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Genetics

Heritable retroviral transgenes are highly expressed in chickens

(transgenic chickens/defective retroviral vector/gene expression)

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ABSTRACT **This report describes expression of heritable reticuloendotheliosis virus (REV) vector ME111 in 20 independent lines of transgenic chickens. The results are strikingly different from studies of Moloney virus in transgenic mice, where restricted expression of inherited proviruses has led to their use primarily as insertional mutagens rather than general agents for gene transfer. In contrast, the REV ME111 provirus is actively transcribed in a variety of tissues from transgenic chickens, is expressed from transcriptional control elements present in the long terminal repeat of the provirus, and codes for active neomycin phosphotransferase II. The REV vector system as applied to the chicken represents a departure from the long-established paradigm of retroviral transgenes in mice and provides a new approach to the study of avian biology.**

Replication-defective vectors derived from reticuloendotheliosis virus (REV) can infect pluripotent stem cells when injected beneath the blastoderm of unincubated chicken eggs (1, 2). Gene transfer at this stage of embryonic development requires the high efficiency of viral infection because the blastoderm contains many thousands of cells (3, 4). This procedure gives rise to mosaic chickens which upon subsequent breeding yield transgenic animals hemizygous for unique provirus insertions. Although these viral transgenes are expressed in somatic cells of the infected embryo, a major question has been whether inherited provirus would remain transcriptionally active.

The first germ-line insertion of experimentally introduced provirus was achieved by infection of the early mouse embryo (5). This and other studies have shown that provirus passed through the mouse germ-line is often transcriptionally inactive (6–12). The ability to manipulate the mouse embryo has led to alternative methods of gene transfer such as nuclear injection of cloned DNA (13, 14) and transfer of genetically altered stem cells into the blastocyst (15, 16). Both of these methods result in predictable expression of heritable transgenes. The unique physiology of the chicken has thus far limited avian gene transfer to the use of retroviral vectors whose expression is critical if they are to be generally used to study avian biology.

This paper describes expression of the REV vector ME111 in second-generation (G_2) transgenic chickens. The results differ dramatically from similar studies of transgenic mice, in which transcription from the long terminal repeat (LTR) of inherited Moloney provirus is usually suppressed (6–12). We find that the ME111 provirus is actively transcribed in transgenic chickens, is expressed from transcriptional control elements present in the LTR of the provirus, and codes for functional neomycin phosphotransferase II (NPT-II).

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MATERIALS AND METHODS

Transgenic Chicken Lines. G_0 transgenic chickens were generated by injection of REV vector ME111 (17, 18) under the surface of unincubated chicken embryo blastoderms (1). Mosaic G_0 males with germ-line insertions were bred with control females to generate lines of G_1 transgenic chickens hemizygous for the proviral transgene. Founders for each line contained unique proviral insertions as determined by Southern blotting (19) (data not shown). G_2 birds were generated by conventional breeding.

Preparation of RNA and Hybridization Probes. Tissues were dissected, rinsed in saline, and frozen in liquid nitrogen. Total RNA was prepared in guanidinium thiocyanate solution (20). Radiolabeled DNA hybridization probes were prepared by random priming (21) of fragments derived from the vector ME111 (Fig. 1*a*). As a positive control duplicate filters were hybridized with a 1.1-kb *Pst* I fragment derived from the plasmid ptl, which contains an α-tubulin cDNA homologous with the five-member α-tubulin gene family (24, 25).

RNA Dot Blot Analysis. Total RNA was denatured in formaldehyde and immobilized on GeneScreen*Plus* membranes (New England Nuclear). Duplicate RNA samples were treated with 2 M NaOH at 65°C as a control for DNA contamination (data not shown). Filters were baked 60 min at 80°C and prehybridized at 65°C for 1 hr in 1 M sodium phosphate, pH 7.2/0.5% SDS/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/1 mM EDTA containing 50 μg of denatured salmon sperm DNA per ml (22). A DNA probe (ME111 or α-tubulin; 5×10^6 cpm) was added and filters were hybridized overnight at 65°C. Filters were washed twice in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.2% SDS for 60 min at 65°C and subjected to autoradiography.

Northern Blot Analysis. Total RNA was passed once over a Stratagene "quick push" oligo(dT)-cellulose column according to the manufacturer's instructions. Two hundred nanograms of RNA from the ME111 cell line or 1 μg of poly(A)$^+$ RNA from chicken tissue was denatured in 50% formamide/6% formaldehyde and size-fractionated in 6% formaldehyde/1% agarose gels at 100 V for 3 hr (23). RNA was transferred overnight to GeneScreen*Plus* membranes by standard capillary blot procedures. Membranes were rinsed for 10 min in 0.3 M NaCl/0.03 M sodium citrate, pH 7. Hybridization conditions were identical to those used in dot blot analysis.

Slot Blot Analysis. RNA slot blots were prepared using a Bio-Rad slot apparatus as described for RNA dot blots. Films were exposed for 18, 24, or 36 hr at room temperature and the slots in each autoradiograph were quantitated by densitometry scanning using an LKB UltroScan apparatus. Silver grain density developed in the film was linear during the period of exposure. The areas under the scan for each slot

Abbreviations: REV, reticuloendotheliosis virus; LTR, long terminal repeat; NPT, neomycin phosphotransferase; HSV, herpes simplex virus; TK, thymidine kinase.

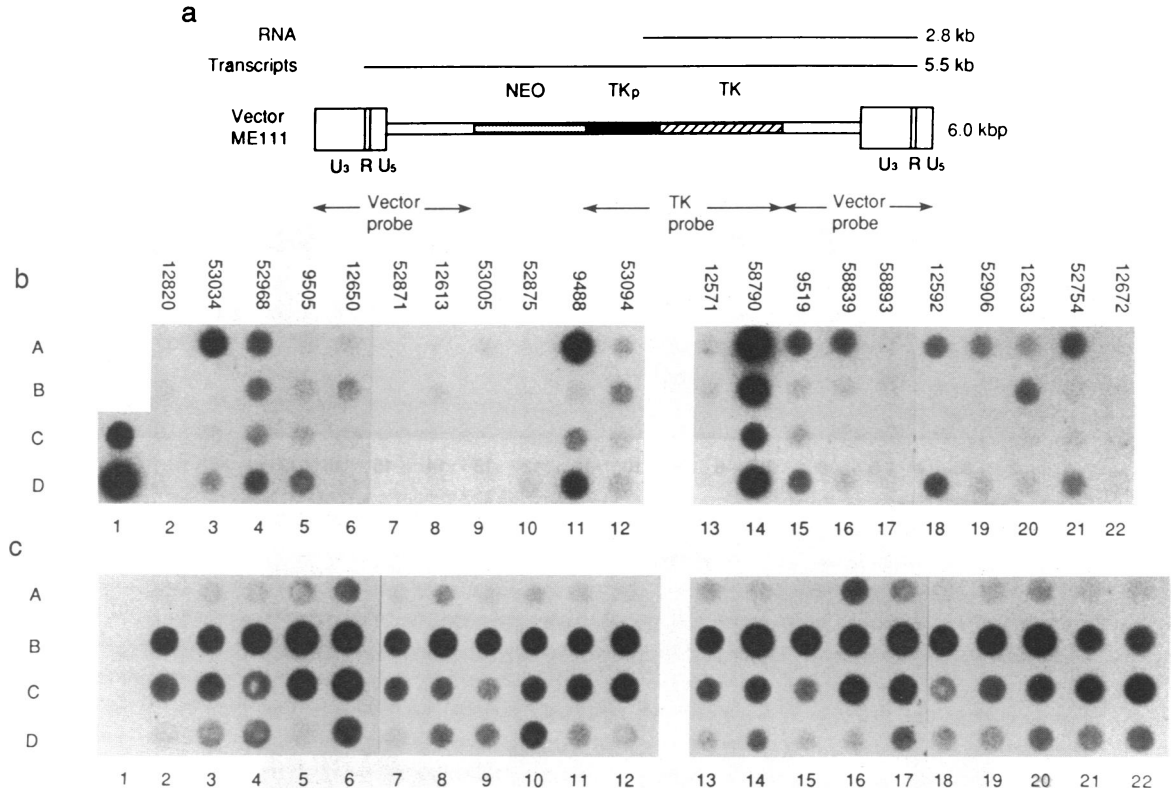

FIG. 1. Dot-blot analysis of ME111 RNA in G_2 transgenic chickens. (*a*) Genome organization of the ME111 vector including the U_3, R, and U_5 regions of the proviral LTR, the NPT sequence (NEO), the herpes simplex virus 1 (HSV-1), thymidine kinase (TK) gene promoter (TKp) and coding sequence, and predicted RNA transcripts. RNA size excludes polyadenylylation. kb, Kilobases; kbp, kilobase pairs. Arrows indicate sequences used as hybridization probes. (*b*) Filters containing total RNA (2–3 μg per dot) hybridized with radiolabeled vector probe. Identification numbers of G_2 transgenic birds carrying different germ-line vector insertion sites are indicated above each column. All samples within a given column derive from the indicated bird. Rows A, B, and C (except C1) contain RNA from liver, brain, and bursa, respectively. Row D, positions 4–7, 10–12, 15–17, 21, and 22 contain spleen RNA, while 2, 3, 8, 9, 13, 14, 18, 19, and 20 contain thymus RNA. C1 and D2 contain 1 and 2 μg of total RNA from the D17 cell line infected with the ME111 vector. Positions 7 A–D contain RNA from a nontransgenic control chicken. (*c*) Duplicate filters hybridized with a 1.1-kb *Pst* I fragment encompassing coding sequences from the five-member α-tubulin gene family (22, 23).

were corrected for differences in probe length and specific activities. Ratios of vector to α-tubulin expression are based upon the average of the three exposures.

NPT Assays. Extracts from liver and spleen of transgenic chickens were prepared by homogenization in 10 mM Tris, ›H 7.0/10 mM NaCl/1.5 mM $MgCl_2$/2 mM phenylmethylsulfonyl fluoride containing pepstatin A (5 μg/ml) and leupeptin (0.5 μg/ml). Tissue homogenates were cleared by centrifugation. Samples (10 μg) of supernatant protein were incubated with kanamycin and [γ-^{32}P]ATP as described (26). NPT-II was obtained from 5 Prime → 3 Prime, Inc.

RESULTS

Dot Blot Analysis of RNA from 20 Lines of Transgenic Chickens. RNAs from 20 lines of second-generation (G_2) transgenic chickens carrying the REV vector ME111 were analyzed by dot blot hybridization. ME111 encodes the Tn*5* NPT-II gene and the HSV-1 TK gene (17, 18). The provirus, its predicted RNA transcripts, and regions of provirus used as hybridization probes are shown in Fig. 1*a*. Total RNA was extracted from liver, brain, bursa, and either spleen or thymus from one bird representing each line. No consistent pattern of tissue specificity was observed. Each of the chicken lines tested contained detectable ME111 RNA in at least one of the tissues examined. Negative results by dot blot analysis may simply indicate low levels of vector RNA expression. RNA samples from bird 58893 were negative for vector RNA by dot blot analysis, while the same samples from a sibling bird (58890) were positive when subjected to Northern blot analysis (see Fig. 2*a*). Hybridization of identical blots with an α-tubulin probe was consistent from bird to bird, confirming that α-tubulin expression is higher in brain than in liver, as previously reported (22). Analysis of RNA from blood of birds 53028 (a sibling of 53034) and 58790 showed low levels of vector RNA which could not account for vector RNA observed in either vascularized tissues or brain (data not shown). As expected, RNA from D17 cells infected with ME111 hybridized to vector probe (Fig. 1*b*, columns 1 C and D), while RNA from a nontransgenic bird did not (Fig. 1*b*, columns 7 A–D). Alkali treatment reduced hybridization to background levels.

Northern Blot Analysis of RNA Transcripts from Three Lines of Transgenic Chickens. ME111 proviral transcripts were further characterized by Northern blot analysis of RNA from birds 58774, 58890, and 53028 (Fig. 2*a*, lanes 6–17). These birds were siblings of birds 58790, 58893, and 53034, respectively, and represent three lines of chickens hemizygous for ME111 provirus at different integration sites. The 5.9-kb transcript that hybridized to vector probe was observed in all tissues examined and is consistent with the size of genome-length polyadenylylated ME111 RNA. Primer extension analysis of RNA from the D17 cell line and transgenic line 58774 was utilized to confirm the transcriptional start site within the 5′ LTR (data not shown). Consistent with this observation the same size transcript hybridized with a TK probe (data not shown). The smaller transcript predicted to originate from the HSV-1 TK promoter was not

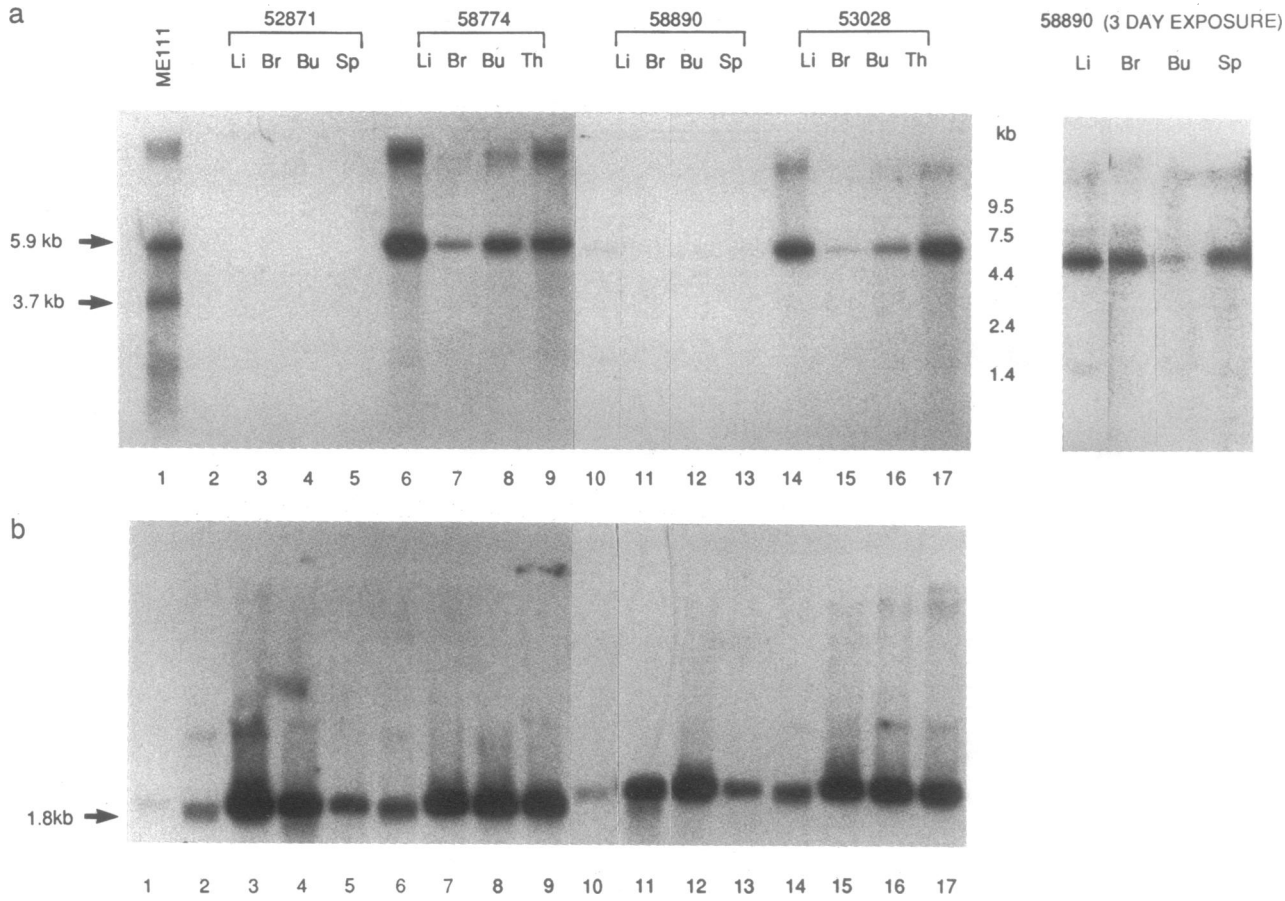

FIG. 2. Northern analysis of REV transgene expression in G_2 transgenic chickens. (*a*) Autoradiographs of Northern blots hybridized with radiolabeled ME111 probe and exposed to x-ray film for 18 hr. The filter containing RNA from bird 58890 was also exposed for 3 days in order to visualize low levels of vector transcript. Lane 1 contains RNA from the D17 cell line infected with ME111. Bird 52871 was a nontransgenic chicken; birds 58774, 58890, and 53028 were G_2 individuals from transgenic lines with different vector provirus insertion sites. Tissues are indicated above each lane: Li, liver; Br, brain; Bu, bursa; Sp, spleen; Th, thymus. (*b*) Filters were stripped of the vector probe and rehybridized to the α-tubulin probe.

observed in RNA from these birds. On the other hand, we detected both the 5.9-kb transcript and a 3.7-kb transcript in RNA from mass cultures of D17 (dog) cells infected with the ME111 vector (Fig. 2*a*, lane 1). The smaller transcript represents polyadenylylated RNA initiated within the HSV-1 TK promoter. No vector transcripts were observed in RNA from the nontransgenic control bird, 52871 (Fig. 2*a*, lanes 2–5). A large transcript was detected in RNA from transgenic chickens and ME111-infected D17 cells by hybridization with the vector probe (Fig. 2*a*), but not with the α-tubulin probe (Fig. 2*b*). Rehybridization of the blot in Fig. 2*a* with a TK probe also revealed this transcript (data not shown). The nature of this RNA is unknown, but it may be a read-through transcript that escapes termination and polyadenylylation at the 3′ end of the provirus. All tissues examined contained the 1.8-kb α-tubulin RNA (Fig. 2*b*).

Slot Blot Analysis of Transgene RNA and Comparison with α-Tubulin RNA. Relative levels of vector RNA and α-tubulin RNA present in liver, brain, bursa, and thymus from bird 58774 (sibling to bird 58790) were compared by slot blot hybridization of identical samples with radiolabeled probes to either vector or α-tubulin RNA. RNA from a nontransgenic bird, 52871, was used as a negative control for vector RNA. Hybridization was measured by densitometric analysis of filter autoradiographs (Fig. 3). Adjusting for probe size and specific activity, the ratios of hybridized vector probe to hybridized α-tubulin probe ranged from 0.075 in brain to 1.14 in liver. We believe these data reflect efficient expression of the ME111 transgene and approximate the relative levels of vector and α-tubulin RNAs in these tissues. The presence of NPT-II encoded by the ME111 vector was confirmed by an enzymatic assay (26). Results are shown in Table 1. Liver and spleen extracts from bird 53028 were 16- and 4.4-fold above respective background levels in tissues from the nontransgenic control. The same tissues from bird 58774 were 13.7- and 7.8-fold above background.

DISCUSSION

Comparison of Retroviral Transgene Expression in Mice and Chickens. The best characterized experimental model of heritable retroviral transgenes is based upon the Moloney murine leukemia virus and its derived vectors (6, 9). Expres-

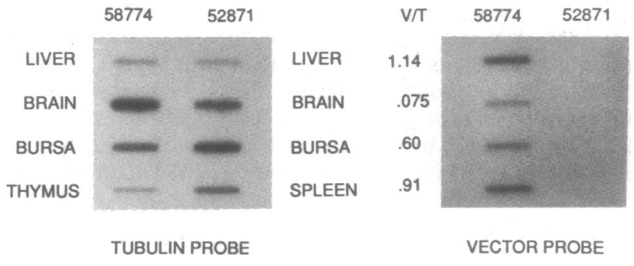

FIG. 3. Slot blot analysis of ME111 transcripts present in total RNA from G_2 transgenic bird 58774 and control bird 52871. V/T is the ratio between the corrected densitometric values of vector (V) and tubulin (T) probe hybridized to RNA from each tissue analyzed. The ratio shown is an average of the three exposures.

Table 1. Expression of NPT-II activity in transgenic chickens

Bird	Tissue	Activity, cpm	% of control
Control	Liver	1,348	100
Control	Liver + NPT-II	19,413	1,440
53028	Liver	22,069	1,640
58774	Liver	18,483	1,370
Control	Spleen	6,577	100
Control	Spleen + NPT-II	55,726	850
53028	Spleen	29,100	440
58774	Spleen	51,270	580

Extracts from liver and spleen of transgenic lines 53028 and 58774 were assayed for NPT-II activity. Activities (cpm) are compared to nontransgenic extracts with and without addition of purified NPT-II (125 pg). Only the first two digits of the figures given are significant.

sion of most Moloney virus transgenes is suppressed *in vivo* because LTR-mediated transcription is restricted in the progeny of mice infected during early embryogenesis (7, 8, 11). Similar results have been observed with Moloney-derived vectors that contain nonviral genes (9, 10). LTR-mediated expression of Moloney virus vectors is also suppressed following infection of embryonic stem cells (16) and embryonal carcinoma cells (27). Even though the MPSVneo vector derived from myeloproliferative sarcoma virus exhibits more efficient expression in embryonal carcinoma cells than Moloney virus vectors (28, 29), passage through the mouse germ line still resulted in blocked expression of the MPSVneo provirus (30). Various factors suggested to affect suppression of transcription from the LTR of Moloney virus transgenes include the stage of mouse embryo infection (8, 9), provirus methylation (7), trans-acting transcription factors (12), cis-acting sequences within the provirus (28, 29), and the provirus integration site (6, 11, 31, 32). However, transcription of some naturally occurring murine endogenous viruses (33) and of Moloney vector transgenes expressed from the mouse β-globin promoter (34) or the HSV-1 TK promoter (35) has been observed. In contrast, the REV LTR of ME111 is an efficient promoter in transgenic chickens, while the HSV-1 TK gene promoter is not. The reason for reduced activity of the ME111 HSV-1 TK promoter *in vivo* is unknown. However, in some cell clones *in vitro* suppression of transcription initiating at the ME111 HSV-1 TK promoter has been observed following drug selection for expression of high levels of RNA from the 5′ LTR promoter (17, 18).

Expression of Experimental and Naturally Occurring Endogenous Provirus in Chickens. Our results are consistent with biological observations of heritable recombinant subgroup A avian leukosis virus transgenes introduced to line 0 chickens by infecting embryos with replication-competent virus (36). Chickens transgenic for the complete virus exhibit viremia (37, 38), while those with spontaneously defective provirus expressing the viral envelope gene show resistance to superinfection by the same subgroup (39, 40). The naturally occurring subgroup E endogenous viruses ev-21 and ev-6 confer similar characteristics to chickens that carry them (39, 40). Because chickens contain numerous germ-line insertions of endogenous provirus (ev1-21), we were concerned that spontaneous expression of complete endogenous virus might result in pseudotyping of the ME111 genome and reinfection of somatic cells. We were able to detect expression of endogenous virus in serum of two lines of transgenic chickens exhibiting high vector expression (families of birds 58790 and 53034). However, we were not able to detect infectious vector in serum from the same birds. Additionally, quantitative hybridization analysis of blood DNA from three generations, and Southern blot analysis of tissue DNAs, did not reveal additional copies of integrated ME111 vector (data not shown). All birds tested negative for REV.

Summary. The 20 lines of transgenic chickens described here represent independent, randomly selected germ-line insertions of the REV vector ME111 (1). The REV NPT-II transgene in chickens is transcribed from the LTR in all tissues analyzed. Variations in ME111 transcription may reflect the influence of host flanking DNA, but provirus expression was not suppressed as generally described for Moloney vectors in mice. Future analysis of cis-acting transcriptional control elements within the LTR and alternative internal promoters of cellular origin will lead to a better understanding of tissue-specific expression of REV transgenes.

The replication-defective ME111 vector can transduce a functional LTR-driven NPT-II gene into the chicken germ line. These observations provide an important *in vivo* approach to the study of avian biology and suggest that retroviral-mediated gene transfer could have broader functional applications in other species as well.

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