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Production of High-Titer RCAS Retrovirus

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Abstract

RCAS (B/P) is a replication-competent avian retrovirus engineered by Hughes et al. (J Virol 61:3004–3012, 1987) and is referred to in this chapter as RCAS for simplicity. The RCAS retrovirus has been widely used as a vehicle for stable transduction of a gene into cells both in the developing chick embryo and tissue/cell culture. It can be used for both gain- and loss-function experiments. The ability of this virus to spread among proliferating cells makes it possible to achieve widespread gene transduction in the developing retina. The transduction efficiency of RCAS is highly depending on the titer of the viral stock, particularly for experiments involving solid tissues such as the developing retina. Here, we describe the procedure that we have used for 15 years to generate RCAS viral stocks with a titer of $1-5 \times 10^8$ pfu/ml.

Keywords

Avian retrovirus; Replication competent; High titer; Retroviral transduction; Chick embryos; CEF cells

1. Introduction

RCAS is a replication-competent avian retrovirus engineered from ALV LTR with splice acceptor retrovirus (1). The natural ability of this virus to spread in a population of dividing cells makes it possible to achieve widespread viral infection and, hence, gene transduction in chick embryos (2). A widespread gene transduction in turn renders it possible for phenotypic changes, provided that the gene is capable of inducing such change. Since the infectious viral particles can be administered into the developing eye after it has assumed its basic structure, RCAS retrovirus can be particularly useful in elucidating genetic regulation of retinal development. RCAS viral infection has no obvious effect on retinal development; we have observed no abnormalities in the developing chick retina either at the gross level or at the microscopic level from hundreds of embryos infected with RCAS or RCAS expressing the green fluorescent protein (RCAS-GFP).

The RCAS system offers a powerful tool for gain-of-function analysis. In gain-of-function studies using embryonic chick eyes, RCAS transduction of various regulatory genes has produced overt, readily detectable phenotypes, including a thickened outer nuclear layer (3), microphthalmia (4), or corneal extrusion (5). Notably, a widespread transduction of certain genes may detrimentally affect the embryos. For instance, infection of chick embryos with

RCAS expressing *neurognin3* (*ngn3*) (6), a gene well known for its proendocrine role by determining which precursor cells in the developing pancreas will become insulin-producing cells of the islets of Langerhans, is embryonic lethal (7). Embryonic lethality also occurs with infection of RCAS expressing *cNSCL1* (8) or *ash1* (5). Compared to the transgenic mouse approach, the RCAS system is more economical and less time consuming.

When engineered to express a dominant negative construct, the RCAS retrovirus can be an effective alternative to other loss-of-function approaches. In our experience, it is particularly potent when coupled with *Drosophila* Engrailed-mediated active repression if the gene of interest is a transcription activator. For example, infection of the developing retina with RCAS expressing the construct of *Drosophila* Engrailed (En) fused with NeuroD lacking the C-terminal region (En-NeuroDAC) results in photoreceptor deficits (9), and widespread infection with RCAS-En-Cbx(HD) is embryonic lethal while that of RCAS-Cbx is tolerated (10).

Like any other experimental system, the RCAS retroviral system has inherent limitations. For example, it creates ectopic expression of a gene (or its dominant negative construct) outside the temporal window and the spatial locale of its normal expression. In addition, the level of expression is not readily alterable or regulated, and the time of onset of gene expression in a specific cell may not be simply calculated from the time of the initial viral administration due to viral spreading through secondary and tertiary infections. Therefore, while RCAS offers opportunities for functional studies on a number of fronts, its limitations should be kept in mind in experimental design and data interpretation.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Use sterilized reagents in cell culture and preparation of viral stocks.

2.1. Chick Embryos

1. Pathogne-free, fertilized chick eggs.
2. Chick egg incubator, set at 38°C with a water reservoir for humidity.
3. Sterile (autoclaved) forceps/tweezers with curve tips (Dumont #7 tweezers).
4. Sterile (autoclaved) razor blades.

2.2. Cell Culture Medium

1. Medium 199.
2. Medium 199 plus 10% fetal bovine serum (Medium 199 + 10% FBS).
3. FBS.
4. Phosphate-buffered saline (PBS).
5. 0.25% Trypsin/EDTA.
6. Cell-Strainer (Falcon 352235).
7. 15-ml plastic tube.
8. Cell culture flasks, 25 and 75 cm².
9. Plastic transfer pipettes.
10. 37°C Water bath.

2.3. Transfection

1. Purified RCAS DNA prepared using a commercial plasmid DNA preparation kit.
2. Fugene 6 (Roche Biochemicals).

2.4. Centrifugation and Ultracentrifugation

1. High-speed centrifuge J2-HS (Beckman Instruments).
2. Ultracentrifuge L-80 (Beckman Instruments).
3. Ultra-Clear centrifuge tubes (Cat# 344058 for SW32Ti rotor, Beckman Instruments).

3. Methods

There are four main steps in producing high-titer RCAS viral stocks: (a) set up primary culture of chick embryonic fibroblast (CEF) cells, (b) transfect the cells with RCAS DNA, (c) harvest cell culture medium containing viral particles, and (d) concentrate the viral particles to obtain a high-titer viral stock. All steps need to be carried out with care to avoid microbial contaminations.

3.1. Establishing CEF Cell Culture

1. Take one day-8 chick embryo (see Note 1) out of egg incubator and clean the center top of its shell with 70% ethanol.
2. Gently knock at the cleaned center top with a pair of sterile forceps to introduce small cracks in the area.
3. Remove the shell pieces.
4. Scoop out the embryo with the curved tips of a pair of sterile forceps and place it in a sterile plastic dish.
5. Decapitate the embryo with a sterile razor blade, remove the gusset with tweezers, and transfer the remaining tissue (body trunk with limbs) into another dish.
6. In a cell culture hood, mince the tissue with a razor blade into fine pieces, and transfer them into a 15-ml tube.
7. Wash the tissue twice with PBS.
8. Add 2.5 ml of 0.25% trypsin/EDTA.
9. Place the tube in a 37°C water bath for 5 min.
10. Bring the tube back in cell culture hood and triturate the tissue ten times with a plastic transfer pipette.
11. Add 10 ml of Medium 199 + 10% FBS. Mix by pipetting.
12. Pass portion (~3 ml) of the cell/tissue suspension through a Cell-Strainer.
13. Transfer 1 ml of the pass through (containing CEF cells) into a 25-cm² flask with 4 ml of Medium 199 + 10% FBS.
14. Place the cell culture flask in a 37°C incubator with 5% CO₂.

¹We find that CEF cell culture from day-8 chick embryo works the best. Cells in culture established with younger embryos peel off culture flasks earlier, thus shortening the time during which virus is harvested. On the other hand, cells in a culture with older embryos often grow slower, lengthening the time required to generate virus.

15. When the CEF cell culture reaches 70% confluence, begin the DNA transfection step as described next.

3.2. Transfecting CEF Cells with Virus DNA

Fugene 6 (see Note 2) is used for transfection of the CEF cells with RCAS DNA. Detailed protocol provided by Roche Biochemicals is followed and is not repeated here.

3.3. Harvesting RCAS Retrovirus

1. When the transfected cell culture becomes confluent (about 2–3 days after DNA transfection), split the 25-cm² flask of CEF cells into two 75-cm² flasks.
2. Culture the cells in 75-cm² flasks with 10 ml of Medium 199 + 10% FBS. Change medium every other day.
3. When the culture is confluent, split the culture 1–5.
4. Change medium every other day with 10 ml of Medium 199 + 10% FBS.
5. When the culture reaches 90% confluence, change medium with 5 ml of Medium 199 + 10% FBS.
6. After 24 h, collect the culture medium from each flask and replenish each flask with 5 ml of Medium 199 + 10% FBS.
7. Centrifuge the collected medium at $8,000 \times g$ (5,500 rpm if using a JA-17 rotor) for 10 min at 4°C to remove cells/cell debris (see Note 3). Combine and store the harvest at –80°C (see Note 4).
8. Continue the harvesting step daily for another 5 days (see Notes 4 and 5).

3.4. Concentrating RCAS Retrovirus (See Note 6)

1. Thaw the collected culture medium (with virus) in a water-filled container at room temperature.
2. Centrifuge the collected medium at $67,000\text{--}81,000 \times g$ (20,000–22,000 rpm for sw41 Ti or sw32 Ti rotor) at 4°C for 20–30 min (see Note 7).
3. Discard the supernatant, followed by one fling of the centrifuge tube.
4. Use the small amount of the medium left in the tube to resuspend the viral particles by repeated pipetting for a few times with a 1-ml plastic pipette. The final volume should be 1% of the original (see Note 8).

²CaCl₂ precipitation is commonly used to transfect cultured cells for the production of retroviruses. We have experimented with different transfection methods and found that results using Fugene 6 are comparable to those using CaCl₂ precipitation. The advantages of Fugene 6 over CaCl₂ precipitation are (a) it needs less DNA, (b) it is easier to perform, and (c) the results are more reproducible. Thus, we recommend Fugene 6 as the transfection reagent in this protocol.

³Some published protocols call for the use of ultracentrifugation at $67,000 \times g$ for removing cells/cell debris. However, we found this to be detrimental to the yield of RCAS retrovirus, as it perhaps removes 90% of the virus along with cells/cell debris.

⁴We routinely combine all harvests into a 500-ml bottle.

⁵At this point, cells start to peel off the culture flask. Otherwise, one may continue the incubation and harvesting for another couple of days until the cells start to peel off.

⁶It is important to have concentrated viral stocks free from microbial contamination for late in vivo and in vitro experiments.

Therefore, cautions are to be exercised to avoid microbial contamination during ultracentrifugation steps. Use autoclaved centrifuge tubes/bottles. When possible, operate in a cell culture hood.

⁷Avoid longer than 30 min of ultracentrifugation at $67,000\text{--}81,000 \times g$, as longer ultracentrifugation induces clumping of the viral particles and inadvertently reduces the yield. Clumping of viral particles is evident by difficulty in resuspending. If clumping occurs with centrifugation for 30 min, reduce the time to 25 or 20 min. In our experiences, a 20-min centrifugation yields ~90% recovery of the viral particles, whereas a 25-min one gives >95% recovery.

5. Store the concentrated virus at -80°C in aliquots of 50 μl (see Notes 9 and 10).

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⁸In our experience, RCAS retrovirus stock with a high titer of $1-5 \times 10^8$ pfu/ml is required for a widespread viral infection of retinal cells by delivering the virus through microinjection into the developing eye. By resuspending into 1% of the original volume, the titer of the viral stock can be as high as 5×10^8 pfu/ml, since the concentration of the retrovirus in the harvested medium is $\sim 5 \times 10^6$ pfu/ml.

⁹Our working experience tells us that the RCAS retrovirus is more stable than MSCV retrovirus. The procedure described here is developed over the past 15 years. It is simple and reliable.

¹⁰Overall, three key points in achieving 98–100% recovery during the viral concentration step are those specified in Notes 3, 7, and 8.