

Using Avian Retroviral Vectors for Gene Transfer

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The promoter regions of the chicken skeletal muscle α -actin (α_{sk} -actin) and the cytoplasmic β -actin genes were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. Replication-competent retroviral vectors were used to introduce these two actin/CAT cassettes into the chicken genome. Chickens infected with retroviruses containing the α_{sk} -actin promoter expressed high levels of CAT activity in striated muscle (skeletal muscle and heart); much lower levels of CAT activity were produced in the other nonmuscle tissues. In contrast, chickens infected with retroviruses containing the β -actin promoter linked to the CAT gene expressed low levels of CAT activity in many different tissue types and with no discernible tissue specificity. Data are presented to demonstrate that the high levels of CAT activity that were detected in the skeletal muscle of chickens infected with the retrovirus containing the α_{sk} -actin promoter/CAT cassette were not due to preferential infectivity, integration, or replication of the retrovirus vector in the striated muscles of these animals.

Avian reproductive physiology makes birds an attractive model system with which to investigate particular aspects of vertebrate developmental biology. Because the chicken embryo is relatively large and because development occurs primarily in ovo, it is more accessible to surgical and biochemical manipulation than are mammalian embryos. We have constructed a series of replication-competent avian retroviral vectors that are capable of introducing DNA inserts into avian cells. These vectors can be used to infect avian cells in culture, to establish somatic infections in vivo, and to generate transgenic chickens.

The RCOS and RCAS vectors express inserted sequences from the retroviral promoter within the long terminal repeat (LTR) via a spliced message. In contrast, the RCON and RCAN vectors, which lack the splice acceptor present in RCOS and RCAS, can be used to express DNA inserts from internal promoters. This makes it possible to separate the expression of the insert from viral gene expression. Previously, we have tested the RCOS and RCAS vectors in cultured fibroblasts. The bacterial gene for chloramphenicol acetyltransferase (CAT) was used to monitor the level of expression (15, 25). The RCON and RCAN vectors were also characterized in cultured fibroblasts by using inserts consisting of the CAT gene linked to either the chicken β -actin or the mouse metallothionein 1 promoter (38). These studies showed that the levels of expression of DNA inserts, whether from the LTR or from an internal promoter, are influenced by proviral sequences within the LTR and *pol* regions. By constructing a series of vectors that contain different LTR sequences and/or different *pol* sequences, we have developed a system that makes it possible to control the level at which a DNA insert will be expressed in infected cells.

We would like to be able to use this retroviral vector system to target the expression of a DNA insert to specific cell types in vivo. In this study, we have somatically infected chickens by using retroviral vectors that contain the CAT gene linked to either the chicken skeletal muscle α -actin

(α_{sk} -actin) or the chicken β -actin promoter. These two actin gene promoters were chosen because they are regulated in a tissue-specific manner and they have been well characterized in the chicken (4, 14, 16, 34, 41). Expression of α_{sk} -actin is restricted to striated muscle (skeletal and cardiac [56]), while β -actin is constitutively expressed in a wide range of cell types (57).

We have found that most chickens infected with retroviral vectors containing the α_{sk} -actin promoter linked to the CAT gene contained high levels of CAT activity in striated muscle. Much lower levels of CAT activity were detected in smooth muscle and nonmuscle tissues. Chickens infected with a retroviral vector containing the β -actin promoter produced low levels of CAT activity in a wide variety of tissues. Chickens infected with a retrovirus vector (RCASBP) that expresses CAT from the viral promoter within the LTR do not show muscle-specific expression of CAT. We discuss the potential applications of this vector system, based on our observations, for future studies in vivo.

MATERIALS AND METHODS

Vectors. Construction of the retroviral vector RCANBP and *Cla*I cassettes containing the chicken α_{sk} -actin (-191 to +24) or β -actin (-277 to +63) promoters linked to the CAT gene has been described before (25, 38, 41). *Cla*I fragments that contained the actin promoter/CAT genes were purified and inserted into the *Cla*I sites of RCANBP (Fig. 1). Recombinant plasmids containing the actin promoter/CAT cassettes in the forward and backward orientation were isolated and purified by banding twice in CsCl₂.

We have previously described the vector RCASCAT3, which expresses the CAT gene via a spliced message from the viral promoter within the LTR (25). The RCASBP/CAT3 vector used in this study was derived by replacing the *pol* region of RCASCAT3 with the *pol* region from the Bryan high-titer strain of Rous sarcoma virus (RSV) (55).

Cell culture. Chicken embryo fibroblast (CEF) cultures were established from decapitated 11-day embryos from line 0 and line 15B₁ specific-pathogen-free chickens. Line 0

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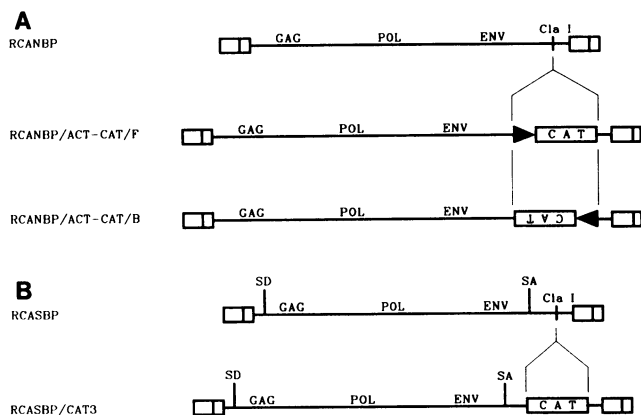


FIG. 1. Retrovirus vectors. (A) Retrovirus vector RCANBP (top) lacks a splice acceptor sequence immediately upstream of the *ClaI* insertion site. If an insert is to be expressed, a functional promoter should be included; RCANBP, containing an actin promoter/CAT cassette in the forward orientation (middle) or in the backward orientation (bottom). (B) The RCASBP vector (top) contains a splice acceptor (SA) sequence immediately upstream of the *ClaI* insertion site. Inserts are expressed as subgenomic mRNAs from the viral promoter within the LTR; RCASBP containing the CAT gene (bottom). *gag*, *pol*, and *env* denote the general location of viral genes. Actin/CAT cassettes or the CAT gene was inserted into unique *ClaI* sites. Open rectangles, retrovirus LTR sequences; solid arrowheads, α_{sk} - and β -actin promoters; CAT, CAT gene; SD, splice donor.

chickens (C/E; susceptible to all avian leukosis virus subgroups except subgroup E) lack endogenous proviruses related to the RCANBP vectors, and line 15B₁ chickens (C/O; susceptible to all avian leukosis virus subgroups) carry only the silent endogenous loci *ev1* and *ev15*. CEF cultures were grown in Leibowitz-McCoy medium (1.75 parts Leibowitz medium to 1 part McCoy medium 5A)–2% tryptose phosphate broth–5% heat-inactivated fetal calf serum–5% heat-inactivated newborn calf serum–5% CO₂. Cells were split 1:3 each time they reached confluence. Early-passage cells were transfected with 10 μ g of cloned retroviral DNA by the calcium phosphate precipitation protocol (27). Retroviral infection was monitored by measuring p27 antigen in the cell culture media by an enzyme-linked immunosorbent assay (ELISA) (48).

Animals. Specific-pathogen-free line 0 eggs were used for all in vivo experiments because this line lacks endogenous proviruses related to the RCANBP vector (1). Fertile eggs were injected at day of set with 50 or 100 μ l of culture medium containing either 10⁶ infected CEF or approximately 10⁵ infectious units of virus from infected CEF cultures (43). Injections were made into the yolk in proximity to the blastoderm (2 to 5 mm), but not into the blastoderm itself. Typical injection sites were localized in pilot studies by using a dye solution. At the time of hatch, blood samples were tested for the presence of the viral capsid antigen p27 by an ELISA (48) and for the presence of proviral DNA by the dot blot procedure (43). Uninfected and infected chicks were wing banded, vaccinated against Marek's disease, and housed according to vector construct in modified Horsfall-Bauer isolators (50). Positive birds were killed at either 2, 15, or 16 weeks of age, and tissue samples were collected and frozen in liquid nitrogen.

CAT assays. Extracts were prepared from tissue samples and adjusted to equivalent protein concentrations as de-

scribed before (41). CAT activities were assayed as described before (38, 41), and the results were quantitated by radioanalytic imaging (Ambis, San Diego, Calif.).

Western transfer (immunoblot) analysis. The detailed procedures for Western transfer and immunodetection have been described before (38). The tissue extracts that were prepared for CAT analysis were subjected to electrophoresis in 12% polyacrylamide gels (49). Samples were prepared for electrophoretic separation by adding 2 volumes of extract to 1 volume of a 3 \times loading buffer. Separated proteins were transferred to a nitrocellulose membrane with an Enprotech blotting apparatus (Integrated Separation Systems, Hyde Park, Mass.). The viral capsid protein p27 was immunochromically detected with a 1:1,000 dilution of rabbit anti-p27 serum (54) followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Kirkegaard and Perry, Gaithersburg, Md.). Alkaline phosphatase activity was detected by using the chromogenic substrates 5-bromo-4-chloro-3-indolylphosphate toluidinium and Nitro Blue Tetrazolium (BRL, Gaithersburg, Md.).

Nucleic acid hybridization. DNA from infected chicken tissues was purified as described before (41). DNA samples were denatured by alkali treatment, neutralized, and transferred to a nitrocellulose membrane with a dot blot apparatus (Schleicher & Schuell). Membranes were baked at 80°C under vacuum for 2 h. Conditions for hybridization and washing have been described before (38). Hybridization probes representing the *gag-pol* region of the retroviral vectors and the coding region of the endogenous β -actin gene were labeled with [α -³²P]dCTP by nick translation.

RESULTS

Retroviral vectors with actin promoter/CAT cassettes. To investigate the behavior of cellular promoters that have been introduced into the avian retroviral vectors, we linked the CAT gene to the chicken α_{sk} -actin or the β -actin promoter and inserted these two actin promoter/CAT cassettes into the replication-competent avian retroviral vector RCANBP (Fig. 1A). RCANBP is a derivative of RCAN (38) that was constructed by replacing the *pol* region of RCAN with the corresponding *pol* region of the Bryan high-titer strain of RSV (55). Recombinant vectors were isolated that contained the actin promoter/CAT genes in the forward and reverse orientations (with respect to viral transcription). Viremic CEF cultures were established by DNA transfection, and infections were monitored by ELISA (48).

Vector infectivity in vivo. Chickens with extensive somatic infections were established by injecting eggs on the first day of incubation with either 10⁶ viremic CEF or 10⁵ infectious units of virus supernatant from infected cell cultures (see Materials and Methods). On the day of hatch, infected chicks were identified by analyzing blood samples for the presence of proviral DNA and viral capsid antigen p27 (Table 1). In this study, we found that injection of viremic CEF was significantly more efficient than virus in producing infected hatchlings (74 versus 45%). Based on previous studies (29, 37), it is unlikely that randomly injected CEF incorporate efficiently into the developing embryo.

Promoter specificity in vivo. Infected chickens were killed at either 2 or 15 to 16 weeks of age, and a variety of tissue samples were collected and assayed for CAT activity. The CAT activities of tissue samples from four chickens infected with retroviral vectors carrying the α_{sk} -actin promoter/CAT cassette in the forward orientation (RCANBP/ α_{sk} CAT/F) and four chickens infected with retroviral vectors carrying

TABLE 1. Infection on day of hatch^a

Inoculum	No. of eggs	No. (%) of eggs positive	
		p27	Proviral DNA
Cells	38	28 (74)	24 (63)
Virus	51	23 (45)	ND ^b

^a Eggs were injected at day of set either with 10⁶ infected CEF (cells) or with filtered supernatants from infected CEF cultures (virus). Blood samples from 1-day-old chicks were tested for viral capsid protein p27 by ELISA. Proviral DNA was detected by dot blot analysis with DNA prepared from blood samples from 1-day-old chicks.

^b ND, not determined.

the same cassette in the backward orientation (RCANBP/ α_{sk} CAT/B) were quantitated by radioanalytic imaging, and the data are compiled in Table 2. We consistently detected the highest levels of CAT activity in striated muscle samples (skeletal muscle and heart). To illustrate the specificity of the promoter in the virus, a representative CAT assay from an RCANBP/ α_{sk} CAT/F chicken is shown in Fig. 2. When the RCANBP vector contained the α_{sk} -actin promoter/CAT cassette in the backward orientation, we did observe relatively high levels of CAT activity in the cardiac muscle and, to a lesser extent, the gizzard. Thus, the specificity of this promoter for adult skeletal muscle alone was relaxed somewhat but not abolished when the cassette was in the backward orientation.

Also listed in Table 2 are the CAT activities in various tissue extracts from four chickens infected with retroviral vectors carrying the β -actin promoter/CAT (β CAT) cassette. In contrast to chickens carrying the α_{sk} -actin promoter constructs, these chickens displayed low levels of CAT activity in a wide range of tissues.

The data in Table 2 can only be used to compare the CAT activities expressed in the various tissues of any one bird. Because not all of the birds were assayed under identical reaction conditions, these data cannot be used to quantitatively compare the levels of CAT expression in the muscle extracts from different birds. To make such a comparison,

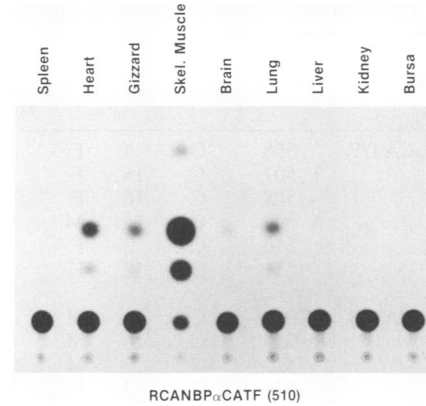


FIG. 2. Tissue-specific CAT expression in infected chickens. Homogenates were prepared from the tissue samples of chicken 510 infected with the retroviral vector RCANBP/ α_{sk} CAT/F. Homogenates were adjusted to an equivalent protein concentration, and CAT activities were assayed as described before (38).

the skeletal muscle extracts prepared from 33 infected chickens were adjusted to an equivalent protein concentration and assayed simultaneously for CAT activity. These data are shown in Table 3. These data show that muscle-specific CAT expression was independent of the mode of infection (cells versus virus), the age of the bird at the time of death, and the sex of the bird. The data clearly demonstrate that high levels of CAT activity were present in skeletal muscle extracts from birds infected by retroviral vectors carrying the α_{sk} -actin promoter but not in birds infected by vectors containing the β -actin promoter.

The vectors are not themselves muscle specific. Although the data obtained from chickens infected with RCANBP/ β CAT vectors suggested to us that vector sequences were not responsible for the muscle-specific expression of CAT in RCANBP/ α_{sk} CAT-infected birds (Tables 2 and 3), previous reports from other laboratories have indicated that RSV

TABLE 2. CAT activities in tissue samples

Vector	Bird no.	% Acetylation of chloramphenicol in:													
		Spleen	Heart	Gizzard	Skeletal muscle	Brain	Lung	Liver	Kidney	Bursa	Provent	Small intestine	Large intestine	Blood	Skin
RCANBP/ α_{sk} CAT/F	506	0	2	8	70	1	1	0	0	ND ^a	ND	ND	ND	ND	ND
	507	0	5	1	76	1	0	0	0	24	0	0	6	0	0
	509	0	0	0	63	0	0	0	0	ND	ND	ND	ND	ND	ND
	510	0	7	3	95	1	3	0	1	ND	ND	ND	ND	ND	ND
RCANBP/ α_{sk} CAT/B	517	0	17	13	71	0	1	0	0	ND	ND	ND	ND	ND	ND
	518	1	56	18	45	2	1	2	1	6	2	1	2	1	5
	521	2	53	15	28	2	2	2	1	8	4	2	2	2	4
	560	0	70	39	72	0	1	0	0	ND	ND	ND	ND	ND	ND
RCANBP/ β CAT/F	525	3	3	3	3	4	3	3	3	3	3	3	3	3	3
	526	1	2	1	1	1	2	1	1	1	ND	ND	ND	ND	ND
RCANBP/ β CAT/B	531	4	7	5	4	5	4	4	3	4	4	4	3	3	3
	537	2	2	2	2	2	2	2	2	2	2	2	2	2	2
RCASBP/CAT3	746	1	1	1	1	0	1	0	0	32	0	0	4	0	22
	747	25	95	57	73	96	40	1	0	95	13	0	1	5	87
	748	1	1	3	0	1	1	0	0	3	1	0	1	0	3

^a ND, not determined.

TABLE 3. Interbird comparison of muscle-specific CAT activity

Vector	Bird no.	Inoculum ^a	Age ^b (wk)	Sex	CAT activity ^c (%)	
					Muscle	Liver
RCANBP/ α_{sk} CAT/F	506	C	16	F	52	0
	507	C	16	F	98	0
	508	C	16	F	0	0
	509	C	16	M	85	0
	510	C	2	F	99	0
	511	C	2	F	0	0
RCANBP/ α_{sk} CAT/B	514	C	16	M	4	0
	517	C	16	M	63	0
	518	C	2	M	32	0
	521	C	2	F	23	0
	556	V	15	F	63	ND ^d
	559	V	15	M	1	ND
	560	V	15	M	82	0
	562	V	15	M	58	ND
	565	V	15	M	40	ND
	566	V	15	F	82	ND
	RCANBP/ β CAT/F	522	C	16	F	0
525		C	2	F	0	0
526		C	2	F	0	0
527		C	16	F	0	0
529		C	16	M	0	0
570		V	15	F	0	ND
572		V	15	M	0	ND
573		V	15	F	1	ND
577		V	15	F	1	ND
580		V	15	F	2	ND
582		V	15	F	2	ND
RCANBP/ β CAT/B	531	C	2	M	0	ND
	536	C	16	M	0	0
	537	C	2	F	0	ND
	538	C	16	F	0	0
	541	C	16	F	0	ND
	542	V	15	F	0	ND

^a Somatic infections were established by injecting eggs with infected cells (C) or viral supernatants (V).

^b Age in weeks at time of sampling.

^c Values are percent acetylation of chloramphenicol.

^d ND, not determined.

infection may exhibit some muscle tropism (7, 13, 23, 24). We assayed CAT activity in extracts of tissues from birds infected with a retroviral vector (RCASBPCAT3) that expresses the CAT gene from the viral promoter within the LTR (Fig. 1B). CAT activity was measured in tissue samples collected from three RCASBPCAT3-infected birds (Table 1). Each RCASBPCAT3-infected bird exhibited a pattern of CAT expression that was both qualitatively and quantitatively distinct from the pattern observed in the RCANBP/ α_{sk} CAT-infected birds. The data show that the CAT gene is not preferentially expressed in striated muscle in birds infected with RCASBPCAT3. Therefore, we conclude that the pattern of CAT expression observed in chickens infected with RCANBP vectors containing either the α_{sk} - or β -actin promoter/CAT gene was not caused by sequences in the retroviral vector. One exception may be the elevated expression of CAT in the bursa of bird 507 infected with RCANBP/ α_{sk} CAT (Table 2).

Distribution of proviral DNA in infected chickens. To determine the extent and distribution of viral infection in the infected birds, we compared the level of proviral DNA in the

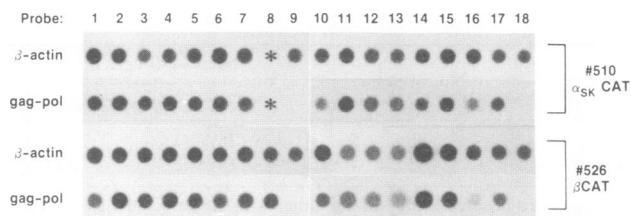


FIG. 3. Analysis of retroviral DNA in infected chicken tissues. Aliquots of DNA prepared from the tissues of two infected chickens (510 and 525) were immobilized on nitrocellulose membranes by the dot blot procedure, and membranes were incubated with [α -³²P]dCTP-labeled hybridization probes. Probes were prepared from DNA fragments representing the *gag-pol* region of the retroviral vector and the coding region of the endogenous chicken β -actin gene. The latter probe does not contain β -actin promoter sequences that are in the RCANBP/ β CAT vectors. Lanes: 1, spleen; 2, heart; 3, gizzard; 4, skeletal muscle; 5, brain; 6, lung; 7, liver; 8, kidney; 9, uninfected CEF; 10, bursa; 11, proventriculus; 12, small intestine; 13, large intestine; 14, blood; 15, skin; 16, nerve; 17, gonad; 18, uninfected CEF. An asterisk indicates that the sample was not available for analysis.

various tissues. Genomic DNA was extracted from tissue samples and transferred to nitrocellulose membranes by the dot blot procedure. Separate filters were hybridized to probes representing the *gag-pol* region of the RCANBP vector and the coding region of the endogenous β -actin gene (Fig. 3). In general, the intensity of the *gag-pol* hybridization signal was directly proportional to the hybridization signal intensity of the endogenous β -actin probe, which shows that the provirus copy number in most tissues was comparable. This shows that the retroviral vectors containing the α_{sk} /CAT cassette did not preferentially infect skeletal muscle cells or their progenitors.

Virus production in infected chickens. Virus production in each tissue was measured directly by quantitating the levels of viral capsid protein p27. Cell homogenates were fractionated by electrophoresis in polyacrylamide gels. The proteins were transferred to nitrocellulose by the Western blot method. p27 was detected immunochemically by incubating the membrane with a polyclonal rabbit anti-p27 serum (54) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG serum and subsequently developing the alkaline phosphatase reaction with chromogenic substrates. The results of an analysis performed on tissue homogenates prepared from chicken 510 infected with RCANBP/ α_{sk} CAT are shown in Fig. 4. Similar results were observed with tissue extracts prepared from RCANBP/ β CAT- and RCASBPCAT3-infected chickens (data not shown). The p27 data show that retroviral proteins were not produced at higher levels in muscle tissue than in nonmuscle tissues. These data, taken together with the proviral DNA data presented in Fig. 3, suggest that virus infection and virus production were comparable in the muscle and nonmuscle tissues of infected chickens. The p27 analyses argue strongly that the retroviral vectors did not preferentially infect skeletal muscle nor was virus production specifically enhanced in muscle tissue.

DISCUSSION

The ability to target gene expression *in vivo* by using tissue-specific promoters has proved to be an advantageous tool for developmental studies in the murine system and certainly would be desirable for the avian system as well. We have demonstrated that the α_{sk} -actin promoter retains ap-

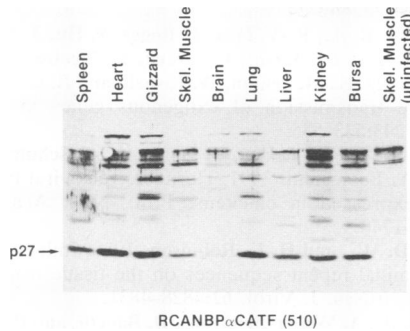


FIG. 4. Quantitation of retroviral protein in infected chicken tissues. Tissue homogenates were prepared from chicken 510 infected with the retrovirus vector RCANBP/ α_{sk} CAT and the skeletal muscle of an uninfected chicken and subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes. The retroviral capsid protein p27 was detected immunochemically with a polyclonal rabbit anti-p27 antibody (54).

appropriate muscle specificity when introduced into the chicken genome by somatic infection with a replication-competent retroviral vector. We have also shown that such somatically infected birds can be generated at reasonably high frequencies (45 to 74%) when embryos are exposed to infectious retroviral vectors on the first day of incubation. Using replication-competent retroviral vectors, we were able to deliver retrovirus vectors efficiently to a wide range of cell types. High-level expression in skeletal muscle from the α_{sk} -actin promoter was independent of the sex of the bird. Expression persisted at high levels in animals killed at 16 weeks of age. However, we did observe that the skeletal muscle specificity of this promoter was slightly reduced when the α_{sk} /CAT cassette was in the backward orientation with respect to viral gene transcription. Birds infected with vectors that carried the α_{sk} /CAT cassette in the backward orientation also had elevated CAT activities in the cardiac muscle of the heart and, to a lesser degree, in the smooth muscle of the gizzard. It is reassuring to find that the pattern of CAT activity that we observed when an α_{sk} -actin/CAT cassette was introduced into the chicken genome with replication-competent retroviral vectors closely resembled the pattern that we reported previously for α_{sk} -actin/CAT cassettes in transgenic mouse lines that were created by DNA microinjection (41). Our earlier characterizations of the β -actin promoter in cultures of CEF infected with RCANBP/ β CAT (38) suggested that the nonmuscle tissues of chickens infected with this retrovirus might have been expected to express higher levels of CAT than those we observed (Tables 2 and 3). However, our CAT data for infected chickens are comparable to, and in agreement with, the levels of CAT activity observed in tissue extracts prepared from several transgenic mouse lines that were established by conventional DNA microinjection of the identical β -actin promoter/CAT sequences (40).

Numerous retroviral vectors can be used to express DNA inserts from internal promoters (for review, see reference 30). Previous studies have directly characterized the behavior of cellular promoters embedded in retroviral vectors (3, 10–12, 18, 31, 35, 38, 47, 52, 53). For the most part, the vectors are replication defective and were designed to permit a single provirus to express the gene of interest and a gene encoding a selectable marker. There is, however, substantially less information that directly addresses the feasibility of using tissue-specific promoters within murine or avian

retroviral vectors to target gene expression to a particular cell type either in vitro or in vivo (8, 17, 19, 20, 32, 45, 51). Our success in targeting gene expression to skeletal muscle in vivo by using retroviral vectors that contain a muscle-specific promoter suggests that gene expression can be targeted to a variety of other avian cell types by constructing similar vectors containing other tissue-specific promoters. In addition, vectors containing inducible promoters, such as those responsive to hormones or metal ions, could simplify experiments involving proteins that are detrimental to the cell or organism when expressed constitutively at high levels, e.g., oncoproteins and toxins.

For technical reasons discussed elsewhere (38), we have been unable to demonstrate directly that muscle-specific transcription initiated properly from the α_{sk} -actin promoter carried within the retroviral vector; however, several observations make this a logical and reasonable interpretation. First, CAT gene expression was not preferentially targeted to skeletal muscle when directed from the viral promoter within the LTR or from an internal β -actin promoter. Second, muscle specificity of CAT expression was observed in birds infected with retroviral vectors containing the α_{sk} -actin/CAT cassette in either the forward or reverse orientation. Third, the analysis of provirus copy number and viral capsid protein levels indicated that the quantitative and qualitative patterns of CAT expression were not a consequence of preferential infectivity or virus production. Nor could the results be attributed to selective proviral stability. Lastly, we have previously demonstrated that RCAN retrovirus vectors containing CAT sequences that are not linked to internal promoters fail to produce appreciable levels of CAT activity in infected fibroblast cultures (38).

We have been unable to demonstrate tissue-specific expression from chicken actin promoters in preliminary experiments in vitro (data not shown). We detected relatively high levels of CAT activity in both chicken fibroblast and myotube cultures that were infected with retroviral vectors containing the CAT gene linked to either the α_{sk} - or β -actin promoter (39). We can speculate that this lack of promoter specificity may reflect the inability of cells in culture to differentiate completely. The inability to fully differentiate in culture may prevent proper transcriptional regulation of actin promoters in vitro. In support of this view, previous studies have shown that actin gene expression in vitro does not accurately reflect actin gene expression in vivo (2, 21, 22, 33, 46). Alternatively, exogenous actin promoter elements in cultured cells may be refractory to the normal in vivo regulatory mechanisms that act during development and serve to suppress or activate the endogenous promoters. Before a decision can be made about the general usefulness of the RCAN and RCAN vectors in vitro, other tissue-specific or inducible promoters need to be tested. In this regard, we have previously demonstrated a three- to fivefold induction of CAT expression following zinc exposure in CEF cultures that were infected with the RCAN vector containing a mouse metallothionein 1 promoter/CAT cassette (38).

The creation of transgenic mice by microinjection of DNA into the pronuclei of fertilized mouse eggs has greatly improved our understanding of gene regulation and of the molecular mechanisms that control developmental processes (for reviews, see references 9, 26, 36, and 58). Differences in the reproductive physiology of mammals and birds make certain aspects of vertebrate development more amenable to experimentation in the avian embryo (28). Unfortunately, the newly fertilized avian egg is not easily accessible, and

attempts to create transgenic birds by microinjection have not been successful (44). As an alternative, we (42, 43) and others (5, 6) have developed retroviral vectors that can be used to introduce foreign DNA into the germ line of chickens. Based on the experiments presented in this article, we believe it will be possible in the near future to target transgene expression to specific tissues of transgenic chickens generated with this vector system. Experiments to test this conjecture are now in progress. However, we would like to point out that the replication-competent vector system should make it possible to address many developmental questions simply by establishing somatic infections in the early avian embryo. In contrast to replication-defective vectors, the replication-competent vectors spread efficiently to essentially all of the cells and tissues of the developing embryo. Experiments can be designed that preselect the level of DNA insert expression and target expression to a particular cell type. This approach eliminates both the cost and time spent on the generation and maintenance of transgenic animals. Unlike transgenes, which are present at a few sites in the genome, somatic infections distribute proviruses to many sites in the genome, and thus positional effects influencing expression at particular integration sites are averaged away, even with a small number of animals.

Obviously, practical applications of retroviral vector-mediated gene transfer in agriculture and medicine will require replication-defective vectors. We are in the process of developing defective vectors and helper function cell lines that are designed to complement our existing replication-competent system. At present, we are trying to overcome some of the typical obstacles associated with defective systems.

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ADDENDUM IN PROOF

It has been recently reported (Z. Liu, B. Moav, A. J. Faras, K. S. Guise, A. R. Kapuscinski, and P. Hackett, *Gene* 108:211-217, 1991) that sequences within intron 1 of the carp β -actin gene improve β -actin promoter function in transgenic embryos; however, these sequences are not required in cells grown in tissue culture.

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