mM DTT, 200 μM each of dATP, dGTP, TTP, and dCTP in a final volume of 50 μl; 2.5 units of *Taq* polymerase were used for each assay. Typically, for each cycle of amplification, the mixture was denatured at 94°C for 2 min, annealed at 55°C for 1 min, and then extended at 70°C for 2 min. From 36 to 40 cycles of amplification were performed and fresh enzyme (2 to 5 units) was added to each tube at the end of every tenth cycle.
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Germline Transmission of Exogenous Genes in the Chicken

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Difficulties associated with in vitro manipulation and culture of the early chicken embryo have restricted generation of transgenic chickens to approaches that use replication-competent retroviruses. The need to produce transgenic chickens in the absence of replicating virus prompted development of a new method of gene transfer into the chicken. Microinjection of the replication-defective reticuloendotheliosis virus (REV) vector ME111 beneath unincubated chicken embryo blastoderms results in infection of germline stem cells. This vector contains genetic information exogenous to the chicken genome, including both the herpes simplex virus type 1 thymidine kinase gene and the Tn5 neomycin phosphotransferase gene. About 8 percent of male birds hatched from injected embryos contained vector DNA in their semen. All four positive males tested passed vector sequences onto their progeny. Analysis of G_1 offspring showed that gonads of G_0 male birds were mosaic with respect to insertion of vector provirus. Thus, primordial germ cells present in the unincubated chicken embryo blastoderm are susceptible to infection by defective REV vectors.

GENE TRANSFER INTO CHICKENS has usually depended on the use of replication-competent retroviral vectors (*1–4*), in part because it is difficult to manipulate the early avian embryo or to grow the embryo in vitro (*5, 6*). Attempts to alter the chicken germline by gene transfer into developing follicles (*7*) or in vitro cultured early embryos (*6*) have not been successful. Documented germline gene transfer results from injection of replicating virus into freshly laid unincubated eggs (*2–4*). Access to the embryo is easy just after ovaposition; however, the embryo has already reached a stage corresponding to a mammalian late blastula or early gastrula. At this time, the embryo consists of a thin layer of many pluripotent cells comprising the blastoderm (*8, 9*). In contrast, the ability to manipulate the early mouse embryo has led to success with a variety of approaches to gene transfer, including microinjection of DNA (*10, 11*), retroviral infection (*12, 13*), and the use of embryonic stem cells that can contribute to the germ-cell lineage of chimeric mice (*14–16*). To generate transgenic chickens in the absence of replicating virus, we have developed a new method of gene transfer based on microinjection of nonreplicating retroviral vectors into embryos of unincubated eggs.

We used the replication-defective reticuloendotheliosis virus (REV) vector ME111 (*17, 18*) to infect susceptible stem cells present in the unincubated chicken embryo. Chickens do not contain endogenous REV-related proviruses that might interfere with detection of newly acquired provirus, even though REV can infect at least some somatic stem cells of the early chicken embryo (*2, 7*). Primordial germ cells appear to reside in the outer layer or epiblast of the blastoderm (*19–22*). Since nonreplicating vectors would infect embryonic cells at about the time of injection, subsequent analysis of adult blood and semen would show whether or not susceptible somatic and germline stem cells were present at this time.

The ME111 vector lacks all viral structural genes and carries both the Tn5 neomycin resistance gene driven by the promoter of the REV long terminal repeat (LTR) and the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene (*17*). C3 helper cells generate stocks of ME111 with titers of about 10^4 TKTU per milliliter (TK transducing units) (*17*). Sequence comparison of the competent parental helper virus, packaging defective helper proviruses, and the vector provirus is shown in Fig. 1. Cells and vector were grown and harvested as previously described (*17, 18*). Ten-microliter volumes of vector-containing cell culture media were injected through the area pellucida into the subgerminal cavity of the blastoderm of unincubated eggs (*23*). Eggs were resealed and allowed to hatch (*24*). DNA from blood and semen of mature birds was analyzed for the presence of integrated proviral vector.

A total of 2599 eggs were injected, of which 38% hatched. DNA from the blood of 760 hatched chicks was analyzed by liquid hybridization with a vector-specific probe (*25*). Of these, 173 chicks contained vector sequences. Of 82 males whose blood was positive, 33 males also carried vector sequences in their semen. DNA blot analysis (*26*) of blood (*27*) and semen DNA confirmed integration of vector provirus. Restriction endonuclease fragments of DNA specific for replicating REV were not observed. Long-term culture assays (*28*), used to test for low levels of virus, detected competent REV in 2 of 14 G_0 birds with vector-positive blood. Sires and progeny described in this report were judged to be virus-negative by the same method. Vector-positive semen from four males was used to inseminate control females. All four transmitted vector sequences to G_1 progeny at a frequency that varied from ~2% to 8%. These results confirm vector-mediated infection of primordial germ cells present in the unincubated chicken embryo.

DNA blot analysis of proviral genome organization in G_0 vector-positive semen and in blood of vector-positive G_1 progeny is shown in Fig. 2. Lanes 3 to 5 in Fig. 2A

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contain Bam HI–digested semen DNA of three G_0 vector-positive birds and show the predicted internal proviral vector Bam HI fragments of 3.7 and 1.65 kb. These bands are absent from negative control semen DNA shown in lane 2. Other bands present in lanes 3 to 5 may represent the 5′ junctions between cellular DNA and integrated proviral vectors.

Figure 2B shows analysis of blood DNA from G_1 progeny of G_0 males 28428 and 87620 whose semen DNA is shown in Fig. 2A, lanes 3 and 4, respectively. DNA from G_1 progeny of G_0 male 28428 is shown in Fig. 2B, lanes 4 to 6; DNA from progeny of G_0 male 87620 is shown in lane 7. Blood DNAs from these four offspring were digested with both Bam HI and Bgl II, Southern blotted, and hybridized with probes derived from the 5′ and 3′ ends of the vector. Progeny DNA in lanes 4, 5, and 7 each show the predicted internal vector DNA fragments of 1.65 and 0.74 kb, also present in the positive control in lane 9. Both internal fragment intensity and the presence of an additional fragment in lane 4 of Fig. 2B indicate that the genome of this bird contains two copies of integrated vector. The strongly hybridizing junction fragments marked by asterisks in Fig. 2B demonstrate the monoclonal nature of the inserted vector DNA and confirm germline passage of the vector provirus from G_0 sires to their G_1 progeny.

DNA from the blood of G_1 progeny, in lane 6 of Fig. 2B, shows the predicted 1.65-kb fragment, a junction fragment of ~0.9 kb, and a third fragment of ~0.65 kb, which is smaller than the predicted 0.74-kb vector fragment present in lanes 4, 5, and 7. The same progeny DNAs, hybridized with a TK gene probe, are shown in Fig. 2C. The predicted TK-hybridizing 1.75-kb fragment is present in lanes 4, 5, and 7 of Fig. 2C, but absent in DNA of lane 6 where a smaller fragment of ~0.9 kb is present instead. Results with the two probes are consistent with the presence of a large deletion of about 0.8 kb between the two Bgl II sites within the proviral vector present in the DNA seen in lane 6 (see Fig. 1).

Analysis of G_1 progeny of G_0 sire 28428 shows that the germline of this G_0-infected male is mosaic with respect to vector insertions. From this male, 11/289 (3.8%) progeny were vector-positive. Other sires gave similar results: 87620, 9/205 (4.4%); 87725, 12/143 (8.4%); and 87658, 2/83 (2.4%). Ten microliters containing about 100 infectious units of ME111 were injected into embryos (~10^4 TKTU/ml as measured on BRLTK$^-$ cells) (*18*). The relative infectivity of this vector on cells of the chicken embryo is unknown. Since other

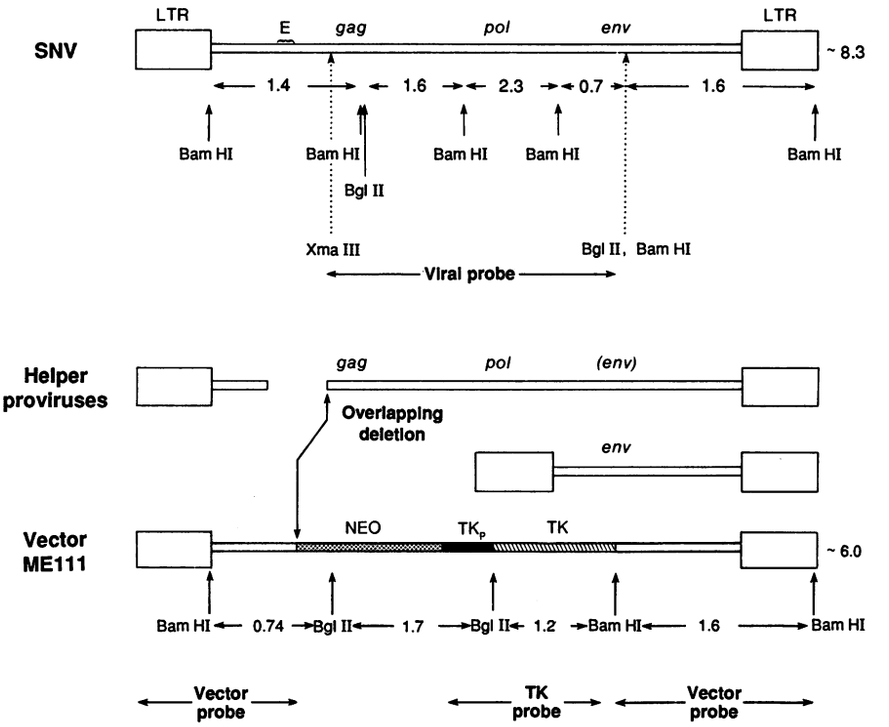

Fig. 1. Sequence similarities among the parental spleen necrosis virus (SNV) provirus, the modified packaging defective helper proviruses, and the ME111 proviral vector. Relevant features of these proviruses include the LTRs, the structural genes of the virus (*gag, pol, env*), the approximate position of the packaging sequence (E), the neomycin phosphotransferase sequences (NEO), and the HSV-1 TK gene. TKp indicates the TK promoter. The (env) sequence in the larger of the two helper proviruses is presumably not expressed because of removal of the 5′ splice donor. All numbers indicate length in kilobases. Overlapping deletions indicated between helper and vector sequences should reduce recombination between these genomes. The REV helper proviruses and the ME111 vector have been described (*17, 18*). The 5′ LTRs of both helper proviruses were derived from SNV. Their coding sequences derived from REV-A. REV-A and SNV share high sequence homology. Bam HI and Bgl II restriction endonuclease fragments indicated are not to scale. Also given are the location of viral, vector, and TK-specific DNA probes.

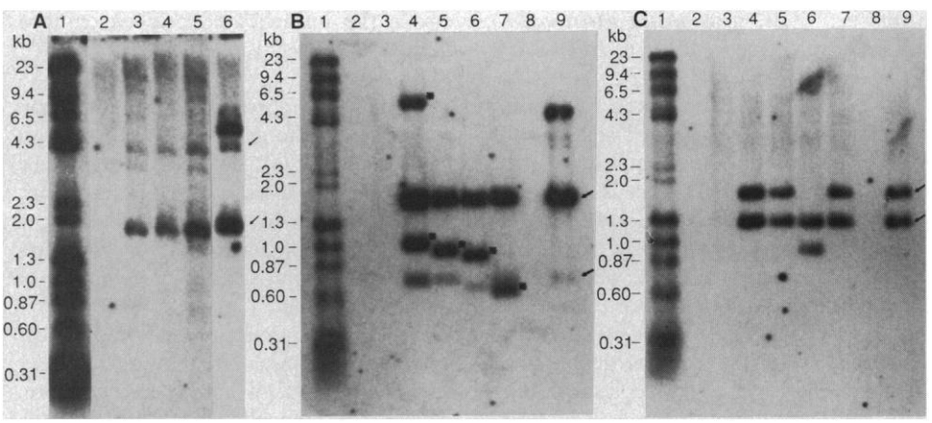

Fig. 2. DNA blot analysis of G_0 vector-positive semen and vector-positive G_1 progeny (*29*). (**A**) Hybridization with vector-specific probe. Lane 2, Bam HI–digested negative control semen DNA; lanes 3 to 5, Bam HI–digested vector-positive semen DNA from three different G_0 chickens; lane 6, Bam HI–digested pME111 DNA. (**B**) Hybridization with vector-specific probe. Lane 3, Bam HI/Bgl II–digested negative control blood DNA; lanes 4 to 6, Bam HI/Bgl II–digested blood DNAs of vector-positive G_1 progeny of male G_0 bird 28428; lane 7, Bam HI/Bgl II–digested blood DNA of vector-positive G_1 progeny of male G_0 bird 87620; lane 9, Bam HI/Bgl II–digested negative control blood DNA and plasmid pME111. Lanes 2 and 8 are blank. (**C**) Hybridization with the TK-specific probe: same as described for (B). Lanes 1 of (A), (B), and (C) contain Hind III–digested λ phage DNA and Hae III–digested φX174 DNA. DNA fragments that derive from internal regions of the vector are marked by arrows. Junction fragments containing the 5′ end of the vector and cellular DNA are marked by asterisks. The largest hybridizing fragments of DNA in lane 6 of (A) and lane 9 of (B) contain vector sequences that remain associated with plasmid DNA after restriction endonuclease digestion.

defective REV vectors have titers reported to be a hundred times that of ME111 (*29*), injection of embryos with replication-defective REV vector could provide a very efficient means of producing germline mosaic chickens. Defective retroviral vectors derived from other viruses such as avian leukosis virus might also be effective (*30*). The progeny of such mosaics could carry proviral insertions at many different sites within the genome.

The embryos used for these studies contained at least 10^4 cells, some of which formed the epiblast at the outer surface of the blastoderm (*31*). Primordial germ cells reside in this layer of cells, but are indistinguishable from somatic stem cells before migrating to the germinal crescent (*19–22*). Our experiments show that injection of the nonreplicating REV vector ME111 beneath the unincubated chicken embryo blastoderm resulted in infection of precursors to both blood and semen. Analysis of blood DNA from progeny of G_0 birds with vector-positive semen confirmed germline transfer of vector sequences. Since replicating helper virus was not detected in the G_0 mosaics used for breeding, nor in their G_1 progeny, the vector sequences present in these animals resulted from infection immediately following injection of the G_0 embryos. This approach provides a way to study cell lineage relations during differentiation (*32, 33*) and vector-mediated gene expression. Chicken embryo epiblast cells have been cultured in vitro (*34*), and generation of chicken chimeras has been accomplished by injecting stem cells into the blastocoel of recipient embryos (*35*). The susceptibility of germline stem cells to REV infection in vivo suggests that a similar approach might be used on stem cells cultured in vitro, paralleling work with murine stem cell lines used to generate transgenic mice (*15*).

Our results identify the unincubated chicken embryo as a source of germline stem cells susceptible to infection by REV vectors and demonstrate the first use of replication-defective REV vectors to transfer heritable, nonviral, genetic information into the chicken germline. The ease and efficiency of this procedure provide both researchers and commercial breeders with a practical method for genetic manipulation of the chicken.

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24. Before injection, freshly laid eggs were held horizontally with respect to their long axis for 5 hours or more at 18° to 20°C so that the blastoderm would lie just beneath the topmost area of the shell. Egg shells were wiped with 70% ethanol before and after a 5- to 8-mm hole was made in the shell with a Dremel (Model 280-5) moto-tool fitted with an aluminum oxide grinding stone (Dremel #924). The shell membrane was removed with a scalpel just before injection. Injection was performed with a Narishige micromanipulator and a Drummond 100-μl digital microdispenser fitted with a glass needle of 50- to 60-μm outer diameter. Needles were pulled using a Kopf Model 720 vertical pipette puller. Ten microliters of an overnight harvest of cell culture media containing ME111 (~10^4 TKTU/ml) were injected beneath the surface of the exposed blastoderm. Injection was monitored using a Wild M5A dissecting microscope. Eggs were resealed with a patch of shell membrane placed over the hole and allowed to dry. The patch was then covered with Devcon Duco cement. Eggs were placed at 37.8°C in a Natureform incubator and allowed to hatch.
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Selective Loss of Hippocampal Granule Cells in the Mature Rat Brain after Adrenalectomy

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Adrenalectomy of adult male rats resulted in a nearly complete loss of hippocampal granule cells 3 to 4 months after surgery. Nissl and immunocytochemical staining of hippocampal neurons revealed that the granule cell loss was selective; there was no apparent loss of hippocampal pyramidal cells or of γ-amino butyric acid (GABA)–, somatostatin-, neuropeptide Y–, calcium binding protein–, or parvalbumin-containing hippocampal interneurons. The hippocampal CA1 pyramidal cells of adrenalectomized animals exhibited normal electrophysiological responses to afferent stimulation, whereas responses evoked in the dentate gyrus were severely attenuated. Corticosterone replacement prevented both the adrenalectomy-induced granule cell loss and the attenuated physiological response. Thus, the adrenal glands play a role in maintaining the structural integrity of the normal adult brain.

ALTHOUGH HORMONES INFLUENCE the survival of neurons in the developing brain (*1*), none have been shown to be necessary for maintenance of the structural integrity of the mature brain. The hippocampus, a brain region involved in learning, memory, and a number of neurological disease states (*2, 3*), is a target of adrenal steroids (*4*) and is thought to play a role in the endocrine functions of the adrenal-hypothalamic-pituitary axis (*5*). Adrenal hormones may exacerbate neurotoxic insults to the hippocampus (*6*), and adrenalectomy can protect hippocampal pyramidal cells

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