Replication-Defective Vectors of Reticuloendotheliosis Virus
Transduce Exogenous Genes into Somatic Stem Cells
of the Unincubated Chicken Embryo

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Replication-defective vectors derived from reticuloendotheliosis virus were used to transduce exogenous
genomes into early somatic stem cells of the chicken embryo. One of these vectors transduced and expressed
the chicken growth hormone coding sequence. The helper cell line, C3, was used to generate stocks of vector
containing about 105 transducing units per ml. Injection of 5- to 20-μl volumes of vector directly beneath
the blastoderm of unincubated chicken embryos led to infection of somatic stem cells. Infected embryos and adults
contained unarranged integrated proviral DNAs. Embryos expressed the transduced chicken growth hormone
gene and contained high levels of serum growth hormone. Blood, brain, muscle, testis, and semen
contained from individuals injected as embryos contained vector DNA. Replication-defective vectors of the
reticuloendotheliosis virus transduced exogenous genes into chicken embryonic stem cells in vivo.

Insertion of genetic information into the chicken provides a new in vivo approach to analyzing gene expression and its
effects on avian physiology. A vector derived from Rous sarcoma virus has been used to transfer additional growth
hormone genes into chicken somatic cells by infection of 7- and 9-day-old embryos (35). More recently, gene transfer
into chicken germ cells (27–29) has been accomplished by injection of day-old embryos with similar replicating Rous
sarcoma virus vectors (18, 33). This approach to avian gene transfer has advantages over DNA microinjection since the
early chicken zygote is difficult to manipulate and even a freshly laid egg contains thousands of cells (10, 20). How-
ever, replicating retroviral vectors have disadvantages. They can result in gene transfer to susceptible cells at various
stages of differentiation long after initial infection of the embryo. This can make it difficult to determine the stage of
development at which gene insertion takes place or the cell lineage relationships within fully differentiated tissues.
Furthermore, replicating vectors also increase the potential for disease states associated with chronic viral infection (16, 24, 38).

Replication-defective retroviral vectors offer an alternative approach (2, 6, 21, 36, 40). Such vectors, derived from
reticuloendotheliosis virus type A (REV-A) (31), are produced by the helper cell line C3 which contains a packaging-
defective helper provirus (40). When transfected with a defective proviral vector, this helper cell assembles infectious
replication-defective vector but little or no competent virus (17). Both replicating REV and the replication-defective
REV vector ME111 have been previously used for gene transfer into chicken somatic cells by injection of virus into
follicles before ovulation (32). We have used a method of
gene transfer based on microinjection of vector into early embryos.

This report describes the transfer of new genetic information, including additional chicken growth hormone (cGH)
coding sequences, into somatic stem cells of the chicken embryo. Chickens do not generally contain endogenous
REV and express endogenous cGH only in the pituitary during late embryogenesis and after hatching (15, 19, 30). In vivo,
these vectors can infect somatic stem cells of day-old chicken embryos, resulting in precociously high levels of
circulating cGH and the presence of vector DNA in a variety of adult somatic tissues.

MATERIALS AND METHODS

Cells. The REV-A helper cell line, C3, was generously provided by H. Temin (40). C3 cells were cultured in
minimal essential medium (Eagle) containing 7% fetal calf serum–400 μg of G418 per ml. Chicken embryo fibroblasts
(CEF) were grown in F-10 medium supplemented with 10% tryptose phosphate broth–5% calf serum. D17 cells
were cultured in minimum essential medium (Eagle)–7% fetal calf serum (40). Buffalo rat liver thymidine kinase (TK)-negative
(BRLtk−) cells were grown in minimum essential medium (Eagle) plus 7% calf serum (39). QT-6 cells were obtained from C.
Moscovici and grown as described previously (23).

Virus infection. BRLtk− cells were infected in medium containing 100 μg of Polybrene per ml. CEF were infected in
normal medium. Cells were usually exposed to virus overnight.

Vectors. The ME111 vector has been previously described (8). The vector SW272/cGH was derived by insertion of cGH
cDNA downstream of the 5′ long terminal repeat (LTR) of the SW272 vector (39).

Vector assays. TK transducing units (TKTU) released by 5 × 105 C3 helper cells stably transfected with vector SW272/
cGH were harvested after 6 h of incubation and were assayed by infection of 105 BRLtk− cells. TK-positive cells
were selected for growth in medium (40) containing 1 × 10−4

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Plasmids and chicken DNAAs. All plasmid DNAAs were propagated by using derivatives of pBR322 and the HB101 strain of *Escherichia coli*. The plasmid pSW272 contains a derivative of the spleen necrosis provirus (SNV), lacks most propagating sequences and contains the herpes simplex virus type 1 (HSV-1) tk gene (40). Chicken genomic DNAAs were isolated from Arbor Acres males of meat breeding lines.

Nucleic acid isolation. Chicken embryo DNA was prepared by solubilizing tissue in buffer containing 100 mM EDTA, 1% sodium dodecyl sulfate, 100 μg of protease K per ml (pH 8). Samples were incubated at 60°C for 15 min, then at 37°C with additional protease (100 μg/ml) for 4 h. The DNA was sheared, adjusted to 200 mM NaCl, and extracted twice with equal volumes of phenol and chloroform-isoomyl alcohol (24:1) and once with 2 volumes of chloroform-isoomyl alcohol. DNA was ethanol precipitated and dissolved in 0.01 M Tris-0.001 M EDTA (pH 8.0). Unsheared DNA was used for Southern blot analysis (34).

Nucleic acid analysis. DNA samples were applied to Gene Screen Plus membranes (New England Nuclear Co.) for dot blot analysis by means of 96-well plexiglass manifolds. DNA on membranes was denatured in 1.5 M NaCl-0.5 M NaOH for 15 min, neutralized in 0.5 M Tris (pH 7.5)-1.5 M NaCl for 1 min, blotted dry, and baked at 80°C for 30 min. Hybridizations were carried out as already described (17). Radiolabeled DNA probe was prepared by the method of random priming (13). Southern blot analysis was performed as described previously (34).

cGH analysis. cGH expression was analyzed either by radioimmunoassay (RIA) (35) or by Western immunoblotting (3).

Transfection. The REV-derived helper cell line, C3, was transfected as previously described (14) with the plasmids pSW272/cGH and pHyG (37). Transfected cells were selected for 10 to 14 days in medium containing 200 μg of hygromycin per ml.

Embryo infection. Shell was removed from the area above the blastoderm of unincubated eggs. A Narishig micromanipulator and a 25-μl Drummond pipette fitted with a glass needle were used to inject 5- to 20-μl volumes of cell culture medium containing vector directly beneath the exposed blastoderm. The titer of vector was about 10⁴ TKU/ml as measured on BRLtk− cells. The relative titer of this vector on chicken embryo cells in vivo is unknown. Eggs were resealed with a patch of shell membrane which was covered with Devcon Duco cement and allowed to dry. Eggs were incubated at 37.8°C.

RESULTS

Vectors ME111 and SW272/cGH. The sequence relationships among SNV, ME111, the cGH transducing vector SW272/cGH, and the packaging-defective helper proviruses present in C3 helper cells are shown in Fig. 1. ME111 has been described in detail elsewhere (8). The parental vector SW272 is derived from SNV and contains the HSV-1 tk gene and promoter in the same transcriptional orientation as the viral promoter (39). The cGH coding sequence was originally derived from a cDNA clone made from chicken pituitary mRNA (35). A DNA fragment *Xba*I to *Nco*I contains the complete coding sequence of the cGH gene but lacks the poly(A) addition signal present at the 3’ end of the cDNA. Using Klenow reagent and blunt-end ligation, the cGH sequences were inserted into the unique *Xba*I site within pSW272 located just downstream of the viral 5’ splice donor and packaging sequence, 555 nucleotides from the 5’ end of the viral RNA transcript (39). The orientation of the cGH coding sequence is the same as that of the viral sequences. Proceeding from the 5’ end of the proviral RNA transcript of SW272/cGH, the first ATG encountered codes for the N-terminal methionine of cGH. SW272/cGH is designed to express cGH mRNA transcripts from the viral promoter.

Transduction and expression of REV vectors in vitro. Careful screening of the C3 helper cells transfected with pSW272/cGH and pHyG yielded clone C3-44 which released 2 × 10⁵ TKU/ml into growth medium but very low levels of competent virus. Competent REV in these cultures, as estimated by infection of cultured CEF, was about 10 infectious units of REV per ml or less (17). Western blot analysis of cGH released by C3-44 cells revealed a predominately single band of protein which comigrated with purified recombinant cGH (Fig. 2). The observed molecular size of cGH was about 23,000 daltons. The estimated concentration of cGH in a 72-h harvest of medium of clone C3-44 was at least 500 ng/ml (data not shown). CEF infected with vector released >40 ng of cGH per ml of growth medium as determined by RIA 3 days after infection (data not shown). Western blot analyses of cGH released by cell lines infected with the SW272/cGH vector are shown in Fig. 2, lanes 13 through 18. Cell lines B56 and B20 derive from the canine cell line D17. Cell lines QT82, QT34, QT15, and QT9 derive from the quail cell line QT-6. All of these cell lines release cGH having the same apparent molecular size as purified recombinant cGH (23 kilodaltons) (35). Approximate levels of cGH expression varied from 2 to 10 ng/ml.

Analysis of DNA from chicken embryos after vector infection. Tissue culture fluid (20-μl volumes) containing the vector SW272/cGH was injected beneath the blastoderm of unincubated chicken embryos. Total embryonic DNA was isolated from vector-injected and uninjected control embryos after 7 days of development and was analyzed by qualitative dot blot hybridization with either a radiolabeled cGH probe (Fig. 3A) or a REV vector probe (Fig. 3B). The cGH probe was used to demonstrate that sufficient DNA was present on the filter for detection at low copy number. Of 25 injected embryos, 13 (52%) hybridized to a radiolabeled probe of vector DNA, whereas control DNA from uninjected embryos did not.

To confirm the presence and correct genome organization of vector sequences in infected 7-day embryos, high-molecular-size DNAs from 10 vector-containing embryos were digested with *Bam*HI endonuclease and subjected to Southern blot analysis (34) (Fig. 4). The embryo DNAs examined included those from Fig. 3B, rows 1a, 2a, 6a, 7a, and 8a. Internal *Bam*HI fragments predicted from the cGH vector sequence are diagrammed in Fig. 1. Digestion of integrated proviral vector sequences of SW272/cGH should yield DNA fragments internal to the provirus of 0.86, 2.3, and 1.6 kilobase pairs (kb). A 5’ junction fragment containing the 5’ LTR of the vector linked to host cellular sequences adjacent to the integration site might also be detected. No 3’ junction fragment containing host DNA sequences would be detected, because a *Bam*HI restriction endonuclease site is located at the 3’ end of the proviral LTR. As shown in Fig. 4A, lanes 3 to 7 and 12 to 16, DNAs from these vector-infected embryos show the expected *Bam*HI DNA fragments of 0.86, 2.3, and 1.6 kb when analyzed with a probe derived from the complete SW272 plasmid DNA, which does not contain cGH sequences. The absence of detectable *Bam*HI fragments containing the junction of cellular DNA and integrated vector DNA indicates multiple sites of vector...
proivirus integration during infection of early embryonic cells. No 0.57-kb BamHI fragment predicted from the structure of unintegrated circular forms of either the vector DNA or helper virus DNA was observed. No 1.4-kb fragment diagnostic of the 5' end of integrated replication-competent proviral SNV DNA was observed (39) (see Fig. 1). BamHI-digested DNA from uninjected whole embryos or from blood of uninjected chickens did not hybridize to the vector probe (Fig. 4A, lanes 2, 8, 11, and 17, respectively).

After removal of the SW272 probe (Fig. 4B), the same filters were hybridized with a viral probe specific for the structural genes of REV to detect the presence of replication-competent virus (Fig. 4C). The parental SNV and REV-A proviruses used to derive the helper cell and vectors described here contain internal BamHI fragments of 1.4, 1.8, 2.2, 0.7, and 1.6 kb (see Fig. 1). Only the 1.6-kb fragment would not be detected by the virus-specific probe (Fig. 1) used in this analysis. No virus-specific BamHI fragments were observed, indicating that endogenous and exogenous REV sequences were not detectable (Fig. 4C). Although this result does not rule out the presence of competent helper virus, it shows that efficient gene transfer takes place via the replication-defective SW272/cGH vector. The dot blot on the right of panel C contains various quantities of plasmid pSW253 which carries the entire REV provirus (5).

The filters shown in Fig. 4C were washed to remove probe (Fig. 4D) and were reanalyzed with a cGH-specific probe (Fig. 4E). The fragments of 0.86 and 2.3 kb in lanes 3 to 7 and 12 to 16 are the predicted cGH-containing vector sequences described in Fig. 1. The two bands (asterisks) of approxi-
ultimately 6.4 kb and approximately 2.7 kb, which are common
to all lanes, represent BamHI fragments derived from the
endogenous cGH gene. As expected, embryo DNAs in lanes
3 to 7 and 12 to 16 contain all four fragments derived from
both the vector and endogenous gene. The 1.6-kb BamHI
fragment present in lanes 3 to 7 and 12 to 16 of Fig. 4A is
missing in Fig. 4E, because this fragment does not contain
cGH sequences.

Dot blot hybridization of DNA from brain, liver, and
muscle of four 14-day embryos infected before incubation
showed that two of the four embryos contained vector-
specific sequences in all three tissues. One embryo con-
tained vector sequences in liver and muscle only, and one
embryo was negative (Fig. 3).

Analysis of serum cGH. Circulating levels of cGH were
determined by RIA of serum from thirty 15-day-old embryos
infected with vector before incubation (Table 1). Concentra-
tions of cGH in serum from 16 of 30 injected embryos (55%)
were at least 10 times the level in uninfected control em-
byos, and they ranged from 18 to 254 ng/ml. All 35 control
embryos contained less than 2 ng of detectable serum cGH
per ml. Western blot analysis of cGH immunoprecipitated
from serum of a number of these embryos is shown in Fig. 2,
lanes 5 to 9. The amount of cGH present in serum from
infected embryos is similar to the amount of cGH produced
in vitro by infected culture cells.

Vector sequences in adult chickens. Southern blot analysis
of DNA isolated from blood, brain, muscle, and testis of an
adult chicken (no. 87725) which had been injected as an
embryo with the ME111 vector is shown in Fig. 5. DNAs
were digested with BamHI and BgIII before analysis. The
four different probes used hybridized with the REV se-
quence present in the vector, HSV-1 tk sequences of the
vector, REV structural gene sequences (absent from the
vector), or endogenous cGH genes. All analyzed DNAs from
bird 87725 contained the predicted DNA fragments of 0.74
and 1.6 kb recognized by the REV vector probe and frag-
ments of 1.2 and 1.7 kb recognized by the tk probe (Fig. 5A
and C, respectively). DNA from blood and brain contained
additional hybridizing fragments which probably include
junctions between vector and cellular DNA at sites of
integration (Fig. 5A, lanes 2 and 3). No REV-specific bands
were observed in any of these tissue DNAs (Fig. 5B).

Hybridization with cGH probe revealed endogenous frag-
ments of ~2.7 and ~6.4 kb (Fig. 5D). D17 cells cocultivated
with blood taken from bird 87725 at 4 weeks of age were
reverse transcriptase-negative after 4 weeks of culture and
did not produce detectable tk gene-transducing activity. Of
14 similarly derived birds, 2 were virus positive as deter-
mined by the same assay (17). Although the presence of low
levels of replicating REV in birds like no. 87725 cannot be
ruled out, these results are consistent with infection of
embryonic stem cells with nonreplicating REV vectors.

Southern blot analysis of DNA from semen and blood
of SW272/cGH-positive and control birds is shown in Fig. 6.
Filters containing BamHI-digested DNAs were hybridized
FIG. 4. Southern blot analysis of DNA from 7-day chicken embryos injected with 20 μl of SW272/cGH vector before incubation. High-molecular-size DNA (15 μg) was digested with BamHI before analysis. The same filter was hybridized to three different probes: pSW272 probe (A), probe removed from panel A (B), virus-specific probe (C), probe removed from panel C (D), and cGH-specific probe (E). Probe hybridized to vector DNA in lanes 9 and 10 of panel A could not be completely removed. Sequences recognized by these probes are illustrated in Fig. 1. Lanes: 1 and 18, HindIII-digested lambda phage DNA. HaeIII-digested φX174 DNA, and BamHI-digested uninjected chicken blood DNA; 2 and 11, DNA from uninjected embryos; 8 and 17, DNA from blood of uninjected chickens; 3 to 7 and 12 to 16, DNA from vector-injected embryos; 9 and 10, BamHI-digested DNA of pSW272/cGH (1 ng) plus uninjected chicken blood DNA. BamHI fragments internal to the proviral vector are marked with arrows in panel A. BamHI fragments containing the endogenous cGH sequence are marked by asterisks in panel E. Dot blot on the right of panel C contains the indicated amounts of pSW253 containing the REV-A provirus (5). Sizes are shown in kilobase pairs (Kb).
with radiolabeled DNA probes for the cGH coding sequence, 5' and 3' vector-specific sequences, or REV virus probe (see Fig. 1). Lane 1 of each panel contains a mixture of bacteriophage lambda and φX174 DNAs digested with HindIII and HaeIII, respectively, and BamHI-digested control chicken blood DNA. Lane 9 in Fig. 6A to C contains BamHI-digested control chicken blood DNA and plasmid DNA of pSW272/cGH. Internal BamHI fragments of vector DNA are indicated by arrows. BamHI fragments derived from the endogenous cGH gene are shown by the asterisks. Internal fragments derived from the complete REV provirus are marked by chevrons. Results obtained by hybridization with the cGH-specific probe are shown in Fig. 6A. BamHI-digested DNAs from control semen and blood (lanes 3 and 6, respectively) contain endogenous fragments of ~2.7 and ~6.4 kb. In lanes 4 and 7, DNAs from the semen and blood, respectively, of the vector-positive male contain an additional 0.86-kb fragment which derives from the vector SW272/cGH and hybridizes to the cGH probe. Although visible in the original autoradiogram, the 2.3-kb BamHI fragment derived from the vector is not well resolved from the strongly hybridizing 2.7-kb BamHI fragment derived from the endogenous cGH gene. Blood DNA appears to contain much less of the 0.86-kb fragment than does semen DNA.

Panel B of Fig. 6 shows results obtained when a similar filter was hybridized with the vector probe. Lanes 4 and 7 show that semen and blood DNA from an infected male contain BamHI fragments of 0.86 and 1.6 kb. These fragments derive from the 5' and 3' ends of the integrated vector DNA, respectively. The additional 2.3-kb internal BamHI fragment of vector DNA containing HSV-1 tk sequences does not hybridize to the vector probe used in Fig. 6B nor does BamHI-digested DNA from semen and blood of uninfected control birds (lanes 3 and 6). No 1.4-kb fragment characteristic of replicating REV was observed. The patterns of semen and blood BamHI DNA fragments hybridizing with these probes are similar to each other and are consistent with the pattern observed in BamHI-digested DNA from infected embryos (Fig. 4).

Panel C of Fig. 6 shows results of hybridization with a virus-specific probe. Lanes 1 to 9 are as described for panels A and B. Lane 10 is blank. Lanes 11 and 12 contain BamHI-digested plasmids pSW279 (39) and pSW253 (5), respectively. DNA in lane 11 has a 5' LTR derived from SNV (with a BamHI site) but a 3' LTR derived from REV-A (without a BamHI site). This provirus also lacks a 310-base-pair packaging sequence (E) located near the 5' end of the provirus. The expected fragments generated by BamHI digestion of this DNA are present at ~1.1 (E'), ~1.8, ~2.2, and ~0.7 kb (visible at longer exposure times). Plasmid pSW253 in lane 12 contains the REV-A provirus and lacks the BamHI site present in the SNV LTR. BamHI digestion of this DNA generates the observed ~1.8- and ~2.2-kb fragments. The large fragment of ~9 kb in lane 12 contains 5' and 3' portions of the provirus and a portion of the gag gene.

FIG. 4—Continued.
sequence. The 0.7-kb fragment is observed at longer exposure times. DNAs in lanes 3, 4, 6, 7, and 9 do not contain sequences detectable with probe derived from the structural genes of REV.

**DISCUSSION**

**Early chicken embryo development.** Fertilization and the first 24 h of chicken embryonic development occur in the oviduct and uterus, concomitant with the accretion of albumen and deposition of the eggshell. During this period, attempts at gene transfer into the embryo must allow for surgical removal after fertilization and either reinsertion to the oviduct or extensive artificial culture (25, 26). Both of these approaches are technically difficult. Alternatively, infection of the embryo just after oviposition represents a strategy well suited to vector-mediated gene transfer. The embryo at this stage is composed of at least 10,000 cells arranged in a disk-shaped blastoderm, one to two cells thick and 2 to 3 mm in diameter (10, 20). The day-old blastoderm floats on the yolk above a fluid-filled subgerminal cavity.

Previous studies have provided a detailed description of early chicken embryo development (10, 20) and insights regarding the developmental potential of cells comprising the embryonic blastoderm of a freshly laid egg (9, 11, 12, 22).

Separated posterior and anterior portions of very young unincubated blastoderm appear totipotent, with similar ability to form embryos in vitro (10). The slightly older blastoderm exhibits cells of both upper epiblastic and lower hypoblastic layers. Separated from the lower layer of cells, the upper epiblastic layer retains its pluripotency, regenerates a new hypoblastic layer, and can subsequently form an early-stage embryo in vitro. When dissociated and grown in culture, epiblast cells form structures resembling embryoid bodies formed by murine teratocarcinomas (22). The hypoblastic, in contrast, survives but does not form embryonic structures (22).

All of the above observations suggest that successful infection of the early blastoderm with REV vectors might result in gene transfer into pluripotent embryonic stem cells. However, the REVs are primarily exogenous viruses in the chicken (41). Even though infected dams can transmit the virus vertically to their offspring by shedding virus into the egg (42), nucleic acid sequences closely related to REV are not endogenous to the chicken genome (Fig. 4C). The biology of virus-host interactions may preclude stable insertion of REV sequences into the chicken genome under natural conditions. Insertion of complete REV proviruses into the chicken genome could adversely affect viability, but even defective proviruses appear to be absent from the chickens analyzed in this study.

**Infection of unincubated chicken embryo blastoderm.** We have used replication-defective REV vectors ME111 and SW272/cGH to test the feasibility of retrovirus-mediated gene transfer in the chicken. The C3 helper cell line has been used to generate titers of about 10⁸ infectious units per ml. The ME111 vector carries the Tn5 neomycin phosphotransferase gene and the HSV-1 tk gene and has been described previously (8). The vector SW272/cGH carries a cDNA sequence encoding the cGH mRNA and the HSV-1 tk gene (see Fig. 1). Clone C3-44 released about 10⁴ TKTU/ml and expressed about 500 ng of cGH per ml of culture medium. Analysis by RIA (35) (data not shown) and Western blotting (3) (Fig. 2) showed that cGH released by C3-44 and transduced by SW272/cGH is similar to natural cGH.

Glass needles (40 to 60 μm diameter) were used to deposit medium containing vector directly above and below the surface of the unincubated embryonic blastoderm. This method resulted in successful transduction of vector sequences into recipient embryos. Estimates of the amount of vector injected into the space beneath the blastoderm are based on the titer on BRL1K cells (≈10⁵ TKTU/ml) and the observation that REV titer on chicken cells could be 10- to 100-fold higher (39). We estimate that between 10³ and 10⁴ TKTU were injected per embryo.

**Vector DNA present in 7-day embryos.** Dot blot analysis of 7-day embryo DNA shown in Fig. 3 indicated that about 50% of injected embryos contained detectable vector sequences. Three different radiolabeled probes were used in Southern blot analysis of high-molecular-mass DNA from 10 embryos to distinguish the REV structural genes, the SW272/cGH vector, and endogenous cGH sequences from each other (see Fig. 1). Since most infectious embryonic cells are likely to have a single copy of vector, these blots indicate that a significant percentage of the embryonic cells may carry vector sequences 7 days after infection. This is most evident in comparisons of endogenous and vector-specific BamHI fragments hybridizing to the cGH probe (Fig. 4E). The lack of BamHI fragments specific for replicating REV (Fig. 4C) confirms that gene transfer is primarily the result of the replication-defective REV vector and not of contaminating helper virus (17). These results show that early embryonic

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**TABLE 1. cGH levels in chicken embryo serum**

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<th>Amt (ng/ml) of cGH</th>
<th>Bird no.</th>
<th>Amt (ng/ml) of cGH</th>
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<td>&lt;0.80</td>
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<td>29</td>
<td>1.4</td>
<td>59</td>
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<td>0.76</td>
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*Embryos of unincubated eggs were injected with 10 μl of medium from cultures of clone C3-44. After 15 days of incubation, serum from each embryo was assayed by RIA for cGH.*
cells are susceptible to REV infection and that they persist during development, comprising a significant fraction of the 7-day embryo.

Expression of cGH in vector-injected embryos. Expression of the endogenous cGH gene occurs late during embryonic development. Caudal cells of the pituitary do not contain immunodetectable cGH until day 12 of embryonic development (19), whereas detectable plasma cGH does not appear until day 17 of incubation (15). Furthermore, response of the pituitary to the cGH secretagogue, thyrotrophin-releasing hormone, is not seen until hatching (7). The absence of endogenous REV and the restricted location and timing of endogenous cGH expression facilitate the distinctions between endogenous and vector-encoded genes and their products.

Expression of the cGH gene in vivo resulted in elevated serum cGH levels in about 50% of infected embryos when measured after 15 days of development (Table 1). Levels of serum cGH in 30 injected embryos varied from <1 ng/ml to 254 ng/ml, whereas none of the 35 un.injected controls had serum cGH levels above 2 ng/ml. Immunoprecipitated serum cGH from infected embryos comigrated with purified recombinant cGH as shown by Western blot analysis (Fig. 2). The relative contribution of somatic tissues to circulating levels of cGH is not known. These results are consistent with infection of embryonic stem cells present in the blastoderm at the time of vector injection and expression of vector-encoded cGH.

Vector DNA in tissues of adult males. Southern blot analysis of DNA from an adult male injected as an embryo with ME111 demonstrated the presence of vector in blood, brain, muscle, and testes (Fig. 5). Analysis of semen DNA by Southern blotting confirmed the presence of integrated un-rearranged vector sequences in a low percentage of the sperm cells from a bird injected with SW272/cGH (Fig. 6). The pattern of BamHI restriction fragments observed (0.86 and 1.6 kb) is consistent with that seen in Southern blot analysis of DNA from infected embryos. Probe containing HSV-1 tk sequences revealed the additional 2.3-kb BamHI

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**FIG. 5.** Southern blot analysis of ~20 μg of *Bam*HI- and *Bgl*II-digested DNA from tissues of ME111-positive adult male 87725. (A through D) Replicate blots hybridized with radiolabeled vector probe (A), virus probe (B), *tk* probe (C), and cGH probe (D). Lanes: 1. *Hind*III-digested lambda phage DNA, *Hae*III-digested δX174 DNA and *Bam*HI- and *Bgl*II-digested negative control chicken blood DNA; 2. blood DNA; 3. brain DNA; 4. muscle DNA; 5. testes DNA; 6. blank; 7. *Bam*HI- and *Bgl*II-digested pME111 (50 pg) and negative control chicken blood DNA. Sizes (in kilobases) are shown at the left of each panel.
vector DNA fragment (data not shown) also seen in Fig. 5A. Vector sequences present in semen are not caused by contaminating blood cells since blood contains lower levels of vector DNA per microgram of total DNA, as shown by Southern blotting. Furthermore, blood cells were not detected in vector-positive semen subjected to microscopic examination nor could vector sequences be detected in negative control semen containing 1% vector-positive blood from a different bird. We did not observe any consistent BamHI fragments representing junctions between cellular and vector sequences, probably because of the polyclonal makeup and low overall percentage of cells carrying the vector. No 1.4-kb BamHI DNA fragment characteristic of replicating SNV was observed (40) (see Fig. 1).

Previous work with replication-competent derivatives of Rous sarcoma virus showed that infection of unincubated chicken embryos resulted in germ line insertion of proviral DNA (28, 29). In contrast, the same approach using competent REV resulted in somatic infection but did not lead to germ line insertion of proviral DNA (29). Similarly, follicular injection of REV vectors resulted in the presence of vector sequences in somatic cells, but germ line transmission of these sequences was not demonstrated (32). We recently confirmed germ line transmission of the replication-defective REV vector ME111 administered as described here to chicken embryos (1). Breeding studies are now in progress to determine whether semen from the chickens infected as embryos with defective REV vectors encoding cGH can accomplish germ line transmission of the vector DNA to progeny.

Conclusion. Replication-defective REV vectors can introduce new genetic information into the chicken by infecting somatic stem cells of the embryo. Susceptibility of these stem cells to infection by REV vectors provides another approach to the in vivo study of avian development (4) and vector-mediated gene expression. The possible applications of this technology are numerous.

ACKNOWLEDGMENTS

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LITERATURE CITED


