Protein extracts were prepared 1, 5, and 12 wk postinoculation; protein concentrations were determined by the Bio-Rad protein assay and assayed for CAT activity. Legs from animals of different ages differ considerably in size. Results of the assays are given both for the entire leg and as specific activity.

a consequence, we cannot exclude the possibility that relatively low levels of receptor protein are present in these tissues. To test the susceptibility of other tissues to infection, the abdominal cavities of 5-day-old line 1494 mice were injected with RCASBP/CAT(A) (0.1–0.2 ml). After 7 days, extracts from the spleen, liver, kidney, and stomach were assayed for CAT activity. Little or no CAT activity was detected in the six animals tested.

In an effort to increase the number of infected myoblasts and muscle fibers, an alternative virus delivery procedure was tested. Two litters of 5-day-old line 1494 mice were injected in the left leg with  $1.5\text{--}2.0 \times 10^5$  RCASBP/CAT(A)-infected CEFs prepared by (i) rapidly thawing frozen infected cells, washing with fresh medium, and concentrating to  $\approx 5 \times 10^6$  cells per ml, or (ii) treating growing cells with trypsin and concentrating to  $\approx 5 \times 10^6$  cells per ml. All of the cells used for injection had been passaged at least eight times after being transfected with cloned vector DNA; in each case the vectors, which are replication competent in avian cells, had infected all of the cells in the culture. The animals were sacrificed 1 wk later, assayed for CAT activity, and compared to assays done on extracts from line 1494 mice infected with viral supernatant alone (Table 4). Injection of infected

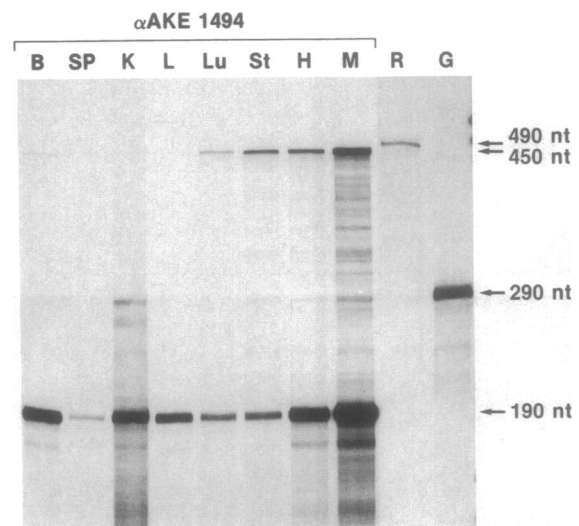


FIG. 4. Analysis of *tv-a* RNA levels in  $\alpha$ AKE line 1494 tissues. RNA preparation and RNase protection assay conditions were as described in *Materials and Methods* and in the legend of Fig. 2. Brain (B), spleen (SP), kidney (K), liver (L), lung (Lu), stomach (St), heart (H), and leg muscle (M) RNAs were hybridized with the receptor (R) and the glyceraldehyde-3-phosphate dehydrogenase (G) RNA probes and digested with RNase; the protected fragments were separated on a 6% acrylamide/7.6 M urea gel.

Table 4. Efficiency of gene delivery by injection of infected CEFs

Virus (vol)	Receptor positive		Receptor negative
	Injected left leg (range)	Uninjected right leg	Injected left leg
RCASBP/CAT(A) supernatant (0.03–0.04 ml)	2,500 (1,400–3,300)	<10	<10
RCASBP/CAT(A)/CEF: thaw/wash (1.5–2.0 × 10 <sup>5</sup> cells)	7,500 (4,700–10,600)	<10	<10
RCASBP/CAT(A)/CEF: growing (1.5–2.0 × 10 <sup>5</sup> cells)	60,800 (35,600–77,400)	<10	<10

Five-day-old line 1494 mice were injected intramuscularly with CEFs infected with RCASBP/CAT(A). Protein extracts were prepared 7 days after injection and assayed for CAT activity. Approximately one-fifth of the protein from the leg muscles was used in each assay.

CEFIs isolated directly from plates increased the level of CAT activity 24-fold over viral supernatant. Injecting recently thawed and washed infected CEFIs produced a 3-fold increase in CAT activity relative to the injection of viral supernatant. All the CAT activity was produced from infected mouse muscle cells since injecting infected CEFIs into the leg muscle of receptor-negative mice did not produce detectable levels of CAT activity. Although we have no direct evidence, we believe that the chicken cells are eliminated by the immune system. The infection was again confined to the injected leg and did not spread to the contralateral leg. The experiment was repeated with RCASBP/AP(A)-infected CEFIs, and considerably more AP-positive muscle fibers and satellite cells were seen throughout the leg (Fig. 3 *D* and *E*) compared to infection with supernatants (Fig. 3*A*).

### CONCLUSIONS

In this report, we have described an experimental system that makes it possible to use ALV-based retroviral vectors for genetic manipulations in mice. This system combines the advantages of ALV vectors and the powerful genetics of the mouse model. By placing the receptor gene under the control of a tissue-specific promoter, the vector can be targeted to the appropriate tissue—in this case, muscle. Additional lines of transgenic mice can be created to express the TV-A protein in other tissues by the use of tissue-specific promoters. It should be possible to further restrict the expression of experimental genes by including an internal tissue-specific promoter in the vector (19). This approach should prove useful for studying cell migration, development, and oncogenesis and for testing specific strategies for gene therapy.

We thank Debbie Swing and Bryn Eagleson for generating, maintaining, and injecting the transgenic mice; Connie Cepko for the RCASBP/AP(A) vector; Alan Kelly and John Leferovich for the muscle sections and expert technical advice; Judy White and Joanna Gilbert for sharing unpublished observations; and Hilda Marusiodis for preparation of the manuscript. The research was sponsored in part by the National Cancer Institute, Department of Health and Human Services, under Contract NO1-CO-74101 with Advanced Biosciences Laboratories; U.S. Department of Agriculture Competitive Grant 92-37204-8041 to M.J.F.; a University of Pennsylvania Research Foundation grant to P.B.; and a National Institutes of Health grant to H.E.V. H.E.V. was an American Cancer Society Research Professor.

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