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Endogenous avian retroviruses contain deficient promoter and leader sequences

(long terminal repeat/DNA-mediated gene expression/promoter assay)

BRYAN R. CULLEN*†, ANNA MARIE SKALKA‡, AND GRACE JU*§

*Department of Molecular Genetics, Hoffmann-La Roche Incorporated, Nutley, New Jersey 07110; †Department of Microbiology, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103; and ‡Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT **A sensitive and quantitative biological assay has been utilized to measure the ability of the exogenous and endogenous avian retroviral long terminal repeats (LTR) to promote gene expression in avian cells. This assay has revealed that the exogenous virus RAV-2 LTR is ≈10-fold more active than the LTRs of endogenous viruses RAV-0, *ev*-1, and *ev*-2. The endogenous viral LTRs show approximately equal activity. Upstream flanking cellular or viral sequences have no significant modulating effect on gene expression in our assay. Unexpectedly, we have detected and localized an additional defect outside of the LTR in the 5′ noncoding leader sequence of *ev*-1 that further decreases gene expression relative to RAV-0 by ≈10-fold.**

Avian retroviruses can be divided into two groups: (*i*) the exogenous retroviruses, which infect avian cells from without and integrate into the DNA of somatic cells, and (*ii*) the endogenous retroviruses, which are propagated as proviral sequences in the germ line of normal chickens. All intact avian retroviruses possess a relatively short, well-defined sequence that is responsible for the control of viral gene transcription. This sequence, termed the long terminal repeat (LTR), is located both upstream and downstream of the viral structural genes in the integrated provirus (1). The LTR contains putative transcription regulatory signals including a "TATA" box, a polyadenylylation-termination signal, and the initiation signal (cap site) (2). We have previously described the molecular cloning and sequence analysis of LTR-containing segments derived from exogenous and endogenous avian retroviral DNA (2–5). The endogenous viral LTRs have been shown to be very similar to each other in nucleotide sequence but to differ significantly from the exogenous viral LTRs. In particular, the endogenous viral LTRs are significantly shorter than the LTR of the exogenous viruses.

Relative amounts of viral-specific mRNA produced by exogenous and endogenous retroviruses in infected cells have been compared (6, 7). The proviruses at two well-characterized endogenous viral loci, *ev*-1 and *ev*-2, are normally transcriptionally silent, producing less than one copy of viral RNA per cell (7). The *ev*-1 locus can be induced by treatment of chicken cells with 5-azacytidine, a methylation inhibitor, resulting in the synthesis of an average of 50 copies of viral RNA per cell. However, no infectious virus is detected (8). BrdUrd activation of the provirus at the *ev*-2 locus results in the production of the endogenous virus RAV-0. RAV-0 is replication-competent and can infect other cells (7). Upon infection, the integrated RAV-0 provirus expresses 1,600–3,000 copies of viral RNA per cell. Although greatly elevated from the noninduced *ev*-2 level, this value is still ≈10% of the amount of viral mRNA transcribed from an integrated exogenous provirus such as RAV-2 (10,000–20,000 copies per cell) (6).

A further important difference between exogenous and endogenous avian retroviruses is their ability to induce neoplastic transformation. The exogenous avian leukosis viruses, such as RAV-2, cause a high incidence of lymphoma in infected chickens (9). The endogenous virus RAV-0 is not associated with lymphoid transformation (4). DNA sequence analysis of genetic recombinants between exogenous and endogenous retroviruses indicates that transforming capability segregates with sequences located at the extreme 3′ end of the exogenous viruses (4). This region of the viral genome includes the U3 promoter region of the LTR. Thus, the U3 region of the LTR may determine both the level of viral transcription and the oncogenic potential of avian retroviruses. Only viruses capable of high levels of transcription appear to cause transformation. This correlation may be explained by the promoter insertion model of leukemogenesis, in which a cellular oncogene is activated by adjacent insertion of an exogenous viral LTR (10).

We have shown previously that cloned avian retroviral DNA is transcriptionally active when microinjected into avian cells (11). To determine the basis for the variation in levels of viral transcription of endogenous and exogenous viruses, we have developed a sensitive biological assay which allows us to compare directly the relative promoter strengths of retroviral LTRs.

MATERIALS AND METHODS

Cells. The quail cell line QCl-3 that we obtained from D. Stacey was originally isolated by R. Fries and is transformed by the replication defective [*env* (−)] Bryan high-titer strain of Rous sarcoma virus [RSV (−) cells]. QCl-3 cells were grown in M199 media, supplemented with 4% fetal calf serum, 1% chicken serum, 10% tryptose phosphate broth, and 1% dimethyl sulfoxide. The media also contained penicillin, streptomycin, and fungizone. Chicken embryo fibroblasts were prepared and used in focus assays as described (11, 12). All tissue culture products were obtained from GIBCO.

Transfection Assay. Cells were transfected by a modification of the DEAE-dextran procedure (13). Tissue culture plates (35 mm) were seeded with 9×10^5 QCl-3 cells 1 day prior to transfection. Plasmid DNA samples were suspended in 0.2 ml of phosphate-buffered saline containing 1 mg of DEAE-dextran per ml (Pharmacia). At the time of transfection, cell monolayers, which were just subconfluent, were washed once with 2.5 ml of phosphate-buffered saline. The DNA samples were then

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Abbreviations: LTR, long terminal repeat; bp, base pair(s); kb, kilobase pair(s); RSV, Rous sarcoma virus; SRA, Schmidt–Ruppin subgroup A; ffu, focus-forming unit(s).

§ To whom reprint requests should be addressed.

added to the cell monolayers and incubated for 30 min at 37°C with occasional gentle shaking. The transfected cells were overlaid with 2.5 ml of M199 growth media and were refed with fresh media 2.5 hr after transfection. Transfected cells producing envelope protein first release infectious transforming virus ≈24 hr after transfection (results not shown). Culture media for focus assay on chicken embryo fibroblasts were taken at 44 hr after transfection to prevent the detection of any recombinant second generation virus in the assay.

Unless otherwise noted, at least two separate plasmid DNA preparations were tested per sample. Each plasmid DNA was isolated both by the cleared lysate method of Norgard *et al.* (14) and by the rapid boil method of Holmes and Quigley (15). The method of DNA preparation had no significant effect on biological activity (results not shown).

Construction of Molecular Clones. All molecular clones tested in the bioassay had structures similar to the prototype construct diagramed in Fig. 1. In each case, the LTR-containing donor cassette, obtained from a particular retroviral DNA clone, was inserted adjacent to the constant *env*-containing acceptor fragment. The acceptor fragment was derived from a λSRA clone containing the entire sequence of the exogenous transforming virus Schmidt–Ruppin subgroup A (SRA) inserted into the *Eco* RI site of the λgtWES·λB vector (16). *Bam*HI digestion products of λSRA were subcloned into the *Bam*HI site of pBR322. One of these subclones, pGJ15, was used for all subsequent constructions. The acceptor fragment in clone pGJ15 consists of the complete *env* and *src* genes, a complete copy of the downstream LTR and a fragment of a second LTR. The unique *Bgl* II restriction site located ≈840 base pairs (bp) upstream of the *env* splice acceptor site was used to insert the various cassettes. The constant SRA acceptor fragment is 5.35 kilobase pairs (kb) in all the molecular clones tested.

The donor cassettes were prepared by digestion with appropriate restriction endonucleases to generate LTR-containing segments with a common 3′ end—namely, the *Bam*HI site located at the beginning of the viral *gag* gene (11). pGJ16 (RAV-2) contains the 1.85-kb *Sal* I–*Bam*HI fragment from λRAV-2 (2). pGJ17 (*ev*-1) was constructed by inserting the 0.96-kb *Bgl* II–*Bam*HI fragment from pGd27 (3), which was derived originally by molecular cloning of the upstream LTR region and flanking cellular DNA at the *ev*-1 locus. The pGJ17 construction retains ≈260 bp of cellular flanking sequences upstream of the viral LTR. pGJ18 (RAV-0) has a structure analogous to the RAV-2 clone pGJ16 and contains a 1.95-kb *Hin*dIII–*Bam*HI segment from pRM1 (4) with a tandem repeat of the RAV-0 LTR.

pGJ19 (*ev*-2) has a control cassette consisting of a 830-bp *Bam*HI–*Bam*HI fragment from pGd100 (5), which includes 130 bp of upstream flanking cellular sequences in addition to the *ev*-2 LTR. pGJ19/REV is similar to pGJ19, but the LTR segment is inserted in the opposite orientation.

Exchange of leader (region III) sequences between clones was accomplished by using the unique *Bst*EII site in the $tRNA^{trp}$ primer binding site and a unique *Kpn* I site in the constant SRA sequence 760 bp downstream of the *Bgl* II/*Bam*HI junction site (Fig. 1). To insert additional cellular flanking sequences into clone pGJ17, a 2-kb *Hin*dIII/*Bgl* II fragment from the *ev*-1 clone pGd27 (3) was inserted at the *Bgl* II site bordering region I to create pGJ17/RL/F.

RESULTS

To quantitate the effects of various LTRs on gene expression, we have developed a modification of the *env* complementation assay previously described (11, 12). Our modified system employs the continuous quail cell line QCl-3 that was established by infection with the Bryan high-titer strain of RSV [RSV (−)]. Because this virus is transformation competent but envelope deficient (src^+ env^-), virions produced by these cells are not infectious due to lack of the *env* gene product (11, 12). Complementation of the *env* defect can occur when QCl-3 cells are transfected with plasmid DNA containing the *env* gene under the control of different retroviral LTR promoters. The number of infectious transforming RSV (−) particles subsequently released is quantitated by a focus assay on chicken embryo fibroblasts. Stacey *et al.* (12) have shown previously that the number of focus-forming units (ffu) released is directly proportional to the amount of *env* mRNA available in the cell.

The strategy employed was to construct clones such that a conserved or constant *env* gene would be under the control of different promoters. The structure of the prototype is shown in Fig. 1. All clones were constructed with the plasmid vector pBR322. The constant region of the clones consists of the *env* and *src* genes derived from the SRA strain of RSV (16). Additionally, this region contains the downstream LTR of SRA, which may be required for correct termination and polyadenylylation of the viral mRNAs. The upstream boundary of the con-

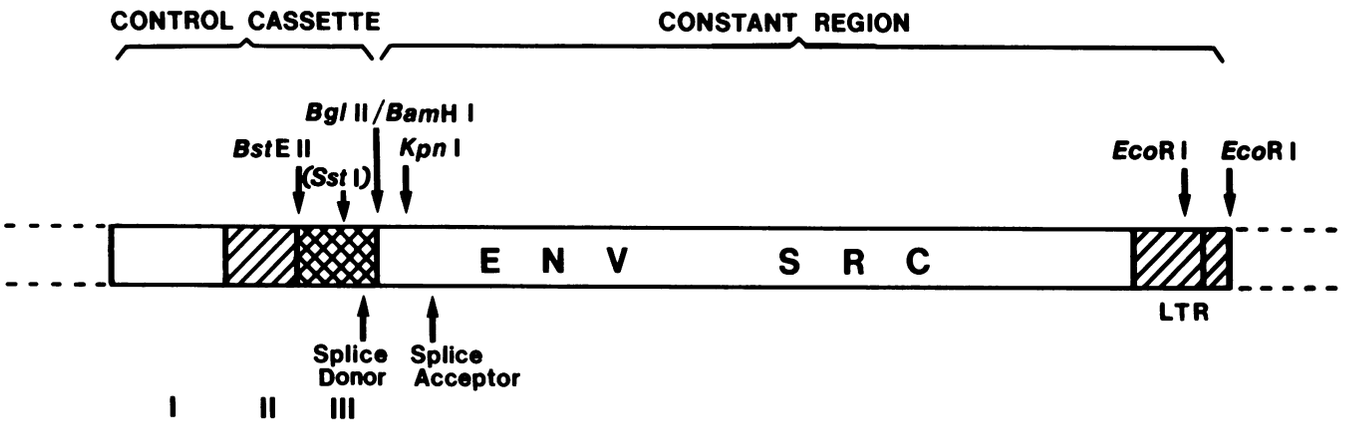

FIG. 1. Structure of the prototype pGJ clone. Each clone consists of a constant *env*-containing region derived from SRA, joined to different LTR containing control cassettes obtained from various endogenous and exogenous avian retroviruses. The control cassettes are subdivided into three functionally distinct regions as described in the text. Important restriction endonuclease sites are shown, including the unique *Sst* I site observed only in the endogenous viruses. The downstream LTR was originally subcloned from a tandemly repeated LTR and thus contains a portion of an additional LTR (16). The approximate positions of the *env* mRNA splice sites are shown. The intron thus delineated consists of parts of the retroviral *gag* and *pol* genes and is ≈980 bp in length. The conserved SRA part of the clones is ≈5.35 kb in size, whereas the size of the control cassettes varies as described in the text.

Table 1. Transforming virus released at 44 hr after transfection of QCl-3 cells with various amounts of pGJ16 DNA

pGJ16 DNA applied, ng	ffu released
4,000	384
950	792
250	1,126
100	552
50	336
25	228

Table 2. Virus released at 44 hr after transfection of QCl-3 cells with equimolar amounts of different retroviral DNA clones

Clone	Upstream LTR	ffu released
pGJ15	None	0
pGJ16	RAV-2	1,128
pGJ17	*ev*-1	16
pGJ18	RAV-0	80
pGJ19	*ev*-2	116
pGJ19/REV	Inverted *ev*-2	0

stant region consists of a *Bgl* II site in the *pol* gene of SRA upstream of the *env* mRNA splice acceptor site. The clone containing the constant region only, without an inserted upstream LTR, was designated pGJ15.

The different control cassettes may be subdivided into three functionally distinct regions as shown in Fig. 1. These regions are: (*i*) the LTR, designated region II; (*ii*) the 5′ noncoding or leader region of the *env* gene, designated region III; and (*iii*) the upstream flanking sequences, designated region I. The RAV-2 and RAV-0 cassettes used in these constructions were derived from molecular clones of circular unintegrated viral DNA molecules. Thus, both the RAV-2 clone (designated pGJ16) and the RAV-0 clone (designated pGJ18) contain LTRs flanked upstream by 1.2 kb of viral sequences. The *ev*-1-derived clone (pGJ17) and the *ev*-2-derived clone (pGJ19) contain LTR regions originally cloned from integrated proviruses and thus are both bounded upstream by cellular DNA. This flanking cellular DNA is ≈230 bp long in the case of pGJ17 and ≈130 bp long in the case of pGJ19. All of the LTRs used have been described and subjected to sequence analysis (2, 5). All of the clones contain a single LTR except pGJ18, which has two tandem copies of the RAV-0 LTR.

Region III in each clone is ≈428 bp and extends from the end of the LTR, marked by a *Bst*EII site in the $tRNA^{trp}$ primer binding site, to a conserved *Bam*HI site close to the 5′ end of the viral *gag* gene (11). This region includes the viral RNA 5′ noncoding region, a short region of the *gag* gene, and the *env* mRNA splice donor site. Ligation of this *gag* gene *Bam*HI site to the *Bgl* II site in the SRA *pol* gene results in a structure closely analogous to an integrated provirus. However, the majority of the *gag–pol* region, which constitutes the *env* gene intron, is deleted. It was previously shown (11) that this deletion does not interfere with the transcription, splicing, and expression of *env* mRNA.

To calibrate the assay, QCl-3 cells were transfected with varying amounts of the RAV-2 clone pGJ16 DNA (Table 1). The results shown, and a more extensive analysis to be reported elsewhere, indicated that ≈500 ng of pGJ16 DNA per 35-mm plate gave a maximal response. The cause of the inhibition at higher concentrations of DNA is not known. To allow comparison of relative biological activities QCl-3 cells were transfected with 500 ng of pGJ16 DNA or equimolar amounts of DNA from other clones in all experiments subsequently performed. Results from a representative transfection experiment com-

Table 3. Comparison of activity of the various retroviral DNA clones used

Group	Clone(s)	Regions compared* I	II	III	Times tested	Ratio of activity	SD	*P* value
1	pGJ17	*ev*-1	*ev*-1	*ev*-1	7	0.0076 (1/131)	±0.0053	<0.001
	pGJ16	RAV-2	RAV-2	RAV-2				
2	pGJ18	RAV-0	RAV-0	RAV-0	7	0.083 (1/12)	±0.069	<0.001
	pGJ16	RAV-2	RAV-2	RAV-2				
3	pGJ19	*ev*-2	*ev*-2	*ev*-2	7	0.106 (1/9)	±0.052	<0.001
	pGJ16	RAV-2	RAV-2	RAV-2				
4	pGJ17	*ev*-1	*ev*-1	*ev*-1	6	0.12 (1/8)	±0.11	<0.001
	pGJ18	RAV-0	RAV-0	RAV-0				
5	pGJ18	RAV-0	RAV-0	RAV-0	7	0.66 (2/3)	±0.27	≈0.02
	pGJ19	*ev*-2	*ev*-2	*ev*-2				
6	pGJ16/eL	RAV-2	RAV-2	*ev*-1	5	0.26 (1/4)	±0.08	<0.01
	pGJ16	RAV-2	RAV-2	RAV-2				
7	pGJ17/RL	*ev*-1	*ev*-1	RAV-2	5	9.2 (9/1)	±3.8	<0.001
	pGJ17	*ev*-1	*ev*-1	*ev*-1				
8	pGJ17/RL/F	Add'l† *ev*-1	*ev*-1	RAV-2	5	0.87 (1/1)	±0.36	≈0.5
	pGJ17/RL	*ev*-1	*ev*-1	RAV-2				
9	pGJ19/eL	*ev*-2	*ev*-2	*ev*-1	2	0.05 (1/20)	ND	ND
	pGJ19	*ev*-2	*ev*-2	*ev*-2				
10	pGJ18/RL	RAV-0	RAV-0	RAV-2	2	1.2 (1/1)	ND	ND
	pGJ18	RAV-0	RAV-0	RAV-0				

Results are expressed as average ratios of activity observed between plasmid DNAs applied at the same time to parallel plates of QCl-3 cells. The observed SD and the number of experiments performed are indicated. The *P* value given is derived by Student's *t* test and indicates the likelihood of the compared DNAs having identical biological activity. At least two different DNA preparations of each clone were used with the exception of clone pGJ18/RL. Groups 9 and 10 do not contain statistical data as these comparisons were only performed twice. ND, not determined.

* Comparison of the origin and nature of the regions that are varied in the clones. Boxes indicate regions that differed between the clones in each group.

† Additional flanking sequences.

paring the activities of the different retroviral clones are shown in Table 2.

Clone pGJ15, which contains no control cassette, and clone pGJ19/REV, which contains an inverted upstream *ev*-2 control region, were both negative for *env* gene expression. As expected, pGJ16 gave the highest amount of *env* expression (Table 3). This level of activity, ≈12-fold higher than that of the RAV-0 clone pGJ18 (group 2), is consistent with the differences in viral RNA expression observed in cells infected by these viruses (6). The clones containing cassettes from the normally transcriptionally silent endogenous loci *ev*-1 and *ev*-2 gave unexpected results. The *ev*-2-derived clone pGJ19 yielded a level of activity slightly higher than pGJ18, whereas the *ev*-1 clone pGJ17 yielded a level of activity ≈1/8th as much (Table 3, groups 4 and 5). The cloned *ev*-2 cassette thus appears free of those factors or sequences that normally limit its expression *in situ* relative to the RAV-0 provirus. In contrast, the *ev*-1 cassette in pGJ17 appears defective relative to the *ev*-2 cassette. Because we have previously shown that the nucleotide sequences of the LTRs of the endogenous viruses *ev*-1, *ev*-2, and RAV-0 are nearly identical (5), these limiting sequences in clone pGJ17 appeared likely to reside in region I or III.

We initially investigated the possibility that region III, the *env* mRNA leader region, was responsible for the defect in the *ev*-1 cassette. This was achieved by inserting the *ev*-1 leader region into the RAV-2 control cassette to produce pGJ16/eL (RAV-2 LTR, *ev*-1 leader) and the RAV-2 leader region into the *ev*-1 cassette to produce pGJ17/RL (*ev*-1 LTR, RAV-2 leader). As the results in Table 3 show, we observed that clone pGJ17/RL gave ≈9-fold higher *env* activity than clone pGJ17 (group 7), whereas clone pGJ16/eL showed a significant drop in activity (group 6). The increase in activity in clone pGJ17/RL is sufficient to give a level approximately equal to that of clone pGJ18 (RAV-0 LTR and leader; Table 3, group 4). The defect in the *ev*-1 cassette was not an artifact of subcloning, as three other independently derived pGJ17 clones showed the same low activity (data not shown).

To analyze further the effect of region III on *env* gene expression we transferred this region from the RAV-2 cassette in pGJ16 to the RAV-0 cassette in pGJ18 to yield pGJ18/RL. No significant increase in *env* activity was observed (group 10). Lastly, to localize the defect in the *ev*-1 leader, a fragment bounded by the conserved endogenous virus *Sst* I site and the *Bam*HI site, which marks the end of the region III, was transferred from the *ev*-1 cassette in pGJ17 to the *ev*-2 cassette in pGJ19 to give pGJ19/eL (*ev*-2 LTR, *ev*-1 leader). Replacement of this 267-bp fragment in pGJ19 resulted in a large decrease in *env* activity (group 9).

Using the clones described above, we have obtained no evidence for the presence of *cis*-acting transcriptional repressors in the host DNA flanking the *ev* loci as proposed (17). A possible explanation for this observation is that the flanking DNA sequence responsible for repression is longer than that included in clones pGJ17 and pGJ19. To examine this possibility we inserted an additional 2 kb of cellular DNA from the *ev*-1 locus in its appropriate position and orientation upstream of the LTR in clone pGJ17/RL to yield pGJ17/RL/F. As shown in Table 3, no significant change in activity was noted.

Our results indicate that the *ev*-2 clone pGJ19 is slightly but significantly more active than the RAV-0 clone pGJ18. It seems possible that the presence of tandem LTRs in pGJ18 may contribute to the observed difference.

DISCUSSION

We have examined the ability of cloned LTRs of the endogenous avian retroviruses *ev*-1, *ev*-2, and RAV-0 and the exogenous virus RAV-2 to act as promoters of an assayable gene, in this case the viral *env* gene. The assay we have employed—namely, complementation of the *env* deficiency in QCl-3 cells—measures transcription indirectly. As all of the clones tested were constructed such that the same *env* gene, containing identical splice and transcription termination signals, was placed under the control of the different LTR segments, the assay should give an accurate measurement of *env* mRNA transcription. Also, unlike other assays that measure the expression of prokaryotic genes under the control of eukaryotic promoters (18, 19), this assay has the advantage of measuring avian LTR promoter activity in avian cells that can normally express the *env* gene. Our results suggest that the endogenous LTRs are equally active promoters but are ≈10% as active as the exogenous virus LTR. Additionally, a defect in the viral mRNA leader region of *ev*-1 was discovered.

In infected susceptible avian cells, exogenous viruses give rise to levels of viral mRNA ≈10-fold higher than that obtained when a susceptible cell is infected by the "endogenous" virus RAV-0 (6). In contrast, proviruses at the endogenous loci *ev*-1 and *ev*-2 are normally transcriptionally silent (7). At least three possible explanations for these differences in transcriptional activity have been suggested: (*i*) *cis*-acting transcriptional repressors in the chicken cellular DNA adjacent to the *ev* loci (17); (*ii*) differences in the sequences of the LTRs (5); and (*iii*) DNA modification, such as methylation (8).

We have recently reported the nucleotide sequence of the LTRs of these exogeneous and endogenous viruses (2–5). These data show that *ev*-1 and *ev*-2 LTRs differ by only three dispersed base changes and that the *ev*-2 and RAV-0 LTRs are identical. However, all three differ greatly from the exogenous viral LTRs in the U3 region thought to contain transcriptional control signals (enhancer/promoter). In the bioassay used here, a direct comparison of the endogenous viral LTRs indicates that they are equivalent in promoter activity, but ≈10% as active as the exogenous viral LTR. This is consistent with results from nucleotide sequence comparisons and correlates closely with the difference observed *in vivo* between RAV-2 and RAV-0 (6). The viral loci *ev*-1 and *ev*-2 are thus further repressed by a second mechanism *in vivo*. Our data show that flanking chicken cellular DNA of up to 2.3 kb has no effect on the promoter activity of the *ev*-1 LTR. Although the effect of these sequences on transcription from the transfected DNA plasmid may be quite different from their influence in chromosomal DNA, this bioassay does not provide evidence for the existence of *cis*-acting transcriptional repressors adjacent to the *ev* loci. Inducibility of the *ev*-1 locus by the cytidine analog 5-azacytidine (8) suggests that transcription may normally be suppressed by methylation. Inhibition of proviral transcription by DNA methylation has been observed in other systems (20). Such methylation would, of course, be removed during molecular cloning of *ev* DNA. We have not examined the possibility that methylation of the input DNA occurs after transfection.

An unexpected result was the observation of a defect in the 5′ viral leader region of *ev*-1 when compared to the other viruses. The *ev*-1 locus is unable to give rise to infectious virus even when induced, and multiple defects in the structural genes of *ev*-1 have been described (21). It appears that the lesion detected in our bioassay, which has been localized to a 267-bp fragment, represents a further defect in *ev*-1. This defect, which might lead to aberrations in translation, in RNA stability, or in *env* mRNA splicing, must be investigated further. Initial results (unpublished data) indicate that the defect is due to minor sequence changes in the 5′ noncoding region. Analysis of this genetic mutation may shed light on the function of eukaryotic mRNA leader sequences.

Exogenous and endogenous viruses differ in their ability to cause lymphoid leukosis in chickens (4). Analysis of genetic recombinants between exogenous and endogenous retroviruses indicates that transforming ability is controlled by sequences encoded at the extreme 3′ end of the RNA genome of the exogenous viruses. This region includes the LTR U3 region thought to contain the retroviral promoter. According to the promoter insertion hypothesis, neoplasia can arise by increased expression of cellular oncogenes (c-*onc*), which are activated by viral LTRs integrated nearby (10). We have shown that the endogenous viral promoters are ≈10% as effective as that of an exogenous virus. Assuming that RAV-0, like RAV-2, is able to integrate next to an oncogene, the level of c-*onc* transcription promoted by the RAV-0 LTR may be below the "threshold" necessary for cellular transformation.

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