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Replication-Competent Retroviral Vectors Encoding Alkaline Phosphatase Reveal Spatial Restriction of Viral Gene Expression/Transduction in the Chick Embryo

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Replication-competent avian retroviruses, capable of transducing and expressing up to 2 kb of nonviral sequences, are now available to effect widespread gene transfer in chicken (chick) embryos (S. H. Hughes, J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave, J. Virol. 61:3004–3012, 1987). We have constructed novel avian retroviral vectors that encode human placental alkaline phosphatase as a marker whose expression can be histochemically monitored. These vectors have been tested for expression by introducing them into the embryonic chick nervous system. They have revealed that the expression of retrovirally transduced genes can be spatially and temporally limited without the need for tissue-specific promoters. By varying the site and time of infection, targeted gene transfer can be confined to selected populations of neural cells over the course of several days, a time window that is sufficient for many key developmental processes. The capability of differentially infecting specific target populations may avoid confounding variables such as detrimental effects of a transduced gene on processes unrelated to the cells or tissue of interest. These vectors and methods thus should be useful in studies of the effect of transduced genes on the development of various organs and tissues during avian embryogenesis. In addition, the vectors will facilitate studies aimed at an understanding of viral infection and expression patterns.

Retrovirus-mediated gene transfer has been used both in vivo (25) and in vitro to misexpress genes in order to test their function (reviewed in reference 6). Murine retroviruses have been used most frequently but are problematic for many in vivo studies as they effect gene transfer in only a limited number of cells. Hughes and colleagues (18, 19, 27) have circumvented these problems in birds by developing a series of retroviral vectors that can function as vehicles for widespread gene transfer into chickens. These vectors, derived from Rous sarcoma virus (RSV), are transformation defective and replication competent, allowing them to spread innocuously throughout a population of dividing cells, such as that composing the early embryo. Up to 2 kb of nonviral sequence can be inserted in place of the RSV *src* gene, where it will be transcribed from the viral promoter and translated from a spliced mRNA. Such vectors hold great promise as vehicles to direct the expression of candidate genes in early embryos in order to assay gene function during development (25, 28).

In addition to their utility in studies of gene function, retroviral vectors can also be used to investigate several aspects of retrovirus-host interactions. For example, it has been difficult to analyze the cellular specificity of transcriptional regulation of retroviruses in vivo because of the indirect nature of many of the assays employed to date. Studies of viral infection and expression that rely on measurements of viral DNA and RNA levels or enzymatic activity in tissue homogenates have suggested that there may be tissue-specific regulation of transcription from the viral promoter (5, 16, 17). However, such analyses typically do not reveal information at the cellular level, thereby precluding analysis of differential expression in different cell types in a complex tissue. In situ hybridization of viral transcripts (12), immunohistochemistry of viral proteins (34), and histochemical detection of virally encoded β-galactosidase (30, 35) have been used to circumvent some of these problems in cells and tissue sections. Nevertheless, there are conflicting reports in the literature relevant to potential problems in transcriptional activation of proviruses. A global block in transcription from viral promoters has been well documented in the case of preimplantation mouse embryos (21) and has also been reported for infected cells derived from pregastrulating chicken (chick) embryos (24). On the other hand, several reports show evidence of transcription and translation of viral sequences in a variety of organs and tissues during early avian embryogenesis (3, 28, 30). The availability of a retroviral vector which can spread and carries a histochemically detectable reporter gene which allows detection of long terminal repeat (LTR) activity at a single-cell level in tissue sections should facilitate studies of the regulation of viral gene expression.

The efficiency of retrovirus-mediated gene transfer throughout developing embryos is influenced by variables other than regulation of transcription from the LTR. The site and the time of infection and the titer of the viral inoculum will greatly affect the rate of viral spread and the ultimate locations where a viral gene will be expressed. An additional parameter is the particular host cell receptor utilized by a strain of avian retrovirus. Different viral envelope proteins, which utilize different host cell receptors and define different viral subgroups, may affect ultimate tissue expression patterns (4, 5, 32). Prior to this report, essentially none of these variables had been tested systematically in early embryos with the aim of maximizing retroviral infection and gene transfer into different tissue types.

This report describes the use of several replication-competent vectors to effect widespread gene transfer in the developing nervous system, as well as methods to limit viral spread to defined regions. Both A- and E-subgroup vectors

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that encode an easily assayable histochemical reporter gene, human placental alkaline phosphatase (PLAP), are described. PLAP expression during the first few days following infection was found to be limited to distinct populations of neural cells by infecting chick embryos at different sites and times with PLAP vectors. The examples presented here illustrate the utility of the PLAP vectors in determining the optimal site and time of infection required to target specific cell types and/or organs during avian embryogenesis. Utilizing these methods with vectors that encode candidate genes of unknown function should provide a powerful technique for testing the role of such genes in the development of distinct tissues or populations of cells within a tissue.

MATERIALS AND METHODS

Viral stocks and plasmids. A combined virus stock, designated AMV-MAV, was obtained as a serum concentrate from viremic chickens infected with replication-defective avian myeloblastosis virus (AMV) and two replication-competent myeloblastosis-associated helper viruses (MAVs) of subgroups A and B; the stock was supplied by Life Sciences, Inc., St. Petersburg, Fla. All other viruses are of the ALV (avian leukosis virus) subtype. Rous-associated virus-60 (RAV-60) was kindly provided by L. Crittenden. WF201 is a recombinant retrovirus (E *env* from RAV-0 cloned into the RAV-1 genome) provided by Brown and Robinson (5). RCASBP(A), provided by S. Hughes, is an unpublished variant of the RCAS vector (18) in which sequences derived from the *pol* gene of the Bryan high-titer strain of RSV were cloned into RCAS(A), a derivative of the Schmidt-Ruppin strain of RSV. The cloning strategy was identical to that described for construction of RCANBP from RCAN (27). Recombinant vectors containing Bryan polymerase yield titers that are approximately 10-fold higher than that of the Schmidt-Ruppin strain. A pBR322 plasmid, pRAV-0, containing the RAV-0 genome was obtained from L. Crittenden. A cDNA encoding the human PLAP sequence (2) was obtained from a plasmid, called pDAP, encoding a murine retrovirus (11).

Retroviral constructions. An array of replication-competent retroviral vectors (RCAS) of the A, B, and D subgroups that were produced by Hughes et al. (18) have a convenient *Cla*I cloning site and splice acceptor for insertion and expression of an exogenous gene. We constructed a fourth vector of the RCAS series of the E subgroup. Two hypervariable regions within the extracellular domain of the *env* gene are of particular importance in determining the host range of different viral subgroups, as shown by the fact that recombinants in these regions have altered host ranges (9). A recombinant RCASBP vector was made by using conserved restriction endonuclease sites, *Kpn*I and *Sal*I, that flank the hypervariable regions as described by others (4, 18). Starting with the A-subgroup variant, RCASBP(A), a new vector of the E subgroup, called RCASBP(E), was constructed by swapping a portion of the A *env* with E *env*. The E *env* sequences were obtained from pRAV-0 by *Kpn*I-*Sal*I digestion and isolation of the 1.1-kb *env* fragment. This fragment was ligated to RCASBP(A) in place of the homologous *Kpn*I-*Sal*I fragment. The recombinants were screened for E *env*-specific sequences by polymerase chain reaction amplification using oligonucleotide primers unique to the E hypervariable regions of the *env* gene (9). The 5′ primer (5′-CTTGATCGCCCCGTGGGTCAATCC-3′) was homologous to sequences in hypervariable region 1. The 3′ primer (5′-TCTGCACATCTCCACAGGTGTAAT-3′) was antisense to sequences in hypervariable region 2. Subgroup specificity was further verified by making viral stocks and testing them on E-resistant (line 0) and E-sensitive (line $15b_1$) chick embryo fibroblasts (CEFs).

In order to clone PLAP into RCASBP vectors, the Cla12 adaptor plasmid described by Hughes et al. (18) was used to obtain *Cla*I ends on PLAP. PLAP was removed from pDAP (11) by *Sal*I digestion and inserted into Cla12 at the *Sal*I site. PLAP was removed from Cla12 by digestion with *Cla*I and inserted into RCASBP(A) or RCASBP(E) at the *Cla*I site. Orientation of PLAP was checked by polymerase chain reaction amplification with an oligoprimer from the 3′ untranslated end of PLAP (5′-GCCACCACCTACAGCCCA GTGG-3′) and an antisense primer within the U_3 region of the LTR of Schmidt-Ruppin A RSV (5′-ATCTCTGCAATG CGGAATTCAGTG-3′).

Chick strains. All animals were obtained as fertilized eggs from the suppliers. For injections with A-subgroup retroviruses, Specific Pathogen Free (S-SPF-Standard) White Leghorn eggs (obtained from SPAFAS, Inc., Norwich, Conn.) were used; these eggs were empirically determined to be resistant to E-subgroup viruses (see addendum in proof). Line $15b_1$ is an inbred strain of chickens that are infectible by all major envelope subgroups; they were used in experiments requiring E-subgroup infection. Line 0 chickens are resistant to E-subgroup viruses. They were routinely used for growing subgroup A viral stocks because they lack endogenous proviral sequences related to ALVs (1), usually of the E subgroup, and thus have the least probability of homologous recombination with exogenous viral genomes that can lead to E-subgroup helper viruses in the A-subgroup stocks. Line 0 and line $15b_1$ were obtained from the USDA Poultry Research Laboratory, Ann Arbor, Mich. CEFs of line $15b_1$ and line 0 were prepared by standard techniques (10).

Virus production. All replication-competent viral stocks of the E subgroup were generated in line $15b_1$ CEFs in the presence of 2 μg of polybrene per ml to increase the efficiency of infection (36). Viruses of the A subgroup were generated in line 0 CEFs without polybrene. CEFs were obtained from the torso of embryonic day 10 (E10) chicks (line $15b_1$ or line 0) by mincing, trypsin digestion (0.05% trypsin, 0.53 mM EDTA in HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-buffered saline solution [GIBCO]), and low-speed pelleting in the presence of high levels of calf serum. A general protocol has been described previously (20). Cells were grown in chick tissue culture medium (10% fetal calf serum and 2% chick serum in Dulbecco's modified Eagle medium), split 1:3 when nearly confluent, and frozen in 15% dimethyl sulfoxide in medium for long-term storage in liquid nitrogen. A 10-cm plate of cells was inoculated with 1 to 10 μl of a concentrated viral stock (approximately 2×10^8 CFU/ml). In cases in which a new viral genome was constructed by recombinant DNA techniques, 5 μg of plasmid DNA was transfected into CEFs by calcium phosphate precipitation followed 4 h later by glycerol shock for 90 s (7). Cells were carried for 7 to 10 days to give the virus time to spread to all cells. The population was then expanded to 20 10-cm plates. When the cells were nearly confluent, the medium was removed and replaced with a half-volume (5 ml) of medium with either high serum (10% fetal calf serum, 2% chick serum) or reduced serum (1% fetal calf serum, 0.2% chick serum). The latter technique is currently our method of choice as the pellets obtained after concentration are considerably easier to resuspend (24a). The next day, the supernatant was collected

and a small aliquot was set aside for titration. The remainder was harvested immediately or frozen at −80°C until a later date. Supernatant was collected from the confluent CEFs on two successive days.

After the supernatant was thawed, it was kept on ice or at 4°C for all subsequent steps. A low-speed, 30-min spin was used to pellet cellular debris; alternatively, filtration through a 0.4-μm Millipore filter is sufficient and may produce better results (24a). A high-speed spin (21,000 rpm in a Sorvall SW28 rotor) for 2 to 4 h at 4°C was used to pellet the virions. The supernatant was carefully removed, and the pellets were resuspended into 1% of the original volume by gentle trituration with a micropipette over the course of 30 to 60 min. Virus was stored at −80°C as aliquots of 20 to 30 μl. Details of virus production, concentration, titration, and helper tests can be found in reference 7.

Virus titration and subgroup specificity. The titers of virus stocks were determined by infecting QT6 quail cells (26) for subgroup A or line $15b_1$ CEFs for subgroup E. Cells were plated at a one-sixth confluence the day before infection. Limiting dilutions of virus were added to duplicate wells in a one-fourth volume of medium; E-subgroup infections were accompanied by 8 μg of polybrene per ml (36). After 4 h, the medium was brought to full volume (with a final concentration of 2 μg of polybrene per ml for the E subgroup). After 48 h, the cells were fixed and immunostained for viral *gag* proteins as described below. The titers of PLAP vectors were determined by PLAP histochemistry as described below. Positive colonies were identified and counted to obtain the number of CFU per milliliter.

E-subgroup virus stocks were determined to be free of contamination by other subgroups as follows. Ten microliters of the concentrated virus stock was added to E-resistant line 0 cells in the presence of 2 μg of polybrene per ml. The cells were passaged for 7 to 10 days to permit the spread of potential contaminants and then fixed and stained by using anti-*gag* antibodies as described below. Parallel cultures of infected line $15b_1$ cells were used as positive controls. All E-subgroup viral stocks were determined to be E specific as judged by their failure to infect line 0 cells in this assay, i.e., no antibody-positive cells were detected.

Virus injection into embryos. Inbred strains of fertilized eggs that were not incubated immediately were stored at 18°C for no more than 3 days. Unincubated S-SPF eggs were stored at 4°C. Eggs were warmed to room temperature before incubation at 38°C in a high-humidity, rocking egg incubator (Petersime, Gettysburg, Ohio). The first 24 h of incubation at 38°C was considered E0. On E1.5 to E2.5, a 20-gauge needle was used to remove 1.5 ml of albumen from the pointed end of the egg. The egg was placed on its side, and small, curved scissors were used to cut through the shell to expose the underlying embryo. Embryo stages were determined according to reference 15.

For virus injection, pipettes were made from pulled glass capillary tubes (Omega Dot, 1-mm outside diameter and 0.75-mm inside diameter; FHC, Brunswick, Maine) that were broken to a tip diameter of approximately 20 to 30 μm. A 1/10 volume of tracking dye (0.25% fast green, filter sterilized) was added to the concentrated virus stock. In the case of E- but not A-subgroup viruses, a 1/10 volume of polybrene (8 mg/ml) was also added to the viral inoculum. It was empirically determined that the optimal concentration of polybrene was 10- to 100-fold higher for in vivo compared with in vitro infections. The injection pipette was attached to a motorized, direct-displacement microinjection apparatus (Stoelting, Wood Dale, Ill.) and backfilled with viral inoculum. The pipette was lowered with a micromanipulator into the appropriate structure of the embryo (otocyst, ventricular system of the brain, or subretinal space of the eye). Approximately 0.1 to 1.5 μl of inoculum was injected and could be followed visually by virtue of the tracking dye. Following injections into the eye cup on E3, the virus inoculum would flow into the neural tube through the optic stalk, which is still in continuity with the ventricular system of the brain at these stages. The dye in the inoculum could usually be traced from the eye through the midbrain and into the hindbrain for injections of 1 to 1.5 μl. In some cases, it was also possible to trace dye into the spinal cord. The window in the shell was sealed with Scotch tape, and the egg was returned to a nonrotating, benchtop incubator (Kuhl, Flemington, N.J.) with high humidity.

Histology. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 to 12 h and rinsed thoroughly in PBS over several hours. The tissue was prepared for sectioning by immersion through graded sucrose solutions (10, 20, and 30% in PBS). It was embedded in 7.5% gelatin–15% sucrose–0.05% sodium azide in PBS and frozen with liquid nitrogen. Tissue was stored at −80°C. Sections were cut at −25°C on a Bright cryostat at 18 to 30 μm, placed onto aminoalkysilane-coated slides (31), and air dried overnight. Sections were postfixed for 15 min with 4% paraformaldehyde and washed thoroughly.

Tissue culture cells were fixed in 4% paraformaldehyde for 15 min and washed in PBS. Thereafter, they were processed in a way similar to that for tissue sections.

For AP histochemistry, whole embryos, cells, or sections were immersed in PBS at 65°C for 30 min to inactivate endogenous AP. The staining procedure has been described previously (11). In some cases, whole-mount embryos were embedded and sectioned as described above. Sections were coverslipped with Gelvatol (33).

An injection artifact was occasionally observed for embryos harvested 12 h after PLAP vectors were injected into the neural tube on E2. In these cases, the entire central nervous system appeared to be completely positive for AP activity when viewed as a whole mount (data not shown). On closer inspection, however, it was obvious that the reaction product was not present in individual cells but was diffusely distributed within the ventricles of the neural tube. Our interpretation of this observation is that the viral inoculum, which was concentrated approximately 100-fold from tissue culture supernatant, is contaminated with AP protein originating from the CEF cells used to produce the virus stock. This diffuse reactivity is usually (but not always) cleared from the ventricular system by 24 h after infection.

We found that whole-mount AP staining may pose a particular problem for neural tissues that are massively infected, even if previously dissected: for example, 4-h whole-mount staining of heavily infected E16 chick retina resulted in an accumulation of reaction product on the retinal surfaces with a failure of the staining reagents to penetrate beyond 50 μm or so. Restaining after sectioning resulted in complete labelling of all retinal layers. Incomplete penetration of the retina is not a problem when a radial cluster of infected retinal cells is flanked by uninfected tissue in either chick (10a) or mouse (11). This supports the idea that this is not a problem with diffusion per se but is unique to heavily infected areas, where a buildup of reaction product on the surface can prevent further diffusion of reagents. There may be other situations in which the reagents have difficulty penetrating through specific, uninfected tissue layers, such as the corneal epithelium of the intact eye.

For anti-*gag* immunohistochemistry, cells or sections were fixed and washed as described above. They were preincubated in blocking solution (10% fetal calf serum, 2% chick serum, 0.05% Triton X-100 in Dulbecco modified Eagle medium) for 30 min. Both primary and secondary antibodies were diluted in blocking solution. Primary antibodies against two different *gag* proteins, MA (matrix protein, p19) or CA (capsid protein, p27) (nomenclature according to reference 22), were used and incubated for 1 h. Anti-CA antiserum (rabbit anti-p27 immunoglobulin G; SPAFAS, Inc.) was diluted 1:2,500; 3C2, a mouse anti-MA monoclonal antibody (29), was collected as a supernatant of hybridoma cells (gift of D. Boettinger) and diluted 1:4 or 1:10. After rinsing in PBS, biotinylated secondary antibodies (1:400 dilution; Vector Laboratories) were applied for 1 h. Additional rinsing in PBS was followed by amplification and detection with either AP histochemistry or diaminobenzidine histochemistry. In the first case, tissue was incubated for 1 h in streptavidin-conjugated AP (Zymed; 1:150) and detected by reacting for 10 to 20 min in X-phosphate detection solution, pH 8.8, as described in reference 34. For diaminobenzidine immunohistochemistry, tissue was incubated for 1 h with the avidin-biotin-peroxidase complex of the Vectastain Kit (Vector Laboratories) and detected with diaminobenzidine following the manufacturer's recommendation. Endogenous peroxidase activity was inhibited by a 30-min incubation in 0.3% H_2O_2 in methanol (−20°C) after the primary antibody step. Sections were coverslipped in Gelvatol.

RESULTS

Retroviral vectors encoding AP. In order to monitor the temporal and spatial spread of virus in embryos, and to assay for successful expression of a nonviral gene ("transgene") in different cell types, vectors that included a marker transgene whose product could be histochemically demonstrated in tissue whole mounts were constructed. The human PLAP gene was cloned into the RSV-derived vector, RCASBP(A), to create a virus named RCASBP/AP(A) and abbreviated APA for this report. A similar vector of the E subgroup was also constructed. In the process of constructing this latter vector, a recombinant version of RCASBP that carried portions of the *env* gene known to confer E-subgroup host range (9) was created. This vector, RCASBP(E), brings to four the total number of subgroups of RCAS vectors currently available (the others include A, B, and D). Cloning PLAP into this vector generated RCASBP/AP(E), which is abbreviated APE for this report. The RCASBP/AP vectors are shown schematically in Fig. 1. Titers of unconcentrated virus were 1×10^6 to 3×10^6 CFU/ml before concentration and 2×10^8 to 4×10^8 CFU/ml after concentration. The stability of PLAP in these viruses was demonstrated in vitro following infection of CEFs (for APA and APE) and QT6 (for APA). Cells were infected at a low multiplicity of infection when stocks were being titrated and were subsequently stained with anti-MA 2 to 3 days postinfection. Positive colonies were scored, and then the dish was stained by AP histochemistry. A comparison between the numbers of colonies that stained positively for each stain was then made. All colonies that were MA^+ were also AP^+. In addition, CEFs were infected at a low multiplicity of infection and passaged for 7 to 10 days. They were then stained by AP histochemistry, and no negative cells could be detected.

Chick cells infected with PLAP vectors showed exquisite morphological detail following histochemical processing, as previously shown for murine cells (11, 14). After infection of the chick embryonic nervous system, complete staining of cell bodies, dendrites, and axons was seen. This point is illustrated by examples from the retina, where the structural details can be appreciated from a whole-mount preparation. For example, Fig. 2A shows a retinal ganglion cell with a well-labelled dendritic arbor; the axon of this cell could be followed for many millimeters as it coursed across the retina to enter the optic nerve. Two examples of a distinctive retinal cell type, the starburst amacrine cell, are shown in Fig. 2B. Many examples of growing axons tipped with growth cones, such as the example in Fig. 2C, could also be seen. Epithelial cells, mesenchymal cells, and muscle fibers, as well as other cell types, were also well labelled by AP.

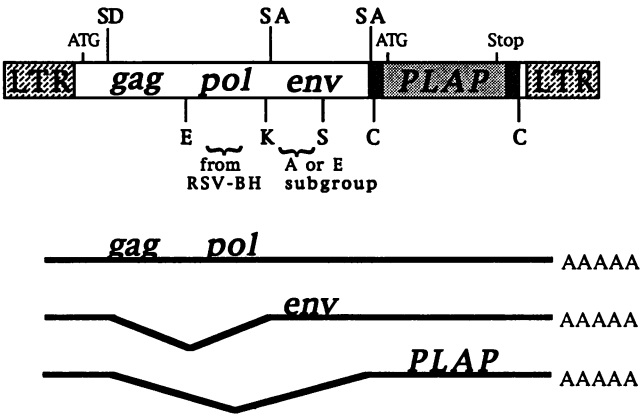

FIG. 1. RCASBP/AP vectors: schematic representation of RCASBP/AP proviral genomes (open bar) and the putative mRNAs (solid lines followed by multiple A's) encoded by the vectors. The restriction sites relevant to the cloning of the constructs are indicated below the diagram of the proviral genome and are as follows: E, *Eco*RI; K, *Kpn*I; S, *Sal*I; and C, *Cla*I. Abbreviations: SA, splice acceptor; SD, splice donor. Polylinker sites and regions reconstructed by Hughes and Kosik (19) are indicated by dark hatched bars.

Targeting different populations of embryonic cells by altering the site and time of infection. To monitor when and where transduced genes are expressed after embryonic infection, a series of injections were performed at different sites and stages of development with the goal of maximizing infection of neural tissues. Both A- and E-subgroup viruses were tested with vectors transducing the PLAP marker gene, which allows easy histochemical detection of the spatial and temporal extent of expression.

(i) Otocyst injections. The otocyst is a placode-derived structure which will go on to form all of the sensory and supporting structures of the inner ear, including both auditory and vestibular organs, as well as the ganglion cells of the VIIIth cranial nerve which will innervate these organs. Injections of APE or APA were targeted into the otocyst (Fig. 2D) at stages 16 and 17 (stages according to Hamburger and Hamilton [15]). After 3 days, this structure was almost exclusively labelled (Fig. 2E). Infected embryos allowed to survive for 7 days, until E10, showed AP labelling that was largely restricted to the inner ear, the surrounding otic capsule, and the VIIIth cranial ganglion. There was a limited amount of labelling within the adjacent central nervous system that appeared to be primarily glial (data not shown). We conclude that targeting the viral injection into the developing otocyst is an effective mechanism for spatially

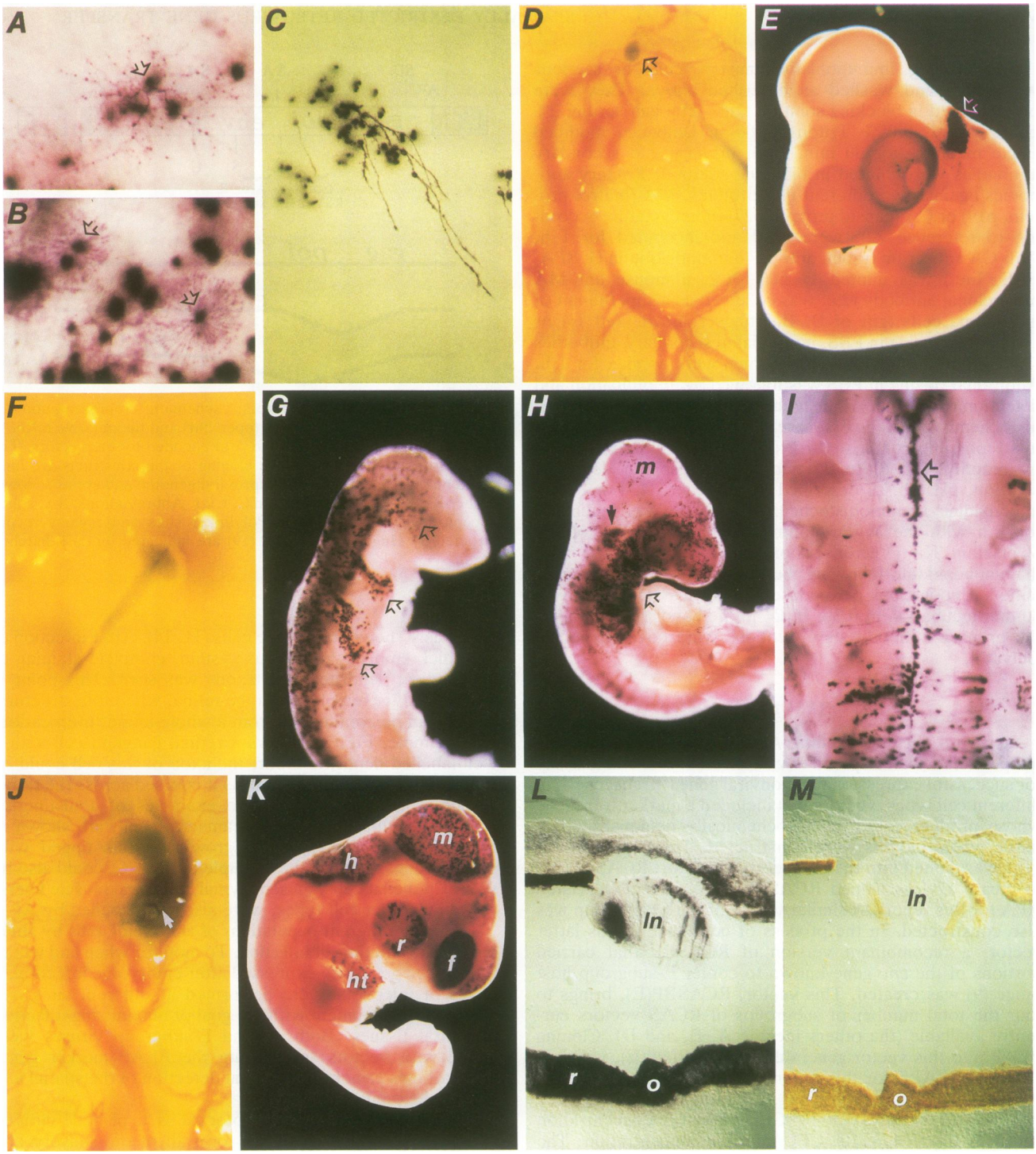

FIG. 2. AP viruses injected into chick embryos at different sites and stages of development. (A and B) Retinas stained as whole mounts for AP after injection of APA at stage 12 and processing on E16. (A) Retinal ganglion cell (arrow). (B) Two starburst amacrine cells (arrows) are evident amidst many radial columns of labelled cells. (C) E6 ganglion cells with growing axons projecting toward the optic stalk were labelled by APA injection into the retina and neural tube at stage 14. (D) Virus injection into the right otocyst (arrow) of a stage 17 embryo is revealed by the presence of fast green as a tracking dye. (E) E5 embryo harvested 22 h after injection of APE into the left otocyst (arrow) at stage 16. (F) Virus injection into the neural tube at stage 10. (G) E2 embryo processed 26 h after injection of APE into the neural tube at stage 10. Neural crest cells are evident all along the length of the neural tube; there are three streams of neural crest cells arising both rostral and caudal to the developing otocyst (arrows). (H) E3 embryo processed 48 h after injection of APE into the neural tube at stage 10. There is heavy labelling of presumed cranial neural crest derivatives (arrow) including mesenchymal cells in the face and branchial arches and cranial nerve ganglia (solid arrow). Most other scattered positive cells in the head are epithelial. Midbrain (m) is negative. (I) Midline cells of the midbrain (arrow) and hindbrain are heavily labelled 48 h after infection with APE at stage 11. (J) Virus injection into the subretinal space (arrow) at stage 14 will fill the eye cup as well as the brain ventricles from a rostral to caudal direction. The tracking dye is visible as far posteriorly as the rostral hindbrain. (K) E5 embryo injected with APA through the eye at stage 14. Note extensive labelling of the forebrain (f), midbrain (m), hindbrain (h), and retina (r) and scattered labelling in the heart (ht). (L and M) Adjacent sections through the retina (r), optic nerve (o), and lens (ln) of an E7 embryo that had been infected with APE on E3. The patterns of staining for AP (L) and anti-MA (M) are similar.

restricting gene transfer to the structures arising from the otocyst and its immediately surrounding tissues.

(ii) Neural tube injections. A systematic analysis of injections into different stages of the neural tube was conducted. Both A- and E-subgroup vectors were tested in the appropriate chick strains. Line $15b_1$ embryos (n = 52) were infected with the subgroup-E vector, APE. Most of the observations that follow have also been confirmed on a less extensive set (n = 14) of S-SPF embryos injected with the subgroup-A vector, APA. Our expectation in doing a time course of virus injection was that the earliest injections would yield the most widespread infections of neural tube derivatives. Surprisingly, this was not the observed result. Injections done prior to stage 7 resulted in nonviable embryos. This result was obtained even when using low-titer, replication-defective viruses that did not contain PLAP. Therefore, the high fatality rate probably reflects the manipulation per se, and/or toxicity in the concentrated tissue culture supernatants, rather than an effect of viral infection on early embryos. Interpretable data were obtained for injections between stages 7 and 17. Within this window, the earlier injections generated widespread infection of the neural crest, while the later injections generated widespread infection of the central nervous system, as described below.

It was of interest to determine when virus expression is first detectable after injection in order to estimate the earliest potential effects of transgene expression. Embryos sacrificed 12 h after infection showed minimal specific labelling with AP (data not shown), while embryos sacrificed at least 21 h after injection showed widespread labelling (Fig. 2G).

(iii) Neural tube injections—stages 7 to 10. Small injections of 0.1 to 0.5 μl were targeted into the developing neural tube at early stages (stages 7 to 10) as shown in Fig. 2F. After 24 h, AP-positive cells are distributed along the length of the embryo, with a concentration in the migratory pathways of the neural crest. Neural crest cells can be observed leaving the region of the hindbrain and streaming towards the developing branchial arches and face (Fig. 2G).

By 48 h after infection, the positive cells in the head are fairly well restricted to the branchial arches and cranial nerve ganglia (Fig. 2H). If a larger viral inoculum (0.5 to 1.5 μl) was used, there was proportionally greater infection of the face, especially in the epithelium. In addition, there was more-widespread labelling of the nasal and maxillary facial mesenchyme and spinal cord neural crest derivatives. In contrast, there was only restricted labelling in the brain following either large or small injections. If the brain was cut along the dorsal midline and splayed open, the full pattern of labelling within the neural tube could be seen. One consistent observation was the striking pattern of positive cells aligned along the midline following injections at stages 9 to 11 (Fig. 2I). These cells extend as a nearly continuous line from the rostral half of the hindbrain into the floor of the midbrain. The pattern breaks up in the diencephalon. In addition to the midline cells, there were scattered cells infected throughout the brain. The most consistent labelling was found in the diencephalon, posterior hindbrain, and spinal cord.

By 3 days after infection, labelling in the majority of the brain and retina was still sparse. Thus, relatively small viral injections (0.1 to 0.5 μl) into the neural tube at stages 7 to 10 can primarily target cells of the neural crest, their derivatives in the cranial ganglia and branchial arches, cells in the ventral midline of the midbrain and hindbrain, and some neurons in the hindbrain and diencephalon.

(iv) Neural tube injections—stages 11 to 13. Injections into the optic cup and neural tube at intermediate stages (stages 11 to 13) were processed 2 to 3 days later. There was some labelling of the cranial neural crest, neural tube, and retina, although none of the structures appeared to be optimally labelled following injections at these stages.

(v) Neural tube injections—stages 14 to 17. Injections into the neural tube at later stages (stages 14 to 17) are shown in Fig. 2J. At these stages, virus was injected behind the retina, which resulted in filling of the ventricular system through the optic stalk (Fig. 2J). Processing 2 to 5 days after injection yielded extensive labelling of cells in the retina, forebrain, midbrain, and hindbrain (Fig. 2K). In sharp contrast to the pattern seen after injections at stages 7 to 10, there was almost no infection of the cranial neural crest or its derivatives. Staining of the interior of the brain also revealed a different pattern. The ventral midline cells that were so obvious from earlier injections were now largely negative (data not shown), although other hindbrain cells were well labelled. This population of hindbrain cells appeared to be distinct from those labelled after stage 7-to-10 injections in that they primarily occupied more-lateral positions.

Comparison of transgene and viral protein expression. Because the RCASBP/AP vectors use a distinct spliced transcript for AP expression (Fig. 1), it was informative to assess the degree to which AP expression reflects general transcription from the viral LTR. To assay this, adjacent sections were processed for AP histochemistry to monitor expression from the spliced transcript or for anti-MA immunohistochemistry to detect protein translated from the full-length transcript. Figure 2L and M show this comparison for an embryo processed 4 days after infection with APE. In general, the distribution of AP and MA was quite comparable in brain, eye, and other tissues in the few cases in which this was examined. This suggests that AP expression appears to be a fairly accurate reflection of general expression from the viral LTR. The more intense appearance of the AP staining relative to MA reflects the membrane-associated distribution of PLAP (i.e., cells are labelled throughout, including within processes), as well as the greater sensitivity of the detection method for this enzyme relative to immunohistochemical detection of MA.

Comparison of the rate of spread with AMV versus ALV retroviruses. The results with infection at stages 7 to 11 with ALV retroviruses suggested that many cells in the developing neural tube may be refractory to retroviral infection and/or expression at early developmental time points. It was of interest to determine whether this was a general phenomenon or whether it could be attributed to specifics of the viral inocula used. To test this, experiments were conducted with several different viral stocks in an attempt to maximize infection of neural tissue at early stages. The most extensive infections were obtained with a high-titer viral stock (AMV-MAV) that contained a mixture of the replication-defective AMV (A subgroup) and two different replication-competent helper viruses, MAV-1 (A subgroup) and MAV-2 (B subgroup). Virus was injected into the ventricles of chicken embryos (S-SPF) at stages 9 and 10, harvested at varying times thereafter, and processed for anti-*gag* immunohistochemistry. The neural tube is not completely closed at the time of injection; therefore, viral inoculum targeted to the neural tube will spill out of the anterior neuropore and the posterior neural fold to infect the surface ectoderm (for an example, see Fig. 2F). Like the RCASBP/AP injections, we found no evidence of viral protein expression 10 h after infection. By 21 h after injection, *gag* protein was detected throughout the central nervous system, in migrating neural

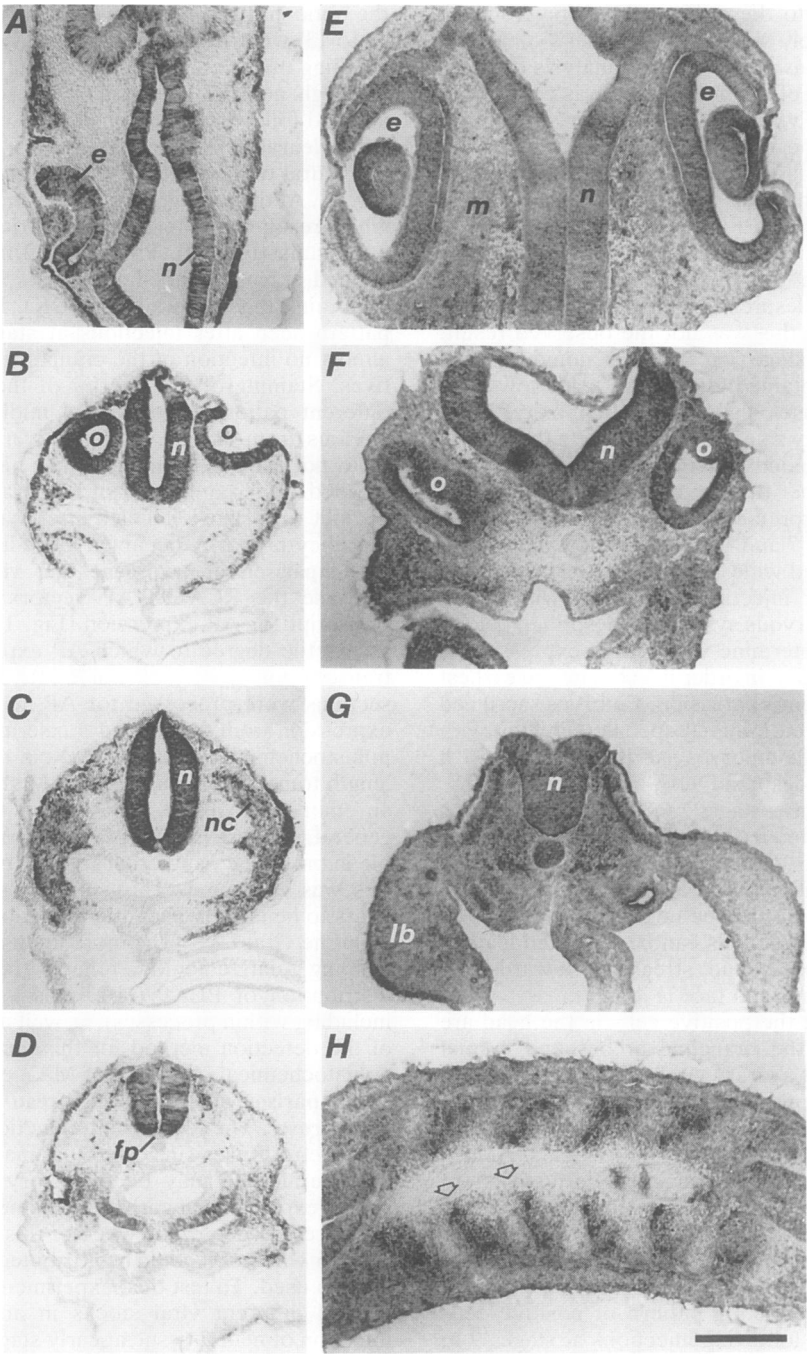

FIG. 3. Histological localization of retrovirus in sections through two embryos harvested either 21 h (left) or 48 h (right) after infection at stage 10 with AMV-MAV. Virus was detected by immunohistochemical staining of the viral *gag* protein, MA, with a biotin-avidin-AP detection protocol. (A) A partially skewed horizontal section through the head showing virus in the neural tube, some mesenchymal tissue, and the left retina and lens. Sections posterior to the midbrain of the same embryo are cut in a transverse plane; shown are sections at the level of the otocysts (B), the cranial neural crest (C), and the spinal cord (D). Sections through a second embryo harvested at 48 h after infection are shown at the level of the eyes (E), the otocysts (F), and the forelimb (G). In panel H, a grazing section through five somites and the notochord reveals a striped pattern which is higher in the anterior half of each somite (arrows) compared with the posterior half. Bar, 250 μm. Abbreviations: e, eye; fp, floorplate; lb, limb bud; m, mesenchyme; n, neural tube; nc, neural crest; o, otocyst.

crest cells, and in surface ectoderm (Fig. 3A to D). The forebrain had a lower percentage of labelled cells than other parts of the central nervous system. By 48 h after infection, most tissues in the embryo were immunolabelled (Fig. 3E to H), showing that viral spread and protein expression are both rapid and extensive. Again, labelling of the forebrain was less extensive. These data clearly demonstrate that beginning at least as early as stage 10, most cells in the avian neural tube can be infected and express from the LTR.

A number of other ALV viral stocks of both the A and the

E subgroups were subsequently used to infect embryos. All appeared to spread more slowly than the AMV-MAV stocks, possibly because of their lower initial titers. In our experiments, the typical titers of replication-competent stocks were 1×10^8 to 4×10^8 CFU/ml after concentration, compared with an estimated titer of greater than 10^{10} CFU/ml for AMV-MAV. Of the replication-competent viruses that were tried, including RAV-60 and WF201 (both E subgroup), APA and APE appeared to spread most slowly. Although the difference in spread rate is difficult to quantify precisely because of variability between specimens, we attempted to compare the different stocks by assessing the extent of infection in retinas injected on E3 (stage 16) and harvested on E6. In contrast to WF201 or RAV-60, which gave greater than 90% labelling (with occasional large patches uninfected), only 40 to 50% of the cells appeared to be labelled with APA. The comparatively slower spread of the RCASBP vectors may account for the success of using these vectors to target gene transfer to different tissues simply by varying the time and site of infection.

DISCUSSION

Novel vectors: RCASBP(E) and AP vectors. Since Hughes et al. (18) have created RCAS vectors of the A, B, and D subgroups, and we have created one of the E subgroup, vectors appropriate for infection of many tissues of the embryonic chick are now available. PLAP vectors of different subgroups are simple to assay and thus offer a simple reagent for testing infectibility through different receptor subgroups. All of the RCAS vectors contain the same LTR, that of Schmidt-Ruppin RSV. Through construction of vectors that vary in other sequences, for example, MAV LTR versus ALV LTR, any additional tissue specificity in replication could be monitored.

In tissues which are infectible by more than one subgroup, the existence of different subgroup vectors offers an additional benefit: the potential for delivering more than one transgene to a single population of cells. Infection of a cell by a replication-competent retrovirus results in a block of superinfection by another virus of the same subgroup by interfering with subsequent receptor binding (reviewed in reference 37). Vectors utilizing distinct receptors do not block each other, thus allowing the possibility of double and triple gene delivery events.

The histochemical detection method for AP reveals superb morphological detail of neurons and their processes, including both axons and dendrites (see also references 11 and 14). The ease of detection in whole embryos, as well as being useful when quickly screening for an optimal infection protocol, aids in identifying patterns of labelling and migration of infected cells. However, deep structures may not be optimally detected by whole-mount staining and may necessitate further dissection or sectioning, followed by restaining, for best results (see Materials and Methods).

Use of retroviruses for gene transfer into early embryos. Using RCASBP/AP vectors, we have shown that it is possible to target gene transfer to selected populations of neurons over the short term (3 to 7 days). We have also been able to achieve precise selectivity over the long term by infecting chimeric embryos with specific envelope-subgroup retroviruses (10). Retrovirus-mediated gene transfer into restricted cell populations or regions of the embryo allows one to focus the effect of a transgene on specific developmental interactions. For these types of experiments, a transgene would replace PLAP and infected cells would be identified by immunohistochemical detection of either the *gag* protein(s) or, when possible, the transgene product.

The promise of this approach was recently realized when the RCASBP vector system was used to analyze gene function in the developing chick limb bud (25). Members of the Hox 4 gene cluster normally show partially overlapping, spatially restricted patterns of expression during limb outgrowth (8). Morgan and colleagues (25) used RCASBP to expand the spatial domain of expression of Hox 4.6, resulting in clear pattern defects in digit formation. In this study, precise targeting of retroviral inoculum to the unilateral limb anlage limited misexpression of the Hox 4.6 transgene to only the injected limb at the critical developmental stages, within 48 h of infection.

Patterns of expression in the developing embryo. After injection of RCASBP/AP viruses at stages 7 to 17, the patterns of AP expression varied as a function of the site and time of injection, as well as the survival time. There did not appear to be a difference in expression between A- and E-subgroup variants. Most of the data were derived from animals sacrificed within a few days of infection and therefore probably reflect the results of initial infection with only a limited amount of spread. After longer survival times, the extent of spread was, in some cases, surprisingly restricted. For example, when virus was targeted to a sufficiently defined region, such as the developing otocyst, the spread appeared to occur primarily cell to cell, rather than diffusely through the animal. This held true for at least as long as 1 week after infection. This probably indicates that immature virus particles, still in the process of budding, attach to adjacent cells and thus only rarely become free particles that are capable of diffusing more widely to attach to distant target cells. With larger injections, such as into the neural tube, the extent of spread was more variable and may have reflected variation in the size of the inoculum as well as the degree to which vascular, epithelial, and/or mesenchymal tissues were exposed to virus at the time of the injection.

Injections of concentrated RCASBP/AP virus into the early neural tube did not result in uniform expression of AP throughout the neuroepithelium when assayed within a few days of infection. Injections performed prior to stage 11 were most effective at achieving expression in neural crest and ventral midline cells, some ventrolateral cells in the hindbrain, and some spinal cord cells. The midline cells in the hindbrain probably correspond to floorplate cells; clonal analysis of these cells revealed clonal expansion and/or migration in a rostrocaudal direction (13) that is consistent with the pattern we saw by retroviral infection. Unexpectedly, other neural tube-derived structures did not express AP with high efficiency, including the forebrain, midbrain, and dorsolateral parts of the hindbrain. Injections performed 12 to 24 h later, at stages 14 to 16, yielded the opposite pattern with respect to head structures. That is, widespread expression was evident in the brain, excepting cells along the ventral midline, which were negative. The cranial neural crest was also negative. It is possible that the entire central nervous system could be fully infected by sequential injections over several stages.

Potential mechanisms underlying restricted nervous system expression. It is of interest to consider the possible mechanisms underlying the different expression patterns in the neuroepithelium exhibited after short survival times. One likely explanation for the decreased expression in cranial neural crest with later injections is that the target population migrates away from the injection site at the brain ventricles (23) and so is not directly available for infection. Since many

cells of this population continue to divide, they probably remain infectible for some time and would most likely be subsequently infected as the virus spreads more widely through the embryo. In the case of the midline floorplate cells, this population may become postmitotic at early stages (by E3), thus blocking successful viral reverse transcription and integration.

More perplexing is the observation that the majority of cells in the brain have undetectable AP expression in the first few days following viral injections at early stages of development. The brains of embryos infected with AMV viral stocks had no such limit to retrovirus-mediated gene expression. The absence of expression with the PLAP vectors could be due to a block at any of a number of steps in the viral life cycle, including binding to receptor, integration, transcription, splicing, and translation. A number of these steps could be assayed independently, and experiments to address this are under way.

It is possible that the lack of early neural tube expression is due to stage- and tissue-specific variation in expression of viral receptors, although we saw no difference in the two different viral subgroups, A and E, that were investigated. In the context of using retroviral vectors to target gene transfer into embryonic tissues, such variability would indicate that viruses of distinct subgroups might be needed to target distinct tissues. Variability in the receptor classes expressed by different cell types, as well as variability in transcription of different LTRs, has been suggested by previous observations of tissue-specific expression of different subgroups of ALVs. For example, in the newly hatched chick, challenge with infectious retroviruses can yield distinct patterns of expression in different tissues that vary systematically with the envelope subgroup and the specific LTR (5). The pathogenic potential of retroviruses is also affected by these variables, a result probably accounted for by the extent of replication through specific target tissues, such as the bursa (4, 32). These studies emphasize the potential importance of using the most appropriate subgroup of retroviral vector when attempting infection and/or gene transfer into distinct tissues in the developing chick. Recent literature (reviewed in reference 5) indicates that subgroup A viruses can efficiently infect a wide range of connective, endocrine, and reproductive tissues including bone marrow, osteocytes, adrenal cortex, thyroid, bursa, gonad, skeletal muscle, and neural tissues (present results) but are less effective in hematopoietic tissues; subgroup B viruses are better than subgroup A in infecting yolk sac macrophages, thymus, and hematopoietic tissues; subgroup E viruses are efficient in infection of bursa, thymus, hematopoietic tissues, and neural tissues (present results).

The experiments using AMV/MAV stocks clearly showed that most cells of the chick central nervous system will express viral *gag* proteins within 21 h of infection with AMV at stage 10. Thus, it appears that post-neurula-stage embryos are capable of transcription and translation of exogenous retroviral sequences. It may be that AMV-based vectors will be found to be more efficient at gene transfer into the developing nervous system than the RCASBP vectors.

Also worth considering is the fact that the RCASBP/AP viruses use a distinct spliced transcript for translation of AP that is different from the transcripts used to make the viral structural proteins (Fig. 1). It is this spliced variant that was assayed by AP histochemistry. Translation of the full-length unspliced genomic transcript that serves as the mRNA for the *gag* structural proteins can be assayed independently to determine whether the absence of AP expression is related to differential splicing. The relative distributions of *gag* versus AP were not found to be markedly different 4 days after infection (see Results), but this has not been systematically studied for all stages of injection. Nonetheless, from the point of view of using RCASBP vectors for gene transfer, it is the specific distribution of the spliced variant, and its resulting protein, that is of biological importance.

Summary and future directions. Replication-incompetent retrovirus vectors have proven to be very useful for lineage analysis, immortalization of primary cells, and functional studies of genes in vitro. The more recently available replication-competent vectors have greatly expanded the utility of retrovirus vectors for gene transfer studies in intact animals in that large numbers of cells can be stably and efficiently transduced. The infection protocols described here, as well as the availability of the PLAP vectors, broaden the potential of the system even further in that partial control of the specificity of infection can now be achieved. Clarification of the role(s) of genes involved at multiple points and in multiple locations during development should thus be possible.

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ADDENDUM IN PROOF

Subgroup resistance of the standard eggs provided by SPAFAS, Inc., should be determined empirically, since the line of chicken can change without notice.

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