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Characterization of Rous Sarcoma Virus Sequences Essential for Viral Gene Expression

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Using the *Escherichia coli lacZ* gene product β-galactosidase as an indicator of gene expression, we analyzed sequences that are required for expression of the Rous sarcoma virus (RSV) genome in avian cells. The RSV long terminal repeat (LTR) and leader region were sufficient to direct the synthesis of high levels of enzymatically active *gag-lacZ* fusion proteins. A portion of U_3 greater than 140 nucleotides upstream from the cap site was essential for gene expression. This element functioned in either orientation, but its activity was attenuated when it was relocated further away from the cap site. The insertion of exogenous LTRs 3′ of *lacZ* augmented the expression of that gene by increasing the level of stable *gag-lacZ* transcripts. Furthermore, 3′ LTRs could partially compensate for certain defects within the 5′ LTR. Insertion of various fragmentary LTRs allowed the identification of at least three synergistically acting domains within the 3′ LTR that influence gene expression. Interestingly, the *gag-lacZ* expression was only stimulated by a 3′ LTR when the exogenous 3′-untranslated region was adjacent. Our results imply that the two LTRs of a provirus interact in a complex manner to promote high levels of stable transcripts. It was also found that *gag-lacZ* expression was independent of viral gene products, suggesting that *trans*-activation is not a key mechanism regulating RSV expression in avian cells.

The retrovirus life cycle includes reverse transcription of the RNA genome into DNA and subsequent integration of the DNA as a linear provirus. The structural genes encoded by the provirus are flanked by long terminal repeats (LTRs) which are derived in a complicated fashion from both ends of the RNA genome during the reverse transcription process. Each LTR has the structure U_3RU_5, where U_3 and U_5 are copied from sequences unique to either the 3′ or 5′ end of the genome and R represents a terminal redundancy (usually quite short) found at the ends of the RNA (3). New viral genomes and mRNAs are synthesized by host RNA polymerase II, with the integrated provirus used as a template (32). The site of transcription initiation, or cap site, is coincident with the 5′ end of R within the upstream LTR (i.e., the LTR that directs viral gene expression); transcripts are polyadenylated at the 3′ end of R within the downstream LTR (33). This functional distinction is not readily explained by features of primary sequence, for as a consequence of reverse transcription, the two LTRs of a given provirus must be perfect repeats.

Several retroviral LTRs have been subjected to intensive scrutiny by both comparative sequence analysis as well as directed mutagenesis, and DNA sequence motifs considered important for correct initiation of eucaryotic transcription have been identified within several U_3 regions that have been examined (for a review, see reference 32). For instance, canonical CAAT and TATA boxes can be identified within the Rous sarcoma virus (RSV) LTR (Fig. 1), although the GC-rich stretches associated with other promoter elements (18) are not detected. It seems that the CAAT element is dispensable but that removal of the TATA motif results in a decreased fidelity of mRNA 5′ ends without a substantial reduction in transcription initiation rate (10). An additional regulatory element has been ascribed to the RSV LTR: a transcriptional enhancer. Enhancers have been found associated with both viral and cellular genes; their distinguishing features are the augmentation of gene expression in *cis* in a manner not strictly dependent on orientation or position relative to the transcribed unit (for a review, see reference 14). Using various assays of transient and stable gene expression, most workers have localized enhancer activity to within roughly the 5′ half of the RSV U_3 region (6, 15, 16, 36).

Intriguingly, some workers have reported that sequences near the 3′ end of the provirus, adjacent to U_3, contribute to the enhancer activity of the RSV LTR (15, 16). It has also been suggested that these sequences, which we refer to as the 3′-untranslated (UT) region, are determinants of viral pathogenicity (24, 30). Additionally, deletion of the region was associated with a reduction in stable mRNA levels (29). Despite efforts to attribute various activities to the 3′-UT, however, no firm role for the region has emerged.

It has also been reported that the accumulation of RSV LTR-directed transcripts is influenced by the presence of viral sequences in *trans* (2). Such a *trans*-activation mechanism is reminiscent of that proposed for human T-cell lymphotropic virus types I and II. A region of the genome of these viruses specifically stimulates the activity of the viral LTR when present in *trans* (27, 28). It has been suggested that a functionally similar protein is translated from an alternative reading frame which overlaps the RSV *gag* gene (2), but this putative *trans*-activator has not been detected.

The terminal regions of the viral genome are richly endowed with signals that are essential for virus replication and gene expression. We sought to establish the boundaries and properties of the transcriptional control elements of the RSV LTRs, as well as to determine whether viral gene expression requires viral gene products. Results of previous work

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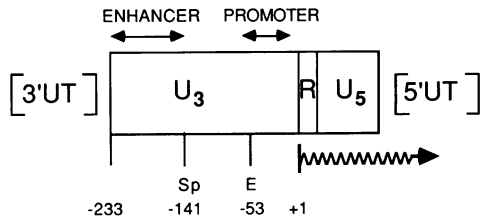

FIG. 1. Structure of the RSV LTR. The schematic of the Pr-RSV-LTR has important structural features indicated, as well as pertinent restriction sites. The promoter region includes the region of homology to the CAAT and TATA consensus sequences (10). The region designated as the enhancer corresponds to a functional domain delineated herein and elsewhere (6, 15). The numbers refer to the published sequence of Pr-RSV-C (25). The bracketed regions indicate the untranslated sequences that flank either the 5′ LTR (5′-UT) or the 3′ LTR (3′-UT). Restricton sites: E, *Eco*RI; Sp, *Sph*I.

demonstrate that in avian embryo fibroblasts, the RSV LTR efficiently directs the expression of a *gag-lacZ* fusion gene that is situated within a proviral context (23). Synthesis of the fusion gene product was readily quantitated by assaying β-galactosidase activity in cell lysates, and levels of enzyme activity reflected rates of RNA synthesis for the plasmids that were compared. Using this assay system, we narrowly defined the LTR sequences that are required in *cis* for efficient *gag-lacZ* expression in avian cells. In addition to a requirement for an intact 5′ LTR, it was determined that a 3′ LTR can greatly influence viral transcript levels and that this activity is modulated by 3′-UT sequences. It was also ascertained that viral gene products are not essential for the transcriptional activity of the RSV LTR.

MATERIALS AND METHODS

Bacterial strains and sources of plasmids. *Escherichia coli* K-12 RV200 (*thi ΔlacX74 rpsL200*) was used for all bacterial manipulations. Plasmids used for the constructions described in this paper were pATV-8, a molecular clone of Pr-RSV-C (13); pRAV-1, a molecular clone of Rous-associated virus-1 (RAV-1) (26); and pRAV-0, a molecular clone of Rous-associated virus-0 (RAV-0) (a gift from P. N. Tsichlis). The *lac* fusion vector pZ-1 (23) requires both transcription and translation initiation signals to yield active β-galactosidase fusion proteins. Also used were pNT-4, a molecular clone of a tdPr-RSV-B derivative (9), and p34, a recombinant between pRAV-1 and pRAV-0 which was generated by homologous recombination in bacteria (J. Coffin, unpublished data). pGEM-1 was obtained from Promega Biotech.

Construction of *gag-lacZ*-encoding plasmids. Restriction endonucleases and T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.), as well as the large (Klenow) fragment of *E. coli* DNA polymerase I (New England Nuclear Corp., Boston, Mass.), were used in accordance with recommendations of the manufacturers. All plasmids contained at least 53 bases of U_3; all of the R, U_5, and leader sequences; and 156 nucleotides of *gag*. These sequences are sufficient to confer a $LacZ^+$ phenotype onto bacteria carrying such plasmids (20); this feature minimized the risk of accumulation of point mutations within the structural gene.

The plasmid pPN-7 (Fig. 2), which has been described previously (23), contained RSV DNA from the *Bgl*II site at nucleotide −1556 in *src* to the *Bam*HI site at nucleotide 532 in *gag* (1 is the mRNA cap site nucleotide) (25). pPN-7a and pPN-8 were derived from pPN-7 by deletion of a *Bgl*II to *Eco*RI fragment (nucleotides −1556 to −53) or an *Sph*I fragment (nucleotides −725 to −141), respectively (Fig. 2). Plasmids pPN-10, pPN-11, and pPN-12 were generated from pPN-7, pPN-7a, and pPN-8, respectively, by the insertion of a *Sal*I fragment of pRAV-1 at the *Sal*I site 3′ of *lacZ*. The RAV-1 fragment (approximately 2 kilobases) extended from a site within the *env* gene and included two tandem LTRs as well as untranslated leader sequences 5′ to the *Sac*I site at nucleotide 255 (26). The fragment also contains a small (about 100 bases) fragment of mouse mammary tumor virus DNA and the 276-base-pair *Bam*HI to *Sal*I fragment of pBR322.

To probe the effect of a downstream LTR in more detail, plasmids were constructed with LTR-containing restriction fragments inserted into pPN-8 or pPN-7 at either the *Bam*HI or the *Sal*I sites immediately 3′ of *lacZ*. All inserts placed the viral sequences 3′ of *lacZ* in the same transcriptional orientation as the upstream viral sequences. The various inserted fragments are diagrammed in Fig. 5 and 6 and are described below; for construction convenience, some fragments contained additional pBR322 sequences. The fragment of pRAV-1 was described above. The inserts that divided the LTR at the *Sph*I site were first subcloned at the *Sph*I site of pBR322 and then removed as *Bam*HI to *Sal*I fragments for insertion 3′ of *lacZ*. pPN-16 and pPN-17 contained a 584-nucleotide *Sph*I fragment (nucleotides −725 to −141), while pPN-19 and pPN-20 contained an *Sph*I fragment extending from −141 to 1006 (in *gag*). A pRAV-1 fragment extending from the *Sal*I site in *env* to the *Eco*RI site at −53 in U_3 was inserted along with the *Eco*RI to *Sal*I portion of pBR322, generating pPN-23 and pPN-24. The pRAV-0 fragment of pPN-27 and pPN-28 contained two LTRs and extended from the *Sal*I site in *env* to the *Xho*I site in *gag*. Insertion of a similar fragment derived from pNT-4, containing a single Pr-RSV-related LTR (9), resulted in pPN-26 and pPN-29. Finally, a *Sal*I to *Xho*I fragment from p34, a RAV-0 and RAV-1 recombinant, was employed in the construction of pPN-30 and pPN-31.

To facilitate manipulations within U_3, pPN-13 was constructed from pPN-7 by deletion of a *Pst*I fragment (nucleotides −1242 to −630); this rendered the *Sph*I site at −141 unique. Nucleotides −140 to −137 were removed by treatment of *Sph*I-linearized pPN-13 with the large (Klenow) fragment of *E. coli* DNA polymerase I. Self-ligation of this blunt-ended fragment produced pPN-13d2; ligation with phosphorylated *Hin*dIII linkers (New England BioLabs) resulted in pPN-13i11 and pPN-13i12. The sequence in the vicinity of nucleotide −141 of these last three plasmids was verified by DNA sequence analysis (17). Two additional plasmids were generated from *Hin*dIII-cleaved pPN-13i11; pPN-13i13 and pPN-13i14 contained a 130-base-pair *Hin*dIII fragment from the *pol* region of pATV-8 (nucleotides 2740 to 2870) inserted in either orientation (see Table 2).

Inversion of the −725 to −141 *Sph*I fragment produced PN-7R (see Fig. 4). pPN-21 was derived from pPN-7, with sequences between −657 and −136 removed and with concomitant insertion of a *Hin*dIII linker. Two *Hin*dIII-*Sph*I fragments were inserted in place of the *Sph*I-*Hin*dIII fragment of pPN-21 (see Fig. 4). The pPN-21i1 insert extended from the *Sph*I site at nucleotide −141 to the *Bal*I site at nucleotide −301, which was converted to a *Hin*dIII site (P. Hopper, personal communication). The pPN-21i2 insertion extended from the *Sph*I site at nucleotide −141 to the *Mst*II site at nucleotide −408 (also converted to a *Hin*dIII site).

Transfection and enzyme assay. Plasmid DNAs were pre-

pared as described previously (23), and the DNA concentration was determined spectrophotometrically. DEAE-dextran-mediated transfection of turkey embryo fibroblasts with 1 to 5 μg of closed circular DNA per well (diameter, 35 mm) or dish (diameter, 60 mm) was essentially as described previously (23), but in some experiments the concentration of the facilitator was decreased to 0.2 mg/ml, resulting in higher levels of enzyme activity per microgram of input DNA. Cultures destined for RNA analysis were transfected in the presence of 80 μM chloroquine.

β-Galactosidase activity was measured 3 days after transfection (23). Briefly, cells were lysed in buffered sodium dodecyl sulfate, and then assay buffer and substrate (*o*-nitrophenyl-β-D-galactoside) were added. Samples were incubated at 37°C until yellow color developed, and hydrolysis of substrate was quantitated spectrophotometrically. Due to some variability from one assay to the next, each experiment included positive and negative control plasmids as reference points. Usually, the raw values, in units of β-galactosidase induced per microgram of input DNA, are presented; in some cases the values have been normalized to percentage of positive control levels (1 U is defined as the A_{420} multiplied by 380 and then divided by minutes of incubation time). All plasmids were tested at least three times, and at least two preparations of each plasmid were tested, although only representative values are presented.

Analysis of transient RNA expression. Total RNA was prepared from cells 72 h posttransfection (23) and subjected to exhaustive DNase I digestion to remove plasmid DNA. Probes used for nuclease protection experiments were radiolabeled, single-stranded RNAs synthesized in vitro by the action of SP6 RNA polymerase (19). The DNA template was a *Bam*HI (nucleotide 532) to *Eco*RI (nucleotide −53) restriction fragment of pATV-8 cloned into pGEM-1 and linearized with *Eco*RI. RNA probes were rid of template DNA by electrophoresis under denaturing conditions.

Mixtures of cellular and probe RNAs were hybridized as described previously (23) in the presence of 20 μg of yeast RNA as carrier. Following ovenight incubation at 50°C, unhybridized material was removed by treatment with a mixture of RNase A and RNase T1 in 0.5 M NaCl–50 mM Tris hydrochloride (pH 7.5). Samples were extracted with phenol and ethanol precipitated, and protected probe fragments were analyzed by electrophoresis through 4% polyacrylamide under denaturing conditions. Dried gels were exposed to Kodak XAR 5 film at −70°C with an intensifying screen.

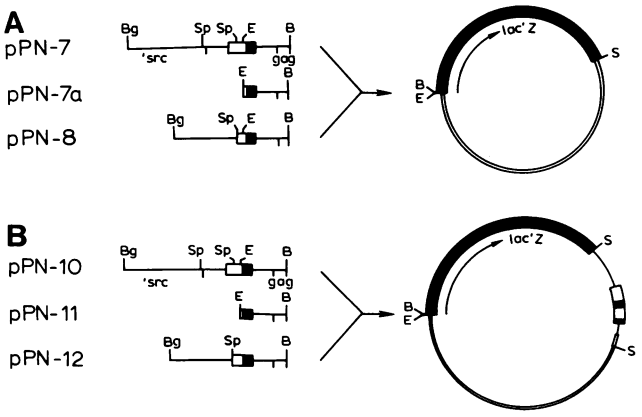

FIG. 2. (A) Plasmids which have LTR sequences solely at the 5′ side of *lacZ*. All viral inserts contained at least 53 bases of U_3 in addition to R, U_5, and leader sequences. All inserts also provide the *gag* amino terminus in the correct reading frame to permit the synthesis of a $p19^{gag}$-β-galactosidase fusion protein (23). (B) A second set of plasmids which have additional LTR sequences 3′ of the indicator gene. Relevant restriction sites are indicated, as is the direction of *lacZ* transcription. Solid bar, *lacZ*; open bar, pBR322 sequences; thin line, viral sequences. LTRs are boxed, with the open portion denoting U_3 sequences. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; Sp, *Sph*I.

TABLE 1. Effect of 3′ LTR insertions

Plasmid	5′ LTR	3′ LTR	β-Galactosidase activity (U/μg)[a]
pPN-7	Intact	−	1.7
pPN-7a	−53 to 101	−	0.01
pPN-8	−141 to 101	−	0.01
pPN-10	Intact	+	9.2
pPN-11	−53 to 101	+	0.02
pPN-12	−141 to 101	+	0.60

[a] A total of 3 μg of each plasmid DNA was used per well (diameter, 35), which had been seeded 1 day earlier with 2.5×10^5 cells. Units of β-galactosidase activity were determined by assaying cultures 3 days after transfection; units were adjusted for the amount of DNA per transfected well.

RESULTS

Sequences required for RSV gene expression. We have previously described the use of the *E. coli lacZ* gene product β-galactosidase as a marker of transient retroviral gene expression (23). A number of plasmids were constructed in which the RSV LTR directed the synthesis of a *gag-lacZ* fusion product. One such plasmid (pPN-7; Fig. 2), contained a single LTR flanked by viral sequences that are normally found adjacent to either the 3′ or the 5′ LTR of an integrated provirus. Transfection of avian embryo fibroblasts with pPN-7 resulted in high levels of β-galactosidase (23) (Table 1). As expected from results presented in other reports (6, 15), deletion of viral sequences upstream of the *Eco*RI site at nucleotide −53 (pPN-7a) or the *Sph*I site at nucleotide −141 (pPN-8; Fig. 2) greatly reduced the levels of β-galactosidase activity elicited in transfected cells (Table 1). Reconstruction of an intact LTR in the absence of viral sequences upstream of the LTR restored enzyme levels to near those of the wild type (data not shown). Thus, the inactivity of pPN-7a and pPN-8 was specifically attributable to deletion of LTR sequences, defining the portion of U_3 from its 5′ boundary at −233 to the *Sph*I site at −141 as essential for *gag-lacZ* expression.

While it seemed most likely that the deletions directly reduced the transcriptional capacity of the LTR, other possibilities were not excluded. As the 5′ ends of the *gag-lacZ* encoding mRNAs derived from the plasmids described above must be identical, we expected that all transcripts were translated with equal efficiency. Dot hybridization analyses of DNA isolated from transfected cell lysates confirmed that all plasmids were introduced into cells with equal efficiency (data not shown). Thus, we conclude that the measured differences in β-galactosidase induced by these plasmids reflect stable levels of *gag-lacZ* mRNA.

Insertion of 3′ LTRs increases stable transcript levels. Normally, a provirus contains two LTRs: one on either side of the structural genes. To test whether insertion of LTRs 3′ of *gag-lacZ* would influence the amount of β-galactosidase synthesized, plasmids pPN-10, pPN-11, and pPN-12 were constructed. These plasmids differed from pPN-7, pPN-7a,

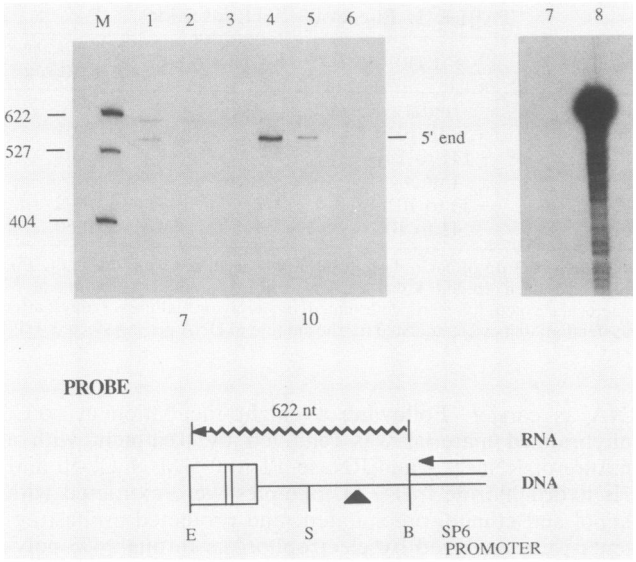

FIG. 3. Nuclease protection analysis of pPN-7- and pPN-10-derived RNAs. Turkey embryo fibroblasts were transfected with 4.0 μg of either pPN-7 or pPN-10 DNA in the presence of 80 μM chloroquine. Three days later, total cellular RNAs were prepared from these cultures and subjected to hybridization with a uniformly labeled RNA probe complementary to viral sequences at positions −53 to 537. The structure of the in vitro-synthesized RNA probe is diagrammed at the bottom of the figure (the probe also includes certain vector-derived sequences). Single-stranded material was removed by digestion with RNase, and hybrids were analyzed by electrophoresis through 4% acrylamide under denaturing conditions. Lanes 1 to 3, 5.0, 1.6, and 0.5 μg of RNA from pPN-7-transfected cells; lanes 4 to 6, similar amounts of RNA as in lanes 1 to 3, respectively, from cells that were transfected with pPN-10; all hybridizations were adjusted to 20 μg of total RNA by the inclusion of the appropriate quantity of yeast RNA as carrier; lane 7, carrier RNA alone; lane 8, 1/50th as much undigested probe as was present in the other lanes; lane M, pBR322 cut with *Msp*I and end-labeled. Numbers to the left of the gel are in bases. Abbreviations: nt, nucleotides; E, *Eco*RI; S, *Sac*I; B, *Bam*HI.

and pPN-8, respectively, by the presence of an LTR-containing fragment of pRAV-1 immediately 3′ to *lacZ*, in the same transcriptional orientation as the upstream LTR (Fig. 2). The permuted pRAV-1 fragment extends from within the *env* gene to the 5′-untranslated leader region and contains two LTRs in tandem. For convenience, the LTR directing *gag-lacZ* gene expression is referred to as the 5′ LTR, with those downstream of *lacZ* referred to as 3′. It should be borne in mind that all plasmids were transfected as circles; thus, this distinction is not absolute but valid only with reference to *lacZ*.

Provision of 3′ viral sequences significantly influenced *gag-lacZ* expression (Table 1). The effect of 3′ LTRs was most pronounced (greater than 50-fold) when the 5′ LTR lacked sequences 5′ of nucleotide −141 in U_3 (compare pPN-8 with pPN-12), suggesting that the LTRs 3′ of *lacZ* partly compensated for the defect. The 3′ LTRs also augmented *lacZ* expression when the 5′ LTR was intact (pPN-7 versus pPN-10), but the effect was not as striking. The most severely truncated LTR of pPN-7a was not activated by the insertion (see pPN-11); it is likely that sequences upstream of nucleotide −53 are an integral part of the promoter and cannot be supplied at a distance.

We have previously established that the β-galactosidase levels induced by pPN-10, pPN-11, and pPN-12 correlate well with the abundance of transcripts initiated at the 5′ LTR of each plasmid (23). While it seemed likely that 3′ LTRs directly increased stable *gag-lacZ* transcript levels, other explanations were possible, such as variation in the stability of different plasmid DNAs. Again, dot hybridization analyses revealed no differences in transfection efficiency (data not shown). Moreover, plasmids bearing multiple LTRs were not more prone to homologous recombination events than plasmids with single LTRs (data not shown). Thus, the 3′ LTRs did not seem to alter the number or stability of transfected DNA molecules, suggesting that the effect was to alter *gag-lacZ* mRNA levels directly. The inactivity of pPN-11 indicates that the 3′ LTRs do not themselves direct transcription of mRNAs that code for β-galactosidase. This suggested that 3′ LTRs augment gene expression by increasing the activity of the 5′ LTR.

Analysis of RNA from transfected cells confirmed that the 3′ LTRs function to increase *gag-lacZ* transcript levels. Parallel cultures were transfected with equal amounts of pPN-10 or pPN-7 DNA. Three days later, RNAs were prepared from one culture transfected with each plasmid, and plasmid-specific RNAs were identified by their ability to protect portions of a uniformly labeled RNA probe (Fig. 3). The intensity of the 537-base fragment protected by the 5′ end of *gag-lacZ* RNA indicated that the ratio of RNAs induced by pPN-10 relative to pPN-7 (between 3:1 and 9:1)

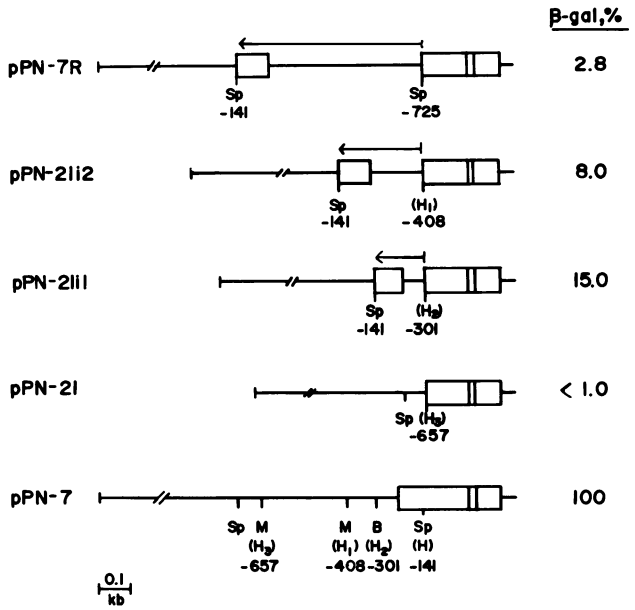

FIG. 4. Inversion of various portions of U_3 relative to the promoter. Details of the construction of these plasmids are given in the text. Arrows indicate that the region so defined was inserted into pPN-21 in an inverted orientation with respect to the promoter. To facilitate manipulations, *Hin*dIII linkers were inserted at various restriction sites, as indicated on the map of pPN-7 at the bottom of the figure (kb, kilobases). The *Hin*dIII site utilized in a particular construction is indicated by subscript numbers. Only the relevant portions of the viral insert are shown (regions not detailed are identical to pPN-7). The open boxes denote LTR sequences. Avian cells were transfected with approximately 3 μg of each plasmid in the presence of 0.2 mg of DEAE-dextran per ml, and 3 days later, cell lysates were assayed for enzyme activity. The data are presented as percent activity (β-gal, β-galactosidase) relative to that of pPN-7. Restriction enzymes: B, *Bal*II; H, *Hin*dIII; M, *Mst*II; Sp, *Sph*I.

TABLE 2. Effect of alterations within the LTR at the *Sph*I site

Plasmid		Sequence alterations (ca. -141)[a]	β-Galactosidase activity, (%)[b]
pPN-13	None:	AGAAAAGGCACCGTGCATGCCGATTGGT	100
pPN-13d2	4 bp Del:	AGAAAAGGCACCGTG CCGATTGGT	78
pPN-13i11	48 bp Ins:	AGAAAAGGCACCGTG<CAAGCTTG>$_6$CCGATTGGT	83
pPN-13i12	72 bp Ins:	AGAAAAGGCACCGTG<CAAGCTTG>$_9$CCGATTGGT	52
pPN-13i13	130 bp Ins:	....ACAAGATCGCGAAGCTTGCCGATTGGT	19
pPN-13i14	130 bp Ins:	....CCAAAAGGAACAAGCTTGCCGATTGGT	9

[a] Abbreviations: Del, deletion; Ins, insertion; bp, base pairs. Inserted sequences are bracketed; subscripts represent the numbers of *Hin*dIII linkers that were inserted. The inserted fragment was a small *Hin*dIII fragment derived from the RSV *pol* gene (25). Underlined nucleotides indicate those shared by pPN-13 and pPN-13i14.

[b] Values are presented as percentage of β-galactosidase activity relative to that of pPN-13, as assayed 3 days after transfection. Background levels of β-galactosidase were less than 3%.

correlated with relative β-galactosidase levels (Table 1). (The band of approximately 580 bases was due to transcripts reading through the LTR 5′ of *lacZ*; no transcripts were initiated at the 3′ LTRs of pPN-10. All the plasmid-borne LTRs that we examined directed polyadenylation inefficiently; manuscript in preparation). It was concluded that the presence of 3′ LTRs increased mRNA levels, and the strong effect observed when the 5′ LTR was truncated suggests that the 3′ LTR provides an enhancer-like activity.

Distance-dependent enhancer activity within U_3. While the −233 to −141 region of U_3 (i.e., the region deleted from pPN-8) may have contained an enhancer element, it was also possible that the deletion disrupted an enhancer. The hallmarks of transcriptional enhancers are the ability to function well in either orientation and at a considerable distance from a tested promoter (14). We wished to determine whether the U_3 sequences from −233 to −141 met these criteria.

For simplicity of interpretation as well as construction, we chose to analyze sequences within the 5′ LTR in the absence of a 3′ LTR. The *Sph*I fragment missing from pPN-8 was reinserted in the opposite orientation (pPN-7R; Fig. 4). This manipulation placed the relevant portion of U_3 more than 400 bases upstream of its normal location. Transfection of cells with pPN-7R resulted in a reduced, but detectable, level of β-galactosidase. It was uncertain, however, whether the poor activity of the plasmid was due to the inversion of U_3 sequences or to their displacement from the cap site. To differentiate these two alternatives, the −233 to −141 segment was inverted and reinserted at various distances from the cap site (pPN-21i1 and pPN-21i2; Fig. 4). The level of β-galactosidase activity increased as the distance between the inverted fragment and the cap site decreased, suggesting that a distal location largely accounted for the low activity of pPN-7R.

Disruption of the *Sph*I site itself could conceivably have a negative effect on LTR function. The simple deletion of base pairs −140 to −137 (the four central nucleotides of the *Sph*I site; pPN-13d2; Table 2), however, did not greatly reduce the levels of enzyme activity. Thus, these few nucleotides are entirely dispensable. Another series of plasmids emphasized that the −233 to −141 sequences need not be positioned precisely with respect to the cap-proximal promoter elements. Insertion of six or nine *Hin*dIII linkers (pPN-13i11 and pPN-13i12, respectively) slightly diminished enzyme activity in transfected cells, to approximately 70 and 50%, respectively (Table 2). However, insertion of a 130-base-pair *Hin*dIII fragment reduced *lacZ* expression to a much greater extent (pPN-13i13 and pPN-13i14; Table 2). This demonstrated that strong distance dependence is not peculiar to the inverted orientation. The difference in gene expression observed when the same fragment was inserted in either orientation (compare pPN-13i13 and pPN-13i14) is likely due to the introduced *pol* sequences. Taken together, the results presented above suggest that sequences on either side of the *Sph*I site interact to form the RSV enhancer element but that this interaction does not require any precise spatial positioning of sequence elements.

The 3′ LTR provides transcriptional enhancement functions. Although our results suggest that transcriptional enhancement plays a role in the 3′ LTR effect, other factors could have been involved. For instance, placement of the viral polyadenylation signal near to the 3′ end of *lacZ* might tend to stabilize the resulting mRNAs. To test this possibility, various LTR fragments were inserted 3′ of *lacZ*, in the same relative orientation as the 5′ LTR. It was anticipated

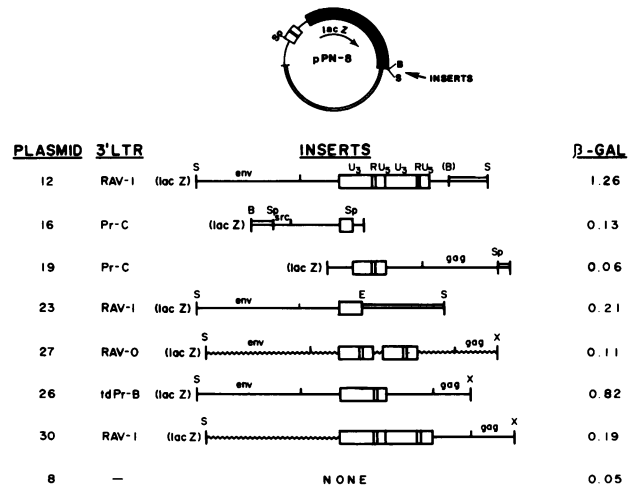

FIG. 5. Structure and activity of various LTR-containing fragments inserted into pPN-8 3′ of *lacZ*. At the top of the figure the plasmid pPN-8, with no viral sequence 3′ of *lacZ*, is shown. Below that are diagrammed the various fragments inserted at the *Bam*HI (B) or *Sal*I (S) sites 3′ of *lacZ*; all 3′ inserts placed the LTRs (boxes) in the same orientation as the 5′ LTR (the position of *lacZ* is indicated). The leftmost column gives the numeric designation of the plasmid (with the prefix pPN- omitted); the next column describes the viral origin of the LTR. The composition of flanking viral sequences is indicated by thin lines (exogenous sequences) or wavy lines (endogenous sequences). pBR322 sequences are indicated by an open bar. Relevant restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; S, *Sal*I; Sp, *Sph*I; X, *Xho*I). Turkey embryo fibroblasts were transfected with 3 μg of each plasmid DNA, and cultures were assayed for β-galactosidase (β-GAL) activity 3 days posttransfection. The right column indicates the level of β-galactosidase activity induced by each plasmid, expressed as units per microgram of input plasmid DNA, adjusting for the slightly different sizes of the plasmids as a consequence of the insertions. The value for pPN-8 was not different from that of the background (i.e., no plasmid).

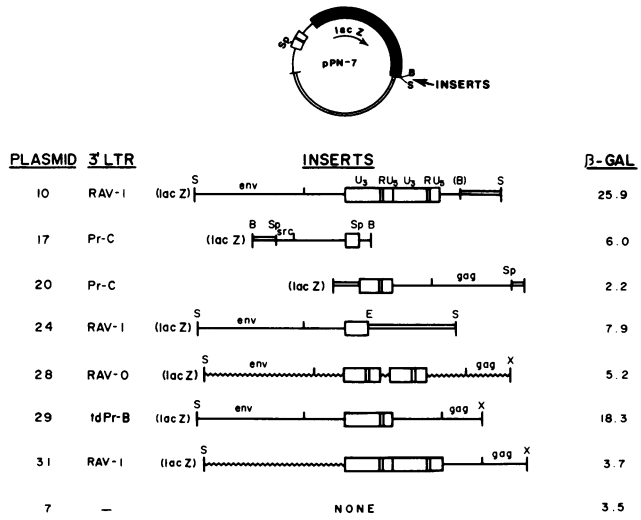

FIG. 6. Structure and activity of various LTR-containing restriction fragments inserted 3′ of *lacZ* in pPN-7. pPN-7 is diagrammed at the top. All conventions are as described in the legend to Fig. 5. Turkey embryo fibroblasts were transfected with 3 μg of each plasmid, and cultures were assayed for β-galactosidase (β-GAL) activity 3 days posttransfection. Values presented represent units of enzyme activity per microgram of input plasmid DNA, adjusted for insert size. Background levels of enzyme activity in this experiment were less than 0.05 U per culture.

that finer localization of the sequences responsible for the 3′ LTR effect might distinguish transcriptional enhancement from RNA stabilization.

We initially tested the various LTR inserts in combination with a 5′ LTR that was truncated at nucleotide −141 (Fig. 5). First, both the RAV-1 fragment and a similar fragment containing a single LTR derived from a tdPr-RSV-B derivative (pPN-26; Fig. 5) elicited roughly equal levels of β-galactosidase activity. Thus, the activation effect of a 3′ LTR was neither specific to the origin of the LTR nor to the presence of two LTRs in tandem.

The activation effect of the 3′ LTR largely resisted further mapping attempts: the region 5′ of the *Sph*I site at −141 exhibited very little activity, and the region 3′ to it was completely incapable of augmenting *gag-lacZ* expression (pPN-16 and pPN-19, respectively; Fig. 5). The latter result tends to discount a requirement for the polyadenylation signal but suggests that the region upstream of the *Sph*I site interacts with sequences that normally lie immediately downstream. When the entire U_3 region of RAV-1, except for the 53 bases nearest to the cap site, was inserted 3′ of lacZ (pPN-23; Fig. 5), β-galactosidase levels increased, confirming that sequences on either side of the *Sph*I site function more efficiently when they are in close proximity to each other. The failure of any of the fragmented LTRs to substitute fully for an intact LTR indicates that the activation phenomenon is complex.

The RAV-0 LTR directs polyadenylation as efficiently as the exogenous LTRs (8, 12), but it does not possess the ability to enhance the expression of linked heterologous genes (7, 8, 36). Insertion of two tandem RAV-0 LTRs 3′ of *lacZ* resulted in far less β-galactosidase activity than when exogenous LTRs were substituted (compare pPN-27 with pPN-12 and pPN-26; Fig. 5). This argues against an important role for polyadenylation in the activation of *gag-lacZ* by 3′ LTRs. Interestingly, a similar fragment derived from a recombinant between RAV-1 and RAV-0 which retained the exogenous LTRs was poorly active in the 3′ position (pPN-30; Fig. 5). This was probably a consequence of the RAV-0 sequences that were present immediately upstream of the LTRs (see below).

To determine whether *gag-lacZ* gene expression from an intact 5′ LTR was similarly affected, the same set of 3′ LTR insertions was evaluated within the context of pPN-7. The insertion of the single Pr-RSV-related LTR, generating pPN-29 (Fig. 6), mimicked that of the RAV-1 LTRs, although the Pr-RSV LTR was somewhat less active. Neither of the *Sph*I-divided LTR fragments greatly increased *lacZ* expression (pPN-17 and pPN-20; Fig. 6). The fragment extending from 5′ of U_3 to the *Eco*RI site at nucleotide −53 stimulated enzyme production to a significant extent (pPN-24; Fig. 6), but it still was not equivalent to an intact exogenous LTR. The RAV-0 LTRs had little effect (pPN-28; Fig. 6), as would be expected if the 3′ LTRs functioned by supplying additional transcriptional enhancement activity. Interestingly, the activity of the RAV-1 LTRs was again attenuated by the juxtaposition of RAV-0 sequences (pPN-31; Fig. 6). The results were consistent with the earlier conclusion that a multicomponent enhancer element resides within the RSV LTR and that the presence of this element 3′ of a gene significantly affects its expression.

LTR activity and viral gene products. It has been proposed that certain promoters, including the RSV LTR, are stimulated in transfected mouse cells when RSV *gag* seqences are present in *trans* (2). Because all the experiments described above were performed in uninfected cells, it was of interest to compare expression in uninfected and infected avian cells. β-Galactosidase activity induced by pPN-7 transfection was, in fact, reduced when cells were preinfected with Pr-RSV-C (Fig. 7). A qualitatively similar but quantitatively less pronounced depression resulted from cotransfection of pPN-7

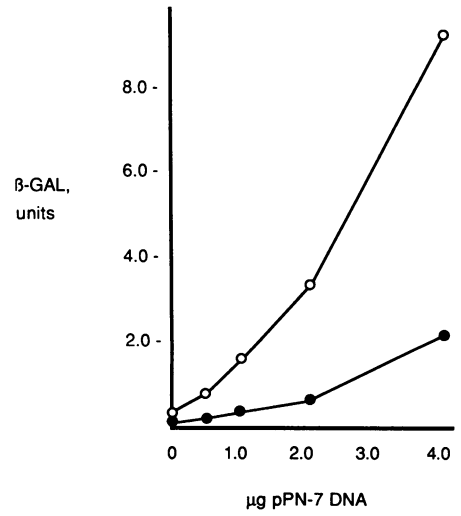

FIG. 7. Expression of pPN-7 in uninfected versus infected cells. Turkey embryo fibroblasts were either infected with Pr-RSV-C (●) or left uninfected (○). Infected cells were exposed to a high titer stock of Pr-RSV-C more than 1 week prior to transfection. One day after cells were seeded at a density of 2×10^5 per well (diameter, 35 mm), cells were transfected with the indicated amounts of pPN-7 DNA. β-Galactosidase (β-GAL) activity was assayed 3 days posttransfection.

TABLE 3. Cotransfection with a *gag*-expressing plasmid has the same effect as transfection of preinfected cells

Plasmid transfected[a]	Cells preinfected	β-Galactosidase activity (U)[b]
pPN-7	No	2.0
pPN-7	Yes	0.49
pPN-7 + pATV-8	No	0.58
None	No	0.04

[a] A total of 1.0 μg of each plasmid DNA was transfected per 1.5×10^5 cells per well (diameter, 35 mm), with sheared salmon sperm DNA added where necessary to ensure that the total amounts of DNA transfected were equal. Cells were either uninfected turkey embryo fibroblasts or parallel cultures that were infected more than 1 week previously with Pr-RSV-C.

[b] β-Galactosidase activity was measured 3 days after transfection.

and pATV-8 DNA (Table 3). The latter plasmid has an intact *gag* gene downstream of an RSV LTR and presumably directs the synthesis of *gag* proteins. Thus, a difference in the transfection efficiency of infected cells relative to that of their uninfected counterparts cannot account for the absence of apparent *trans*-activation of *gag-lacZ* by RSV sequences. We could find no evidence for significant *trans*-activation of transcription or translation of viral mRNA by RSV sequences, but the depression of gene expression (probably a consequence of sequestering of mRNA by *gag* proteins) might have obscured a small effect.

DISCUSSION

We situated a marker gene within a proviral context, i.e., flanked by LTRs and other noncoding sequences. Following transfection of avian fibroblasts with such constructs, expression of a *gag-lacZ* fusion was monitored both by assay for β-galactosidase activity and by direct examination of transcript levels. An intact 5′ LTR was required for efficient expression of the marker gene, and within the LTR a region was identified which was required in an orientation-independent but distance-dependent fashion. Expression was further increased by provision of additional LTRs 3′ of *lacZ*. Interestingly, it was found that deletions which rendered a 5′ LTR unable to promote detectable levels of β-galactosidase activity could be partially complemented in *cis* by insertion of a second LTR 3′ to the marker gene.

Delineation of an RSV enhancer element. Analysis of deletion mutants demonstrated that sequences between nucleotides −233 and −141 were obligatory for LTR activity but could function in either orientation, partly conforming to the definition of a transcriptional enhancer (14). The function of this region was not completely independent of position, however, as moving the element greater than 100 nucleotides away significantly impaired function (Fig. 4 and Table 2). While analyses of the simian virus 40 (SV40) enhancer element have revealed that its function declines with distance from the promoter (35), it is not clear that the region of U_3 between nucleotides −233 and −141 is equivalent to the SV40 enhancer element. Rather, it seems likely that the *Sph*I site might lie within an enhancer that is composed of multiple synergistically acting domains that have some flexibility with regard to distance and orientation. Such a modular organization has indeed been proposed for the RSV enhancer (15).

The sequence that forms the *Sph*I recognition site at nucleotide −141 does not itself appear to be essential for LTR activity, as plasmids carrying deletion or insertion mutations at that site were capable of conferring high levels of *gag-lacZ* expression (Table 2). This site lies within the longest stretch of alternating purine and pyrimidine residues within U_3; such sequences are capable of assuming a Z-DNA conformation under the appropriate circumstances (34). It has been suggested that transcriptional enhancement is associated with regions of Z-DNA (22). The data in Table 1 offer no support for this hypothesis and rule out an absolute requirement for the central 4 base pairs of the *Sph*I site, which is in agreement with results of a previous report regarding deletion of these bases (6). Coincidentally, the sequences introduced adjacent to the altered *Sph*I site in pPN-13i14 conserved 8 of 9 nucleotides that are normally found upstream of that site (underlined bases, Table 2), suggesting that these 9 bases were not the crucial nucleotides that were lacking from pPN-8. Other investigators (16) have also found that a small deletion (ca. 10 base pairs) at the *Sph*I site did not significantly reduce LTR activity. Thus, it seems unlikely that this restriction site forms a specific binding site for a single protein or complex of proteins, as such a regulatory element would be expected to have precise spatial requirements.

Examination of the nucleotide sequence of the Pr-RSV-C U_3 region (25) reveals an imperfect direct repeat, with the sequence at nucleotides −168 to −154 (GCCTTACAAG GAAAG) repeated with four mismatches at positions −105 to −91 (GCCTTATTAGGAAGG). It has been pointed out (15) that a portion of this repeat bears homology to the adenovirus E1A enhancer repeat AGGAAGGTGA (11). In addition, the sequence at positions −118 to −111 (GTG GTACG) bears homology to the SV40-type enhancer consensus sequence (GTGGWWWG, where W represents either A or T) (37), and a related sequence is present a few base pairs upstream at −129 to −123 (GTGGTAG). While the importance of these various sequence motifs remains to be determined, it is clear that certain of the core elements alone are not sufficient to activate the RSV promoter; for pPN-8, which retains one copy of the adenoviruslike element as well as both the SV40-like repeats, was incapable of *gag-lacZ* expression.

Effect of 3′ LTRs on transcript levels. Modulation of *gag-lacZ* expression by a 3′ LTR could occur by several mechanisms. Nuclease mapping demonstrated that β-galactosidase activity correlated wth mRNA levels (Fig. 3); and because all plasmids were transfected with equal efficiency, it was likely that the 3′ LTRs influenced stable transcript levels directly. As the 3′ LTR lies within the *gag-lacZ* transcript, its presence could affect the rate of mRNA synthesis or its stability (or both). Unfortunately, attempts to measure transcription rates via nuclear run-on transcription experiments were unsuccessful due to the relative insensitivity of the technique (data not shown). It should be noted that β-galactosidase activity appears to be at least as sensitive an indicator of gene expression as direct RNA analysis. Plasmids such as pPN-12, which produce low levels of *gag-lacZ* mRNA (23; P. A. Norton and J. M. Coffin, manuscript in preparation) induce levels of β-galactosidase that are substantially above those of the background.

It also seemed unlikely that 3′ LTRs could direct the synthesis of translationally active *gag-lacZ* mRNA. Indeed, it was observed that the highly truncated 5′ LTR, which lacks all sequences 5′ of position −53, was insensitive to the insertion of 3′ LTRs (pPN-11). This also demonstrates that certain 5′ LTR components cannot be supplied at a distance. This result agrees with that of Mitsialis et al. (21), who found that proviral structures with highly defective 5′ LTRs are transcriptionally inactive.

Our inability to fully map the 3′ determinant(s) which increased transcript levels came as a surprise. To summarize, the region between nucleotides −233 and −141 was poorly active in the absence of adjacent LTR sequences. Sequences between −141 and −53 operated only in conjunction with the −233 to −141 moiety, as the −141 to 102 fragment was completely inactive. Provision of a polyadenylation signal alone did not detectably influence *gag-lacZ* expression, but some component 3′ of position −53 was essential for full LTR function. The incremental increases in enzyme activity with insertion of a larger LTR fragment define a minimum of three interacting domains of the exogenous 3′ LTR; these are −233 to −141, −141 to −53, and −53 to the 3′ boundary of U_5. Our results do not indicate whether the domains are functionally equivalent, as has been suggested by other data (15).

Interestingly, substitution of an endogenous-type 3′-UT region largely abrogated the stimulatory effect of exogenous 3′ LTRs. This also demonstrated that the 3′-UT must act in a position-dependent fashion, as the exogenous sequence was adjacent to the 5′ LTR in pPN-31. It has been reported that deletion of the exogenous 3′-UT region is correlated with reduced levels of viral RNA (29), but it was not shown whether the endogenous 3′-UT was functionally equivalent. It has also been suggested that the region modulates viral pathogenicity (24, 30). The nonpathogenic endogenous virus RAV-0 contains only a well-conserved sequence between *env* and U_3, while the exogenous viruses examined contain additional sequences, some of which are unrelated to each other (1, 4, 31). It is possible that the additional sequences present in the exogenous viruses relay information between the 5′ and 3′ LTRs. Alternatively, insertions in this region may disrupt an element within the conserved sequence which interferes with 3′ LTR function.

In summary, while the LTR serves to direct efficient transcription initiation, our data indicate that the same sequences function in a less direct fashion and at a substantial distance to increase viral gene expression. Both activities appear to rely on the integrity of the LTRs within a proviral unit. It is unclear whether other transcriptional units possess a similar organization of controlling elements. A mechanism that would bias LTR activities in favor of proviral transcription rather than expression of flanking cellular sequences might be advantageous to both the virus and the host cell. Such a regulatory system requires a means of distinguishing the two identical LTRs of a provirus; we have discovered a sequence within the provirus near the 5′ LTR which may fulfill this function (Norton and Coffin, in preparation).

The RSV LTR does not require viral gene products to function. A portion of the genome of human T-cell lymphotropic virus types I and II appears to specifically stimulate LTR activity in *trans* (27, 28), and a similar mechanism has been proposed for RSV (2). Levels of RSV LTR-directed transcripts, as well as transcripts initiated at a heterologous promoter, were increased in NIH 3T3 cells when RSV sequences were present in *trans* (2). We thus asked whether *gag-lacZ* expression was stimulated in preinfected avian cells. The data in Fig. 7 led us to the opposite conclusion: β-galactosidase activity is depressed in infected cells relative to uninfected cells, perhaps because of sequestering of *gag-lacZ* mRNA in virions. The proposed *trans*-activation does not play a major role in the expression of RSV in avian fibroblasts, which is in contrast to the situation with the human retroviruses.

It has been suggested that the stimulation of expression of transfected DNA in preinfected avian cells is due to an increase in DEAE-dextran-mediated uptake (5). This explanation cannot account for the data regarding the cotransfection of pPN-7 and pATV-8 (Table 3). The inconsistency between our data and those reported previously (2) could be resolved if the apparent *trans*-activation was, in fact, due to RNA stabilization and not increased transcription initiation. Stabilization of viral RNA by virtue of bound viral proteins might simultaneously reduce translatability. The observable consequences of such a mechanism would be an increase in the levels of stable RNA, along with a decrease in the translation of that RNA (Fig. 7). Further analyses should provide a definitive resolution to the problem.

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