JOURNAL OF VIROLOGY, Feb. 2002, p. 1980–1985 Vol. 76, No. 4
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.4.1980–1985.2002

NOTES

Regulated Gene Expression in the Chicken Embryo by Using Replication-Competent Retroviral Vectors

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Received 7 September 2001/Accepted 19 November 2001

Rous sarcoma virus (RSV)-derived retroviral vector could efficiently deliver the green fluorescent protein (GFP), which is driven by the internal cytomegalovirus enhancer/promoter, into restricted cell populations in the chicken embryo. RSV-derived vectors coupled with the *tet* regulatory elements also revealed doxycycline-dependent inducible GFP expression in the chicken embryo in ovo.

Although the avian embryo has long been a popular model for studying vertebrate development, its usefulness for genetic studies using modern molecular methods has been limited compared with the mouse and zebra fish. Because of a long reproductive cycle and other drawbacks, transgenic approaches are not routinely available for the chick. Instead, 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 (5–7, 22–24). Although these approaches are of value for genetic studies of the early embryo, it is desirable and often essential to control gene expression more precisely for the study of later developmental events. Because many tissues are generated and become populated by distinct cell populations only gradually during development, simply varying the site and time of transfection with viral vectors may not sufficiently control gene expression. Instead, the use of tissue or cell type-specific enhancer/promoters and inducible gene expression systems would be exceedingly valuable for genetic studies in the chicken embryo. Such strategies may also be implemented in gene therapy protocols in the avian system in which a therapeutic gene is delivered.

The *tet* regulatory system has been a reliable system by which the expression of a transgene can be suppressed or activated by tetracycline or its analogs such as doxycycline (DOX) (10, 11). This system has been shown to work in different organisms from *Dictyostelium* to rodent (2, 3, 16, 17, 26, 32). In the mouse embryo, fibroblast growth factor 7 FGF-7 was induced in the fetal lung by administration of DOX to the mother (29). If the transgene that constitutes the *tet* regulatory system could be efficiently introduced into the chicken embryo, it would be a powerful model that would take advantage of the accessibility of the chicken embryo to experimental perturbations in ovo.

In an attempt to develop such a method, we have employed RSV-derived avian retroviral vectors with internal regulatory elements. It was previously shown that high levels of chloramphenicol acetyltransferase (CAT) activity can be specifically detected in striated muscle in the chick with retroviral vectors driven by the skeletal muscle α-actin promoter (24). Therefore, RSV-derived replication-competent retroviral vectors can be used when coupled with internal regulatory elements for transgene expression. Currently, the most popular avian retroviral vectors are RCASBP vectors, in which genes of interest are expressed by alternative splicing of a proviral RNA (5, 22) (Fig. 1). RCANBP vectors, which are derived from RCASBP vectors, do not possess the splice acceptor site downstream of *env* to drive transgene expression by an internal promoter (5, 23, 24). Because RCANBP vectors as well as RCASBP vectors possess all viral genes required for their replication, recombinant viruses can be easily propagated without helper viruses, and they can spread in the embryo by secondary infection. Thus, we chose RCANBP vectors as vehicles for the transgene cassette.

The human cytomegalovirus (CMV) enhancer/promoter from pEGFP-N1 (Clontech, Palo Alto, Calif.) and the tetracycline-responsive element (TRE) linked to the minimal CMV promoter from pTRE2 (Clontech) were used as internal regulatory elements. The enhanced green fluorescent protein (EGFP) from pEGFP-N1 or pEGFP-C2 (Clontech) was used as a reporter gene. The reverse tetracycline-controlled transactivator (rtTA) was obtained from pTet-On (Clontech). These components were initially subcloned into adapter plasmid Cla12Nco (15) or Slax13Nco (22). Then, the constructs were cloned into a *Cla*I site of either RCANBP(A) or RCANBP(B) retroviral plasmids. A RCANBP(B) plasmid was generated by exchanging the *Kpn*I-*Sal*I fragment from RCAN(B) (5, 23) with that of RCANBP(A). We generated RCANBP vectors carrying a transgene cassette including

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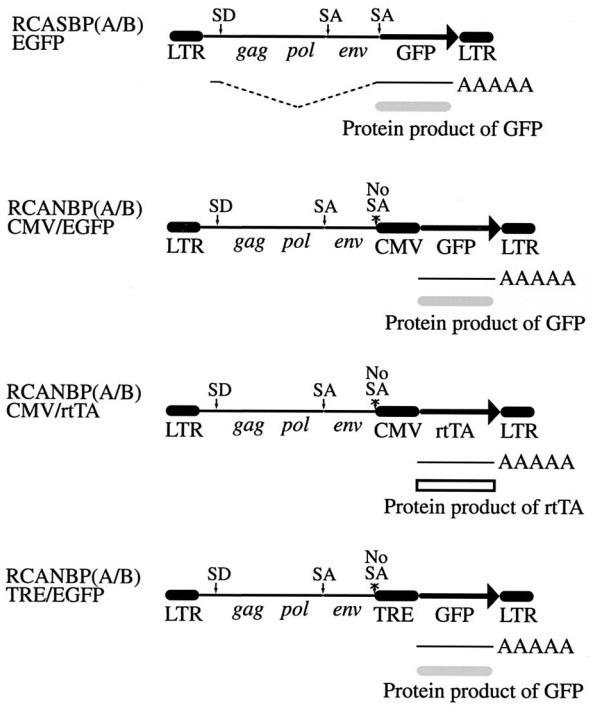

FIG. 1. Retroviral vectors. RCASBP(A)EGFP and RCASBP (B)EGFP [RCASBP(A/B)EGFP] are replication-competent retroviral vectors carrying a GFP reporter gene. GFP is produced from one of the alternative splice variants for the viral mRNA that is transcribed under control of the viral promoter within the long terminal repeat (LTR). RCANBP retroviral vectors lack a splice acceptor (SA) sequence downstream of the *env* region. Accordingly, expression of a transgene depends on the activity of an internal promoter. RCANBP(A/B) CMV/EGFP carries the CMV promoter and a GFP reporter gene. 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. *gag*, *pol*, and *env* denote the location of viral genes. SD, splice donor.

the human CMV promoter driving a GFP reporter gene (RCANBP/CMV/EGFP), the rtTA driven by the CMV promoter (RCANBP/CMV/rtTA), and the TRE promoter/GFP cassette (RCANBP/TRE/EGFP), respectively (Fig. 1). Although the CMV promoter/GFP cassette was placed in the forward and reverse orientations with respect to viral transcription, we found efficient GFP expression in DF-1 chick fibroblasts (13, 27) only by RCANBP with the cassette in a forward orientation (data not shown). In previous studies using promoter/CAT cassettes, higher levels of CAT activity were observed with the cassette in a forward orientation (23); the specificity of the chicken skeletal muscle α-actin promoter was relaxed somewhat when the cassette was in a backward orientation (24). Therefore, we only used RCANBP retroviral vectors with the transgene cassette in a forward orientation. During the propagation of viruses, we observed efficient GFP expression in DF-1 cells transfected with RCASBP/EGFP and RCANBP/CMV/EGFP, but not with RCANBP/TRE/EGFP, as expected (data not shown).

High-titer viruses were generated by standard procedures (22). Typical virus titers were 1×10^8 to 5×10^8 CFU/ml. Viruses were injected into the neural tube and onto the surface ectoderm of embryos at Hamburger and Hamilton stage 8 to 10 (12) via a pulled micropipette. In preliminary studies using more than 100 embryos infected with RCASBP/EGFP vectors, GFP appeared to be expressed more intensely by the virus with the B-subgroup envelope, RCASBP(B), than by the virus with the A-subgroup envelope, RCASBP(A), although there were no obvious differences in virus spread as confirmed by immunostaining against the viral *gag* protein p19 (data not shown). Accordingly, GFP expression was primarily examined in embryos infected with viruses carrying the B-subgroup envelope.

We initially examined the GFP expression pattern in embryonic day 8.5 (E8.5) chick embryos infected with RCASBP/EGFP and RCANBP/CMV/EGFP. During a series of experiments, more than 50 embryos for each viral infection were initially screened, and then 10 embryos with RCASBP/EGFP and 12 embryos with RCANBP/CMV/EGFP were further analyzed in detail. Living embryos were removed from the shell at E8.5 and initially observed by using an epifluorescence dissecting microscope with a GFP plus filter (Leica, Nussloch, Germany). The GFP expression pattern in embryos infected with RCANBP/CMV/EGFP appeared to differ from that in embryos infected with RCASBP/EGFP (Fig. 2A to H). In embryos infected with RCANBP/CMV/EGFP, strong green fluorescence was specifically observed in restricted regions such as the retinal pigmented epithelium (RPE), the liver, and proliferating zones in developing bones (Fig. 2D and H). By contrast, GFP expression with RCASBP/EGFP was widespread but was excluded from the RPE and the embryonic liver (Fig. 2B and F).

Embryos were then fixed with 4% paraformaldehyde and cryosections (8 to 10 μm thick) were examined with an epifluorescence microscope equipped with a fluorescein isothiocyanate filter (Zeiss, Oberkochen, Germany). GFP-expressing cells were identified in a majority of neural retinal cells, lens cells, and the corneal epithelium in the developing chick eye following infection with RCASBP/EGFP (Fig. 2I and J). By contrast, GFP was expressed predominantly in the RPE in embryos with RCANBP/CMV/EGFP (Fig. 2K and L). Virtually all the RPE expressed GFP, whereas neural retina only expressed GFP irregularly and weakly (Fig. 2L). In embryos with RCANBP/CMV/EGFP, the intensity of green fluorescence was much higher in the pupillary regions of the RPE than in the central region of the RPE (Fig. 2L). Transverse cryosections at the lower thoracic level revealed that GFP is ubiquitously expressed in the spinal cord, the dorsal root ganglion (DRG), the vertebra, and the mesenchyme in embryos with RCASBP/EGFP (Fig. 2N). In similar cryosections from embryos with RCANBP/CMV/EGFP, green fluorescence was also intense but was restricted to specific regions such as the dorsomedial region of the DRG and the dorsal root (Fig. 2P). Only sparse green fluorescence was observed in cryosections of the liver with RCASBP/EGFP (Fig. 2R). By contrast, virtually all liver cells intensely expressed GFP in embryos infected with RCANBP/CMV/EGFP (Fig. 2T). Levels of GFP expression were also assessed by immunoblotting for GFP, confirming the results obtained by fluorescence observation (Fig. 3O, lanes b and c).

To determine whether restricted and specific GFP expression in embryos following infection with RCANBP/CMV/EGFP was driven by the internal CMV promoter or by viral spread, the distribution of the viral *gag* protein p19 was exam-

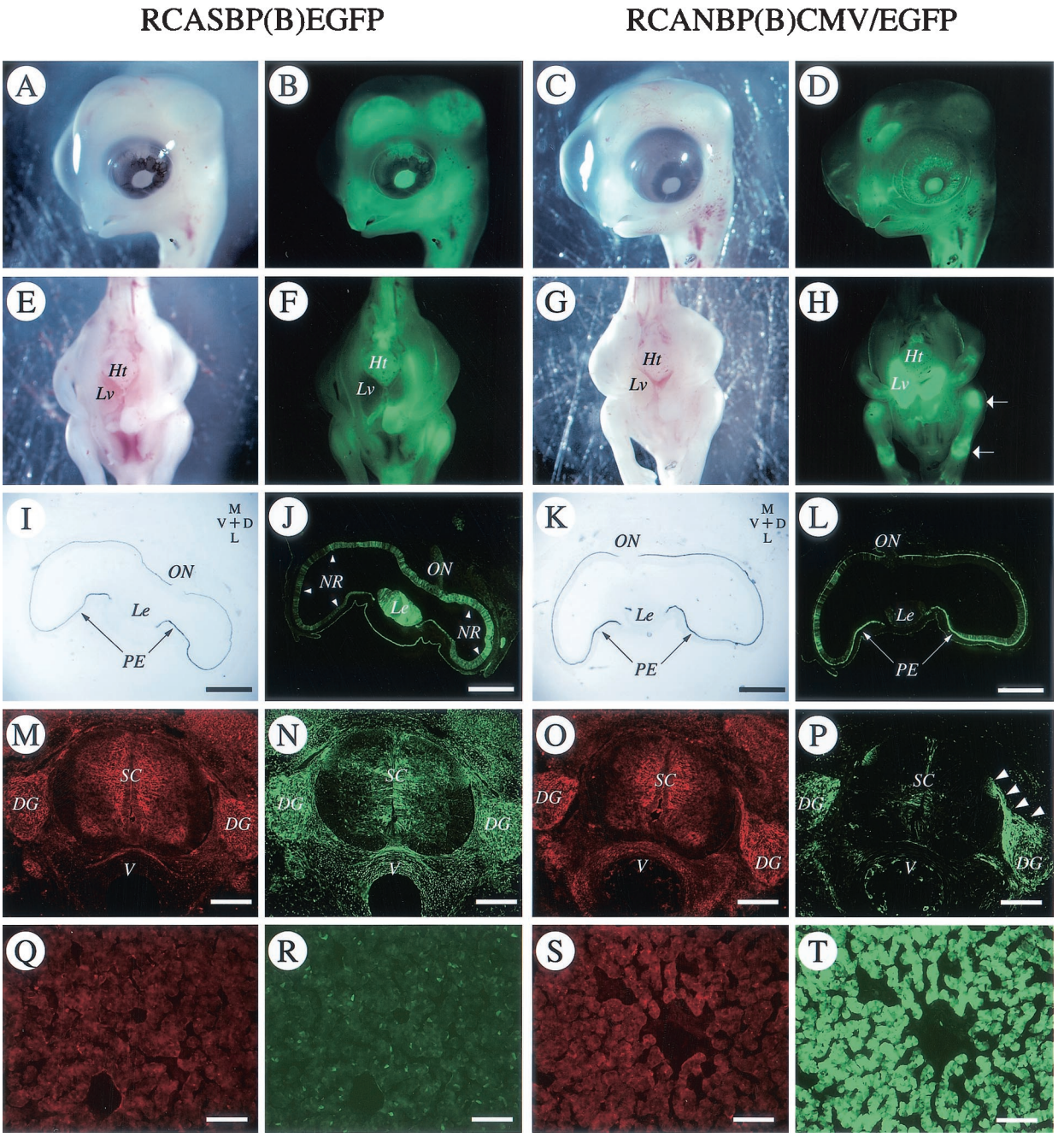

FIG. 2. GFP expression driven by the internal CMV promoter on E8.5 embryos. Embryos and cryosections are shown after infection with either RCASBP(B)EGFP (A, B, E, F, I, J, M, N, Q, and R) or RCANBP(B)CMV/EGFP (C, D, G, H, K, L, O, P, S, and T). Heads (A to D) and bodies (E to H) are shown with an epifluorescence dissecting microscope. Arrows (H) indicate proliferating zones in developing bones. (E to H) Ht, heart; Lv, liver. (I to L) Horizontal sections of the eye. Arrows (I, K, and L) indicate the pigmented epithelium. Arrowheads (J) indicate the neural retina. Le, lens; NR, neural retina; ON, optic nerve; PE, pigmented epithelium. Bars, 1 mm. (M to P) Comparison of the expression patterns of GFP (N and P) and the viral *gag* protein p19 (M and O) in the spinal cord. Arrowheads (P) indicate the dorsal root and the dorsomedial region of the DRG. DG, DRG; SC, spinal cord; V, vertebra. Bars, 200 μm. (Q to T) Comparison of the expression patterns of GFP (R and T) and p19 (Q and S) in the liver. Bars, 100 μm.

ined. For immunostaining against the viral p19 *gag* protein, sections were incubated with the antiviral p19 *gag* protein (AMV-3C2) monoclonal antibody (Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City) and visualized using Texas red-labeled antimouse immunoglobulin G (Molecular Probes, Eugene, Oreg.). Immunoreactivity for p19 was strongly deteced in the ventricular zone of the spinal cord and in the DRG in embryos infected with either RCASBP/EGFP or RCANBP/CMV/EGFP (Fig. 2M and O). In the eye, p19 was localized to the vitreal side of the neural retina (corresponding to the ganglion cell layer), in the basal side of the neural retina, and in the RPE after infection with

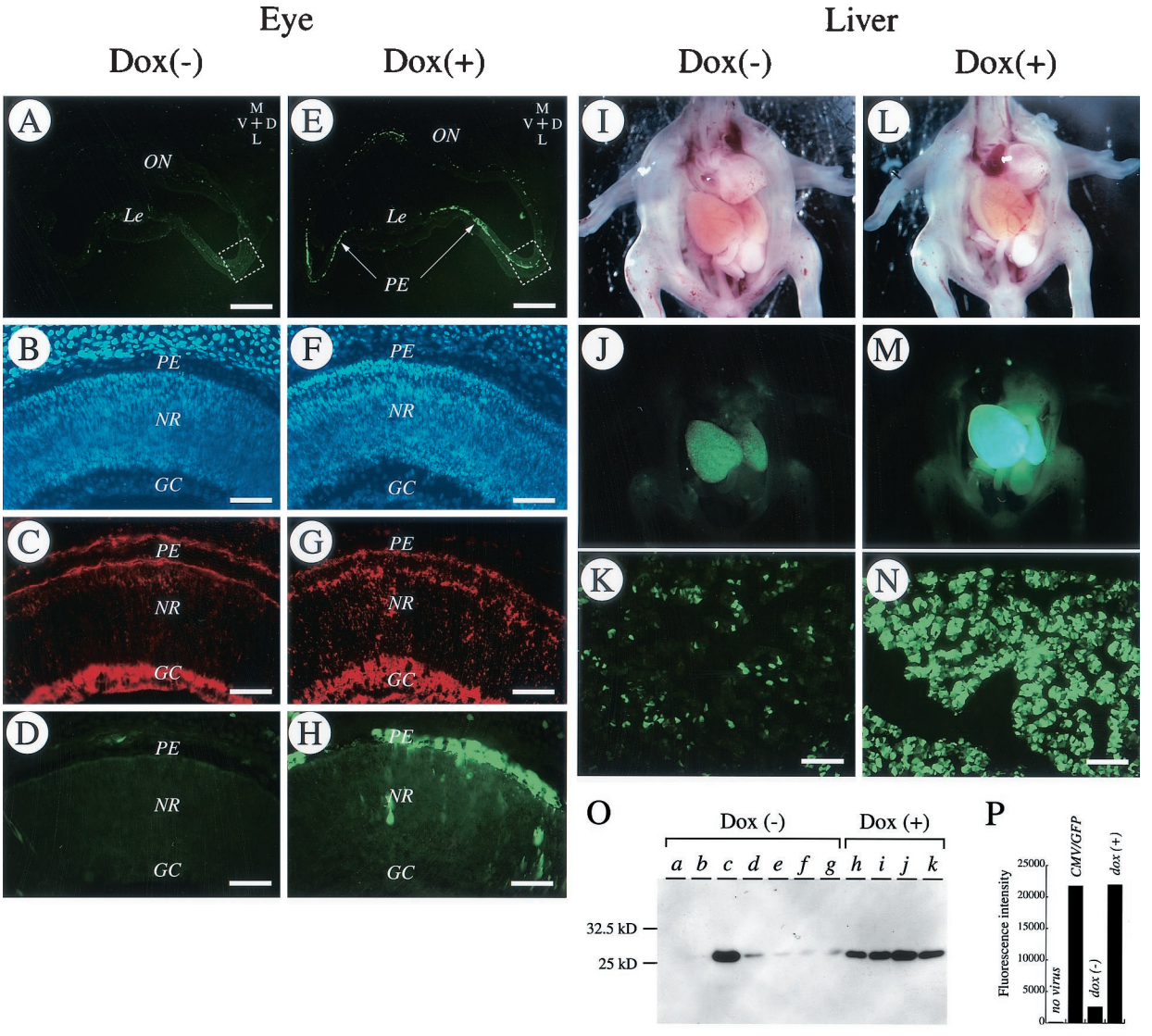

FIG. 3. DOX-induced GFP expression in the chicken embryo. The eyes of embryos without DOX (A to D) or with DOX (E to H) are shown following infection with both RCANBP(B)CMV/rtTA and RCANBP(A)TRE/EGFP. (A and E) GFP expression in eyes. Arrows (E) indicate the pigmented epithelium. Dotted boxes are shown in the following panels (B to D and F to H) with higher magnification. Le, lens; ON, optic nerve; PE, pigmented epithelium. Bars, 1 mm. Retinas are shown with DAPI (4′,6′-diamidino-2-phenylindole) staining (B and F), immunostaining against p19 (C and G), and GFP fluorescence (D and H). GC, ganglion cell layer; NR, neural retina; PE, pigmented epithelium. Bars, 50 μm. Embryos treated without (I and J) or with (L and M) DOX are shown following infection with both RCANBP(B)CMV/rtTA and RCANBP(A)TRE/EGFP. GFP expression is also shown in cryosections of the liver from embryos without (K) and with (N) DOX. Panels J and K represent the highest background expression of GFP without DOX. Bars, 100 μm. (O) Immunoblot analysis of liver lysates for GFP. Liver lysates were prepared from embryos infected without virus (lane a), with RCASBP(B)EGFP (lane b), with RCANBP(B)CMV/EGFP (lane c), and with both RCANBP(B) CMV/rtTA and RCANBP(A)TRE/EGFP (lanes d to k). Embryos without (lanes a to g) or with (lanes h to k) DOX are shown. The 2.7-kDa protein bands correspond to GFP. (P) Relative fluorescence intensity for GFP. Four samples in each experimental group were measured, and the averages are shown.

either of the viruses (results are not shown for either virus, since they are similar to the results shown in Fig. 3C and G). Interestingly, p19 was only weakly expressed in the liver with either RCASBP/EGFP or RCANBP/CMV/EGFP (Fig. 2Q and S). These data indicate that the two viruses spread similarly in the embryo. In embryos infected with RCANBP/CMV/EGFP, low levels of GFP expression were seen in the spinal cord, the ventral-lateral regions of the DRG, and the neural retina where p19 was abundantly detected, whereas GFP was strongly expressed in the liver where little p19 was detected. Therefore, GFP expression following infection with the RCANBP/CMV/EGFP retroviral vector was driven by the internal CMV promoter.

These results suggest that use of the internal CMV promoter with retroviral vectors will be of value for introducing heterogeneous genes into restricted regions such as the RPE and the liver, where conventional retroviral vectors do not function efficiently. The CMV promoter has been reported to determine tissue- or cell type-specific expression in transgenic mice (1, 18, 31). For example, in the mouse embryo the CMV

promoter drove *lacZ* expression in the RPE and along spinal nerves in a pattern consistent with that of pre-Schwann cells (18). These data are consistent with our observation that GFP expression occurred in the RPE and in the dorsal root. Although gene expression patterns need to be carefully evaluated based on the different developmental schedules of the chick and mouse, retroviral-mediated gene transfer with the internal human CMV promoter in the chicken embryo appears to mimic the situation in transgenic mice embryos carrying the CMV promoter to drive a reporter gene.

We next examined whether the *tet* regulatory system functions in the avian embryo with this viral vector. RCANBP/CMV/rtTA and RCANBP/TRE/EGFP were mixed and injected into the embryo. In our preliminary experiments, infection with RCASBP carrying different transgenes (the GFP gene or *bcl*-2) with the same type of envelope resulted in only a few DF-1 cells which expressed both GFP and Bcl-2, whereas the use of the two different envelopes significantly increased the number of double-labeled cells (data not shown). It was also shown that Bcl-2 and c-Myc were expressed efficiently in chicken embryonic fibroblasts with RCASBP vectors with different subgroups (9). Therefore, for dual infection, these two viruses possessing different envelope subgroups, A envelope or B envelope, were used. To achieve a lower background and maximal induction, a retroviral vector carrying the CMV promoter/rtTA cassette with the B envelope and the virus carrying the TRE promoter/GFP cassette with the A envelope were combined. DOX (100 μg) was administered to embryos at E7.5 and E8, and embryos were removed from the shell at E8.5 and subjected to GFP fluorescence observation. Since TRE-mediated gene expression depends on the level of rtTA expression as well as DOX concentration (16), both the RPE and the liver in which the CMV promoter significantly drives GFP expression were examined. Following preliminary experiments with more than 100 embryos, 24 embryos with DOX and 17 embryos without DOX were further analyzed in detail.

After exposing the embryos for 24 h to DOX, GFP expression in the RPE was clearly induced (Fig. 3A and E). Many cells located in the pupillary region of the RPE expressed GFP with an intense fluorescence in the presence of DOX (Fig. 3E, F, and H). By contrast, GFP was expressed in only a few cells in the absence of DOX (Fig. 3A, B, and D). GFP expression was not observed in the RPE of embryos infected with only RCANBP/TRE/EGFP, either with or without DOX (data not shown). The distribution of p19 was shown to be similar to that in embryos infected with either RCASBP/EGFP or RCANBP/CMV/EGFP (Fig. 3C and G). Taken together, these data indicate that the internal TRE promoter drives GFP expression dependent on rtTA expression controlled by the CMV promoter in the presence of DOX.

DOX-dependent transgene expression was also clearly observed in the developing liver (Fig. 3I to N). The liver expressed GFP at a relatively high level within 24 h following DOX administration (Fig. 3 M), and GFP was expressed in virtually all cells in the liver (Fig. 3N). Treatment with lower doses of DOX (1 or 10 μg) resulted in only moderate GFP expression, suggesting that GFP expression is induced by DOX in a dose-dependent manner (data not shown). A lower-level expression of GFP was also detected in the liver without DOX (Fig. 3J). Examination of transverse sections showed that a few cells expressing GFP were scattered in the liver (Fig. 3K).

To further assess DOX-mediated induction of GFP expression in the developing liver, the levels of GFP expression were examined by immunoblotting. Livers were dissected from embryos and homogenized in sonication buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl [pH 8.0], 200 mM NaCl). Following five cycles of freezing and thawing, lysates were centrifuged at 13,000 × *g* for 20 min at 4°C, and the supernatants were collected. The lysates (10 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon P membranes (Millipore, Milford, Mass.). The membranes were incubated with the anti-GFP antibody (Roche Molecular Biochemicals, Indianapolis, Ind.). After the membranes were incubated with peroxidase-conjugated antimouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, Pa.), the protein band for GFP was visualized by chemiluminescence detection (Amersham, Arlington Heights, Ill.). Immunoblot analysis also showed significant GFP induction in the presence of DOX (Fig. 3O, lanes h through k). Only faint bands were detected in tissues from embryos in the absence of DOX or with RCASBP/EGFP (Fig. 3O, lanes b and d through g).

The lysates (10 μg) were also set in 96-well plates, and GFP fluorescence was directly detected and analyzed with the FMBIO II Multi-View scanner (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). An approximately 10-fold induction was observed within 24 h after embryos were exposed to DOX by directly measuring the fluorescence intensity of GFP (Fig. 3P). Both the immunoblot and fluorescence intensities revealed that GFP expression was induced to a level similar to that when driven by the CMV promoter (Fig. 3O, lanes c and h through k, and 3P).

Several factors are likely to be important for determining basal and induced levels of transgene expression. First, capability of dual virus infection may be an important factor in determining whether DOX-mediated inducible gene expression occurs in a target cell population. Because retroviral vectors carrying different envelope subgroups were combined, if cells predominantly express a receptor for either the A envelope or the B envelope, only one transgene cassette will be introduced efficiently. In the case of the TRE/EGFP cassette, there may be increased background expression dependent on where the cassette is integrated into the genome of the host cell. Since it was not clear which type of receptor is expressed in a given cell population during development, the efficiency of dual virus infection may vary among cell types. The response to DOX may also vary among cells or tissues. For example, accessibility to DOX in various tissues might be affected by development of the vascular system. Furthermore, different cells may vary in the responsiveness of the *tet* regulatory system (14). Finally, the level of rtTA expression may more precisely determine induced levels of transgene expression. In the developing eye, the CMV promoter was more active in the pupillary regions of the RPE than in the central region of the RPE (Fig. 2L). This is consistent with the observation that DOX-induced gene expression occurred predominantly in the pupillary regions of the RPE, owing to the use of the CMV promoter to drive rtTA expression in the chicken embryo.

The fact that low levels expression of GFP were detected in the liver without DOX suggests that the *tet* regulatory system may not provide complete "off-state" expression in some situations, as described previously (8, 14, 16). Nonetheless, this system has been used to study memory mechanisms (19–21) and for the creation of mouse models of genetic disorders such as prion disease (30), cardiomyopathy (25), and Huntington's disease (33). Therefore, the *tet* regulatory system coupled with retroviral gene transfer described here will be used in variety of situations in the avian in which the phenotype to be controlled depends on the relative levels of an active transgene. This may include, for example, examining the function of genes which are toxic and cause severe malformations or embryonic lethality at a later stage of development.

In conclusion, the method of using an internal regulatory element with RSV-derived retroviral vectors provides a valuable tool for exploring gene function in specific and restricted cell populations and/or at specific and limited periods of development. This method can be employed in combination with other approaches such as transplantation (6) or in ovo electroporation of viral plasmids (4, 28) to provide greater flexibility in the design of genetic experiments in the chicken embryo.

We thank S. H. Hughes for providing the adapter plasmid Cla12Nco and the RCASBP(A/B), RCANBP(A), and RCAN(B) retroviral plasmids, B. A. Morgan for the adapter plasmid Slax13Nco, J. E. Johnson for his advice on the use of retroviral vectors, and Y. Tsuji and M. Hashimoto for advice about the *tet* regulatory system.

This research was supported by Grants-in-Aid for the Encouragement of Young Scientists (11770006 and 13770013) to N.S.

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