Generation of tissue-specific transgenic birds with lentiviral vectors

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Communicated by Susumu Tonegawa, Massachusetts Institute of Technology, Cambridge, MA, September 27, 2005 (received for review January 12, 2005)

Birds are of great interest for a variety of research purposes, and effective methods for manipulating the avian genome would greatly accelerate progress in fields that rely on birds as model systems for biological research, such as developmental biology and behavioral neurobiology. Here, we describe a simple and effective method for producing transgenic birds. We used lentiviral vectors to produce transgenic quails that express GFP driven by the human synapsin gene I promoter. Expression of GFP was specific to neurons and consistent across multiple generations. Expression was sufficient to allow visualization of individual axons and dendrites of neurons *in vivo* by intrinsic GFP fluorescence. Tissue-specific transgene expression at high levels provides a powerful tool for biological research and opens new avenues for genetic manipulation in birds.

avian | genetics | lentivirus | synapsin | neuron

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 (Chestertown, 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

Conflict of interest statement: No conflicts declared.

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Abbreviation: Hsyn, human synapsin I gene.

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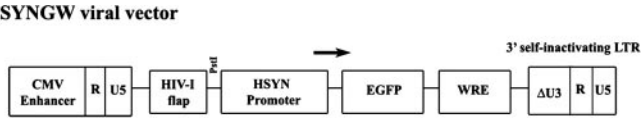

Fig. 1. Diagram of the relevant regions of the HsynGW vector used to generate mosaic quails. The 5′ CMV enhancer is used to express genomic viral RNA during the production of vector particles but is excluded from the integrated proviral DNA in the bird's genome. The position of the restriction site PstI used for Southern blot analysis of proviral integration is indicated. The arrow located on top of the HSyn promoter box marks the start site of transcription of EGFP. The woodchuck hepatitis virus posttranscriptional regulatory element (WRE) was included to increase the level of EGFP transcription. The U3 region of the viral 3′ LTR (ΔU3) contains a deletion that minimizes the endogenous transcriptional activity of the LTRs of the integrated provirus.

broken flush to 20 μm o.d. Embryos were observed with a dissecting microscope at $\times$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 a relative humidity of 45% until hatching, and were turned periodically. Eggs hatched after 18 days of incubation and were subsequently kept in a heated brooder. Three weeks later, founder mosaic quails were placed in a cage until they reached sexual maturity at ≈7 weeks, at which time they were mated to wild-type quails. Eggs from transgenic founders were collected, placed in the incubator, and examined at different stages of development. 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 μl of blood.

Analysis of Transgene Copy Number and Expression Profile. Genomic DNA from blood was extracted from whole blood samples by overnight digestion in proteinase K followed by phenol chloroform extraction (13). DNA was digested with the restriction enzyme PstI overnight at 37°C. PstI cuts once inside the integrated provirus between the 5′ viral LTR and the Hsyn promoter (Fig. 1). Southern blot hybridization was performed with a ^{32}P-labeled DNA probe against the GFP sequence.

To examine 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.

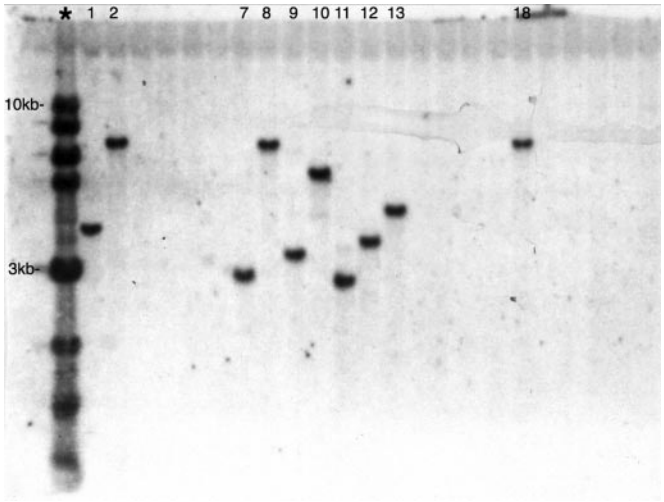

Fig. 2. Transgenic animals carry single copies of the HsynGW provirus. Southern blot analysis of genomic DNA from 23 progeny of Hsyn mosaic founders. *, Lane with molecular weight standards (1-kb ladder, New England Biolabs). All other lanes contain genomic DNA from individual F_1 quails. Genomic DNA extracted from quail blood or embryos was digested with the restriction enzyme PstI, which cuts once inside the lentiviral vector. The probe used hybridizes to a 500-bp section of GFP. Transgenic birds were found to carry only single copies of the integrated provirus as indicated by the presence of a single band per lane on the Southern blot (lanes 1, 2, 7–13, and 18).

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 (F_0) mosaics to wild-type quails. The progeny of F_0 mosaics were screened by Southern

Table 1. Germ-line transmission rates in mosaic and transgenic quails

Generation/sex	No. of progeny examined	No. of progeny carrying transgene	No. of progeny expressing GFP	Germ-line transmission frequency, %
F_0/M	16	5	5	31
F_0/M	47	8	7*	17
F_0/M	12	1	1	8
F_0/M	35	0	0	
F_0/F	12	4	4	33
F_0/F	4	1	1	25
F_1/M	17	10	10	59
F_1/M	8	5	5	62

M, male; F, female; F_0, mosaic founder; F_1, first generation transgenic.
*In one transgenic embryo GFP expression was not observed.

blot analysis to test for the presence of the transgene (Fig. 2). Germ-line transmission rate ranged between 8% and 33%, depending on the founder (results are summarized in Table 1). One founder mosaic did not produce any transgenic offspring. Of the 16 transgenic offspring found, each carried a single copy of the transgene (as assessed by Southern blotting).

Expression Pattern in F_1 and F_2 Transgenics. GFP expression was first visible in transgenic embryos after 60 h of incubation. At this time, GFP was visible in the soma of cells in the rostral spinal cord and forebrain. GFP expression increased steadily through early development. By 72 h of incubation, GFP fluorescence was present in the axon and dendrites of cells in the brain and spinal cord. After 4 days of incubation, individual neurons in the forebrain of could be identified (see Fig. 4 *A* and *B*). Confocal microscopy performed on the intact living embryo at this stage revealed the axons and cell bodies of single neurons of the forebrain (Fig. 4*B*). By embryonic day 6, the brain, spinal cord, and peripheral nervous system showed strong GFP expression (Fig. 3). The innervation of the limb buds was visible both in the intact live embryo (Fig. 3*A*) and in tissue sections (Fig. 3*D*). The eyes of the transgenic embryos were particularly well labeled with GFP (Fig. 4 *C* and *D*). GFP expression in the retina was visible through the pupil. In addition, axons innervating the iris and cornea could be imaged easily in the live embryo (Fig. 4 *C* and *D*). The observed temporal expression pattern of GFP in the transgenic quails is similar to that of the endogenous synapsin gene I in chicken embryos (14).

The level of GFP expression was comparable in 15 of 16 F_1 transgenics. However, one embryo that carried a transgene copy as assessed by Southern blot analysis did not display any detectable GFP expression. A positional effect may have been responsible for the lack of expression in this animal, because the site of transgene integration on a chromosome can exhibit strong effects on transgene transcription (15).

Two transgenic quails (F_1) carrying different insertions were allowed to develop to sexual maturity. These quails were mated with wild-type quails to determine whether GFP expression levels and tissue specificity were consistent across multiple generations. Progeny were screened by Southern blot analysis to test for the presence of the transgene, and by fluorescence microscopy to check for GFP expression. The transgene was transmitted to F_2 progeny at ratios that approached those expected from the Mendelian inheritance of individual alleles: 59% ($n = 17$) and 62% ($n = 8$) for each parent, respectively. Examination of embryonic, juvenile, and adult quails revealed comparable spatial and temporal GFP expression pattern in F_1 and F_2 transgenics.

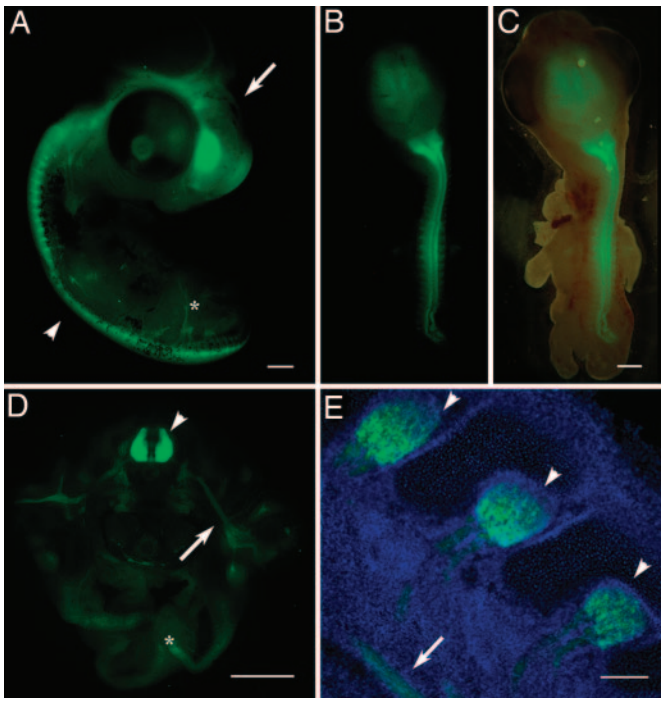

Fig. 3. GFP expression in an embryonic day 6 transgenic quail. (*A*) Profile view of GFP expression in an intact embryo. Arrow indicates head; arrowhead indicates spinal cord. GFP fluorescence in the retina can be seen through the pupil. At this stage, the sensory and motor innervation of the developing limb buds begins to be visible (*). (*B*) GFP fluorescence in the brain and spinal cord. (*C*) Merged view of fluorescence and bright field of the same embryo shown in *B*. (*D*) Cross-sectional view at the level of the forelimbs. Arrowhead indicates the spinal cord. Arrow indicates a bundle of motoneuron axons innervating the developing limb bud. The signal observed in the embryo's ventral surface (*) is due to autofluorescent signal originating from the skin. (*E*) Sagittal section of three spinal ganglia (arrowheads). A short section of the spinal cord labeled by GFP occupies the bottom left of the panel (arrow). Neurons are labeled by intrinsic GFP fluorescence (green), and nuclei of all cells are labeled by Hoechst 33258 (blue). (Scale bars: *A*, *C*, and *D*, 1 mm; *E*, 100 μm.)

Tissue specificity in both F_1 and F_2 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. 5*D*). 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

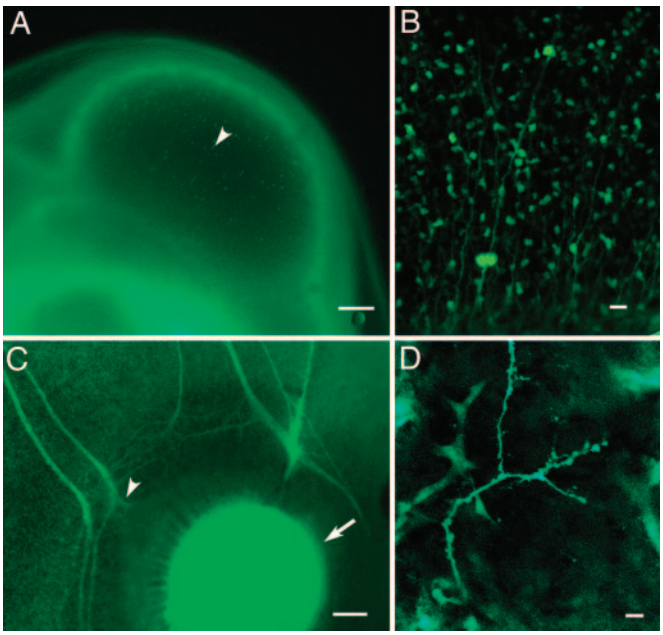

Fig. 4. Imaging of GFP-expressing neurons in live embryos. (*A*) GFP-expressing neurons in the brain of embryonic day 4 transgenic quail at low magnification. Arrowhead indicates region magnified in *B*. (*B*) Cell bodies and axons of GFP-labeled neurons in a live embryo, viewed by confocal microscopy. (*C*) Eye of live embryonic day 6 quail. Arrow indicates GFP-positive retina viewed through the pupil. Arrowhead indicates axons innervating the cornea and iris. (*D*) Individual axon innervating the cornea, viewed by confocal microscopy. (Scale bars: *A* and *C*, 250 μm; *B* and *D*, 10 μm.)

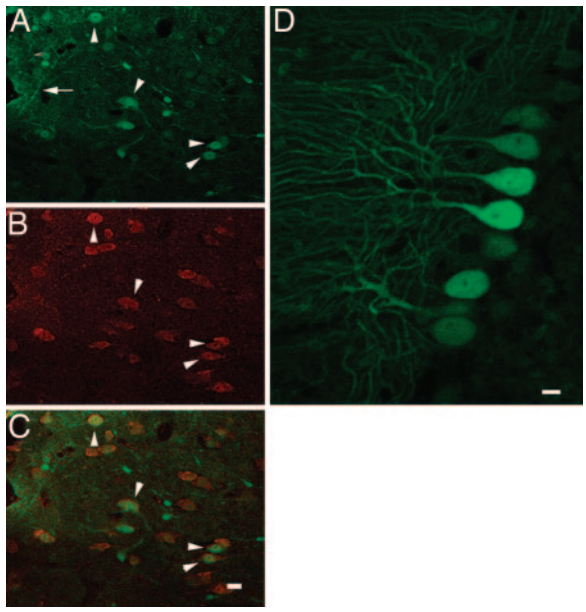

Fig. 5. Selective expression of GFP in neurons from HsynGFP transgenic quails. Shown is immunohistochemistry against GFP (*A*) and neuron-specific marker NeuN (*B*) in sections from the forebrain of transgenic quails. (*C*) A merged image of *A* and *B*. Immunohistochemistry was performed against GFP because intrinsic GFP fluorescence fades after fixation. Tissue sections were viewed with a confocal microscope. (*B*) NeuN staining was observed in the nuclei and perinuclear cytoplasm of neurons. Neuron processes were labeled only by GFP (see arrow in *A*). In most cases, GFP-positive cell bodies were also NeuN-positive (exemplary cells are marked by arrowheads in *A*, *B*, and *C*). Some cells with neuron morphology (i.e., bearing axons and dendritic arbors), such as the Purkinje cells of the cerebellum (*D*), were GFP-positive, but not NeuN-positive. This result was expected because previous works have shown that NeuN antibodies do not label all neurons. (Scale bar: 10 μm.)

GFP at high levels. Importantly, the transgene was expressed selectively in neurons, a pattern we expected based on the transcriptional activity of the synapsin promoter that we engineered into our vector. Achieving the reliable spatial and temporal expression pattern of a transgene is a critical step in the development of transgenic technologies in birds. Our vector contains two elements that we expect to contribute to the faithful expression of GFP in neurons: a highly conserved promoter and a recombinant viral backbone engineered not to interfere with transgene expression.

For our promoter, we chose a region of the transcriptional regulatory element of the Hsyn, an element known to produce neuron-specific expression *in vitro* (17, 18). Although the avian synapsin homolog has not been sequenced, the promoter region for the synapsin I gene is highly conserved in several mammalian species (19). In another study (11), the promoter region for phosphoglycerol kinase was used to direct ubiquitous expression in transgenic chickens. In contrast, previous studies with transgenic birds have often relied on viral promoters such as the SV40 promoter (4) and the CMV promoter (5). It is difficult to predict the expression pattern of these promoters because they show no similarity to any known endogenous promoter in birds. When a lentiviral vector carrying the CMV promoter was used to produce transgenic chickens, transgene expression was primarily in the pancreas and to a lesser extent in the liver, skin, muscle, and lining of the intestine (10). In addition, the level of transgene expression in these tissues was variable between different transgenic lines.

In addition to the promoter used, the viral backbone can also affect transgene expression. Viral sequences of retroviral-based vectors have been shown to affect gene expression in two ways. (*i*) Oncoretroviral elements and associated sequences are silenced during development by *de novo* methylation of cytosine residues. 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 Hysn 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).

B.B.S. thanks A. Stolfi for assistance with egg injections and H. Y. Chung and L. Smith for help with figures.

1. Jaenisch, R. (1988) *Science* **240,** 1468–1474.
2. Sang, H. (2004) *Mech. Dev.* **121,** 1179–1186.
3. Bosselman, R. A., Hsu, R. Y., Boggs, T., Hu, S., Bruszewski, J., Ou, S., Kozar, L., Martin, F., Green, C., Jacobsen, F., *et al.* (1989) *Science* **243,** 533–535.
4. Mozdziak, P. E., Borwornpinyo, S., McCoy, D. W. & Petitte, J. N. (2003) *Dev. Dyn.* **226,** 439–445.
5. Harvey, A. J., Speksnijder, G., Baugh, L. R., Morris, J. A. & Ivarie, R. (2002) *Poultry Sci.* **81,** 202–212.
6. Mizuarai, S., Ono, K., Yamaguchi, K., Nishijima, K., Kamihira, M. & Iijima, S. (2001) *Biochem. Biophys. Res. Commun.* **286,** 456–463.
7. Lois, C., Hong, E. J., Pease, S., Brown, E. J. & Baltimore, D. (2002) *Science* **295,** 868–872.
8. Hofmann, A., Kessler, B., Ewerling, S., Weppert, M., Vogg, B., Ludwig, H., Stojkovic, M., Boelhauve, M., Brem, G., Wolf, E. & Pfeifer, A. (2003) *EMBO Rep.* **4,** 1054–1060.
9. Hofmann, A., Zakhartchenko, V., Weppert, M., Sebald, H., Wenigerkind, H., Brem, G., Wolf, E. & Pfeifer, A. (2004) *Biol. Reprod.* **71,** 405–409.
10. McGrew, M. J., Sherman, A., Ellard, F. M., Lillico, S. G., Gilhooley, H. J., Kingsman, A. J., Mitrophanous, K. A. & Sang, H. (2004) *EMBO Rep.* **5,** 728–733.
11. Chapman, S. C., Lawson, A., Macarthur, W. C., Wiese, R. J., Loechel, R. H., Burgos-Trinidad, M., Wakefield, J. K., Ramabhadran, R., Mauch, T. J. & Schoenwolf, G. C. (2005) *Development* **132,** 935–940.
12. Bellairs, R. & Osmond, M. (1998) *The Atlas of Chick Development* (Academic, San Diego).
13. Sambrook, J. & Russel, D. W. (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Woodbury, NY).
14. Plateroti, M., Vignoli, A. L., Biagioni, S., Di Stasi, A. M., Petrucci, T. C. & Augusti-Tocco, G. (1994) *J. Neurosci. Res.* **39,** 535–544.
15. Kioussis, D. & Festenstein, R. (1997) *Curr. Opin. Genet. Dev.* **7,** 614–619.
16. Mullen, R. J., Buck, C. R. & Smith, A. M. (1992) *Development* **116,** 201–211.
17. Thiel, G., Greengard, P. & Sudhof, T. C. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3431–3435.
18. Sauerwald, A., Hoesche, C., Oschwald, R. & Kilimann, M. W. (1990) *J. Biol. Chem.* **265,** 14932–14937.
19. Schoch, S., Cibelli, G. & Thiel, G. (1996) *J. Biol. Chem.* **271,** 3317–3323.
20. Jahner, D., Stuhlmann, H., Stewart, C. L., Harbers, K., Lohler, J., Simon, I. & Jaenisch, R. (1982) *Nature* **298,** 623–628.
21. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. & Verma, I. M. (1998) *J. Virol.* **72,** 8150–8157.
22. Padgett, C. A. & Ivey, W. D. (1959) *Science* **129,** 267–268.
23. Herskowitz, I. (1987) *Nature* **329,** 219–222.
24. Tiscornia, G., Singer, O., Ikawa, M. & Verma, I. M. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 1844–1848.
25. Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., Nerbonne, J. M., Lichtman, J. W. & Sanes, J. R. (2000) *Neuron* **28,** 41–51.
26. Niell, C. M. & Smith, S. J. (2004) *Annu. Rev. Physiol.* **66,** 771–798.
27. Chen, C. & Tonegawa, S. (1997) *Annu. Rev. Neurosci.* **20,** 157–184.
28. Clayton, N. S. & Dickinson, A. (1998) *Nature* **395,** 272–274.
29. Horn, G. (2004) *Nat. Rev. Neurosci.* **5,** 108–120.
30. Konishi, M. (2003) *Annu. Rev. Neurosci.* **26,** 31–55.
31. Nottebohm, F. (1999) in *The Design of Animal Communication*, eds. Hauser, M. D. & Konishi, M. (MIT Press, Cambridge), pp. 63–110.
32. Harvey, A. J., Speksnijder, G., Baugh, L. R., Morris, J. A. & Ivarie, R. (2002) *Nat. Biotechnol.* **20,** 396–399.
33. Ivarie, R. (2003) *Trends Biotechnol.* **21,** 14–19.
34. Konishi, M., Emlen, S. T., Ricklefs, R. E. & Wingfield, J. C. (1989) *Science* **246,** 465–472.
35. Stern, C. D. (2005) *Dev. Cell* **8,** 9–17.
36. Brown, W. R., Hubbard, S. J., Tickle, C. & Wilson, S. A. (2003) *Nat. Rev. Genet.* **4,** 87–98.