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Gene transfer into the chicken embryo: Temporal regulation of transgene expression with the *tet* regulatory system

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Summary. Recent methodological developments using viral and non-viral vectors have allowed for efficient gene transfer into the chicken embryo which is a classical model for studying vertebrate development. Because a number of interesting and important events occur at specific time of development, to understand molecular mechanism undergoing such events it is necessary to control transgene expression temporally as well as spatially. One of most popular strategies for temporal control of transgene expression is the *tet* regulatory system in which transgene expression can be controlled by the *tet* operator sequence in the presence of doxycycline. Recent progress suggest that gene transfer techniques in combination with the *tet* regulatory system can allow transgene expression at defined time of development in the chicken embryo. Thus, these strategies can provide a valuable tool to address specific biological questions in vertebrate morphogenesis.

Key words — chicken embryo; doxycycline; inducible gene expression; *in ovo* electroporation; replication-competent retroviral vector; *tet* regulatory system.

INTRODUCTION

The avian embryo has long been a popular and an excellent animal model for studying vertebrate development. A major advantage of the avian system is that the embryo can be 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.

Despite its classical manipulative advantages and expanded applications as an experimental model system, the usefulness of the avian model for developmental studies has been limited until recently compared with the other vertebrates such as the mouse and fish. Over a past quarter century, development of genetic approaches has made it possible to investigate molecular mechanisms in a specific biological event during embryogenesis and organogenesis as well as those in many other fields of biology. To understand the molecular mechanisms involved in developmental processes, gain-of-function and/or loss-of-function of specific genes are badly needed. However, there have been only a few successful examples to generate transgenic bird to date[1]. Because of a long reproductive cycle and other drawbacks, transgenic approaches are not routinely available in the chick. In stead, non-transgenic approaches of gene transfer are reasonable and necessary to take advantage of the avian system. These approaches include viral-mediated gene transfer such as the adenovirus system[2] and the retrovirus system[3,4]. More recently, *in ovo* electroporation has emerged as a new excellent technique to deliver exogenous genes into the chick embryo[5-7].

Although these approaches are useful for examining gene function in early embryos, it is desirable and often essential to be able to control gene expression more precisely for the study of later developmental events. Because many tissues are generated and become

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populated by distinct cell populations only gradually during development, simply varying the site and time of transfection with either viral vectors or non-viral vectors may not effectively control gene expression. To address molecular mechanism at a specific time point of development inducible gene expression systems would be exceedingly valuable. One possible candidate is the *tet* regulatory system by which the expression of a transgene can be suppressed or activated by tetracycline or its analogs[8,9]. In this short article, I shall briefly introduce a couple of popular strategies for gene transfer into the avian embryo and especially focus on current available methods for temporal control of transgene expression using the *tet*-on system in the chick embryo.

The *tet* regulatory system and animal application

In the *tet* regulatory system, transgene expression is turn on or off by the treatment of tetracycline. A critical component is the regulatory protein based on the Tet repressor protein (TetR) which blocks transcription of the transgenes by binding to the *tet* operator sequences (*tet*O) in the absence of doxycycline (Dox), a tetracycline derivative. A "reverse" Tet repressor (rTetR) which was generated by four amino acid changes in TetR can bind to *tet*O in the presence of Dox. Thereby, transgene under the control of *tet*O is transcribed by the addition of Dox. Both repressors are genetically engineered to activators known as the tetracycline-controlled transactivator (tTA) and reverse tTA (rtTA), respectively, and resulting activators may act on the tetracycline-response element (TRE) which is composed of the *tet*O repeats and the minimal CMV promoter. To turn on transgene expression, rtTA can be used in the *tet* regulatory system. By the addition of Dox transgene is expressed in this *tet*-on system (Fig. 1). This method has been shown to work in animal models[10], where it has been used to study memory mechanisms[11-13] and for the generation of mouse models of genetic disorders[14-16]. In a study during developmental period, FGF-7 was shown to be induced in the fetal lung by administration of Dox to the mother[17]. These suggested a great possibility for the chick system because, if these components (rTetR and *tet*O) can be efficiently introduced into the embryo, the chick embryo may allow Dox treatment better than other animal models with its accessibility.

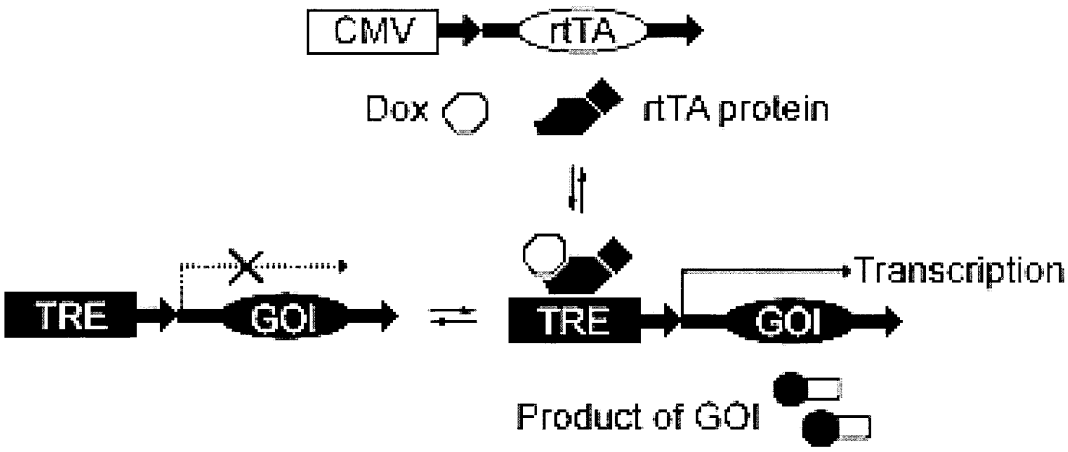

Fig 1. Overview of the *tet*-on system. The rtTA protein can only drive an experimental gene expression via binding TRE in the presence of doxycycline (Dox).

How can we introduce foreign genes into the chicken embryo?

(1) Avian replication-competent retroviruses

Rous sarcoma virus (RSV)-derived replication-competent avian retroviral vectors, developed by Hughes and colleagues, have been extensively used for *in vivo* delivery of genes of interest into the avian embryo[3,4]. This method has been widely employed and modifications have been introduced to meet specific needs. 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. 2). 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[18,19]. An inserted gene is expressed by alternative splicing of a proviral RNA whose transcription is controlled by the constitutive viral LTR enhancer (Fig. 2). In many 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 successfully 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 death[20].

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. 2). Thus, this type is a strong candidate to create the *tet* regulatory system in the avian embryo. 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/the green fluorescence protein (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 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.

Although RCANBP vectors can allow limited size of DNA as transgene, an internal tissue-specific enhancer/promoter is applicable 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 promoter[21]. 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 embryo[22]. 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.

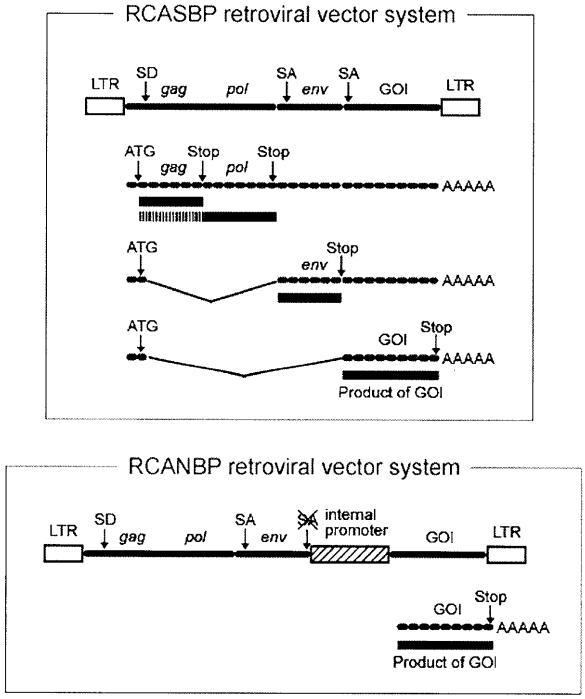

Fig 2. Replication-competent avian retroviral vector system.
In the RCASBP retroviral vector system, genes of interest (GOI) are expressed by alternative splicing of a proviral RNA. GOI are produced from one of the alternative splice variants for the viral mRNA that is transcribed under control of the 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.

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.

Concentrated virus solution is usually injected into the embryo with a pulled glass needle. For maximal gene transduction 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[23,24], the virus efficiently transduces cells during mitosis. Therefore, 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 according to Hamburger (HH)[25]. 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 HH 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.

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* and *in vivo*[26,27]. 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.

(2) *in ovo* electroporation

Although the replication-competent avian retroviral vector series enable very efficient transduction of transgenes, a couple of disadvantages disturb universal usage of the RCAS/RCAN vector system in the chick developmental studies. One of major problems is transgene size constraints. Recent progress in gene transfer into living embryo has been emerged with the development of electroporation[5]. Electroporation has been usually used for introducing DNA into wide variety of cultured cells such as bacteria and eukaryotic cells. But conventional procedures were achieved by exposure to high voltages, which usually cause massive cell death suggesting that it was not suitable for the living embryo. Improvements of electroporator enabling to handle low voltages made it possible to deliver DNA into the living embryo without apparent toxicity.

In ovo electroporation reveals several characteristics for transgene expression. First, it is possible to use large DNA as a transgene. Therefore, varieties of expression vectors, which may contain a long enhancer/promoter element or large genes of interest such as certain receptors, are available. Second, transgene can be expressed very rapidly following electroporation. For example, in the neural tube we can usually detect the expression of GFP as early as 3~4 hours after electroporation. Third, the target tissues and organs for gene transfer are restricted or controlled by where the electrodes are placed in the embryo since the target area must be positioned between two electrodes.

Due to its unique features *in ovo* electroporation can be easily modified according to individual experimental design. For example, plasmid constructs of RSV-derived vectors can be directly introduced into the avian embryo by *in ovo* electroporation[28]. 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. Furthermore, transgene expression may last for longer period compared with the common expression plasmid.

Expression of transgenes in the chicken embryo with the *tet*-on system

These gene transfer techniques using viral and non-viral vectors have been improved very recently and now provide a satially controlled gene delivery system in the chick embryo. As mentioned above, 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. These requests and needs can be addressed by the use of the *tet*-on system in the avian embryo with its excellent accessibility (Fig. 3).

Our group initially tried to use the *tet* regulatory system with RSV-derived retroviral vectors. Because the RCANBP retroviral system allows one to deliver distinct tansgenes by using different envelope subgroups as already mentioned in the text, RCANBP vectors carrying a transgene cassette including rtTA driven by

the CMV promoter (RCANBP/CMV/rtTA), and the TRE promoter/GFP cassette (RCANBP/TRE/GFP), have been prepared. Following dual infection, GFP expression was clearly induced in the developing liver in the presence of Dox. In other region where the CMV promoter can efficiently drive transgene expression such as the retinal pigmented epithelium, GFP was significantly expressed after exposing the embryo for 24 hours to doxycycline[22]. Therefore, the *tet*-on system mediated by the RSV-derived replication-competent retroviral vectors can be used for a variety of experiments that require inducible gene expression in the chicken embryo.

In ovo electroporation was also revealed to be available for the *tet*-on system in the living embryo[29]. After both plasmids carrying the TRE/GFP casette and rtTA driven by the motoneuron specific *HB9* promoter[30] were transfected into neural tube at E2 by *in ovo* electroporation, GFP expression was specifically induced in cervical motoneurons at E4 in the presence Dox. Approximately 5-20% of motoneuron express GFP very intensely whereas the remaining motoneurons never express GFP, suggesting that, although the transgene is induced in limited numbers of motoneurons, once activated, cells express a large amount of the protein product of the experimental gene. In the study we also examined two tetracycline responsive elements, TRE2 and TRE-Tight (TRET), for inducible GFP expression. In the absence of Dox, the TRET promoter exhibited less background expression than the TRE2 promoter. Thus, TRE elements may be a key to control background expression[29].

The *tet*-on system is also suitable to transfer cytotoxic genes into the embryo, because cytotoxic genes are not expressed in host cells or target tissues unless Dox is added. We tried to create the expression system for Bax, which is a pro-apoptotic protein that is required for programmed cell death (PCD). To introduce Bax specifically into motoneurons during the period of early PCD, we have taken advantage of *in ovo* electroporation coupled with the *tet* regulatory system. In the presence of Dox, foreign Bax was clearly introduced into motoneurons in the cervical region and subsequent analysis revealed that naïve Bax appears to enhance PCD only when motoneurons are stressed by the presence of death signal[29]. Moreover, apparent cytotoxic molecules, gain-of-function mutants of Bax[31] were also successfully introduced into motoneurons exclusively at the time of PCD by the addition of Dox[29]. Because these molecules clearly let motoneurons, which should be healthy before the period of PCD, to die if those are introduced by the conventional methods, the *tet* regulatory system may open the door to address the function of cytotoxic molecules such as Bax at defined time (the cell death period).

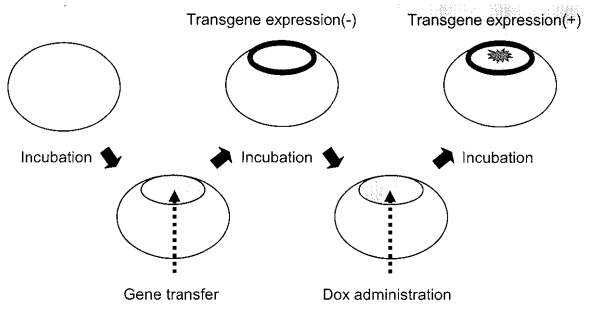

Figure 3. Overview of transgene induction in the chicken embryo.

CONCLUDING REMARKS

RSV-derived retroviral vectors and *in ovo* electroporation are a valuable tool for exploring gene function in specific and restricted cell populations as well as at specific and limited periods of development by using the *tet* regulatory system. These methods can be employed in combination with other approaches such as transplantation 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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