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Transcriptional Interaction between Retroviral Long Terminal Repeats (LTRs): Mechanism of 5′ LTR Suppression and 3′ LTR Promoter Activation of c-*myc* in Avian B-Cell Lymphomas

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Chicken syncytial viruses induce bursal lymphomas by integrating into the c-*myc* locus and activating *myc* expression by 3′ long terminal repeat (LTR) promoter insertion. In contrast to wild-type proviruses, in which transcription initiates predominantly in the 5′ LTR, these *myc*-associated proviruses exhibit a predominance of transcription from the 3′ LTR and little transcription from the 5′ LTR. Most of these proviruses contain deletions within the 5′ end of their genome that spare the 5′ LTR. We report the identification of a 0.3-kb viral leader sequence that modulates 5′ and 3′ LTR transcriptional activities. In the presence of this sequence, transcription from the 5′ LTR predominates, but in its absence, the 3′ LTR promoter becomes activated, resulting in a high level of *myc* expression. This viral sequence does not behave like a classical enhancer; it activates transcription only when located downstream from the promoter and in the sense orientation. In this regard, it resembles the recently described human immunodeficiency virus RNA enhancer. This study suggests that retroviruses contain internal sequences which directionally activate the 5′ LTR promoter to facilitate transcription of the viral genome and that deletion of these sequences is one step in the activation of the 3′ LTR of *myc*-associated proviruses in avian bursal lymphomas.

In the proviral form, the retroviral genome is flanked by two long terminal repeats (LTRs), a 5′ LTR and a 3′ LTR. Despite the sequence identity of these two LTRs, they have distinct functions in retroviral replication. The 5′ LTR serves as the sole promoter to transcribe the viral genome, whereas the 3′ LTR provides a polyadenylation signal to define the end of the viral genome. Herman and Coffin have reported that the promoter activity of the 5′ LTR is at least 50 times higher than that of the 3′ LTR (34). Clearly, therefore, the transcriptional activities of the two LTRs are differentially modulated in the provirus. Several mechanisms, including transcriptional interference, have been proposed to explain this differential activity (16, 34, 51).

Although the 3′ LTR promoter contributes little to the transcription of viral genes, it does play a significant role in proto-oncogene activation by nonacutely transforming retroviruses. Activation of proto-oncogenes is a key step in retrovirus-induced oncogenesis. Several mechanisms by which retroviruses activate proto-oncogenes have been identified, including LTR promoter insertion and LTR enhancer insertion (reviewed in reference 37). In LTR promoter insertion, the proto-oncogene is transcribed from either the 5′ or the 3′ LTR promoter. Activation of a proto-oncogene by transcription from the 3′ LTR is typified by avian leukosis virus (ALV) and chicken syncytial virus (CSV) activation of c-*myc* in chicken bursal lymphomas (25, 33, 50, 53). In these tumors, the proviruses are integrated upstream of the c-*myc* coding exons and in the same transcriptional orientation as c-*myc*. The proviral 3′ LTR initiates transcription of the downstream *myc* sequences and thus eliminates cellular regulation of *myc* expression.

Further analysis of these *myc*-activating CSV and ALV proviruses turned up two curious but consistent findings. (i) Transcription from the 5′ LTR of these proviruses is reduced to very low or undetectable levels (27, 33, 67). This transcription pattern contrasts with that observed in replicating retroviruses and suggests that up regulation of the 3′ LTR requires shutdown of the 5′ LTR. (ii) These proviruses frequently suffer large deletions which map to the leader-*gag* region near the 5′ LTR (27, 59, 67). If these deletions remove viral sequences (e.g., enhancers) necessary for transcription from the 5′ LTR, they could cause shutdown of the 5′ LTR promoter.

We have previously isolated and characterized a CSV provirus involved in c-*myc* activation (67). This provirus, 713, is inserted in the first c-*myc* intron in the same transcriptional orientation as c-*myc*. It contains a deletion encompassing ~80% of the viral sequences but has two intact LTRs. In the tumor, abundant truncated c-*myc* transcripts linked to the 3′ LTR were detected but no viral transcripts from the 5′ LTR were detected. When the 5′ LTR was isolated and linked to a chloramphenicol acetyltransferase (CAT) construct, however, it was fully functional (57). The inactivity of the 5′ LTR in the tumor, therefore, was not due to mutations within the LTR. This suggests that LTR activity can be modulated by surrounding viral and/or cellular sequences and is consistent with findings on other systems, such as ALV, Moloney murine leukemia virus, and *gypsy* (3, 11, 38, 41, 43, 46, 51, 64, 71). The identification of these sequences is of fundamental importance for understanding viral transcriptional regulation and viral interaction with host genes.

In this report, we show that the lack of detectable transcripts from the 5′ LTR of provirus 713 is due to inhibition of transcriptional initiation within the 5′ LTR. This inhibition is caused by deletion of the viral leader sequence and by

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promoter interference from the 3′ LTR. It is not caused by the genetic background of the tumor or by the surrounding *myc* sequences. The 254-bp CSV leader sequence immediately downstream of the primer-binding site (PBS) enhances transcription from the 5′ LTR in the sense orientation but not in the antisense orientation. Enhancement of transcription from the 5′ LTR leads to a concomitant decrease in transcription from the 3′ LTR of provirus 713. Thus, down regulation of transcription from the 5′ LTR by deletion of the CSV leader may affect c-*myc* activation by the 3′ LTR of provirus 713.

MATERIALS AND METHODS

Plasmid construction. Plasmid p713*myc* was constructed by cloning the entire insert from λ713 between the *Sma*I and *Sst*I sites in vector pGEM-4Z (Promega, P2161). The *Cla*I site in *myc* exon 3 was deleted, and the *Sma*I sites in *myc* intron 1 were converted to *Cla*I sites by insertion of the *Cla*I linker sequence CATCGATG.

Plasmid p713GH was constructed by inserting the *Cla*I-*Cla*I fragment from p713*myc* upstream of a bovine growth hormone cDNA. This fragment contains provirus 713 as well as flanking *myc* intron sequences. The bovine growth hormone cDNA was kindly supplied by F. Rottman, Department of Molecular Biology and Microbiology, Case Western Reserve University.

Plasmid p5′LTR was derived from the *Cla*I-*Cla*I fragment of p713*myc*. The viral sequences between the *Bam*HI sites in provirus 713 were deleted, leaving a solo LTR flanked by *myc* intron sequences. This LTR contains 475 bp from the 5′ LTR and 35 bp from the 3′ LTR. This LTR and the adjoining *myc* intron sequences were then inserted upstream of the bovine growth hormone cDNA to construct plasmid p5′LTR-GH.

To prepare plasmid p5′LTR*env*, the 5′ LTR of provirus 713 and the adjoining inverted *env* sequences and *myc* intron sequences were isolated from the *Cla*I-*Cla*I fragment of p713*myc* (as a *Cla*I-*Hin*cII fragment). This 5′LTR*env* fragment was then inserted upstream of the bovine growth hormone cDNA to construct plasmid p5′LTR*env*GH.

Viral sequences were restored to the 5′ end of provirus 713 by inserting the sequences at the *Bam*HI site in the 5′ LTR. The viral sequences restored to provirus 713 were obtained from spleen necrosis virus clone pB101 (5). The 1.4-kb and 0.3-kb fragments are 1,381 and 327 bp, respectively. The 1.4-kb fragment extends from the *Bam*HI site in the 5′ LTR to the first *Bam*HI site in *gag*. The 0.3-kb fragment extends from the *Bam*HI site in the 5′ LTR to the *Sal*I site in the leader.

A CSV proviral clone was kindly provided by D. Robinson (Department of Molecular Biology and Microbiology, Case Western Reserve University). This provirus was inserted into p5′LTR to add the *myc* intron sequences which flank provirus 713. Provirus CSVΔ0.3 was constructed by inserting the linker sequence GAT CCG GAC TGA ATC CGT AGT ATT TCG GTA CAA CAT TTG GGG GCT CGT CCG GGA TG between the *Bam*HI site in the 5′ LTR and the *Sal*I site in the leader. This deleted the viral sequences between the PBS and the *Sal*I site.

CAT gene expression plasmid pSVCAT$_{pro}$ was purchased from Promega (28). This plasmid has the simian virus 40 (SV40) promoter driving transcription of the CAT gene and the SV40 polyadenylation signal terminating transcription. Because the SV40 enhancer elements are deleted from the promoter in pSVCAT$_{pro}$ (pSV0CAT), this vector can be used to assess the abilities of sequences to enhance transcription from the SV40 early promoter. The *Bam*HI-*Sal*I fragment from the spleen necrosis virus leader sequence was inserted in the sense and antisense orientations at the *Bgl*II, *Stu*I, or *Bam*HI site. The *Bgl*II site is 139 bp upstream of the transcription initiation site; the *Stu*I site is 46 bp downstream of the initiation site; the *Bam*HI site is downstream of the polyadenylation site—1,695 bp downstream of the initiation site.

Cell culture and transfections. D17 cells are an immortalized canine cell line derived from a lung metastasis of an osteosarcoma (ATCC CCL183) (58). This cell line is permissive for expression of reticuloendotheliosis viruses (2, 70). The D17 cells were grown in a 1:1 mixture of M199 and Dulbecco modified Eagle medium (low glucose) with 5% fetal bovine serum, 1% penicillin-streptomycin (GIBCO 600-5075AE), and 1% amphotericin B (Fungizone; GIBCO 600-5295AE). The cells were grown at 37°C in a 5% CO_2 atmosphere.

For stable transfection of D17 cells, a confluent plate of cells was split into 12 plates at 16 h before transfection. These cells were then transfected with both the plasmid DNA (60 μg/15-cm-diameter plate) and SV2neo^r DNA (20 μg/15-cm-diameter plate) by using the calcium phosphate precipitation method (29). The transfected cells were then selected with G418 (800 μg/ml; GIBCO 860-1811), and the resistant colonies were pooled.

Chicken embryo fibroblasts were grown in a 1:1 mixture of M199 and Dulbecco modified Eagle medium (low glucose) with 5% fetal bovine serum, 1% chicken serum, 1% penicillin-streptomycin (GIBCO 600-5075AE), and 1% amphotericin B (GIBCO 600-5295AE). The cells were grown at 37°C in a 5% CO_2 atmosphere.

RNA isolation and Northern (RNA) blotting. RNA was isolated from cells either by pelleting through a cesium chloride cushion or by the acid guanidinium thiocyanate-phenol-chloroform extraction method (12, 13). mRNA was isolated from total RNA prepared by one of these methods. The mRNA was isolated by using oligo(dT) cellulose to bind the mRNA (4).

For Northern analysis, mRNA was fractionated on denaturing (formaldehyde) agarose gels and transferred to nitrocellulose (44). The nitrocellulose blots were then baked at 80°C for 1 to 2 h and subsequently hybridized with the appropriate probe.

Nuclear run-on assays. Nuclei were isolated from stably transfected D17 cells by the methods of Greenberg and Bender and Groudine et al. (30, 31). The isolated nuclei were stored at −70°C.

The run-on assays and hybridization of the run-on products were done as described by Linial et al. (40).

RNA secondary-structure analyses. Potential RNA secondary structures were derived by analysis of RNA sequences as described by Zuker and Stiegler, with modifications described by Turner et al. (68, 72).

CAT assays. The CAT plasmids were linearized by *Sal*I digestion before transfection. Chicken embryo fibroblasts were cotransfected with the CAT plasmid (10 μg/10-cm-diameter dish) and with human growth hormone (hGH) plasmid pXGH5 (5 μg/10-cm-diameter dish) by calcium phosphate precipitation. At 48 h after transfection, the cells were harvested for CAT assays and the medium was harvested for hGH assays. The CAT activity in the extracts was analyzed by the liquid scintillation method (49). The CAT activity levels were adjusted for transfection efficiency by using the results of the quantitation of hGH in the medium.

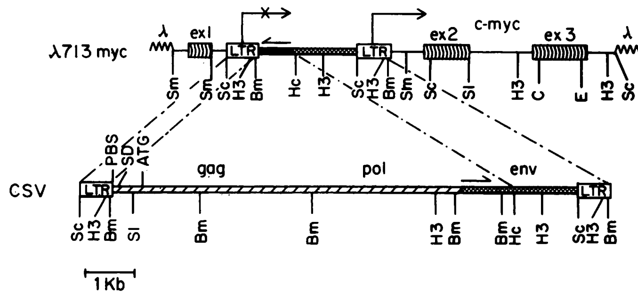

FIG. 1. Restriction endonuclease map of a wild-type CSV provirus and *myc*-associated provirus 713. The lambda clone from which provirus 713 was isolated is shown in the upper diagram. The arrows above the LTRs indicate the transcriptional activities of the respective LTRs. Colinear homologous regions of the two proviruses are connected by dashed lines. About 80% of the CSV viral sequences are deleted from provirus 713. Provirus 713 also contains a portion of the *env* gene in the antisense orientation (■). Abbreviations: Bm, *Bam*HI; C, *Cla*I; E, *Eco*RI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I; Sm, *Sma*I; Sl, *Sal*I; ex1, ex2, and ex3, exons of the c-*myc* locus; SD, splice donor site; ATG, initiation condon of the viral *gag* gene.

The medium harvested from the cells for hGH assays was filtered through a 0.45-μm-pore-size filter to remove any debris. The amount of hGH in the medium was quantitated by using reagents supplied by Nichols Institute Diagnostics (hGH transient gene expression assay system 40-2205) (63).

RESULTS

The structure and expression of provirus 713 in the B-cell lymphoma. As reported previously, provirus 713 is inserted in the first c-*myc* intron in the same transcriptional orientation as c-*myc* (Fig. 1) (67). The structure of provirus 713 was deduced by DNA sequencing and restriction enzyme mapping. The provirus has a deletion which removes ~80% of the internal viral sequences, including the *gag* gene, the *pol* gene, and part of the *env* gene. Both LTRs, however, are intact. The 5′ end of the deletion lies 18 nucleotides downstream of the 5′ LTR and coincides with the 3′ end of the PBS. In addition to this deletion, the provirus contains an inversion of a portion of the *env* gene.

The transcriptional expression of provirus 713 contrasts with that of an intact CSV provirus (67). With an intact CSV provirus, abundant transcription from the 5′ LTR was detected and little transcription from the 3′ LTR was detected; however, with the 713 provirus, no transcription from the 5′ LTR was observed but abundant transcription from the 3′ LTR was seen. The abundant transcripts from the 3′ LTR were linked to *myc* exons II and III and resulted in overexpression of *myc* transcripts in the tumor cells.

Expression pattern of provirus 713 in nonlymphoid cells. To determine whether the unusual transcription pattern of provirus 713 was imposed on it strictly by the B-cell environment, provirus 713 transcription was assessed in D17 cells, a canine osteosarcoma cell line (58). D17 cells were used (i) because reticuloendotheliosis viruses (of which CSV is one) replicate and express efficiently in this cell line (2, 70), (ii) because D17 cells are nonlymphoid, and (iii) because the avian v-*myc* probe does not cross-hybridize to canine c-*myc* under stringent conditions. To facilitate this study, the insert from lambda 713 was transferred into plasmid vector pGEM-4Z, resulting in the construct p713*myc*. p713*myc* was transfected into D17 cells, and the transcription pattern of provirus 713 was analyzed by Northern blot hybridization with a CSV LTR probe, an internal viral probe (713V), and a v-*myc* probe. As shown in Fig. 2, the transcription pattern of provirus 713 in D17 cells is similar to that observed in the tumor, i.e., a paucity of viral genomic transcripts and a large amount of *myc* and LTR transcripts. Thus, the lack of 5′ LTR transcription is not restricted to the B-cell lymphoma environment and can be reproduced in vitro.

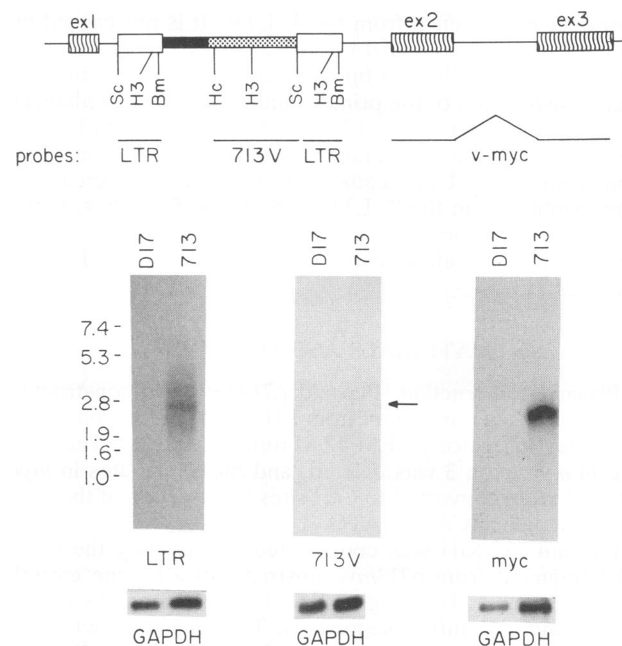

FIG. 2. Northern blot analysis of D17 cells stably transfected with p713*myc*. The map of p713*myc* is shown in the diagram at the top. Poly(A) RNA was isolated from untransfected D17 cells (lane D17) or from p713*myc*-transfected D17 cells (lane 713) and blotted and hybridized to the three probes indicated. If full-length viral transcripts were transcribed from the 5′ LTR, a 2.7-kb band would be expected on hybridization with the 713V probe (arrow). The RNA level in each lane was monitored by subsequent hybridization of each filter with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe. Abbreviations: Bm, *Bam*HI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I. The numbers to the left of the leftmost gel are sizes in kilobases.

Expression of provirus 713 in a non-*myc* environment. The unusual transcription pattern of provirus 713 could also be dictated by the c-*myc* locus. The c-*myc* locus contains a complex array of transcriptional regulatory elements near the first exon-intron junction which might influence proviral transcription (7, 8, 65). To test this possibility, the 713 provirus was removed from the *myc* locus and cloned upstream of a bovine growth hormone cDNA (GH). This construct, p713GH, when transfected into D17 cells, exhibits a transcription pattern similar to that observed with p713*myc*—a poverty of viral genomic transcripts and a large amount of GH and RU5 transcripts (Fig. 3, 713V and GH). The sizes of the GH transcripts are consistent with the notion that transcription originates in the 3′ LTR, and this assumption is confirmed by hybridization with the downstream intron fragment (panel I). Thus, the abundance of transcripts from the 3′ LTR and the lack of transcripts from the 5′ LTR are determined not by the *myc* locus but apparently by provirus 713 itself.

Mechanism of 5′ LTR suppression in provirus 713. The lack

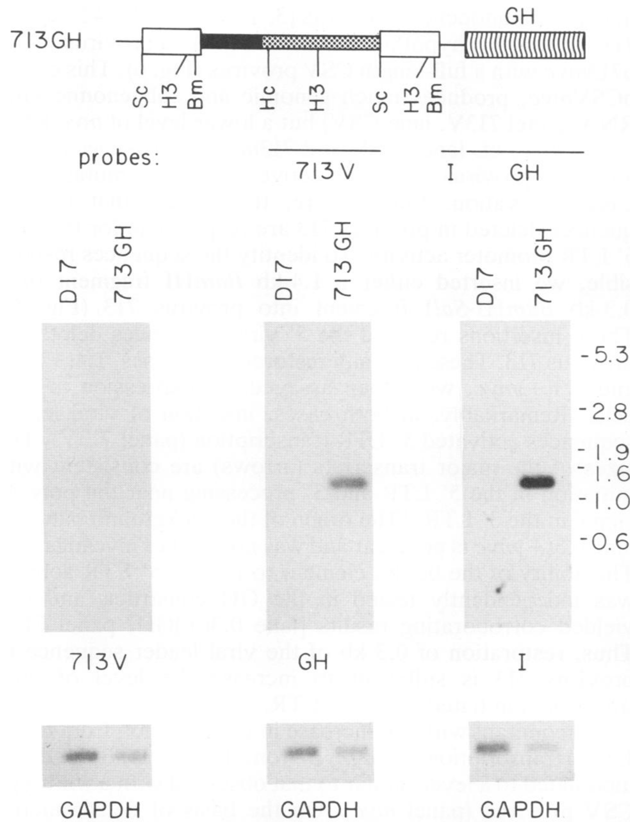

FIG. 3. Northern blot analysis of D17 cells stably transfected with p713GH. Poly(A) RNA was blotted and then hybridized to the three probes indicated. The RNA level in each lane was monitored by subsequent hybridization of each filter with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe. Abbreviations: Bm, *Bam*HI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I. The numbers to the right of the rightmost gel are sizes in kilobases.

of detectable transcripts from the 5′ LTR could be secondary to RNA instability, a transcriptional elongation block, or suppression of transcriptional initiation. To test these possibilities, a nuclear run-on experiment was conducted by using nuclei prepared from D17 cells stably transfected with p713*myc*. As shown in Fig. 4B, the p713*myc* sequences were divided into four probes: a *myc* probe, an LTR probe, and two internal viral probes (V1 and V2). These probes were hybridized with nascent RNAs labelled with [α-^{32}P]UTP. Because only nascent RNAs are labelled, this experiment assayed the quantity of new RNAs being synthesized and was independent of RNA stability. Furthermore, because the quantity of transcripts labelled is proportional to the number of polymerases loaded on the template, an elongation block is manifested by an abrupt drop in RNA synthesis between adjacent template sequences. As shown in Fig. 4A, an abundance of transcripts was detected from the LTR and *myc* regions but few were detected from the V1 and V2 regions. The results, after correction for the sizes of the blotted DNA fragments, are summarized in Fig. 4B. The sensitivity of these transcripts to α-amanitin confirms their synthesis by RNA polymerase II. These data suggest that the lack of detectable transcripts from the 5′ LTR is most likely due to suppression of transcriptional initiation. While the above-described experiment does not rule out RNA instability as an additional factor, the severity of suppression at the initiation level suggests that it is the primary cause.

Modulation of transcription from 5′ and 3′ LTRs. The preceding data suggest that the suppression of the 5′ LTR promoter is at the level of transcriptional initiation; however, sequencing of the 5′ LTR revealed no structural defect (67). To confirm that the 5′ LTR of provirus 713 is functionally competent, the 5′ LTR was inserted upstream of the GH gene (p5′LTR-GH) and checked for the ability to drive transcription of GH (Fig. 5). The 5′ LTR is fully competent to direct transcription of the GH gene. This confirms the

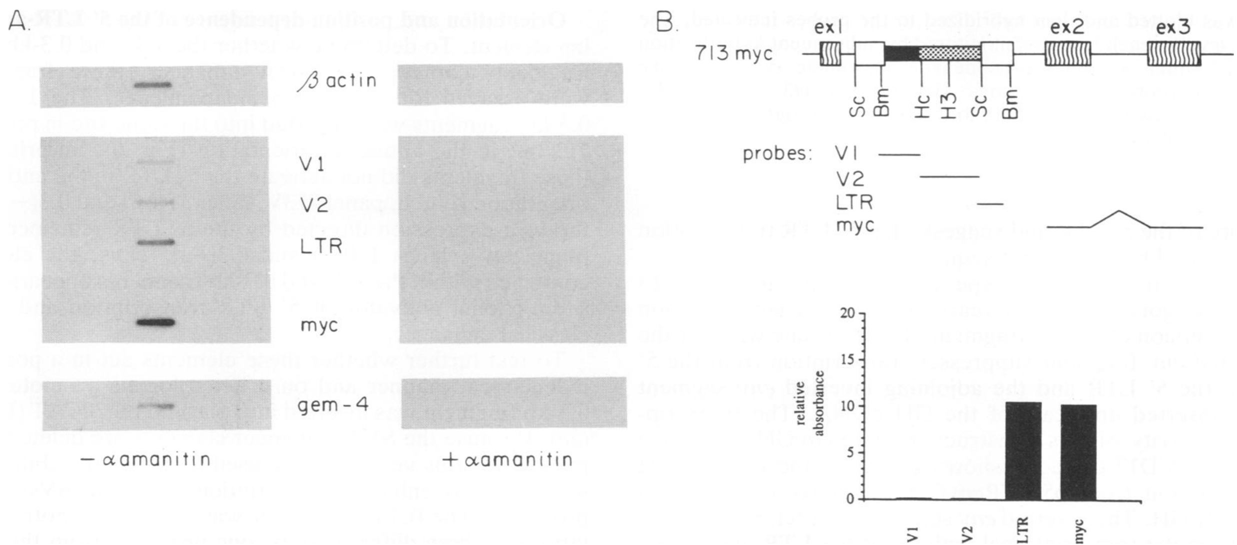

FIG. 4. Nuclear run-on analysis of D17 cells stably transfected with p713*myc*. The probes used for this assay are diagrammed in panel A. V1 and V2 are internal viral probes which contain little of the LTR sequence. The LTR probe contains the U3, R, and U5 regions (*Sac*I to *Bam*HI). The *myc* probe contains exons 2 and 3. (A) Results of hybridization of run-on products to probes. Denatured probes (5 μg of plasmid DNA) were immobilized on nitrocellulose and then hybridized with 10^7 cpm of [α-^{32}P]UTP-labelled RNA per ml. Synthesis of nascent transcripts was inhibited by addition of α-amanitin (2 μg/ml) to the nuclear run-on reaction. (B) Quantitation of hybridization results. The results were quantified by scanning densitometry and then adjusting for the number of UTP molecules per probe. These results are normalized to the hybridization to the plasmid vector alone (gem-4)—i.e., each unit on the ordinate of the graph equals the absorbance of gem-4. Abbreviations: Bm, *Bam*HI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I.

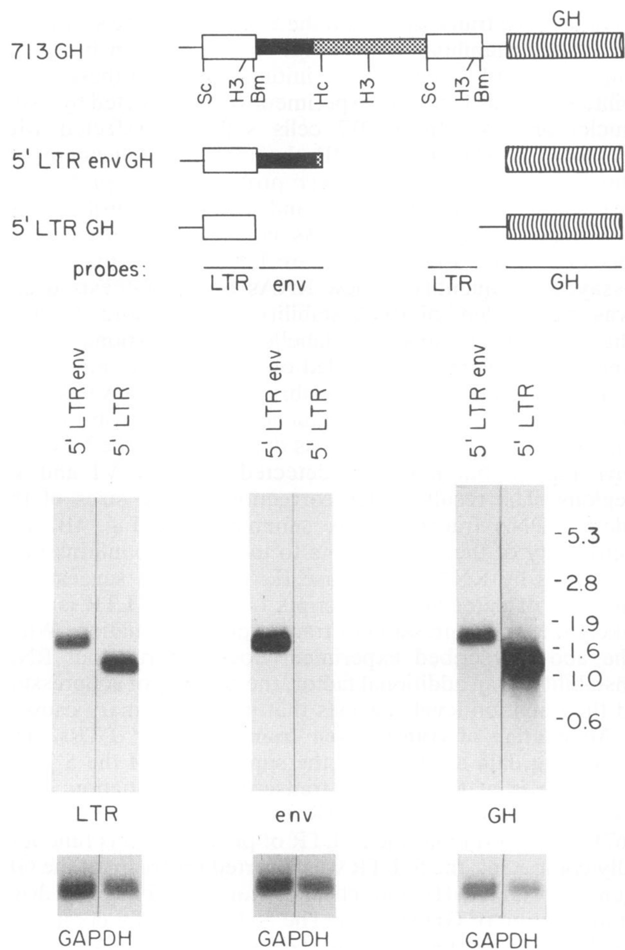

FIG. 5. Northern blot analysis of D17 cells stably transfected with p5′LTR*env*GH or p5′LTR-GH. Poly(A) RNA from each cell line was blotted and then hybridized to the probes indicated. The RNA level in each lane was monitored by subsequent hybridization of each filter with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe. Abbreviations: Bm, *Bam*HI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I. The numbers to the right of the rightmost gel are sizes in kilobases.

fidelity of the 5′ LTR and suggests that 5′ LTR transcription is regulated by other viral sequences.

Compared with a wild-type CSV provirus, provirus 713 has undergone two major rearrangements: a large deletion and inversion of an *env* fragment. To determine whether the inverted *env* fragment suppresses transcription from the 5′ LTR, the 5′ LTR and the adjoining inverted *env* segment were inserted upstream of the GH cDNA. The transcriptional activity of this construct, p5′LTR*env*GH, was then assayed in D17 cells. As shown in Fig. 5, the level of GH transcription from p5′LTR*env*GH is similar to that from p5′LTR-GH. The inverted *env* sequence, therefore, has little effect on the transcriptional activity of the LTR and apparently does not affect the transcriptional inhibition of the 5′ LTR in provirus 713.

Alternatively, the transcriptional inhibition of the 5′ LTR in provirus 713 might be explained by deletion of *cis* regulatory elements. Sequences downstream of the transcriptional initiation site regulate transcription in several retroviruses, including ALV, Moloney murine leukemia virus, and human immunodeficiency virus (3, 11, 17, 34, 41, 42, 61, 66, 71). To test this hypothesis, we first replaced provirus 713 in p713*myc* with a full-length CSV provirus (Fig. 6). This clone, pCSV*myc*, produces much genomic and subgenomic viral RNA (panel 713V, lane CSV) but a lower level of *myc* RNA (panel *myc*, cf. lanes CSV and 713*myc*). This suggests that an intact provirus is an ineffective insertional mutagen for c-*myc* activation. Furthermore, this argues that the sequences deleted in provirus 713 are responsible for the high 5′ LTR promoter activity. To identify the sequences responsible, we inserted either a 1.4-kb *Bam*HI fragment or a 0.3-kb *Bam*HI-*Sal*I fragment into provirus 713 (Fig. 6). These insertions restored the 5′ viral sequences deleted in provirus 713. These partially restored proviruses, 1.4(+)*myc* and 0.3(+)*myc*, were then assayed for expression in D17 cells. Remarkably, in both cases, insertion of viral leader sequences activated 5′ LTR transcription (panel 713V). The sizes of the major transcripts (arrows) are consistent with initiation in the 5′ LTR and 3′ processing near the poly(A) signal in the 3′ LTR. [The origin of the background bands in lane 0.3(+)*myc* is not clear and was not further investigated.] The ability of the 0.3-kb element to restore 5′ LTR activity was independently tested in the GH construct, and this yielded corroborating results [lane 0.3(+)GH, panel 713]. Thus, restoration of 0.3 kb of the viral leader sequence to provirus 713 is sufficient to increase the level of viral transcripts initiated in the 5′ LTR.

Concomitant with the increase in transcription from the 5′ LTR, transcription of c-*myc* from the 3′ LTR is down modulated to a level similar to that observed with a wild-type CSV provirus (panel *myc*). On the basis of densitometric tracing, the down modulation is about 5- to 10-fold. An inverse relationship between *myc* expression and viral expression is evident in Fig. 6. This finding suggests that transcription from the 5′ LTR interferes with transcription from the 3′ LTR. Furthermore, inactivation of transcription from the 5′ LTR appears to facilitate proviral expression of the c-*myc* gene by 3′ LTR promotion.

Orientation and position dependence of the 5′ LTR-activating element. To determine whether the 1.4- and 0.3-kb viral fragments contain a classical enhancer, these fragments were assayed for orientation independence. The 1.4- and 0.3-kb fragments were inserted into the same site in provirus 713 but in the antisense orientation (Fig. 6). Surprisingly, these fragments did not activate the 5′ LTR in the antisense orientation [Fig. 6, panel 713V, lanes 1.4(−) and 0.3(−)] and the *myc* expression directed by the 3′ LTR remained high [panel *myc*, lanes 1.4(−) and 0.3(−)]. Thus, the element contained within the 1.4- and 0.3-kb fragments appears to be a directional activator of 5′ LTR transcription and not a classical enhancer.

To test further whether these elements act in a position-independent manner and on a heterologous promoter, the 0.3-kb fragment was inserted into plasmid pSV0CAT (Fig. 7) (28). Because the SV40 enhancer elements are deleted from pSV0CAT, this vector can be used to assess the abilities of sequences to enhance transcription from the SV40 early promoter. The 0.3-kb fragment was inserted in both orientations at three different sites: one upstream from the transcriptional start site (*Bgl*II), one immediately downstream from the start site (*Stu*I), and one after the poly(A) signal (*Bam*HI). The CAT activity of these plasmids was assayed after transfection into chicken embryo fibroblasts. As shown in Fig. 7, the CSV leader sequence activates CAT expression only when inserted immediately downstream from the start site and in the sense orientation. An RNase protection

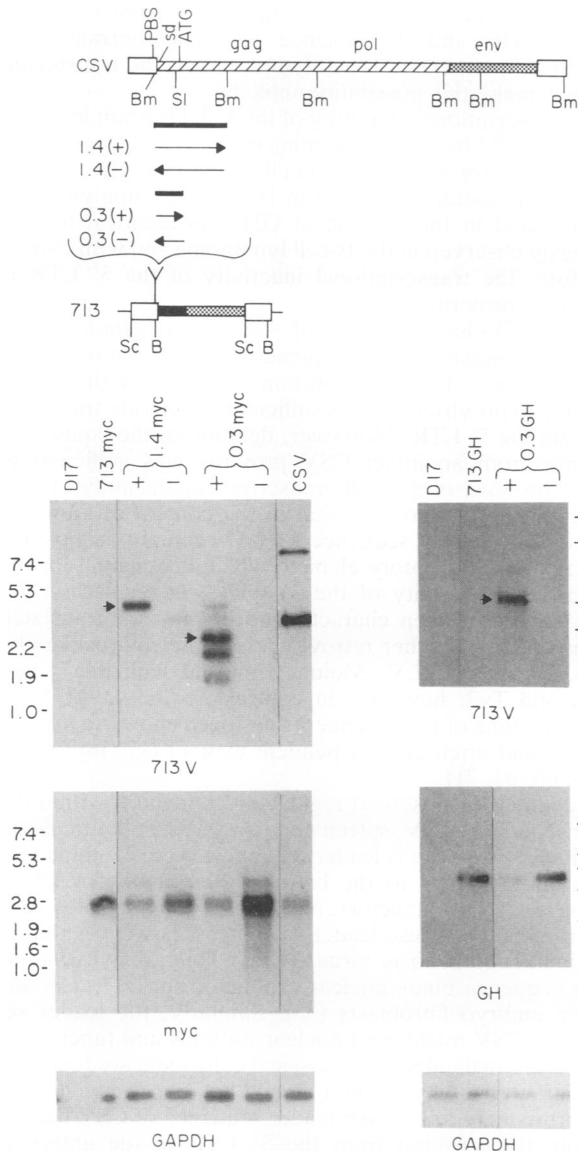

FIG. 6. Northern blot analysis of transcription from provirus 713 derivatives with viral leader sequences inserted. Either 1.4-kb (*Bam*HI-*Bam*HI) or 0.3-kb (*Bam*HI-*Sal*I) fragments of the 5′ viral sequence were inserted at the 5′ *Bam*HI site of provirus 713 in either the sense (+) or the antisense (−) orientation. Poly(A) RNA from each cell line was blotted and then hybridized with a viral genomic probe (713V) and a *myc* or GH probe. The RNA level in each lane was monitored by subsequent hybridization of each filter with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe. Lane CSV contained RNA from D17 cells transfected with pCSV*myc*, a p713*myc*-based plasmid in which provirus 713 was replaced by the full-length CSV provirus. Abbreviations: Bm, *Bam*HI; H3, *Hin*dIII; Hc, *Hin*cII; Sc, *Sac*I; Sl, *Sal*I. The numbers to the sides are sizes in kilobases. sd, splice donor site.

experiment confirmed that the difference in CAT activity was reflected by the level of CAT transcripts (data not shown). These data confirmed the unusual position- and orientation-dependent nature of the transcriptional element within the 0.3-kb fragment.

Function of the 5′ LTR activating element in a wild-type CSV provirus. The above data showed that the CSV leader

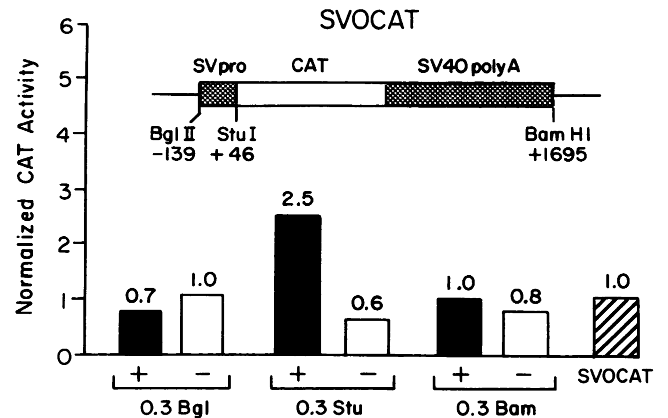

FIG. 7. Effect of the 0.3-kb viral leader sequence on SV40 early promoter activity in chicken embryo fibroblasts. The fragments containing the SV40 promoter (SVpro) and polyadenylation site (SV40polyA) are indicated. The SV40 enhancer elements are deleted from the promoter in pSV0CAT. The viral leader sequence was inserted into this vector in both the sense (+) and the antisense (−) orientations at the *Bgl*II, *Stu*I, and *Bam*HI sites. The positions of these sites relative to the mRNA start are indicated. Chicken embryo fibroblasts were transfected with each construct. The effect of the leader sequence on the SV40 promoter was quantitated by liquid scintillation measurement of CAT activity in chicken embryo fibroblast extracts. The results are adjusted for transfection efficiency and normalized to the CAT activity of the pSV0CAT vector. The plus and minus signs indicate, respectively, the sense and antisense orientations of the 0.3-kb insert.

sequence is necessary for transcription from the 5′ LTR in a deleted provirus. To test whether this holds true for the wild-type CSV provirus as well, 254 bp of the leader sequence were deleted from a full-length CSV provirus. The 5′ end of the deletion coincides with the 3′ end of the PBS—the same point at which the deletion in provirus 713 begins. The 3′ end of the deletion coincides with the *Sal*I site in the leader. Deletion of this sequence severely cripples the ability of the 5′ LTR to transcribe viral genes (Fig. 8). This suggests that the 254-bp leader region is functionally important for wild-type provirus transcription.

DISCUSSION

Retroviral proviruses are complex transcription units which contain a promoter in each LTR. The modulation of the relative transcriptional activity of these tandem promoters is essential for viral replication and for activation of host proto-oncogenes by promoter insertion. The mechanisms by which proviruses regulate the LTR promoters are poorly defined; however, a few studies have provided some clues to the interactions between tandem promoters. In tandem promoters, the transcriptional activity of one promoter frequently interferes with the activity of the other. The promoter occlusion model of transcriptional interference, which was initially described in *Escherichia coli*, defines a unidirectional form of transcriptional interference in which the upstream promoter interferes with transcription from the downstream promoter by blocking the access of RNA polymerase to the downstream promoter (1, 16, 32, 55, 69). The epigenetic suppression model of transcriptional interference, which was first proposed by Emerman and Temin to explain the interaction between promoters of selectable genes, describes a bidirectional form of transcriptional interference in

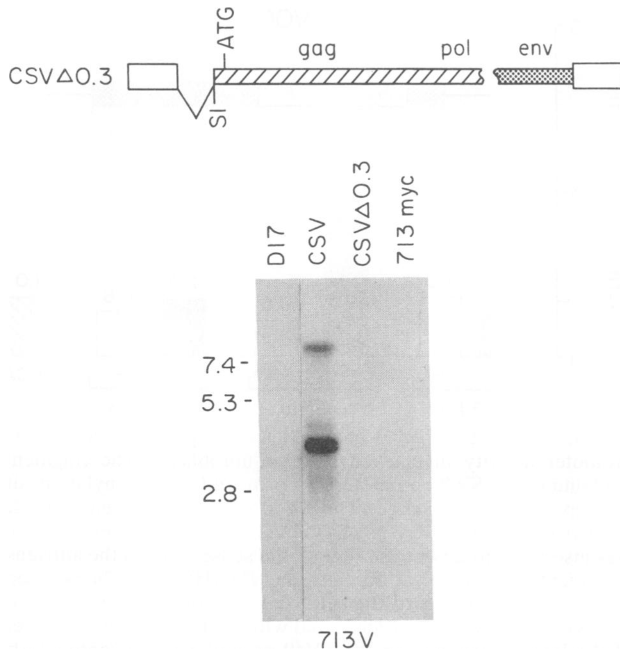

FIG. 8. Northern blot analysis of transcription from a wild-type CSV provirus and a CSV provirus with a deleted leader sequence (CSVΔ0.3). The deletion begins immediately 3′ of the PBS and extends to the *Sal*I site (SI). Poly(A) RNA from stably transfected D17 cells was blotted and then hybridized with a viral genomic probe (713V). The number on the left are sizes in kilobases.

which the selected promoter interferes with transcription from an adjacent upstream or downstream promoter (21–23). Promoter competition, as observed in *Saccharomyces cerevisiae* and *Drosophila melanogaster*, defines a bidirectional form of transcriptional interference in which the promoter with the highest affinity for a limiting factor inhibits transcription from adjacent promoters (20, 26, 36, 46, 52, 64).

A striking feature of retroviral modulation of tandem promoters is the discrimination between promoters in apparently identical LTRs. In replicating wild-type avian leukosis viruses, the transcriptional activity of the 5′ LTR promoter is more than 50-fold greater than that of the 3′ LTR (34). This differential activity of the 5′ and 3′ LTRs is at least partially accounted for by transcriptional interference via promoter occlusion (16).

In contrast to replicating proviruses, *myc*-associated proviruses in avian B-cell lymphomas exhibit a high level of transcription from the 3′ LTR and little or no detectable transcription from the 5′ LTR (27, 67). Structural analysis of *myc*-associated proviruses in B-cell lymphomas reveals that most have deletions which map to the 5′ end of the viral genome (25, 27, 48, 53, 59, 67). If these deletions alter the 5′ LTR, they might account for the reduced transcriptional activity of the 5′ LTR. To assess this possibility, we isolated and characterized *myc*-associated CSV provirus 713. This provirus has an intact 5′ LTR which is fully functional when removed from the provirus (57, 67; Fig. 5).

The 5′ LTR promoter of provirus 713 appears to be suppressed at the level of transcriptional initiation. Nuclear run-on analyses of p713*myc* detected few nascent transcripts from the 5′ LTR of provirus 713. Although premature termination of transcription within the 5′ LTR cannot be ruled out by this assay because of the redundancy of the 5′ and 3′ LTRs, the abundant transcription from the 5′ LTR in p5′LTR*env*GH and the absence of small aberrant LTR transcripts in either the B-cell lymphoma or the transfected D17 cells make this possibility unlikely.

The transcriptional inactivity of the 5′ LTR is not imposed on provirus 713 by the surrounding *myc* sequences or by the lymphoid environment of the B-cell lymphoma. The proviral transcription pattern observed in D17 cells, a nonlymphoid cell line, and in the context of GH was identical to that previously observed in the B-cell lymphoma and with c-*myc*. Therefore, the transcriptional inactivity of the 5′ LTR is inherent to provirus 713.

Provirus 713 has a deletion of ~80% of its genome. This deletion encompasses viral sequences essential for transcription from the 5′ LTR. Restoration of 0.3 kb of the leader sequence to provirus 713 was sufficient to activate transcription from the 5′ LTR. Moreover, deletion of the equivalent sequence from an intact CSV provirus was sufficient to reduce the level of 5′ LTR transcripts significantly. These findings suggest, therefore, that in the context of an intact provirus, the leader sequence of CSV contains a positive transcriptional regulatory element which is essential for the transcriptional activity of the 5′ LTR. *cis* regulatory elements have also been characterized in the 5′ untranslated regions of several other retroviruses and retroviruslike elements, including ALV, Moloney murine leukemia virus, *gypsy*, and Ty2; however, in contrast to the 0.3-kb CSV sequence, none of these elements has been shown to act in a position- and orientation-dependent manner (17, 18, 24, 41, 46, 56, 60, 64, 71).

To define the conserved regulatory element(s) within the leader sequence, CSV, spleen necrosis virus, and reticuloendotheliosis virus type A leader sequences were examined for regions homologous to the binding sites of known DNA-binding transcription factors. No potential binding sites were conserved among these leader sequences; however, studies of reticuloendotheliosis virus type A have shown that its leader sequence binds nuclear proteins from D17 cells and chicken embryo fibroblasts (35). Similarly, the leader sequence of CSV might bind nuclear proteins and function as a transcriptional enhancer. Classical enhancers are functionally characterized by orientation and position independence (6). Surprisingly, the 0.3-kb leader sequence of CSV did not promote transcription from the 5′ LTR in the antisense orientation. Equally provocative is the fact that this sequence enhances transcription only when placed downstream from an SV40 promoter and in the sense orientation, although the activation (2.5-fold) of the SV40 promoter is somewhat less than that of the CSV LTR (greater than 10-fold). This difference could be due to the promoter specificity of the 0.3-kb leader sequence. Although it is less likely, the leader sequence could also have a differential stabilizing effect on *myc* and CAT RNAs. At any rate, the 0.3-kb leader sequence therefore does not contain a classical enhancer but an element which positively regulates 5′ LTR transcription in an orientation- and position-dependent manner.

The orientation- and position-dependent regulatory properties of the CSV leader sequence are reminiscent of those described for the *trans*-acting response (TAR) element of HIV. The TAR element is an RNA enhancer, increasing both the rate of transcriptional initiation and the processivity of RNA polymerase (9, 15, 39, 45, 62). Like the CSV leader sequence, it enhances transcription only when placed downstream from a promoter and in the sense orientation (47, 54). The TAR element enhances transcription from the human

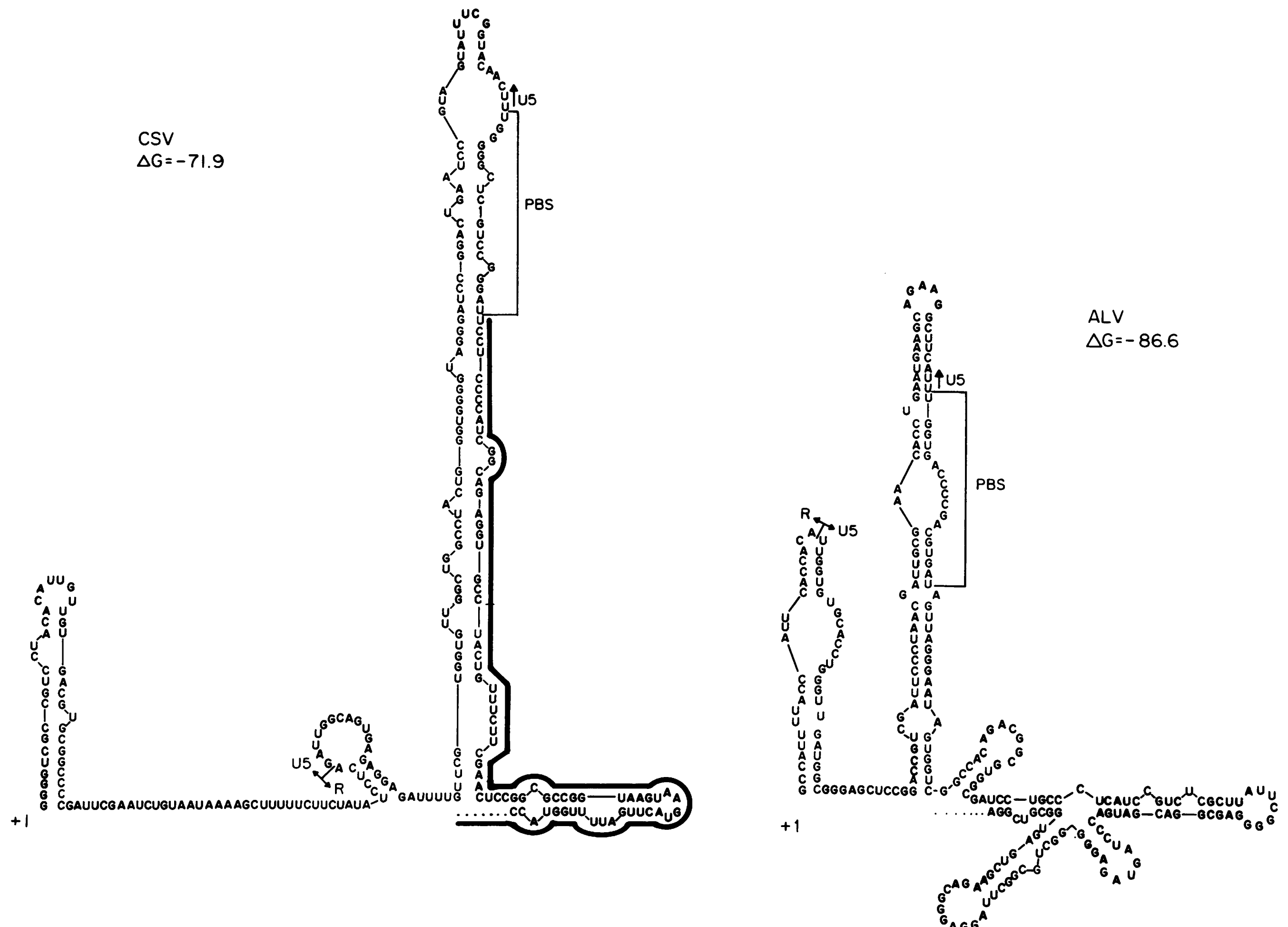

FIG. 9. Potential RNA secondary structures for the leader sequences in transcripts from CSV and ALV. The secondary structures were derived by the algorithm of Zuker and Stiegler with modifications described by Turner et al. (68, 72). The PBS, direct repeat regions (R), and unique 5′ regions (U5) are indicated. The CSV sequences deleted in provirus 713 are highlighted in the CSV structure (left).

immunodeficiency virus LTR through the interaction of its transcript with cellular transcription factors and the HIV *tat* gene product. Similarly, even though CSV does not encode a Tat-like protein, the CSV leader sequences could promote 5′ LTR transcription via interactions with cellular transcription factors. The interaction between TAR and its associated proteins is dictated by both the primary sequence and the hairpin structure of the TAR transcript (9, 15, 19, 39, 45, 47). Computer analysis of CSV leader sequences has shown that stable hairpin structures could also form in transcripts of this region (Fig. 9) (14). This structure is disrupted by the deletion within provirus 713, suggesting that the integrity of this structure is important for the transcriptional activity of the 5′ LTR (67). Intriguingly, a similar structure is also present in the corresponding region of ALV (Fig. 9). This secondary-structure conservation suggests that RNA enhancers are a common feature of retroviral regulation of 5′ LTR transcription. The presence of an RNA enhancer within the viral leader would allow the provirus to distinguish between promoters in identical LTRs and preferentially to promote transcription from the 5′ LTR.

Interestingly, concomitant with the activation of the 5′ LTR in provirus 713, transcription from its 3′ LTR declined significantly. This finding is consistent with the previous findings of transcriptional interference between retroviral 5′ and 3′ LTRs (16, 34). Furthermore, it provides experimental support for the notion that inactivation of the 5′ LTR is important for retroviral activation of a proto-oncogene by 3′ LTR promoter insertion. Within provirus 713, inactivation of the 5′ LTR is probably accomplished by a combination of two mechanisms: (i) deletion of an essential transcriptional element in the leader sequence and (ii) epigenetic suppression of the 5′ LTR by the transcriptional activity of the 3′ LTR. According to this hypothesis, the deletion of the leader sequence would reduce the potency of the 5′ LTR promoter such that the 3′ LTR promoter would be stronger (owing to the presence of an enhancer immediately before the 3′ LTR [10, 35]); the increased transcriptional activity of the 3′ LTR would then further repress transcription from the 5′ LTR by epigenetic suppression. In sum, we have identified a CSV leader sequence that is required both for the transcriptional dominance of the 5′ LTR over the 3′ LTR and for proviral transcription from the 5′ LTR. While the promoter activity of a solo LTR should also be augmented by this element, its effect is most pronounced when the LTR is negatively interfered with by a downstream promoter, as in the context of an intact provirus.

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