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Replication-Competent Retrovirus Vectors for the Transfer and Expression of Gene Cassettes in Avian Cells

CHRISTOS J. PETROPOULOS AND STEPHEN H. HUGHES*

ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, Maryland 21702-1201

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We have constructed a series of replication-competent retrovirus vectors to introduce and express gene cassettes in avian cells. To characterize these vectors, we inserted the coding sequences for the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the chicken β-actin gene promoter or the mouse metallothionein 1 gene promoter. In all cases, we found the structure of integrated proviruses to be stable during serial cell passage in vitro. Chloramphenicol acetyltransferase activity was detected biochemically and immunocytochemically in infected cells. Cassettes were inserted in the vectors in the same or in the opposite orientation with respect to viral transcription. Although both orientations were functional, the cassettes inserted in the forward orientation were usually expressed at higher levels than the corresponding backward constructions. The level of expression was strongly influenced by surrounding proviral sequences, particularly by the transcriptional enhancer elements within the retrovirus long terminal repeat sequences. Expression was higher with vectors that contained the polymerase (*pol*) region of the Bryan high-titer strain of Rous sarcoma virus. Inclusion of the Bryan *pol* region also improved vector replication in the chemically transformed quail fibroblast line QT6.

We have previously described the construction and nomenclature of a series of replication-competent avian retrovirus vectors (RCAS, replication-competent, avian leukemia virus [ALV] long terminal repeat [LTR], splice acceptor retrovirus vector; RCOS, replication-competent, endogenous Rous-associated virus type O [RAV-O] LTR, splice acceptor retrovirus vector; RCAN, replication-competent, ALV LTR, no splice acceptor retrovirus vector; RCON, replication-competent, RAV-O, no splice acceptor retrovirus vector) that derive from the Rous sarcoma virus (RSV) and the endogenous RAV-O (9, 11, 12). These vectors were constructed by replacing the v-*src* gene of the Schmidt-Ruppin A (SR-A) strain of RSV with a unique *Cla*I site that can be used for the insertion of DNA. Several adaptor plasmids that can be used to convert virtually any DNA segment to a *Cla*I fragment have been constructed (12, 13). These vectors can be used, both in vitro and in vivo, to introduce DNA sequences into avian cells (9, 12, 23–25, 33). The principle difference between the vector pairs RCAS/RCAN and RCOS/RCON is the origins of their LTR sequences. RCAS and RCAN contain RSV LTRs and replicate efficiently, whereas RCOS and RCON contain the LTR sequences of the endogenous retrovirus RAV-O and grow to lower titers.

The RCOS and RCAS vectors were designed to express inserted sequences as spliced, subgenomic transcripts initiating from the promoter within the proviral LTR. We have previously demonstrated that DNA sequences inserted in RCOS and RCAS are stably transferred and expressed in infected chicken embryo fibroblast (CEF) cultures (9, 12). For many gene transfer applications it is important to separate the transcription of the inserted gene from the transcription of the viral genes. The RCON and RCAN vectors differ from the RCOS and RCAS vectors in that they lack the splice acceptor sequences that are present immediately upstream of the *Cla*I cloning site of RCOS and RCAS. Consequently, sequences inserted into RCON and RCAN are not expressed from the promoter within the viral LTR unless functional splice acceptor sequences are supplied within the inserted DNA segment. The RCON and RCAN vectors were designed to accept and transfer gene cassettes that contain an internal promoter. RCON and RCAN were developed for two primary applications: to facilitate the study of tissue-specific promoters and to direct the expression of a DNA insert to a specific cell or tissue type. Since the RCON and RCAN vectors are replication competent, these types of studies can be performed either in vitro or in vivo in the absence of helper virus or a selectable marker.

In this study we have characterized the expression of several promoter-CAT cassettes that have been stably introduced into cultured avian cells by using the RCON and RCAN vectors. We have constructed and tested RCON and RCAN derivatives that contain a resident chloramphenicol acetyltransferase (CAT) gene immediately adjacent to the *Cla*I insertion site. Promoter sequences can be inserted directly into this series of CAT vectors, eliminating the cloning step in which the promoter is linked to the CAT gene prior to insertion into either the RCON or the RCAN vectors. We have also constructed and characterized a set of RCON and RCAN derivatives that contain *pol* sequences derived from the Bryan high-titer (BH) strain of RSV. The Bryan *pol* region elevates expression of promoter-CAT cassettes introduced into the vectors. Vectors containing Bryan *pol* also replicate more efficiently in QT6 cells than the corresponding parental vectors.

MATERIALS AND METHODS

Construction of recombinant plasmids. The construction and detailed maps of RCON, RCAN, and the adaptor plasmid Cla12 have been described previously (9, 11, 12). RCON/PCAT and RCAN/PCAT (PCAT stands for promoter-CAT) were constructed by embedding the CAT gene into

* Corresponding author.

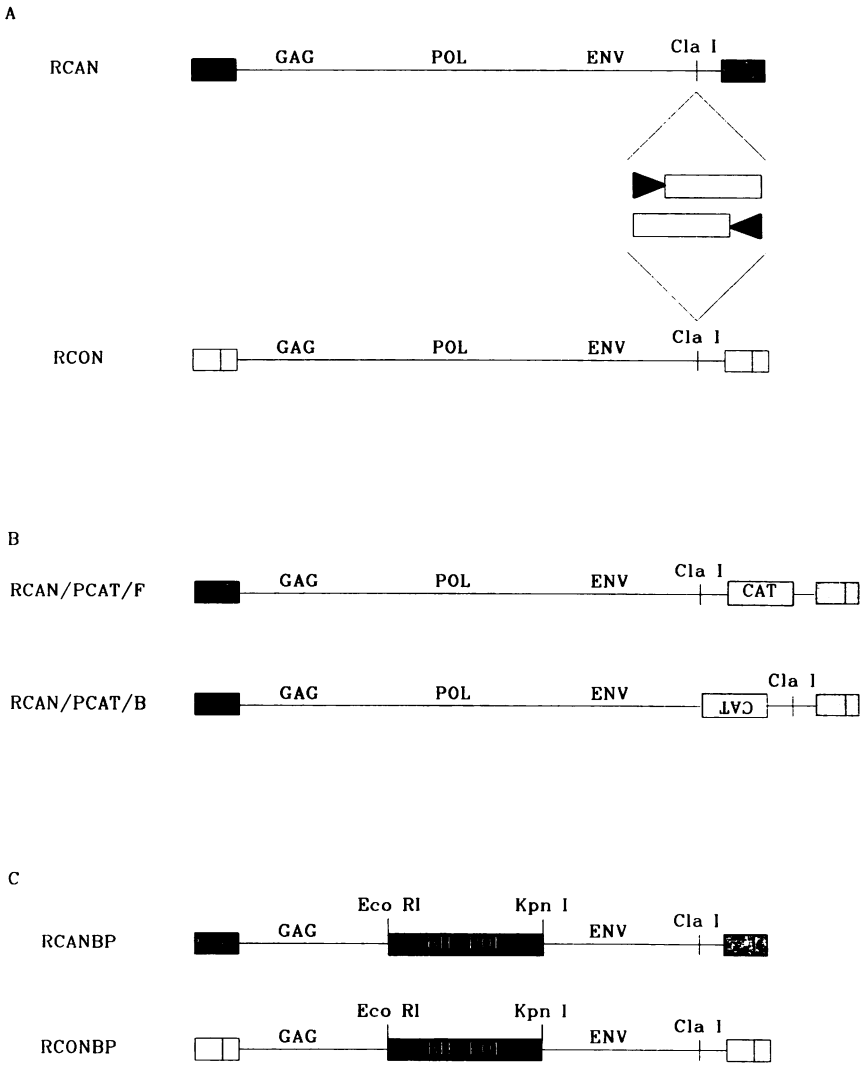

FIG. 1. Retrovirus vectors. (A) Proviral structures of RCON and RCAN are presented schematically. Complete plasmid maps of the retrovirus vectors RCON and RCAN are published (9, 11, 12). The LTR sequences of RCAN derive from the SR-A strain of RSV (shaded boxes). The LTR sequences of RCON derive from the endogenous retrovirus RAV-O (open boxes). Gene cassettes consisting of a promoter (solid triangle) linked to any sequence of interest (open rectangle) can be inserted in either orientation at a unique *Cla*I site positioned downstream of the structural genes *gag*, *pol*, and *env*. (B) The bacterial CAT gene has been inserted adjacent to the *Cla*I sites in RCON and RCAN to generate the retrovirus vectors RCON/PCAT and RCAN/PCAT. Promoters inserted in the *Cla*I site of RCAN/PCAT/F drive CAT transcription in the same orientation as the viral genes, whereas in RCAN/PCAT/B promoters drive CAT transcription in the reverse orientation with respect to viral gene transcription. (C) RCON and RCAN vectors containing the *pol* region (solid rectangle) of the BH-RSV are illustrated. The *pol* regions of RCON/RCAN and RCONBP/RCANBP are flanked by conserved *Eco*RI and *Kpn*I recognition sequences.

the RCON and RCAN parent vectors. A *Sau*3AI fragment containing the CAT gene was cloned into the *Bam*HI site of the adaptor plasmid ClaBB. ClaBB resembles Cla12 but contains the recognition sequences for the restriction endonucleases *Bam*HI, *Xho*I, *Mlu*I, *Apa*I, and *Bgl*II flanked by *Cla*I sites (13). The CAT insert was purified as a *Cla*I to *Xho*I fragment (approximately 800 bp) by digestion with the restriction endonuclease *Taq*I. The *Taq*I fragment containing CAT was cloned into the *Cla*I sites of RCON and RCAN, and plasmids containing the CAT insert in both the forward and the backward orientations were purified. In both orientations, the *Cla*I cloning site was restored (*Cla*I to *Cla*I ligation) upstream (5′) of the CAT open reading frame but was destroyed (*Xho*I to *Cla*I ligation) downstream (3′) of the CAT open reading frame (Fig. 1). Retrovirus vectors containing CAT sequences in the same transcriptional orientation as the viral genes were designated PCAT/F (F stands for forward), whereas those containing CAT sequences in the opposite orientation were designated PCAT/B (B stands for backward). The PCAT vectors are capable of accepting promoter regions of up to about 1.2 kb in size.

The chicken β-actin promoter region (−277 to +63) was inserted into the adaptor plasmid Cla12 at the *Hin*dIII site to generate the plasmid Cla12/340βACT. A *Kpn*I to *Bgl*II fragment containing the metallothionein 1 (MT1) gene promoter (−600 to +38) was subcloned in pAJ10 (*Kpn*I to *Bam*HI). pAJ10 is a derivative of the pBluescript KS+ plasmid that contains the multiple cloning sites flanked by *Cla*I sites (13a). The chicken β-actin promoter and the MT1 promoter were purified as *Cla*I fragments and were cloned

into the *Cla*I sites of RCON/PCAT/F, RCON/PCAT/B, RCAN/PCAT/F, and RCAN/PCAT/B. Promoter-CAT junctions were confirmed by nucleic acid sequencing with an antisense CAT oligonucleotide primer.

An RCAN/v-*src* vector (19) that contained the *pol* region (*Eco*RI to *Kpn*I) of the BH strain of RSV was obtained (31). RCONBP and RCANBP (BP for Bryan *pol*) vectors were constructed by moving a 4.7-kb *Sac*I to *Kpn*I fragment that contains the *gag-pol* region of RCAN/v-*src* into RCON and RCAN, respectively. To construct a β-actin promoter-CAT cassette within a *Cla*I fragment, the β-actin promoter from Cla12/340βCAT (*Cla*I to *Sac*I) and the bacterial CAT gene from Cla12CAT1 (*Sac*I to *Cla*I [described in reference 20]) were inserted into the *Cla*I site of pBR322. The promoter-CAT junction was confirmed by nucleotide sequence analysis. The β-actin-CAT cassette was purified as a *Cla*I fragment and was inserted into the *Cla*I sites of RCONBP and RCANBP. Recombinant vector plasmids with the actin-CAT cassettes in both orientations were isolated.

Cell culture and DNA transfection. CEF cultures were established from 11-day embryos of line EV-0. QT6, a chemically transformed quail fibroblast line (17), was obtained from P. Shank (Brown University, Providence, R.I.). CEF and QT6 cultures were grown in 100-mm dishes in Dulbecco's minimal essential medium (GIBCO, Inc.) supplemented with 5% fetal bovine serum (HyClone, Inc.), 5% newborn bovine serum (Advanced Biotechnologies, Inc.), 10% tryptose phosphate broth (GIBCO), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were passaged 1:3 at confluence with trypsin DeLarco (pH 6.8). QT6 and early-passage CEF cultures were transfected with 10 μg of plasmid DNA by using the calcium-phosphate precipitation method (14).

To measure induction of the MT1 promoter by heavy metal ions, cells were plated at approximately 50% confluence in Dulbecco's minimal essential medium containing 2% fetal bovine serum. After 3 h cells were shifted to Dulbecco's minimal essential medium containing 0.5% fetal bovine serum. $ZnSO_4$ was added 3 h later to a final concentration of 100 μM. No $ZnSO_4$ was given to control cells. Cells were harvested 12, 18, 24, and 36 h after the addition of $ZnSO_4$.

RT assays. Media from 100-mm dishes were cleared of cell debris by centrifugation at 3,000 × *g* for 10 min at 4°C. The supernatants were spun at 35,000 rpm in a Beckman SW40 rotor for 60 min at 4°C to pellet the virus. Viral pellets were assayed for reverse transcriptase (RT) by measuring the incorporation of [α-^{32}P]dGTP into acid-precipitable material with an oligo(dG) primer and a poly(rC) template. Each pellet was resuspended in a 100-μl RT reaction mixture [50 mM Tris-Cl, 20 mM dithiothreitol, 12 mM $MgCl_2$, 60 mM NaCl, 0.1% Nonidet P-40, 5 μg of oligo(dG) per ml, 10 μg of poly(rC) per ml (pH 8.3)]. Reaction mixtures were incubated for 1 h at 37°C in the presence of 1 μl of 1 mM dGTP–0.1 μl of [α-^{32}P]dGTP (Amersham; 800 Ci/mmol). RT reactions were terminated by the addition of 1 ml of cold 10% trichloroacetic acid. Precipitates were collected on Whatman GF/C filters, and the incorporation of [α-^{32}P]dGTP was measured by counting the filters in a scintillation counter.

Southern transfer analysis. Confluent cells were rinsed three times with calcium-free, magnesium-free phosphate-buffered saline (PBS). Cells from each 100-mm dish were lysed in 2 ml of guanidine thiocyanate solution. Cell lysates from 10 dishes were pooled and extracted twice with phenol-chloroform (1:1). Aqueous phases were adjusted to 0.2 M sodium acetate, and total nucleic acid was precipitated at room temperature with an equal volume of isopropanol. Precipitates were collected by centrifugation and dissolved in 5.0 ml of water. Preparations were adjusted to 1× STE (100 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA [pH 7.8]), treated with 2,500 U of RNase T_1 at 37°C for 30 min, and extracted with phenol-chloroform, and the DNA was recovered by isopropanol precipitation.

Restriction endonuclease digestions were performed according to the manufacturers' specifications (*Nco*I, Boehringer Mannheim; *Eco*RI, New England BioLabs) by using 20 μg of DNA. Following agarose gel electrophoresis, the gels were soaked in 0.5 N NaOH–1.5 M NaCl for 45 min and then in 1.0 M NaCl–1.0 M Tris base–0.7 N HCl for 60 min. DNA was transferred overnight to nitrocellulose membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were baked in a vacuum oven for 1 h at 80°C and were hybridized to nick-translated DNA probes in 50% formamide at 42°C. Filters were washed in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 55°C for 2 h.

CAT assays. Cells were rinsed three times with PBS and were scraped from 100-mm culture dishes in 1.0 ml of TNE buffer (0.04 M Tris-Cl, 0.15 M NaCl, 0.001 M EDTA [pH 7.4]). Cells were collected by sedimentation for 2 min at 4°C in a microcentrifuge, resuspended in 0.2 ml of 0.25 M Tris-Cl (pH 7.8), and lysed by three successive cycles of freezing and thawing. Lysates were cleared of cell debris by sedimentation in a microcentrifuge for 5 min at 4°C. The relative protein concentrations of cell lysates were measured spectrophotometrically by using a modified Bradford protocol (Bio-Rad) and were adjusted by adding 0.25 M Tris-Cl (pH 7.8).

Cell lysates (180 μl) were incubated for 1 to 2 h at 37°C in a solution containing 70 μl of 2 M Tris-Cl (pH 7.8), 40 μl of 4 mM acetyl coenzyme A (Pharmacia), and 5 μl of [^{14}C] chloramphenicol (New England Nuclear; 60 Ci/mmol, 0.1 mCi/ml). Reaction mixtures were extracted with 0.5 ml of ethyl acetate. The organic phases were evaporated to dryness, dissolved in 30 μl of ethyl acetate, spotted onto thin-layer chromatography plates (silica gel IB2; J. T. Baker), and chromatographed for 1 h (190 ml of chloroform and 10 ml of methanol). CAT chromatographs were quantitated by using a radioanalytic imaging system (Ambis, San Diego, Calif.).

Western transfer analysis. Virus was collected from 10 ml of cell culture supernatant by centrifugation as described above. Virus pellets were solubilized in 50 μl of sample buffer (50 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 5% sucrose, 0.005% bromophenol blue) and were subjected to electrophoresis in 12% polyacrylamide gels (27). Separated proteins were transferred to a nitrocellulose membrane with an Enprotech blotting apparatus (Integrated Separation Systems, Hyde Park, Mass.). To prevent nonspecific binding of antibodies, the filters were incubated in PBS containing nonfat dry milk (10%, wt/vol) for a minimum of 4 h at room temperature. The viral capsid protein p27 was detected immunochemically with rabbit anti-p27 sera (28) followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Kirkegaard and Perry, Gaithersburg, Md.). Incubation with anti-p27 sera was performed overnight at 4°C by using a 1:1,000 dilution in rinse buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1% Tween 20) containing 1.0% nonfat dry milk. Filters were subsequently washed three times (each time for 5 min) in 100 ml of rinse buffer. Incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG was performed for 2 h at room temperature by using a 1:666 dilution. Filters were again washed three times in rinse buffer, and alkaline phosphatase activity was de-

tected by using the chromogenic substrates 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) as specified by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.).

Immunofluorescence. Cells were fixed and prepared for immunofluorescent staining as described previously (33), except that cells were grown on glass coverslips. Cells were incubated simultaneously with a 1:500 dilution of polyclonal rabbit anti-CAT antisera and a 1:200 dilution of monoclonal mouse anti-p19 antisera at 37°C for 1 h. After incubation, the cells were rinsed five times with PBS and were again incubated simultaneously, but with a 1:200 dilution of rhodamine-labeled goat anti-rabbit IgG and fluorescein-labeled sheep anti-mouse IgG for 1 h at 37°C. Cells were rinsed five times with PBS and mounted in a 50% glycerol-PBS solution.

RESULTS

PCAT vectors. Detailed descriptions of the construction of the plasmids that contain the replication-competent avian retrovirus vectors, RCON and RCAN, have been published (9, 11, 12). To facilitate the characterization of foreign promoters by using RCON and RCAN, we have modified these vectors by embedding the CAT coding sequences within each vector adjacent to the *Cla*I cloning site (see Materials and Methods) (Fig. 1). This modification eliminates a subcloning step that is required to link a foreign promoter to CAT sequences before the resultant promoter-CAT cassette can be inserted into either RCON or RCAN. The new vectors are referred to as RCON/PCAT or RCAN/PCAT. RCON/PCAT and RCAN/PCAT vectors that carry the CAT gene in either the forward (PCAT/F) or backward (PCAT/B) orientation, with respect to transcription of viral genes, were constructed (Fig. 1).

Function of internal promoters in infected cells. To functionally characterize promoters that have been introduced into avian cells with this type of retrovirus vector, we chose the chicken β-actin promoter and the mouse MT1 promoter. Actin gene expression is tissue specific and developmentally regulated. The β-actin promoter is transcriptionally active in a variety of non-muscle cell types that include fibroblasts (32). Transcription from the MT1 promoter can be induced by exposure to heavy metal ions such as Zn and Cd (10). We cloned the β-actin promoter and the MT1 promoter into each member of the PCAT vector series. Early-passage cultures of CEFs were transfected with 10 μg of plasmid DNA by the calcium-phosphate precipitation method, and cells were passaged each time they reached confluence. Retrovirus infection was monitored just prior to each cell passage by assaying the culture media for the presence of RT activity. At each passage, cells were harvested and cell lysates were assayed for CAT activity. The results of a representative experiment with RCAN/PCAT vectors carrying the β-actin and MT1 promoters inserted in the forward and backward orientations are shown in Fig. 2. RT and CAT activities were easily detected after the first cell passage (4 to 5 days posttransfection). RT levels gradually increased during passages 1 to 10, but consistent declines were observed by passage 15. CAT activities in infected cells approached their highest levels by the second passage and generally maintained these levels through passage 15. We have consistently observed that cells infected with vectors containing promoter-CAT cassettes in the forward orientation produce higher levels of CAT activity than cells infected with the corresponding backward construction (Fig. 2).

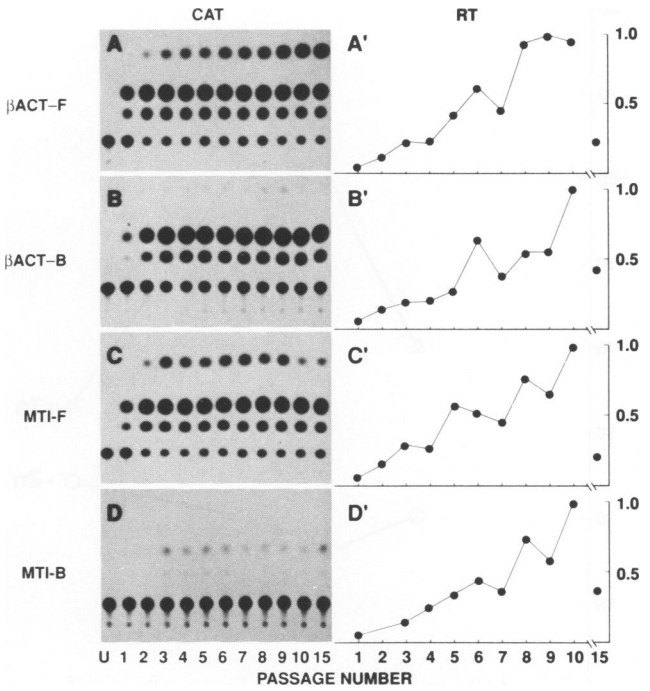

FIG. 2. CAT and RT levels in infected cells. Early-passage CEF cultures were transfected with RCAN/PCAT plasmid DNA containing either the chicken β-actin or the MT1 gene promoter. Cells were split 1:3 each time they reached confluence. CAT and RT activities were assayed at passages 1 to 10 and again at passage 15. CAT activities are shown in the panels on the left, and corresponding RT activities are plotted in the panels on the right. RT activities have been normalized for each culture. A value of 1.0 represents approximately 2.5×10^4 to 5.0×10^4 cpm above background. (A and A') RCAN/βPCAT/F; (B and B') RCAN/βPCAT/B; (C and C') RCAN/MT1PCAT/F; (D and D') RCAN/MT1PCAT/B.

No differences in CAT activity were observed when these experiments were performed either by inserting the β-actin promoter directly into the PCAT vectors or by first linking the β-actin promoter to the CAT gene and inserting the β-actin promoter-CAT cassette into the parental RCON and RCAN vectors (data not shown).

Zinc induction of the MT1 promoter in infected cells. To test whether expression of the MT1 promoter-CAT cassette could be induced by heavy metal ions, cells infected with RCAN/MT1PCAT/F were exposed to 100 μM $ZnSO_4$. CAT activity was measured in cell lysates prepared after 12, 18, 24, and 36 h of $ZnSO_4$ exposure and was compared with the CAT activity of lysates prepared from infected cells that were not treated with $ZnSO_4$ (Fig. 3). The data show a significant transient increase in the level of CAT activity in cells exposed to $ZnSO_4$. The highest level of induction (threefold) was observed 18 to 24 h after the addition of $ZnSO_4$. By 36 h the level of CAT activity in treated cells had returned to nearly that in control cells. Similar experiments using 25 and 50 μM $ZnSO_4$ demonstrate that transient induction of the MT1 promoter in infected cells is also dose dependent (data not shown).

Intact proviruses contained by infected-cell DNAs. Retrovirus genomes are inherently unstable. Unless they are under positive selection, sequences inserted into vectors derived from retroviruses can be lost after several rounds of replication (see reference 1 for a discussion). We have previously

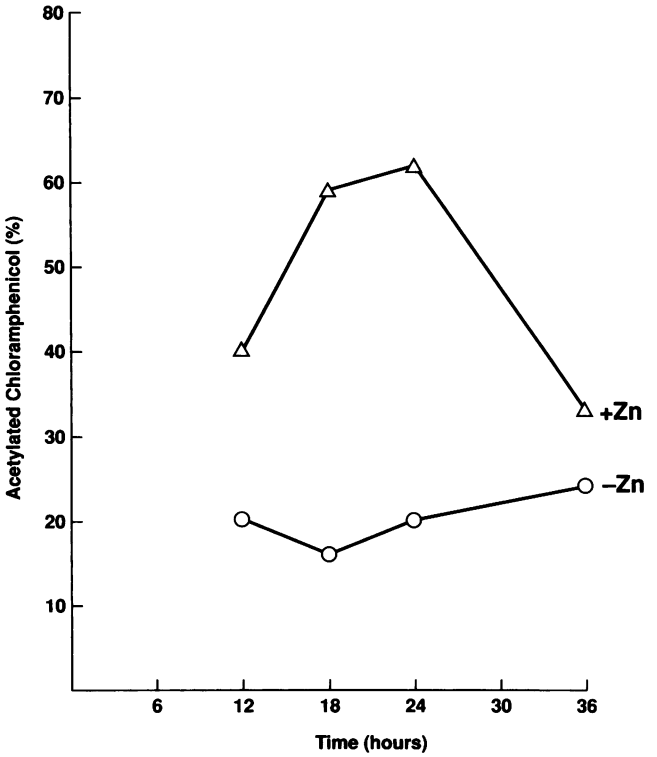

FIG. 3. Zinc induction of the MT1 promoter-CAT gene. CAT activity was measured in lysates prepared from CEF cultures infected with the RCAN/PCAT vector containing an MT1 promoter-CAT cassette. Cells were exposed to 100 μM $ZnSO_4$ (triangles) and compared with untreated controls (circles).

described several modifications that have significantly improved the stability of our vector system in the absence of selection (11, 12).

We tested the stability of RCON/PCAT and RCAN/PCAT vectors that contained either the chicken β-actin promoter or the mouse MT1 promoter after serial cell passage in CEFs. To determine whether any of the viruses had undergone gross rearrangement, DNA was isolated after 10 passages and proviral sequences were mapped by using the restriction enzyme *Nco*I. Digested DNA was separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled probe. The probe was prepared from CAT and RSV DNA fragments that were mixed after they were labeled in separate nick translation reactions. The data for RCAN/PCAT-infected cultures are shown in Fig. 4. Although a small amount of large extraneous fragments, which may represent partial digestion of viral DNA segments, was observed, the majority of proviral structures appeared complete and unrearranged. Evidence of extensive proviral instability was not seen. To estimate the average number of proviruses per haploid genome, we compared the signal intensities of proviral bands with that of the 2.3-kb genomic c-*src* band. The latter is detected by the v-*src* portion of the RSV DNA hybridization probe. On the basis of this comparison, we estimate the proviral copy number at one per haploid genome.

Cassette expression in infected cells influenced by LTR sequences. As noted above, the RCAN and RCON vectors differ in the origins of their LTRs. RCAN LTRs derive from the SR-A strain of RSV, a pathogenic avian sarcoma virus

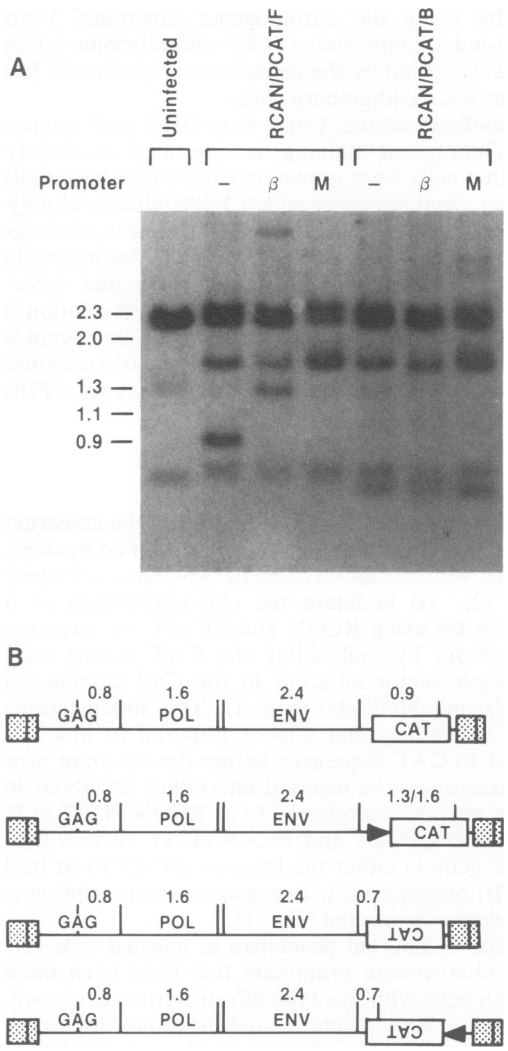

FIG. 4. Southern transfer analysis of PCAT proviral DNA. (A) Genomic DNA was purified from cells infected with RCAN/PCAT viruses. DNA was digested with *Nco*I, separated by agarose gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was incubated with a ^{32}P-labeled probe consisting of CAT sequences and a *Sal*I DNA fragment containing the entire sequence of the SR-A strain of RSV. In addition to LTR, *gag*, *pol*, and *env* sequences, this *Sal*I fragment also contains v-*src* sequences that primarily hybridize to a 2.3-kb *Nco*I fragment of genomic DNA containing c-*src* sequences. –, no promoter; β, β-actin promoter; M, MT1 promoter. (B) Schematic illustration of RCAN/PCAT/F and RCAN/PCAT/B proviral structures showing the positions of *Nco*I recognition sequences and the sizes (in kilobases) of the predicted digestion products.

(35). The RCON vector (9) was created by replacing the LTRs of RCAN with the corresponding LTR sequences of the nonpathogenic, endogenous RAV-O (2, 18). The pathogenicity of avian retroviruses has been correlated with the transcriptional enhancer activity of their LTR sequences (3–5, 16, 21, 36). In agreement with these reports, we have previously demonstrated that avian retrovirus vectors containing RAV-O LTRs are substantially less pathogenic than the corresponding vectors that contain SR-A RSV LTRs (23, 24). Furthermore, we have reported that cells infected with a retrovirus vector that expresses CAT from an RSV LTR

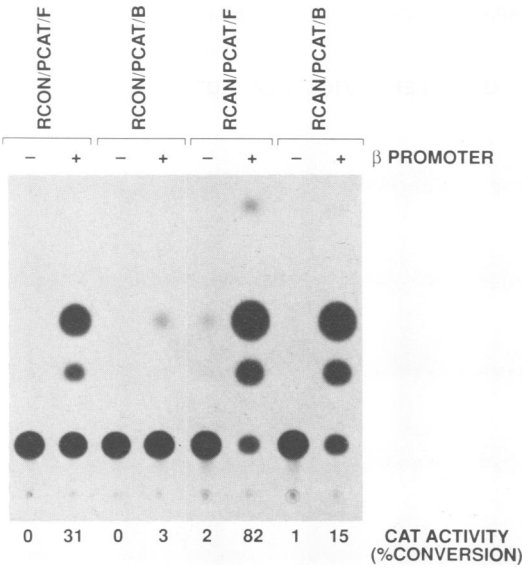

FIG. 5. LTR sequence influence on expression of gene cassettes. CAT activity was assayed in CEF cultures that were transfected with retrovirus vectors containing the LTR sequences of RAV-O (RCON/PCAT) or the LTR sequences of RSV (RCAN/PCAT). Cells infected with retrovirus vectors that contained the chicken β-actin promoter linked to the CAT gene (+) were compared with those lacking the β-actin promoter (−). Vectors containing the CAT cassette in either the forward orientation (F) or backward orientation (B) were tested. Transfected cells were split 1:3 each time they reached confluence, and CAT assays were performed on passage 9 cells. CAT activities were quantitated by radioanalytic imaging.

promoter (RCAS/CAT) contain 30- to 50-fold higher levels of CAT than do cells infected with a corresponding vector (RCOS/CAT) that expresses CAT from the RAV-O LTR promoter (9, 12).

To determine whether cellular promoters within proviruses are responsive to enhancers within the LTRs, we compared the levels of CAT activity in cell lysates prepared from the CEF cultures infected with the RCON/PCAT and RCAN/PCAT vectors carrying the β-actin promoter or the MT1 promoter. As shown in Fig. 5, we were able to detect higher levels of CAT activity in cells infected with the RCAN/βPCAT constructions than in those infected with the corresponding RCON/βPCAT counterparts. Similar increases were observed for PCAT vectors containing the MT1 promoter (data not shown). In agreement with the data presented in Fig. 2, higher levels of CAT activity were detected in the cells infected with viruses containing promoter-CAT sequences in the forward orientation than in cells infected with viruses carrying the same sequences in the backward orientation.

Two observations argue strongly that the CAT activity observed in these experiments was not produced by the illicit translation of CAT sequences in either the genomic or subgenomic viral mRNAs initiated by the LTR promoter. First, little, if any, CAT activity was detected in lysates prepared from cells infected with any of the parent PCAT vectors that lacked the promoter inserts. Second, significant levels of CAT activity were detected in the lysates prepared from cells infected with viruses containing the promoter-CAT sequences in the backward orientation. Since the RCON/PCAT and RCAN/PCAT vectors differ solely in their LTR sequences and each infected-cell culture contained roughly an equivalent number of proviruses (data not shown, but see Fig. 8A), we believe that the difference in CAT activity observed between cells infected with RCAN/PCAT vectors and cells infected with RCON/PCAT vectors reflects an activation of the β-actin and MT1 promoters by the enhancer elements within the RSV LTRs of RCAN/PCAT.

CAT protein uniformly expressed by infected cells. We have used immunofluorescence microscopy to examine whether the CAT activity detected in cell lysates was uniformly produced by all of the cells or whether subpopulations of cells that produced CAT protein at significantly different levels existed in the cultures. Cells infected with the RCAN/PCAT viruses containing the chicken β-actin or the mouse MT1 promoter were fixed, and immunofluorescent staining with polyclonal rabbit anti-CAT antisera was done. Uniform immunofluorescent staining of the CAT protein was observed in both RCAN/βPCAT- and RCAN/MT1PCAT-infected cultures. By contrast, no CAT protein was detected in uninfected cells (data not shown). Immunofluorescent staining with a monoclonal mouse anti-p19^{gag} antiserum indicated that the vast majority of cells were infected by the retrovirus vectors and were producing viral proteins (data not shown). Again, no immunofluorescent staining was observed in uninfected control cells. The homogeneous distribution of both CAT and p19 immunofluorescence in infected cultures suggests that the CAT activity detected biochemically in cell lysates represents the ubiquitous and reasonably uniform expression of CAT protein in infected cells. Our results indicate that a majority, if not all, of the infected cells simultaneously produce both CAT protein and viral proteins irrespective of the orientation of the actin-CAT cassette.

Expression of internal cassettes increased by BH-RSV *pol* sequences. The BH strain of RSV (BH-RSV) replicates more efficiently than other strains of RSV (31). Several observations suggest that this is the consequence of increased viral transcription. Although the precise mechanism for this increase is not clearly understood, sequences mapping within the BH-RSV *pol* region appear to be important (31). To measure what effect, if any, the BH-RSV *pol* region has on the expression of gene cassettes inserted within the RCON and RCAN vectors, the SR-A RSV-derived *pol* region of RCON and RCAN was replaced with the *pol* region of BH-RSV (see Materials and Methods). The Bryan *pol* derivatives of RCON and RCAN are referred to as RCONBP and RCANBP (BP stands for Bryan *pol*), respectively (Fig. 1).

To compare RCON and RCAN with their Bryan *pol* derivatives, a β-actin promoter-CAT cassette was inserted into each of the vectors, and the resulting vectors (forward orientation only) were transfected into CEF cultures and the chemically transformed quail fibroblast line, QT6 (17). Following cell passages 3, 6, and 9, cells were harvested to assay for CAT activity and, at the same time, culture media were collected to test for the presence of RT. CAT activities from passage 3 and passage 6 cells did not differ significantly from those detected in passage 9 cells. The latter are presented in Fig. 6 together with RT data. In both CEF and QT6 cultures, the Bryan *pol* region generally increased CAT and RT levels by about fivefold when it was inserted into the RCON and RCAN vectors. Although Fig. 6 does not illustrate a fivefold increase in CAT activity when the RCAN/βCAT vector is compared with the RCANBP/βCAT vector in CEF cultures, it was apparent when the CAT assays were repeated with serial dilutions of the cell extracts (data not shown).

Vector replication in QT6 cells improved by BH *pol* sequences. We have noted in preliminary experiments that

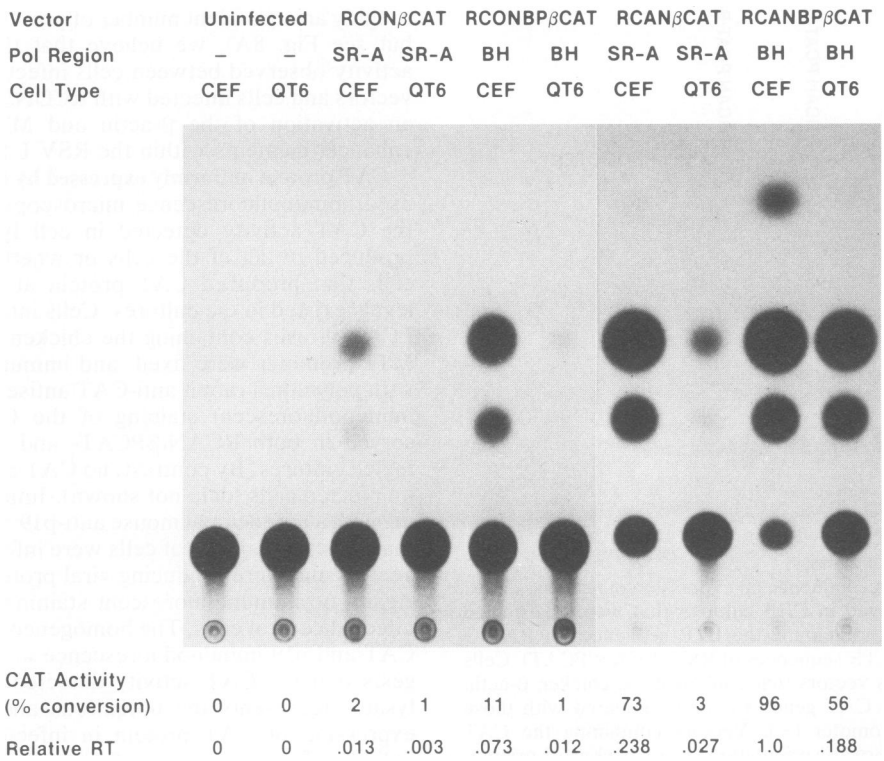

FIG. 6. Evidence that BH-RSV *pol* sequences improve expression in CEF and QT6 cells. CAT and RT levels were measured in CEF and QT6 cultures transfected with retrovirus vectors that express a β-actin promoter-CAT cassette. Vectors containing the *pol* regions of the SR-A strain of RSV or the BH strain of RSV were compared.

inserts carried by the RCON and RCAN vectors are expressed at lower levels in QT6 cells than in CEFs. A direct comparison of CAT and RT levels in infected CEF and QT6 cultures is presented in Fig. 6. CAT and RT activity in RCON/βCAT- and RCAN/βCAT-infected QT6 cells were consistently 5- to 10-fold lower than in parallel CEF cultures. Similar results were also obtained with the corresponding RCONBP/βCAT and RCANBP/βCAT viruses that contain *pol* sequences from BH-RSV. Although all of the vectors tested did not perform as well in QT6 cells as they did in CEFs, Fig. 6 clearly demonstrates that QT6 cells infected with the retrovirus vectors containing the Bryan *pol* region produced more RT and CAT activity than QT6 cells infected with retrovirus vectors containing the SR-A RSV *pol* region.

In addition to CAT and RT assays, we have also analyzed virus production by immunoblot analysis of the capsid protein p27 in cell supernatants (Fig. 7). In agreement with the CAT and RT data, infection of CEF and QT6 cells with RCANBP produced more p27 than the corresponding RCAN infections. This difference was particularly marked in QT6 cells. With both RCAN and RCANBP, we found higher levels of p27 in CEF cultures than in the corresponding QT6 cultures.

We have compared the proviral DNA in infected CEF and QT6 cultures by using *Eco*RI restriction endonuclease digestion and filter hybridization analysis (Fig. 8). Extensive proviral rearrangements or deletions were not evident. The data indicate that in CEF cultures, the Bryan *pol* vectors did not accumulate at higher proviral copy numbers than the original RCON and RCAN vectors (Fig. 8A). This implies that the Bryan *pol* region does not increase the level of expression in infected CEF cultures simply by increasing the number of proviruses in infected cells. Rather, this observation is consistent with a transcriptional activation model but does not rule out other mechanisms.

In contrast to the results obtained in CEF cultures, the data in Fig. 8B show that the amount of proviral DNA in

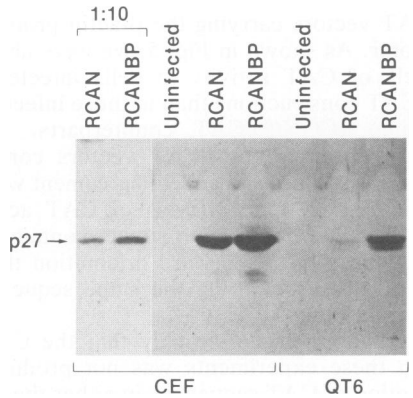

FIG. 7. Levels of p27 viral capsid protein in cell culture supernatants. Virus pellets prepared from cell culture supernatants by centrifugation were solubilized and subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred to a nitrocellulose membrane and the p27 capsid protein was detected immunochemically with rabbit anti-p27 sera followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. Infections are listed at the top of each lane, and cell types are listed below. 1:10 dilutions of samples prepared from the supernatants of infected CEFs are also shown.

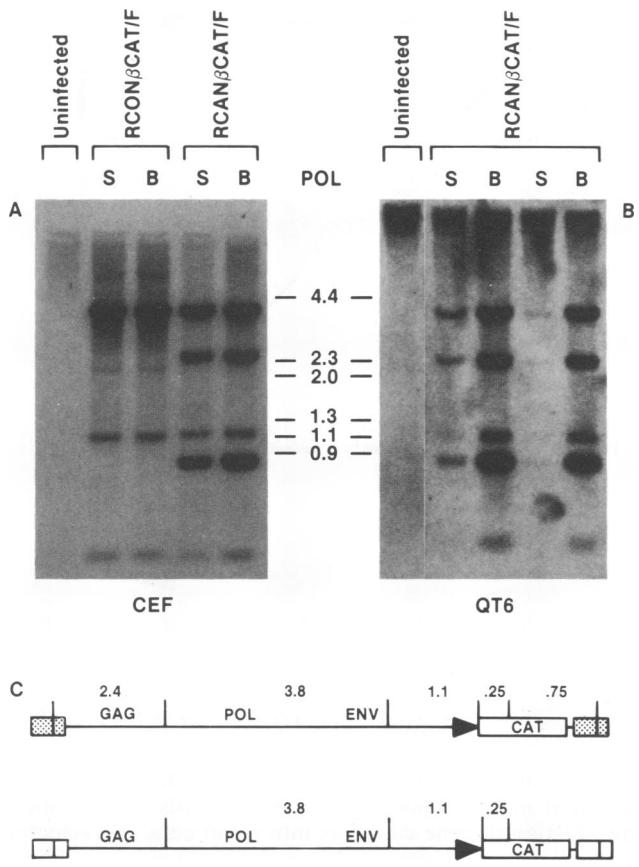

FIG. 8. Southern transfer analysis of BH *pol* vector DNA. DNA was isolated from cells infected with retrovirus vectors containing the *pol* regions of SR-A RSV (S) or BH-RSV (B). DNA (20 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with a ^{32}P-labeled probe that consisted of RSV DNA and CAT DNA sequences (as described in the legend to Fig. 4). (A) Southern transfer analysis of CEF cultures infected with RCON/βCAT/F and RCAN/βCAT/F and their Bryan *pol* vector counterparts, RCONBP/βCAT/F and RCANBP/βCAT/F. (B) Southern transfer analysis of QT6 cultures infected with either RCAN/βCAT/F or its Bryan *pol* counterpart, RCANBP/βCAT/F. The results of two separate experiments are shown. (C) Schematic illustration of RCON (or RCONBP) and RCAN (or RCANBP) proviral structures showing the position of *Eco*RI recognition sequences and the sizes (in kilobases) of the predicted digestion products.

QT6 cultures was much higher when the *pol* region of RCAN was derived from BH-RSV. The data shown are the results of two separate experiments in which QT6 cells were transfected with either RCAN or RCANBP viruses that contained the β-actin-CAT cassette. These data suggest that in QT6 cultures, proviruses containing the BH-RSV *pol* region were established and/or maintained in a larger fraction of the cell population than proviruses containing the SR-A RSV *pol* region. Taken together, the data presented in Fig. 6, 7, and 8 argue that BH *pol* sequences markedly improve virus replication in QT6 cells. Consequently, BH *pol* sequences elevate expression of DNA inserts in QT6 cells, both because the infection is more efficient and because the transcription of the insert is enhanced in those cells that do become infected.

DISCUSSION

In this study we have used a series of closely related replication-competent retrovirus vectors to introduce functional gene cassettes into avian cells. Cassettes may consist of virtually any promoter linked to an open reading frame or other sequences of interest. These vectors permit the inserted DNA to be expressed independently of the endogenous viral genes, which are under the control of the LTR promoter. However, we found that internal promoter activity may be influenced significantly by proviral sequences, such as enhancer elements within the LTR. Because of packaging constraints and the presence of a complete set of viral *gag*, *pol*, and *env* sequences, the sizes of DNA fragments that can be inserted into RCON and RCAN and into their PCAT derivatives are limited to about 2.0 and 1.2 kb, respectively.

We chose to test these vectors initially in cultured fibroblasts, using cassettes constructed from the promoter regions of two well-characterized eukaryotic genes linked to a reporter gene. Cassettes consisting of the chicken β-actin promoter and the MT1 promoter linked to the bacterial CAT gene were efficiently expressed in infected cells. CAT expression from the MT1 promoter was induced threefold in the presence of zinc. In general, proviruses were stable throughout serial cell passage. In CEF cultures we consistently observed low proviral copy numbers; approximately one provirus per haploid genome. Cells infected with vectors carrying promoter-CAT cassettes inserted in the same transcriptional orientation as the endogenous retrovirus genes (*gag*, *pol*, and *env*) expressed higher levels of CAT than cells infected with vectors containing the cassette in the reverse orientation. Since the cassettes we have constructed lack polyadenylation signal sequences, one possible explanation for this observation is that CAT mRNAs generated from cassettes inserted in the backward orientation lacked poly(A) tails and therefore were less stable or were less efficiently translated. We expect that mRNAs produced from cassettes inserted in the forward orientation were polyadenylated at the poly(A) site in the proviral 3′ LTR. Alternatively, interference by transcription initiated within the LTR promoter may have reduced the levels of CAT mRNA that were transcribed from cassettes in the reverse orientation. A third possibility is that the CAT mRNAs produced from cassettes in the reverse orientation were inaccessible for translation because they annealed with complementary viral genomic and subgenomic mRNAs. With respect to the latter two possibilities, we found no evidence to suggest that vectors containing cassettes in the backward orientation replicated less efficiently than the corresponding vectors carrying cassettes in the forward orientation.

Numerous studies have mapped determinants of retrovirus pathogenicity and tissue tropism within the LTRs (see reference 34 for a discussion). At least some of the determinants within the LTR are transcriptional enhancers. The interaction of retrovirus enhancer elements with species-specific and cell-type-specific *trans*-acting transcription factors probably plays a major role in determining the location and extent of retrovirus transcription and consequently of retrovirus replication (22). Since the RCON and RCAN vectors were designed to dissociate transcription of the DNA insert from viral transcription by permitting inserts to be expressed from foreign promoters, we have asked whether an LTR enhancer within the vector can influence the level of expression from inserted promoters. The LTRs of RCON are from a relatively nonpathogenic, endogenous

retrovirus, RAV-O (2, 18). The LTRs of RCAN are from the highly pathogenic SR-A strain of RSV (35). These two LTRs are known to differ, in that the SR-A RSV LTR contains a powerful transcriptional enhancer that the RAV-O LTR lacks (3–5, 16, 21, 36). When promoter-CAT cassettes were introduced into fibroblasts by using the RCAN vectors (RCAN, RCAN/PCAT, and RCANBP), we detected high levels of CAT and RT in infected cultures. When the same cassettes were tested in the corresponding RCON vectors (RCON, RCON/PCAT, and RCONBP), CAT activity and RT were 5- to 50-fold lower (Fig. 5 and 6). By examining proviral copy numbers, we have ruled out the possibility that SR-A RSV LTRs increase CAT activity simply by elevating the number of integrated proviruses per cell. We currently believe that the SR-A RSV LTR increased the expression of CAT in RCAN-infected cells by stimulating transcription from the β-actin and MT1 promoters. Using a strand-specific CAT sequence probe for RNA protection analyses (S1 nuclease and RNase digestion), we have detected much higher levels of CAT transcripts in the RNA prepared from RCAN/βCAT-infected cells than was found in RNA from RCON/βCAT-infected cells (data not shown). Using the same approach, we have also detected CAT transcripts in cells infected with vectors containing the βCAT cassette in the reverse orientation. Unfortunately, we have not been able to accurately quantitate the amount of CAT mRNA that is correctly initiated from the internal β-actin promoter in infected cells.

The ability of BH-RSV to replicate more efficiently than other strains of RSV appears to map within the *pol* region of the virus (31). Although the exact mechanism is not understood fully, preliminary studies indicate that this region serves to increase viral transcription. By substituting the *pol* regions of the RCON and RCAN vectors with the corresponding region of the BH strain of RSV, in each case we were able to increase the expression of inserted β-actin promoter-CAT cassettes by fivefold. An examination of proviral copy numbers has ruled out the possibility that in chicken cells, the elevated levels of CAT activity were the result of increased numbers of integrated proviruses per cell. Although we have not investigated what effect the Bryan *pol* region has on viral RNA levels in cells infected with our vectors, we have shown that cells infected with retrovirus vectors containing BH *pol* produce significantly more p27 capsid protein than corresponding vectors containing SR-A *pol*.

Our studies indicate that the established quail cell line QT6 is not as readily infected by the RCON and RCAN viruses as are CEFs. Consequently, the levels of promoter-CAT cassette expression were significantly lower in QT6 cultures. On the basis of the amount of proviral DNA in infected cells and p27 capsid protein levels, the RCANBP vector appears to replicate much more efficiently in QT6 cells than the vectors lacking the Bryan *pol* region. In QT6 cultures, a β-actin-CAT cassette was expressed at much higher levels when the retrovirus vector contained the BH-RSV *pol* region. We recommend that the versions of the RCON and RCAN vectors that contain the BH-RSV *pol* region be used for experiments done in QT6 cells.

The RCON and RCAN vectors that have been characterized in this study offer an efficient means of introducing gene cassettes into avian cells. By constructing vectors from cloned copies of replication-competent retroviruses, we have developed a retrovirus gene transfer system that can be used in the absence of helper virus or genetic selection. This strategy has also been used by others to create replication-competent vectors from cloned copies of the genomes of RSV (6, 7, 15, 26), spleen necrosis virus (8), and the Moloney murine leukemia virus (29, 30). The vectors characterized in this report will be useful tools for the stable introduction of gene cassettes into avian cells. By selecting appropriate promoters, we have begun to use this vector system to successfully target gene expression to specific cell types in vivo (19a).

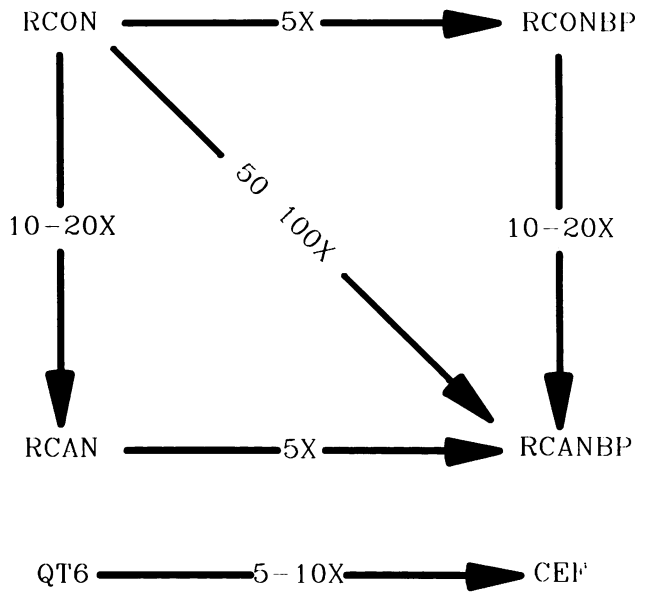

FIG. 9. Summary diagram of the relative expression levels obtained with the RCON/RCAN vector series.

The effects of proviral sequences on the level of expression of DNA inserts from internal promoters are summarized diagrammatically in Fig. 9. The capability of this vector system to express DNA inserts throughout a wide range (50- to 100-fold) of levels can be applied to investigate the effects of quantitative changes in gene expression. The latter should prove to be particularly useful for the study of genes involved in cell growth, differentiation, and oncogenesis.

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