

Targeted Gene Misexpression in Chick Limb Buds Using Avian Replication-Competent Retroviruses

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The methods and applications for using avian replication-competent retroviruses to target gene misexpression in the developing limb bud of the chicken are described. These viruses provide the means to exploit the strengths of the chick as a model system in experimental embryology in conjunction with a genetic approach for ectopically expressing a gene of interest. The applications and strengths of the system are detailed. All the steps required to produce a virus carrying a transgene of interest and the methodologies behind designing and carrying out misexpression strategies are outlined, and some useful techniques for analyzing infected embryos are described. © 1998 Academic Press

The chicken embryo has always had a central role as a classical embryological model system. The chicken egg is easy and cheap to maintain. Moreover, the embryo contained within can be accessed from very early stages of development and is sturdy enough to continue to develop even after surgical manipulations. The dynamics of chick development are also favorable to experimental embryologists as most of the important organizing events both in the primary axes and in patterning of various organs are achieved during the first 4 to 5 days of incubation. One embryonic structure that has been particu-

larly extensively studied is the formation of the limb. One advantage of the system is that the limb buds are external to the embryo and hence are accessible from the earliest stages. The developing limbs can be first identified as discrete outgrowths from the side of the embryo that become visible around 2.5 days of incubation. Most of the patterning of the limb takes place in the following 2 days, and by 6 days of incubation all the major structures of the limb are evident.

One of the greatest strengths of the chicken system is the ability to combine experimental embryology with modern genetic methods. The types of genetic approaches currently exploited in the mouse system, such as transgenics and knockouts, are not available with the chick system. The development of methods using avian retroviruses to target ectopic gene expression *in ovo* has therefore provided a very powerful experimental tool for the chick system.

In this article we concentrate on the methods and applications of using avian replication-competent retroviruses for targeted misexpression of genes in the chick limb bud. Since these vectors spread within the host after infection, genes can be misexpressed over large domains. Avian replication-defective viruses, that do not spread and, as a consequence, are particularly useful in lineage studies are also available but have not, as yet, been widely exploited to study chick limb development. The use of replication-defective viruses for studying the chick visual system is described elsewhere in this issue.

All the techniques detailed in this article are based around the use of replication-competent retroviral

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vectors—RCASBP—originally designed and developed by Hughes and colleagues (1–3). These viruses were derived from an avian oncogenic virus, Rous sarcoma virus (RSV), that contains a host-derived oncogene, *src*. The retroviral vector is constructed by inserting a transgene in place of the *src* oncogene and is amplified as a conventional bacterial plasmid. Therefore large quantities of proviral DNA can be prepared easily. The proviral DNA can then be transfected into a suitable host cell line which produces large quantities of infectious virus particles that are secreted into the medium. This viral supernatant is harvested and concentrated and used directly to infect embryos (4–6). Alternatively, infected tissue culture cells can be pelleted and used directly *in ovo* to locally deliver transgenes encoding secreted proteins (7).

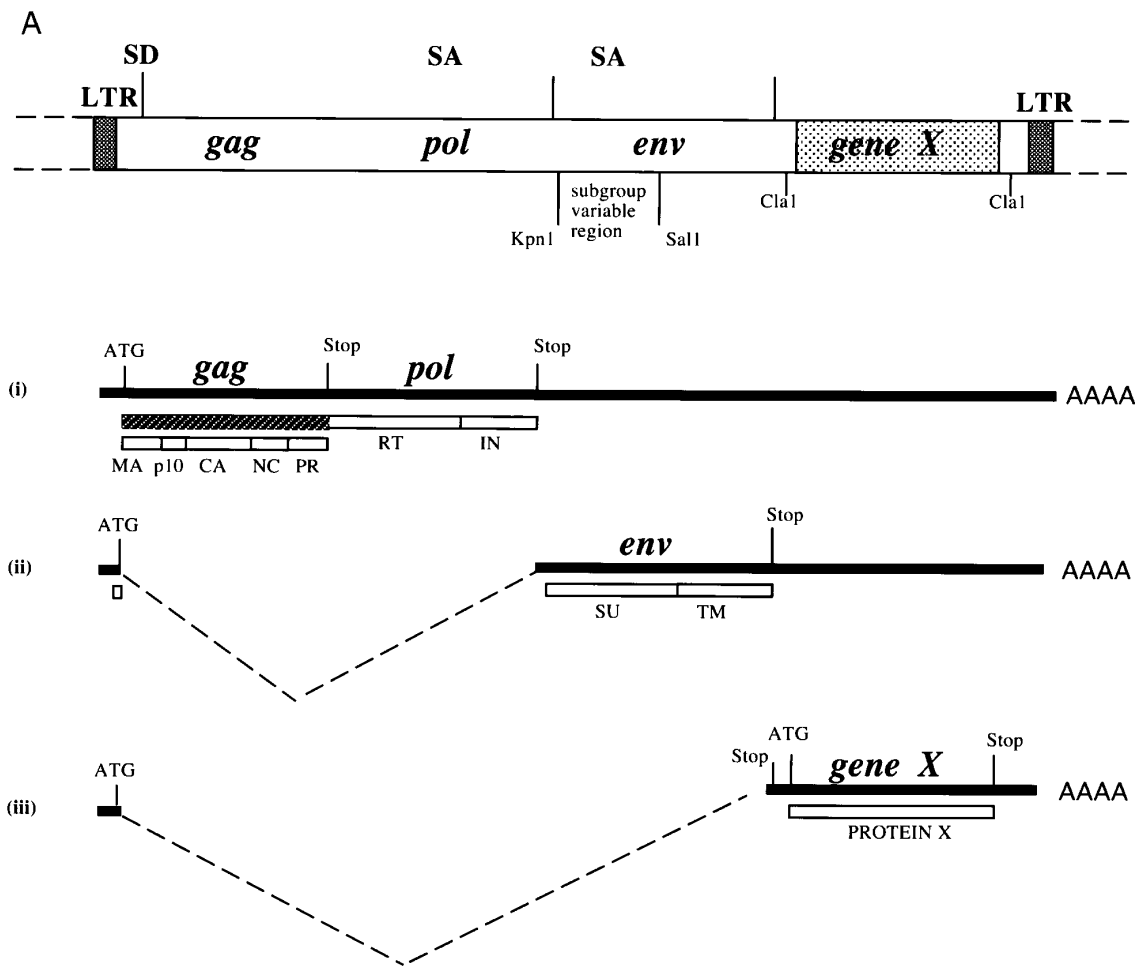
GENERAL RETROVIROLOGY AND APPLICATIONS OF THE SYSTEM

The provirus of RSV is the double-stranded DNA form of the retrovirus that has recombined into the host cell chromosome after reverse transcription of the single-stranded viral genome RNA. The retroviral infectious unit or particle consists of two identical strands of genomic RNA surrounded by a protein core. The protein core becomes covered by an additional membranous envelope, or coat, embedded with viral-specific surface proteins as the virus particle buds off from the host cell surface. [For more details of the virus life cycle see (8) and references therein.]

Three distinct viral mRNA transcripts are generated from the integrated RSV-based RCASBP viruses (Fig. 1A). A full-length transcript may be either packaged into new virions as genomic RNA or translated as a mRNA for a polyprotein that is proteolytically cleaved to produce the viral core proteins—MA (matrix), p10, CA (capsid), NC (nucleo-protein), PR (protease)—encoded by the *gag* gene. Using a translational frameshift the same mRNA encodes for the reverse transcriptase (RT) and integrase (IN) enzymes. An alternative splice generates a shorter transcript encoding the viral envelope proteins: SU (surface/receptor-binding) and TM (transmembrane). The third alternative-splice transcript encodes the transgene inserted in place of the *src* oncogene carried by the parent RSV virus.

The ability of the retroviral virus particle to infect chicken embryo cells is dependent on the viral surface protein (SU), tethered to the cell surface by the transmembrane protein (TM), recognizing a protein that it uses as a receptor on avian cells. Differences in SU define five subgroups of RSV-based retroviruses designated envelope subgroups A, B, C, D, and E. Each subgroup is defined by which receptor protein it interacts with on host cells (the viral tropism) and its ability to block other members of the same subgroup from superinfecting a host cell (the viral interference). The variable regions responsible for distinguishing the viral subgroups are all included in a 1.1-kb *KpnI*–*ClaI* fragment within the *env* gene (Fig. 1A). This fragment, isolated from proviruses of different strains, has been inserted in the RCASBP vector to make viral vectors of different subgroups. RCASBP is available in subgroups A, B, D, and E. Only subgroups A, B, and E are used routinely in the laboratory. The A subgroup receptor has been identified (9) but the receptors for subgroups B, D, and E remain to be identified. Since little is known about the identity of the viral receptors, their tissue distribution is not known and therefore tissue susceptibility to infection has been only partially characterized by empirical data. In our experiments no differences have been observed between subgroups A, B, and E in their ability to infect cells of susceptible chick limb buds. An important note, however, is the observation [(8) and unpublished data] that the infectivity of both the B and E coat viruses (but not A) can be increased by injecting the virus in the presence of Polybrene (Sigma) and therefore it is routinely added to B- and E-type viral inocula. In our hands a final concentration of 80 ng/ml is sufficient although working concentrations 10- to 100-fold higher have also been described [(8) and references therein].

Strains of chickens exist that differ in their susceptibility to infection with viruses of different subgroups. C/E (line 0) chickens (and any cell lines derived from them) are resistant to infection by viruses of the E-envelope subgroup but can be readily infected by viruses of the A and B subgroups. C/A,B cells are resistant to infection with A and B subgroup viruses and C/0 (line 15b) cells are not resistant to any of the five subgroups. The existence of strains with different resistance and sensitivity to infection can be exploited for some applications. It is possible to graft infected cell pellets derived from a sensitive strain into the limb bud of an insensitive strain. This provides a convenient



B

pSLAX13

ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC ATC GAT TCT AGA CCA
 T3 Primer Cla I

CTG TGG CCA GGC GGT AGC TGG GAC GTG CAG CCG ACC ACC ATG GCC ATG ATT
 Nco I

ACG AAT TCG AGC TCG CCC GGG GAT CCT CTA GAG TCG ACC TGC AGC
 EcoR I Sma I BamH I Pst I

CCA AGC TTA TCG ATA CCG TCG ACC GTC GAC CTC GAG GGG
 Hind III Cla I Xho I

GGG CCC GGT ACC CAA TTC GCC CTA TAG TGA GTC GTA TT
 Apa I Kpn I T7 Primer

FIG. 1. (A) RCASBP retroviral vector (top). Avian leukemia virus long terminal repeat (LTR) sequences (dark shading) flank the retroviral *gag*, *pol*, and *env* genes and the site used to insert the transgene (*gene X*). The *KpnI*–*SalI* sites were used to insert envelope genes of different subgroups (A, B, E). The *ClaI* sites are used to insert the transgene (light shading). See text for details. SD, splice donor, SA, splice acceptor. The three retrovirally derived RNA species are shown [(i), (ii), (iii)], with the splicing indicated. The ATG represents the initiator methionine and Stop, the stop codon. The protein products of each gene are indicated by open bars underneath their respective mRNA. Multiple protein products encoded by a single gene are generated by proteolytic cleavage. MA, matrix; CA, capsid; NC, nucleoprotein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface (receptor binding); TM, transmembrane. (B) Multiple cloning site of pSLax13 shuttle vector. Useful restriction sites are underlined. The 5' end of the transgene coding sequence is inserted incorporating the ATG of the *NcoI* site as the ATG, start methionine. Any of the more 3' sites can be used at the 3' end of the transgene construct (i.e., *EcoRI*–*BamHI*–*HindIII*). The flanking *ClaI* sites are used for cloning directly into RCASBP. The T3 and T7 primer sites are useful if sequencing the insert.

way to generate a local source of transgene expression while avoiding subsequent spread of the virus following application of the graft (7).

The replication-competent avian vectors are able to carry out all the functions of the virus life cycle in addition to encoding an additional transcript of the inserted transgene. It is therefore possible for the virus to spread from cell to cell after the initial infection following injection into the embryo. This can result in a widespread infection in the host embryo, and therefore a broad misexpression of the transgene, following the initial infection. Integration of the provirus into the host chromosome is tied to the cell cycle; hence the extent of the subsequent viral spread then becomes, at least in part, a function of the rate of cell division of the population of cells originally infected. Therefore, if the progenitor cell population of the limb-forming regions can be infected before they begin to grow into a bud it is possible to infect all the cells of the wing or leg that develops. Since the replication-competent virus can spread after initial infection, the successful infection procedure is less dependent on an initial high-titer virus stock to generate a widespread infection of the host embryo. An additional advantage of the replication-competent virus is that it is much easier to make high-titer viral stocks with these vectors than with the replication-incompetent vectors. One important disadvantage with replication-competent viruses, however, is that there is a constraint on the size of the insert. It has been determined empirically that RCASBP vectors have a maximum insert size of approximately 2.4 kb. Some success has been reported (C. Cepko, personal communication) with inserts of up to 2.6 kb or even larger, but this is generally not recommended.

The family of retroviral vectors used for all the applications described herein has been denoted RCASBP for replication-competent, avian leukemia virus long terminal repeat, splice acceptor, Bryan high-titer polymerase. A vector RCANBP is also available that is similar to RCASBP but has no splice acceptor. This vector can be a useful tool for troubleshooting injection strategies with certain constructs (see Methods).

MATERIALS

Dissecting Tools

Dumont "Inox" forceps, 12.5 cm (FST Inc., No. 11210-10)

Dumont No. 5 forceps, 11 cm (FST Inc., No. 11250-20)

Graefe forceps with angled tip (FST Inc., No. 11052-10)

Perforated spoon, Moria MC-18 (FST Inc., No. 10370-18)

Iris scissors (straight), 12 cm (FST Inc., No. 14068-12)

Iris scissors (curved), 12 cm (FST Inc., No. 14069-12)

Plasmids

Shuttle vector: pSlax13

Retroviral vectors: RCASBP(A), RCASBP(B), RCASBP(E), RCASBP(A)-AP, RCASBP(B)-AP, RCANBP(A), RCANBP(B)

pRCS whole-mount *in situ* probe template

Sequencing Primers

T3, T7

RCAS5' (ACGCTTTTGTCTGTGTGCTGC)

Miscellaneous Equipment

Rotating incubator (Petersime, Gettysburg, OH)

Benchtop egg incubator (Kuhl, Flemington, NJ)

Eggs, obtainable from a supplier such as SPAFAS (Norwich, CT) [These chickens are normally of a strain (line 22) that is infectable with A-, B-, and E-type viruses. Strains C/0 (line 15b) and C/E (line 0) for producing chicken embryo fibroblasts (CEFs) are obtainable from USDA, Avian Disease and Oncology Laboratory, 3606 East Mount Hope Road, East Lansing, Michigan 48823.]

Tygon tubing (Norton Co., Akron, OH)

0.05-ml Hamilton gastight syringe No. 705, (Hamilton Co., Reno, NV)

18G11/2 syringe needle (luer mount) (Becton Dickinson, Franklin Lakes, NJ)

20G11/2 syringe needle (luer mount) (Becton Dickinson)

Leica MZ6, or equivalent, dissecting microscope

Leica KL 1500, or equivalent, fiberoptic light source with a polarizing filter fitted

Egg trays

3C2 hybridoma supernatant (10) (Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa)

Micromanipulator (Prior, England)

Universal Box sealing tape (61500-Clear, United Stationers Supply Co., Des Plaines, IL)
Heavy Mineral oil (Sigma)
Polybrene (Sigma, No. P-4515)

METHODS

Construction of Viral Vector

Overview

The RCASBP vector carrying the transgene is constructed by inserting the coding sequence of the gene of interest 3' of the *env* gene (Fig. 1A), into the site originally occupied by the host-derived oncogene *src* in RSV. This strategy places the 5' untranslated region of the *src* gene immediately 5' to the ATG, initiator methionine, of the transgene open reading frame (ORF). The retention of the viral 5' untranslated region (UTR) ensures high levels of translation of the transgene and eliminates the possibility of flanking sequences endogenous to the transgene having a deleterious effect on the efficiency of misexpression. The 3' untranslated regions of mRNAs sometimes contain sequences responsible for transcript stability. Since the viral transcripts are known to be stable and the role, if any, of the 3' UTRs of the gene to be inserted are usually not known, it is prudent to remove all the extraneous sequence 3' of the stop codon of the gene to be inserted.

The RCASBP vector is a large (11 kb), low-copy-number plasmid. The construction of inserts is therefore most easily carried out using a Bluescript (Stratagene)-based shuttle vector, pSlax13 (7) (Fig. 1B). pSlax13 was constructed by placing a *Cla*I-adapter fragment into a Bluescript derivative. This has the advantage of allowing all the cloning steps to be carried out in a relatively small, high-copy-number vector.

The 5' UTR of *src* that is contained in the adapter contains the splice acceptor and sequence elements required for efficient translation of the transgene transcript. To use these sites efficiently, the coding sequence of the transgene must be cloned in-frame with the ATG of the *Nco*I site in the *Cla*I-adapter fragment (Fig. 1B). Any of the more 3' restriction sites (e.g., *Eco*RI-*Sma*I-*Bam*HI, etc.) can be used at the 3' end of the sequence. After the transgene has been inserted into the *Cla*I-adapter sites and the integrity of the sequence confirmed by sequenc-

ing, the entire *Cla*I-adapter fragment, including the inserted transgene, is cloned into a *Cla*I-linearized RCASBP vector.

Virus Construct Cloning Strategies

The following strategies are commonly used methods for generating virus constructs. Any given construct can be generated in a number of different ways and therefore the list of methods described is intended as a guideline and is by no means exhaustive. The exact method used to generate the correct transgene construct is entirely the choice of the researcher and will vary depending on the sequence with which you are working.

PCR is the simplest way to generate an insert with compatible ends for cloning directly into pSlax13. A high-fidelity *Taq* polymerase such as Vent DNA polymerase (NEB) or the KlenTaq kit (Clontech) is recommended. Using regular *Taq* DNA polymerase is not advised as the likelihood of errors being placed in the coding sequence of the insert is significantly higher. The entire insert can, in principle, be generated in one step by designing 5' and 3' primers for either end of the transgene ORF. Even with higher-fidelity thermostable polymerases, however, using PCR to generate the entire length of the insert will increase the chance of errors in the coding sequence. The amount of PCR-generated material in the insert can be reduced by replacing internal fragments ("cassettes") flanked by convenient restriction sites with the identical fragment from the original cDNA clone. Alternatively, the same internal cDNA clone fragments can be ligated together with 5' and 3' PCR-generated fragments in a four-way ligation. To remove all the extraneous sequence 3' of the stop codon, one of the restriction sites present in the pSlax13 cloning site can be incorporated into the 3' primer immediately after the stop codon.

Depending on the sequence that surrounds the initiator methionine (ATG) of your gene of interest one of three strategies can be used to generate a *Nco*I-compatible 5' end for cloning into the pSlax shuttle vector.

1. When the ATG start codon is followed by a G, forming the sequence-NATGG, the 5' PCR primer can be designed to generate an artificial *Nco*I site followed by the transgene coding sequence. Incorporation of at least 18 bases of transgene sequence into the primer is usually sufficient to ensure a high-fidelity PCR product. At least 3 (arbitrary) bases

should be incorporated into the primer 5' of the *NcoI* site to ensure efficient digestion with the enzyme, e.g., NNNCCATGG(N)₁₄.

2. In some genes the ATG start codon is part of a genuine *NcoI* site: CCATGG. In these cases a 5' PCR primer can be designed from the wild-type sequence with at least 3 bases flanking 5' of the *NcoI* site to ensure efficient cutting with the enzyme, e.g., NNNCCATGGN₁₄.

3. For sequences that contain neither a genuine *NcoI* site nor a NNATGG half-site, an *NcoI*-compatible end can be generated using any one of a number of restriction enzymes that cut N₁/N₅ nucleotides downstream of their recognition sequence. Enzymes of this type, such as *Alw26I*, *BsaI*, and *BsmBI*, are available from most restriction enzymes suppliers. As with the other strategies, addition of a minimum of an extra 3 bp 5' to the restriction site will ensure efficient digestion, and incorporating at least 18 bp of wild-type sequence into the primer ensures fidelity of the PCR product.

The integrity of the construct should be confirmed in the pSlax13 vector by sequencing. *ClaI* digestion of the pSlax13-transgene construct releases a fragment that can be ligated directly into a *ClaI*-linearized and phosphatased RCASBP vector. The ligation step with the RCASBP vector often works with only low efficiency. Since the cloning is not directional it is necessary to check the orientation of the positive clones. This can be done easily with a diagnostic *NcoI* or *BamHI* digest. The RCASBP vector has these sites situated asymmetrically either side of the cloning site. The correct orientation of the insert can also be confirmed by sequencing using a primer situated just 5' prime to the cloning site, RCAS5' (see Materials).

Viral Production

Overview

Viral supernatants are produced by transfecting a primary cell line of chicken embryo fibroblasts (CEFs) with the cloned RCASBP-retroviral construct containing your gene of interest. On infection the viral DNA is integrated into the host cell genome. The host cell's transcriptional machinery is used to produce many viral mRNAs, which either are translated into viral structural proteins and enzymes and the protein encoded by the transgene or are packaged as retroviral genomic RNA. The as-

sembled virus particle buds off the host cell membrane and is then capable of infecting other cells. For a more detailed description of the retroviral life cycle see Morgan and Fekete (8).

Since the RCAS viral construct is capable of self-replication and, after being secreted into the medium, of infecting other uninfected cells in the tissue culture plate, high-titer supernatants can be produced using a simple transfection protocol because even if the original transfection efficiency is relatively low, through subsequent rounds of viral replication and reinfection all the cells in the plates become infected. After a few days in culture, the medium in which the cells are grown becomes increasingly virus rich. Viral supernatants are harvested by simply collecting the medium from the infected plates. These unconcentrated supernatants usually contain virus titer in the range 10⁵ to 10⁷ infectable units (IU)/ml. To ensure a good infection and subsequent viral spread, supernatants are normally concentrated by ultracentrifugation to produce a concentrated viral stock. Concentrated viral stocks commonly have titers around 1–5 × 10⁸ IU/ml but they can be as high as 1 × 10⁹ IU/ml.

Initially, to generate a viral supernatant a suitable producer cell line must first be prepared. Commonly, primary cultures of CEFs produced from a strain infectable with the virus subtype being prepared are used. Line C/0 embryos are used for subgroups RCASBP-A and RCASBP-B. To generate RCASBP-E-type virus CEFs prepared from line C/E (strain 15b) should be used.

Preparation of Primary Cell Line

Before starting, ethanol-sterilize the instruments. Wear gloves and ethanol-sterilize the gloves after you put them on. Work in a tissue culture hood and have trypsin, serum, Dulbecco's modified Eagle's medium (DMEM), etc., solutions ready.

Reagents

- Ethanol-sterilized dissection instruments
- DMEM with glutamine (Gibco-BRL, Gaithersburg, MD)
- Fetal calf serum (FCS) (Sigma, St. Louis, MO)
- Chicken serum (CS) (Sigma)
- 0.25% trypsin/1 mM EDTA (Gibco-BRL)
- Dimethyl sulfoxide (DMSO) (Sigma)
- 125- or 250-ml Erlenmeyer flask
- One dozen eggs incubated for 10 days (line C/0)

for RCASBP A and B subtypes, line C/E for RCASBP E subtype)

Incubate eggs until Day 10 [approximately stage 36 (11)]. Remove an embryo from an egg and lay in a 10-cm petri dish. Remove the limbs and head using a sterile razor blade and eviscerate with forceps. Collect four or five embryo trunks in a 10-cm Petri dish and mince the tissue well with a razor blade. It is important to mince the tissue thoroughly as this will greatly increase the efficiency at which the trypsin solution dissociates the cells.

Collect the chopped embryos into an Erlenmeyer flask containing 10 ml of 0.25% trypsin/1 mM EDTA solution (GIBCO). Shake for 15 min on a rotator. It helps to shake by hand every few minutes. Gradually the small chunks of tissue will begin to disappear. Do not overtrypsinize as cells will die. Instead, let any big chunks settle and transfer the supernatant to a 50-ml centrifuge tube (e.g., Corning or Falcon sterile plastic tube).

Add an equal volume of 100% fetal calf serum (FCS), mix, and leave to stand for 5 min in the tissue culture hood to allow any remaining tissue chunks to settle. Decant this solution into a fresh 50-ml tube and centrifuge at 1000 rpm for 5 min in a Sorvall RC3B or equivalent rotor. Resuspend the cell pellet in 10 ml of 100% FCS, spin again, and resuspend in DMEM supplemented with 10% FCS and 2% CS.

Count the number of cells in the suspension using a conventional hemocytometer and plate the entire preparation onto the appropriate number of 10-cm tissue culture dishes at three different concentrations: 10^7 cells/dish, 10^6 cells/dish, and 3×10^5 cells/dish. Monitor the cells daily. They will eventually recover and begin to divide very rapidly. As soon as the healthiest dishes (i.e., those with fewest dead and fastest-growing cells) become close to confluent they are ready for harvesting. It is important to harvest the cells before they become confluent on the dish. The cells are harvested by light trypsinization, care being taken not to overtrypsinize. Remove the medium, rinse the cells with two washes of PBS, and add 1 ml trypsin solution. As soon as the cells begin to lift off the dish and separate add 3 ml DMEM (+10% FCS +12% DMSO) and continue to mix the cells in this solution until no cell clumps are present. Place 0.5-ml aliquots into vials and freeze on dry ice. In our experience cells frozen slowly on dry ice recover and grow more vigorously in culture than cells flash-frozen in liquid nitrogen. CEFs can generally be maintained for 4–6 weeks in culture.

Transfection and Viral Supernatant Production

Reagents

DMEM with glutamine (Gibco-BRL, Gaithersburg, MD)
FCS (Sigma)
CS (Sigma)
Penicillin–streptomycin solution (Pen–Strep) (Gibco-BRL)
2.5 M CaCl_2
2× BES: 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES: Calbiochem), 280 mM NaCl, 1.5 mM Na_2HPO_4 , pH 6.95, 800 ml H_2O [Adjust the pH to 6.95 with 1 M NaOH at room temperature and bring the final volume to 1000 ml with distilled water. Filter-sterilize through a 0.45- μm filter and store in aliquots at -20°C]

Start with a freshly thawed aliquot of a CEF line produced from a suitably permissive host strain for the vector subtype to be produced. Seed the cells onto 10-cm dishes and culture overnight in 10 ml DMEM (supplemented with 10% FCS and 2% CS and Pen/Strep) so that the cells have reached approximately 70–80% confluence the following day.

Add 10 ml of fresh medium 2–4 h before the transfection. To prepare the DNA for transfection dilute approximately 10 μg of RCASBP-transgene plasmid DNA with distilled water to a final volume of 450 μl . Add 50 μl of 2.5 M CaCl_2 solution and mix well. Add 500 μl of 2× BES, mix well, and incubate at room temperature for 20–30 min. After this incubation period, add the DNA solution dropwise onto the dish of cells while slowly swirling the medium in the plate. Incubate overnight.

The following day, the cells should be confluent and can be split to a 15-cm plate. After a further day in culture the cells should be sufficiently dense to split to two 15-cm plates. At this point a small aliquot of the trypsinized cells can be used to seed a well on a 24-well cell culture plate (Costar, Cambridge, MA). After a day of culture these cells can be stained with the 3C2 monoclonal antibody (mAb) which recognizes the viral MA antigen (see protocol below for Titration of Virus). All the cells on the plate should stain for the viral MA antigen, confirming that the transfection procedure was successful. Finally, when the two 15-cm plates reach confluence, split each plate 1 in 3, to six 15-cm plates. These six

plates will be used to produce the viral supernatants for concentration.

Allow the cells on each of the six plates to reach confluence and then wait another 36–48 h so that the cells become superconfluent. At this stage the cells can be seen to pile up on one another and the cell processes appear as a meshwork on the surface of the dish. Replace the medium with 10 ml DMEM (+)1% FCS + 0.2% CS + Pen/Strep (reduced or 1/10 serum medium). After 24 h, harvest the supernatant by pipetting the medium into a 50-ml centrifuge tube. The supernatants from each of the six plates can be combined into two 50-ml centrifuge tubes. Freeze the solution by dropping the tubes into liquid nitrogen or placing on dry ice and store at -70°C . Replace the medium with a further 10 ml of 1/10 serum medium. Repeat the harvesting step for 2 more days to give a total of 3 days of harvests. Since the titers of the viral supernatant may often vary over the harvesting period the medium pooled on each day is processed separately to obtain the highest possible titer from each harvest.

Concentration and Storage of Viral Supernatants

Thaw the viral supernatants by placing the 50-ml centrifuge tubes in a breaker containing water in a 37°C water bath. Mix the tubes periodically, and as soon as the solutions have thawed place the tubes on ice. Filter the supernatants through a $0.45\text{-}\mu\text{m}$ filter to remove the cell debris. Use a glass-fiber pre-filter to prevent clogging of the $0.45\text{-}\mu\text{m}$ filter with cellular debris. Transfer the filtered viral supernatants to $1 \times 3 \frac{1}{2}$ -in. Ultra Clear centrifuge tubes (Beckman No. 344058 or equivalent). Place 30 ml of supernatant per tube. If you had six plates of infected cells and took harvests every 3 days you should have sufficient virus supernatant to fill all six centrifuge tubes in one rotor. Ultracentrifuge spin at 21,000 rpm for 3 h in a SW-28 rotor. When the spin is complete, carefully pour the supernatant from each tube into a beaker and touch the last drop to the side of the beaker so that a minimum of solution remains in the tube. Often no viral pellet is obvious. Place the tubes on ice in a bucket or tray and shake the tubes on a rotary shaker to resuspend the virus. Combine the concentrated viral suspension from each day of harvest into a single 1.5-ml microcentrifuge tube and store the concentrated virus at -70°C . Aliquot 10–30 μl into 0.5-ml microcentrifuge tubes to avoid repeated freeze–thaw of the stocks.

Titration of Virus

Before starting an injection protocol it is important to know the approximate titer of the concentrated viral stock. This can be determined by infecting CEFs with serial dilutions of the virus stock and assaying for viral infection using the 3C2 monoclonal antibody. For simplicity the antibody stain detection can be carried out using the Vectastain and DAB substrate kits. It is important that concentrated viral stocks have a titer no less than 1×10^8 IU/ml to obtain a robust infection and therefore broad regions of transgene misexpression in the embryo.

Reagents

MST blocking solution: DMEM containing 10% CS and 0.2% Triton

PBT–PBS with 0.1% Tween 20 (for 1 liter $1 \times$ PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 , pH to 7.4)

Vectastain Kit (Vector Labs, Burlingame, CA)

DAB Substrate Kit for Peroxidase (Vector Labs)

3C2 monoclonal antibody (10)

Biotinylated anti-mouse IgG (Vector Labs)

Split CEFs 1–6 into a 24-well multiwell plate with DMEM (supplemented with 10% FCS and 2% CS and Pen/Strep). Fill enough wells to have four wells per virus to be titered plus an additional well as an uninfected control. The following day, make dilutions of the viral stocks in DMEM of 10^{-3} , 10^{-5} , 10^{-6} , and 10^{-7} . Add 100 μl of diluted virus to each well and incubate for 48 h.

Following this culture period, wash the cells twice in PBS and fix the cells for 15 min in 4% paraformaldehyde/PBS. After fixation wash the cells three times in PBS and preblock in MST for 30 min. Add 0.5 ml of the 3C2 monoclonal antibody (diluted 1/5 in MST) and incubate at room temperature for 30 min. Wash the cells three times with MST, 5–10 min for each wash. Add 0.5 ml of the secondary antibody, biotinylated anti-mouse IgG diluted 1/400 in MST, and incubate at room temperature for 30 min.

During the secondary antibody incubation, make the A/B solution (Vectastain Kit). Add 2 drops of solution A and 2 drops of solution B to 10 ml of PBT. Mix well and let stand for 30 min.

Remove the secondary antibody solution and wash the cells three times with PBT for 5–10 min each wash. Add 0.5 ml of the A/B solution to each well

and incubate for 30 min. Wash three times with PBT for 5–10 min each wash.

Stain for antibody detection using the DAB Substrate Kit for Peroxidase. Add 2 drops buffer stock solution to 5 ml distilled water, and mix well. Add 4 drops of DAB stock solution and mix well. Add 2 drops peroxide solution and mix well. Add 0.5 ml of the final DAB/peroxide solution to the wells. An adequate signal is often apparent after several minutes. When the stain is judged sufficient the reaction can be stopped by several washes with PBT.

Cells positive for the 3C2 epitope and therefore infected with virus stain brown. The cells infected with the 10^{-3} dilution of virus should be uniformly infected while the uninfected plate serves as a negative control. Discrete clones of infected cells should be obvious in the wells infected with higher dilutions of virus. If one clone of infected cells is seen with the 10^{-7} dilution then the titer is taken to be 1×10^8 IU/ml, if three clones are observed then the titer is taken to be 3×10^8 IU/ml, etc.

Injection Protocols

Incubating and "Windowing" Eggs

Eggs are incubated in a humidified rotating incubator (Petersime) at 37°C. Periodic rotation of the eggs helps prevent the developing embryos from sticking to the shell. This can be a problem, particularly at later stages of development. To gain access to the embryo place the egg on its side in an egg tray and carefully poke two holes in the eggshell—one at the broader end of the egg and the other at the upper side (facing the microscope objective) of the egg—with a pair of broad forceps. Using a 20G11/2 needle (Becton Dickinson) attached to a 5-ml syringe (Becton Dickinson) gently remove 1 ml of albumin from the egg through the hole at the end of the egg. Removing this small amount of albumin from the egg lowers the embryo away from the upper surface of the egg and allows for easier manipulation of the embryo during subsequent steps. Finally, the embryo is revealed by making a "window" in the upper surface of the shell by carefully removing pieces of shell from around the hole made in the upper side of the egg. With forceps, remove enough of the shell so that the embryo can be clearly seen and manipulated.

Needle Preparation

Injection needles are prepared by pulling borosilicate glass capillaries ($1 \times 0.75 \times 100$ mm, Catalog No. 30-30-0, FHC, Brunswick, ME) on a Flaming

Brown Micropipette Puller (Model P80/PC, Sutter Instruments) or similar. In general, needles with relatively long, gradual tapers that become sealed at the very tip work well. Needles should be inspected under a dissecting scope before use and the desired needle point produced by using forceps to break off the sealed end. Needle tips with a fine beveled edge work particularly well. It is sometimes possible to generate a beveled edge at the tip of the needle by grasping the sealed end of the capillary with the forceps and breaking the tip in one smooth downward motion. Needles can often break accidentally or become clogged during an injection protocol and therefore it is usually necessary to prepare a stock of pulled needles prior to starting injections.

Apparatus Setup

The injection setup consists of a gastight Hamilton syringe attached to a 18G11/2 needle mounted in a micromanipulator. The glass capillary needles are attached to the injection apparatus using a piece of Tygon tubing and the whole setup is filled with heavy mineral oil (Sigma). The oil allows for greater control over the volumes injected.

Fill the Hamilton syringe slowly with mineral oil, carefully avoiding the formation of air bubbles in the barrel. Fill an 18G11/2 needle (Becton Dickinson) with mineral oil and place onto the end of the Hamilton syringe. Avoid letting air into the joint. Attach the syringe setup to the micromanipulator and place a $\frac{1}{2}$ -in. length of Tygon tubing (Norton) to the end of the syringe. A pulled needle can then be attached to the open end of the tubing. Fill the needle with mineral oil slowly until a small droplet is just visible at the tip.

The needle can be backfilled with viral solution by placing a 5- to 10- μ l aliquot onto Parafilm (Greenwich, CT) and lowering the needle into the solution. Load the needle by lightly lifting the plunger to generate slight negative pressure. The viral solution can be dyed by adding 1/40 vol of filter-sterilized 1% Fast Green solution (Baker, Phillipsburg, NJ), which makes it easy to monitor. When the needle is loaded, adjust the plunger so there is a slight positive pressure on the needle before removing it from the viral solution. Any remaining viral solution can be stored on ice for use later.

Injection Targeting Strategies

Overview

An important consideration when designing an experimental injection protocol is that the virus can

only integrate into the genome of a host cell during the mitotic (M) phase of the cell cycle. This aspect of the infection mechanism may place an important limitation on the time of infection. For this reason, if complete, global infection of the limb bud is required the viral injection protocol is carried out at stage 10, many hours before the outgrowth of the limb bud occurs. Injections at this stage are technically more demanding than injections at later stages, for example, injections in the wing and leg region at stage 17 when limb bud tissue is clearly visible. Infection at this later time results in only a limited infection of the limb. The resulting regionalized infection can be very useful for some applications.

Another important note regarding choice of infection time is the number of hours required for the transgene expression levels to become high enough to have a physiological effect. Morgan and Fekete (8) determined that the MA (matrix) core protein is detectable by immunohistochemistry within 18 h of infection but is not detectable after 10 h. It may be possible to study the individual dynamics of protein expression for a particular transgene if an antibody raised against that protein is available. As an alternative, tags such as the Myc epitope (8) and the Flu/HA (12CA5) and Flag (Kodak, Rochester, NY) tags (6) have been used to successfully monitor protein expression levels from viral constructs.

Targeting the Wing and/or Leg Buds

Incubate eggs in a humidified, rotating incubator for approximately 36 h or until they reach Hamilton/Hamburger stage 10. The stage 10 embryo is rather small and has little contrast and so can be hard to identify clearly under normal tungsten illumination. It is very helpful to have a polarizing or daylight-correction filter for the light source.

When you have located the embryo, orient the egg under the microscope so the tail is facing you. Using forceps, gently tear away the vitelline membrane that covers the surface of the embryo to expose the injection site. Extreme care must be taken to avoid damaging the embryo. If the embryo becomes torn do not continue to work with the egg and start afresh. Some workers choose not to remove this membrane and inject directly through this covering. This method, while simple, can lead to many clogged needles and difficulty monitoring virus spread.

Using the micromanipulator bring the needle over

the injection site at an angle of approximately 45°. Figure 2A shows the regions of the lateral plate mesoderm that should be targeted to obtain complete infection of either the wing or leg bud. Extend the needle into the embryo. There will be some resistance to penetration of the needle but it should pass into and through the embryo easily. If the embryo becomes very distorted as you try to penetrate, then the needle is too blunt and you should prepare a new one. Since there is usually some resistance to penetration of the needle, when it does pierce the embryonic tissue it usually passes through the thin layer of cells and into the yolk below. Therefore, carefully withdraw the needle until it can be seen to slightly lift the embryo from the surface of the yolk. The viral inoculum can then be injected into the embryo by very gently tapping the syringe plunger. If the embryo has been correctly targeted the viral solution will appear as a sharp dot that slowly spreads from the needle tip. If the virus is flowing into the subembryonic yolk it will spread quickly and appear as a more diffuse patch. After the initial infection withdraw the needle and repeat the injection procedure. The number of injections required to lead to complete infection of the limb bud is dependent to some extent on the titer of the virus being injected, but usually around five such injections is sufficient.

When the injection is complete cover the window in the eggshell with clear tape and place the egg into a humidified benchtop incubator at 37°C. During the incubation period a mortality rate of around 10% is normal.

Targeting a Subpopulation of Cells in the Limb

One of the great advantages of viral-mediated misexpression is the ability to target a subpopulation of cells in the developing limb bud. The growth characteristics of the limb bud are particularly useful for this purpose. The limb buds arise as outgrowths from the lateral plate mesenchyme at stage 17. The developing limb bud induces a specialized structure along the distal tip of the limb ectoderm, the apical ectodermal ridge (AER). Subsequent outgrowth of the limb is dependent on the proliferation of a population of cells directly subjacent to the AER, the progress zone. Therefore, cells infected in the progress zone of a relatively early-stage limb (e.g., stage 18, see Fig. 2B) will give rise to a clone of cells that extends in a stripe along the proximodistal (PD) axis of the limb bud. Interestingly, lateral spread of the

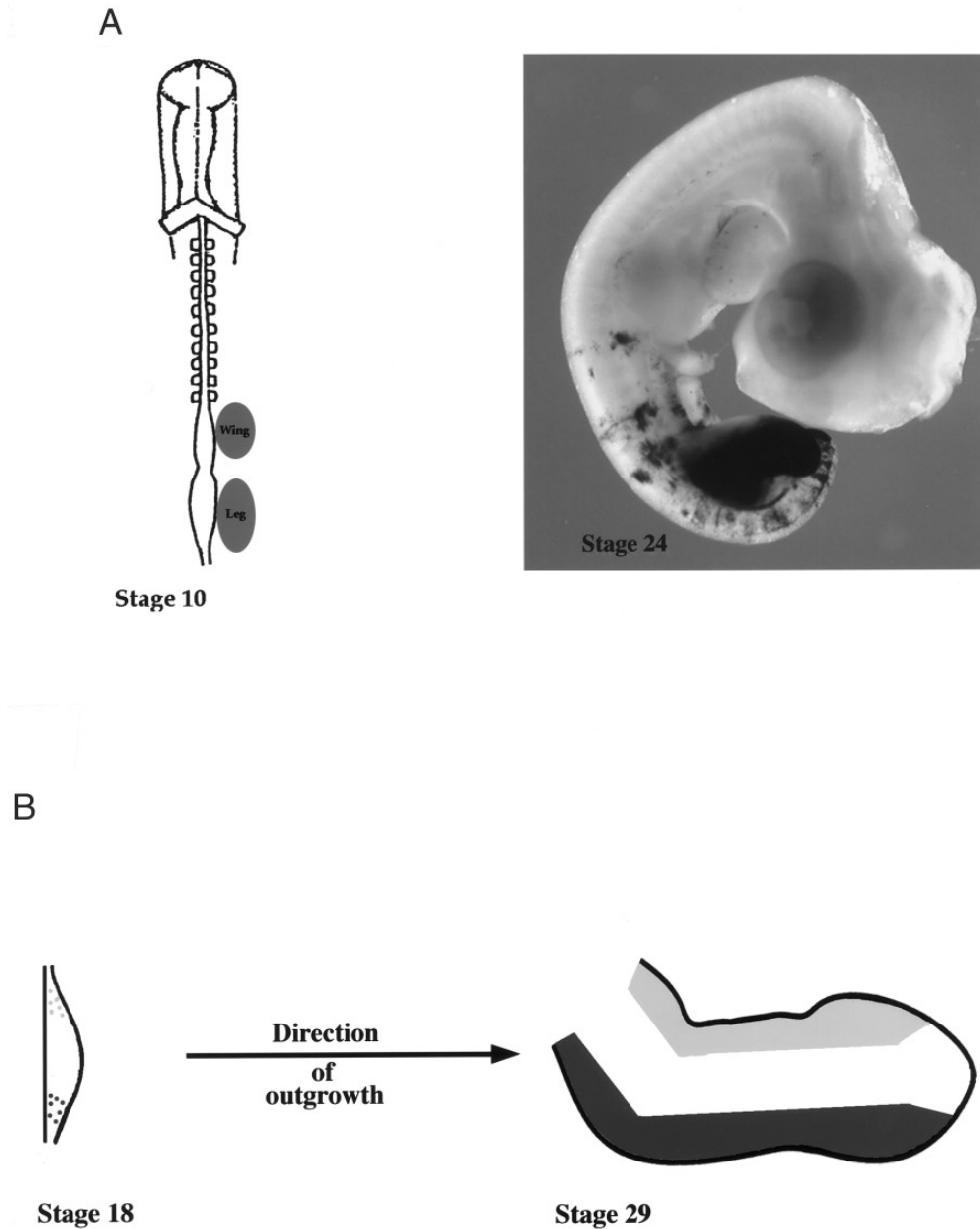


FIG. 2. Strategies for targeting the chick limb bud. (A) Diagram of a Hamburger/Hamilton stage 10 embryo with the approximate area of cells destined to become incorporated into either the prospective wing or leg bud indicated (shaded). Targeting viral infection to these regions of the embryo will commonly lead to complete infection of the respective wing or leg bud at later stages. A representative example of such an infected embryo, incubated until stage 24, is shown on the right. In this case, cells of the prospective leg bud were infected as indicated in the diagram. The extent of viral infection was analyzed by detecting viral transcripts by RNA whole-mount *in situ* hybridization with the RCASBP-specific probe pRCS (see Materials). (B) A diagram indicating the type of viral spread produced when a stage 18 limb bud is infected and viral spread is analyzed at stage 29. A broad band of infection is observed along the proximodistal axis of the limb when the developing limb bud is infected in either an anterior (light shading) or posterior (darker shading) domain. The lateral (anteroposterior) extent of the viral spread is minimal.

virus along the anteroposterior (AP) axis is relatively slight. In this manner, it is possible to generate broad bands of misexpression along the PD axis in anterior, medial, or posterior domains (see Fig. 2A). In contrast, injections in the limb bud at a later stage and more proximally, outside the progress zone, will generate discrete patches of infection in the proximomedial regions of the limb bud.

Many examples of targeted misexpression of a virus in the limb bud now exist in the literature. For example, Riddle *et al.* targeted an RCASBP-Wnt7a virus to the ectoderm by pooling a layer of virus between the ectoderm and vitelline membrane at stage 18 (5). Laufer *et al.* were able to infect relatively early-stage lateral plate ectoderm at stages 7–11, generating clones of infected cells that spanned the dorsal and ventral ectoderm of the limb bud, often without substantial spread of the virus into the underlying mesenchyme (6).

Use of Infected Cell Pellets

As described in the introduction, it is possible to implant a pellet of virus-infected cells into a host limb bud to assay the function of secreted transgene proteins (7). By infecting a susceptible tissue culture cell line [e.g., line 15b (C/0)] and transplanting into a nonsusceptible host strain [e.g., line 0 (C/E)] a localized source of a particular protein can be administered to an ectopic location without subsequent spread of the virus.

To prepare infected cell pellets, transfect a single 60-mm dish containing line 15b CEFs using the protocols described previously. When the cells have reached 50–90% confluence lightly trypsinize them from the plate and spin at 1000 rpm for 5 min in a benchtop ultracentrifuge. Resuspend the pellet in 1 ml of DMEM, transfer to a microcentrifuge tube, and spin at 2000 rpm for 2 min. Incubate the tube for 30 min at 37°C and then respin at 2000 rpm for 2 min. The cell pellet fragments can be lightly stained in medium containing 0.01% Nile blue sulfate (Aldrich, Milwaukee, WI) vital dye and then embedded into the limb bud of a line 0 chicken embryo.

Analysis of Injected Embryos

Detection of Human Alkaline Phosphatase

A convenient way of practicing and developing the injection technique that best suits your experiment is to use the RCASBP alkaline phosphatase vector

(RCASBP-AP). In this construct the human alkaline phosphatase gene has been cloned into the RCASBP vector. Alkaline phosphatase activity produced after infection and spread of the virus can be easily identified in the whole embryo, *in situ*, following a simple protocol.

Reagents

Alkaline phosphatase buffer: 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.024 g levamisole (Sigma)/50 ml buffer (optional: levamisole is an inhibitor of endogenous alkaline phosphatases).

Detection solution: To 5 ml of alkaline phosphatase buffer add 25 μ l nitroblue tetrazolium (NBT, Sigma N-6876) solution [0.075 g/ml in 70% dimethylformamide (DMF)] and 62.5 μ l 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP, Sigma B-6149) solution (0.05 g/ml in 100% DMF) PBT–PBS with 0.1% Tween 20 (for 1 liter 1 \times PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, pH to 7.4).

Fix the embryos in 4% paraformaldehyde/PBS, overnight at 4°C or room temperature for 2 h. Six-well tissue culture plates (Corning) are useful containers for the embryos. All the subsequent steps can be carried out in these dishes using volumes of 5–10 ml depending on the age of the embryos.

Wash the embryos thoroughly with PBT, 5 times for 15 min, and incubate at 65°C, in PBS, for 30 min to inactivate endogenous alkaline phosphatase activity.

Preequilibrate the embryos in alkaline phosphatase buffer for 30 min and then add the detection solution. Incubate in the dark. Signal is usually visible within 30 min but may require incubation overnight. To stop the reaction, wash in TE (10 mM Tris–HCl, pH 8, 10 mM EDTA) followed by three washes in PBS/10 mM EDTA.

Detection of Viral Infection by Whole-Mount Immunohistochemistry

In experiments in which an area of the limb has been targeted and appears affected by transgene misexpression it is important to confirm viral infection of the affected area using the 3C2 antibody (10).

Materials

PBT–PBS with 0.1% Tween 20 (for 1 liter 1 \times PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, pH to 7.4)

Vectastain Kit**DAB Substrate Kit for Peroxidase**

TBST–10× TBS: 8 g NaCl, 0.2 g KCl, 25 ml 1 M Tris–HCl, pH 7.5, 75 ml H₂O [Dilute to 1× and add 0.1% Tween 20.]

3C2 monoclonal antibody (see Materials)

Biotinylated anti-mouse IgG

Fix the injected embryos at room temperature for 2 h or overnight at 4°C, in 4% paraformaldehyde. Postfixation, wash the embryos in PBT twice for 5 min, then dehydrate in 100% methanol and bleach in 3% hydrogen peroxide/methanol for 1 h.

Rehydrate the embryos through a stepwise series of 75% methanol/25% PBT, 50% methanol/50% PBT, 25% methanol/75% PBT, and finally two washes in 100% PBT. Move the embryos to TBST and wash twice for 15 min, then preblock in TBST/10% horse serum for 1 h. Incubate overnight in 3C2 monoclonal antibody diluted 1/5 in TBST/1% horse serum.

Remove the antibody solution and wash twice with TBST for 5 min. Wash an additional five times, 1 h each wash.

Incubate overnight with the secondary antibody, biotinylated anti-mouse IgG diluted 1/400 in TBST/1% horse serum. Remove the antibody solution, wash twice with TBST for 5 min, and then wash an additional five times, 1 h each wash. As was described in the Titration of Virus protocol, the 3C2 antibody stain can be developed effectively using the Vectastain Kit and Dab Substrate Kit for Peroxidase essentially following the manufacturer's instructions.

Prepare the A/B solution by adding 2 drops of solution A and 2 drops of solution B to 10 ml of PBT. Mix well and leave to stand for 30 min. Incubate the embryos in the A/B solution for 30 min and then wash three times for 10 min each wash.

To prepare the stain developing solution using the DAB Substrate Kit for Peroxidase add 2 drops of Buffer stock solution to 5 ml distilled water and mix well. Then add 4 drops of DAB stock solution, mix well and add 2 drops of peroxide solution, and mix well. Add the stain solution to the embryos and when a good stain is obvious stop the reaction with several washes of PBT.

The epitope for the 3C2 monoclonal antibody appears to be very stable since a modified version of the staining procedure used on tissue culture cells (see Titration of Virus) was able to detect 3C2-positive (and therefore virus-infected) cells in wax sec-

tions of embryos previously processed through the whole-mount RNA *in situ* protocol (6). The 3C2 epitope is therefore maintained even after the steps of the whole-mount *in situ* procedure and wax embedding protocol. For some applications it may be advantageous to be able to confirm virus infection, and so presumably transgene misexpression, after analysis of affected limbs with molecular markers.

Detection of Viral Message by RNA Whole-Mount *In Situ* Hybridization

A powerful method to analyze possible phenotypes following virus misexpression is to look for perturbation of wild-type gene expression patterns by whole-mount *in situ* hybridization. A protocol for this method has been described elsewhere (7). The same technique can also be used to detect viral transcripts by using an RCASBP transcript-specific probe, pRCS. This probe can be particularly useful to distinguish viral message (and viral spread) from endogenous gene expression.

Troubleshooting with RCANBP Viral Vector

In some instances the RCANBP vector can be very useful for troubleshooting problems with viral constructs. The RCANBP vector is essentially identical to the RCASBP vector except that it contains no splice acceptor in front of the inserted transgene. A comparison of the titer obtained with identical inserts cloned into both the RCANBP and RCASBP constructs can indicate the source of problems in generating high-titer virus stocks. That an RCANBP viral construct can be grown to high titer while in the same time frame the RCASBP construct cannot suggest that the protein encoded by the transgene is interfering with the normal proliferation of the host cell or production of the virus itself. If both the RCANBP and the RCASBP constructs fail to generate a high-titer viral stock then the transgene DNA sequence may itself be interfering with the production of the virus. Similarly the RCANBP vector can indicate potential toxic effects of the transgene protein *in ovo*. As mentioned earlier, the mortality rate following injection is usually around 10% of injected embryos. A higher rate of mortality may indicate a toxic effect of transgene expression. Side-by-side injection of the RCANBP version of the same transgene may be useful in identifying such an effect.

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