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Retroviral infection coupled with tissue transplantation limits gene transfer in the chicken embryo

(chimera/nervous system)

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ABSTRACT **Gene transfer into early embryos is a powerful methodology for unraveling the molecular bases of developmental processes. One can attempt to minimize widespread effects of an exogenous gene by using tissue- or region-specific promoters in the few instances where they are available. We have developed a method that bypasses the requirement for specific targeting sequences to achieve regionally restricted gene transfer. Intraspecific chimeras have been created by transplantation of restricted portions of a chicken embryo from a donor strain to a host strain. The donor cells are infectable with a recombinant retroviral vector that carries the exogenous gene, whereas the host cells are not. We have demonstrated the feasibility of this approach using a histochemically distinct reporter gene, human placental alkaline phosphatase. The expression of retrovirally transduced alkaline phosphatase was limited to a transplanted hemiprosencephalon (forebrain and eye) in embryonic chickens. This technique can be applied to many other organ systems during avian embryogenesis to test the function(s) of molecules that are normally controlled through spatial and/or temporal regulation, such as many of the growth factor receptors or homeobox-containing proteins.**

Genes that may play a role in vertebrate development are rapidly being isolated through a variety of screens. One approach to study their function is to perturb their normal pattern or level of expression. Currently, the methods for achieving this vary with different experimental organisms. For example, retroviral-mediated gene transfer is currently available for use in some species, such as birds and mammals. Until recently, the retroviral vectors used to effect gene transfer into embryos have been replication-defective and, therefore, of limited value in infecting a large number of cells (for review, see ref. 1). For the mouse, there are well-developed alternative techniques to direct the misexpression or knockout of a candidate gene using transgenic animals (for review, see refs. 2 and 3). The chicken, in contrast, has enjoyed only limited success (4) due to its long generation time and poor efficiency of achieving stable transgenics by microinjection. Yet the chicken remains an important model system for studying early development because of its accessibility to experimental manipulations *in ovo* (for review, see ref. 5). It was therefore a significant technological advance when Hughes and colleagues (6–8) reported the development of replication-competent avian retroviral vectors that can carry up to 2 kb of nonviral sequence.

When a replication-competent vector is introduced into a developing embryo, it will eventually spread throughout most of the organism. It is easy to imagine situations where excessive spread of a transgene could be detrimental to the embryo or to the interpretation of an experiment. In such situations it may be beneficial to limit the spread of the vector, and in turn the transgene, to specific structures or regions of interest. To achieve this, we devised a method to precisely limit viral spread. The method involves the creation of chimeric chicken embryos in which the donor and host tissues are derived from strains differing in their susceptibility to infection by retroviral subgroups. In the resulting chimeras, only the donor tissue is infected by a particular subgroup of retrovirus and, therefore, it is the only region of the embryo to express the transgene. The chimeric approach for limiting gene transfer may prove especially valuable for studying genes that have multiple distinct functions during development or for genes that are normally controlled through spatial and/or temporal regulation of expression.

Insight into developmental mechanisms can also be gained from a variety of transplants in which donor tissue has been indelibly marked by infection with histochemically detectable virus. Two such vectors encoding human placental alkaline phosphatase (PLAP) are now available (9) that could be used in combination with transplantation protocols exemplified here to facilitate analysis of classical developmental problems. Examples include tracing migration patterns, studying cell origins of widely disseminated cell types, or determining cellular phenotypes arising in male–female chimeras (for review, see ref. 10). Transplantation of dissociated donor cells either heterochronically or heterotopically could also be used to study cell commitment (11). This technique eliminates the potential problems inherent in chimeras composed of different species (such as chicken–quail) while additionally providing an improved method for distinguishing donor from host.

MATERIALS AND METHODS

Viral Stocks and Plasmids. WF201 (12) is a recombinant retrovirus (*E-env* from RAV-0 cloned into RAV-1 genome) provided by Brown and Robinson (University of Massachusetts Medical School, Worcester). RCASBP(A) provided by S. Hughes (National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD) is an unpublished variant of the RCAS vector (7) in which sequences derived from the *pol* gene of the Bryan high-titer strain of Rous sarcoma virus (RSV) were cloned into RCAS(A), a derivative of the Schmidt–Ruppin strain of RSV. To alter the envelope subgroup of RCAS, *env* sequences between the conserved restriction endonuclease sites, *Kpn* I and *Sal* I, were swapped as described (7, 13). Specifically, an E-envelope variant, called RCASBP(E) was created by replacing the *A-env* sequences of RCASBP(A) with the homologous *Kpn* I–*Sal* I fragment of *E-env* from RAV-0. Vectors encoding PLAP were created (9) by cloning PLAP cDNA, originally supplied by S. Udenfriend (Roche Institute of Molecular

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Abbreviations: AP, alkaline phosphatase; CEF, chicken embryo fibroblasts; E, embryonic day; MA, matrix protein of avian leukosis virus (p19 gag); PLAP, placental alkaline phosphatase; S-SPF, standard specific pathogen free.

Biology, Nutley, NJ), from pDAP plasmid (14) into the *Cla* I site of RCASBP(E) or RCASBP(A); the resulting vectors were called RCASBP/AP(E) and RCASBP/AP(A), respectively. In these vectors, the PLAP gene is transcribed from the promoter in the viral long terminal repeat; the mRNA encoding PLAP is a spliced product that utilizes a splice donor that is 3′ to the ATG of the *gag* gene and a splice acceptor that is 5′ to the *Cla* I cloning site, upstream of the ATG of PLAP.

Chicken Strains. A summary of the relevant chicken and virus strains is given in Table 1. All animals were obtained as fertilized eggs from the suppliers. Standard specific-pathogen-free (S-SPF) eggs were from a closed flock of White Leghorn chickens and were obtained from SPAFAS (Norwich, CT). Line 0, line $15b_1$, and line 7_2 strains were obtained from the U.S. Department of Agriculture Poultry Research Laboratory (Ann Arbor, MI).

Cell and Virus Culture. Because most commercially available eggs are E-subgroup-resistant, it was necessary to generate chicken embryo fibroblasts (CEFs) from line $15b_1$ cells to grow E-subgroup virus stocks. Line 0 CEFs were used for growing all other viral stocks and for helper tests because they lack endogenous avian leukosis virus proviruses (15). CEFs were obtained from the torso of embryonic day (E) 10 chickens and cultured as described (9, 16). CEFs were inoculated either by infection with competent virus or by calcium phosphate transfection followed 4 hr later by a 90-sec glycerol shock (17). Cells infected with E-subgroup virus were cultured in the presence of Polybrene (2 μg/ml) to increase the efficiency of infection; this is not necessary for A-subgroup virus (18). After 7–10 days of culture expansion, supernatants were harvested on two successive days, concentrated, and titered as described (9). For titrations, infected cells were fixed 48 hr after infection and either immunostained for viral gag proteins or, in the case of RCASBP/AP(E)-infected cells, processed for alkaline phosphatase (AP) histochemistry (9). Positive colonies were identified and counted to obtain the titer, defined as colony-forming units per ml (cfu/ml). Virus titers were typically $1–3 \times 10^6$ cfu/ml before concentration and $2–4 \times 10^8$ cfu/ml after concentration. E-subgroup virus stocks were determined to be free of contamination by other subgroups based upon their failure to infect line 0 cells (9).

Embryo Surgery. Eggs were handled and embryos were prepared for surgery as described (9). Embryos were staged according to Hamburger and Hamilton (19). Concentrated virus was injected by direct displacement (9). For stage 8–11, 0.1–0.3 μl was injected into the neural tube. For stage 13–16, 0.2–1.0 μl was injected into the subretinal space. Transplants of the prosencephalon were made at stage 9–11 (stage 10 was optimal) from line $15b_1$ donor embryos into line 0 host embryos to generate intraspecific chimeras. Visualization was aided by injection of india ink beneath the embryo. Transplants were dissected using electrophoretically etched tungsten wire needles, transferred using a 5-μl capillary tube, and maneuvered into the homotopic position of the host using tungsten needles. Embryos and transplants were kept moist during the procedure by addition of L-15 medium. After all embryo manipulations, the shell was sealed with tape and the egg was placed in a nonrotating incubator. Donor embryos were also returned to the incubator; after 24 hr they were harvested to assay the spread and expression of virus.

Cell Dissociation. E6 retinas were obtained from line $15b_1$ donor embryos that had been infected with WF201 on E2.5. Retinas were dissected and dissociated into a single-cell suspension by digestion with papain (Cooper/Worthington; 33 units/ml) as described (20), except that Hanks'-buffered saline solution replaced Earle's balanced salt solution. Cells were resuspended in bovine serum albumin at 2 mg/ml in Hepes-buffered saline solution with calcium and magnesium. Cells were kept at 37°C for 0–3 hr, resuspended in 0.025% fast green/Polybrene (0.8 mg/ml) at 2×10^4 cells per μl; ≈0.1 μl was injected into each embryo at stages 14–16, filling the subretinal space and the brain ventricles. Approximately 10–20% of the calculated number of cells actually leave the pipette with these injections; the remainder presumably adhere to the glass micropipette.

Histology. Embryos were fixed with 4% (wt/vol) paraformaldehyde, processed through graded sucrose solutions, embedded in gelatin/sucrose, frozen-sectioned, and post-fixed as described (9). Tissue culture cells were fixed for 15 min with 4% paraformaldehyde and washed in phosphate-buffered saline (PBS). AP histochemistry was carried out as described (14). Embryos and cells were incubated for 4 hr in an AP-detection solution; sections were incubated for 20–40 min. Tissue was rinsed in 20 mM EDTA/PBS for at least 1–4 hr and stored at 4°C. The procedure for anti-gag immunohistochemistry was as described (9). Briefly, after preincubation in serum-containing medium, tissue or cells were incubated for 1 hr in primary antibody, a mouse monoclonal antibody, 3C2 (21), against one of the gag proteins of avian leukosis virus matrix protein (MA; nomenclature according to ref. 22). Biotinylated anti-mouse secondary antibody was amplified and detected by AP histochemistry as described (23). Uninfected retinas of several different strains of chicken (line 0, line $15B_1$, line 7_2, and S-SPF) were stained as whole mounts for anti-gag immunoreactivity to determine the degree of background staining. Whole retinas (E16–19) were dissected into Hanks'-buffered saline solution and fixed in 4% paraformaldehyde for 4 hr. They were processed for anti-MA immunohistochemistry as described (9) except 0.3% Triton X-100 was used with each antibody incubation, and all

Table 1. Infectivity of A- and E-subgroup retroviruses on selected lines of chicken

	Chicken line			
	E-subgroup		A,B-subgroup	
Virus strain	Donor (line $15b_1$; C/O)	Host (line 0*; C/E)	Donor (line 0*; C/E)	Host (line 7_2; C/A,B,E)
WF201	+	−		
RCASBP(E)	+	−		
RCASBP/AP(E)	+	−		
RCASBP(A)			+	−
RCASBP/AP(A)			+	−

+, Virus can infect the indicated line of chicken; −, virus cannot infect the indicated line of chicken; C/O, the chicken line is resistant to no known avian retrovirus subgroup; C/E, the chicken line is resistant only to the E-subgroup of avian retroviruses; C/A,B,E, the chicken line is resistant to the A-, B-, and E-subgroups of avian retroviruses.

*S-SPF could be used as an alternative; however, the genotype of S-SPF eggs can vary and one should empirically determine subgroup resistance.

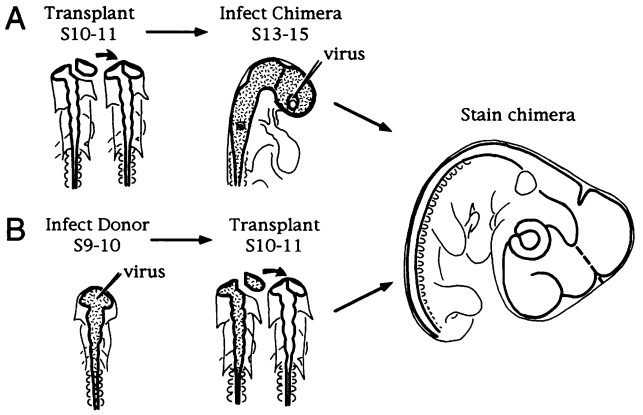

FIG. 1. Schematic of transplant and infection protocols. Shading depicts the diffusion of the carrier dye into the neural tube at the time of virus injection. Dotted line rostral to the midbrain indicates the presumed position of the junction between donor and host neural tissue. (*A*) Transplantation precedes viral inoculation. (*B*) Transplantation follows viral inoculation.

incubation times were lengthened to stain throughout the depth of the tissue. Minimum incubation times were as follows: primary antibody (5 days), secondary antibody (3 days), streptavidin-AP (3 days), PBS rinses (several changes over 24 hr), and predetection buffer (1 hr). Detection times were usually limited to 20 min. In all cases retrovirus-infected retinas were stained in parallel as positive controls.

RESULTS

Choice of Chicken and Virus Strains. The sensitivity of a strain of chicken to a particular strain of virus is determined by whether the chicken cells carry a receptor for a viral surface glycoprotein that is necessary for entry of the virion into the host cell cytoplasm. The viral glycoprotein is encoded by the viral *env* gene and has been used as the basis for subdivision of chicken retroviruses into subgroups A through E (for review, see ref. 24). Viral subgroups can also vary in other aspects of replication. For example, A-subgroup viruses tend to replicate faster than E-subgroup viruses due to sequences encoded outside of the *env* gene. Two of the retroviruses used here, WF201 and RCASBP/AP(E), are replication-competent recombinants in which E-subgroup *env* sequences have been cloned into A-subgroup viruses. These recombinants thus retain the A-specific sequences that enable rapid spread while endowing the virus with E-subgroup host range. The schemes for combining either E- or A,B-subgroup vectors with the appropriate donor and host strains of chicken are indicated in Table 1. The examples demonstrated in this paper have utilized the E-subgroup scheme, where E-sensitive line $15b_1$ embryos are used as donors and E-resistant line 0 embryos are used as hosts. An alternative A,B-subgroup scheme is provided in the event that a particular tissue or cell type is more readily infected with one of these subgroups.

Detection of A,B- or E-subgroup viruses is possible by immunohistochemistry using antibodies directed against the viral gag proteins. For RCASBP/AP vectors (9), the viruses also carry a histochemically detectable gene, PLAP, and therefore, infected cells are readily detected using AP histochemistry on embryo whole mounts or sectioned material.

Prosencephalon Transplants. Unilateral transplantation of the prosencephalon was used to test the feasibility of a chimera/retroviral infection protocol to spatially limit gene transfer. Chimeric embryos were produced by transplanting the right half of the prosencephalon from a donor embryo into the equivalent vacated position of a host embryo at stages 10–11 (Fig. 1). In the initial pilot experiment, the donor tissue was "labeled" the following day (stage 13–15) by infection with WF201. The virus injections were done after transplantation so that brain tissue derived from both donor and host embryos had equal access to the viral inoculum. The transplant and injection procedure is schematically shown in Fig. 1*A*. Resistant line 0 embryos were likewise injected with virus as negative controls. At E8, viral infection and expression were monitored immunohistochemically in tissue sections using an antibody to one of the *gag* gene products, the p19 matrix protein, MA. As expected, resistant line 0 embryos were negative when assayed several days after infection (data not shown). Similar injections into susceptible strains resulted in heavy infection of the retina, forebrain, and midbrain with various amounts of infection in more

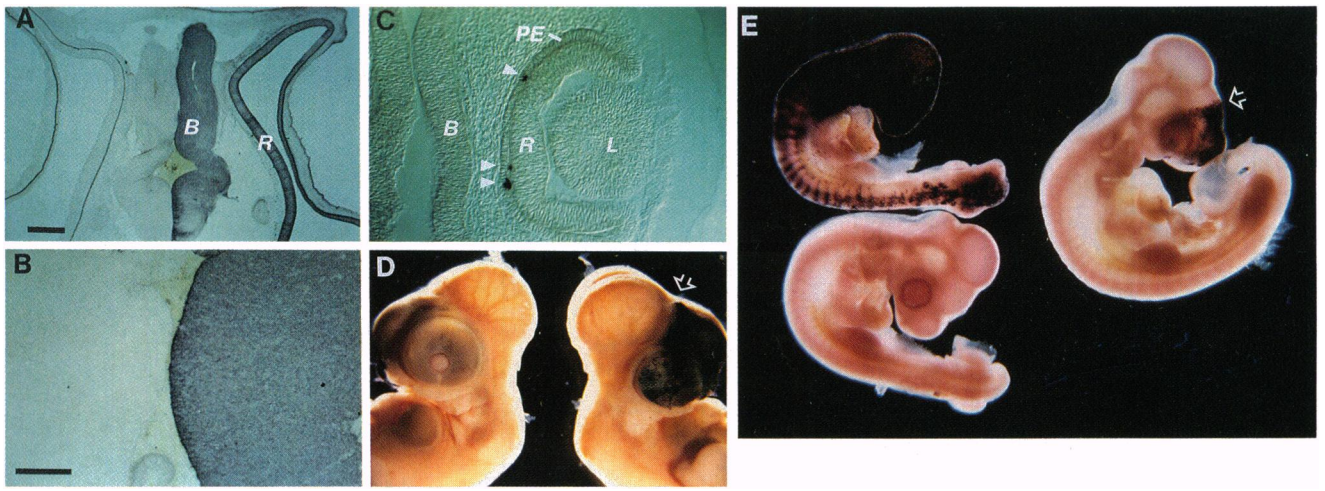

FIG. 2. (*A* and *B*) Horizontal sections through an E8 chimeric embryo stained with anti-MA antibody. The transplant was done at stage 10 and WF201 was injected on E3. (Bars: *A*, 500 μm; *B*, 200 μm.) B, brain; R, retina. (*C*) Dissociated WF201B-infected E6 retinal cells (arrowheads) transplanted into the subretinal space of an E3 host embryo and detected by anti-MA antibody. B, brain; L, lens; PE, incipient pigmented epithelium; R, retina. (*D* and *E*) AP expression in infected chimeric embryos produced by hemiprosencephalon transplantation. (*D*) The left and right sides of the head of a chimeric embryo processed on E5. (*E*) All three embryos were injected with RCASBP/AP(E) and processed on E4. (*Upper Left*) Line $15b_1$ embryo injected at stage 8^+. (*Lower Left*) Line 0 embryo injected at stage 8^-. (*Right*) Chimeric embryo in which the prosencephalon of a line $15b_1$ donor embryo injected at stage 10 was homotopically transplanted into a line 0 host embryo at stage 11. The border between presumed donor and host tissue is indicated by an arrow in *D* and *E*.

posterior central nervous system and surrounding tissues (data not shown).

Infection in the chimeras, on the other hand, should be limited to tissue derived from the donor prosencephalon: retina, optic nerve, diencephalon, telencephalon, and perhaps surrounding mesenchymal and epithelial tissue. Of three chimeras assayed on E8, two displayed precisely this labeling pattern (Fig. 2 *A* and *B*). In the third chimera, the transplant evidently did not incorporate; the right eye and forebrain failed to develop, and the chimera was negative for anti-MA staining.

Dissociated Cell Transplants. As an alternative to the transplantation of tissue chunks, retrovirally labeled tissue could be dissociated into single cells prior to transplantation. Such a method could, if properly controlled, be used as a marking technique to study clones derived from individual transplanted progenitor cells. As an example of this approach, labeled retinal cells were dissociated and transplanted heterochronically as follows. Retinas of donor embryos were infected at stages 11–15 with WF201. Four days later, the retinas were dissociated into single-cell suspensions. Aliquots of the cells were assayed for viral infection by immunocytochemistry; >90% of the cells were positive for anti-MA immunoreactivity. Small aliquots (several hundred cells) of these E6 retinal cells were introduced into the subretinal space of an E3 host embryo. Four hours later, embryos were fixed, sectioned, and immunostained to localize the transplanted cells. Individual cells or small groups of cells were found scattered throughout the subretinal space, as shown in Fig. 2*C*.

Gene Transfer into Chimeric Embryos. The pilot experiments clearly demonstrated that retroviral infection appeared to be limited to donor-derived tissue. To demonstrate that expression of a nonviral transgene could likewise be spatially restricted, RCASBP/AP(E) was used to target the marker gene, PLAP, to susceptible donor tissue. The donor tissue was labeled by retroviral infection either before (n = 10) or after (n = 11) transplantation of the prosencephalon as diagrammed in Fig. 1. Animals were processed at intervals of 1–4 days after transplantation. A total of 11 transplants were scored as successful based on having two well-formed eyes on the day of sacrifice; 3 embryos failed to incorporate the transplant; the remaining 7 embryos died without being analyzed. Both line $15b_1$ embryos (n = 52) and line 0 embryos (n = 6) were injected with RCASBP/AP(E) and processed in parallel with the chimeras, along with uninjected control embryos (n = 10) of various strains.

Chimeras judged to have successful transplants were fixed and processed as whole embryos for AP histochemistry. In each case, the right but not the left side of the head rostral to the midbrain showed evidence of heavy infection; an example is shown in Fig. 2*D*. The specificity of infection can best be appreciated by a direct comparison of infected line $15b_1$, line 0, and chimeric embryos, such as that shown in Fig. 2*E*. The different patterns of expression of the marker gene are striking. In the $15b_1$ embryo, AP activity was high in ectoderm and mesoderm of the head, including neural-crest-derived structures, in spinal cord, and in trunk neural crest and its derivatives. In line 0 infections, the majority of the embryo appeared not to express AP, although some staining was detected. The animal shown in Fig. 2*E*, for example, had faint AP activity in the left otocyst (data not shown) that was higher than that found in uninjected controls. A small region of staining was observed overlying the left eye in two other injected line 0 embryos. However, these staining patterns were in sharp contrast to those of chimeric embryos, where the staining was intense and restricted to the right side of the head. The results of staining infected chimeric embryos indicated that the spread of virus was severely limited and appeared to be restricted to tissues that are likely to be derived from the donor.

Viral Detection in Alternative Chicken Strains for Transplantation. When using RCAS vectors to test gene function, detecting the expression of a transgene using a specific antibody is not always possible. In such cases, the expression of viral structural proteins can be followed immunohistochemically to at least allow determination of potential sites for transgene expression. As a control for background staining, anti-MA antibodies were used to stain uninfected embryos of the different potential host strains listed in Table 1. Retinas were processed as whole mounts to simultaneously screen $\approx 10^8$ cells per retina. Uninfected retinas of both S-SPF and line 7_2 strains showed detectable staining of small clusters of cells, presumably due to endogenous proviral expression. Approximately 50% of these retinas showed at least one positive cluster; the positive cells represent a tiny fraction of the total retinal cells, usually $<10^{-6}$. A systematic analysis of other parts of the embryo has not been attempted, although scattered sampling suggests that endogenous MA immunoreactivity was similarly rare. We conclude that these lines could be used as hosts to create chimeras in which a large chunk of infected tissue is transplanted. However, they could present a problem for interpreting the results of dissociated cell transplants of the type demonstrated in Fig. 2*C*, if MA immunoreactivity is the only assay for detecting transplanted cells. Additional drawbacks of line 7_2 eggs are that they have relatively low fertility rates, are more sensitive to experimental manipulation than the other strains, and are not readily available all year. In contrast, >60 uninfected line 0 retinas were stained and found to be free of labeling. These eggs are available year-round, and although there is some seasonal variability in their fertility rate and sensitivity to experimental manipulation, they are much hardier than line 7_2. It is not clear what accounts for the difference in endogenous MA immunoreactivity between chicken strains, but the absence of staining in line 0 retinas may be related to the fact that this strain is missing endogenous proviruses (15).

DISCUSSION

Use of Retroviruses for Gene Transfer into Early Embryos. Using the RCASBP/AP(E) vector, we have shown that it is possible to target gene transfer to selected populations of neurons over the long term by infecting chimeric embryos with specific envelope-subgroup retroviruses. A homologous vector of the A-subgroup, RCASBP/AP(A), is also available for testing host range and constructing chimeras. Retroviral-mediated gene transfer into restricted cell populations or regions of the embryo gives greater flexibility in the design of experiments for testing gene functions and for studying cell–cell interactions. For gene-transfer experiments, the gene of interest would replace PLAP and infected cells would be identified, if possible, by the transgene product. If not, we describe immunohistochemical detection methods for localizing a gag protein encoded by the same retroviral vector(s).

In some cases, it is possible to limit the spread of a retrovirally transduced gene to a restricted subset of cells over the short term (several days) by simply varying the site and time of infection (9). For example, we were able to differentially target neural crest verses neural tube by varying injection parameters. Similarly, Morgan *et al.* (26) were able to target most mesenchymal cells of the developing limb bud unilaterally using an RCASBP vector encoding the murine *Hox-4.6* gene. Anecdotally, we have found that injections that enter the vascular system result in widespread infection of endothelial and cardiac cells (unpublished observations).

We imagine that in many cases specific targeting will not be possible by simply manipulating the site and time of infection. For example, within the central nervous system, we were

unable to find injection parameters that restricted gene transfer to specific rostrocaudal domains (unpublished observations). Yet this axis is important in defining the major structural and functional components of the brain. Furthermore, a number of genes have been shown to have rostrocaudal domains of expression in the developing brain that often correspond to precise anatomical boundaries, including a variety of homeobox-containing genes (for review, see ref. 27). To unravel the possible functional roles of these and other genes and to specify the cellular interactions involved, it may prove necessary to misexpress them in defined spatial domains. The chimeric protocol described in this paper holds promise for carrying out such studies. In this protocol, restriction of gene transfer to specific rostrocaudal domains is attainable by transplantation and is limited only by the accuracy with which one can transplant a defined region and the extent to which that region can be completely infected with retrovirus by the desired stage of development. The surgical approach is quite feasible since transplants as small as one rhombomere in length have been accomplished (28). Additional improvements in vectors and/or injection parameters may be necessary to obtain high levels of expression in specific early-forming brain regions, such as the developing rhombomeres. It may also be possible to use retroviral vectors to target antisense messages, thereby obtaining spatially restricted gene knockouts that may complement and extend analyses done using embryonic stem cell knockouts in mice.

Viral Labeling for Transplantation. Retroviruses make good marking reagents for transplantation experiments because they efficiently infect target cells, are stably inherited, and appear to be innocuous in terms of effects on developmental processes. Injection of replication-competent virus into early embryos can lead to extensive labeling of donor cells for homospecific transplantation of individual cells, groups of cells, or regions of an embryo. When PLAP is used as a marker gene, this technique can be used as an effective way to track the transplanted cells and their progeny. The chimeric animals that can be created by this technique are similar in many respects to chicken–quail chimeras. The variety of applications already established for chicken–quail chimeras thus should be equally appropriate for homospecific chimeras. These would include studies of cell commitment, plasticity, migration patterns of cell populations, the origin of widely dispersed cell types (such as lymphocytes, cephalic tissues, limb mesoderm, and neural crest), and organogenesis (for review, see ref. 10). The homospecific chimeras could in addition be used to study the cellular bases and hormonal regulation of intergender behavior by constructing male–female chimeras within the same species.

Homospecific chimeras have several potential advantages over the chicken–quail system. There may be fewer problems with immune rejection of the transplant (see ref. 29) although we have yet to test this by allowing chimeric animals to survive to adulthood. In addition, the overall size and growth rate of the chicken strains are similar; thus the use of chicken chimeras avoids the potential problems encountered by differential growth of chicken and quail embryos. *In vitro* reaggregates of chicken and quail retinal cells do not mix during subsequent proliferation and development (30); it is possible that this reflects differential affinity and/or sorting that may complicate *in vivo* experiments in which cell–cell interactions are important. These potential problems may be avoided by homospecific chimeras, although the possibility of differential affinity between infected and uninfected cells remains to be tested. Finally, tracing the transplanted cells by their expression of AP gives added morphological detail (9, 13) compared with nuclear markers (5) and is easier to use than antibodies that distinguish chicken from quail (25). The improved detection method is especially advantageous for determining cell types in the central nervous system.

Note Added in Proof. SPAFAS (Norwich, CT) has recently changed the genotype of their S-SPF eggs. These eggs no longer routinely have E-subgroup resistance. We recommend that their subgroup specificity be empirically determined before using them as host embryos.

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