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Z. R. Donaldson, S.-H. Yang, A. W.S. Chan and L. J. Young
[Abstract] [Full Text] [PDF]

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D. J. Adams and L. van der Weyden
*Physiol Genomics*, August 1, 2008; 34 (3): 225-238.
[Abstract] [Full Text] [PDF]

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Lentiviral vectors: are they the future of animal transgenesis?

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Lentiviral vectors have become a promising new tool for the establishment of transgenic animals and the manipulation of the mammalian genome. While conventional microinjection-based methods for transgenesis have been successful in generating small and large transgenic animals, their relatively low transgenic efficiency has opened the door for alternative approaches, including lentiviral vectors. Lentiviral vectors are an appealing tool for transgenesis in part because of their ability to incorporate into genomic DNA with high efficiency, especially in cells that are not actively dividing. Lentiviral vector-mediated transgene expression can also be maintained for long periods of time. Recent studies have documented high efficiencies for lentiviral transgenesis, even in animal species and strains, such as NOD/scid and C57Bl/6 mouse, that are very difficult to manipulate using the standard transgenic techniques. These advantages of the lentiviral vector system have broadened its use as a gene therapy vector to additional applications that include transgenesis and knockdown functional genetics. This review will address the components of the lentiviral vector system and recent successes in lentiviral transgenesis using both male- and female-derived pluripotent cells. The advantages and disadvantages of lentiviral transgenesis vs. other approaches to produce transgenic animals will be compared with regard to efficiency, the ability to promote persistent transgene expression, and the time necessary to generate a sufficient number of animals for phenotyping.

lentiviral vector; integration; progeny; transgenic animals
direct use to inject or transfect mammalian cells. In general, the insert is removed from the vector, which has been thought to hamper the integration of the DNA. Table 1 shows a comparison of the factors involved in the plasmid DNA microinjection approach compared with the lentiviral vector system (which will be discussed below).

The number of copies of plasmid DNA that can be integrated varies from one to several hundred copies, which can be found as concatemers with a predominant head-to-tail orientation (3, 4, 77). Inhibitory effects from nearby genomic areas can alter the expression of the transgene (1), which is why larger genomic fragments are often used that include insulator sequences to maximize the production of transgene-expressing transgenic animals (113). The unrestricted size limitation is the single most important advantage to creating transgenic animals through direct incorporation of plasmid DNA. Even extremely large genomic DNA pieces have been cloned into yeast artificial chromosomes (YAC; 1- to 2-Mb insert size) or bacterial/P1-derived artificial chromosomes (BAC/PAC; <300-kb insert size) before transgenic applications. The details regarding the construction and advantages of artificial chromosomes have been reviewed elsewhere (33). However, there can be problems with the use of YAC/BAC/PAC because of rearrangements, shearing, and the technical nature of handling large constructs.

Nucleic acids generally integrate randomly (113), but there have been several attempts to achieve direct integration into more discrete sites. Hollis et al. (50) co-injected a donor plasmid DNA and phage ΦC31 integrase mRNA into the pronucleus to demonstrate site-specific integration by the plasmid DNA during the generation of transgenic mice. Although these investigators were able to produce a transgenic mouse, only 1 of 105 (or <1%) of the pups was found to be transgenic (50). This study illustrates that site-specific integration is possible, but further investigation is warranted to enhance the efficiency of this approach. Moreover, it remains to be determined whether this integration site is sensitive or resistant to epigenetic modifications that affect gene expression.

Even with the disadvantage of a low transgenic efficiency using pronuclear microinjection, this approach still remains the most simple and least labor-intensive routine method to produce transgenic animals, particularly in mice.

### Integrating Viral Vectors: Lentiviral Vectors

There are a plethora of viral vector systems that have been developed for gene therapy applications, but the vast majority of these vectors are nonintegrating, which precludes their usefulness for transgenesis. The best characterized integrating viral vectors originate from the *retroviridae* family, the members of which are ideal for the genetic manipulation of mammalian cells because of their intrinsic ability to integrate into genomic DNA. At present, the use of simple retroviruses based on murine Moloney leukemia virus (MLV) (61, 92, 133) is becoming overshadowed by more sophisticated lentiviral vector systems that have superior efficiency in modifying cells in vitro and in vivo compared with MLV-based vectors. For this reason, the main topic of discussion will focus on the lentiviral vector system and its potential role in transgenesis.

The genetic composition and the applications for transgenesis of complex retroviral (or lentiviral) vectors are illustrated in Fig. 1. The lentivirus genome derived from immunodeficiency viruses, such as human immunodeficiency virus-1 (HIV-1), has been split into multiple fragments to minimize the potential formation of replication-competent viruses. These components are broken into the following categories: 1) transfer or integrating vector, 2) structural and packaging, and 3) envelope plasmids. Since the transfer (integrating) vector plasmid is the only component that is transferred to the transgenic animal, the molecular composition of this plasmid will be described in this section, while the details regarding the packaging/structural and pseudotyping plasmids and detailed pro-
Prom, viral or cellular promoter; ZP, zona pellucida.

For the purposes of transgenesis, the primary benefit in using the lentiviral vector system is its efficient ability to integrate into the host genome (91, 100). However, proliferative stimuli before the administration of the lentiviral vectors or cells actively undergoing division have been shown to significantly enhance lentiviral vector transduction efficiency (93, 100–102). The latter characteristic could be a distinct advantage for lentiviral transgenesis compared with pronuclear microinjection by allowing for more rapid integration into the genome and reducing the potential for genetic mosaicism. A comparison between the lentiviral vector system and the pronuclear microinjection method can be found in Table 1.

TECHNIQUES USED IN THE MANIPULATION OF MALE- AND FEMALE-DERIVED EARLY PLURIPOTENT CELLS IN TRANSGENIC ANIMAL PRODUCTION

Both male- and female-derived cells have been used to generate transgenic animals. The differences in the approach being used with lentiviral vectors will be summarized and placed into context for each cell type.

Female-Derived Cells

In this section, the classic nucleic acid-based pronuclear injection approach will be discussed and compared with more modern approaches using lentiviral vectors. In general, fertilized zygotes have been manipulated at the single cell stage as well as unfertilized oocytes.

Pronuclear microinjection. This is the classic method to generate transgenic animals, and few changes have been made in the pronuclear injection method using DNA as the vector since the pioneering studies by Gordon et al. (35). The details of this approach have been extensively reviewed elsewhere (12, 113, 134). This procedure involves the transferring of genetic material by microinjecting DNA into the pronucleus of fertilized one-cell eggs, which are subsequently implanted into the oviduct of pseudopregnant surrogate females following mating with a vasectomized male. This method remains the predominant approach to produce transgenic animals because of its proven track record in generating a variety of transgenic animals, including mice, rats, pigs, chickens, cows, goats, and rabbits (5, 24, 31, 35, 40, 126).

Even with the successes described for this method, it has been fraught with a low transgenic efficiency. Embryo survival following microinjection is species dependent. It ranges from low viabilities in cows (17%) (14) to more moderate-to-high survival rates in rats and mice (31–87%) (7, 31, 126). In addition, integration of the constructs is often poor, and the generation of transgenic founder rats and other large animals, including cows and pigs, averages from 1 to 5% (7, 31, 126), whereas transgenic founder mice can be generated at efficiencies ranging from 5 to 20% (7, 134). In general, there is an ~50% probability that any given transgenic line will express the transferred gene product (134). It is important to note that the production of founder transgenic mice has been shown to be strain dependent, and that certain inbred strains of mice, such as C57Bl/6, can have an extremely low efficiency (0.5–
Table 2. Summary of lentiviral vector applications using female and male pluripotent cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Promoter</th>
<th>Transgene</th>
<th>% Embryo Viability (No. of Viable Treated Cells/Total Cells)</th>
<th>% Born (No. of Birth/Transferred Embryos)</th>
<th>No. of F0 Transgenic/Total Examined (Transgene Expression)</th>
<th>No. of F1 Transgenic/Total Examined (Transgene Expression)</th>
<th>F0 Vector Copy No.</th>
<th>Method</th>
<th>Ref. Nos.</th>
</tr>
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<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>UbC</td>
<td>GFP</td>
<td>90% (210/233)</td>
<td>17% (22/130)</td>
<td>13/22 (9)</td>
<td>1–7</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 74</td>
</tr>
<tr>
<td>Rat</td>
<td>UbC</td>
<td>GFP</td>
<td>57% (65/114)</td>
<td>43% (28/65)†</td>
<td>13/28 (12)</td>
<td>1–4</td>
<td>18/64 (18)</td>
<td>PVZ</td>
<td>Ref. 131</td>
</tr>
<tr>
<td>Rat</td>
<td>CAG</td>
<td>GFP</td>
<td>100% (121/121)</td>
<td>50% (60/121)</td>
<td>13/60</td>
<td>1–4</td>
<td>68/139</td>
<td>PVZ</td>
<td>Ref. 81</td>
</tr>
<tr>
<td>Rat</td>
<td>SYN</td>
<td>GRIP1</td>
<td>40% (20/50)</td>
<td>55% (11/20)</td>
<td>5/11 (2)</td>
<td>&gt;1</td>
<td>15/44 (9)</td>
<td>PVZ</td>
<td>Ref. 90</td>
</tr>
<tr>
<td>Rat U6</td>
<td>DAZL</td>
<td>shRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>UbC</td>
<td>GFP</td>
<td>86% (231/270)</td>
<td>21% (92/446)</td>
<td>63/73 (58)</td>
<td>1–21</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 74</td>
</tr>
<tr>
<td>Mouse</td>
<td>UbC</td>
<td>GFP</td>
<td>81% (86/106)</td>
<td>15% (11/74)</td>
<td>11/15 (37)</td>
<td>2–15</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 74</td>
</tr>
<tr>
<td>Mouse</td>
<td>UbC</td>
<td>GFP</td>
<td>NP</td>
<td>13 (19/146)</td>
<td>19/31 (19)</td>
<td>1–12</td>
<td>NP</td>
<td>INFZ</td>
<td>Ref. 74</td>
</tr>
<tr>
<td>Mouse</td>
<td>CAG</td>
<td>GFP</td>
<td>NP</td>
<td>10% (3/29)</td>
<td>3/3 (3)</td>
<td>N/D</td>
<td>NP</td>
<td>INF M</td>
<td>Ref. 105</td>
</tr>
<tr>
<td>Mouse</td>
<td>CAG</td>
<td>GFP</td>
<td>NP</td>
<td>25% (28/110)</td>
<td>15/23 (2)</td>
<td>1–8</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 52</td>
</tr>
<tr>
<td>Mouse</td>
<td>CMV</td>
<td>GFP</td>
<td>NP</td>
<td>18% (7/40)</td>
<td>7/7 (56)</td>
<td>N/D</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 52</td>
</tr>
<tr>
<td>Mouse</td>
<td>Rho</td>
<td>GFP</td>
<td>NP</td>
<td>22% (13/60)</td>
<td>9/11 (1)</td>
<td>N/D</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 52</td>
</tr>
<tr>
<td>Mouse</td>
<td>K12</td>
<td>GFP</td>
<td>NP</td>
<td>30% (18/60)</td>
<td>14/18 (1)</td>
<td>N/D</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 52</td>
</tr>
<tr>
<td>Mouse</td>
<td>K12</td>
<td>GFP</td>
<td>8% (5/60)</td>
<td>5/5 (2)</td>
<td>N/D</td>
<td>NP (3/40)</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 110</td>
</tr>
<tr>
<td>Mouse</td>
<td>PGK</td>
<td>GFP</td>
<td>95% (187/197)</td>
<td>NP</td>
<td>1/2*</td>
<td>N/D</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 110</td>
</tr>
<tr>
<td>Mouse</td>
<td>PGK</td>
<td>GFP</td>
<td>93% (94/101)</td>
<td>NP</td>
<td>4/7 (0)</td>
<td>N/D</td>
<td>NP</td>
<td>PVZ + Pr</td>
<td>Ref. 110</td>
</tr>
<tr>
<td>Mouse</td>
<td>UbC</td>
<td>GFP</td>
<td>80% (163/203)</td>
<td>20% (20/1363)</td>
<td>31/32 (31)</td>
<td>3–1</td>
<td>12/12</td>
<td>INVI Z</td>
<td>Ref. 141</td>
</tr>
<tr>
<td>Mouse</td>
<td>UbC</td>
<td>GFP</td>
<td>64% (97/151)</td>
<td>18% (78/797)</td>
<td>17/17 (17)</td>
<td>1–14</td>
<td>10/14</td>
<td>PVZ</td>
<td>Ref. 141</td>
</tr>
<tr>
<td>Mouse</td>
<td>CAG</td>
<td>GFP</td>
<td>61% (51/83)</td>
<td>34% (53/156)</td>
<td>NP/53</td>
<td>3–11</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 95</td>
</tr>
<tr>
<td>Mouse</td>
<td>CAG</td>
<td>GFP</td>
<td>100% (81/81)</td>
<td>43% (78/180)</td>
<td>NP</td>
<td>1–9</td>
<td>NP</td>
<td>INF B</td>
<td>Ref. 95</td>
</tr>
<tr>
<td>Mouse</td>
<td>H1</td>
<td>GFP shRNA</td>
<td>NP</td>
<td>2/5</td>
<td>13–21</td>
<td>2/15 (2)</td>
<td>NP</td>
<td>INF T</td>
<td>Ref. 128</td>
</tr>
<tr>
<td>Mouse</td>
<td>U6</td>
<td>CD6 shRNA</td>
<td>NP</td>
<td>16/32 (8)†</td>
<td>2–6</td>
<td>2–6</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 111,112</td>
</tr>
<tr>
<td>Mouse</td>
<td>U6</td>
<td>CD25 shRNA</td>
<td>NP</td>
<td>11/42</td>
<td>N/D</td>
<td>N/D</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 111,112</td>
</tr>
<tr>
<td>Mouse</td>
<td>U6</td>
<td>P51 shRNA</td>
<td>NP</td>
<td>5/22</td>
<td>N/D</td>
<td>N/D</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 111,112</td>
</tr>
<tr>
<td>Mouse</td>
<td>U6</td>
<td>Scl11a shRNA</td>
<td>NP</td>
<td>3 (2)</td>
<td>N/D</td>
<td>N/D</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 63</td>
</tr>
<tr>
<td>Mouse</td>
<td>U6</td>
<td>Ryk shRNA</td>
<td>NP</td>
<td>45% (18/40)</td>
<td>8/18 (8)</td>
<td>1–5</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 75</td>
</tr>
<tr>
<td>Mouse</td>
<td>CMV</td>
<td>hGM-CSF</td>
<td>100% (95)</td>
<td>37% (35/95)</td>
<td>24/35 (24)</td>
<td>1–8</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 107</td>
</tr>
<tr>
<td>Chicken</td>
<td>CMV</td>
<td>GFP</td>
<td>27% (20/73)†</td>
<td>N/A</td>
<td>1/1</td>
<td>1–5</td>
<td>20/44 (5)‡</td>
<td>SGC</td>
<td>Ref. 80</td>
</tr>
<tr>
<td>Chicken</td>
<td>CMV</td>
<td>lacZ</td>
<td>27% (20/73)†</td>
<td>N/A</td>
<td>13/19</td>
<td>1–4</td>
<td>724/87 (13)‡</td>
<td>SGC</td>
<td>Ref. 80</td>
</tr>
<tr>
<td>Chicken</td>
<td>PGK</td>
<td>GFP</td>
<td>4% (19/473)</td>
<td>N/A</td>
<td>3/66</td>
<td>N/D</td>
<td>4/637 (4)</td>
<td>SGC</td>
<td>Ref. 15</td>
</tr>
<tr>
<td>Chicken</td>
<td>OVA</td>
<td>miR24</td>
<td>N/A</td>
<td>1/NP</td>
<td>N/D</td>
<td>19/463 (7)</td>
<td>SGC</td>
<td>Ref. 73</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>OVA</td>
<td>hIFNβ1a</td>
<td>N/A</td>
<td>1/NP</td>
<td>1</td>
<td>2/NP (1)</td>
<td>SGC</td>
<td>Ref. 73</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>EREOVA</td>
<td>hIFNβ1a</td>
<td>N/A</td>
<td>3/NP</td>
<td>1</td>
<td>12/NP (3)</td>
<td>SGC</td>
<td>Ref. 73</td>
<td></td>
</tr>
<tr>
<td>Quail</td>
<td>SYN</td>
<td>GFP</td>
<td>10% (8/80)</td>
<td>8/8 (2)†</td>
<td>1</td>
<td>19/143 (18)</td>
<td>SGC</td>
<td>Ref. 117</td>
<td></td>
</tr>
<tr>
<td>Porcine</td>
<td>PGK</td>
<td>GFP</td>
<td>19% (46/244)</td>
<td>32/46 (30)</td>
<td>1–20</td>
<td>NS</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 48</td>
</tr>
<tr>
<td>Porcine</td>
<td>CMV</td>
<td>GFP</td>
<td>82% (120/147)</td>
<td>33% (40/120)</td>
<td>37/40 (35)</td>
<td>1–5</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 137</td>
</tr>
<tr>
<td>Porcine</td>
<td>PGK</td>
<td>GFP</td>
<td>NP</td>
<td>3 (3)</td>
<td>1–2</td>
<td>14/26 (9)</td>
<td>NP</td>
<td>PVZ</td>
<td>Ref. 47</td>
</tr>
<tr>
<td>Bovine</td>
<td>PGK</td>
<td>GFP</td>
<td>45 ± 22% (227)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 48</td>
</tr>
<tr>
<td>Bovine</td>
<td>PGK</td>
<td>GFP</td>
<td>40 ± 17% (227)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 48</td>
</tr>
<tr>
<td>Bovine</td>
<td>PGK</td>
<td>GFP</td>
<td>58 ± 7% (182)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 30</td>
</tr>
<tr>
<td>Bovine</td>
<td>PGK</td>
<td>GFP</td>
<td>35 ± 6% (248)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 30</td>
</tr>
<tr>
<td>Bovine</td>
<td>PGK</td>
<td>GFP</td>
<td>14 ± 7% (126)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 30</td>
</tr>
<tr>
<td>Bovine</td>
<td>UbC</td>
<td>GFP</td>
<td>30% (42/139)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>PVZ</td>
<td>Ref. 30</td>
</tr>
<tr>
<td>Bovine</td>
<td>UbC</td>
<td>GFP</td>
<td>25% (12/48)</td>
<td>50% (48/98)</td>
<td>4/4 (4)</td>
<td>4/12</td>
<td>1/1 (1)</td>
<td>PVO</td>
<td>Ref. 49</td>
</tr>
<tr>
<td>Bovine</td>
<td>UbC</td>
<td>GFP</td>
<td>22% (9/357)</td>
<td>22% (417)</td>
<td>0/4 (0)</td>
<td>NS</td>
<td>NS</td>
<td>PVO</td>
<td>Ref. 49</td>
</tr>
</tbody>
</table>

Continued
### Table 2—Continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Promoter</th>
<th>Transgene</th>
<th>No. of F0 Transgenic/Total Examined</th>
<th>% Embryo Viability (No. of Viable Born/Transgene Expression)</th>
<th>Method</th>
<th>Ref. No(s.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>EF</td>
<td>LacZ</td>
<td>N/A</td>
<td>N/A</td>
<td>SSC (p)</td>
<td>Ref. 88</td>
</tr>
<tr>
<td>Mice</td>
<td>EF</td>
<td>LacZ</td>
<td>23/23 (23)f</td>
<td>5.8%</td>
<td>SSC (p)</td>
<td>Ref. 88</td>
</tr>
<tr>
<td>Rat</td>
<td>EF</td>
<td>LacZ</td>
<td>42/730 (42)</td>
<td>1</td>
<td>SSC (a)</td>
<td>Ref. 114</td>
</tr>
</tbody>
</table>

NS, studies were not performed to produce F0 and/or F1 progeny; NP, specific nos. were not provided in the study, but the experiments were performed; N/A, not applicable; N/D, not determined.

Promoters: UbC, human ubiquitin C promoter; CMV, cytomegalovirus promoter; PGK, phosphoglycerokinase promoter; EF, elongation factor-1 promoter; CAG, chicken β-actin promoter with CMV enhancer; CMV, cytomegalovirus; SV40, simian virus 40; K14, human keratin K14 promoter; RG, red pigment gene; Rho, rhodopsin; K12, keratin-12 gene; OVA, ovalbumin promoter; CMV, cytomegalovirus; RRE, Rev responsive element; 3.5 kb, 3.5 kilobase pair; LCR cloned 5′ to the OVA (3.5 kb); H1, human H1 polIII promoter; U6, mouse U6 polIII promoter.

1a; miR24, ScFv-Fcmini-antibody; GFP, green fluorescent protein; hIFN-1α, human interferon-1α; lacZ, bacterial lacZ; hGM-CSF, human granulocyte macrophage colony stimulating factor; shRNA, short hairpin RNA molecule.

Physiol Genomics 33% transgenesis. Did not provide the specific no. of embryos transduced with each distinct vector (lacZ and GFP). Only 6 of 19 founders were examined, and 3 of the 6 examined were found to be transgenic.

d Infection was performed using a range of multiplicities of infections (MOI) between 8 and 2,000. The low dose (MOI 8 – 80) only produced 1 transgenic mouse of 8 live births (12.5%), decreasing the overall transgenic. i Only 2 of 3 mice were shown (the 3rd mouse was assumed to be transgenic and GFP positive). j Gene expression was noted by Northern blot or ELISA in some of the animals * Not all animals have been shown to be very high (generally >70%) in all of the lentiviral vectors, and others have been shown to be less than 1.45% compared with other strains, such as crossed F1 C57BL/6 hybrids to SJL or C3H (3–5%) (126). For this reason, the pronuclear approach is limited when attempting to isolate transgenic animal lines back-crossed onto specific genetic backgrounds, which are difficult to modify by this approach. This difficulty often leads to a lengthy breeding strategy to generate an acceptable expressing transgenic line. These latter issues as well as the need to obtain special training and equipment to perform the technically challenging pronuclear injections have led researchers to explore the use of viral systems as an alternative system to manipulate pluripotent cells to generate higher transgenic efficiencies.

Other than a proof-of-concept study using wild-type Moloney leukemia retroviruses demonstrating chromosomal integration following the pronuclear injection method (123), there has been little interest in the utilization of this method with replication-defective retroviral and lentiviral vectors because of the availability of simpler systems for viral transduction as discussed below.

**Viral vector-mediated gene transfer.** The present methods, using both retroviral and lentiviral vector-mediated gene transfer, have been to either 1) incubate fertilized cells minus their zona pellucida or early embryos with the vector system or 2) directly inject the vectors into the perivitelline space or cytoplasm of zygotic cells. Details regarding the efficiency of transgenic animal production using these viral vector systems as well as the ability to promote long-term and, possibly, organ-specific expression in the context of lentiviral integration are discussed below.

**Retroviruses.** Many of the pioneering retrovirus studies were performed by Jaenisch and colleagues in the 1970s and early 1980s (54–57). These investigators incubated zona pellucida-digested fertilized mouse eggs or early-stage embryos with wild-type MLV to demonstrate the proof of concept that retroviruses could readily integrate and pass through the germ lines allowing for their propagation into subsequent generations. However, the resulting progeny demonstrated genetic mosaicism and viral silencing (57). Nearly three decades have passed, and the development and use of replication-defective retroviral vectors have been tested in cows (14), pigs (11), and monkeys (13), but the problems related to transgene silencing have persisted even though transgenic efficiency is higher than for pronuclear injections with DNA. To circumvent the problems associated with the simple retrovirus vectors derived from MLV, more complex retroviral vectors, also known as lentiviral vectors, designed from various strains of immunodeficiency virus from humans and horses have been examined.

**Lentiviruses.** UBQUITOUS CELLULAR AND VIRAL PROMOTERS. Lois et al. (74) and Pfeifer et al. (105) opened the door for the use of replication-defective lentiviral vectors for transgenic applications (see Table 2 for a list of studies using lentiviral transgenesis). Lois et al. performed a proof-of-concept study in mice and rats showing that the injection of YSV-G-pseudotyped lentiviral vectors into the perivitelline space of fertilized oocytes could significantly increase the production efficiency of transgenic mice. Pfeifer et al. (105) also demonstrated that lentiviral vectors can be used to efficiently manipulate zona-free embryos to produce founder transgenic mice. The embryo viability following lentivector transduction has been shown to be very high (generally >70%) in all of the
species studied, including mice (74, 107, 110), rats (74), pigs (137), and cows (48).

Confirming studies in mice (52, 107) and rats (81, 131) reported similar transgenic efficiencies (68–73% in mice and 22–46% in rats). However, strain-specific differences in the transgenic efficiencies have been noted between inbred Lewis (33%) (131) and Dahl SS (22%) (81) vs. outbred Sprague-Dawley (53–59%) rats (74, 131). Subsequent breeding of the founder mice (52, 141) and rats (81, 131) resulted in 56–74% and 28–50%, respectively, of the F1 population being transgenic with >93% of the F1 mice and rats continuing to express green fluorescent protein (GFP) (52, 110, 131). To produce organ-specific expression following lentiviral transgenesis using ubiquitous promoters, Okada et al. (95) manipulated zona pellucida-denuded embryos at the blastocyst stage to generate transgenic animals that expressed GFP exclusively in the placenta. The manipulation of these germ cells at this stage in development was found to reduce the embryonic lethality associated with placental defects following delivery of the lentiviral vectors overexpressing Ets2, Mapk1, Mapk11, and Mapk14 (95). These studies showed that lentiviral vector administration at different stages in embryo development could affect the site of expression following mammalian differentiation even with the use of a promoter that is normally capable of ubiquitous activity.

Studies in larger animals, including pigs (48, 137) and cows (30, 34, 49), have also shown a very high level of efficiency of transgene integration in founder animals using lentiviral vectors. Pigs and cows have exhibited transgenic rates of 70–93% (48, 137) and 100% (49), respectively. A high rate of transgene expression was also observed in the founder pigs (64%) (47) and cows (100%) (49). Unlike the studies in mice, rats, and pigs using microinjection of the viral constructs in fertilized eggs, unfertilized bovine oocytes were used because of previous work by Hofmann et al. (48) demonstrating higher yields of GFP-expressing blastocysts after injection. In contrast, birds have been reported to exhibit a variable rate of transgenesis during founder production, from 4 to 27% (15, 73, 80, 117). The complexities of handling the embryos during subgerminal injection followed by the culturing of the manipulated embryos in a surrogate egg shell until hatching may play a role in the poor efficiencies in birds independently of the problems associated with transduction using lentiviral vectors. Even with the low rate of transgenesis in birds (Table 2), the germline transmission from the F0 population was observed to propagate the integrated lentiviral vector into subsequent generations at rates up to 45% (15, 73, 80, 117).

As shown in Table 2, various cellular and viral promoters have been examined to assess transgene expression. Presently, five different ubiquitous promoters have been studied, including human ubiquitin C (UbC), cytomegalovirus (CMV), chicken β-actin with a CMV enhancer sequence (CAG), phosphoglycerokinase (PGK), and the elongation factor–1α (EF). The UbC and PGK promoters have been reported to drive the expression of reporter molecules in transgenic rats at >69% in F0 rats (74, 131), mice (74, 141), pigs (47, 48), and cattle (49) as well as >92% in F1 mice (110). Both the UbC and PGK promoters are generally considered to drive only weak transgene expression in the context of lentiviral vectors. More robust transgene expression can be achieved using the viral CMV and cellular CAG and EF promoters, and lentiviral vectors containing these promoters have been used to generate transgenic animals at high efficiency in F0 pigs (>94%) (137) and mice (100%) (95, 107) and at 50% in F1 mice (52).

These studies using the lentiviral vector containing ubiquitous promoter elements have shown an extremely efficient ability to modify early progenitor cells to produce transgenic animals with the capability to regulate gene expression on a persistent basis, in some cases for several generations. One species that could not generate transgenic founders using lentiviral vectors has been rhesus macaques (139). However, the reasons for this remain to be determined. It may be due to differences in the timing of the vector injection, or the vector sequences were not compatible with efficient integration into the rhesus macaque genome using a human lentiviral vector system (66). Further work is still needed to maximize transduction efficiency depending on the species being manipulated, and it may require the testing of alternative lentiviral vectors from different species, such as simian immunodeficiency virus (64, 89), to tailor specific strains for certain animals.

TISSUE-SPECIFIC PROMOTORS. The lentiviral vector system would have far greater utility if cell- and tissue-specific expression could be achieved in the transgenic animals. To address this issue, tissue-specific promoters were cloned into the lentiviral vectors to restrict transgene expression in the transgenic animals. Transgenic founders were produced at high efficiencies in mice (>70%) (52, 74) and rats (56%) (90), similar to the rates seen in transgenic animals generated using ubiquitous promoters. Lower efficiencies (13%) were found in F0 pigs (48) using the basal keratinocyte-specific K14 promoter, but both of the founder transgenic pigs produced specific expression of GFP in various layers of the skin epithelium. In mice, Lois et al. (74) found that mice carrying the lentiviral integrants with myogenin promoter have an expression profile consistent with the published findings on the distribution of myogenin (16), specifically the skeletal muscles in the tongue, jaw, chest, and limbs but not the cardiac or smooth muscle, or other non-muscle-derived tissues. Similar tissue-specific expression was found in the thymus using the T lymphocyte proximal lck promoter (74).

Other studies have observed persistent neuron-specific expression of GFP using the synapsin-1 (SYN) promoter from F0 and F1 transgenic rats (90) and F1 quails (117). However, there was ectopic expression of the transgene in the testes using the SYN promoter (90) as well as variable gene expression in different layers of the quail brain (117). In the study by Scott and Lois (117), silencing mechanisms could not be excluded as the reason for differential gene expression patterns in the neurons. Similar non-tissue-specific expression was noted by Ikawa et al. (52) in which retinal-specific expression of GFP was observed using red pigment (RG) and rhodopsin (Rho) promoters, but the corneal epithelial-specific promoter, K12, was found to have expression in the eye but also in other tissues, including the brain, skin, heart, and kidney.

Larger promoter (2.8–3.5 kb) regions from the ovalbumin (OVA) gene were used to study the expression of human IFNβ1a and miR24 (73) in chickens to determine whether cis-acting DNA elements could minimize the epigenetic effects. These transgene products were found to be exclusively expressed in the oviduct as determined by Northern blot analysis, but the level of expression was highly variable in the F1 chickens, likely because of position effects of the proviral
The importance of the lentiviral vector system has been extensively analyzed, similar to that found in F0 transgenic rats overexpressing their genetic background, as determined by Southern blot analysis. In one group, transgenic mice overexpressing CD8 shRNA led to a significant reduction in fluorescence intensity in double-transgenic (GFP+/GFP shRNA−) founder transgenic mice with various shRNA constructs. This was in contrast to control mice, but the loss in the number of mature T cells was not directly correlated with the decreased level of gene expression (111, 112), likely because of a position effect of the proviral integrants or other epigenetic modifications.

RNA interference. Transgenic approaches have largely involved the overexpression of various transgenes, but the advent of RNA interference in mammals has allowed for the production of transgenic animals with the ability to decrease expression of specific genes (42, 115, 121). Recently, short hairpin RNA (shRNA) molecules have been used to knock down gene expression using lentiviral transgenesis, with low-to-moderate transgenic efficiencies (13–53%) observed in founder mice and rats harboring shRNA constructs (21, 63, 75, 111, 112, 128). Tiscornia et al. (128) used transgenic GFP mice to demonstrate that shRNA targeted against the GFP gene would significantly reduce fluorescence intensity in double-transgenic (GFP+/GFP shRNA−) founder and progeny F1 mice.

Because of the ease and efficiency of lentiviral transgenesis, more sophisticated studies using RNA interference (RNAi) knockdown technology have been pursued. Rubinson et al. (111, 112) were able to generate 23–50% founder transgenic mice with various shRNA constructs in the lentiviral vector. In one group, transgenic mice overexpressing CD8 shRNA led to tremendous silencing of CD8 expression by 87–94% compared with control mice, but the loss in the number of mature CD8+ T cells (20–100%) did not directly correlate with the decreased level of gene expression (111, 112), likely because of a position effect of the proviral integrants or other epigenetic modifications. Subsequent studies by Kissler et al. (63) demonstrated that RNAi silencing of Scl11a1, which downregulates Nramp1 protein, reduced the frequency of type 1 diabetes mellitus following the genetic modification of nonobese diabetic (NOD) zygotes. The NOD is a mouse strain that is notoriously difficult to manipulate using standard pronuclear injections. Lu et al. (75) discovered that Ryk was a novel biological receptor for Wnt in vivo by chronic knockdown of Ryk using shRNA expressed by lentiviral vectors. In this study, 38% of the mice were transgenic, but there was mosaicism in their genetic background, as determined by Southern blot analyses, similar to that found in F0 transgenic rats overexpressing shRNA to Dazl (21). This further breeding was necessary to segregate the integrants to obtain progeny with only a single copy of the lentiviral vectors (75).

Overall, lentiviral vectors with various types of expression cassettes have demonstrated extreme flexibility to generate small and large transgenic animals with relatively high efficiencies. These studies have documented that the lentiviral vector system can regulate gene expression using over- and underexpression constructs, even in animal species and strains that were previously found to be relatively resistant to genetic manipulation using standard pronuclear DNA injection methods (63, 107). Large numbers of founder animals using lentiviral transgenesis can be generated without the need to generate individual transgenic lines in a time-consuming and laborious process. This would provide investigators the ability to expedite their phenotypic analyses of lentiviral vector-generated transgenic animals within a short (<7 wk) period of time (119). The importance of the lentiviral vector system has been clearly demonstrated by numerous investigators over the past few years, but several problems associated with this approach still need further study and will be discussed below (see issues still to consider regarding transgenesis using lentiviral vectors).

Nuclear transfer using genetically modified embryonic or somatic donor cells. This approach involves the removal of the nucleus from mature oocytes and transfer of genetically manipulated embryonic or somatic cells under the zona pellucida. An electrical pulse is applied, resulting in the fusion of the two cell membranes and release of the donor nucleus into the oocyte. It is important to note that the donor embryonic or somatic cells are generally transfected with a plasmid DNA construct or transduced with an integrating viral vector system to express a selectable marker used to isolate a clonal population of transgenic cells. Since lentiviral vectors are extremely efficient in transducing cells in vitro, the amount of time needed to select for transgenic cells would be reduced, as shown by Golding et al. (34) in goats and Hofmann et al. (49) in cows. In both of these studies, however, only one transgenic birth was obtained, and it appears that the limiting step remains the physical transfer of the nucleus into recipient cells, which is 1–3% efficient in the generation of transgenic animals for most species (122).

Even though this method is about as efficient as pronuclear injection (94), there are a number of advantages in this approach vs. the classic pronuclear injections. First, the sex as well as the genetic background of the animal can be predetermined by choosing the appropriate genetic material. Second, this approach can bypass problems associated with mosaicism. Third, the donor embryonic stem or somatic cells can be manipulated in cell culture to control the level of specific gene expression and/or delete the function of a particular gene. Subsequently, these donor cells can be selected, propagated, and then stored for future transgenic applications. These benefits are offset by the major drawback associated with this approach, which is the major loss of embryos during pregnancy and a higher rate of neonatal deaths (29, 138). In the end, lentiviral vectors may only play a role in mediating the genetic modification of the donor nuclei before the injection and generation of transgenic animals.

Male-Derived Cells

In recent years, there has been an interest in investigating whether male cells, i.e., spermatozoa and spermatogenic stem cells, can be used to direct the transfer of exogenous DNA into female cells to generate transgenic animals. Alternative cells that have been genetically modified to produce transgenic animals from mice and other species are described below.

Sperm cell-mediated transgenesis. It has been found that exogenous DNA can be bound and taken up by sperm cells, allowing them to transfer their own as well as the internalized foreign DNA to unfertilized oocytes. The initial proof-of-concept study was performed by Brackett et al. (6), in which they transfected rabbit spermatozoa with SV40 DNA and used it to inseminate unfertilized ova in the female uterus. Nearly two decades later, Lavitrano et al. (70) extended this approach by producing transgenic mice using spermatozoa transfected with plasmid DNA. At that time, this was a major advance in the field, since this was more efficient (2- to 5-fold) than the...
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manipulation of female-derived cells. These findings were controversial (8), but there are now many studies demonstrating the production of transgenic animals from other species (120). Replacement of the live spermatozoa with membrane-damaged or “dead” unfertilized sperm cells could produce increased transgenic efficiencies (103). Other studies have shown that intracytoplasmic sperm injections of DNA, even with large DNA fragments between 0.25 and 0.5 Mb in YAC (85, 86), at the metaphase II stage can produce transgenic animals at high efficiencies (35%) compared with the conventional pronuclear microinjection method (<1% efficiency) (33).

In terms of viruses, high transgenic efficiency was observed by De Miguel and Donovan (22) using retroviral vectors in sperm, but it was difficult to determine whether their efficiency was any better than plasmid DNA, which was not compared. No studies have examined the potential effectiveness of the use of lentiviral vectors to modify sperm cells. Conceivably, if retroviral vectors prove to be superior to the use of naked DNA in sperm cells, then it is likely that lentiviral vectors would be even more effective and provide another step forward in transgenic efficiency using this approach. In the end, this method still requires refinement to isolate sperm cells as well as to optimize the conditions needed to maximize efficiency of the transduction of these particular cells using lentiviral vectors.

Spermatogonial or germine stem cells. An alternative method to manipulating spermatozoa has been to isolate and genetically modify spermatogonial stem cells (SSC) in vitro using plasmid DNA (58) and retroviral vectors (87). Subsequently, these cells are implanted into the seminiferous tubules of recipient male testes to produce transgenic spermatozoa. Studies by Nagano et al. (87) demonstrated that MLV-based vectors could transduce murine SSC, leading to the generation of transgenic offspring, of which only 4.5% of the pups inherited and expressed the marker gene.

To circumvent some of the problems associated with MLV vectors, recent studies have examined the use of lentiviral vectors as a vector to manipulate SSC from mice (88) and rats (41, 114). Nagano et al. (88) found that first-generation lentiviral vectors pseudotyped with VSV-G resulted in 30–50% higher transduction efficiency into mouse SSC compared with MLV-based retroviral vectors. The transplantation of the lentiviral vector-modified SSC derived from mouse pups resulted in the establishment of spermatogonial colonies in 100% of the recipient mouse testes. Unfortunately, the transmission of the lentiviral vector gene sequences was not assessed in subsequent mouse generations in this study. On the other hand, SSC from rats were manipulated by Hamra et al. (41), who found that 59% of the progeny were transgenic and derived from the cultured donor cells, and 50% (13 of 26) of those pups were transgenic with the lentiviral vector. In a more recent study, Ryu et al. (114) found lower transgenic efficiencies using lacZ (4.2%)- and GFP (6.3%)-expressing lentiviral vectors in the testes of recipient Sprague-Dawley rats than in the mouse studies. However, they observed that GFP was expressed in the subsequent progeny for at least three generations without silencing. It is important to note that the use of certain promoters following transduction into the SSC may play a pivotal role in determining whether a successful transgenic outcome is achieved. Transgenesis using the CMV promoter was not observed to induce expression of the reporter gene in mice (88). Similar results were seen in a study using the CMV promoter in rats [<8% of the animals expressed the transgene in F1 rats (41)]. Swapping of the promoters to EF resulted in 100% (31 of 31) of the transgenic founder mice transplanted with the lentiviral vector-transduced SSC continuing to express β-galactosidase protein (88).

Overall, these are encouraging results compared with earlier experiences using MLV vectors (87) or pronuclear DNA injections (46), but the use of SSC appears far less efficient in male progenitor cells than with female germ cells. However, it remains an important and simple approach to develop transgenic animals having specific gene-targeted deletions, and improvements in manipulating and engrafting SSC into recipient testis are continuing to evolve.

ISSUES STILL TO CONSIDER REGARDING TRANSGENESIS USING LENTIVIRAL VECTORS

It is clear that lentiviral vectors have a strong future for transgenesis in various small to large animal species, but there are several lingering issues that require further investigation. Some of these problems cannot be solely attributed to the lentiviral vector system but to the field of transgenics in general.

Genetic Mosaicism: Effect of Lentiviral Integrant Copy Number

Several studies using mice (21, 51, 74), rats (131), and birds (117) have indicated that genetic mosaicism occurs using viral constructs, even though zygotes were used at the single cell stage. It is somewhat surprising to find mosaic founders with the use of fertilized single cell zygotes in mice (74) and rats (21, 131), but there are two potential scenarios that could lead to the creation of transgenic animals with a different genetic makeup: 1) the lentiviral vector integration process is not complete before the initial cell division, and 2) there may be prolonged persistence of the lentiviral preintegration complex.

To reduce the time frame for vector administration into the perivitelline space to nuclear translocation for genomic integration, the vector must undergo a complicated series of steps. The pseudotyped lentiviral vector must 1) bind to its receptor and 2) become internalized, 3) uncoated, and 4) reverse transcribed, and 5) the preintegration complex must be translocated into the nucleus and 6) integrated into the genome. Although wild-type lentiviruses take 1–2 h to integrate (44), VSV-G-pseudotyped lentiviral vectors have been shown to require at least 12 h for initial signs of integration, and most of the integration can take up to 48 h or longer (132). The elimination of several steps before the reverse transcription step could help to accelerate the integration following administration to the cell. To address this possibility, Yang et al. (141) produced an envelope-free lentiviral vector system for direct injection into the cytoplasmic space of the zygote, which would be able to circumvent many of the steps before the uncoating of the vector particle. This enabled these investigators to produce transgenic mice at an extremely high efficiency rate (97%) compared with the standard injection into the perivitelline space using the envelope-free (2.2%) or the VSV-G-pseudotyped lentiviral vector (46%). Southern blot analyses were not applied in this study, so it is not known whether this
approach resulted in less mosaicism. There was, however, a major drawback with this approach, since it had an adverse effect on the development of the modified embryos. These effects appeared to be due to the toxicity associated with the injection of a crude vector preparation rather than the invasiveness of the injection (141). Contaminating elements in the vector preparation could readily be eliminated by purifying the concentrated lentiviral vectors before injection (118, 129).

Another cause of genetic mosaicism could be the relatively long half-life of the lentiviral vector, which has been shown to be in the range of 8–9 h in vitro (144). There was an earlier study by Park et al. (100) that showed increased transduction into mouse hepatocytes following a partial hepatectomy 7 days after vector administration in vivo. This study suggests the possibility that lentiviral preintegration complexes could persist for several rounds or more of cell division before integration, resulting in genetic mosaicism. More information is needed to better understand the time frame of the survival of infectious lentiviral vector particles following introduction in the perivitelline space or cytoplasm of cell embryo to better control the factors contributing to variable integration into the transgenic animals. Since not all studies found this type of variable patterning, it would be interesting to know whether intrinsic vector-related sequences can be used to eliminate mosaicism by enhancing or destabilizing the preintegration complexes.

In general, the number of integrants found in lentiviral vector-produced transgenic animals has ranged between 1 and 31 copies in various studies. Unlike the concatamers found using plasmid DNA, only individual integration events have been reported using lentiviral vectors. The multiple proviral integrants observed in the founder animals have been segregated to produce transgenic mice (51) and rats (131) with only individual copies. Further studies of these animals have demonstrated that the individual copies of integrated lentiviral vectors are sufficient to elicit transgene expression and eliminate the variable gene expression found in the F0 animals (131). The issue of genetic mosaicism remains a potential problem in transgenic animal production, and further investigations are needed to determine the factors mediating this process.

Variability of Transgene Expression: Role of Epigenetic Modifications

Regardless of the promoter-transgene combination, mosaic gene expression within distinct regions of specific organs or specific populations of cells in mice (63, 107, 124) and birds (80, 117) has been observed. Kissler et al. (63) found that GFP expression was cell type dependent, where 34% of the CD4(+) T cells were positive, whereas only 11% of B cells and 17.5% of granulocytes expressed GFP. Epigenetic silencing was associated with mosaic gene expression, since progeny F1 mice demonstrated similar variegation. Using the CMV promoter, Punzon et al. (107) detected variable protein secretion of human granulocyte macrophage colony in transgenic mice that was independent of copy number and may have been the result of position effects of the integrant, leading to the activation of promoter silencing mechanisms. Complete and irreversible shutdown of GFP expression was observed in transgenic mice containing a modified tet-inducible promoter system, and this was believed to be induced by specific DNA sequences known as the Kruppel-associated box (KRAB) (124). In this study, the epigenetic silencing only occurred in the first few days of embryo development in the absence of the inducer, doxycycline, which allowed for the binding of the recombinant tet-repressor fusion protein, tTRKRAB, onto its target site in the promoter region. In the presence of doxycycline during this critical period, the silencing phenomenon could be avoided, and reversible expression of GFP could be achieved (124). This study definitively shows that cis-acting DNA elements can play a major role in epigenetic silencing of the promoters and are important factors to consider when generating transgenic animals.

In birds, expression of GFP was found in only 10% of quail neurons using the synapsin promoter (117), and variable transgene expression was detected in transgenic founders and progeny chickens by McGrew et al. (80). These examples appear to contradict the previous belief that lentiviral vector-mediated expression is more resistant to epigenetic silencing, such as methylation (83, 106) or histone deacetylation (96, 97), than when using MLV-based counterparts (57, 123) or pronuclear injection of plasmid DNA (17, 67). From these findings, it would appear that the DNA sequences found within the lentiviral vector constructs are responsible for the variegated expression and transgene silencing following integration.

For this reason, lentiviral vectors in recent studies were designed with specific deletions in the 5′-LTR (99, 146) in a process known as “self-inactivation” (or SIN) to eliminate the negative promoter interference between the LTR and the internal promoters. MLV-based vectors were methylated in the promoter region of the LTR by a variety of sophisticated cellular mechanisms, which evolved to eliminate the biological effects of genomic intruders (57, 123). However, it is clear that other vector-related sequences within either the remnant LTR or the expression cassette (promoter-transgene combination) can initiate the mobilization of silencing complexes to shut down gene expression from the proviral integrant, similar to the MLV vectors (25). Most of the transgenic F0 animals studied to date have contained multiple integrants, which likely led to a desirable situation whereby some of the integrants were able to escape the silencing mechanisms and somewhat subvert the variability found in the transgene expression.

To address silencing issues in the context of transgenic animals, Hofmann et al. (47) bred progeny to segregate their proviral integrants and discovered that ~33% of the F1 progeny were affected by hypermethylation of CpG dinucleotides within the human PGK promoter region, leading to low-level gene expression. An additional finding was that the site of proviral integration could markedly affect the level of GFP expression (47), and that the effects on transgene expression may be associated with a phenomenon known as position effect variegation, in which integration within particular chromosomal regions results in altered transgene expression (60). Although HIV has been shown to integrate a small percentage (2%) within 1 kb of CpG islands compared with MLV vectors (17%), this may be enough to provide the signals to promote vector silencing (140). It may be possible to overcome these negative cis-acting effects by the surrounding chromatin through the use of insulators or matrix/scaffold attachment regions (MAR/SAR) (26, 27, 45, 125), which can protect the expression cassettes from epigenetic shutdown. However, the
insertion of insulators or MAR/SAR into the lentiviral vector, usually into the 3\'-LTR, may compromise vector titer and reduce the efficiency of transgenesis (see below).

Another factor involved in vector silencing could be the role of histone deacetylation, which was found to be an important mechanism in promoting silencing of lentiviral transgenes in mouse embryonic stem cells (76). Hofmann et al. (47) found that histone deacetylases, which were dependent on trichostatin A, did not play a major role in the maintenance of lentiviral vector silencing in their F1 transgenic pigs. However, recent findings by Wang et al. (136) indicate that histone function may play a critical role in determining where the lentivirus integrates into the genome.

There are still many issues to consider regarding vector silencing with respect to the activation and recruitment of silencing machinery, which may be dependent on the inserted transgene as shown by Dalle et al. (20), who found that GFP, which has 60 CpG islands, potentiated the silencing of the β-globin gene. Elimination of the CpG islands by codon optimization in a GFP variant (dmGFP) was found to have markedly lower fluorescence intensity, by 2- to 14-fold, compared with wild-type GFP. However, none of the transduced cells in vitro or transgenic mice expressing the variant dmGFP was found to be silenced, unlike the integrants expressing the wild-type GFP. Most of the lentiviral transgenesis studies that have been performed to date have used GFP as the transgene marker, so much of the silencing found in these studies may need to be reexamined using codon-optimized transgenes without CpG islands or other recognition sites for silencing (20).

In the end, the selection of a specific internal promoter can be an important factor in the context of lentiviral transgenesis. Most of the cellular promoters discussed throughout this review have been able to maintain persistent transgene expression in either the founder or subsequent progeny for extended periods of time, although there were negative effects on silencing with the viral CMV promoter following transduction into either spermatogonia stem cells (41) in vitro or chicken eggs in vivo (80). The silencing mechanisms mediating the downregulation of the CMV promoter have not been fully elucidated, but the experimental results are consistent with previous gene therapy studies using this particular promoter in the liver (29, 36, 62), brain (79), and airway epithelia (142). Similar injection methods using the cellular PGK promoter in chickens (15) and cattle (49) were found to have ubiquitous expression in all tissues analyzed (15) with a constant level of GFP fluorescence (49). It is possible that the location of the proviral integration may have specific effects on particular promoter regions that would complicate the transgenesis approach using lentiviral vectors. There remains little doubt that further work on variable gene expression and silencing effects will be needed to validate the use of specific promoters, and that specific cellular promoters will likely prove to be superior over general viral promoters for lentiviral transgenesis.

**Packaging Size**

Empirically, early generation lentiviral vectors were able to produce functional vector particles even with a genome size of 16 kb (68). In reality, however, lentiviral vectors of this size may not be useful for biological applications, since the viral titers are dramatically reduced by up to 1,000-fold compared with lentiviral constructs of 5–7 kb. To calculate the cloning capacity, it is important to include the backbone of the lentiviral vector that is essential for the production of functional vector, which is generally ~1.6 kb (without WPRE) or ~2.2 kb (with the WPRE). From the previous studies by Kumar et al. (68), the maximal size of the lentiviral vectors was estimated to be ~13.5 kb.

As the vector genome sizes were increased to 7–9 kb, the vector titer was only reduced by 10–30% compared with the smaller vectors. In terms of transgenesis, bacterial lacZ-containing vectors (~6 kb), which are ~50% larger than GFP-containing vectors (~4 kb), were found to have an approximate twofold reduction in avian transgenesis (80), implicating a size-dependent effect on transgenic efficiency. However, rodent transgenesis using vector constructs similar to the lacZ vectors in the chicken study (80) had 45% transgenic efficiency in the founders, comparable (45%) with previous avian studies using smaller GFP-expressing lentiviral vectors with heterologous promoters. The differences in the transgenic efficiencies were likely attributable to differences in the vector stock titers (3-log orders between lacZ vs. GFP) or the use of an alternative non-primate-derived immunodeficiency viral vector system from horses (equine infectious anemia virus; EIAV) (80), in which the size of the expression cassette may have a greater effect on transgenesis compared with more widely used HIV-based vectors.

Expression cassettes (promoter-transgene) markedly larger than 4 kb would likely have reduced transgenesis rates using lentiviral vectors because of effects on vector integration, so studies are needed to determine the upper limit of the lentiviral vector packaging capacity. At the present time, however, the issue of packaging size may not be a major limitation, since promoter-transgene constructs <10 kb within the scientific community are fairly prevalent, so this approach should become a routine method in animal transgenesis.

**Vector Toxicity**

Early studies by Burns et al. (10) using VSV-G-pseudotyped retroviral vectors demonstrated fusogenic and other cell toxicity on cultured cells. It remains to be determined what optimal vector dose would promote efficient integration into pluripotent cells from different species; it may require the use of alternative viral glycoproteins that are less harmful to the cell (19, 59, 98, 108). The recent study by Yang et al. (141) demonstrating highly efficient transgenesis using an envelope-free lentiviral vector injected into the zygotic cytoplasm may circumvent some of the problems associated with the vector-related toxicity caused by the VSV-G envelope protein. However, further refinement of the technique to physically deliver the vector into the cytoplasm will be required to minimize the adverse effects on embryo development as noted by the authors using this approach (141).

**Lentiviral Vector Integration and Its Implication for Gene Therapy**

In light of recent gene therapy studies documenting leukomogenesis in young children treated with retroviral-manipulated hematopoietic stem cells (37, 38), the method of lentiviral transgenesis may have an alternate use as a preclinical model...
to screen lentiviral vector constructs for problems related to insertional mutagenesis.

Previously, Montini et al. (84) demonstrated that MLV-based retroviral vectors activated a dose-dependent acceleration of tumor onset, and clinical trials using MLV-based retroviral vectors found integration to occur within the LMO2 region, resulting in leukemogenesis (37, 38). However, there may be several mitigating factors that preclude replication-defective lentiviral vectors as a potential prooncogenic vehicle. First, lentiviral vectors, even at high loads, did not exacerbate tumorigenesis in vitro (84), and there have been no observations of oncogenesis in lentivector-treated animals in vivo. Second, high HIV-1 viral loads in humans have not been conclusively shown to promote tumorigenesis as a direct result of lentivector integration (53). These findings appear to support the safety of lentivector vectors derived from immunodeficiency viruses vs. their predecessor, Moloney leukemia retroviruses. As yet, there have been no reports of oncogenesis as a result of lentivector transgenesis in different animal species.

However, in utero and early neonatal administration of lentivector vectors derived from horses was found to potentiate tumor formation in mouse livers and lungs in vivo, although this was not the case with lentivector vectors derived from humans (127). This may suggest that the human lentivector vectors are safer than those of other species, but this still may not be a true indicator of their nononcogenic potential, since MLV vectors were only found to be leukemogenic after transplanting transduced hematopoietic stem cells into human infants (37, 38). Presently, it remains to be determined how differences in lentivector integration compared with the prooncogenic MLV can lead to the avoidance of insertional mutagenesis, even in the face of multiple integration events throughout the genome (Table 2).

It was initially believed that retroviral integration, including lentivirus, was completely random, but previous studies by Schroder et al. (116) in 2002 demonstrated integration of wild-type HIV-1 into actively transcribing genes and regional integration hotspots in which a small 2.4-kb area accounted for 1% of the integration sites using a cell culture system. This was subsequently validated by other groups (72, 82, 136, 140). Consistent with the wild-type HIV study, De Palma et al. (23) found that lentivector vectors based on HIV-1 integrated within transcriptional units, but these investigators found that the integrated lentivector vector had an attenuated ability to promote anomalous transcription because of the location of the proviral integrant, and even in the face of multiple integration events throughout the genome (Table 2).

The points should help to alleviate fears about using lentivector vectors based on HIV as a method for transgenesis, especially for animals that will be subsequently handled by animal care and laboratory staff and used in a variety of animal and human studies. There is heightened public awareness regarding the consumption and biomedical use of genetically modified products, and as such, fear and resistance could have an adverse impact on biological products obtained from animals generated using lentivector vectors. To alleviate biosafety concerns, clinical trials should elucidate the value of lentivector vectors as a powerful and safe tool and address any potential problems, such as vector mobilization and the formation of replication-competent lentivector vectors (2, 51, 65, 71, 78). These studies as well as future research can demonstrate the complete safety of the lentivector vectors for use as a method of animal transgenesis for biomedical studies and human consumption.

**Biosafety**

Because of the enormous amount of work in the past decade on the biology of lentivector systems, initial biosafety fears about the use and production of transgenic animals have been drastically reduced. It is clear, however, that unlike simple DNA constructs, viral vectors are more complex and could promote pathogenesis in humans, particularly those vectors predisposed to causing human disease, such as HIV. The split genome format of the lentivector systems presently being employed in these types of studies are replication defective and, as such, treated as a level 2 biohazard. Although the precautions needed with this vector system are more involved than for the use of plasmid DNA, contaminating vector can be eliminated from most surfaces by simple cleaning of the affected areas with mild detergents and/or bleach followed by an ethanol wash. To date, there is an extremely low probability of generating replication-competent lentiviruses using the lentivector system with three or more plasmids (28), which substantiates the safety of this system for users as well as animals.

**PERSPECTIVES**

Lentivector vectors have drawn significant interest from outside of the gene therapy field and have emerged as a promising new system for transgenic applications. It is becoming more
clear that the innate ability of lentiviral vectors to integrate into the host genome with high efficiency can be exploited by investigators to modify early pluripotent cells for transgenic animal production in various species, including those that have been experimentally difficult to manipulate using traditional pronuclear DNA injections, such as inbred strains of mice and rats as well as larger farm animals. The high efficiency in generating large numbers of founder transgenic animals, especially in mice and rats, would allow for more rapid screening of phenotypic changes without the need of laborious, time-consuming breeding protocols to isolate transgenic lines with defined integration sites following pronuclear injections. Even with the distinct advantages of using lentiviral vectors to create transgenic animals, several problems remain with regard to packaging limitations, transgene silencing, vector-related toxicity, and genetic mosaicism.

In consideration of the successes that have already been achieved using lentiviral vectors, it is clear that this system will continue to be improved to mitigate many of the present problems, and this system will emerge as an important technique for animal transgenesis. For now, the lentivector system in the field of transgenics remains a highly promising application, and this method could revolutionize animal transgenesis by providing investigators with an alternative method to genetically modify animals at a more expedient and cost-effective manner for biomedical as well as agricultural and pharmaceutical research.

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