Generation of tissue-specific transgenic birds with lentiviral vectors

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Transgenesis has proven to be one of the most powerful tools for modern biology (1). Genetic experiments using transgenic mice, fish, worms, and flies have revolutionized the study of developmental biology, neurobiology, and immunology, among other fields. Unfortunately, transgenic tools remain unavailable for many popular research species. In particular, useful transgenic birds have been difficult to produce, although numerous attempts have been made for >25 years (2).

One major obstacle to genetic manipulation in birds has been achieving reliable expression of the transgene. Foreign DNA can be efficiently introduced into avian genomes by infecting the early embryo with oncoretroviral vectors (3). A number of groups have successfully produced transgenic chickens using this general method (4, 5, 6). However, in transgenic birds produced by using oncoretroviral vectors, transgene mRNA and protein product are present at low or undetectable levels, possibly due to developmental silencing (6).

Another class of retroviruses, the lentiviruses, is not silenced during embryonic development. Transgenic mice and rats generated by using lentiviral vector show reliable transgene expression (7). Lentiviral vectors have also been used to generate transgenic pigs and cattle (8, 9), and, in principle, they should allow for the generation of transgenic birds. Indeed, promising results were obtained in chickens with the use of recombinant equine infectious anemia virus (EIAV), a type of lentivirus (10). Transgenic chickens were generated that expressed GFP in some tissues; however, the pattern of GFP expression was inconsistent with the expected activity of the viral promoter used. Recently, another study reported the use of HIV-1-based lentiviral vectors to generate transgenic chickens that ubiquitously express GFP under the control of the phosphoglycerol kinase (PGK) promoter (11). Interestingly, the frequency of germ-line transmission among chicken founders reported in this study was <1%, whereas the rate of transgenesis in mice is ∼80% (7). These reports demonstrate the potential strengths of lentiviral vector-based avian transgenesis. However, to be useful, both of the following should apply: a system for the production of transgenic birds should be efficient and gene expression should be predictable and reliably controlled by the regulatory sequences of the transgene.

Here, we describe the efficient generation of transgenic birds with neuron-specific expression using lentiviral vectors derived from HIV-1. Vectors derived from HIV-1 have been shown to allow faithful tissue-specific expression in transgenic mice (7). To test whether lentiviral vectors could be used to direct transgene expression specifically in neurons, we used the promoter sequence from the human synapsin I gene (Hsyn). Lentiviral vectors engineered to contain Hsyn driving GFP were introduced into Japanese quail embryos. This method produced mosaic founder quails that expressed GFP in neurons and transmitted the transgene to their progeny, which expressed high levels of GFP selectively in neurons. In transgenic animals, the axons and dendrites of developing neurons were easily detectable by fluorescence microscopy.

Our technique can be modified for use in other avian species and can be used to alter the expression of endogenous genes. Birds are important model organisms for many problems in biology but are not more widely used because effective methods for genetic manipulation in these animals do not exist. Lentiviral transgenesis is a versatile and powerful tool that will provide a molecular approach to studying biological questions in birds and will make possible new avenues for research in a group of popular research animals for which modern genetic techniques were previously unavailable.

Methods

Construction of Lentiviral Vectors. We have developed a vector for neuron-specific transgene expression based on FUGW, a self-inactivating lentiviral vector derived from the HIV-1 (7). We replaced the ubiquitin-C promoter region of FUGW with a regulatory sequence that lies −570 to −93 bp from the transcription start site of the Hsyn. The resulting construct is called HsynGW (Fig. 1). Recombinant HsynGW virus was prepared and stored as described (7). We titrated the virus on primary cultures from newborn rat cortex and confirmed that GFP expression in vitro was specific to neurons.

Production of Mosaic and Transgenic Quails. Freshly laid Japanese quail (Coturnix coturnix japonica) eggs were purchased from CBT Farms (Chesertown, MD) and arrived the next morning by express courier. Eggs were placed on their sides for 1 h before injection to allow the embryo to float to the top of the yolk. Before windowing, egg shells were disinfected with 70% ethanol. To gain access to the embryo, a 4-by-4-mm window was drilled at the top of the eggshell with a handheld rotary tool (Dremel, Mount Prospect, IL), and the shell membrane was removed with forceps. Viral vector solution of HsynGW (10^7 infectious particles per microliter) was loaded into a pulled glass capillary (Sutter Instruments, Novato, CA; o.d. = 1 mm, i.d. = 0.75 mm) with a tip that had been scored with a ceramic tile (Sutter) and...
broken flush to 20 μm o.d. Embryos were observed with a dissecting microscope at ×16 magnification, and 3 μl of vector solution was injected into the subgerminal cavity below the embryo with an oil hydraulic injection system (CellTram oil, Eppendorf). To allow visualization of the injection site, 5% phenol red in PBS was added to the viral solution. Injections were considered successful if the viral solution spread horizontally in a circle below the embryo and if the perimeter of the viral solution reached the borders of the area opaca (for a useful atlas of avian embryo anatomy, see Bellairs and Osmond, ref. 12). More than 90% of injections were successful according to these criteria. After a successful injection, eggs were sealed to prevent microbial contamination and fluid loss during incubation.

To seal the eggshell, a round glass coverslip was placed over the shell window and was attached to the egg with a biocompatible silicone elastomer (Kwik-Cast, WPI Instruments, Waltham, MA). Eggs were placed blunt end up into a forced air incubator (Brinsea, Titusville, FL), with a temperature of 38°C and 16444/100% humidity. Embryos were examined both with epifluorescent and confocal microscopes. To genotype hatchlings, we nicked the alar vein on the wing of 5-day-old animals and collected 70% glycerol, and examined under epifluorescence or with a confocal fluorescent microscope. To examine whether GFP expression, juvenile and adult quail were transcardially perfused with 3% paraformaldehyde in PBS. Brains were cut into 50-μm sections on a vibrating microtome. Sections were counterstained with Hoechst 33258 (Sigma–Aldrich; 1.2 μg/ml) for 5 min at room temperature, mounted in 50% glycerol, and examined under epifluorescence or with a confocal fluorescent microscope. To examine whether GFP expression was confined to neurons, we performed double immunocytochemistry by incubating sections overnight at 4°C with a Rabbit polyclonal anti-GFP antibody (Abcam, Cambridge, MA; dilution 1:1,000) and mouse monoclonal anti-NeuN (Chemicon; dilution 1:500). Both antibodies were diluted in a blocking solution containing 0.1% Triton X-100 and 10% normal goat serum. The next day, sections were washed with PBS three times for 1 h each and incubated with a fluorescein-conjugated anti-rabbit secondary antibody (Fl-1000, Vector Laboratories) and a Texas red-conjugated anti-mouse antibody (Ti-2000, Vector Laboratories) at room temperature for 1 h in blocking solution. Finally, sections were washed with PBS three times for 1 h each, mounted in 50% glycerol, and examined under epifluorescence or with a confocal fluorescent microscope.

Results
Production of Mosaic Quails and Germ-Line Transmission of the Transgene. Mosaic quails were produced by infecting the blastodiscs of unincubated eggs with concentrated HsynGW lentiviral vector. At this stage, the quail blastodisc is a thin sheet consisting roughly of 40,000 cells. We infected 80 embryos with this method, and, of these, 8 hatched and developed to adulthood. Because the vector particles are too large to diffuse throughout all layers of the blastodisc, this method infects only a percentage of the cells of the embryo. Therefore, it is expected that these founder quails will be mosaic for the presence of the transgene. Accordingly, we expected only a percentage of the somatic tissue of each quail to carry the transgene. To examine the expression of the transgene in mosaic founders, we observed tissue sections from two adult quails (>50 days old).

In mosaic animals, GFP expression was confined to the peripheral and central nervous system. In tissue sections from the brain, we could observe GFP expression in the cell bodies of neurons, axons, and dendrites. In particular, the dendritic fan and soma of Purkinje cells of the cerebellum and the axons of projection cells in the hippocampus were brightly fluorescent. The neurons of the forebrain and optic tectum were also well labeled. Although individual cell bodies were easily distinguished, the high density of labeled neurons made it difficult to identify the processes of individual neurons. As expected, only a percentage (~10%) of neurons in the mosaic founders were GFP-positive (data not shown).

To examine the transmission rate of the transgene to the progeny, we bred six adult founder (F0) mosaics to wild-type quails. The progeny of F0 mosaics were screened by Southern
Table 1. Germ-line transmission rates in mosaic and transgenic quails

<table>
<thead>
<tr>
<th>Generation/sex</th>
<th>No. of progeny examined</th>
<th>No. of progeny carrying transgene</th>
<th>No. of progeny expressing GFP</th>
<th>Germ-line transmission frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0/W</td>
<td>16</td>
<td>5</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>F0/M</td>
<td>47</td>
<td>8</td>
<td>7*</td>
<td>17</td>
</tr>
<tr>
<td>F0/S</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>F0/M</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F0/F</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>F1/M</td>
<td>17</td>
<td>10</td>
<td>10</td>
<td>59</td>
</tr>
<tr>
<td>F1/M</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>62</td>
</tr>
</tbody>
</table>

*In one transgenic embryo GFP expression was not observed.

Tissue specificity in both F1 and F2 generations was examined in whole-mount sections from day 6 embryos and in tissue sections from the brains of juvenile and adult quails. GFP-positive neurons were clearly visible throughout the brain, spinal cord, and peripheral nervous system of transgenic animals, but not in controls. Sections from the cerebral hemispheres, cerebellum, and optic tectum were processed for immunohistochemistry with antibodies against GFP and NeuN, a nuclear protein specific for many types of mature neurons (16). No GFP-positive cells were observed outside the central or peripheral nervous system. NeuN was observed in the cell nuclei and perinuclear cytoplasm of cells in the forebrain, cerebellum, and optic tectum. All NeuN-positive cells expressed GFP, and most GFP-positive cells were also labeled by NeuN (Fig. 5 A–C). Some cells, such as Purkinje neurons of the cerebellum, were GFP-positive, but not NeuN-positive (Fig. 5D). This result was expected because some cell types, including Purkinje neurons, are not well labeled by the NeuN antibody (16). However, wherever GFP-positive cells were not also labeled with NeuN, cell morphology clearly indicated that these GFP-labeled cells were neurons. The observation that GFP-expressing cells always had a neuronal morphology, were labeled with the neuN antibody, and were restricted to the nervous system demonstrates that transgene expression was specific to neurons.

Discussion

In this study, we have demonstrated that lentiviral vectors can be used to efficiently produce transgenic quails that express...
Methylation stimulates the formation of heterochromatin, which blocks the transcriptional activity of the region surrounding the integrated retrovirus, and ultimately results in low or undetectable levels of transgene expression. This effect had been clearly documented in mice (20), and there is evidence suggesting this effect may also occur in birds (6). (ii) Retroviral LTRs contain internal promoters and enhancers, which may interfere with the expression of the transgene in both oncoretroviral and lentiviral based vectors. The vector used in our study has been engineered to minimize the transcriptional activity of the LTRs (21), and it was shown to faithfully allow tissue-specific transgene expression without developmental silencing (7). Thus, recombinant HIV-derived lentiviral vectors are an effective vector to allow tissue-specific expression in both transgenic mammals and birds.

We chose quails as a bird model for transgenesis because of their widespread use in developmental studies and for a number of practical reasons (22). Quails are excellent breeders, require less space to house than chickens, and develop rapidly. Incubation lasts 18 days, and hatchlings become sexually mature after 7 weeks. Eggs are easy to obtain from farms by mail so it is not necessary to maintain a breeding colony solely to produce eggs for the generation of mosaics. Transgenics of other avian species would require more time and space to breed. Because the organization of the avian embryo is well conserved at the time of oviposition, we do not anticipate any major obstacles to the generation of transgenics in other species, as suggested by previous experiments in chickens (10, 11).

Transgenesis with lentiviral vectors will allow for the molecular dissection of physiological processes in birds with a level of precision unattainable with other methods. Lentiviral transgenesis can be used to interfere with normal gene expression in a
number of ways. (i) Genes of interest can be ectopically expressed to modify the development or function of cells. (ii) Dominant-negative constructs can be introduced to block normal gene function (23). (iii) Lentiviral vectors carrying short interfering RNAs (siRNAs) can down-regulate endogenous mRNAs and can be used against genes for which no dominant negative constructs are known (24). These powerful manipulations make lentiviral transgenesis a useful genetic tool to study complex biological processes in birds.

For our initial experiments we chose a marker that enables the visualization of individual neurons in the developing embryo. Using the Hyn promoter to drive expression of GFP in the neurons of quails, we were able to label individual cells beginning 60 h after incubation. By 72 h, GFP had diffused into and labeled the dendrites and axons of neurons in the forebrain and spinal cord. Because their neurons are well labeled and because of the early age at which GFP expression begins, these birds will be useful for in vivo imaging studies of neural development. Strains of transgenic mice with neuron-specific GFP expression have been valuable for in vivo studies of synaptogenesis and neuromuscular junction development (25). However, imaging mouse pups during embryonic development is difficult because the mother must be killed and offspring do not survive long outside the womb. The study of embryonic development is easier in birds than in mammals, because the avian embryo can be continuously viewed over hours and days both in the shell and in artificial culture systems. Current techniques for in vivo imaging in chick embryos require the injection of dyes or electroporation of plasmids (26), but these invasive techniques can disrupt normal development. Transgenic or mosaic birds could offer a powerful advantage in experiments where current methods of cell labeling cannot be used.

We anticipate that avian transgenesis would be particularly useful for the study of behavioral neurobiology. Experiments with transgenic, mutant, and knock-out mice have been extremely valuable in studying the molecular basis of instinctual behavior as well as learning and memory (27). However, for the study of many behaviors, avian species are the preferred model organisms. Avian species exhibit a wide range of well-studied behaviors, such as food hoarding (28), filial imprinting (29), sound localization (30), and vocal learning (31). Transgenesis with lentiviral vectors will allow for the precise molecular dissection of these behaviors.

In addition to its use in basic science research, avian transgenesis has potential commercial applications, specifically in the production of therapeutic proteins. Transgenic birds generated by using oncoretroviral vectors have been shown to express low levels of transgene in the egg whites of laid eggs (32). The chicken ovalbumin promoter has been suggested as a regulatory sequence for directing protein expression in egg whites (33). Using lentiviral vectors containing the ovalbumin promoter, transgenic chickens could be engineered to produce high levels of therapeutic protein in their egg whites, providing a high-yield source of biopharmaceuticals.

In summary, we have developed a method for the generation of transgenic animals with tissue-specific expression in a group of species for which genetic experiments were previously not feasible. Although birds have historically been useful for studying many important problems in development and neurobiology (34, 35), research in mice (and in other animals in which genetic and molecular experiments are possible) has dominated these fields. However, for many questions in biology, avian species remain the model organisms of choice. We anticipate that lentiviral transgenesis will greatly improve our ability to study these questions and will be an asset to the growing field of avian genetics (36).

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