

GENE DELIVERY INTO THE CHICKEN EMBRYO BY USING REPLICATION-COMPETENT RETROVIRAL VECTORS

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Abstract : Rous sarcoma virus (RSV)-derived retroviral vectors have allowed for efficient gene transfer into the chicken embryo which is a classical model for studying vertebrate development. Current evidence reveals that this method can be used for regionally restricted expression, inducible expression, and for interfering with endogenous gene function, suggesting that gain-of-function and loss-of-function strategies for specific genes can be achieved spatially and temporally in the avian embryo. Thus, retroviral-mediated gene transfer into the chicken embryo coupled with a wide variety of strategies is now an important tool to address specific biological questions in the vertebrate.

Key words : replication-competent retroviral vector, chicken embryo, promoter, inducible gene expression, doxycycline

INTRODUCTION

The avian embryo has long been a popular and an excellent model for studying vertebrate development. The egg is inexpensive and easy to maintain in the laboratory and the embryo is accessible throughout development. This accessibility allows a number of experimental manipulations such as chick-quail grafting, cell and tissue transplantation, treatment with chemical and biological factors, for investigating specific events at a defined time during development. Furthermore, recent studies also indicate that the chick system is a good model to investigate cancer cell intravasation^{1,2)}. Thus, the chick system is useful in cancer biology as well as for vertebrate development.

Despite its classical manipulative advantages and expanded applications as an

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experimental model system, the usefulness of the chick model for genetic studies using modern molecular biological methods has been limited compared with the other vertebrates such as the mouse and zebrafish. To understand the molecular mechanisms involved in developmental processes, gain-of-function and/or loss-of-function of specific genes are often necessary. Such approaches have had limited success with the chick³⁾. Because of a long reproductive cycle and other drawbacks, transgenic approaches are not commonly available in the chick. Instead, Rous sarcoma virus (RSV)-derived replication-competent avian retroviral vectors, devel-

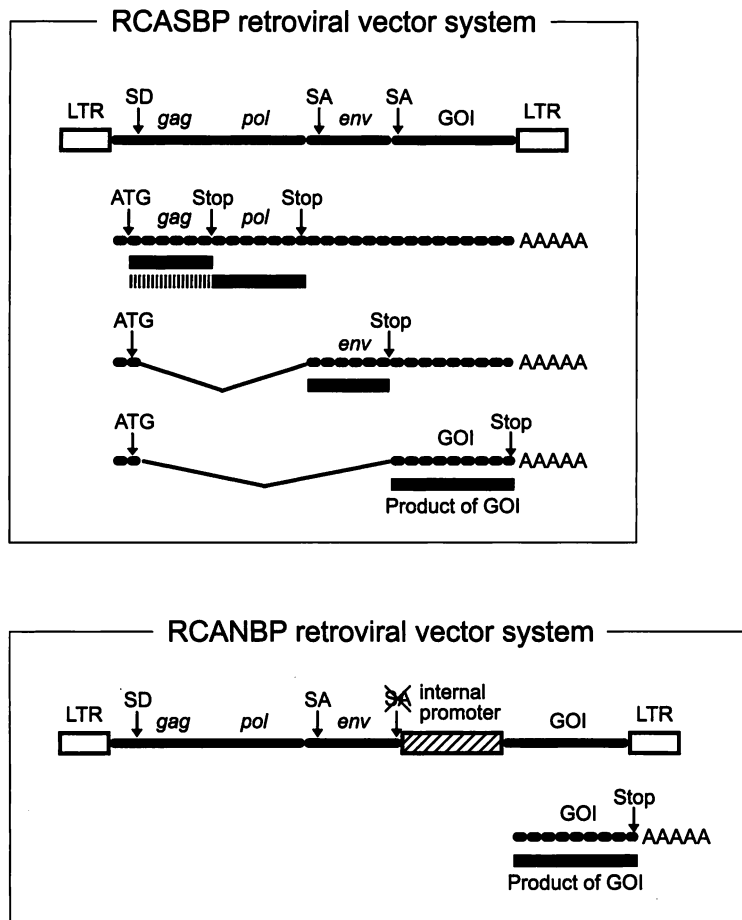


Fig. 1. Replication-competent avian retroviral vector system.

In the RCASBP retroviral vector system, genes of interest (GOI) are expressed by alternative splicing of a proviral RNA. GOI are produced from one of the alternative splice variants for the viral mRNA that is transcribed under control of the viral promoter within the long terminal repeat (LTR). In the RCANBP retroviral vector system, a splice acceptor (SA) sequence is removed from downstream of the *env* region. Accordingly, expression of GOI depends on the activity of an internal promoter. *gag*, *pol*, and *env* denote the location of viral genes. SD, splice donor.

oped by Hughes and colleagues, have been extensively used for *in vivo* delivery of genes of interest into the avian embryo^{4,5}). This method has been widely employed and modifications have been introduced to meet specific needs. In this paper, I shall briefly review current progress of the avian replication-competent retroviral vector system.

GENERAL ASPECTS OF AVIAN REPLICATION-COMPETENT RETROVIRUSES

A series of avian replication-competent retroviral vectors are derived from the Schmidt-Ruppin A strain of Rous sarcoma virus. In these vectors, foreign genes are cloned into a unique *Cla*I recognition site in place of the RSV *src* oncogene that is not required for viral propagation (Fig. 1). Because these vectors still have all viral genes required for their replication, recombinant viruses can be easily propagated without helper viruses and they can spread in the embryo efficiently by secondary infection. Among these vectors, RCASBP vectors (Replication-Competent, ALV LTR, Splice acceptor, Bryan Polymerase) are popular and most favorable in developmental biology. This vector, which allows high levels of transgene expression in the chicken embryo, can be easily grown to high titer in avian cells such as DF-1 fibroblast^{6,7}). An inserted gene is expressed by alternative splicing of a proviral RNA whose transcription is controlled by the constitutive viral LTR enhancer (Fig. 1).

Alternatively, RCANBP vectors, which lack the splice acceptor site downstream of the *env* region, can be used to drive transgene expression by an internal promoter (Fig. 1). Although the transgene cassette can be placed either in a forward or a backward orientation in the RCANBP vectors, we have confirmed that, in the case of the CMV promoter/GFP cassette, GFP expression is not observed when the vector carries the cassette in a backward orientation. Thus, the vector should possess the transgene cassette in a forward orientation.

In both RCASBP and RCANBP vectors, the size of an experimental sequence is approximately from 2.2 kb to 2.4 kb. Although a few successful examples with larger inserts have been reported, large inserts as well as any sequences that are toxic to the cell will be generally removed from the virus during propagation.

INFECTION OF THE CHICKEN EMBRYO BY RSV-DERIVED VECTORS

Although the RSV-derived retroviral vector can be used in a series of transgenic mouse lines that express the viral receptor, *tv-a*, under the control of specific enhancers⁸), I intend to focus on gene transfer into the chick embryo system in this review. To introduce RSV-derived vectors into the chicken embryo, a couple of strategies are currently available.

A conventional procedure is the injection of concentrated virus solution into the embryo with a pulled glass needle (Fig. 2A). For maximal gene transduction

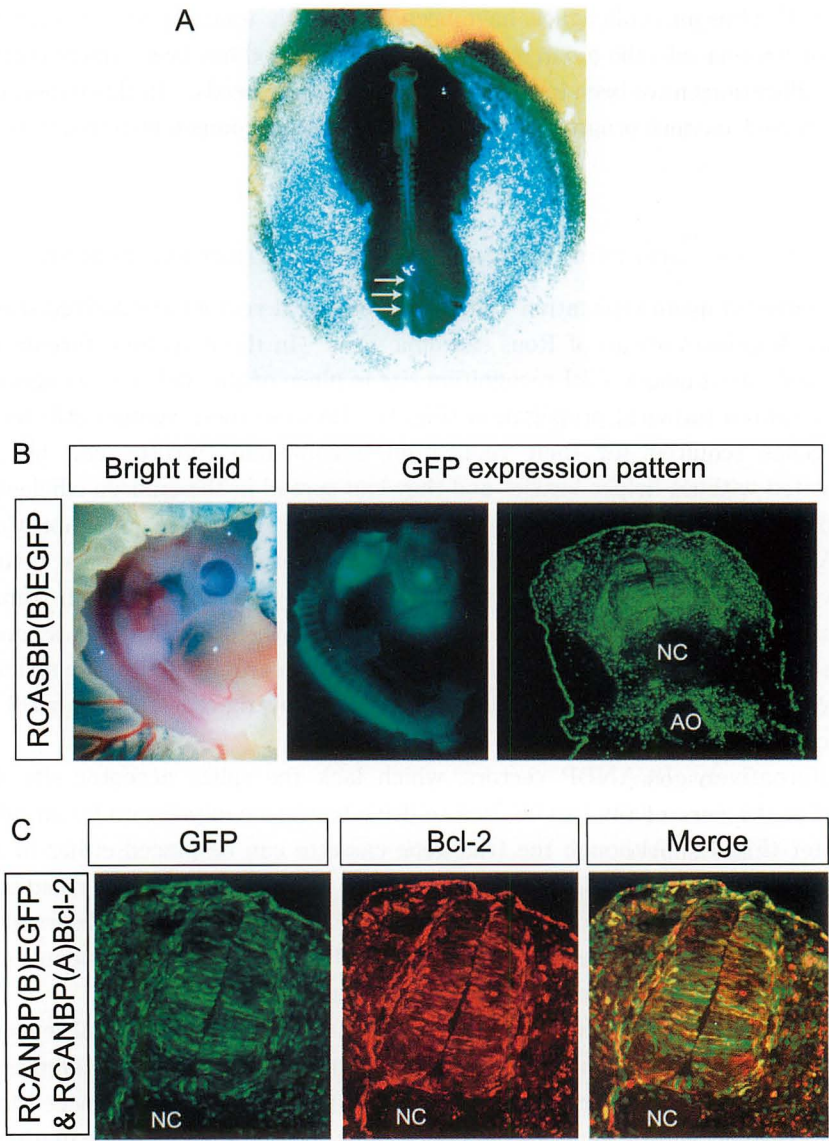


Fig. 2. Infection of the chicken embryo by RSV-driven vectors. **A**, Viral solution is injected into the HH stage 9-10 chicken embryo by using a pulled glass needle (arrows). **B**, GFP is clearly delivered into the E4.5 embryo by RCASBP retroviral vectors. Photomicrographs (Bright field and GFP expression) of a living embryo infected with RCASBP(B)EGFP show a bright field view and GFP expression. GFP immunoreactivity in a transverse section of a HH stage 24 embryo infected with RCASBP(B)EGFP is also shown. NC, notochord ; AO, aorta. **C**, GFP and Bcl-2 can be delivered into the spinal cord following dual viral infection, RCANBP(B)EGFP and RCANBP(A)Bcl-2. NC, notochord.

efficiency in target tissues, it is important to consider the time of infection as well as the location of injection. Although a few reports have revealed that Rous sarcoma virus can infect nondividing cells such as neurons^{9,10}, the virus efficiently transduces cells during mitosis. Using this character, it may be possible to deliver the transgene predominantly into cell populations that differentiate at later developmental periods. The structure of the embryo also affects infection of the vectors. For example, efficient gene transfer into the spinal cord requires viral injections between stage 8 and 10 (Fig. 2B). If the viral vector with sufficient titer can be prepared and injected into the embryo at this stage before the neural tube is closed completely, most cells in the spinal cord can be infected. Injections at stage 10 or later induce a limited infection in the spinal cord, thereby leading to inefficient transgene expression in postmitotic neurons such as ventral motoneurons.

Alternatively, plasmid constructs of RSV-derived vectors can be directly introduced into the avian embryo by *in ovo* electroporation¹¹. Recently, *in ovo* electroporation has emerged as a new approach for efficient gene transfer into living avian embryos^{12,13}. When the viral plasmid construct is introduced into the embryo, expression of the transgene initially occurs from the plasmid itself. Thereafter, a recombinant virus is generated in initially transfected cells and spreads over the neighboring cells. One of the advantages of this strategy is that transgene expression occurs rapidly compared with the conventional viral infection protocol, since a viral plasmid can work as an expression plasmid before the recombinant virus expresses a transgene through a viral life cycle.

The members of the Rous sarcoma virus family have been classified into five major envelope subgroups (A-E). Therefore, RSV-derived vectors with different subgroups are available. With the use of different subgroups, double transgenes can be introduced into the single cells both *in vitro*¹⁴ and *in vivo* (Fig. 2C). When two genes are transferred by using the vectors carrying the same envelope subgroup, the majority of cells express only one of the two genes and only a few cells can express both genes. In contrast, many of cells can express two genes when the respective vectors have different envelope subgroups (Fig. 2C).

EXPRESSION OF TRANSGENES IN THE CHICKEN EMBRYO

Once a RSV-derived vector infects avian cells, transgene expression primarily depends on the activity of the viral LTR or an internal specific enhancer in the target cells. In the case of RCASBP that uses the viral LTR for transgene expression, a vector with a subgroup B envelope is favorable for introducing higher levels of transgene expression as compared with a vector carrying a subgroup A envelope. In general, levels of transgene expression infected by RCASBP vector are less than those from the same vector construct when transfected by *in ovo* electroporation. With *in ovo* electroporation, a large number of plasmids can be transferred into a single cell, whereas a limited number of proviral DNA may be integrated into the

genome of a host cell. Thus, *in ovo* electroporation can allow high levels of transgene expression from RCASBP vectors. However, in most experiments such as gain-of-function or ectopic expression of a specific gene, levels of expression by these RCASBP vectors are sufficient to investigate the function of a particular gene. Using RCASBP(B) vector we have introduced the anti-apoptotic gene, Bcl-2, into motoneurons in the cervical spinal cord and found that Bcl-2 can rescue motoneurons from programmed neuronal death¹⁵. In some situations, the use of an internal ribosomal entry sequence (IRES) was shown to increase transgene expression^{16,17}.

RESTRICTED GENE EXPRESSION

The RSV-LTR enhancer generates widespread and constitutive expression in avian cells infected with RCASBP. Although this is useful in many gain-of-function experiments, it is desirable and often essential to be able to restrict gene expression to a particular tissue or cell type. Several strategies have shown regionally restricted expression by using RSV-derived vectors.

To restrict gene expression in a specific tissue, it is advantageous to use an internal tissue-specific promoter to drive transgene expression. A previous study demonstrated that high levels of CAT activity can be specifically detected in striated muscle in the chick after hatching using RCAN vectors driven by the skeletal muscle α -actin promoter¹⁸. More recently, we also confirmed that the RCANBP vector carrying an internal CMV promoter delivers GFP into restricted populations of specific tissues in the chicken embryo¹⁹. Therefore, an internal promoter coupled with a RSV-derived vector can be used to drive gene expression in restricted cell populations of the chicken embryo. However, some enhancer/promoter regions placed in the RCANBP vector often fail to drive tissue specific expression, although the same enhancer region can drive gene expression in specific cell populations following *in ovo* electroporation. Therefore, the success of this approach may be determined by both the specificity and the structure/sequence of the enhancer/promoter region.

An alternative approach is to express a transgene by a conventional viral infection protocol with the restriction of the spread of a recombinant virus to a particular target tissue. This is commonly achieved by the regionally targeted injection at a defined stage of development. A more elegant procedure introduced by Fekete and Cepko involves retroviral gene transfer system combined with tissue transplantation based on host strain restrictions to limit gene transfer to restricted cell populations²⁰. It is also possible to introduce a viral plasmid by *in ovo* electroporation into limited anatomical regions using microelectrodes¹¹.

INDUCIBLE GENE EXPRESSION

Although tissue specific gene delivery is a useful tool to investigate the function

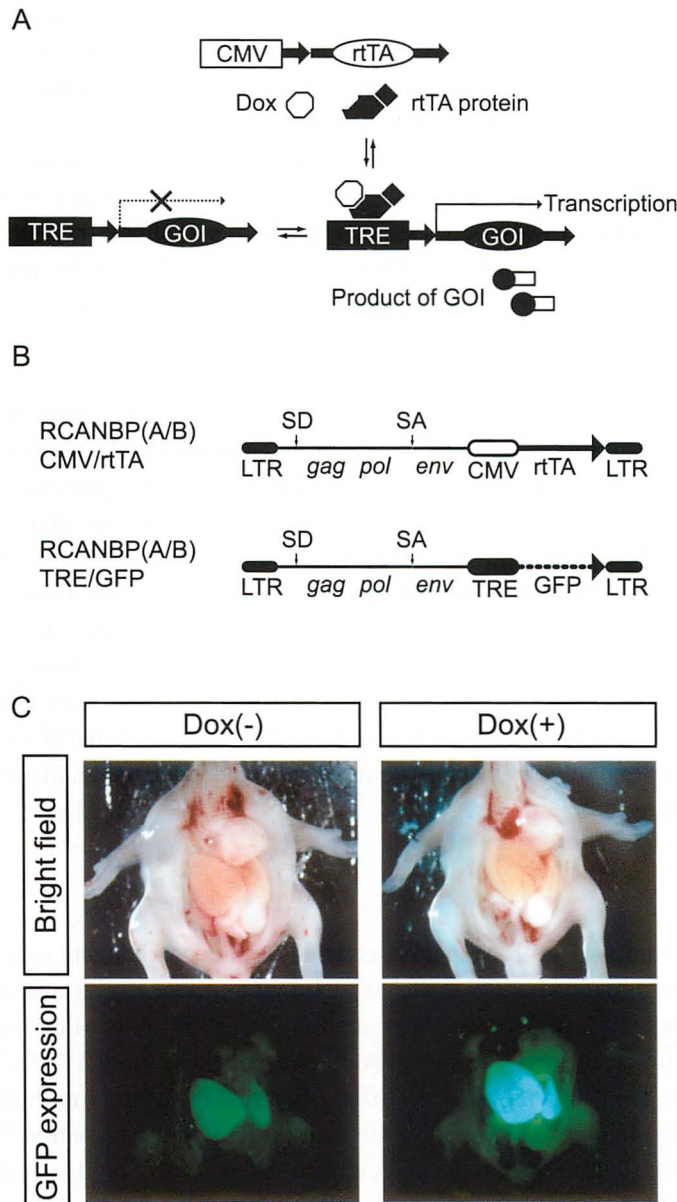


Fig. 3. Doxycycline-induced GFP expression in the chicken embryo.

A. The rtTA protein can only drive an experimental gene expression via binding TRE in the presence of doxycycline (Dox). B. RCANBP(A/B)CMV/rtTA carries the CMV promoter and the rtTA. RCANBP(A/B)TRE/EGFP carries the TRE promoter and a GFP reporter gene. C. The embryo is allowed to survive to E7 following infection of RCANBP(B)CMV/rtTA and RCANBP(A)TRE/EGFP. Within 24 hours after doxycycline treatment, GFP expression is clearly induced in the developing liver.

of a particular gene, it would be more valuable if one could control the time of gene expression. Since many developmental events occur during a narrow time window, some experimental genes are best expressed exclusively at a defined stage but not at other earlier or later stages of development.

To develop an inducible gene expression system in the chicken embryo, our group has tried to use the *tet* regulatory system with RSV-derived retroviral vectors. The *tet* regulatory system provides one method by which the expression of a transgene can be suppressed or activated by tetracycline or its analogs^{21,22}. A summary of gene induction mechanisms is shown in Figure 3A. If the transgene that constitutes the *tet* system could be efficiently introduced into the chick embryo, it would be a powerful model that could take advantage of the accessibility of the chick embryo to experimental perturbations *in ovo*. Because the RCANBP retroviral system allows one to deliver distinct transgenes by using different envelope subgroups (Fig. 2C), RCANBP vectors carrying a transgene cassette including the reverse tetracycline-controlled transactivator (rtTA) driven by the CMV promoter (RCANBP/CMV/rtTA), and the TRE promoter/GFP cassette (RCANBP/TRE/GFP), have been generated (Fig. 3B). Following dual infection, GFP expression is clearly induced in the developing liver in the presence of doxycycline (Fig. 3C). In other region where the CMV promoter can efficiently drive transgene expression, GFP is significantly expressed after exposing the embryo for 24 hours to doxycycline¹⁹. Therefore, RSV-derived replication-competent retroviral vectors can be used for a variety of experiments that require inducible gene expression in the chicken embryo.

LOSS-OF-FUNCTION EXPERIMENTS

RSV-derived vectors have also been used to silence endogenous gene activity. Most attempts have used expression of dominant-negative mutants of the target gene. If dominant-negative mutants are available, it may allow one to study specific gene function by a loss-of-function experiment using RSV-derived vectors.

It has also been shown that *neuregulin-1* function in developing chicken embryos is perturbed by delivery of the ribozyme using a RSV-derived vector²³. Hammer-head ribozymes that are catalytic RNA enzymes specifically bind to and then cleave target RNAs. In this study, ribozyme-tRNA cassettes, which are designed for cutting *neuregulin-1* mRNA, are introduced into the embryo by a RSV-derived vector. Although it is still difficult to design ribozymes suitable for a particular gene, this method may provide a possible means for disrupting specific genes in the chicken embryo.

Another approach reported previously is that delivery of antisense RNA by a RSV-derived vector results in a decrease of LIFR β expression in the developing spinal cord of the chicken embryo²⁴. Expression of antisense RNA for specific genes by a RSV-derived vector may be an alternative method to disrupt endogenous

gene function. However, there have been few successful examples reported using this approach.

Recently, a RSV-derived vector designed to express short hairpin RNA sequences was generated²⁵. This vector could reduce GAPDH expression in mammalian cells. Although it remains unknown whether RNA interference by using a replication-competent RSV-derived vector can silence specific target sequences in the avian embryo, this may provide an opportunity to create universal loss-of-function experiments in the chicken embryo.

CONCLUDING REMARKS

RSV-derived retroviral vectors are a valuable tool for exploring gene function in specific and restricted cell populations as well as at specific and limited periods of development. This method can be employed in combination with other approaches such as transplantation or *in ovo* electroporation of viral plasmids to provide greater flexibility in the design of genetic experiments in the chicken embryo. Further progress in this area will provide opportunities to address a number of biologically important questions in the vertebrate embryo.

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