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EMBO reports

scientific report

Efficient production of germline transgenic chickens using lentiviral vectors

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An effective 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 G_2 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

EMBO reports (2004) **5**, 728–733. doi:10.1038/sj.embor.7400171

INTRODUCTION

Development of an efficient method for genetic modification of chickens has proved a significant technical challenge (Sang, 1994; Zajchowski & Etches, 2000). The earliest methods developed were based on the use of avian retroviruses: replication-competent vectors derived from avian leucosis virus (ALV; Salter & Crittenden, 1989) and replication-defective vectors derived from reticuloendotheliosis virus (Bosselman *et al*, 1989). More recently, an ALV replication-defective vector has been used to produce transgenic birds at low frequency. Approximately 1% of hatched males proved to be germline transgenic and these birds in turn transmitted the vector to fewer than 1% of their offspring. Transgene expression from these vectors has only been detected 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%; Mozdziak *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; Pfeifer *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.

RESULTS

Production of G_0 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

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Received 27 January 2004; revised 27 February 2004; accepted 26 April 2004; published online 11 June 2004

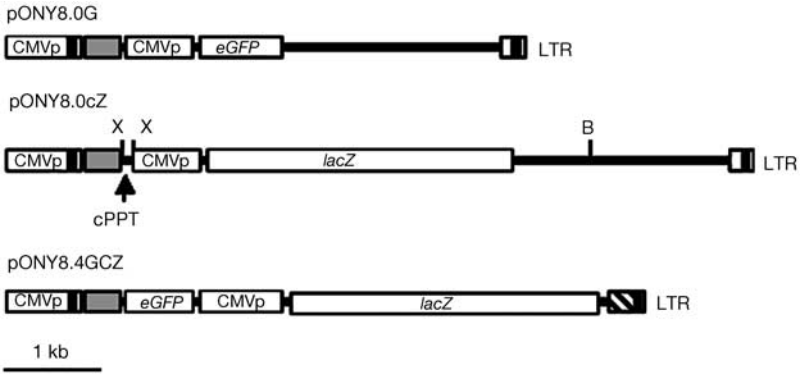

Fig 1 | Schematic representation of the EIAV vectors used in this study. The grey box represents the EIAV packaging signal, and the diagonally lined box in pONY8.4GCZ represents the MLV U3 region. Restriction sites (*Xba*I [X], *Bst*EII [B]) used for Southern blot analysis are indicated.

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 G_0 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 G_0 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 5×10^7 TU/ml, but that transduction frequency increases if higher titres are used.

Germline transmission from G_0 males

Semen samples were collected from the 12 G_0 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 G_1 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 G_1 transgenic birds and transmission to G_2

We predicted that the G_1 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 G_0 cockerels, transduced with pONY8.0cZ (experiments 1 and 2), were selected for further analysis of their transgenic offspring (Table 2). Genomic DNA from individual G_1 birds was analysed by Southern blot. Samples were digested separately with *Xba*I and *Bst*EII, 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 G_1 bird and the number of different insertions present in the offspring of each G_0 were analysed. An example of this analysis is shown in Fig 2A,B and the results are summarized in Table 2. The majority of G_1 birds carried single proviral insertions but several contained multiple copies, with a maximum of four detected in one bird. Some offspring of each G_0 bird carried the same proviral insertion, indicating that they were derived from the same germ cell precursor.

Three male G_1 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 G_2 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 G_2 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 G_2 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 G_1 and G_2 transgenic birds

The vectors pONY8.0cZ and pONY8.4GCZ carried the reporter gene *lacZ* under control of the human cytomegalovirus (CMV)

Table 1 | PCR analysis of hatched chicks and germline transmission from founder cockerels

Experiment	Construct (viral titre TU/ml)	Bird no.	Genome equivalents		Germline transmission Transgenics/ total
			CAM	Semen	
1	pONY8.0cZ[7] (5×10^7)	1-1	0	0.05	1/14 (7%)
		1-2	0.01	–	–
		1-3	0	–	–
		1-4	0.01	0.5	16/55 (29%)
		1-5	0.01	0.1	ND
2	pONY8.0cZ (5×10^8)	2-1	0.1	–	–
		2-2	0.1	1.0	4/20 (20%)
		2-3	0	0.01	ND
		2-4	0.1	0.5	19/67 (28%)
		2-5	0	–	–
		2-6	0.05	–	–
		2-7	0.05	–	–
		2-8	0.05	0.5	15/60 (25%)
3	pONY8.4GCZ (7.2×10^8)	3-1	0	0.05	1/25 (4%)
		3-2	0	0.05	3/64 (5%)
		3-3	0.01	–	–
		3-4	0.01	0.05	4/100 (4%)
		3-5	0.01	0.1	9/82 (11%)
		3-6	0.01	–	–
4	pONY8.0G (9.9×10^9)	4-1	0.05	1.0	20/44 (45%)

Table 2 | Estimation of the number of provirus insertions in the genome of G_1 birds

Bird no.	Total G_1 analysed	Number of birds with *N* insertions				Total no. of independent insertions
		1	2	3	4	
1-4	14	11	3	0	0	10
2-2	4	3	1	0	0	4
2-4	14	11	2	1	0	14
2-8	14	10	1	2	1	19

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 G_1 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 an adult pONY8.0cZ G_1 bird were stained (Fig 3B), revealing high levels of transgene expression throughout the exocrine pancreas, the epithelium of the skin and villi of the small intestine. Expression analysis of GFP in sections of tissue from a pONY8.0G G_1 bird detected expression in the pancreas, skin and breast muscle (Fig 3C) and weak expression in the intestine (data not shown). These results show that transgenic birds produced with the same EIAV vector but carrying different reporter genes showed similar patterns of expression.

Western analysis of tissues from G_1 birds carrying different single proviral insertions of pONY8.4GCZ detected *lacZ* expression, in a pattern similar to that seen in the pONY8.0cZ transgenic birds (supplementary Fig 1A online). However, staining of tissue sections revealed a more extensive pattern of expression than was observed in birds transgenic for pONY8.0cZ. β-Galactosidase activity was detected additionally in the smooth muscle of the intestine, in blood vessels underlying the epidermis and in tubular gland cells of the oviduct (Fig 4). An enzyme-linked immunosorbent assay (ELISA) determined that β-galactosidase protein levels were higher in pONY8.4GCZ birds in all tissues analysed than in pONY8.0cZ birds (supplementary Fig 1B online).

To establish whether transgene expression was maintained after germline transmission, expression in G_2 birds carrying the vectors pONY8.0cZ and pONY8.0G was examined. Western analysis was carried out on tissue extracts from two G_1 cockerels that each had a single proviral insertion, and two G_2 offspring from each cockerel (Fig 5A). β-Galactosidase protein levels and patterns of expression are very similar in the parent and two offspring. Staining of tissue sections from a G_2 bird demonstrated expression patterns comparable with those observed in the parent (supplementary Fig 2 online). GFP fluorescence was readily detected in live G_1 chicks carrying pONY8.0G, and the G_2 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 (G_0), G_1 and G_2 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

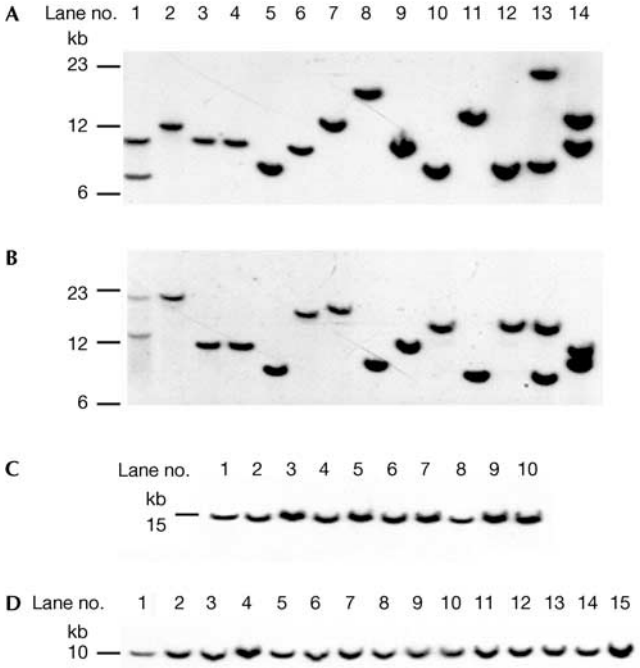

Fig 2 | Southern transfer analysis of genomic DNA from individual birds to identify proviral insertions. Genomic DNA samples were digested with *Xba*I (**A**,**C**,**D**) or *Bst*EII (**B**) and hybridized with a probe for *lacZ*. (**A**,**B**) Analysis of 14 G_1 offspring of G_0 bird no. 1-4 (Table 1) revealed multiple proviral insertions in the G_1 birds. (**C**) Analysis of G_1 bird no. 2-2/6 (lane 1) and nine of his G_2 offspring (lanes 2–10) and (**D**) G_1 bird no. 2-2/19 (lane 1) and 14 of his G_2 offspring (lanes 2–15).

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 G_1 birds is maintained after germline transmission to G_2. 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. Owing to the low level of somatic chimerism, analysis here was more informative in the G_1 generation, which can be produced when G_0 birds reach sexual maturity, approximately six months after hatching. These results indicate that the use of lentiviral vectors will overcome

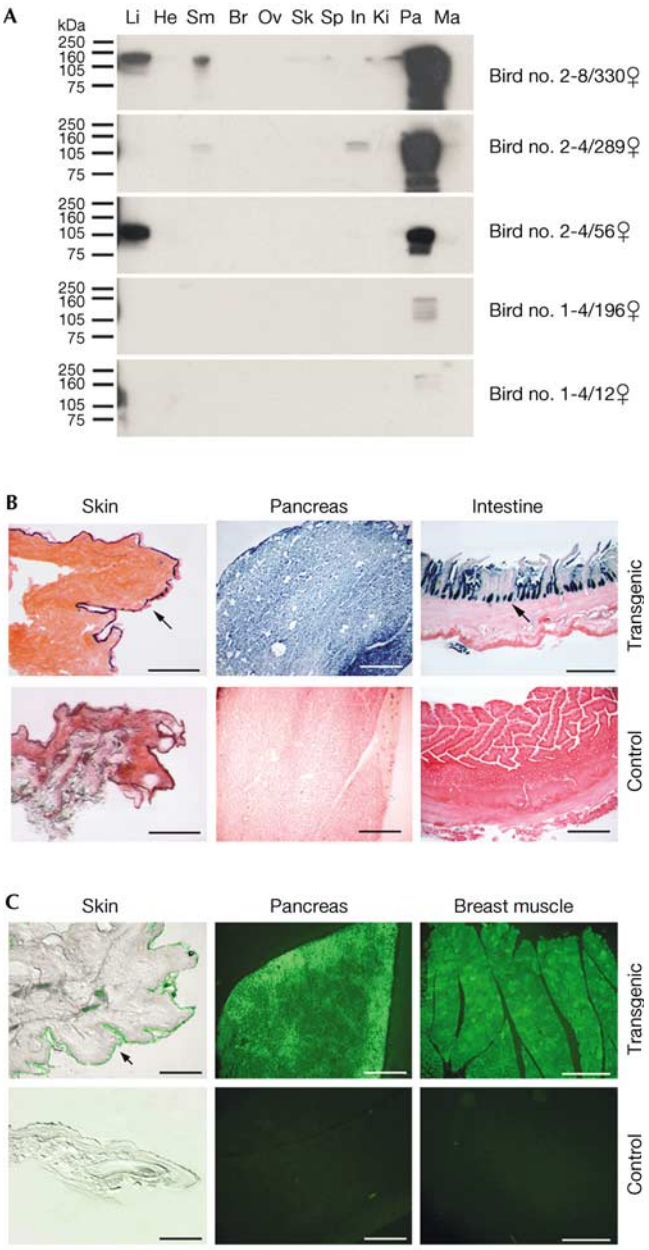

Fig 3 | Reporter gene expression in pONY8.0cZ and pONY8.0G G_1 transgenic birds. (**A**) Western blot analysis of liver (Li), heart (He), skeletal muscle (Sm), brain (Br), oviduct (Ov), skin (Sk), spleen (Sp), intestine (In), kidney (Ki), pancreas (Pa) and bone marrow (Ma) protein extracts from five adult G_1 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 G_1 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.

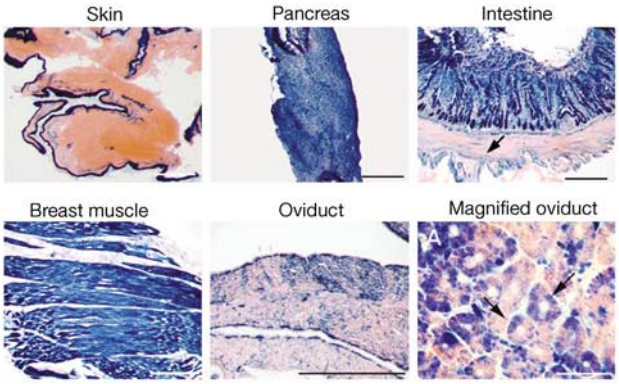

Fig 4 | Reporter gene expression in pONY8.4GCZ G_1 transgenic birds. Sections of tissues from a single-copy G_1 bird were stained for β-galactosidase activity (arrow indicates smooth muscle of intestine). Scale bars, 0.5 mm (except final panel). In the higher magnification of the oviduct section, arrows identify cells lining the tubular gland. Scale bar, 0.05 mm.

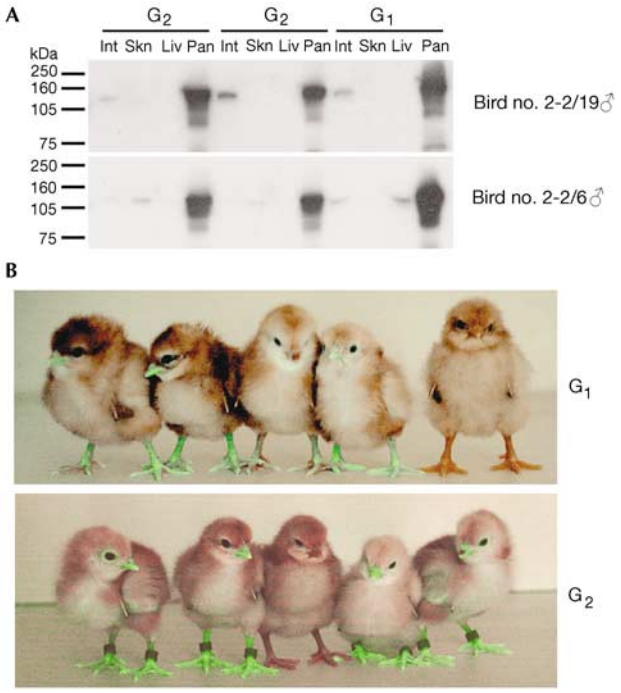

Fig 5 | Reporter gene expression in G_2 transgenic birds. (**A**) Western analysis of protein extracted from intestine (Int), skin (Skn), liver (Liv) and pancreas (Pan) of G_1 cockerels 2-2/19 and 2-2/6 and two G_2 offspring of each bird. (**B**) Top panel: five G_1 offspring of bird number 4-1. The four birds on the left are transgenic for pONY8.0G and express eGFP. The bird on the right is not transgenic. Lower panel: five G_2 offspring of bird number 4-1/66. The bird in the centre is not transgenic.

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 pONY8.0cZ and pONY8.0G have been described previously (Corcoran *et al*, 2002). The vector pONY8.4GCZ 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 6,000*g* for 16 h at 4 °C followed by ultracentrifugation at 50,500*g* 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 surrogate shell *ex vivo* 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 ×), a tenfold dilution (0.1 ×) and a 100-fold dilution (0.01 ×) as described previously (Sherman *et al*, 1998). Primers used were as follows: 5′-CGAGATCCTACAGTTGGCGCCCGAACAG-3′ and 5′-ACCAG TAGTTAATTTCTGAGACCCTTGTA-3′. The number of proviral insertions in individual G_1 birds was analysed by Southern transfer. Genomic DNA extracted from whole blood was digested with *Xba*I or *Bst*EII. 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 ^{32}P-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% gluteraldehyde 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 $MgCl_2$ 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 PDVFA membranes. Membranes were incubated with mouse anti-β-galactosidase antibody (Promega, Southampton, UK) at 1:5,000 dilution and donkey anti-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).

ACKNOWLEDGEMENTS

We thank R. Mitchell, F. Thomson and M. Hutchison for excellent technical support, M. Espasa and K. Binley for providing the pONY8.0G vector and F. Wilkes for the pONY8.4GCZ vector. This work was supported by the Biotechnology and Biological Sciences Research Council (UK), Oxford Biomedica (UK) Ltd and Viragen Incorporated (Florida, USA).

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