Efficient transgenic rat production by a lentiviral vector

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Abstract

Efficient transgenic rat production by a lentiviral vector. Am J Physiol Heart Circ Physiol 293: H881–H894, 2007. First published February 23, 2007; doi:10.1152/ajpheart.00060.2007.—A lentiviral construct for an enhanced green fluorescent protein (eGFP) driven by a chicken β-actin promoter, cytomegalovirus enhancer, and intronic sequences from rabbit β-globin (CAG) was used to produce transgenic lines of rats for evaluation of the usefulness of this approach in gene function studies. Fertilized eggs were collected from inbred Dahl S and outbred Sprague-Dawley rats, and ~100 pl of concentrated virus were micro-injected into the perivitelline space of one-cell embryos. Of 121 embryos injected, 60 pups (49.6%) were born. Transgenic rates averaged 22% in Dahl S and 14% in Sprague-Dawley rats. Copy number ranged from one to four in the founders, and the inheritance of the transgene in a subsequent F1 population was 48.2%. The small number of insertion sites enabled us to derive inbred transgenic lines with a single copy of the transgene within one generation. Sequencing of each transgene insertion site revealed that they inserted as single copies with a preference for the introns of genes. The CAG promoter drove high levels of eGFP expression in brain, kidney, heart, and vasculature, making it very suitable for exploring the cardiovascular function of newly discovered genes. The pattern of eGFP expression was similar across five different F1 transgenic lines, indicating that the expression of the transgene was independent of its chromosomal position. Thus lentiviral transgenesis provides a powerful tool for the production of transgenic inbred rats and will enhance the usefulness of this species in gene discovery and target validation studies.

The rat is still the most widely studied animal model in the fields of hypertension, cardiovascular physiology, diabetes and metabolic disorders, behavioral studies, and toxicity testing. This species is particularly attractive, because it is large enough for invasive studies and sufficient quantities of blood and tissue can be collected for most analysis; also, a vast body of physiological and toxicological data have accumulated for this animal. There are also a large number of very well-characterized inbred rat models of cardiovascular and metabolic disease, including the Dahl salt-sensitive, spontaneously hypertensive, and Fawn Hooded rat models of hypertension and renal disease and the BB, GK, and Zucker models of diabetes and lipid disorders. The availability of these model systems, advances in rat genomic resources, and sequencing of the rat (6), human (13), and mouse (33) genomes have greatly accelerated the use of inbred rat models for discovery of the genetic basis of complex diseases. Indeed, the Rat Genome Database (http://rgd.mcw.edu/) lists >1,000 quantitative trait loci that have been identified in the rat. In many cases, these regions have been narrowed to a manageable number of genes through the creation of congenic strains (2, 12, 18, 29, 31). The next step in the discovery process is the identification of sequence variants in candidate genes and target validation by use of transgenic techniques. Unfortunately, the advances in rat genomics have not been accompanied by parallel progress in transgenic technology. The use of the rat as a model system for genetic discovery has been hampered by the lack of efficient techniques for the production of transgenic rats because of the lack of stem cell lines for gene targeting and the greater difficulty and poorer success rate in generating transgenic rats than mice with use of pronuclear injection (8, 16, 17, 19).

Recently, lentiviral systems have been developed to more efficiently introduce up to 18-kb constructs into the genome. This system has been used to produce transgenic mice (23) and has been applied to other mammalian organisms (9, 15). Two reports have demonstrated that it is possible to produce transgenic rats using lentiviral constructs (14, 32). However, the procedure for routine use in the rat has not been optimized, and the efficiency of the procedure, the sites and number of integration sites, the heritability of the transgene, the ability to create transgenic lines, and the pattern and levels of expression in various organs remain to be determined.

In the present study, we used a lentiviral construct expressing an enhanced green fluorescent protein (eGFP) reporter gene driven by a chimeric CAG promoter consisting of a human cytomegalovirus immediate-early enhancer, a chicken β-actin transcription start site, and a composite intron, including sequences from rabbit β-globin, to generate transgenic rats (21, 23). Subsequently, we determined the number and exact chromosomal location of proviral integration sites and the rate of the integration of the transgene into the germline and evaluated the feasibility of deriving stable transgenic lines in two different genetic backgrounds, Dahl S (SS) and Sprague-Dawley (SD) rats. We also extensively analyzed the pattern of eGFP expression in various organs across several different transgenic lines to determine whether expression of the transgene is position dependent.

MATERIALS AND METHODS

General

SS and SD rats were housed in a temperature-controlled (21–22°C) room with lights on from 0600 to 1800. The SS rats were fed a low-salt chow (AIN76A) containing 0.4% NaCl (catalog no. 113 755, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin; and 3Laboratory of Genetics, The Salk Institute, La Jolla, California

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A lentiviral vector expressing eGFP driven by a ubiquitous CAG promoter was used to generate the eGFP transgenic rats (Fig. 1A). The generation of this vector has been described in detail previously (23). This vector contained long terminal repeats (LTRs) with a self-inactivating mutation (37); a central polypurine tract of HIV-1 (4, 35); the CAG promoter, which consists of a chicken β-actin transcription start site; a human cytomegalovirus immediate-early enhancer; and a composite intron, including sequences from rabbit β-globin (21, 23).

The eGFP cDNA in this vector was derived from the bioluminescent jellyfish Aequorea victoria, and a posttranscriptional regulatory element from the woodchuck hepatitis virus (W) (36) was also included in the construct. The lentiviral particles were produced by cotransfection of four plasmids, the transfer vector, packaging plasmids (expressing gag/pol and rev), and a plasmid coding for the G protein of the vesicular stomatitis virus into 293T cells, using the calcium-phosphate method (23). The virus was harvested after 4 days and concentrated by ultracentrifugation (68,000 g). The functional titer of the virus was determined by quantification of eGFP fluorescent cells by fluorescence-activated cell sorting after transduction of 293T cells (23). The lentivirus was injected at a concentration of 8 × 10⁸ transducing units/ml. We failed to obtain transgenic animals when we injected the same virus with a 10-fold lower titer.

**Generation of Lentiviral eGFP Transgenic Rats**

Superovulation was induced in 5- to 6-wk-old SS and SD female rats by intraperitoneal injection of 20 IU of pregnant mare serum gonadotropin (Sigma, St. Louis, MO) followed 48 h later by intraperitoneal injection of 20 IU of human chorionic gonadotropin hormone (Sigma), and they were mated with males of the same strain. On the next day, one-cell embryos were collected from the females with plugs (8, 11, 16, 17).

Micropipettes with a tip diameter of 5 μm were used to inject the embryos with the lentivirus. The eggs were placed in 5–10 μl of M-2 medium (Sigma) on a microscope slide covered with paraffin oil. The egg was held with a holding pipette, the tip of the injection pipette was pushed through the zona pellucida, and the viral solution (20–100 pl) was injected into the perivitelline space (between the zona pellucida and the cytoplasmic membrane; Fig. 1B). Then the embryos were transferred to M-16 medium (Sigma), incubated for 1–2 h at 37°C in a 5% CO₂ incubator, and transferred to the oviduct of a pseudopregnant female rat (8–12 per oviduct), as previously described (16). The females were rendered pseudopregnant by mating with vasectomized males.

**Genotyping, Identification of Transgene Copy Number, and Breeding Scheme to Establish the Transgenic Lines**

Transgenic animals carrying the lentiviral eGFP DNA were identified by PCR (Fig. 1C). DNA was extracted from tail tissue using the phenol-chloroform method (30). PCR amplification was performed using the primer pair 5'-GAGCTAAACGGCCACAAGTTC and 5'-GTCGCCCTCGAACTTCACCTC, which amplifies a 291-bp region of eGFP. The reactions were denatured at 94°C for 3 min and cycled 35 times at 94°C for 30 s, 55°C for 40 s, and 72°C for 50 s.

The lentiviral transgene copy number in the founders and their offspring (F₁ generation) was determined by Southern blot. DNA was digested with BamHI, which cut the transgene only once upstream from the probe region (Fig. 1, A and D). After digestion, the DNA was separated on an 0.8% agarose gel, transferred to a nylon membrane, hybridized overnight at 65°C to a 748-bp BamHI I-Sal I fragment of the p156KR plasmid (23) encompassing the open reading frame of eGFP, labeled with [³²P], and subjected to autoradiography.

**Cloning and Sequencing of the Lentiviral-CAG-eGFP Insertion Sites by Linker-Mediated PCR**

A linker-mediated (LM)-PCR protocol, originally developed for cloning murine leukemia virus (34) and vertebrate transposon (33) integration sites, was adapted for amplification of lentiviral-CAG-eGFP insertions. The LM-PCR protocol (Fig. 2) is similar to a method recently described by Bryda et al. (1). DNA samples (2 μg) were digested with Nla III. An aliquot (10 μl) of digested fragments was ligated to linkers created by annealing two oligonucleotides, linker(+) (5'-GTAATTACGACTCATTAGGGCTCGTTAGGGGACCATG-3') and Nla III linker(−) (5'-phosphate-GTCCCTTAAGCGGATCC-3) for the site-CAG-eGFP vector is shown in Fig. 1A in the 5'-to-3' orientation. In a primary PCR, Gag-2 (reverse) (5'-GCTTTAATCTGACGCTTCGCACC-3') or Pre (forward) (5'-CCCTCAGAGCAGTGCGATCC-3') was used, along with linker primer (5'-GTAATTACGACTCATTAGGGG-3'), to amplify the 5'-genomic DNA-lentivirus or 3'-lentivirus-genomic DNA junction.

**Fig. 1. Strategy for production and identification of lentiviral enhanced green fluorescence protein (eGFP) transgenic rats.** A: CAG-eGFP lentiviral vector construct consisting of a viral long-terminal repeat (LTR), a central polypurine tract (PPT) of the lentivirus, a chicken β-actin promoter (CAG), a sequence encoding for eGFP, and a posttranscriptional regulatory element of the woodchuck hepatitis virus (W). Dotted lines, rat chromosome; thick red line, hybridization line 63 (D, right). B: Southern blot of CAG-eGFP in founders (F₀, left) and in the first filial generation (F₁, right) generated from DNA extracted from two nontransgenic littermates (unmarked lanes). D: Southern blot of CAG-eGFP in founders (F₀, left) and in the first filial generation (F₁) generated from founder 63 (right). Note 2 faint nonspecific background hybridization bands (++) in all lanes. Founder 63 had 3 copies of the transgene, each incorporated at a different genomic site, as reflected by the 3 distinct hybridization bands (white chevrons): 6.5, 3.5, and 2.8 kb. Subsequent cross-breeding of this founder with a nontransgenic partner produced a number of F₁ CAG-eGFP transgenic rats, each with the 3.5- or 2.8-kb hybridization band, indicating that the 2 separately segregating transgenes were located on different chromosomes. F₁ animals with a single 3.5-kb Southern band were the transgenics with the same chromosomal insertion site and were pooled into the same experimental group (line 63H) for analysis of chromosomal insertion site on the expression of the transgene (see Fig. 7, C and D). Transgenic rats exhibiting the 2.8-kb hybridization signal were also pooled to represent the small number of animals, no F₁ offspring carrying the 6.5-bp band were identified. Numbers adjacent to arrows indicate molecular weight. E: position-specific PCR genotyping of founders (1- and 2-digit numbers) and F₁ generation animals (4-digit numbers). One primer specific to the lentivirus and the second primer specific to genomic sequence adjacent to the lentivirus integration site were used to genotype offspring across generations. F₁ animals of line 63 (D, right) are the same animals examined by PCR in GL-014 insert. PCR products specific to GL-014 insert (3.5 kb) appeared only in the 7 transgenic lines carrying the 3.5-kb inserts (line 63H) and not in 4 animals with a smaller (2.8-kb) insert (line 63I), thus confirming that the 3.5-kb hybridization bands reflected the same transgenic insertion site. * DNA from animal 4025 (line 1) could not be amplified. F: eGFP protein expression analyzed in transgenic (+) and nontransgenic (−) rats by Western blot using polyclonal anti-eGFP antibody on extracts from kidneys (K), heart (H), skeletal muscle (M), and brain (B). Distinct signals are seen in extracts from organs obtained from transgenic animals, but not from nontransgenic controls.

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After PCR, the reactions were diluted (1:50) and reamplified in a PCR using Gag-1 (reverse) (5'-GCTCTCGACCCCATCTTCTC-3') or LTR (forward) (5'-TAAGACCAATGCTTACAAGGACG-3'), along with a nested linker primer (5'-AGGGTCTCGCTTAAGG-GAC-3'), to amplify across the 5' or 3' junction, respectively. The PCR products were separated on a 2% agarose gel. The bands were excised, extracted, and subcloned into a sequencing plasmid with use of the pGEM-Teasy vector system (Promega, Madison, WI). Plasmids harboring the subcloned PCR products were sequenced using M13 (forward) (5'-GTAAAACGACGGCCAG-3') or M13 (reverse) (5'-CAGGAAACGCTTATGAC-3') primer. Individual sequence Basic Logic Alignment Search Tool (BLAST) results were obtained using the University of California, Santa Cruz, genome browser, rn4 assembly (Nov. 2004).

**Figure 2.** Linker-mediated PCR (LM-PCR) method for cloning CAG-eGFP proviral integration sites. Founder phenol-chloroform-extracted genomic DNA is digested with *N*laIII linker (forward) and reverse primers (1-factor repetition) followed by Tukey's post hoc test was used to determine whether there was a significant effect of transgenic line, organ, or transgene copy number on eGFP expression. P < 0.05 was considered to be significant.

**RESULTS**

**Transgenic Rates, Copy Number, Chromosomal Insertion Sites, and Germline Transmission of the Transgene**

Six of nine (66.7%) prepubescent SS females that were stimulated to ovulate and mated with SS males produced 138 single-cell embryos. Of 121 single-cell embryos injected with the lentiviral vector and transferred to six SD recipients, 60 live pups (49.6%) were born. As determined by PCR (Fig. 1C), 22% carried the proviral insertion and were transgenic founders. Each litter had at least one transgenic founder. The transgenic success rates within the six SS litters ranged from 9.1 to 40%. The transgenic rates for eggs collected from female SD rats were similar and averaged 14%.

The number of copies of the transgene ranged from one to four in the founders (Fig. 1D, Table 1). Using a modified LM-PCR strategy, we also identified the exact chromosomal integration sites (Fig. 2). This approach, which is a well-established method for cloning single-copy murine leukemia retrovirus or transposons inserts (3, 34), involves cloning and sequencing of each of the 3', 3'-flanking regions surrounding each transgene insertion site. It has also been recently adapted to the identification of lentiviral insertion sites by Bryda et al. (1). The LM-PCR method yielded 28- to 574-bp junction sequences, which were compared with the current draft of the rat genome. The results confirmed that all the integrations were single copy (no tandem repeats) on different chromosomes, and there was no evidence of plasmid or lentiviral sequences in any of the cloned flanking regions. Subsequently, we determined the exact chromosomal address for each lentiviral gene by 1 single nucleotide (Table 2). The presence of a 5-bp target site duplication in proviral integrations demonstrated that the integration of the CAG-eGFP transgenes was mediated by the lentivirus integrase. The mapped proviral integration copy number obtained in four of five of the founders obtained using the LM-PCR method exactly matched the proviral copy number determined by the Southern blots. The only exception was founder 63, in which we were able to clone only one of the three integration sites identified in the Southern blots (Tables 1 and 2). These insertions were missed in the LM-PCR assay most likely because of the random nature of the genomic sequence, the dependence of the LM-PCR method on restriction fragment

![Fig. 2. Linker-mediated PCR (LM-PCR) method for cloning CAG-eGFP proviral integration sites. Founder phenol-chloroform-extracted genomic DNA is cleaved with *N*la III into 3 types of fragments: 1) 5' junctions between genomic DNA and provirus, 2) 3' junctions between provirus and genomic DNA, and 3) random short genomic fragments (step 1). The 5'-phosphate of *N*la III linker (--) allows for efficient ligation (step 2); 3'-phosphate (3'-P) prevents extension of the linker during PCR, which in turn prevents amplification of the random genomic fragments (which are at a ~10^{-3} molar excess over the junction fragments) and allows for junction-specific amplification of proviral integration sites in 2 rounds of PCR (steps 3 and 4). Secondary, nested PCR products are subcloned and sequenced before the sequences are compared with genome databases (steps 5–7).](http://ajpheart.physiology.org/Downloadedfrom)
lengths, and/or PCR amplification biases. A similar LM-PCR protocol, recently reported by Bryda et al., that uses different restriction enzymes and additional linear amplification step before linker ligation also demonstrated incomplete efficiency in mapping proviral integration sites.

Interestingly, 5 of 11 of the insertion sites identified in the founders incorporated within introns of known genes. One proviral integration site (GL-005) could not be mapped to the current build (National Center for Biotechnology Information rn4 build, Nov. 2004) of the rat genome. This likely reflects gaps known to be present in the current draft of the rat genome.

To determine whether the transgenes incorporated into the germline and could be transmitted to subsequent generations, founders were backcrossed with wild-type SS or SD partners (Table 1). Seven of the eight founders (87.5%) passed at least one copy of the transgene to 48.1% (n = 139) of their F1 progeny. These results indicate that the transgene was incorporated into the germline in most of the founders and at least one copy was transmitted to the progeny in an expected Mendelian fashion.

Table 1. Founder copy number and germline transmission rate

<table>
<thead>
<tr>
<th>Founder ID No.</th>
<th>No. of Transgene Copies by Southern Blot</th>
<th>No. of Insertions Cloned by LM-PCR</th>
<th>No. of F1 Pups</th>
<th>No. of F1 Transgenics</th>
<th>No. of Insertions Transmitted to F1</th>
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<tr>
<td>1</td>
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<td>4</td>
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<td>20</td>
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<td>1</td>
</tr>
<tr>
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<td>9</td>
<td>5 (44)</td>
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<td>3</td>
<td>1</td>
<td>40</td>
<td>19 (48)</td>
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<td>43</td>
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<td>NA</td>
<td>4</td>
<td>2 (50)</td>
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</tbody>
</table>

Values in parentheses are percentages. Founders 63 and 97 are Sprague-Dawley rats; others are Dahl S rats. Founder 28 was euthanized before reproduction. LM-PCR, linker-mediated PCR; NA, not analyzed.

The transgenic F1 progeny of founders 1, 10, 18, 63, and 97 were further examined by Southern blot for transgene copy numbers and segregation rates (Fig. 1D, right; Table 1). With the exception of line 18, the F1 progeny inherited a single copy of the transgene, indicating that in the multicopy founders the transgenes incorporated separately on different chromosomes. Founder 18 produced offspring (n = 5) with two or three copies of the transgene at four different proviral integration sites (data not shown). Some multiple proviral integrations in founders 1, 10, and 63 were not transmitted to the F1 population, perhaps because the number of F1 animals was insufficient to capture all the potential genotypes, or, more likely, because there was some degree of mosaicism in the germ cell population of the founders with multiple insertions.

Growth Rate, Reproduction, and General Appearance of CAG-eGFP Lentitransgenic Rats

Growth rates of the transgenic males and females were not different from those of nontransgenic littermates (data not shown).
shown). Similarly, reproduction rates did not differ between the transgenic rats and their nontransgenic littersmates: on average, each transgenic litter had 10 ± 1 pups, and this litter size was within the normal fertility range for our SS and SD rat colonies. The transgenic animals appeared healthy, and histological examination of sections of the brain, heart, kidney, and skeletal muscle revealed no abnormalities (data not shown).

Pattern of eGFP Expression in Organs of Lentitransgenic Rats

The pattern of eGFP expression was evaluated in transgenic founders (F₀) and their F₁ progeny. Expression of eGFP was clearly evident at all stages of development, including two-cell embryos 94 h after injection in culture, 14-day embryos, 2- and 12-day-old pups, and mature rats (Fig. 3). Young, hairless pups were entirely green; green fluorescence was observed in the adult transgenic animals in the areas of the body not covered by hair, i.e., ears, eyes, facial skin, tongue, paws, and tail. All 28 organs examined exhibited green fluorescence (Table 3). The pancreas, salivary gland, skeletal muscle, kidney, and brain exhibited the most intense fluorescence; adipose tissue, spleen, and prostate and seminal vesicles or uterus and ovaries demonstrated weaker intensities (Fig. 4). This pattern of transgene expression was consistently observed in the organs of all the transgenic founders and their offspring, regardless of gender. All the PCR-positive transgenic animals expressed eGFP, indicating that eGFP expression was independent of its chromosomal integration site. The specificity of the transgene product was also confirmed by Western blot using a polyclonal anti-eGFP antibody on homogenates obtained from the kidney, heart, skeletal muscle, and brain (Fig. 1F). GFP protein was detected in all the organs of transgenic rats; no eGFP protein signal was detected in any of the nontransgenic littersmates.

Intraorgan Pattern of eGFP Expression

In the brain, eGFP was expressed throughout the cortex and white matter (Fig. 5). In the heart and soleus muscle, eGFP expression was uniformly distributed among the myocytes. In the kidney, eGFP was expressed in the renal cortex and the medulla. Fluorescence was not observed in corresponding sections of these organs collected from nontransgenic littersmates.

Cellular Distribution of Transgenic eGFP in Various Organs

The fluorescence intensity in the confocal images of the four organs examined in F₁ transgenic rats was very robust and well above the background fluorescence levels in the organs of nontransgenic rats. In the heart (Fig. 6A), eGFP was expressed in cardiomyocytes. However, the intensity of the fluorescence was not uniform in all cells. This uneven expression pattern among cardiomyocytes was confirmed after a cardiac section was immunostained with an eGFP antibody (not shown). A similar uneven pattern of eGFP expression was also seen in sections prepared from the soleus muscle (Fig. 6B). eGFP fluorescence was present in the myocytes, but its intensity varied among the cells. In the kidney, eGFP expression was most intense in the proximal tubules in the renal cortex and less intense in the glomeruli (Fig. 6C) and thick ascending loop and collecting tubules in the outer medulla. In the brain, eGFP was expressed in neurons and the surrounding tissue (Fig. 6D). Remarkably, the cross sections of the blood vessels of the four organs revealed a very intense staining of the endothelium, whereas little of the eGFP was found in the smooth muscles in the walls of the vessels. This may reflect the fact that α-actin, rather than β-actin, is predominantly expressed in this tissue.

Quantitative Analysis of eGFP Expression: Lentitransgene Position and Copy Number Effects

To determine the effect of chromosomal insertion site of the transgene on eGFP expression, we compared the tissue concentrations of eGFP protein in homogenates of organs prepared from rats of five different transgenic lines carrying a single copy of the transgene at different chromosomal locations. We also measured the fluorescence intensity of the organs after fixed illumination. Southern blots were used to select the animals to be included in this analysis (Fig. 1D, right). Such selection was possible because of 1) a small transgene copy number (i.e., 2–4) in our founders, 2) lentitransgene incorporation at a given chromosomal site as a single copy, 3) distinguishable sizes (>0.7 kb) of the transgenic inserts on the Southern blots, and 4) Mendelian segregation of most of the transgenic inserts into the F₁ generation. The unique identity of each insertion within each line was further confirmed by PCR genotyping based on amplification of genomic sequences adjacent to the lentitransgene integration site (Fig. 1E). As shown in Fig. 1, D (right) and E, most of the F₁ offspring had a single copy of the transgene. PCR products specific to the GL-014 insert (3.5 kb) appeared only in the seven transgenic animals carrying the 3.5-kb insert (line 63H), and not in the four animals with a smaller (2.8-kb) insert (line 63L), thus confirming that the 3.5-kb hybridization bands reflected the same transgenic insertion. Following a similar strategy, animals from other lines (1, 10, and 97) carrying a single copy of the insert were also identified (Fig. 1E) for the analysis of the effect of chromosomal integration site on GFP expression levels. The founder of line 18 produced offspring with two copies of the transgene (n = 5), indicating that, in this line, the inserts may have been incorporated at the same chromosome (not shown). Thus only animals from this line were included in the analysis of the copy number effect (Fig. 1E).

The tissue concentrations of eGFP were significantly different between organs within a transgenic line, and the level of expression in any particular organ was statistically significantly different between the lines. Among the animals of five transgenic lines, the animals of line 97 (SD strain) expressed the lowest and the animals of line 10 (SS strain) the highest (1.8-fold higher than line 97) organ concentrations of eGFP (P < 0.05). However, in transgenic lines 1 (SS) and 63H and 63L (both SD), levels of eGFP protein expression were similar in all organs and were not different from those detected in line 97. Thus the influence of position on the levels of eGFP expression between the lines was not as great as the differences in eGFP expression between the different organs within a given rat or individuals of a given strain. Across the five organs examined, eGFP concentration was highest in the soleus muscle and lowest in the brain (P < 0.05). The concentration of eGFP was lower in renal tissue than in skeletal muscle but higher (P < 0.05) in renal tissue than in cardiac tissue or the
Fig. 3. Fluorescence imaging of transgenic eGFP expression in F₀ animals during development. Presence (+) or absence (−) of the transgene detected by PCR genotyping is shown. A and B: rat embryos in culture 94 h after injection visualized under white light and fluorescence, respectively. C and D: white light images of 14-day rat embryos and 2-day-old pups, respectively. C' and D': fluorescent light images of embryos and pups shown in C and D. E and F: fluorescent images of 2- and 12-day-old pups in mixed (white and fluorescence) light. G and H: fluorescent images of adult CAG-eGFP transgenic rats.
an 80% transgenic efficiency. Using the lentiviral system in the mouse, Lois et al. (14) and Pfeifer et al. (23, 24) reported at least one transgenic founder was identified in every litter studied. In comparison, using the lentiviral system these features allowed for a much higher success rate for capillary has to be advanced into the nucleus. Taken together, the embryo than pronuclear injection, where the injection into the subzonal space is far easier and much less invasive for these transgenic rats was engineered to be expressed outbred and inbred strains of rats, Van den Brandt et al. (32) reported 46% and 30% transgenic efficiencies, respectively. Our overall transgenic efficiencies of 22% and 14% for SS and SD rats are lower than those reported previously. This may be due to differences in the transduction efficiency between mouse and rat embryos and/or differences in the ability of the virus to transduce different strains of rats. However, the most likely causes are technical differences in the type, concentration, and amount of live virus injected in the subzonal space among the studies and differences in the transgenic efficiency of the viral preparation, which varies from batch to batch.

In the present experiment, Southern blots were used to determine the copy number of the transgene incorporated, which was confirmed by cloning and sequencing the integration sites in the founders. Copy number ranged from one to four in the founders. Sequencing of each lentitransgenic insertion site revealed that the transgenes inserted as single copies on different chromosomes, with a preference for the introns of genes. These results contrast with the multiplicity tandem incorporation of a transgene, which is often reported after pronuclear injection of “naked” DNA in rats (8, 17, 19, 27). On the other hand, the present findings are entirely consistent with a large body of evidence from a variety of studies indicating that lentivector-based transgene integrates at each genomic site as a single copy (9, 14, 23, 24). This mechanism-based transgene insertion was confirmed in the present study from our chromosomal insertion site sequencing results, which indicated the presence of a 5-bp target site duplication in the proviral integrations characteristic of the actions of the lentiviral integrase.

The transgene copy number determined in the present study is in the range previously reported by others using lentiviral-driven transgenesis in rats and mice (23, 32). Higher copy numbers of a lentitransgene incorporation were reported by Lois et al. (14) for the rat: 1–21 (average 6.6). These results raised serious concerns as to whether the lentiviral technique could be used to develop transgenic lines, since the large number of transgenes inserted would require an intensive and time-consuming breeding scheme to establish stable lines that transmit a fixed number of transgenes to subsequent generations. However, in the founders generated in the present study, only one to four copies of the transgene inserted on different chromosomes that segregated independently. Therefore, it was relatively easy to derive transgenic lines with a single transgene copy in an F1 generation to study the effects of chromosomal position on eGFP expression. We believe that the differences between the copy number inserted in the present study and that reported by Lois et al. likely reflect procedural differences in the concentration and amount of virus injected into the perivitelline space more than any endemic problem with the lentiviral transgenic technique. Indeed, in the present study, when we diluted the viral stock by a factor of 10, we were unable to obtain any transgenic animals, similar to the study and that reported by Lois et al. likely reflect procedural differences in the concentration and amount of virus injected into the perivitelline space more than any endemic problem with the lentiviral transgenic technique. Indeed, in the present study, when we diluted the viral stock by a factor of 10, we were unable to obtain any transgenic animals, similar to the finding of Verma et al. (28). Thus it seems likely that empirical determination of a range of concentrations of each batch of virus will be required to optimize the procedure to achieve the copy number and transgenic efficiency desired when different constructs are used.

In contrast to the jellyfish, where the native GFP is sequestered in cytoplasmic lumisomes (22), the eGFP used in these transgenic rats was engineered to be expressed

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<th>Table 3. Subjective estimation of fluorescence intensity in transgenic organs</th>
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</tr>
<tr>
<td>Duodenum, skin, stomach, ileum, colon, brain, testis</td>
</tr>
<tr>
<td>Heart, prostate, seminal vesicles, epididymis, vas deference, uterus, ovary</td>
</tr>
<tr>
<td>Spleen, aortic arch, brown and adipose tissues</td>
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Organs were obtained from F1 transgenic rats of the 5 transgenic lines evaluated quantitatively in Fig. 7. Organ fluorescence intensity was evaluated independently by 2 investigators after visualization of eGFP fluorescence (see Fig. 7 legend).

cerebral cortex. This organ-dependent pattern of eGFP expression levels was reproducible in different individuals of the five transgenic lines examined.

The pattern of fluorescence intensity between different organs was consistent with that obtained from measurement of the protein levels in the homogenates of the five transgenic lines. Expression of eGFP in the various organs was lowest in animals from line 97 (except skeletal muscle) and intermediate in animals from lines 63L and 1 (Fig. 7D). However, the overall effect of the line (i.e., the position) on organ eGFP fluorescence was statistically insignificant, indicating a lack of position effect. Within a line, the fluorescence intensity also differed among organs: it was highest in the skeletal muscle, intermediate in the kidney, and lowest in the heart and brain in all five of the transgenic lines.

Expression of eGFP in various organs was dependent on the number of incorporated transgenes (Fig. 7E). Concentrations of eGFP in the kidney and skeletal muscle were higher in the F1 rats carrying two copies of the transgene (line 18, n = 4) than in transgenic lines with a single copy of the transgene. This difference in eGFP expression between the lines with one and two copies of the transgene was also readily apparent when the fluorescence intensities recorded from the kidney and skeletal muscle of animals from line 18 were compared with those recorded in rats from the other lines.

**DISCUSSION**

The present study examined the feasibility of using a lentiviral construct for an eGFP driven by a CAG promoter to create transgenic lines of rats. Fertilized eggs were collected from SS and SD rats, and the virus was microinjected into the perivitelline space of one-cell embryos. Injecting the virus into the subzonal space is far easier and much less invasive for the embryo than pronuclear injection, where the injection capillary has to be advanced into the nucleus. Taken together, these features allowed for a much higher success rate for injecting the embryos and a better survival rate of the embryos. This is reflected by a high number of live births per injected embryo and a transgenic efficiency of 10–40%, which is several times higher than results from pronuclear injections (8, 16, 17, 19). A least one transgenic founder was identified in every litter studied. In comparison, using the lentiviral system in the mouse, Lois et al. (14) and Pfeifer et al. (23, 24) reported 80% transgenic efficiency. Using the lentiviral system in
Fig. 4. Fluorescent images of brain, heart, kidney, soleus muscle, adrenal gland, and eye of F1 generation (rat 4021, line 1) CAG-eGFP transgenic (Tsg) rats (left rows) and nontransgenic (Non-Tsg) littermates (right rows). Inset: dorsal view of brain.
throughout the cytoplasm. Because of its high solubility, allowing distribution throughout the cytosol, it could be expected that high levels of eGFP expression in multiple organs, particularly the brain or heart, could have a potentially toxic effect. However, we obtained very high embryo survival rates after injection and transfer, as well as very high subsequent transgenic success rates. Furthermore, despite high levels of eGFP expression throughout the body, even during very early embryonic stages, the CAG-eGFP transgenic rats grew at normal rates, appeared healthy, and

![Fluorescent images of cryosections of brain, heart, kidney, and soleus muscle of an F1 CAG-eGFP transgenic (rat 4021, line 1) and nontransgenic rat. No green fluorescence was observed on sections of organs from nontransgenic littermate.](image)

![Cellular distribution of eGFP fluorescence in heart (A), soleus muscle (B), kidney (C), and brain (D) of a CAG-eGFP transgenic animal (rat 4021, line 1). Images were obtained by confocal fluorescence microscopy. Magnification ×200. Insets: confocal fluorescent images of corresponding tissues from organs of nontransgenic littermate. Yellow arrows in A, B, and C point to walls of blood vessels; red arrow in C points to a glomerulus.](image)
were able to reproduce. These results indicate that, similar
to observations in the mouse (22), eGFP can be used in the
rat as a convenient reporter gene, and it can be studied
throughout the life span of the rat.

Pattern of eGFP Transgene Expression

One of the persistent problems in transgenic technology is
that the pattern and intensity of the expression of the transgene
often varies among transgenic lines. Epigenetic influences on
transgene expression may be due to a cis-acting regulatory
mechanisms stemming from the chromosomal location of the
insertion site, the vector sequence, the degree of lentiviral
integrant methylation, and the host genetic background (10, 19,
27). As a result, transgene expression may be position depen-
dent, or, often, complete inactivation of transcription underlies
the phenotypic variability among the transgenic lines.

To determine the influence of chromosomal position on
transgene expression, we used an LM-PCR to determine chro-
mosomal location of each transgenic insert and created five
independent transgenic lines that carry a single copy of the
transgene integrated at different locations in the genome. Our
expression analysis showed that CAG-eGFP transgene expres-
sion was more dependent on the organ and the transgene copy
number and less dependent on position. The relative lack of
position effect on the lentiviral CAG-eGFP expression is sup-
ported by the following findings: 1) the organ transgene ex-
pression was very robust and followed a similar pattern in all
transgenic lines; 2) there was only an 1.8-fold difference in
eGFP expression between the two most extreme lines; and 3)
eGFP was expressed at a very similar level in three of the five
lines.

A ubiquitous chimerical CAG promoter containing chicken
β-actin promoter sequences was used to drive the lentitrans-
genetic eGFP expression into a broad range of tissues. It was
expected that this promoter would drive expression in a wide
range of cell types, because the β isoform of actin is one of the
most abundantly expressed proteins in all animal cells (5, 25).
There is a high degree of sequence conservation of the β-actin
promoter between the rat and chicken (5, 25). This promoter
has also been shown to be ubiquitously expressed in transgenic
mice produced by pronuclear injection (22).

Our findings indicate that GFP expression driven by this
promoter was not affected by developmental stage and its
expression pattern was sustained in the next generation. Fur-
thermore, a novel design of the lentiviral vector used in this
study included deletion of the essential enhancer and promoter
regions from the LTRs of the lentiviral vector. Thus it is
possible that this modification rendered the vector resistant to

Fig. 7. Quantitative analysis of eGFP fluorescence intensity in various organs
obtained from 5 independent CAG-eGFP transgenic lines of rats. A and B:
representative examples of fluorescent images of brain, heart, and kidney of 2
transgenic animals representing lines 18 and 97, respectively, acquired for
quantification of eGFP fluorescence intensity (D and E) using MetaMorph
Morphometric software. C and D: comparison of organ concentrations of eGFP
and corresponding epifluorescence intensities, respectively, across the 5 CAG-
eGFP transgenic lines of rats. Number of animals in each line is shown in
parentheses. Lines 97, 63H, and 63L represent Sprague-Dawley transgenic
lines; lines 1 and 7 are derived from Dahl S transgenic founders. Animals of
lines 63H and 63L are offspring of founder 63 and have 3.5- and 2.8-kb inserts,
respectively. In C, transgenic line and organ had a significant effect on eGFP
expression (P < 0.05). In D, the organ, but not the transgenic line, had a
significant effect on eGFP expression (P < 0.05). E: comparison of organ
concentration of eGFP in F1 progeny of transgenic animals carrying 2 copies
(line 18, n = 4) with that in F1 progeny carrying 1 copy (n = 30) of the
transgene. There was a significant difference in mean values between the
animals with 1 copy and those with 2 copies of the transgene (P < 0.05).
silencing by trans-acting factors (repressors), which bind to the LTRs (20, 23, 24). Consistent lentitransgene expression during development and in subsequent transgenic generations indicates that this type of lentivector in the rat escapes epigenetic and DNA methylation-dependent silencing (10). It is also possible that the resistance of the lentitransgene to epigenetic silencing was due to the fact that this lentivirus (HIV-1) normally is not infectious for rodents, so no defense mechanisms have evolved in the rat during evolution.

The LM-PCR-based method of sequencing of insertion sites allowed us to determine the exact chromosomal insertion site of each transgene in the present study. However, because of the random nature of the genomic sequence, the dependence of the LM-PCR method on restriction fragment lengths, or PCR amplification biases, some insertions were missed in the present LM-PCR assay. A similar LM-PCR protocol, recently reported by Bryda et al. (1), which uses an additional linear amplification step before linker ligation, also demonstrated incomplete efficiency in mapping proviral integration sites. Thus multiple approaches or, at least, multiple restriction enzymes may be required to clone all proviral integration sites.

Interestingly, approximately half of the 11 lentiviral-CAG-eGFP transgenes were inserted in the intronic regions of known genes. The fact that gene-containing sequences constitute only 3–5% of the whole genome (6, 13) indicates that lentiviral vectors do not randomly incorporate in the genome but, instead, preferentially insert in the gene-rich areas. Similar preferences for lentiviral integration into active genes have been reported in cultured human cells (7, 26). To our knowledge, the relatively high percentage of intronic insertions observed in these CAG-eGFP transgenic rats is the first evidence suggesting gene preference of lentiviral transgenes in a whole animal setting.

The preference of lentiviral vectors for integration into genes has important implications for use of these vectors for germline and somatic gene transfer applications. Since the intronic areas of an endogenous gene may contain cis-acting regulatory sequences important for proper endogenous gene expression, the preferential incorporation of the lentitransgenes may raise some concerns regarding the usefulness of lentivectors in functional genomics. These genomic regulatory elements could influence lentitransgene expression, or, vice versa, regulatory elements present in the lentitransgene vector could influence the transcription of endogenous genes. To avoid this potential problem, as presented in this report, the LM-PCR method for sequencing each lentivector insert or an equally efficient strategy recently described by Bryda et al. (1) can readily be used for identification of transgenic founders with no intronic insertions.

In summary, the present results indicate that a lentiviral vector system can be used as a simpler and more efficient method for the production of transgenic rats than the traditional approach using pronuclear injection of DNA. Our CAG-eGFP lentiviral vector produced high transgenic rates and stable transgene transmission to the offspring and exhibited a consistent pattern of transgene expression in two strains of rats that was preserved after germline transmission. The small number of insertion sites enabled us to derive transgenic lines with a single copy of the transgene within one generation of backcross breeding. Tissue concentrations of eGFP protein and intensity of fluorescence of various organs in rats carrying a single copy of the transgene were expressed at similar levels in various organs across five independent transgenic lines, indicating that expression of the transgene was not positionally dependent. Our chimeric CAG promoter drove high levels of eGFP expression in all the organs examined. Although it will be desirable in some studies to change the promoter to obtain tissue-specific expression of the transgene, the global nature of expression obtained with the CAG promoter indicates that it is very suitable for first-pass screening studies of the function of newly discovered candidate genes affecting blood pressure and cardiovascular function about which little or nothing of the pattern of expression or the mechanisms of action of the expressed protein will be known. Overall, lentiviral transgenesis provides an important new tool to produce transgenic rats allowing this comprehensively studied species to be more widely used in studies of gene function and for the target validation studies in inbred rat models of complex disease.

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GRANTS

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