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Efficient production of germline transgenic chickens using lentiviral vectors

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INTRODUCTION

Development of an efficient method for genetic modification of chickens has yet to be developed. An efficient technology, enabling production of transgenic birds at high frequency and with reliable expression of transgenes, will have many applications, both in basic research and in biotechnology. We investigated the efficiency with which lentiviral vectors could transduce the chicken germ line and examined the expression of introduced reporter transgenes. Ten founder cockerels transmitted the vector to between 4% and 45% of their offspring and stable transmission to the G2 generation was demonstrated. Analysis of expression of reporter gene constructs in several transgenic lines showed a conserved expression profile between individuals that was maintained after transmission through the germ line. These data demonstrate that lentiviral vectors can be used to generate transgenic lines with an efficiency in the order of 100-fold higher than any previously published method, with no detectable silencing of transgene expression between generations.

Keywords: lentiviral vector; genetic modification; chick

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RESULTS

Production of G0 transgenic birds

Three different self-inactivating EIAV vectors (Fig 1) were used, pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G). These vectors have previously been used to transduce a number of tissues in several animal model systems, both in vitro and in vivo (Rholl et al., 2002; Bienemann, 2003). The vector preparations were concentrated to give titres of approximately $10^7$–$10^{10}$ transducing units per millilitre (TU/ml). A 1–2 μl volume of

at low levels (Rapp et al., 2003), probably because of host silencing of the viral sequences. An improvement in the frequency of production of germline transgenic birds (to one in 15 males) has been shown using a spleen necrosis virus-based vector, although the germline transmission frequency was still low (0.9%; Mozdzioz et al., 2003). Several non-viral methods for genetic modification of the avian germ line have been described (Sang, 1994; Zajchowski & Etches, 2000), but so far the frequencies obtained are even lower than those obtained using retroviral vectors. The inefficiency of any of the available methods for production of transgenic birds inhibits exploitation of transgenic technologies in poultry. There are many possible applications, including expression of pharmaceutical proteins in eggs, modification of production traits for poultry breeding, and investigation of genes involved in vertebrate development, for which the chick is becoming an increasingly useful model (Brown et al., 2003).

A new group of vectors has been developed recently, derived from members of the lentivirus class of retroviruses. These have potential advantages over those derived from oncoretroviruses, including the ability to infect non-dividing cells (Naldini et al., 1996). More significantly, from the perspective of their use in the production of transgenic animals, transgenic mammals have been generated efficiently using human immunodeficiency virus-based vectors, and reliable tissue-specific expression of a reporter gene was seen after germline transmission (Lois et al., 2002; Pleifer et al., 2002; Hofmann et al., 2003). The possible advantages of a lentiviral vector system for genetic modification of animals that have proved recalcitrant to genetic manipulation prompted us to test the ability of equine infectious anaemia virus (EIAV) vectors to transduce the chicken germ line.
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concentrated vector was injected into the subgerminal cavity below the developing embryonic disc of newly laid eggs. These embryos consist of an estimated 60,000 cells, approximately 50 of which are thought to give rise to primordial germ cells (Karagenc et al., 1996). Preliminary analysis of transduced embryos, by staining for expression of the reporter gene lacZ, indicated a low level of transduction of somatic cells of the embryos (supplementary Fig 1 online). Injected embryos were cultured to hatch, and genomic DNA was extracted from the chorioallantoic membrane (CAM) of hatched G0 chicks and then analysed by PCR to detect vector sequence. The approximate copy number of the vector with respect to the amount of genomic DNA present was estimated (see Methods). All chicks were raised to sexual maturity, and genomic DNA from semen samples was similarly screened by PCR.

To determine the efficiency with which the EIAV vectors could transduce chick embryo cells, four experiments were carried out (Table 1). A total of 73 eggs were injected in the four experiments from which 20 (27%) chicks hatched. The results of the PCR screen of hatched male and female chicks from each experiment are shown in Table 1. A total of 14 out of 20 G0 birds contained vector sequences at levels estimated to be between 0.5 and 0.01 copies per genome equivalent. The vector pONY8.0cZ transduced the chick embryos more efficiently than the vector pONY8.4GCZ when injected at a similar concentration, possibly because of the presence of the viral cPPT sequence, which is involved in nuclear import of the reverse transcribed viral genome. The results also show that transgenic birds can be produced using titres as low as 10^7 TU/ml, but that transduction frequency increases if higher titres are used.

**Germline transmission from G0 males**

Semen samples were collected from the 12 G0 males when they reached sexual maturity. The results of PCR screens of genomic DNA extracted from these samples are given in Table 1. These showed that vector sequences were present in the germ line of each cockerel, even in those that had been scored as not transgenic when screened at hatching. This was confirmed by breeding from 10 of the 12 cockerels by crossing to stock hens and screening their G1 offspring to identify transgenic birds. All ten cockerels produced transgenic offspring, with frequencies ranging from 4% to 45%. The results show efficient production of transgenic birds and suggest a germline transduction frequency approximately tenfold higher than that of somatic tissues.

**Analysis of G1 transgenic birds and transmission to G2**

We predicted that the G1 birds resulted from separate transduction events of individual primordial germ cells and that different birds would have independent provirus insertions, representing transduction of single germ cell precursors. Four G0 cockerels, transduced with pONY8.0cZ (experiments 1 and 2), were selected for further analysis of their transgenic offspring (Table 2). Genomic DNA from individual G1 birds was analysed by Southern blot. Samples were digested separately with XbaI and BstEII, which are restriction enzymes that cut within the integrated EIAV provirus but outside the probe region (Fig 1). They were then hybridized with probes to identify restriction fragments that would represent the junctions between the proviral insertions, and the genomic DNA at integration sites. The number of proviral insertions in each G1 bird and the number of different insertions present in the offspring of each G0 were analysed. An example of this analysis is shown in Fig 2A,B and the results are summarized in Table 2. The majority of G1 birds carried single proviral insertions but several contained multiple copies, with a maximum of four detected in one bird. Some offspring of each G0 bird carried the same proviral insertion, indicating that they were derived from the same germ cell precursor.

Three male G1 offspring of bird 2-2 (2-2/6, 2-2/16 and 2-2/19) were crossed to stock hens to analyse transmission frequency to the G2 generation. Cockerels 2-2/6 and 2-2/19 had single proviral insertions, and the ratios of transgenic to non-transgenic offspring, 14/30 (47%) and 21/50 (42%), did not differ significantly from the expected mendelian ratio. Cockerel 2-2/16 had two proviral insertions, and 79% (27/34) of the G2 offspring were transgenic, reflecting the independent transmission of two insertions. Southern transfer analysis was used to compare the proviral insertion present in birds 2-2/6 and 2-2/19 with 9 and 14 of their G2 offspring, respectively (Fig 2C,D). Identical restriction fragments were observed in parents and offspring, indicating that the proviruses were stable once integrated into the genome.

**Transgene expression in G1 and G2 transgenic birds**

The vectors pONY8.0cZ and pONY8.4GCZ carried the reporter gene lacZ under control of the human cytomegalovirus (CMV)
immediate-early enhancer/promoter (CMVp) and pONY8.0G carried the reporter enhanced green fluorescent protein (eGFP), also controlled by CMVp. Protein extracts were made from a range of tissues from seven pONY8.0cZ G1 birds, each containing a different single provirus insertion, and were analysed by Western blotting. A protein of the expected molecular mass (110 kDa) was detected in some tissues of each transgenic bird. Expression was consistently high in the pancreas and lower levels of protein were present in other tissues, including the liver, intestine and skeletal muscle. The analysis of five of these birds is shown in Fig 3A. The pattern of expression was consistent between the individual birds but the overall amounts of protein varied. Sections of tissues from each cockerel (Fig 5A). \( \beta \)-Galactosidase protein levels and patterns of expression are very similar in the parent and two offspring. Staining of tissue sections from a G2 bird demonstrated expression patterns comparable with those observed in the parent (supplementary Fig 2 online). GFP fluorescence was readily detected in live G1 chicks carrying pONY8.0G, and the G2 offspring of one of these birds showed a similar level of expression (Fig 5B, supplementary Fig 3 online).

**DISCUSSION**

We have demonstrated that the lentiviral vector system that we tested is an efficient method for the production of germline transgenic birds. The high success rate may be due to a number of factors, including the ability of lentiviral vectors to transduce non-dividing cells, the use of the VSV-G pseudotype, previously used to introduce a retroviral vector into quail (Mizuarai et al, 2001) and lentiviral vectors into pigs and cattle (Hofmann et al, 2003), and the high titres used compared with previous transgenic studies. The chick embryo in a laid egg is a disc consisting of a single layer of cells, lying on the surface of the yolk, with cells that move through the embryo to form the hypoblast layer below the embryonic disc (Eyal-Giladi & Kochav, 1976). Primordial germ cells begin to migrate at this stage, from the embryonic disc, through the subgerminal cavity and onto the hypoblast below (Urven et al, 1988; Karagenc et al, 1996). This migration through the suspension of viral particles in the cavity may account for the higher frequency of germ cell transduction compared with that of somatic cells.

Expression of the reporter gene lacZ was detected in founder (G0), G1 and G2 birds. The expression of lacZ was directed by human CMVp (nucleotides \(-726\) to \(+78\)), an enhancer/promoter generally described as functioning ubiquitously in many cell types. This is usually the case if it is used in cell culture transfection experiments, but expression from the CMVp in transgenic mice, generated by pronuclear injection, varies between tissues. Expression between different transgenic lines was found to vary 100,000-fold, and between tissues within a
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high-expressing line it varied over a 10,000-fold continuum (Furth et al., 1991). In particular, it has been reported that the CMVp transgene shows predominant expression in exocrine pancreas in transgenic mice (Zhan et al., 2000). We have shown that the pattern of expression of both lacZ and GFP in embryos and birds is predominantly in the pancreas, although it is expressed at varying levels in most tissues, mirroring the expression patterns seen in transgenic mice. Expression from the vector pONY8.4 was significantly higher than from pONY8.0, possibly as a result of an increase in mRNA stability in the former resulting from removal of instability elements in the env region (data not shown). The expression pattern seen in G1 birds is maintained after germline transmission to G2. These results indicate that transgene-specific expression, from transgenes introduced using lentiviral vectors, is maintained after germline transmission, confirming and extending the results described in mice, pigs and rats.

The study described here is an evaluation of the possible application of lentiviral vectors for the production of transgenic birds. We have shown that we can obtain a high frequency of germline transgenic birds, stable transmission from one generation to the next, and a pattern of transgene expression that is maintained after germline transmission, confirming and extending the results described in mice, pigs and rats.

Fig 1 | Southern transfer analysis of genomic DNA from individual birds to identify proviral insertions. Genomic DNA samples were digested with XbaI (A,C,D) or BstEII (B) and hybridized with a probe for lacZ. (A,B) Analysis of 14 G1 offspring of G0 bird no. 1-4 (Table 1) revealed multiple proviral insertions in the G1 birds. (C) Analysis of G1 bird no. 2-2/6 (lane 1) and nine of his G1 offspring (lanes 2–10) and (D) G1 bird no. 2-2/19 (lane 1) and 14 of his G1 offspring (lanes 2–15).

Fig 2 | Reporter gene expression in pONY8.0CZ and pONY8.0G G1 transgenic birds. (A) Western blot analysis of liver (Li), heart (He), skeletal muscle (Sm), brain (Br), oviduct (Ov), skin (Sk), spleen (Sp), intestine (In), kidney (Kl), pancreas (Pa) and bone marrow (Ma) protein extracts from five adult G1 birds each containing single, independent insertions of pONY8.0CZ. β-Galactosidase protein detected as described in Methods. (B) Sections of skin, pancreas and intestine from G1 bird no. 2-2/19 stained for β-galactosidase activity and comparable sections of a non-transgenic control bird (arrows indicate epidermis of skin and villi of intestine). Scale bars, 0.5 mm. (C) Sections of skin, pancreas and breast muscle from a single-copy transgenic and a control bird were visualized for GFP fluorescence (arrow indicates epidermis of skin). Exposure conditions were identical for each transgenic and control pair, but differed between tissue types. Scale bars, 0.5 mm.
many of the problems encountered so far in the development of a robust method for production of transgenic birds. The application of this method for transgenic production will allow many transgene constructs to be tested to determine those that express in appropriate tissues and at required levels. The use of lentiviral vectors may overcome the problems associated with transgene incorporation and expression using oncoretroviral vectors. The development of an efficient method for production of transgenic birds is particularly timely as the chicken genome sequence is due to be completed shortly, and the value of the chick as a model for the analysis of vertebrate gene function is increasing (Brown et al., 2003).

MEthods

eIAv vectors and preparation of virus stocks. The vectors pONY8.0CGZ and pONY8.0G have been described previously (Corcoran et al., 2002). The vector pONY8.4CGZ has a number of modifications, including alteration of all ATG sequences in the gag-derived region to ATTG, to allow expression of eGFP downstream of the 5’ long terminal repeat (LTR). The 3’ U3 region has been modified to include the Moloney leukaemia virus U3 region. Vector stocks were generated by FuGENE6 (Roche, Lewes, UK) transfection of HEK 293T cells plated on 10 cm dishes with 2 μg of vector plasmid, 2 μg of gag/pol plasmid (pONY3.1) and 1 μg of VSV-G plasmid (pRV67; Rholl et al., 2002). At 36–48 h after transfection, supernatants were filtered (0.22 μm) and stored at −70°C. Concentrated vector preparations were made by initial low-speed centrifugation at 6,000g for 16 h at 4°C followed by ultracentrifugation at 50,500g for 90 min at 4°C. The virus was resuspended in formulation buffer for 2–4 h, aliquoted and stored at −80°C.

Production and analysis of transgenic birds. Approximately 1–2 μl of viral suspension was microinjected into the subgerminal cavity beneath the blastodermal embryo of newly laid eggs. Embryos were incubated to hatch using phases II and III of the culture system (Perry, 1988). DNA was extracted from the CAM of embryos that died in culture at or after more than 12 days of development using the Puregene genomic DNA purification kit (Flowgen, Asby de la Zouche, UK). Genomic DNA samples were obtained from CAM of chicks at hatch, blood samples from older birds and semen from mature cockerels. PCR analysis was carried out on 50 ng DNA samples for the presence of proviral sequence. To estimate copy number, control PCR reactions were carried out in parallel on 50 ng aliquots of chicken genomic DNA with vector plasmid DNA added in quantities equivalent to that of a single-copy gene (1 ×) and a 10-fold dilution (0.1 ×) and a 100-fold dilution (0.01 ×) as described previously (Sherman et al., 1998). Primers used were as follows: 5’-CGAGATCTACAGTGGCCCGGAACAG-3’ and 5’-ACCAGTAGTGAATTTCTGAGACCCTTGTA-3’. The number of proviral insertions in individual G1 birds was analysed by Southern transfer. Genomic DNA extracted from whole blood was digested with XbaI or BstEII. The digested DNA was resolved on a 0.6% (w/v) agarose gel and then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Amersham, UK). Membranes were hybridized with 32P-labelled probes for the reporter gene lacZ or eGFP at 65°C. Hybridization was detected by autoradiography. All experiments, animal breeding and care procedures were carried out under license from the UK Home Office.

Expression analysis. Adult tissues were isolated, fixed for 30 min in 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate-buffered saline (PBS) and were cryo-embedded and sectioned at 14 μm. β-Galactosidase activity was detected by incubating at 37°C in 5 mM potassium ferricyanide, 5 mM potassium
ferrocyanide, 2 mM MgCl₂ and 0.5 mg/ml X-gal for 90 min. GFP images of hatchlings were captured using a Fujifilm digital camera (Nikon 60 mm lens) shot through a GFSP-S lens system (BLS Ltd, Czech Republic). Selected tissues were snap-frozen and total protein was extracted by homogenization in PBS containing protease inhibitors (Complete Mini, Roche, Lewes, UK). Protein concentration was determined by Bradford assay. Either 50 μg (Fig 5) or 100 μg (Fig 3) of protein extract were resolved on 12% polyacrylamide gels (Invitrogen, Paisley, UK) and transferred to PVDF membranes. Membranes were incubated with mouse anti β-galactosidase antibody (Promega, Southampton, UK) at 1:5,000 dilution and donkey ant mouse IgG-HRP antibody (Santa Cruz Biotech) at 1:2,000 dilution and visualized with the ECL -galactosidase antibody (Promega, Southampton, UK) at 1:2,000 dilution and donkey ant mouse IgG-HRP antibody (Santa Cruz Biotech) at 1:2,000 dilution and visualized with the ECL western blotting detection system (Amersham Biosciences, Amersham, UK). ELISA was performed using the β-gal Elisa kit (Roche, Lewes, UK).

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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