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RETROVIRAL VECTORS TO STUDY CELL DIFFERENTIATION

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1. ABSTRACT

Retroviral vector-mediated gene transfer has been contributing to studies in developmental biology, genetics and clinical science. Retroviruses are ideal tools for gene transfer into dividing cells both *in vitro* and *in vivo*, which has led to their expanding use in developing systems. In this review we will primarily review their use for ectopic gene expression and lineage analysis to study cell differentiation in murine and chick embryos.

2. INTRODUCTION

Studies of cell differentiation have used two categories of retroviral vectors: replication-competent and replication-incompetent. The proviral genome of a replication-competent vector contains all of the components needed for replication, integration and viral particle formation; such vectors produce new viruses in infected cells, and spread out from the points of injection. Replication-incompetent retroviral vectors are lacking one or more viral genes essential for replication; they do not spread out from the initially infected cells (or their progeny) unless assisted by competent (i.e., helper) viruses. Vectors include those derived from Rous sarcoma/avian leukemia virus RSV/ALV or murine leukemia virus (MLV).

Gene transfer mediated by replication-competent viral vectors is the most commonly used technique to overexpress exogenous genes in the chick embryo. Used in combination with classical experimental embryological techniques to manipulate the chick embryo, ectopic gene expression by viral vectors has played an important role in the last few years to reveal the functions of numerous genes in cell differentiation in various tissues. Viral vectors have been used as a complementary approach to transgenic techniques for studies using a gain-of-function approach. Together with the loss-of-function approach using embryonic stem cell gene targeting, gain-of-function analysis using retroviral vectors can provide a more complete understanding of developmental genes in birds and mammals.

Gene transfer mediated by replication-incompetent viral vectors has played an equally important role in developmental studies, particularly in revealing lineage relationships among different cell types. Replication-incompetent vectors are ideal for this use because they can provide a transgene that will label all progeny of an infected cell but will not be passed to unrelated cells. Lineage analysis using viral vectors has been undertaken in both rodents and chicks. Replication-incompetent retroviral vectors can also be used in gain-of-function studies where they are especially valuable for looking at the effect of gene transfer into a small number of cells in the midst of an otherwise normal environment. Because they provide persistent expression of a transgene in a restricted number of cells, starting from a desired stage, without disrupting the overall pattern of gene expression in the tissue, insight can be gained about the cell-autonomous nature of a gene's action. Examples of this approach include studies exploring the role of various transcription factors in neural specification and differentiation.

3. STRUCTURE OF RETROVIRAL VECTORS

3.1. Structure of replication-competent retroviral vectors

Most of the reported replication-competent vectors used in developmental biology are based on the RSV/ALV-derived vector, RCAS, and its derivatives (see a

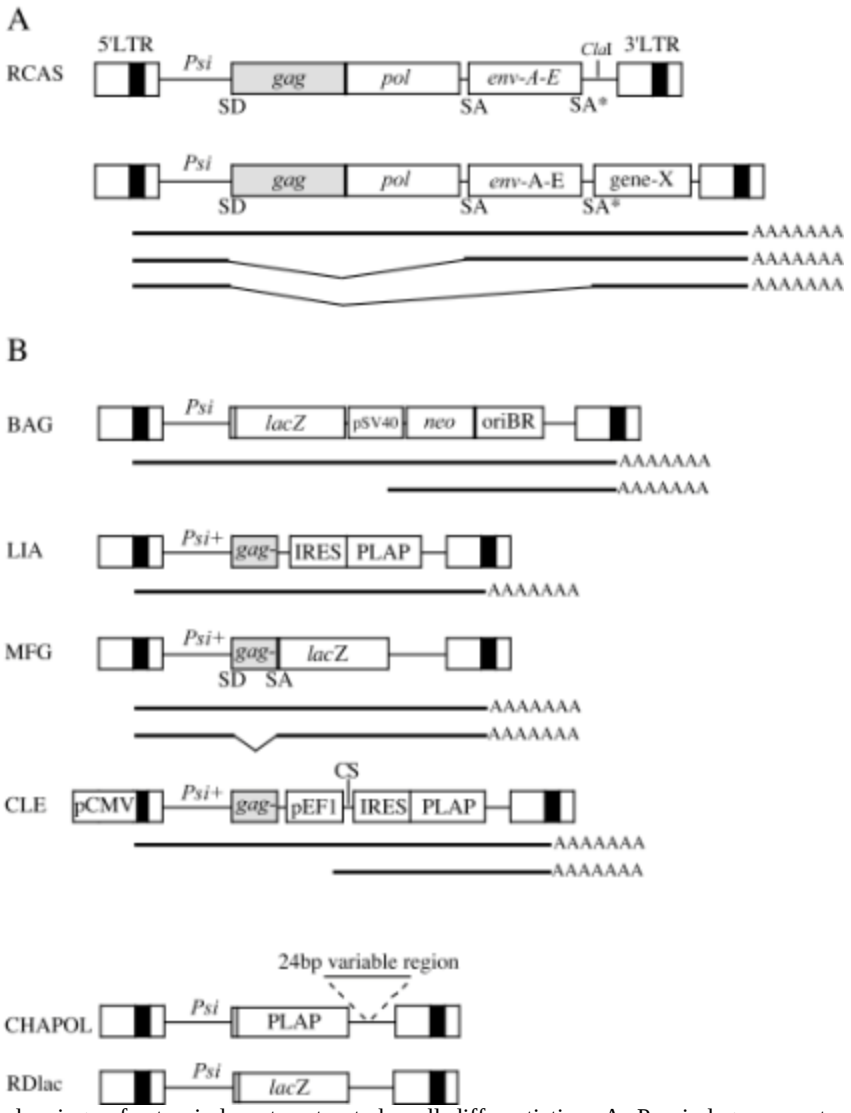

Figure 1. Schematic drawings of retroviral vectors to study cell differentiation. A, Proviral genome structure of replication-competent viral vector, RCASBP. B, Proviral genome structures of MLV-derived replication-incompetent vectors. C, Proviral genome structures of ALV/RSV-derived replication-incompetent vectors. Black lines under proviral genome indicate mRNAs with polyA tails. *Psi*: packaging signal, *Psi*+: extended packaging signal, CS: cloning site, PLAP: human placental alkaline phosphatase, *lac*Z: beta-galactosidase, SD: splice donor, SA: splice acceptor, SA*: splice acceptor of *src*, pEF1: promoter of *Xenopus* elongation factor 1, pCMV: promoter of cytomegalovirus, IRES: internal ribosome entry site, oriBR: replication origin of pBR322, *neo*: neomycin resistant gene, *gag*-: incomplete *gag* gene.

review (1, 2), Figure 1A,). RCAS, replication-competent avian leukemia virus LTR with splice acceptor is derived from the Schmidt Ruppin-A (SR-A) strain of Rous sarcoma virus (3). RCAS has a similar proviral structure to Rous associated virus (RAV) in that it contains all essential components for replication: 5' and 3'LTRs, a packaging signal, *gag*, *pol* and *env* genes, but no *src* oncogene. In place of *src*, RCAS has a unique *Cla*I site following the strong splice acceptor site of *src* located between the *env* gene and the 3'LTR. The exogenous gene is inserted into the *Cla*I site and is translated from a spliced transcript using the splice donor in the *gag* gene and the *src* splice acceptor. RCAN (replication-competent avian leukemia virus LTR with no splice acceptor) is similar in structure to RCAS but lacks the *src* splice acceptor. If RCAN is to be used for transgene expression, an internal ribosome entry site (IRES) must first be cloned upstream of the exogenous gene. Alternatively, because RCAN will not splice the message for the exogenous gene, it can be used as a control for non-specific effects of viremia in developing animals. RCASBPs are a series of RCAS-derived vectors that have a *pol* gene from the Bryan high titer strain of RSV, allowing them to replicate more efficiently than the prototype RCAS (4). Many of the published papers use RCASBP vectors, but refer to them as RCAS. In either vector, exogenous sequence up to 2.0-2.4k base pairs can be inserted at the

*Cla*I site. Infection of cells or tissues with RCAS-derived vectors can be detected by immunostaining with anti-*gag* monoclonal antibody (5).

There are five major envelope subgroups in RSV/ALV: A, B, C, D and E. The viral envelope protein encoded by the *env* gene is responsible for the range of infectable host cells (tropism) as well as the ability of viruses to block each other from superinfecting the same cell (interference). RCAS and RCASBP have an A-type envelope gene, so they are also called RCAS(A) and RCASBP(A), respectively. RCASBP(B), RCASBP(D) and RCASBP(E) were generated by substitution of the appropriate variable region from different ALV *env* genes that encode the protein surface domains responsible for the tropism of B, D and E subgroups, respectively (1).

Replication-competent retroviral particles are generated by transfection of appropriate host cells with plasmid DNA that contains the proviral genome. The host cells must be susceptive for the envelope subgroup, and should not have endogenous virus in their genome to avoid recombination. In general, it is easier to produce a higher titer virus stock with replication-competent retroviral vectors than with incompetent vectors. In the case of RCASBP made in chick embryonic fibroblasts (CEFs), titers usually range from 10^6 to 10^7 infectious units (IU)/ml, and can be concentrated by ultracentrifugation to titers of 10^7 to 10^9 IU/ml (1). By comparison, replication-incompetent viruses are typically about 100-fold lower in titer.

3.2. Structure of replication-incompetent retroviral vectors

Replication-incompetent retroviral vectors derived from ALV or MLV are commonly used to study cell differentiation in chicks and rodents, respectively (6). Figure 1B shows a schematic diagram of typical replication-incompetent vectors derived from MLV. Vectors lacking almost the entire coding region of the endogenous viral genome have an advantage over replication-competent vectors in that they can carry up to approximately 10kb of exogenous sequence. Most MLV-derived vectors retain the N-terminal region of the *gag* gene because this region is known to enhance packaging of viral RNA (7). Some vectors are designed to express two exogenous genes to facilitate detecting infected cells. The exogenous genes can be expressed in several ways: translation as a fusion protein with the N-terminal peptide of *gag* (e.g., BAG(8)); translation from IRES (e.g., LIA(9)); alternative splicing (e.g., MFG(10)); or transcription from an internal promoter. Examples are shown in figure 1B.

To apply replication-incompetent retroviral vectors for lineage analysis, libraries of heterogeneous viral particles have been used. The MLV-derived BAG library contains fragments of *Arabidopsis* genomic DNA in addition to the b-galactosidase gene as a reporter (11). RSV-derived CHAPOL contains alkaline phosphatase as a reporter, as well as a tag consisting of 24 base pairs of degenerate oligonucleotide. After histological analysis of infected tissues, the tag can be analyzed by PCR to assist in assigning infected cells to a clone (12).

The viral particles of replication-incompetent vectors can be generated in at least three ways: (1) transient or (2) stable transfection of proviral DNA into packaging cells or (3) cotransfection with plasmids that express viral genes. Packaging cells are stably transfected with *gag*, *pol* and *env* genes but lack a packaging signal which directs the RNA .into the virion. For RSV-derived vectors, packaging cells with envelope types A or B are available. For MLV-derived vectors, packaging cells with amphotropic or ecotropic envelope are available. Both avian and murine viral vectors can be pseudotyped with the VSV-G protein to produce pantropic viral particles (13). That is, VSV-pseudotyping allows for infection of a wide variety of animals (14, 15), including frogs(16) and fishes (17).

The potential appearance of replication-competent helper virus is a critical issue in the production of replication-incompetent viral particles, especially for lineage analysis and gene therapy. The replication-competent helper virus, which has all the genetic components required for replication, is probably generated by recombination between the transfected proviral DNA (containing packaging sequences) and the viral genome carried by the packaging cells (containing gag, pol and env genes). Contamination with even a small amount of helper virus can result in spread of replication defective viral vectors from initially transfected cells to unrelated cells in the host. This is especially problematic for lineage analysis, which requires transmission of virus only to the direct descendants of infected cells. Accordingly, replication-incompetent viral stocks must be tested for the absence of replication-competent helper virus before they can be used for lineage analysis.

The enhancer and promoter regions of the viral LTRs have been manipulated to improve the specificity and/or efficiency of replication-incompetent vectors. For example, altering the 3'LTR has enhanced tissue-specific expression from internal promoters, and has provided a means to trap gene promoters using viruses (18). Removal of the enhancer region of the 3'LTR causes inactivation of the 5'LTR in infected cells. This is because the proviral genome of an infected cell derives its 5'LTR enhancer and promoter regions from the 3'LTR of the former generation, due to strand-jumping during reverse transcription. By removing the 3'LTR enhancer, the virus becomes self-inactivating for LTR-mediated transcription, thereby allowing an internal promoter, or a nearby enhancer in the host genome, to function without interference. Because of strand-jumping, the enhancer region of the 5'LTR can be manipulated in producer cells without affecting the LTR genomic structure that is packaged in viral particles and subsequently transferred to infected host cells. Replacing parts of the 5'LTR with the enhancer and promoter of cytomegalovirus (Figure1B) has been reported to increase the viral titers obtained from producer cells (18).

4. ADVANTAGES OF RETROVIRAL VECTORS TO STUDY CELL DIFFERENTIATION

A clear advantage of using retroviral vectors to study cell differentiation is the ease with which one can use the same virus stock *in vitro* and *in vivo* to infect various cell types and tissues. Although recent progress in gene

manipulation techniques in mouse enables stage- or position-specific gene misexpression, retroviral vectors are still the most flexible tools to stably introduce transgenes to embryos at various positions and stages. Optimization of infection parameters is important. Depending on the purpose of the experiment, the time, position, and amount of virus can be varied to optimize the size of the infected domain, the percentage of infected cells, and the time course of spread (1).

The host cell requirements of retroviral vectors can be advantageous to study the effect of transgenes on cell differentiation. Unlike adenoviral or lentiviral vectors, most retroviral species require mitosis of host cells to be integrated into the genome (19). As a result, post-mitotic cells are insensitive to retroviral vectors, making it possible to specifically target progenitor cells. Once integrated into the genome of a host cell, the proviral DNA is stably transmitted to the progeny of the host cell after it divides. Replication-competent vectors are suitable for overexpression of transgenes because they can spread out from the initially infected cells provided the surrounding cells continue to divide. For this reason, they are valuable for perturbing more global patterning genes. In contrast, replication-incompetent vectors will not spread, so they are suitable to deliver transgenes into single progenitors or small populations of cells to study cell fate specification.

Another advantage of retroviruses is that the experimenter has some control over the range of host cells that will be infected. Susceptibility of host embryo or tissue will be blocked if there is endogenous expression of viral envelope genes. For example, chicks of the SPF-11 strain express E-type envelope gene, making them resistant to infection by RCASBP(E), whereas the line 0 strain lacks endogenous virus and is susceptible to E-subgroup. This difference in susceptibility between chick strains can be exploited to limit infection. For example, the domain of infection can be limited by implantation of infected cells or tissue from a susceptive donor embryo to a non-susceptive host embryo (20).

To maximize both the onset and longevity of gene transfer, one can combine retroviral gene transfer with electroporation. Low-voltage *in vivo* electroporation is a recently developed technique to introduce plasmid DNA into early embryos (21). In this approach, transgene expression can be detected within 2 hours. This stands in contrast to retroviral infection, in which protein expression is not strongly induced in most infected cells until about 18 hours. On the other hand, with electroporation the transgene is unlikely to be stably integrated into the host cell, resulting in a transient misexpression followed by dilution and loss over time as the embryo grows. One can harness the advantages of both techniques by electroporating the plasmid DNA that includes a provirus complete with flanking LTRs (22). The DNA serves first as a transient source for transcription and translation when present in the cytoplasm, but the DNA will eventually incorporate into the host genome for stable transfection. This approach was recently used to study cell fate specification in the retina (Chen and Cepko, personal communication).

5. APPLICATIONS OF RETROVIRAL VECTORS

5.1. Applications of replication-competent retroviral vectors

Retroviral vectors have enhanced the usefulness of the chick embryo as a model for developmental biology. Clearly advantageous because of its accessibility to classic embryonic surgery and transplantation techniques, the chick embryo no longer suffers from a lack of convenient transgenic approaches that have been so successful in the mouse. RCAS vectors are powerful tools for ectopic- or over-expression of ligands, receptors, signal transduction molecules and transcription factors both *in vivo* and *in vitro*. Several examples from the recent literature are presented to illustrate the power of retroviral gene transfer into the chick embryo.

RCAS vectors propelled the study of various aspects of limb bud development, including limb bud induction and patterning. Fibroblast growth factors play important roles in limb induction. In the developing limb bud, FGF-8 and FGF-10 are expressed in the apical ectodermal ridge (AER) and underlying mesenchyme, respectively. Ectopic expression of FGF-8 or FGF-10 by RCASBP(A) in the flank caused ectopic limb formation, confirming their essential role in limb outgrowth (23, 24).

Viral vectors have contributed to understanding the molecular basis of patterning the limb along its anterior-posterior (A-P) axis. The zone of polarizing activity (ZPA) lies at the posterior margin of the limb bud and is critical for A-P patterning. The ZPA will induce mirror-image duplication of the limb when grafted to the anterior margin of a recipient limb bud. Riddle *et al* showed that *sonic hedgehog*, a homologue of the *Drosophila hedgehog* gene, is expressed in the ZPA. They infected chick embryo fibroblasts with RCASBP(E)-*shh* and grafted them into the anterior limb bud of E-type resistant hosts to provide a focal source of signaling and to minimize spread of virus throughout the limb (25). The Shh-expressing graft mimicked the ZPA, producing mirror-image duplicate limbs and altering *Hoxd*-13 expression in the tissue surrounding the transplant.

Understanding the molecular basis of dorsal-ventral axis specification in the limb has also benefited from virus-mediated gene transfer. Wnt-7a and Lmx1 are expressed in the dorsal ectoderm and mesenchyme in the developing limb bud, respectively. Overexpression of Lmx-1 in the ventral limb mesenchyme using RCASBP(A) causes dorsalization of the ventral part of limb. When Wnt-7a is ectopically expressed in the entire ectoderm of the limb bud using RCASBP(A), Lmx-1 is induced in the underlying mesoderm which again dorsalizes the ventral limb. En-1, the mouse homologue of *Drosophila engrailed* transcription factor gene, is expressed in the ventral limb ectoderm (26). Ectopic expression of En-1 in the dorsal ectoderm using RCASBP(A) represses the expression of Wnt7a and Lmx1 and disrupts AER morphology (27).

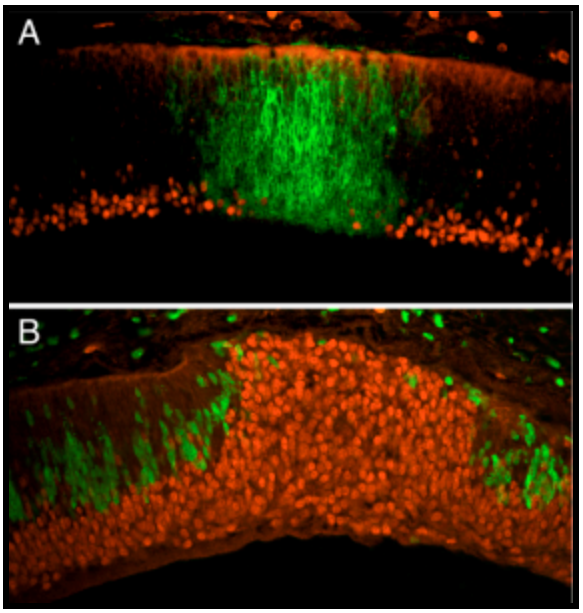

Figure 2. Application of avian replication-competent vectors to study cell differentiation. Forced expression of Delta1 and dominant-negative (dn) Delta1 alters retinal cell fate (28). RCAS-*delta1* (A) and RCAS-*delta1*dn (B) was injected into the optic vesicle of the chick embryo at stage 8-12 and analyzed at E5-E6. Nuclei of retinal neurons are shown by red fluorescence with anti-Islet1/2 in A and B. A virus infected patch is shown by green fluorescence using *in situ* hybridization in A (green). Proliferating cells are detected by BrdU incorporation in B (green). Forced expression of Delta1 blocks production of neurons in retina (A), while forced expression of Delta1dn promotes premature differentiation of neurons and prevents cell division (B). Reprinted from Current Biology, 7(9), Henrique, Hirsinger, Adam *et al*, "Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina", 661-670, Copyright (1997), with permission from Elsevier Science.

Retroviral overexpression in neurons has furthered our understanding of cell differentiation. To analyze the role of the Notch signaling pathway in vertebrate neuronal differentiation, a gene encoding Notch ligand, *delta-1*, or its dominant-negative form, were integrated into RCASBP vectors, and the virus was injected into developing eye (28) or ear (29). In retina, overexpression of Delta-1 caused continued cell division and generated a patch lacking differentiated neurons (Figure 2A). In contrast, cells expressing a dominant-negative form of Delta-1 were less proliferative than controls and predominantly differentiated into ganglion cells (Figure 2B). Using replication-incompetent vectors, Bao and Cepko (9) showed that the constitutively active form of Notch causes abnormal growth of immature cells (Figure 4). These data demonstrated an inhibitory role of Notch in neurogenesis by preventing the differentiation of retinal neurons. In the chick inner ear, dominant-negative Delta-1 downregulated the expression of a Notch ligand, Ser1, which is expressed in supporting cells (29). These data indicated that the normal function of Delta-1 in hair cells was to mediate the lateral induction of Ser1 in the surrounding supporting cells.

The establishment of left-right (L-R) asymmetry is another field in which retroviral vectors have recently contributed (30). While studies in mammals have benefited from the identification of genes that cause situs problems in mouse mutants and inherited human syndromes, studies in chick have relied on misexpression via bead-implantation and RCAS vectors. In mammals, one current model to explain the initial break in symmetry is focused on directional fluid movement at the node (the nodal flow hypothesis). However, in the chick there is no evidence for nodal flow, and so the mechanism for initiating L/R asymmetry is still unclear. The first molecular asymmetry in the chick embryo is revealed by asymmetric expression of FGF-8 (right side) and Shh (left side) around node. On the left side of the node, Shh induces the expression of the BMP-antagonist Car; Car then allows Nodal expression in left lateral plate mesoderm (LPM). Nodal, in turn, induces Pitx2 expression in left LPM. Ordering these genes into a left-side pathway was accomplished by expressing each of them ectopically on the right side using infection with RCAS vectors or implantation of RCAS-infected cells (31-35). These manipulations induced the appropriate downstream genes and caused laterality defects.

Ectopic- or over-expression studies of exogenous genes using RCASBP have also contributed to several other fields, including feather morphogenesis, vascularization, and myogenesis. Using RCASBP, Noramly and Morgan (36) showed that lateral inhibition by BMPs controls the regular spacing of the feather placodes. During feather placode formation, BMP-2 appears first in a diffuse pattern and is then concentrated into the placodes. Forced expression of BMP-2 or BMP-4 suppresses feather bud formation, while forced expression of Noggin, a BMP inhibitor, causes loss of the interbud region. In feather development, one of the earliest markers of A-P patterning is the expression of Wnt-7A expression in the posterior region of the bud. Overexpression of Wnt-7A using RCASBP disrupts A-P patterning and yields elongation of feather buds (37). In vascularization research, quail vascular endothelial growth factor was overexpressed using RCASBP, causing hypervascularization (38). In myogenesis, expression of an activated form of Raf using RCASBP prevented differentiation of myoblasts (39, 40).

5.2. Lineage analysis using replication-incompetent retroviral vectors

Replication-incompetent retroviral vectors are providing a valuable contribution to lineage studies in neural tissues, including the brain, retina and inner ear (41, 42). Lineage analysis allows one to study the distribution and the fates acquired by daughter cells generated from a single progenitor cell. In contrast to fate mapping, which can be accomplish by labeling a small group of cells, in lineage analysis it is important to mark single progenitor cells to follow their progeny. Within the central nervous system, direct microscopic observation of cell fate acquisition or injection of marker molecules into single progenitors is extremely difficult because there are so many

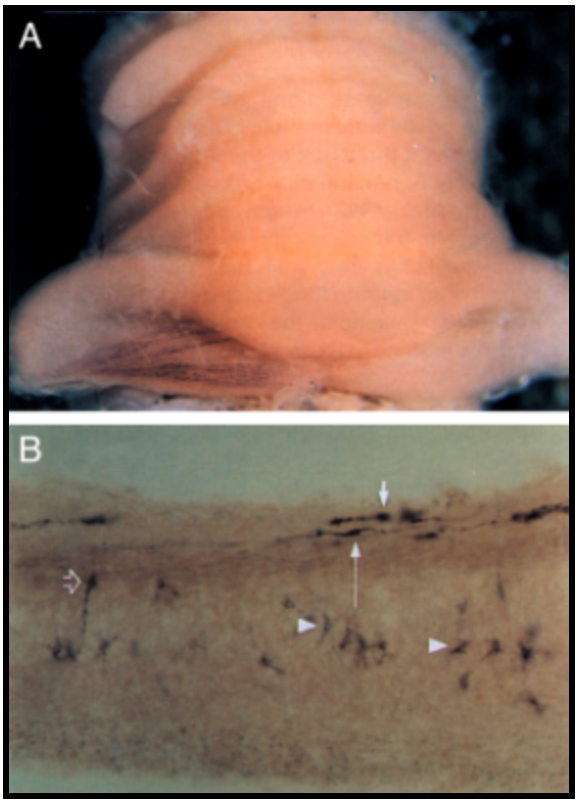

Figure 3. Lineage analysis of neural progenitors in chick cerebellum using replication-incompetent avian vector (47). Whole-mount view (A) and a transverse section (B) of cerebellum with a virus-infected clone containing granule cells at different stages of differentiation: unipolar (short arrow), bipolar (long arrow), migrating inward (open arrow) and in the internal granule layer (arrowheads). The clones generated from the external granule layer contain granule cells that disperse long distances from their site of origin. Reprinted from Neuron, 12(5), Ryder and Cepko**,** "Migration patterns of clonally related granule cells and their progenitors in the developing chick cerebellum", 1011-1029, Copyright (1994), with permission from Elsevier Science.

cells, there is extensive cell migration, and most cells are too small to target or to observe directly over a long time. An exception is the frog retina, where it has been possible to track the progeny of single injected cells because they remain in tight radial clusters (43).

These problems can be overcome with replication-incompetent retroviral vectors. Retroviral particles can easily infect progenitors in a wide variety of tissues. The proviral DNA of replication-incompetent retroviral vectors is integrated into the host chromosome only in mitotic progenitor cells. Because the virus gains access to the nucleus late in mitosis, after S- phase when the nuclear envelope breaks down (19), the provirus will be inherited by only one daughter of the original infected cell. Thereafter, the vector with its marker gene will be inherited by all the progeny without dilution. To increase the number of clones that can be reliably identified in a single specimen, mixtures of viral vectors carrying different histochemical marker genes can be used. For the most extreme complexity of viral markers and unambiguous clonal identification, there are libraries of viral vectors where each member of the library carries a different variable region.

For lineage analysis, a virus stock of optimized titer is injected at the desired place and time into the target tissue. The embryo is allowed to develop until cell differentiation is complete, and then the distribution and identity of cells expressing the histochemical marker gene is evaluated, usually from histological sections. If a viral library is used, each positive cell can be dissected after histochemical analysis, and the variable region in the proviral genome can be amplified using PCR.

Retrovirus-mediated lineage analysis was first performed in rodent retina (8), in which undifferentiated neuronal cells do not migrate tangentially. When the viral stock was injected into developing retina, marked cells were obviously clustered into clones. Single clones were observed that contained both neurons and glia. In aggregate, the cell types within a clone were found in ratios that were appropriate given their birthdates and their overall proportions in the retina (44, 45). These data showed that the developing retina contains multipotent progenitors that can generate all the neuronal cell types as well as glial cells.

Mixtures or libraries of viruses are used for lineage analysis in brain regions where there is extensive cell migration, including striatum (46), cerebral cortex (11), cerebellum (47), telencephalon (48), and diencephalon (49). These reports show that various neuronal types and glial cells can be clonally related, and that neuronal progeny can migrate away from the original location of the progenitor, sometimes beyond functional boundaries (Figure 3).

The sensory epithelium of the inner ear contains two major classes of cells distributed in a regular array: the mechanosensory transducers, called hair cells, and the surrounding epithelial cells, called supporting cells. Retrovirus-mediated lineage analysis has shown that these two cell types can be generated from a common progenitor in the chick ear (50), a finding that was recently confirmed using a viral library (51).

5.3. Studies of cell differentiation using replication-incompetent retroviral vectors

Replication-incompetent retroviral vectors have also been used for gain-of-function studies to explore the molecular mechanisms underlying cell differentiation in mouse neural tissue. The approach can provide complementary information to the loss-of-function approach most commonly pursued using null mutant mice. In contrast to overexpression by replication-competent retroviral vectors, using defective virus means that the transgene of interest can be introduced into a small number of mitotic cells to analyze the fate of their progeny in the

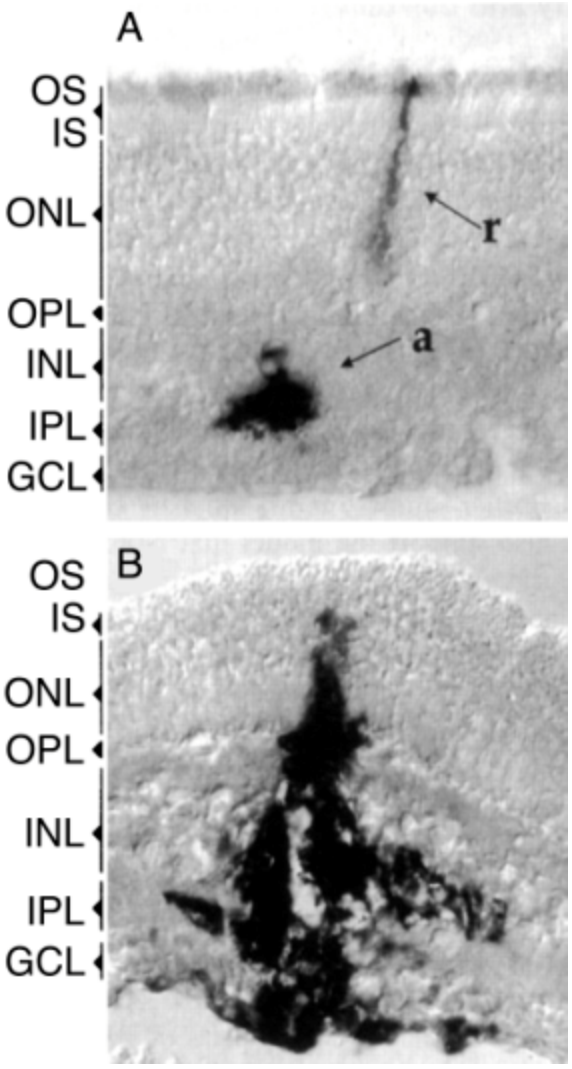

Figure 4. Analysis of the cell-autonomous effect of Notch using a replication-incompetent murine vector (9). (A) LIA or (B) LIA with a constitutive active form of Notch were injected into the mouse retina at P0, and animals were analyzed 3 weeks later. Clones transducing the active form of Notch are larger in size than control, and contain premature neurons. OS, outer segment layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; a, amacrine cell; r, rod photoreceptor. Reprinted from The Journal of Neuroscience, 17(4), Bao and Cepko**,** "The expression and function of Notch pathway genes in the developing rat eye", 1425-1434, Copyright (1997), with permission by the Society for Neuroscience.

context of a normal environment. The greatest advantage of this approach is that it can reveal cell-autonomous effects of a transgene on cell differentiation without disrupting the overall context of development. Another advantage over transgenic approaches is that viral vectors can often be delivered at the desired time and place, as with lineage analysis.

In mice, the viral vectors frequently used in this kind of approach are derived from MLV. The replication-incompetent viral vector, LIA, uses a bicistronic message to express both the transgene, which is inserted into a cloning site downstream of the 5'LTR, and alkaline phosphatase, which follows IRES (Figure 1B). Infection with the experimental virus can be compared to infection with a control virus that has only the alkaline phosphatase marker gene. Using various histochemical techniques, the proportion of each cell type, the frequency of cell death and effects on mitosis can be analyzed in marked clones.

The transcriptional control of neuron-glia cell fate determination and neural differentiation has been analyzed using replication-incompetent retroviral vectors. Many basic helix-loop-helix (bHLH) transcription factor genes are expressed in the developing brain in specific cell types. bHLH genes are activators or repressors of transcription that regulate the expression of other bHLH genes. Retroviral studies have shown that many activator bHLH genes can block gliogenesis and some of them can also stimulate neurogenesis. For example, the activator bHLH gene, *neuroD*, is normally expressed in a subset of undifferentiated retinal cells. When this gene is introduced into the developing retina using LIA, transduced clones lack Müller glial cells, contain fewer bipolar neurons and have more amacrine neurons than clones formed by control viruses (52). These results show that *neuroD* blocks gliogenesis and preferentially stimulates differentiation into amacrine neurons. In postnatal mouse cerebral cortex, cells transduced with viral vectors expressing other activator bHLH genes, *ngn1*, *ngn2* and *mash1*, form clones containing neurons, as compared to control vectors, which produce virtually no neurons (53).

Retroviral studies also examined the roles of repressor bHLH genes in neurogenesis. For example, a repressor bHLH, Hes1, is expressed in neural precursors in the central nervous system. Cells infected with a viral vector carrying *hes1* remain as undifferentiated precursors (54). In contrast, retinal precursors infected with virus carrying two other repressors, *hes5* and *hes6*, produced clones consisting almost entirely of Müller glial cells and rod photoreceptor cells, respectively. These results showed that Hes1 represses neuronal differentiation from precursors, while Hes5 and Hes6 stimulate gliogenesis and neurogenesis, respectively (55, 56).

6. PERSPECTIVES

The application of retroviral vectors to study cell differentiation will be extended by further progress in virus engineering, including manipulations that alter host cell specificity. Retroviral studies are expanding from rodents and avians to other animal species such as *Xenopus* and zebrafish by using VSV-G pseudotyping. VSV-G pseudotyping has already been used for production of transgenic animals, insertional mutagenesis and gene trapping in those species (16). Because of the receptor-independence and stability of the envelope protein, VSV-G pseudotyping also improves both infection efficiency and maximal viral titer of murine and avian viral stocks.

Pseudotyping with genetically engineered envelope genes is advancing and has already been used to study cell differentiation (29). Moreover, using *in vitro* DNA shuffling and selection in cell culture, MLV envelope genes with ultracentrifuge resistance and new tropism have been generated (57, 58). There are also renewed attempts to target viral particles to specific cell types using modified envelope genes.

Alteration of the transcriptional specificity of transgenes using internal promoter and self-inactivating LTRs have also been described, and no doubt these vectors will soon be applied to developing cells and embryos. Although the LTR promoters work in a wide range of tissues, proviral genomes inserted into host chromosomes are sometimes inactivated (59). Internal promoters were shown to prevent this silencing in some cases *in vivo* (14). Viral vectors with tissue-specific internal promoters will also be useful to study cell differentiation.

Lentivirus-based vectors will expand the use of retroviral vectors to post-mitotic cells such as neurons (19). Unlike MLV- or ALV-derived vectors, which can infect only mitotic cells, vectors derived from lentiviruses, such as human immunodeficiency virus, can infect post-mitotic cells (60).

In summary, retroviruses have become commonplace in the toolkit of the avian developmental biologist, with recent advances extending their value to those working with rodents, frog and fish. We anticipate many additional contributions of this methodology to the study of cell fate specification and pattern formation.

7. ACKNOWLEDGMENT

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