protein has a very long half-life, the morphological response of that cell will be correspondingly slower. For example, REF cell lines transformed with SV40 T-antigen (unpublished observations) took significantly longer to revert to a more untransformed phenotype than REF cell lines transformed with a mutant p53 construct.

Use of LAP267

Although LAP348 has proven to be effective in the establishment of reversibly transformed cell lines, it is probably difficult to use LAP348 in stable cell lines when the test gene is toxic or inhibitory to cell growth. In this case it would be advantageous to use a system where the test gene was normally off but could be rapidly turned on using some stimulus. LAP267 may be of particular use in these settings because of its very low level of activity in the absence of IPTG. Although it should be possible to use direct selection techniques for LAP267, in general, LAP267-expressing cell lines have been produced by cotransfection with a constitutively expressed drug resistance marker. A second gene can then be introduced into the LAP267 cells using a second selectable marker. It should be noted that LAP267 expression may be toxic to cells since it has been very difficult to produce cell lines expressing this protein, although mouse Ltk- cells, human 293 cells, and 3T3 cells have been used successfully. The description of the LAP267 vectors is included for completeness. To date, the best results have been obtained with a variant of mouse Ltk- cells which have been transformed with pHβA267. LAP267-expressing cells can be used to express a test gene by cotransfection and coselection with the HSVtk gene. These transfections are carried out at 39 ° to minimize the activity of LAP267 since it appears activate transcription at a low level at 37 °.
with retrovirus vectors. This chapter summarizes the basis for this technique and details the strategies and current methods in use in our laboratory.

**Transduction of Genes via Retrovirus Vectors**

A retrovirus vector is an infectious virus that transduces a nonviral gene into mitotic cells *in vivo* or *in vitro*. These vectors utilize the same efficient and precise integration machinery of naturally occurring retroviruses to produce a single copy of the viral genome stably integrated into the host chromosome. Those that are useful for lineage analysis have been modified so that they are replication incompetent and thus cannot spread from one infected cell to another. They are, however, faithfully passed on to all daughters once viral integration has taken place, making them ideal for lineage analysis.

Retroviruses use RNA as their genome, which is packaged into a membrane-bound protein capsid. Immediately following entry into a cell, they produce a DNA copy of their genome via reverse transcriptase, a protein included in the capsid. The DNA copy is integrated into the host cell genome and is thereafter referred to as a "provirus." Complete synthesis of an integration-competent DNA viral genome requires S phase, and integration occurs during M phase. Thus, only mitotic cells will serve successfully as hosts for retroviral integration. Since integration occurs during the M phase and since there is only one DNA copy of the viral genome produced per infectious virion, only one daughter of the first mitosis following infection will inherit the viral genome. However, all subsequent daughters of that cell will inherit the viral genome.

Most vectors began as proviruses that were cloned from cells infected with a naturally occurring retrovirus. Although extensive deletions of proviruses were made, vectors retain the cis-acting viral sequences necessary for the viral life cycle. These include the Ψ packaging sequence (necessary for recognition of the viral RNA for encapsidation into the viral particle), reverse transcription signals, integration signals, viral promoter, enhancer, and polyadenylation sequences. A cDNA can thus be expressed in the vector using the transcription regulatory sequences provided by the virus. Since replication-incompetent retrovirus vectors usually do not encode the structural genes whose products comprise the viral particle, these proteins must be supplied through complementation. The structural proteins, products of the *gag*, *pol*, and *env* genes, are typically supplied by "packaging"

cell lines. These lines are usually stable lines that contain the *gag*, *pol*, and *env* genes as a result of the introduction of these genes by transfection. However, these lines do not contain the packaging sequence, $\Psi$, on the viral RNA that encodes the structural proteins. Thus, the packaging lines make viral particles that do not contain RNA encoding *gag*, *pol*, or *env* proteins.

Retrovirus vector particles are essentially identical to naturally occurring retrovirus particles. They enter the host cell via interaction of a viral envelope glycoprotein (a product of the viral *env* gene) with a host cell receptor. The murine viruses have several classes of *env* glycoprotein which interact with different host cell receptors. The most useful class for lineage analysis of rodents is the ecotropic class. The ecotropic *env* glycoprotein allows entry only into rat and mouse cells via the ecotropic receptor on these species. It does not allow infection of humans, and thus is considered relatively safe for gene transfer experiments. Until 1988, the packaging line most commonly in use was the $\Psi 2$ line. It encodes the ecotropic *env* gene and, in our experience, it makes the highest titers of vectors, relative to other packaging lines (for unknown reasons). However, it can also lead to the production of helper virus (discussed below). Second generation ecotropic packaging lines, $\Psi$CRE and GP+E-86, and third generation packaging lines, $\Omega E$ and Bosc 23, have not been reported to lead to production of helper virus to date. Any of the ecotropic packaging lines can be used to produce vector stocks for lineage analysis. Regardless of the packaging line used, however, all stocks should be assayed for the presence of helper virus.

Avian viruses also have multiple envelope types, called subgroups A–E. Two packaging lines, Q2bn and Isolde, are available for producing avian retroviruses and both make subgroup A envelope. Subgroup A envelope allows infection of most commercially available chicken strains. However, one thing to keep in mind is that commercially available chickens are not monitored or bred for infection of subgroup A viruses. If you have trouble with efficiency of infection, you might want to use another source of embryos in case the A envelope receptor is limiting.

It has been known for some time that nonretroviral enveloped viruses can contribute their envelope glycoprotein to retroviruses and thus endow a retrovirus with a different host range. The vesicular stomatitis virus (VSV) envelope protein, G protein, has been expressed in a murine retrovirus vector such that a broad host range virion carrying the neo gene was produced. This virus was shown to infect and express in zebrafish, chum salmon, hamster, and canine cells in vitro. The vector unfortunately expressed poorly in the fish lines and thus newer generation vectors need to be designed to overcome this limitation. Such vectors are currently being developed.

Production of Virus Stocks for Lineage Analysis

Replication-incompetent vectors that encode a histochemical reporter gene, such as Escherichia coli lacZ, are the most useful for lineage studies as they allow analysis of individual cells in tissue sections or whole mounts. Stocks of such vectors are most easily obtained from packaging lines stably transduced with the vector genome. One can also produce stocks transiently by transfection of particular packaging cells, such as the Bosc 23 or Q2bn, or by cotransfection using the vector expressing VSV-G when one wishes to obtain the broadest host range. (A detailed description of protocols for making vector stocks, for titering and concentrating virus stocks, and checking for helper virus contamination has been published and will not be given here.) When one can use stable lines, as in the case when infecting murine or rat cells, it is best to obtain lines that make high titered stocks of lineage vectors from the laboratories that have created stable lines. We have placed $\Psi$2 and $\Psi$CRE producers of BAG, a lacZ virus that we have used for lineage analysis, on deposit at the ATCC in Rockville, Maryland. They can be obtained by anyone and are listed as ATCC CRL Nos. 1858 ($\Psi$CRE BAG) and 9560 ($\Psi$2 BAG). Similarly, $\Psi$2 producers of DAP, a vector encoding human placental alkaline phosphatase (PLAP, described further below), is available as CRL No. 1949. Both of these vectors transcribe the reporter gene from the viral LTR promoter and are generally useful for expression of the reporter gene in most tissues. We compared

the expression of lacZ driven by several different promoters\textsuperscript{14,15} and found that the LTR was generally the most reliable and non-cell type specific. This is an important consideration as it is desirable to see all of the cells descended from an infected progenitor and thus a constitutive promoter is the most useful for lineage studies. However, even with constitutive promoters, it has been noted that some infected cells do not express detectable $\beta$-Gal protein, even among clones of fibroblasts infected \textit{in vitro}. Thus, it is important to restrict conclusions about lineage relationships to cells that are marked and not to make assumptions about their relationships to cells that are unmarked.

For lineage applications it is usually necessary to concentrate the virus in order to achieve sufficient titer. This is typically due to a limitation in the volume that can be injected at any one site. Viruses can be concentrated fairly easily by a relatively short centrifugation step. Virions also can be precipitated using polyethylene glycol or ammonium sulfate, and the resulting precipitate is collected by centrifugation. Finally, the viral supernatant can be concentrated by centrifugation through a filter that allows only small molecules, to pass (e.g., Centricon filters). Regardless of the protocols that are used, it must be kept in mind that retroviral particles are fragile, with short half-lives even under optimum conditions. One exception to this are the viruses bearing VSV-G, which are very stable. In order to prepare the highest titered stock for multiple experiments, we usually concentrate several hundred milliliters of producer cell supernatant. These concentrated stocks are titered and tested for helper virus contamination, and can be stored indefinitely at $-80^\circ$ in small (20- to 50- aliquots).

\textbf{Replication-Competent Helper Virus}

The replication-competent virus is sometimes referred to as the helper virus as it can complement ("help") a replication-incompetent virus and thus allow it to spread from cell to cell. It can be present in an animal through exogenous infection (e.g., from a viremic animal), expression of an endogenous retroviral genome, or through recombination events between two viral RNAs encapsidated in retroviral virions produced by packaging lines. The presence of a helper virus is an issue of concern when using replication-incompetent viruses for lineage analysis as it can lead to horizontal spread of the marker virus, creating false lineage relationships. The most likely source of helper virus is the viral stock used for lineage analysis. The genome(s) that supplies the gag, pol, and env genes in packag-


ing lines does not encode the $\Psi$ sequence, but can still become packaged, although at a low frequency. If it is coencapsidated with a vector genome, recombination in the next cycle of reverse transcription can occur. If the recombination allows the $\Psi^{-}$ genome to acquire the $\Psi$ sequence from the vector genome, a recombinant that is capable of autonomous replication results. This recombinant can spread through the entire culture (although slowly due to envelope interference). Once this occurs, it is best to discard the producer clone as there is no convenient way to eliminate the helper virus. As would be expected, recombination giving rise to helper virus occurs with greater frequency in stocks with a high titer and with vectors that have retained more of the wild-type sequences (i.e., the more homology between the vector and packaging genomes, the more opportunity there is for recombination). Note that a murine helper genome itself will not encode a histochemical marker gene as apparently there is not room, or flexibility, within murine viruses that allows them to be both replication competent and capable of expressing another gene like $\text{lacZ}$. The way that spread would occur is by a cell being infected with both the $\text{lacZ}$ virus and a helper virus. Such a doubly infected cell would then produce both viruses. In the case of avian viruses, a replication-competent helper virus can carry and express a nonviral gene of $<2.5$ kb, but it is unlikely to do so unless a very specific recombination takes place. The likelihood of problems with avian viruses can be minimized using line O chickens, which do not encode any endogenous avian viruses.

When performing lineage analysis, there are several signs that can indicate the presence of helper virus within an individual animal. First, if an animal is allowed to survive for long periods of time after inoculation, particularly if embryos or neonates are infected, the animal is likely to acquire a tumor when the helper virus is present. Most naturally occurring replication-competent viruses are leukemogenic, with the disease spectrum being at least in part a property of the viral LTR. Second, if one analyzes either short or long term after inoculation, the clone size, clone number, and spectrum of labeled cells may be indicative of helper virus. For example, the eye of a newborn rat or mouse has mitotic progenitors for retinal neurons, as well as mitotic progenitors for astrocytes and endothelial cells. By targeting the infection to the area of progenitors for retinal neurons, we only rarely see infection of a few blood vessels or astrocytes as their progenitors are outside of the immediate area that is inoculated and they only get infected by leakage of the viral inoculum from the targeted area. However, if the helper virus was present, we would see infection of a high percentage of astrocytes, blood vessels, and eventually, other eye tissues since virus spread would eventually lead to infection of cells outside of the targeted area. A correlation would be expected to exist between the
percentage of such nontargeted cells that are infected and the degree to which their progenitors are mitotically active after inoculation, due to the fact that infection requires a mitotic target cell. If tissues other than ocular tissues were examined, one would similarly see evidence of virus spread to cells whose progenitors would be mitotically active during the period of virus spread. In addition, the size and number of "clones" may also appear to be too large for true "clonal" events if the helper virus were present. This interpretation of course relies on some knowledge of the area under study.

_Determination of Sibling Relationships_

When performing lineage analysis, it is critical to unambiguously define cells as descendents of the same progenitor. This can be relatively straightforward when sibling cells remain rather tightly and reproducibly grouped. An example of such a straightforward case is the rodent retina, where the descendents of a single progenitor migrate to form a coherent radial array. In such a system, lineage analysis can be performed using one or two distinct histochemical marker viruses, as described below. For more complex systems, particularly where cell migration is important, many more markers are needed, as described in a later section (see Clonal Analysis Using the PCR/Library Method).

If only a single histochemical marker virus is used, a standard virological titration can be performed in which a particular viral inoculum is serially diluted and applied to tissue. In the retina, the number of radial arrays, their average size, and their cellular composition were analyzed in a series of animals infected with dilutions that covered a 4-log range. The number of arrays was found to have a first order relationship to the dose or virus, whereas the size and cell composition of the radial arrays were independent of the viral dose. Such results indicate that the working definition of a clone, in this case a radial array, fulfilled the statistical criteria expected of a single hit event.

There are several difficulties involved with the use of a single marker virus. First, there must be a wide range of dilutions that can be injected to give countable numbers of events, which is required for determination of a first-order relationship between clone number and viral dose. This type of analysis also relies critically on controlling the exact volume of the injection. These problems may be avoided in cases where it is possible to perform the analysis solely on infections with very small amounts of virus, although generating large amounts of data under these conditions is tedious.

Another potential problem with the dilution approach is that aggregates may form during virus concentration that are not separated by dilution. Thus, even at low dilution, a “clone” may be the result of infection of adjacent cells by two or more members of such an aggregate. In addition, it is difficult to calculate an error rate for the assignment of clonal boundaries, which may make interpretation difficult, particularly of events which occur rarely in the data set.

For these reasons, additional viral vectors with histochemically distinguishable marker genes have been developed for use in mixed infections. In addition to addressing the problems mentioned earlier, use of two marker viruses can often provide a much better initial idea of what clonal boundaries are likely to be by simple visual inspection of the tissue. By using two markers, errors arising from formation of viral aggregates can be visualized since some of these aggregates will include two distinguishable virions. Various criteria for clonal boundaries can be tested, and error rates for each can be computed. Extensive dilutions are not needed.

Two viruses that have been used for this approach encode cytoplasmically localized versus nuclear localized β-Gal. This virus pair is useful when the cytoplasmically localized β-Gal is easily distinguished from the nuclear-localized β-Gal.17,18 We have found that this is not the case in rodent nervous system cells, as the cytoplasmically localized β-Gal quite often is restricted to neuronal cell bodies and is therefore difficult to distinguish from nuclear-localized β-Gal. To overcome this problem, we created the above-mentioned DAP virus,13 which is distinctive from the lacZ-encoding BAG virus. Another virus that is currently under construction is one encoding the green fluorescent protein.19 This protein may not only give another easily distinguishable histochemical tag, but may enable studies of clone growth in a live animal.

To perform the two-marker analysis in the rodent retina, a stock containing BAG and DAP was made by growing a Ψ2 producer clone for BAG and a Ψ2 producer clone for DAP on the same dish.13,20 The resulting supernatant was concentrated and used to infect rodent retina. The tissue was then analyzed histochemically for the presence of blue (due to BAG infection) and purple (due to DAP infection) radial arrays. If radial arrays were truly clonal, then each one should be only one color. Analysis of 1100 arrays indicated that most were only one color. However, 5 comprised blue cells and purple cells, presumably from infection of adjacent progenitor cell bodies.

cells with BAG and DAP. (Infection of one cell with both BAG and DAP would not be a problem since the resulting cells would be clonally related.) The 5/1100 figure is an underestimate of the true frequency of incorrect assignment of clonal boundaries as sometimes two BAG or two DAP virions will infect adjacent progenitor cells; the resulting arrays will be a single color, but not clonal. A closer approximation of the true frequency can be obtained using the following formula (for derivation, see Field-Berry et al.13).

\[
\% \text{ error} = \frac{(\text{number of bicolored arrays})[(a + b)^2/2ab]}{\text{number of total arrays}}
\]

where \(a\) and \(b\) are the relative titers of the two viruses in the virus stock. The relative titer of BAG and DAP used in the coinfection was 3 : 1, and thus the value for percentage errors in clonal assignments in the rodent retina was 1.2%.

Such a low error rate shows that the choice of clonal boundary was reasonable and that viral aggregation during concentration (or at any other step) was not a problem for this viral stock. To take an extreme counter example, if every array were the result of infections by two particles, 6/16 of the arrays would be composed of both blue cells and purple cells. (For a stock with relative viral titers of 1:1, half the arrays would have blue cells and purple cells.) In analyzing the composition of arrays (e.g., by cell type), one need not worry, as one would if using one marker, that rare cell type combinations may be due to occasional errors in assignment of clonal boundaries. If such rare arrays are always of one color (when the error rate is very low), then they are very likely clones.

The error rate being computed here is the rate of "lumping" errors, that is, the frequency with which the criteria defining a clone lumps together daughters of more than one progenitor. However, this computation does not allow assessment of "splitting" errors, where clones that are more spread out (presumably owing to migration) are split by the definition of a clone into two or more subclones. If the criteria used to define clonal relationships are found to generate too many lumping errors, a more restrictive definition can be made, and then this definition can be tested for percentage lumping errors. This process can be done iteratively until the error rate reaches a level that is acceptable relative to the point that is being tested. However, it should be kept in mind that the more restrictive a definition is for clonal relationships, the more it is prone to generate splitting errors. Splitting errors may become obvious in animals injected with very dilute viral stocks; for example, one blue array in a large, otherwise unlabeled area is probably a clone, but it may be divided into several by too restrictive clonal definitions. Thus, combining two-marker analysis with
a few dilution experiments may be useful to help balance splitting and lumping errors. If it is necessary to avoid completely both splitting and lumping errors, a much greater number of vectors and more tedious detection method must be used, as detailed below (see Clonal Analysis Using the PCR/Library Method).

The value of 1.2% for lumping errors in assignment of clonal boundaries places an upper limit of 1.2% as the frequency of aggregation for this viral stock since this figure will include errors due to both aggregation and independent virions infecting adjacent progenitors. The presence of helper virus would probably increase the error rate, as it would have the effect of enlarging apparent clone size and creating overlap between clones of different markers. The percentage of errors on injecting this stock in other areas of an animal would depend on the particular circumstances of the injection site and on the multiplicity of infection (MOI, the ratio of infectious virions to target cells). Most of the time the MOI will be quite low (e.g., in the retina it was approximately 0.01 at the highest concentration of virus injected). Injection into a lumen, such as the lateral ventricles, should not promote aggregation or a high local MOI, but injection into solid tissue could present problems since the majority of the inoculum may have access to a limited number of cells at the injection site. By coinjecting BAG and DAP, one can monitor the frequency of these events and thus determine if clonal analysis is feasible.

The above analysis, and similar ones using avian vector stocks,21,22 were performed using viruses that were produced on the same dish and concentrated together. This was done because we felt that the most likely way that two adjacent progenitors might become infected would be through small aggregates of virions. We grew both virions together on the same dish in order for the assay to be sensitive to any aggregation that might occur prior to concentration, but this is probably not necessary in most cases. Aggregation most likely occurs during the concentration step, as one often can see macroscopic aggregates after resuspending pellets of virions. Thus, when the two-marker approach is used to analyze clonal relationships, it is best to coconcentrate the two vectors together in order for the assay to be sensitive to aggregation arising from this aspect of the procedure. (Although aggregation of virions may frequently occur during concentration, it apparently does not frequently lead to problems in lineage analysis, presumably because of the high ratio of noninfectious particles to infectious

particles found in most retrovirus stocks. It is estimated that only 0.1–1.0% of the particles will generate a successful infection. Moreover, most aggregates are probably not efficient as infectious units; it must be difficult for the rare infectious particle(s) within such a clump to gain access to the viral receptors on a target cell.)

Several methods can be used to determine the ratio of two genomes present in a mixed virus stock (e.g., BAG plus DAP). The first two methods are performed in vitro and are simply an extension of a titration assay. Any murine virus stock is normally titered on NIH/3T3 cells to determine the amount of virus to inject. The infected NIH/3T3 cells are then either selected for the expression of a selectable marker when the virus encodes such a gene (e.g., neo in BAG and DAP) or are stained directly, histochemically, for β-Gal or PLAP activity without prior selection with drugs. If no selection is used, the relative ratio of the two markers can be scored directly by evaluating the number of clones of each color on a dish. Alternatively, selected G418-resistant colonies can be stained histochemically for both enzyme activities and the relative ratio of blue versus purple G418-resistant colonies computed. A third method of evaluating the ratio of the two genomes is to use the values observed from in vivo infections. After animals are infected and processed for both histochemical stains, the ratio of the two genomes can be compared by counting the number of clones, or infected cells, of each color.

When all the above methods were applied to lineage analysis in mouse retina and rat striatum, the value obtained for the ratio of G418-resistant colonies scored histochemically was almost identical to the ratio observed in vivo. Directly scoring histochemically stained, non-G418-selected NIH/3T3 cells led to an underestimate of the number of BAG-infected colonies, presumably because such cells often are only faint blue, whereas DAP-infected cells are usually an intense purple. In vivo, this is not generally the case as BAG-infected cells are usually deep blue.

Regardless of which method is used to score sibling relationships, one further recommendation to aid in the assignments is to choose an injection site that will allow the inoculum to spread. If one injects into a packed tissue, the viral inoculum will most likely infect cells within the injection tract, and it will be very difficult to sort out sibling relationships (i.e., too many lumping errors). In addition, one must inject such that the virus has clear access to the target population; the virus will bind to cells at the injection site and will not gain access to cells that are not directly adjoining that site.

The procedures described below are those that we have used for infection of rodents and chick embryos, histochemical processing of tissue for
β-Gal and PLAP visualization, and preparation and use of a library for the polymerase chain reaction (PCR) method.23

II. Procedures

Infection of Rodents

Injection of Virus in Utero

The following protocols may be used with rats or mice. Note that a clean, but not aseptic, technique is used throughout. We routinely soak instruments in 70% ethanol (v/v) before operations, use the sterile materials noted, and include penicillin/streptomycin (final concentrations of 100 units/ml each) in the lavage solution. We have not had difficulty with sepsis using these techniques.

Materials

Ketamine hydrochloride injection (100 mg/ml ketamine)
Xylazine injection (20 mg/ml)
Animal support platform
Depilatory
Scalpel and disposable sterile blades
Cotton swabs and balls, sterile
Tissue retractors
Tissue scissors
Lactated Ringer’s solution (LR) containing penicillin/streptomycin
Fiberoptic light source
Virus stock
Automated microinjector
1- to 5-μl micropipettes
3-0 Dexon suture
Tissue stapler

1. Mix ketamine and xylazine 1:1 in a 1-ml syringe with a 27-gauge needle; lift the tail and hindquarters of the animal with one hand and with the other inject 0.05 ml (mice) or 0.18 ml (rats) of anesthetic mixture intraperitoneally.

One or more additional doses of ketamine alone (0.05 ml for mice and 0.10 ml for rats) are usually required to induce and/or maintain anesthesia, particularly if the procedure takes over 1 hr. Respiratory arrest and sponta-

neous abortion appear to occur more often if a larger dose of the mixture is given initially or if any additional doses of xylazine are given.

2. Remove hair over entire abdomen using depilatory agent (any commercially available formulation, such as Nair, works well); shaving of remaining hair with a razor may be necessary. Wash skin several times with water, then with 70% ethanol, and allow to dry.

3. Place animal on its back in support apparatus. For this purpose, we find that a slab of styrofoam with two additional slabs glued on top to create a trough works well for this purpose. With the trough appropriately narrow, no additional restraint is then needed to hold the anesthetized animal.

4. Make a midline incision in the skin from xyphoid process to pubis using a scalpel and retract; attaching retractors firmly to the styrofoam support will create a stable working field. Stop any bleeding with cotton swabs before carefully retracting the fascia and peritoneum, and incising them in the midline with scissors (care is required here not to incise the underlying bowel). Continue incision cephalad along the midline of the fascia (where there are few blood vessels) to expose entire abdominal contents. If it is necessary to expose the uterus, gently pack the abdomen with cotton balls or swabs to remove the intestines from the operative field, being careful not to lacerate or obstruct the bowel. Fill the peritoneal cavity with LR, and lavage until clear if the solution turns at all turbid.

Wide exposure is important to allow the later manipulations. During the remainder of the operation, keep the peritoneal cavity moist and free of blood; dehydration or blood around the uterus increases the rate of postoperative abortion.

5. Elevate the embryos one at a time out of the peritoneal cavity and transilluminate with a fiberoptic light source to visualize the structure to be injected. For lateral cerebral ventricular injections, for example, the cerebral venous sinuses serve as landmarks.

When deciding on a structure to inject, keep in mind that free diffusion of virus solution throughout a fluid-filled structure lined with mitotic cells is best for ensuring even distribution of viral infection events throughout the tissue being labeled. The neural tube is an example of such a structure; when virus is injected into one lateral ventricle, it is observed to quickly diffuse throughout the entire ventricular system.

6. Using a heat-drawn glass micropipette attached to an automatic microinjector, penetrate the uterine wall, extraembryonic membranes, and the structure to be infected in one rapid thrust; this minimizes trauma and improves survival. Once the pipette is in place, inject the desired volume of virus solution, usually 0.1–1.0 μl. Coinjection of a dye such as 0.005% (w/v) trypan blue or 0.025% fast green helps determination of the accuracy
of injection and does not appear to impair viral infectivity; coinjection of the polycation Polybrene (80 μg/ml) aids in viral attachment to the cells to be infected.

The type of instrument used to deliver the virus depends on the age of the animal and the tissue to be injected. At early embryonic stages, the small size and easy penetrability of the tissue makes a pneumatic microinjector (such as the Eppendorf 5242) best for delivering a constant amount of virus at a controlled rate with a minimum of trauma. Glass micropipettes should be made empirically to produce a bore size that will allow penetration of the uterine wall and the tissue to be infected. At later ages (late embryonic and postnatal), a Hamilton syringe with a 33-gauge needle works best.

When injecting through the uterine wall, all embryos may potentially be injected except those most proximal to the cervix on each side (injection of these greatly increases the rate of postoperative abortion). In practice, it is often not advisable to inject all possible embryos, if excessive uterine manipulation would be required. At the earliest stages at which this technique is feasible [embryonic day (E) 12 in the mouse or E13 in the rat], virtually any uterine manipulation may cause abortion, so any embryo which cannot be reached easily should not be injected.

7. Once all animals have been injected, lavage the peritoneal cavity until it is clear of all blood and clots, ensure that all cotton balls and swabs have been removed, and move retractors from the abdominal wall/fascia to the skin.

Filling the peritoneal cavity with LR with penicillin/streptomycin before closing increases survival significantly, probably by preventing maternal dehydration during recovery from anesthesia as well as preventing infection.

8. Using 3-0 Dexon or silk suture material on a curved needle, suture the peritoneum, abdominal musculature, and fascia from each side together, using a continuous locking stitch. After closing the fascia, again lavage using LR with penicillin/streptomycin.

9. Close the skin using surgical staples (such as the Clay-Adams Autoclip) placed 0.5 cm apart. Sutures may also be used, but these require much more time (often necessitating further anesthesia, which increases abortion risk) and are frequently chewed off by the animal, resulting in evisceration.

10. Place animal on its back in the cage and allow anesthesia to wear off. Ideally, the animal will wake up within 1 hr of the end of the operation. Increasing time to awakening results in increasing abortion frequency. Food and water on the floor of the cage should be provided for the immediate postoperative period.
11. Mothers may be allowed to deliver progeny vaginally or the offspring may be harvested by cesarean section. Maternal and fetal survival are approximately 60% at early embryonic ages of injection, and increase with gestational age to virtually 100% after postnatal injections.

*Injection of Virus Using ex Utero Surgery*

Injections into small or delicate structures (such as the eye) require micropipettes that are too fine to penetrate the uterine wall. In addition, it is impossible to precisely target many structures through the rather opaque uterine wall. These problems can be circumvented, although with a considerable increase in technical difficulty and decrease in survival, by use of the *ex utero* technique. The procedure is similar to that just detailed with the following modifications to free the embryos from the uterine cavity:

1. The technique works well in our hands only with outbred, virus-antigen free CD-1 and Swiss-Webster mice, but even these strains may have different embryo survival rates when obtained from different suppliers or different colonies of the same supplier. This variability presumably results from subclinical infections which may render some animals unable to survive the stress of the operation. We have had no success with this technique in rats.

2. After the uterus is exposed and *before* filling the peritoneum with LR, incise the uterus longitudinally along its ventral aspect with sharp microscissors. The uterine muscle will contract away from the embryos, causing them to be fully exposed, surrounded by their extraembryonic membranes.

3. Only two embryos in each uterine horn can be safely injected, apparently because of trauma induced by neighboring embryos touching each other. Thus, all other embryos must be removed. Using a dry sterile cotton swab, scoop out each embryo to be removed, with its placenta and extraembryonic membranes, and press firmly against the uterine wall where the placenta had been attached for 30–40 sec to achieve hemostasis. It is very important to stop all bleeding before proceeding. From this point on, the embryos must be handled extremely gently, as only the placenta is tethering an embryo to the uterus, and it detaches easily.

4. Fill the peritoneal cavity with LR, and cushion each embryo to be injected with sterile cotton balls soaked in LR. Keeping the embryos submerged throughout the remainder of the procedure is essential to their survival.

5. The injection should then be done with a pneumatic microinjector and heat-pulled glass micropipette. This may usually be done by puncturing

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the extraembryonic membranes first and then the structure to be injected; for some very delicate injections it may be necessary to make an incision in the extraembryonic membranes, which is then closed with 10-0 nylon suture after the injection.

6. At the time of desired fetal harvest or at the latest early on the last day of gestation, sacrifice the mother by cervical dislocation, rapidly incise the abdomen, and deliver the fetuses. If survival to a postnatal time point is desired, it is necessary to foster the pups with another lactating female. This is best done with a mouse that has delivered at the same time as the experimental animal, but we have successfully fostered pups with mothers that delivered several days to a week previously. Attempts to reanesthetize the experimental mother for delivery, thus allowing her to survive and obviating the need for fostering, have been unsuccessful due to poor survival of the pups. This is probably due to the deleterious effect of the anesthesia on the pups, as well as poor milk production by the mother after multiple operations.

*Infection of Chick Embryos*

*Retroviral Injections into Chicken Embryos*

*Materials*

- Rotating, wooden egg incubator, optional (Petersime, Gettysburg, OH)
- Benchtop egg incubator with plastic egg trays (Kuhl, Flemington, NJ)
- Fertilized White Leghorn chicken eggs, specific pathogen free (SPF) (SPAFAS, Inc., Norwich, CT). Stored at 4–12° until needed.
- 70% ethanol
- Small curved scissors, previously soaked in ethanol
- Fine forceps, previously soaked in ethanol
- 20-gauge needle with a 5-ml syringe
- Clear packing tape
- Egg holder, homemade wooden or plastic rings, 2-inch diameter, one-half inch high
- Dissection microscope
- Fiber optic light source with green filter (optional)
- Micromanipulator
- Microinjector with motorized, direct-displacement advance (optional) (Stoelting, Wood Dale, IL)
- Evacuated mineral oil to fill tubing of microinjector and micropipettes
- Approximately 10 micropipettes (Omego Dot, 1 mm outer diameter, 0.75 mm inner diameter), broken with fine forceps to an outer diameter of 10–20 mm.
Pipette puller
Fast green (0.25% in water), filter sterilized and stored frozen as aliquots
Concentrated virus, stored at \(-80^\circ\), thawed, and placed on ice
Polybrene (0.8–8 mg/ml in water), not required for A-subgroup viruses but may help; titer for your application by performing infections \textit{in vivo} in the site you wish to infect

1. Warm the eggs to room temperature for 1–2 hr and then incubate them on their sides in a humidified, rocking incubator at 37.5–38\(^\circ\). The first 24 hr of incubation is considered embryonic day 0 (E0). A nonrocking (benchtop) incubator is adequate for injections and survival to E19, provided that albumin is removed as detailed in steps 2–4.
2. On E1 or E2, remove a batch of eggs from the incubator, drench with 70% ethanol, and air dry.
3. Lay the egg on its side in an egg holder, and use the 20-gauge needle attached to the 5-ml syringe to jab a small hole in the side of the egg facing up. Jab a second hole in the large end of the egg and insert the needle about 0.5 cm into the albumin at a steep angle to avoid hitting the yolk. Withdraw 1.5 ml of albumin and discard.
4. Seal the holes with tape. Place the egg on its side in a humidified, nonrotating, benchtop incubator.
5. Use small curved scissors to open and enlarge the hole on the top of the egg. Cutting through tape prevents small pieces of shell from falling into the egg. Make a hole approximately 1 cm in diameter, locate the embryo, and then enlarge the hole as necessary (typically about 2 cm wide) to expose the embryo for injection. This step can be done in advance, on E1 or E2, or it can be done immediately before injection. If done in advance, the hole in the shell is resealed with tape.
6. On the day of injection, mix 1 part fast green, 1 part polybrene (we use 1 part 0.8 mg/ml for subgroups E and A), and 8 parts concentrated virus. Spin for approximately 10 sec in a microfuge (10,000 rpm) to pellet large aggregates. Keep the virus stock on ice for the 2–4 hr required to inject a batch of embryos (usually about two to three dozen per experiment).
7. Insert a micropipette into the microinjector tubing that was previously filled with heavy mineral oil, avoiding air bubbles. Attach the micropipette to the micromanipulator, and use gentle pressure to fill the micropipette with oil to the tip.
8. Place 3–5 μl of viral stock on a piece of Parafilm, and lower the tip of the micropipette into the viral stock. Pull back on the Hamilton syringe of the microinjector to fill the pipette with virus. Place the tip of the filled micropipette in sterile saline whenever there is a lag in the procedure.
9. Open the hole in the side of the egg, stabilize the egg with an egg holder, and illuminate the embryo with fiber optics under a dissection microscope. Optimal contrast is obtained by illuminating the embryo from an oblique angle; a green filter can be added to enhance contrast. Embryos younger than E4 can usually be injected directly. Older embryos (E4 and later) usually require some additional preparation because of a thickening and increased vascularization of the embryonic membranes. With fine forceps, tear the serosa, avoiding or diverting the allantois if possible. Otherwise, make an additional tear in the allantois, avoiding the blood vessels as much as possible. Tease open the amnionic membrane and drape the torn edges over the edge of the hole in the shell to stabilize the embryo during injection.

10. Lower the micropipette into the desired structure or cavity of the embryo using the micromanipulator. For neural tube injections, the tip is lowered directly through the overlying amnion and epidermis until it enters the ventricles of the neural tube. The location of the inoculum is readily observed because of the presence of carrier dye.

11. Inject with the motorized microinjector, usually 0.1–1.0 μl; this usually takes 5–50 sec.

12. Reseal the hole with tape and return to the incubator.

Human Placental Alkaline Phosphatase as a Histochemical Marker Gene

Human placental alkaline phosphatase (PLAP) has only recently been adapted for use as a histochemical marker for lineage studies. As such, neither its benefits nor its potential drawbacks have been exhaustively analyzed, and this should be kept in mind as this enzyme begins to enjoy wider use. Our laboratory has undertaken a systematic lineage analysis in the postnatal mouse retina and rat striatum comparing β-Gal and PLAP and has seen very little difference between the two. Thus for these particular combinations of tissue and vectors (BAG and DAP), there appears to be no effect of ectopic PLAP expression on the choice of cell fate or survival. We do not yet know whether this conclusion will hold for other tissues. In the chick retina and cerebellum, for example, clonal analysis does show differences between β-Gal and PLAP in the ease of detection of different cell types. It is not yet clear whether these differences reflect cell-specific inactivation of the promotor or the enzymes, differential distribution of the two enzymes within intracellular compartments, or (most worrisome) the perturbation of cell fate or survival.

Human PLAP was initially chosen as a potential histochemical marker for several reasons. Among the variety of isoenzymes of alkaline phosphatase that have been studied, human PLAP is by far the most heat stable,
by a factor of about 100.\textsuperscript{25} Thus, although many tissues express endogenous alkaline phosphatase(s), it is possible in most cases to greatly reduce this background reactivity by preincubating the tissue at 65° for 30 min. In addition, PLAP is resistant to a variety of substances that act to inhibit other isoenzymes of alkaline phosphatase.\textsuperscript{26} We have tested the following inhibitors on mouse and chick neural tissue as recommended by Zoellner and Hunter: 0.5 mM levamisole (L[\(-\)]-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole), 2 mM mercuric chloride, 5 mM L-leucylglycylglycine, 1 mM EDTA, 1 mM L-phenylalanine-glycyl-glycine, 0.2 M lysine HCl, or 0.3 mM sodium arsenate. Levamisole was the most useful in reducing the background staining in brains, although it also reduced PLAP staining slightly in some cases; it was less effective in retinas. None of the inhibitors was nearly as effective as heat treatment in reducing background in the central nervous system. Nonetheless, it is certainly possible that their use may facilitate detection in other tissues. Another benefit of PLAP as a marker is the fact that its activity is probably minimal at normal intracellular pH since this is considerably below the optimal pH for enzyme function. This may suggest that ectopic expression of PLAP during development is not likely to perturb normal physiological processes, but this remains to be tested systematically for different tissues.

\textit{Double-Staining of Infected Tissues for \(\beta\)-Galactosidase and Alkaline Phosphatase Activities}

The following protocol was adopted for the double staining of \(\beta\)-Gal and PLAP in nervous system tissue. Cells expressing \(\beta\)-Gal will be rendered a bright blue, whereas cells expressing PLAP will be rendered purple due to the presence of NBT in the reaction. The order in which the staining is done is critical since \(\beta\)-Gal is inactivated by the heat treatment that is required to inhibit endogenous alkaline phosphatases. Obviously, if only a single marker is needed, the protocol can be minimally adapted for use with either enzyme individually.

Detecting both enzymes in the same cell is not always possible using the following protocols because the PLAP reaction product is so intense that it usually obscures the \(\beta\)-Gal reaction product. For example, it is difficult to detect cells cotransfected with both genes \textit{in vitro}. This is not a problem when the two enzymatic markers are used in retroviral vectors for lineage mapping studies because individual cells are very unlikely to be infected with both vectors and express both enzymes. (Even if they


were, the interpretation of the results would not be affected since one is still observing a clone.) If detection of cells containing both enzymes is necessary, use of different chromogenic substrates might circumvent this difficulty. PLAP can be reacted with naphthol-AS-BI-phosphate/New Fuchsin, which produces a red stain,\(^ {27}\) and NBT can be added to the β-Gal reaction to produce a purple precipitate. Red/blue or red/purple might be distinguishable from the corresponding single stains; we have not tested these possible combinations.

We have found that performing immunohistochemistry in conjunction with either β-Gal or PLAP histochemical staining has not been very satisfactory. The colored precipitates block fluorescence, thus preventing the use of fluorescently conjugated antibodies. Using HRP-conjugated antibodies, it is difficult to distinguish blue/brown double-labeled cells from brown-labeled ones whereas purple and brown/purple cells would probably look identical. Situations in which the subcellular localization of β-Gal and an antigen are significantly nonoverlapping (e.g., a nuclear antigen) may allow for detection of both.\(^ {28}\) In addition, one can try to limit the intensity of the β-Gal or PLAP reaction product by shortening the time of the reactions to allow for more sensitivity in detecting an antigen within the same cell by HRP-conjugated antibodies.\(^ {29}\) Alternatively, antibodies exist for both β-Gal (rabbit polyclonal, Cappel; mouse monoclonal, Boehringer Mannheim) and PLAP (rabbit polyclonal, Zymed; mouse monoclonal, Medix), although using them is obviously more time-consuming than relying on histochemical reactions.

Different species, tissues, or parts of tissues (i.e., brain regions) can have varying amounts of background labeling.\(^ {30-32}\) This is especially noticeable with alkaline phosphatase reactions, but is also true for β-Gal. We recommend always including negative controls in order to assess the extent of background for a particular tissue or region of interest. Important variables that affect the signal to noise ratio include type of fixative, length of fixation, length of washes, length of heat treatment, prolonged exposure to light, and prolonged storage after staining. For a particularly problematic


tissue, it may prove advantageous to try different inhibitors of endogenous alkaline phosphatases (see above), as well as different substrates.

**Solutions**

Phosphate-buffered saline (PBS; 10×): 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄, and 2 g KH₂PO₄ in 1 liter H₂O. Adjust to pH 7.2–7.4. Dilute 1:10 before using.

0.5% glutaraldehyde: 25% stock (Sigma) can be stored at −20°C and frozen/thawed many times. Make dilution immediately before use.

4% paraformaldehyde: 4 g solid paraformaldehyde, 2 mM MgCl₂, and 1.25 mM EGTA (0.25 ml of a 0.5 M EGTA stock, pH 8.0) in 100 ml PBS, pH 7.2–7.4. Heat about 80 ml H₂O to 60°C and add paraformaldehyde; add enough NaOH to get paraformaldehyde in solution. Cool to room temperature, add 10 ml 10× PBS, adjust pH with HCl, add MgCl₂ and EGTA, and make up to 100 ml with H₂O. The solution can be stored at 4°C for 1–2 weeks.

X-Gal detection buffer: 35 mM potassium ferrocyanide (can vary from 5 to 35 mM), 35 mM potassium ferricyanide (can vary from 5 to 35 mM), 2 mM MgCl₂, 0.02% Nonidet P-40 (NP-40) (diluted from 10% stock solution), and 0.01% sodium deoxycholate (diluted from 10% stock solution) in PBS. This buffer can be stored for at least 1 year at room temperature in a foil-covered container.

X-Gal stock (40×): 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethylformamide. Store at −20°C in a glass container covered with foil.

X-Gal reaction mix: Make a 1:40 dilution of X-Gal stock into X-Gal detection buffer. Make dilution immediately before using. Final concentration of X-Gal is 1 mg/ml.

X-P detection buffer (Buffer 3, Genius Kit, Boehringer-Mannheim): 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂. Store at room temperature. Tends to precipitate over several weeks. This does not seem to noticeably affect the staining.

X-P stock (100×): 10 mg/ml 5-bromo-4-chloro-3-indolyl phosphate. X-P, also referred to as BCIP in H₂O. Store in the dark as aliquots at −20°C. Can be frozen and thawed several times.

NBT stock (50×): 50 mg/ml nitro blue tetrazolium in 70% dimethylformamide, 30% H₂O. Store at −20°C in a glass container covered with foil. Does not freeze at this temperature.

X-P reaction mix: 50 μl X-P stock (100×), 100 μl NBT stock (50×), and 50 μl 50 mM levamisole (100×) (if desired) in 5 ml X-P detection buffer. Make fresh immediately before using. Final concentrations:
X-P, 0.1 mg/ml; NBT, 1 mg/ml; and levamisole, 0.5 mM. Levamisole was ineffective at reducing background in chick cerebellum, while slightly inhibiting PLAP, and was therefore not used in this tissue. Any other desired inhibitor (see introduction) would also be added to the X-P reaction mix.

Gelatin subbing solution for slides: 2 g gelatin and 0.1 g chromium potassium sulfate (chrome alum) in 200 ml H2O. Heat H2O to 60°. Dissolve chrome alum, then gradually dissolve gelatin. Filter before use. Can increase or decrease percentage of gelatin. Load slides in racks, dip quickly, and air-dry overnight.

Gelatin/sucrose embedding medium: 7.5% gelatin (porcine skin, Sigma), 15% sucrose, and 0.05% sodium azide in 1× PBS. Dissolve gradually at 60°, with stirring. The medium solidifies at room temperature to a transparent gel. Store at room temperature. Liquefy in microwave with frequent swirling before embedding samples.

Gelvatol mounting medium: Make up according to the protocol given by Rodriguez and Deinhard. Instead of Elvanol, we use Vinol grade 205, polyvinyl alcohol resin (Air Products and Chemicals, Inc., Allentown, PA).

Whole Mount Staining Procedure

The primary protocol shown was worked out for staining of intact mouse retinas that had been double-infected with BAG and DAP. The order in which staining is done is critical since β-Gal is inactivated by the heat treatment that is required to inhibit endogenous alkaline phosphatases. The incubation times for X-Gal and X-P reactions are variables that may require adjustment for different tissues. Longer times may be necessary for large or dense chunks of tissue, although there is a trade off as the background staining of either enzyme intensifies with increasing reaction time. Whole chick embryos (E7) and chick brains (E10) were found to be incompletely reacted in their centers after 4-hr incubation periods. For tissues that are difficult to stain completely as whole mounts, one can stain as sections (see next section). Alternatively, at least for the X-P reaction, one can do a 3- to 4-hr whole mount stain to locate cells of interest, dissect out and section only those areas of interest, and restain the sections for 20–30 min to obtain optimal staining.

1. Dissect the tissue into PBS containing 2 mM MgCl2 (PBS + Mg2+) on ice.

2. Fix in 0.5% glutaraldehyde in (PBS + Mg\(^{2+}\)) for not longer than 45 min on ice. Glutaraldehyde (0.5%) decreases the alkaline phosphatase activity in chick cells stained \textit{in situ} (but not in chicken embryo fibroblasts stained \textit{in vitro}). Therefore, fixation of chick whole mounts is typically done in 4% paraformaldehyde in PBS for 2–4 hr at 4\(^\circ\). In areas where background alkaline phosphatase activity is a problem, increasing the time in 4% paraformaldehyde, even up to several days, can decrease endogenous background without significantly decreasing PLAP activity. However, such long fixation times may decrease \(\beta\)-Gal activity.

3. Rinse in (PBS + Mg\(^{2+}\)) five times for 5 min. Rinsing overnight is fine; but waiting for several days at this step may decrease \(\beta\)-Gal activity.

4. Stain in X-Gal reaction mix for 2–4 hr at 37\(^\circ\). (Tris buffer was tried in place of PBS, with no success.)

5. Rinse many times in PBS until the solution no longer turns yellow. This usually takes about five changes. An overnight rinse is fine. It is important to remove X-Gal since residual \(\beta\)-Gal activity in the presence of X-Gal and NBT (added for the following reaction) may enable \(\beta\)-Gal\(^+\), PLAP\(^-\) cells to turn purple. Chick retinas and cerebella have been kept in PBS at 4\(^\circ\) for at least 1 month at this point with no appreciable loss of signal in subsequent X-P staining. X-Gal staining can be easier to examine prior to carrying out the X-P reaction as background alkaline phosphatase staining can obscure X-Gal signal somewhat, particularly in whole mounts.

6. Heat tissue in PBS at 65\(^\circ\) for 30 min. This is usually done by floating the dish containing the tissue in a water bath preset to 65\(^\circ\). For staining of embryonic chick diencephalon (one of the areas of the brain with the highest background), this step was increased to 1.5 hr.

7. Preincubate in X-P detection buffer for 15 min. Extending the time of this step results in diffusion of the alkaline phosphatase reaction product.

8. Incubate in X-P reaction mix for 3 hr at room temperature. Since background staining increases in light, cover with foil during and after staining.

9. Rinse in 20 mM EDTA in PBS for 2–4 hr. Background can be due to endogenous AP or other reactions which generate hydride ions and thus reduce NBT to form a purple precipitate. We have noted that background staining comes up more slowly in the presence of EDTA. Tissue can be stored in the dark at 4\(^\circ\) in PBS + EDTA or 30% sucrose in PBS + EDTA for many months, although the background clearly increases over time.

10. Embed in Paraffin wax using minimum necessary times for the tissue of interest. For mouse retina, which is approximately 250 \(\mu\)m thick, the following procedure was used. Dehydrate through graded ethanols: 50%, 70%, 95%, 100%, 100% for 20 min each. Clear in xylene, 2 \(\times\) 15 min. Infiltrate with 1:1 mix of xylene and Paraffin, 65\(^\circ\) for 30 min. Paraffin,
2 × 15 min. Embed in Paraffin. Clearing and Paraffin embedding is not recommended for tissues fixed with paraformaldehyde. In paraformaldehyde-fixed chick tissues, both the β-Gal and the PLAP reaction products are very sensitive to xylene treatment; even relatively short exposures to xylene causes the reaction product to diffuse into the surrounding tissue. In some cases, this was true in the ethanol washes as well. Frozen sections are a workable alternative (see next section on the preparation of tissue for cryostat sectioning). Other embedding protocols, particularly aqueous-based procedures, may be worth testing. It is worth noting that strong staining in glutaraldehyde-fixed material can even withstand preparation for electron microscopy.28

11. Section onto slides coated with gelatin. Silane-treated slides are equally effective.34

12. Remove Paraffin with xylene and mount with Permount. For frozen sections, fix sections to slides with 4% paraformaldehyde for 15 min. Rinse with PBS, then mount in Gelvatol (+ EDTA if desired).

Storing slides at −80° helps prevent background staining from increasing.

Protocol for Staining Sections

This protocol was worked out for embryonic rat striatum.20 Fixation and staining times may need to be altered for other areas of interest.

1. Fix tissue by perfusion and follow with immersion in 4% paraformaldehyde at 4° for 8 hr. Rinse briefly in PBS, then sink in 30% sucrose in PBS containing 2 mM MgCl₂ (PBS + Mg²⁺) at 4°. Fixation times will vary with size of tissue. Perfusion may not be necessary for all tissues, especially in embryonic animals. Shorter fixation times may be preferable, as X-Gal staining may be decreased by lengthy fixation.

2. Embed brain in OCT or gelatin/sucrose mounting medium and freeze on liquid N₂. Gelatin/sucrose embedding gives better frozen sections for embryonic tissue than does OCT. Paraffin embedding destroys β-Gal activity, but cells in culture treated as if to Paraffin embed retain PLAP activity.

3. Cut cryostat sections and mount on gelatin-coated slides; air-dry overnight. Sections up to 90 mm thick (the thickest we have tried) have been successfully stained.

4. Fix sections to slides in 4% paraformaldehyde for 10–15 min at 4°.

5. Rinse slides in (PBS + Mg²⁺) twice, for 10 min each, at 4°.

6. Stain slides in X-Gal reaction mix for 6 hr at 37°.

7. Rinse slides in PBS three times, for 10 min each, or until solution is no longer yellow. Slides can be left in PBS overnight. (See comments in Whole Mount Staining Procedure, step 5.)
8. Transfer slides to preheated PBS at 65° and heat for 30 min.
9. Rinse slides in X-P detection buffer for 10 min.
10. Stain slides in X-P reaction mix for 12 hr. Since background staining increases in light, cover with foil during and after staining.
11. Rinse slides in PBS + 20 mM EDTA three times for 10 min. Mount in Gelvatol (+ EDTA if desired). (See comments on background staining in Whole Mount Staining Procedure, step 9. Storing slides at −80°C helps prevent background staining from increasing.)

Clonal Analysis Using the PCR/Library Method

As described earlier in the section on determination of clonal boundaries, there are situations where a great deal of migration can lead to splitting errors. In these situations, a larger number of distinguishable viruses can lead to identification of sibling relationships with a greater degree of certainty. We thus devised a method in which a large number of viruses (a "library") could be produced and injected into a single animal.\(^{23}\) PCR was then used to distinguish the individual library members. In our first libraries, we used insertion of 50–300 irrelevant DNA fragments to create libraries in murine BAG\(^{12}\) and DAP\(^{13}\) vectors and in avian RDlac\(^{35}\) and CHAP\(^{21}\) vectors. We have also made a library using degenerate oligonucleotides and the PCR products are sequenced.\(^{35a}\) This library is of far greater complexity (at least 10\(^4\) members) and is still being tested, but looks very promising for use in situations where more certainty is desirable. The protocols used for creating a murine library in BAG and for amplifying the products from single cells are given below.

Preparation of a Retroviral Library for PCR Analysis

In principle, any retroviral plasmid can be used to make a library. We started with the BAG plasmid which contains a unique cloning site (XhoI) downstream of the reporter gene. The insertion of DNA into this site does not appear to interfere with expression of the upstream genes. The inserted DNA was genomic DNA from *Arabidopsis thaliana* digested with *MboI*. The digested DNA was run on an agarose gel and DNA fragments less than 450 bp in size were used as inserts.\(^{23}\)

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Before making retroviruses with any of these constructs, approximately 100 constructs were identified whose inserts were conveniently distinguishable by size or by their pattern of digestion using restriction enzymes. PCR products were prepared from bacterial colonies, the products were separated on agarose gels, and the approximate size of each PCR fragment was recorded. PCR products were sorted by size, and each product was digested with a mixture of restriction enzymes with four base recognition sites that were chosen because they were inexpensive and compatible within the same digestion buffer (MspI, RsaI, AluI, CfoI, and MseI). The size and restriction pattern of each tag were recorded, and approximately 100 constructs were chosen that were distinguishable in this standard assay.

Retroviruses were next prepared from the 100 constructs by transfecting minipreparation DNA, purified using crushed glass, into a packaging cell line. The 100 DNAs were transfected pairwise into 50 dishes of the ~CRIP amphotropic packaging line so that each transfected plate contained a mixture of cells producing two viral constructs. The supernatant of each transfected plate was used to infect a dish of the ecotropic packaging cell line ~2. Infected ~2 producer cells were selected by growth in medium containing G418 for 7 to 10 days, and the population of resistant colonies was raised to confluence.

Viral supernatants were recovered and titered on NIH/3T3 cells in 6-well tissue culture dishes. DNA from the infected NIH/3T3 cells was amplified using PCR to evaluate the passage of the genetic tags. After the titering reaction, 0.5 ml of a solution of 0.5% Tween 20 and 200 μg/ml proteinase K in 1× PCR buffer was added to each plate of cells. After incubation for 60 min at 65°, the proteinase solution was transferred to a 0.6-ml microfuge tube and incubated at 85° for 20 min, then at 95° for 10 min. The undissolved X-Gal precipitate was pelleted by centrifugation at 10,000 rpm for 10 min, and 10-μl samples of the supernatant were used as templates for 50-μl PCR reactions as described earlier.

The 50 viral supernatants were then mixed in approximately equal quantities and concentrated by centrifugation. The concentrated stock was titered, tested for helper virus, and checked by infection of rodent retina. It was then used to perform experiments in the cerebral cortex.

The method just described can be easily modified in several ways. Any convenient restriction site in a replication-incompetent vector can be used for insertion of tags so long as it does not interfere with expression of the reporter gene or transmission of the virus. Any source of DNA that is not

present normally in the host tissue or vector can be used. Any set of enzymes can be chosen for the restriction enzyme characterization of the inserts, and any range of insert sizes can be used if the longer ones are not so long that their size would cut down on the efficiency of the PCR. Finally, it is possible to transfect the entire library as one plasmid preparation into producer cells rather than going through the tedium of making individual stable producers or producer populations. We have used this method in making extremely complex libraries using oligonucleotides as tags.\textsuperscript{35a}

\textit{Tissue Analysis for PCR-Based Clonal Assignments}

Animals are infected and processed histochemically for the appropriate reporter gene as described in the previous sections. Usually sections are made for this analysis, but in special circumstances, whole mounts can be used. Once the histochemistry is complete, the labeled cells are analyzed and their position as well as the outline and hallmarks of the tissue are recorded. The goal is to create a permanent record of the morphology and relative location of the labelled cells since the PCR analysis will destroy the cells and surrounding tissue. A standard microscope and camera lucida device allows morphological details of cells to be drawn at high magnification, whereas cell location can be plotted at low magnification. Alternatively, cells can be photographed or plotted on a computerized system (e.g., CARP\textsuperscript{37}). The anatomical analysis is often the most time-consuming aspect of the entire analysis.

\textit{PCR of Labeled Cells}

After anatomical analysis, PCR amplification of the tags that distinguish the vectors allows clonal analysis. For these experiments, start with two "nested" pairs of oligonucleotides specific for the region of the vector in which the inserts were placed. PCR parameters for each oligonucleotide pair must be optimized, especially the Mg\textsuperscript{2+} concentration in the PCR buffer.\textsuperscript{38}

\textit{Materials}

Histological sections prepared and stained as described in the preceding section, and coverslipped in Gelvatol
Sterile distilled water
1× PCR buffer
Two nested pairs of oligonucleotides (as 20 μM solutions)

\textsuperscript{37} C. P. Austin and C. L. Cepko, Development (Berlin) 110, 713 (1990).
\textsuperscript{38} K. B. Mullis and F. A. Faloona, this series, Vol. 155, p. 335.
Deoxynucleotide solution (20 mM each in dATP, dTTP, dCTP, dGTP)
Proteinase K, 10 mg/ml solution, in sterile, distilled water
Tween 20, 10% solution, in sterilized distilled water
Mineral oil (light), from Sigma
Disposable, breakable razor blades and blade holder (Fine Scientific Tools)
600-μl microfuge tubes (autoclaved and silanized) or 96-well microtiter dishes and lids (e.g., Falcon No. 3911 and 3913)
Automated thermal cycler
Dissecting microscope
50-ml centrifuge tubes

*Note:* All solutions and containers must be assembled and stored using the most stringent precautions to prevent contamination with DNA that could be amplified in the PCR. A dedicated set of solutions should be handled with dedicated, positive displacement (or other contamination-resistant) pipetting devices in a separate laboratory remote from the thermal cycler. Good discussions of minimizing PCR contamination are available elsewhere. 38

**Dissect Cells and Digest Tissue**

1. Prepare a lysis solution. For 50 samples, mix
   50 μl, or optimum amount, of 10× PCR buffer
   25 μl of 20 μM solution of each of the outermost oligonucleotides
   10 μl of 10 mg/ml solution of proteinase K (final concentration is 0.2 mg/ml)
   25 μl of 10% Tween 20 solution (final concentration is 0.5%)
   375 μl distilled water

2. Soak off coverslips in distilled water in a clean, sterile 50-ml centrifuge tube. After the slide has soaked about 30 min, the coverslip can be carefully pried off with a razor blade. After the coverslip comes off, soak the tissue about 5 min more to remove traces of Gelvatol.

3. Pipette 10 μl of the lysis solution into each 0.6-ml microfuge tube (or well of a 96-well microtiter dish).

4. Break off a fresh fragment (2–5 mm wide at the edge) of the breakable razor blade in the blade holder.

5. Under the dissecting microscope, locate a labeled cell. Well-stained cells can be seen with a low magnification objective (0.8×), but lighter cells may only be seen with a high (5×) magnification objective.

6. Using the razor blade, cut a fragment of tissue that includes the nucleus of the labeled cell. If labeled cells are widely scattered, they can be dissected one at a time in chunks that contain approximately 1000 unlabeled cells. Chunks are typically <500 μm in each dimension, but larger pieces can be used (PCR sensitivity may be less with larger pieces since they do not dissolve as well). If labeled cells are immediately adjacent, dissect several cells in one chunk.

7. Transfer the tissue piece, on the razor blade, to the lysis solution. Confirm under the microscope that the labeled cell is in the lysis solution. It is important to keep careful notes of which cell goes where—drawings are helpful here.

8. Cover the lysis solution with 100 μl of mineral oil, and cap the tube (or cover the microtiter plate).

9. Replace the blade fragment with a fresh one, and dissect the next cell (return to step 5). While dissecting cells, prepare negative controls. Intersperse samples that contain no tissue, or unlabeled tissue, among the positive samples.

10. After all cells have been dissected, and all lysis samples are covered with mineral oil, cover the tubes (or plate).

11. Transfer samples to a thermal cycler. Digest for 2–3 hr at 65°. Inspect a few samples after this time to confirm that the tissue is totally dissolved. If not, digest longer (overnight at 37° is alright). The X-Gal precipitate does not dissolve, but does not interfere with the PCR.

12. Once the tissue is digested, heat it to 85° for 20 min, then 95° for 5 min. This inactivates the proteinase K and denatures the genomic DNA. The samples are now ready for the PCR.

First PCR Reaction

1. Prepare PCR solution. For 50 samples, use
   100 μl (or optimum amount) of 10× PCR buffer
   50 μl of 20 μM solution of each of the outermost oligonucleotide primers
   10 μl of the mixed deoxyribonucleotide solution (20 mM of each dNTP)
   7.5 μl (37.5 U) of Amplitaq DNA polymerase (Perkin Elmer)
   800 μl of distilled water

These reactions can be scaled up or down proportionately. Taq polymerase from other manufactures can be used but should be tested first.
2. Put the samples in the thermal cycler and start the PCR reaction, which should begin with an initial denaturation at 92–94° for 3 min. PCR conditions need to be optimized, but will comprise 45 repetitions of denaturation (92–94° for 30–45 sec), annealing (55–70° for 1–3 min), and extension (72° for 1 min).

3. Once the samples have reached >85°, uncover them and add 20 µl of the PCR solution to each sample. The "hot start" enhances the sensitivity and specificity of the PCR. The added PCR solution does not have to be mixed in. It will sink beneath the oil and join the aqueous phase.

4. Cover the samples and allow the PCR reaction to proceed.

Second PCR Reaction

The samples now contain large amounts of amplified target DNA and should not be opened or even transported into the clean laboratory. Since contamination of them is not a concern, they may be handled in the main laboratory. The sensitivity of the second PCR reaction is not critical, and Taq polymerase from any manufacturer can be used.

1. Prepare the following solution. For 50 samples, use
   10 µl mixed deoxyribonucleotide solution (20 mM of each dNTP)
   200 µl (or optimum amount) of 10× PCR buffer
   50 U Taq DNA polymerase
   1800 µl sterile distilled water
   20 µl of each of the internal pair of oligonucleotide primers (final concentration is 0.2 µM)

The lower concentration of primers and deoxyribonucleotides used in the second reaction does not affect the PCR sensitivity or product yield.

2. Pipette 40 µl of this solution into 50 tubes (or microtiter wells).

3. Transfer 2–4 µl of the product of each PCR reaction into each tube (or well). Keep samples carefully labeled, and take note of any pipetting errors.

4. Run the second PCR for 25–35 cycles. Conditions for the second PCR will also have to be optimized, but will likely be similar to those used for the first reaction. If the same thermal cycler is used for both the first and second PCR reactions, be sure not to open any of the samples of the second reaction near the machine.

5. Separate the PCR products on 3% NuSieve/1% Seakem agarose gels. Use 1× TBE as the running buffer, and include pBR/MspI or ΦX/HaeIII DNA size standards. Anticipate that 40–70% of the samples will produce a band. Suspect a problem if yields are consistently <40%; suspect contamination if many contiguous samples show the same product.
Direct Restriction Enzyme Digestion of PCR Products

DNA inserts may be distinguished by size or sequence analysis. Restriction mapping offers the most convenient assay and is sufficiently specific for libraries with 100–300 different inserts. We chose five restriction enzymes with four-base recognition sequences (CfoI, RsaI, AluI, MseI, MspI) that were active in similar buffer conditions (50–100 mM NaCl). The mixture cuts small DNA fragments frequently, allowing them to be easily distinguished. Before loading the diagnostic gel or prior to restriction digestion, sort the PCR products by size. When samples of similar initial size are run side by side on the gel, it allows the most direct comparison of the restriction fragments.

1. Prepare the following solution (for 50 samples):
   - 250 U of each restriction enzyme
   - 150 µl of 10× restriction enzyme buffer, appropriate for the enzymes chosen.
   - 15 µl 10 mg/ml bovine serum albumin
   - Sterile distilled water to make a total of 500 µl
2. Pipette 10 µl of this solution into individual wells of a microtiter dish (tubes can also be used).
3. Add 20 µl of one of the PCR reaction products to each well.
4. Cover the microtiter plate and incubate at 37°C for 3 hr.
5. Terminate the reactions by adding loading buffer, and separate the products of the reaction on a 3% Nusieve/1% Seakem agarose gel.
6. Record which samples contain the same tags and the overall number of tags seen. Compare this information to the original plots of cell location to get clonal information.

Statistical Analysis

The tentative conclusion from the PCR analysis is that cells containing the same tag are members of the same clone. The confidence of this conclusion rests on (1) the number of clones in a given experiment, k, and (2) the number of tags in the library, n. There is a surprisingly large probability that the same tag will appear in two different clones by coincidence, and this probability needs to be considered in the clonal analysis.

The probability of "coincidental double infections" by one tag can be calculated using binomial theory or computer-simulated using a Monte Carlo simulation. Either method requires the assumption that all tags are present in equal ratios, and thus the ratios of tags in the library must be verified experimentally. Some typical results are shown in Table I, to provide guidelines.
### Table I

<table>
<thead>
<tr>
<th>Number of tags (n)</th>
<th>k = 3</th>
<th>k = 5</th>
<th>k = 10</th>
<th>k = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.14</td>
<td>0.40</td>
<td>0.86</td>
<td>0.99</td>
</tr>
<tr>
<td>80</td>
<td>0.04</td>
<td>0.12</td>
<td>0.43</td>
<td>0.90</td>
</tr>
<tr>
<td>85</td>
<td>0.035</td>
<td>0.11</td>
<td>0.41</td>
<td>0.88</td>
</tr>
<tr>
<td>100</td>
<td>0.030</td>
<td>0.10</td>
<td>0.36</td>
<td>0.85</td>
</tr>
<tr>
<td>250</td>
<td>0.012</td>
<td>0.04</td>
<td>0.16</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Probability that >1 clone shows the same tag = 1 - \( \frac{n!}{(n-k)!n^k} \).

To evaluate clonal data, it is important to evaluate the actual complexity of the viral library (i.e., how many tags are seen in total).\(^{40}\) The best results demand a highly complex library, with very few clones labeled in each experiment (three to four clones). Under these conditions the probability is maximized that observed patterns faithfully reflect clonal patterns. However, be skeptical of patterns that are seen rarely, as they may only reflect rare coincidental double infections instead of true clonal events.

### Reagents and Solutions

10× PCR buffer: 10 mM Tris buffer, pH 8.3 (purchase as premixed crystals from Sigma), 50 mM KCl, 0.01% gelatin, and 1.5–2.5 mM MgCl₂.

Mixed deoxyribonucleotide solution: Deoxyribonucleotides may be purchased from Pharmacia as separate 100 mM solutions of dATP, dCTP, dGTP, and dTTP. Mix them 1:1:1:1:1 with distilled water to make a working mixture that is 20 mM in each. Store 10-μl aliquots at -70°C.

Proteinase K: Can be purchased from many manufacturers, dissolved in sterile distilled water to make a 10-mg/ml solution, and stored as 20-μl aliquots at -70°C.

Oligonucleotide primer solutions: Deprotected oligonucleotides can be passed over a NAP-10 ion-exchange column (Pharmacia) and eluted with sterile distilled water. Adjust the concentration of the effluent to 20 μM by measuring absorbance at 260 nm. We routinely

use oligonucleotides without further purification. Oligonucleotides should be stored as 25- to 50-μl aliquots at -20°.

Note: Reagents, instruments, and glass microscope slides should be handled with scrupulous technique and UV-irradiated when needed to destroy contaminating DNA.

Introduction

Identification of the temporal and spatial patterns of gene expression can provide important clues about gene function. This holds particularly true in embryonic development. In situ hybridization makes it possible to localize transcripts directly in cells within the context of their tissues and organs.\(^1\)\(^2\) Assuming that a protein is expressed in the cells where the encoding transcripts accumulate, roles for gene products consistent with the timing of appearance and observed sites of expression can be proposed.

In situ hybridization relies on the annealing of labeled nucleic acid probes to DNA or RNA sequences within a tissue section or, in the case of whole mount protocols, within an organ or fragment of tissue.\(^3\) The considerations for successful in situ are thus not dissimilar to those of effective membrane nucleic acid hybridization. The signal to noise ratio depends on the specificity, length, complexity, and accessibility of the probe to the hybridization substrate. In the case of in situ hybridization, accessibility gains considerable importance, as the tissue section has a thickness that must be penetrated by the probe without compromising histological integrity.

The in situ hybridization technique has been adapted effectively for studies in a variety of organisms ranging from Drosophila to humans. This method can be employed using DNA or RNA probes to hybridize to a variety of tissue targets including endogenous genes (DNA), viral genes

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