JOURNAL OF VIROLOGY, June 1996, p. 3834–3843 Vol. 70, No. 6
0022-538X/96/$04.00+0

Avian Retroviral RNA Element Promotes Unspliced RNA Accumulation in the Cytoplasm

ROBERT A. OGERT, LINDA H. LEE, AND KAREN L. BEEMON*

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Received 20 December 1995/Accepted 11 March 1996

All retroviruses need mechanisms for nucleocytoplasmic export of their unspliced RNA and for maintenance of this RNA in the cytoplasm, where it is either translated to produce Gag and Pol proteins or packaged into viral particles. The complex retroviruses encode Rev or Rex regulatory proteins, which interact with *cis*-acting viral sequences to promote cytoplasmic expression of incompletely spliced viral RNAs. Since the simple retroviruses do not encode regulatory proteins, we proposed that they might contain *cis*-acting sequences that could interact with cellular Rev-like proteins. To test this possibility, we initially looked for a *cis*-acting sequence in avian retroviruses that could substitute for Rev and the Rev response element in human immunodeficiency virus type 1 expression constructs. A *cis*-acting element in the 3′ untranslated region of Rous sarcoma virus (RSV) RNA was found to promote Rev-independent expression of human immunodeficiency virus type 1 Gag proteins. This element was mapped between RSV nucleotides 8770 and 8925 and includes one copy of the direct repeat (DR) sequences flanking the RSV *src* gene; similar activity was observed for the upstream DR. To address the function of this element in RSV, both copies of the DR sequence were deleted. Subsequently, each DR sequence was inserted separately back into this deleted construct. While the viral construct lacking both DR sequences failed to replicate, constructs containing either the upstream or downstream DR replicated well. In the absence of both DRs, Gag protein levels were severely diminished and cytoplasmic levels of unspliced viral RNA were significantly reduced; replacement of either DR sequence led to normal levels of Gag protein and cytoplasmic unspliced RNA.

All retroviruses generate multiple mRNAs from a single primary RNA transcript (reviewed in references 15, 16, 24, 58, and 63). A fraction of the viral unspliced RNA is capped, polyadenylated, and transported to the cytoplasm, where it is either translated to generate Gag and Pol proteins or packaged into virus particles. Another fraction of the primary RNA transcripts is spliced in the nucleus to generate subgenomic mRNAs, including *env* mRNA in all retroviruses and more than 30 additional, alternatively spliced mRNAs in the complex retroviruses. While retroviral intron-containing RNAs are transcribed, posttranscriptionally processed, exported from the nucleus, and translated by cellular machinery, they differ from most cellular mRNAs in that they are not spliced to completion before export to the cytoplasm. Posttranscriptional regulatory mechanisms have evolved to allow retroviruses to achieve a necessary balance of spliced and unspliced RNAs in the cytoplasm. These mechanisms include control of splicing via inefficient 3′ splice sites, as well as a variety of exonic and intronic *cis*-acting regulatory sequences (1, 3, 27, 28, 33, 39, 40, 47, 57, 64).

Retroviruses also need to circumvent the normal cellular machinery in order to transport their unspliced, intron-containing RNA from the nucleus to the cytoplasm, where it is stably maintained and translated. The complex retroviruses, including human immunodeficiency virus (HIV) and other lentiviruses and human T-cell lymphotropic virus, encode posttranscriptional regulatory proteins (Rev and Rex, respectively), which bind to *cis*-acting viral RNA sequences (Rev or Rex response elements [RRE]). These *cis*-acting elements and viral proteins are both necessary for stable cytoplasmic expression of incompletely spliced viral RNAs (references 21 and 55; reviewed in references 16, 24, and 63). The complex retroviruses also contain *cis*-acting repressive sequences (CRS) which inhibit cytoplasmic expression of incompletely spliced RNAs in the absence of Rev or the RRE by nuclear retention and destabilization (14, 36, 51, 53).

Rous sarcoma virus (RSV) is a relatively simple avian retrovirus which produces only three mRNAs; it does not encode a posttranscriptional regulatory protein like Rev or Rex (reviewed in references 15 and 58). In addition to the unspliced *gag/pol* mRNA, RSV has two spliced mRNAs for *env* and *src* gene products. These mRNAs are alternatively spliced from a common 5′ splice site at nucleotide (nt) 397 to either the *env* (nt 5078) or the *src* (nt 7054) 3′ splice site. While RSV does not have CRS (53), it does have a *cis*-acting negative regulator of splicing element in the *gag* gene, which is capable of retaining heterologous unspliced RNA in the nucleus (3). Nevertheless, the unspliced RNA of RSV does not appear to be retained in the nucleus or to be unstable (3, 6, 7, 59); this suggested that it may have a different mechanism of nucleocytoplasmic export than cellular mRNAs.

Recently, several viral elements that play a role in posttranscriptional regulation of unspliced RNAs have been identified. A *cis*-acting element from the 3′ untranslated region (UTR) of Mason-Pfizer monkey virus (MPMV), a subgroup D retrovirus, was found to confer Rev-independent expression in HIV type 1 (HIV-1) constructs containing either *gag* and *pol* genes or *env* genes (11). This element was termed a constitutive transport element (CTE) because it increased cytoplasmic levels of unspliced RNA. A similar element has been identified in the closely related simian retrovirus type 1, which allows replication of HIV-1 mutants lacking Rev and the RRE (66). Unspliced viral RNAs from hepatitis B virus and herpes simplex virus have also recently been shown to have *cis*-acting elements necessary for their expression (31, 35).

Although RSV does not encode regulatory proteins, we

* Corresponding author. Phone: (410) 516-7289. Fax: (410) 516-5213. Electronic mail address: KLB@JHUVMS.HCF.JHU.EDU.

asked whether it might contain a *cis*-acting CTE sequence that could replace the *trans*-acting Rev protein in promoting expression from HIV-1 expression constructs. Initial results showed Rev-independent HIV-1 Gag protein expression upon insertion of sequences from the 3′ UTR of RSV into an HIV-1 expression construct. Subsequently, the RSV element was mapped between RSV nt 8770 and 8925; this region contains one copy of a direct repeat (DR) sequence (17, 52). The other copy of the DR sequence is located between the *env* and *src* genes of RSV. RSV replication was found to be inhibited by removal of both DRs and restored by replacement of either DR, in agreement with earlier studies which proposed that the DR was necessary for RNA packaging (56). We have also observed a significant decrease in levels of packaged virion RNA in the absence of either DR sequence. However, our data suggest that the primary effect of the DR elements is to promote cytoplasmic accumulation of unspliced viral RNA, which is necessary both for synthesis of virion proteins and for packaging of viral RNA.

MATERIALS AND METHODS

Plasmid construction. pCMVrev (54) and pCMVgagpol-RRE were obtained from M. L. Hammarskjold, University of Virginia. pCMVgagpol-RRE was derived from pSVGagPol-RRE (54) by replacement of the simian virus 40 (SV40) promoter by the cytomegalovirus intermediate early promoter. pCMVgagpol-RRE-8707 was generated by inserting an RSV fragment containing nt 8707 to 101 (RSV coordinates according to the work of Schwartz et al. [52]) into a unique *Bam*HI site downstream of the RRE in pCMVgagpol-RRE. This RSV fragment contains the complete 3′ UTR and long terminal repeat (LTR) and was generated by PCR amplification from RSV genomic plasmid pPrC with *Pfu* polymerase (Stratagene) and *Bam*HI-engineered primers spanning RSV nt 8707 to 8724 (5′) and nt 85 to 101 (3′). Construct pCMVgagpol-RRE-8891 was generated similarly by insertion of RSV nt 8891 to 101, containing the RSV LTR and the E element (52), into the *Bam*HI site of pCMVgagpol-RRE. This RSV fragment was generated by PCR with primers spanning RSV nt 8891 to 8904 (5′) and nt 85 to 101 (3′).

The RSV element present in the 3′ UTR of RSV was further mapped with construct pRSV2CAT-IR-1+, which we obtained from M. T. McNally. This construct was made by ligating an end-repaired fragment containing the chloramphenicol acetyltransferase (CAT) gene into the repaired *Sal*I site of pRSV2 (40), which contains a multicloning site between the RSV LTR promoter and the SV40 early poly(A) site (lacking the SV40 intron). pRSV2CAT-IR-1+ contains the CRS inhibitory region IR-1 from the HIV-1 *gag* gene, characterized by Maldarelli et al. (36). The IR-1 fragment (HIV-1 BH10, nt 709 to 2003 [49]) was end repaired and ligated in both orientations into a blunted *Xba*I site downstream of the *cat* gene in pRSV2CAT. Various primers with *Bam*HI sites were used to construct PCR fragments corresponding to regions within the 3′ UTR of RSV (nt 8707 to 101) as depicted in Fig. 2. These PCR fragments were ligated into the unique *Bam*HI site downstream of the IR-1 fragment in pRSV2CAT-IR-1+. The upstream DR (DR1) and flanking sequences (RSV nt 6863 to 7037) were similarly cloned into pRSV2CAT-IR-1+.

Plasmid pAPrC, containing a full-length nonpermuted copy of the Prague C (PrC) strain RSV genome, was obtained from Meric and Spahr (42) and was previously modified to generate a unique *Aat*II site (6). This construct was further modified by removal of the redundant *gag* sequences downstream of the 3′ LTR to generate pPrC, which was obtained from J. Cammarata. To construct pPrC, a linear fragment of pAPrC was generated by partial *Bst*EII digestion. This fragment was then digested with *Cla*I, and a *Cla*I linker (CCATCGATGG) was inserted to replace the *Bst*EII-*Cla*I fragment downstream of the 3′ LTR in pAPrC.

The *Kpn*I-*Cla*I fragment from pPrC (nt 4995 to 110), containing RSV *env* and *src* genes and the 3′ LTR, was subcloned into pBluescript KS+ (Stratagene) to generate pDL1. Next, the *src* gene and both DRs flanking it were deleted from this subclone (Δ6897-8960) by PCR recombination (30) to generate pDL2. A PCR fragment corresponding to RSV nt 6752 to 6896 was generated with a 5′ primer spanning nt 6752 to 6770 and a 3′ primer spanning nt 6883 to 6896, which also contained the polylinker sequence 5′ CGTTAACGGCGCCGACGTCGAC 3′. A 5′ primer spanning RSV nt 8961 to 8974 and containing the partially complementary polylinker sequence 5′ CGCCGTTAACGGTCACCGCGCGC 3′ and a 3′ primer spanning RSV nt 32 to 55 were used to produce PCR fragment 8961-55. Recombinatorial PCR between half-reactions containing PCR fragments 6752-6896 and 8961-55, generated as described above, produced a 565-bp fragment containing the newly generated 34-mer polylinker 5′ GTCGACGTCG GCGCCGTTAACGGTCACCGCGCGC 3′ in place of RSV nt 6897 to 8960. This PCR fragment was digested with *Bst*BI and *Rsr*II and cloned into the corresponding sites in pDL1 to generate plasmid pDL2. The *Kpn*I-*Cla*I fragment from pDL2 was ligated to the 7.7-kb *Kpn*I-*Cla*I fragment of pPrC to produce pPrCΔDR. pPrCΔDR contains the PrC RSV genome bearing a 2-kb deletion spanning nt 6897 to 8960, which removed the *src* gene and both copies of the DRs flanking *src*. A PCR fragment containing the upstream DR (DR1) (RSV nt 6897 to 7016) was generated with a 5′ primer spanning RSV nt 6897 to 6912 containing a *Sal*I site and a 3′ primer spanning RSV nt 7002 to 7016. This PCR fragment was digested with *Sal*I and cloned into the *Hpa*I-*Sal*I sites within the pPrCΔDR polylinker to produce plasmid pPrC-DR1. Similarly, a PCR fragment containing RSV nt 8770 to 8925 was generated with a *Sal*I-engineered 5′ primer spanning RSV nt 8770 to 8785 and a 3′ primer spanning RSV nt 8908 to 8925. A PCR fragment spanning nt 8770 to 8925 was cloned into the pPrCΔDR polylinker as described above to produce plasmid pPrC-DR2, which contains the downstream DR (DR2) from RSV. The sequences of all viral constructs generated by PCRs were verified by DNA sequencing.

Cell culture and DNA transfection. Secondary chicken embryo fibroblasts (CEFs) were cultured in Medium 199 containing 2% tryptose phosphate, 1% calf serum, 1% chick serum, penicillin, and streptomycin (all from Life Technologies). CEFs were transfected with either 5 μg of plasmid DNA per 6-cm dish or 10 μg of plasmid DNA per 10-cm dish in Medium 199 containing 200 μg of DEAE-dextran per ml. After 5 h, cells were subjected to a 10% dimethyl sulfoxide shock for 2 to 5 min depending on cell density. To control for transfection efficiency, pMyc23 (40) was cotransfected with the RSV constructs, and pCMV110, a cytomegalovirus–β-galactosidase expression vector obtained from Tom Hope, was transfected with the CAT constructs.

For measurement of RNA stability, actinomycin D (dactinomycin; Calbiochem) treatment was carried out 48 h after transfection. A final concentration of 1 μg of actinomycin D per ml was used for 6 h with harvest of RNA at 2-h intervals. This treatment was previously shown to reduce RNA synthesis by >99% (6).

CAT assays. CAT activity was measured by the method of Gorman et al. (29) and quantitated with an InstantImager (Packard). Briefly, CEFs were scraped from 6-cm dishes, microcentrifuged for 5 min, and resuspended in 10 mM Tris, pH 7.5. Cells were lysed by three 5-min freeze-thaw cycles. Cell lysates were incubated with [^{14}C]chloramphenicol (Amersham) for 1 h at 37°C, and products were resolved by thin-layer chromatography. As a control, a cytomegalovirus–β-galactosidase expression plasmid was transfected in parallel and assayed from the same cell lysates with an assay system obtained from Promega.

Viral infection and reverse transcriptase assays. CEFs on 6-cm culture dishes were transfected with RSV genomic plasmids pPrC, pPrCΔDR, pPrC-DR1, and pPrC-DR2. Cells were transferred 72 h later onto 10-cm dishes and allowed to reach confluency (about 1 week after transfection). Reverse transcriptase assays were performed as previously described (32). To assay viral infectivity, equal titers of virus from each transfected dish (except for the pPrCΔDR construct which gave negligible levels of reverse transcriptase activity) were used to infect CEFs on 6-cm plates which were about 15% confluent. Reverse transcriptase assays of the infected cells were performed every 48 h.

Protein isolation and Western blots (immunoblots). Cellular and viral proteins were isolated with radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM sodium phosphate buffer [pH 7], 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, and 1% Trasylol). Electrophoresis and blotting were carried out as previously described (45), with SDS–15% polyacrylamide minigels (Hoefer) for electrophoresis. After electrophoretic transfer of proteins to Hybond membranes (Amersham), the membranes were blocked with 1% casein–bovine serum albumin in phosphate-buffered saline overnight at 4°C. HIV-1 Gag proteins were detected on Western blots with purified immunoglobulin G (IgG) from the sera of HIV-positive donors, obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, and peroxidase-labeled goat anti-human IgG (Sigma). Recombinant HIV-1 p24/25^{gag} protein used as a positive control for Western blots was also obtained from the NIH AIDS Research and Reference Reagent Program. Blots were developed with the enhanced chemiluminescence reagent kit (Amersham). RSV Gag proteins were detected with polyclonal rabbit anti-avian myeloblastosis virus p19^{gag} serum obtained from D. P. Bolognesi, Duke University, and peroxidase-labeled goat anti-rabbit IgG (Sigma).

RNA isolation and RNase protection assays. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (13) approximately 48 h after transfection. Viral RNA was harvested from cell culture supernatants 48 h after transfection. Supernatants were clarified by centrifugation at 750 × *g* for 10 min and then centrifuged with a Beckman ultracentrifuge SW41 Ti rotor for 1 h at 35,000 rpm. Pelleted virus was resuspended in 1 ml of solution D containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol and processed as described previously (13).

Transcription of antisense RNA probes and RNase protection analysis were performed as previously described (6, 41). The viral splice donor probe was a T7 transcript of an *Eco*RI-*Bam*HI fragment (RSV nt −53 to 532) cloned into pGEM-2 (Promega) as previously described (3). The *myc* riboprobe used as a control contains the exonic portion of the pmyc23 5′ splice site riboprobe that was described previously (40); its template was generated by deletion of a *Sal*I fragment. Riboprobes (250,000 dpm each) were hybridized to viral, total cellular, nuclear, or cytoplasmic RNA for 16 h at 55°C. RNase digestions utilized 10 U of RNase T_1 per ml (Calbiochem) and 10 μg of RNase A per ml (Calbiochem) and were carried out for 1 h at room temperature. Electrophoresis was on 6%

polyacrylamide gels containing 8 M urea, and quantitation was carried out on an InstantImager (Packard). In calculations of the relative amounts of the two RNA species, adjustments were made for differences in the size and base compositions of the protected fragments of the probe.

Cell fractionation. Cytoplasmic RNA and nuclei were isolated by a citric acid cell fractionation procedure described previously (7, 8). Cytoplasmic RNA was prepared by resuspending trypsinized CEFs in 25 mM citric acid and subjecting them to 10 strokes with a Dounce homogenizer (Kontes). Nuclei were pelleted by spinning at 2,000 rpm for 8 min in a Sorvall HB4 rotor and further purified by two centrifugations through 0.25 M–0.88 M sucrose step gradients. The first centrifugation gradient contained 25 mM citric acid, and the second contained reticulocyte standard buffer (10 mM Tris [pH 7.4], 10 mM NaCl, and 3 mM $MgCl_2$). Cytoplasmic RNA and nuclear RNA were processed as described above.

RESULTS

RSV 3′ UTR promotes Rev-independent expression of HIV-1 Gag proteins. Expression of HIV-1 Gag proteins normally requires the presence of both the viral Rev protein and the *cis*-acting RRE sequence (16, 21, 24, 55, 63). To search for *cis*-acting sequences in RSV that could substitute for the HIV-1 Rev-RRE complex, RSV fragments encompassing the entire genome were inserted into the HIV-1 *gag-pol* expression construct that had been used previously by Bray et al. (11) to demonstrate activity of the MPMV CTE. Initially, these constructs were tested for Gag protein expression in COS cells; however, none of the RSV constructs resulted in detectable Gag protein expression here (data not shown). To test the possibility that the putative RSV CTE might be active in avian but not mammalian cells, we next inserted fragments of the RSV genome into the pCMVgagpol-RRE expression vector and monitored HIV-1 Gag protein expression by Western blotting of proteins from transfected CEFs.

HIV-1 $Pr55^{gag}$ expression was detected in the absence of Rev when the entire RSV 3′ UTR and LTR (nt 8707 to 101) were inserted between the RRE and the rabbit β-globin poly(A) addition signal in this construct to generate pCMVgagpol-RRE-8707 (GP-8707) (Fig. 1, lane 4). Deletion of the 5′ portion of the RSV 3′ UTR (nt 8707 to 8891) from this construct to generate pCMVgagpol-RRE-8891 (GP-8891) decreased HIV-1 Gag protein expression (Fig. 1, lane 2), suggesting that part or all of the putative CTE was within RSV nt 8707 to 8891. Appreciably higher levels of both the $Pr55^{gag}$ precursor and processed Gag polypeptides were present when a Rev expression construct (pCMVrev) was cotransfected with GP-8891 (Fig. 1, lane 3). The parental CMVgagpol-RRE construct failed to express Gag proteins in the absence of Rev expression (data not shown).

Proteins for Western blotting were obtained from cells lysed without prior removal of associated viral particles; thus, the processed Gag proteins observed are thought to be present in budding virions. Higher Gag expression levels were correlated with more processed Gag proteins (Fig. 1), suggesting that a higher proportion of Gag precursors was assembling into particles. All of the *gag-pol* transcripts in the experiment shown in Fig. 1 contain a poly(A) site in the RSV LTR upstream of the β-globin intron and its poly(A) site to minimize potential complications due to splicing from HIV-1 splice sites to globin splice sites.

In direct contrast to these results showing that the RSV element was active in CEFs but not in COS cells, we detected Gag expression with an HIV-1 construct containing the MPMV CTE in COS cells but not in CEFs (data not shown). In summary, the 3′ UTR of RSV was found to promote HIV-1 *gag* gene expression in the absence of Rev, suggesting that RSV may contain a CTE-like element similar to that of subgroup D retroviruses (11, 66).

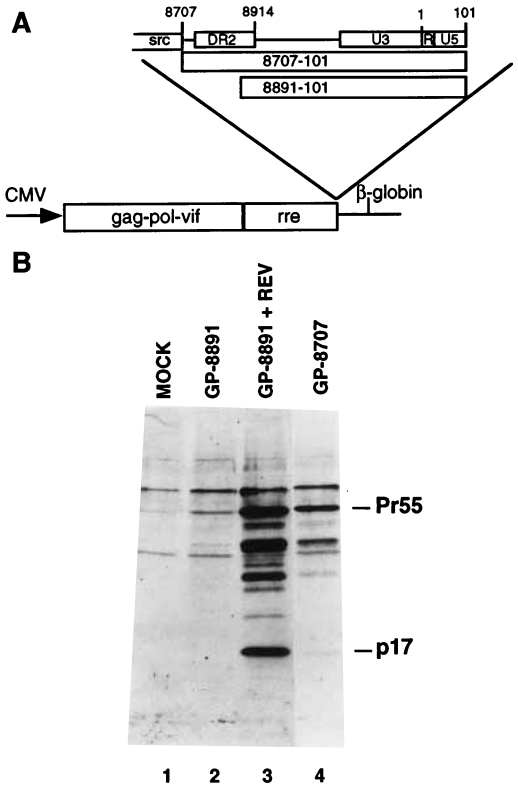

FIG. 1. The 3′ UTR of RSV promotes Rev-independent HIV-1 Gag protein expression. (A) Schematic representation of CMVgagpol-RRE and the 3′ UTR of RSV. Constructs GP-8707 and GP-8891 were produced by inserting RSV nt 8707 to 101 and 8891 to 101, respectively, downstream of the RRE in the CMVgagpol-RRE construct as depicted. CMV, cytomegalovirus. (B) Western blot with anti-HIV-1 antibodies from CEFs transfected with no DNA (lane 1), GP-8891 (lane 2), GP-8891 and the pCMVrev expression plasmid (lane 3), and GP-8707 (lane 4). Pr55 is the HIV-1 Gag precursor protein; p17 is the Gag matrix protein. A recombinant HIV-1 $p24^{gag}$ protein was coelectrophoresed as a marker (data not shown).

RSV element is localized to nt 8770 to 8925, which contain one copy of a DR sequence. To further map the *cis*-acting RSV element that promoted Rev-independent expression of HIV-1 Gag proteins, a CAT construct containing the HIV-1 IR-1 inhibitory region was used. It has been reported that the IR-1 region within the HIV-1 *gag* gene inhibits *gag* and reporter gene expression; this inhibition is overcome when Rev is present in *trans* and the RRE is present in *cis* (14, 36, 51, 53). We asked whether sequences from the RSV 3′ UTR would promote CAT expression from the CAT-IR-1 construct in the absence of Rev and the RRE. Figure 2 depicts a subset of the RSV sequences that were inserted between the IR-1 region and the SV40 early poly(A) site within this CAT construct.

When the IR-1 region was inserted in the antisense (−) orientation downstream of the *cat* gene, maximum CAT activity was observed; however, when the IR-1 region was inserted in the sense (+) orientation, CAT expression was inhibited 47-fold relative to CAT-IR-1(−) (Fig. 2A). Cotransfection of a Rev expression construct into RRE-containing CAT-IR1+ constructs alleviated the repression (data not shown). To identify any RSV *cis*-acting sequences capable of overcoming the IR-1-mediated inhibition of *cat* gene expression, various regions from the 3′ UTR of RSV were inserted into pRSV2CAT-IR-1+ in the sense orientation, and CAT activity was monitored. All CAT activity levels were expressed as fold activation relative to

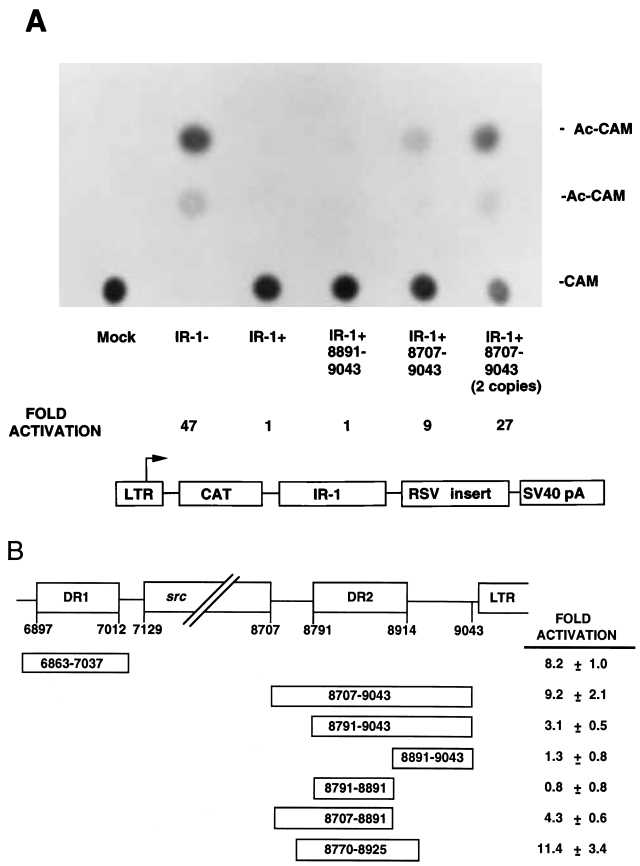

FIG. 2. Mapping of the RSV element required to promote HIV-1 gene expression with a reporter gene construct. (A) Schematic of the CAT reporter gene construct containing the *gag* HIV-1 IR-1 repressive sequence, used to identify the essential region in the 3′ UTR of RSV RNA. Representative CAT assays with CEFs transfected with the constructs indicated are depicted. Fold activation shown is relative to pRSVCAT-IR-1(+) repressed control. (B) Schematic drawing of the PrC RSV genome spanning nt 6863 to 9043; regions which were cloned into the CAT reporter gene construct are depicted. All values are reported as the fold activation over the pRSVCAT-IR-1(+) construct which was run in parallel with each CAT assay. Values depicted are normalized to a cotransfected β-galactosidase construct and represent the mean ± standard deviation obtained from at least three separate transfections for each construct tested.

that of the inhibited control plasmid pRSV2CAT-IR-1+, which was assayed in parallel.

Initially, the entire RSV 3′ UTR between the 3′ end of the *src* gene and the 5′ end of the LTR (nt 8707 to 9043) was tested and found to give ninefold enhancement over the control IR-1+ construct (Fig. 2A). When two tandem copies of the UTR were present, CAT activity was increased 27-fold over IR-1+ levels (Fig. 2A). However, no significant activation of CAT activity was observed with the 3′ half of the 3′ UTR (nt 8891 to 9043), termed the E element (52). These results are consistent with those shown in Fig. 1, suggesting that the entire RSV 3′ UTR can override IR-1-mediated inhibition to promote gene expression (*gag* or *cat*), while the 3′ half of this region lacks this ability. Further, this suggests that the RSV LTR which was present in the HIV-1 constructs used in Fig. 1 to supply a poly(A) site is not necessary for this activity.

In a series of additional CAT-IR-1+ constructs, the 5′ end of the RSV element promoting CAT activity was localized between nt 8770 and 8791, and its 3′ end was localized between nt 8891 and 8925 (Fig. 2B). The minimal region tested which produced CAT activity comparable to that of the entire 3′ UTR spanned nt 8770 to 8925 and was 11-fold more active than the control. This region contains one copy of a DR sequence (DR2), spanning nt 8791 to 8914 (17, 18, 52, 61). When constructs containing RSV fragments starting at the 5′ boundary of the DR (8791-9043) or ending just upstream of its 3′ boundary (8707-8891) were tested, CAT activity was reduced relative to that of constructs containing short sequences flanking DR2 on both sides, such as the 8770-8925 construct. Combining these endpoints to generate nt 8791 to 8891 (minimal DR as defined by Schwartz et al. [52]) without any flanking sequences did not increase CAT activity over that of the control CAT-IR-1+ construct. Thus, it appears that the boundaries of the CTE-like element defined by this assay are close to those of the DR sequence as defined originally by Czernilofsky et al. (18).

The other copy of the DR sequence (DR1), bearing 82% homology to DR2 (18), is present upstream of the *src* gene at nt 6897 to 7012 in PrC RSV. This upstream DR1 plus flanking sequences (nt 6863 to 7037) was also tested in the CAT-IR1+ assay and showed levels of CAT activity similar to that of DR2 (eightfold enhancement) (Fig. 2B). In summary, both the upstream (DR1) and downstream (DR2) RSV DRs flanking the *src* gene were observed to activate CAT activity appreciably over the level inhibited by IR1+. Further, two tandem copies of the 3′ UTR were found to give about threefold more CAT activity than a single copy (Fig. 2A).

Viral replication requires at least one copy of the DR sequence. The results described above showed that an RSV *cis*-acting element, containing a DR sequence, was capable of promoting HIV-1 *gag* gene expression and of overcoming the inhibitory effect of the HIV-1 IR-1 region in the absence of Rev expression. We turned next to study the effect of this element on replication of RSV. Since two copies of the DR are present in RSV (17, 18, 52, 61), but only one copy of this sequence is present in other avian retroviruses (9, 46, 62), we asked whether one copy was sufficient for viral replication. Starting with a nonpermuted proviral clone of PrC RSV, we first generated a deletion mutant (pPrCΔDR) lacking both DR sequences as well as the *src* gene which they flank (Δ6897-8960). A short polylinker sequence was inserted at the site of the deletion. We then inserted either the upstream (DR1) or the downstream (DR2) DR into the polylinker of the deleted construct to generate variant viral constructs with a single copy of the DR (pPrC-DR1 and pPrC-DR2). Schematic drawings of these viral constructs are shown in Fig. 3.

Virus isolated from transfected cells was assayed for reverse transcriptase activity and used to infect CEFs, and replication was monitored by additional reverse transcriptase assays. Observations of reverse transcriptase activity depicted in Fig. 4 showed that the wild-type virus and both of the single DR constructs replicated efficiently; in contrast, replication of viral mutant pPrCΔDR, which has neither direct repeat, was not detectable for up to 10 days after infection (Fig. 4) or transfection of CEFs (data not shown). While the constructs with a single DR replicated quite well, their growth frequently lagged slightly behind that of the wild-type virus but eventually attained comparable levels of reverse transcriptase production (Fig. 4). However, the rate of replication was equivalent for pPrC-DR1 and pPrC-DR2, suggesting that the two DRs are indistinguishable in terms of viral replication. The higher initial replication rate observed with the wild-type RSV over that of the constructs with a single DR sequence could be due to the presence of the additional DR sequence and/or the *src* gene. A construct containing two tandem copies of DR1 and DR2 but lacking the *src* gene replicated at a level intermediate between

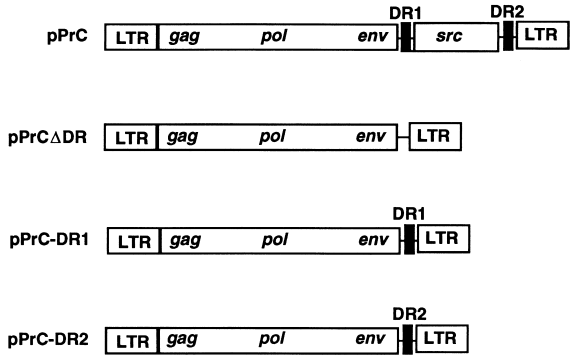

FIG. 3. Schematic diagrams of wild-type PrC RSV genome and deletion mutants generated from it. pPrC, wild-type RSV construct; pPrCΔDR, RSV construct with deletion of both DRs and *src* (deletion of nt 6897 to 8960); pPrC-DR1, RSV construct containing one copy of DR1 and flanking sequences (nt 6897 to 7016); pPrC-DR2, RSV construct containing one copy of DR2 and flanking sequences (nt 8770 to 8925).

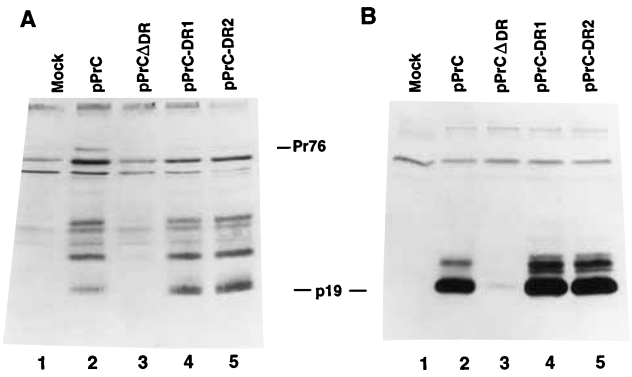

FIG. 5. RSV deletion mutant pPrCΔDR exhibits severely diminished levels of Gag proteins. CEFs were transfected with the viral constructs shown in Fig. 3, and proteins were harvested 48 h posttransfection from both cells (A) and isolated viral particles (B). Western blots shown were probed with polyclonal antibodies raised against avian myeloblastosis virus Gag protein p19. Lanes 1, mock; lanes 2, pPrC wild type; lanes 3, pPrCΔDR; lanes 4, pPrC-DR1; lanes 5, pPrC-DR2.

that of constructs with a single DR and the wild-type RSV construct (65), suggesting that both *src* and the additional DR sequence are contributing to the higher replication level. In conclusion, this experiment shows that one copy of either DR sequence is essential for viral replication. A similar result was obtained previously with constructs bearing slightly different deletions made from the Schmidt-Ruppin A (SR-A) strain of RSV (56).

Viral protein expression is greatly reduced for an RSV mutant lacking both copies of the DR sequence. To study the mechanism of action of the RSV DR sequence in RSV, we analyzed steady-state levels of viral protein and RNA by using the constructs shown in Fig. 3. Transient transfections of wild-type pPrC RSV and viral constructs pPrCΔDR, pPrC-DR1, and pPrC-DR2 were performed, and cellular proteins were harvested 48 h posttransfection. Western blots of total cellular proteins were probed with rabbit anti-avian myeloblastosis virus $p19^{gag}$ antibodies; Fig. 5A depicts a representative Western blot. Equivalent levels of the processed viral Gag p19 matrix protein were observed for wild-type RSV (lane 2) and viral constructs containing either DR1 or DR2 sequence (lanes 4 and 5). In contrast, viral mutant pPrCΔDR lacking both DR sequences had barely detectable levels of intracellular Gag proteins (lane 3). The partially and completely processed Gag proteins observed are thought to be present in cell-associated virus particles.

Viral particles were also isolated from culture media 48 h posttransfection, and levels of viral proteins in these particles were determined. Western blots of viral proteins depicted in Fig. 5B showed similar levels of $p19^{gag}$ for wild-type RSV (lane 2) and for viruses with one copy of either DR sequence (lanes 4 and 5). However, severely diminished levels of $p19^{gag}$ were observed with the viral mutant lacking both DRs (lane 3). Thus, the presence of at least one DR is necessary for efficient expression of RSV Gag proteins from our proviral constructs. Two copies of the DR, present in wild-type RSV, were not associated with higher levels of Gag protein expression than was one copy in these assays. Consistent with this, avian leukosis viruses replicate efficiently with a single copy of the DR sequence.

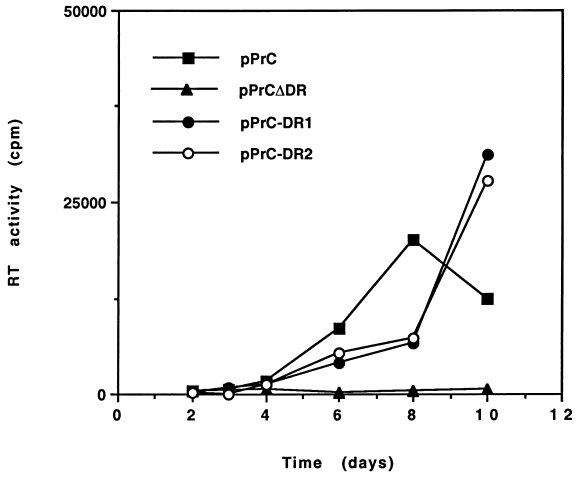

FIG. 4. RSV replication requires at least one copy of the DR sequence. The results of reverse transcriptase (RT) assays performed on virus particles isolated from the culture supernatants of CEFs infected with each virus are shown. Constructs used for the original transfections are shown in Fig. 3.

A viral mutant lacking DR1 and DR2 shows reduced cytoplasmic levels of unspliced RNA. Since Gag protein expression was severely restricted in the viral mutant lacking both DRs and this restriction was alleviated by insertion of a single DR sequence, we next studied steady-state viral RNAs produced by these same viral constructs in an attempt to identify the primary site of action of the DR element. For these studies, CEFs were transiently transfected with viral constructs, and RNA was isolated from the cells and from viral particles 48 h posttransfection. RNA was probed by RNase protection assays using an antisense riboprobe complementary to the common 5′ splice site, allowing resolution of unspliced RNA and total spliced mRNA (*env* and *src*). In these experiments, the relevant comparison is between the construct lacking both copies of the DR (pPrCΔDR) and those with a single copy of the DR sequence (pPrC-DR1 and pPr-DR2); the wild-type virus (pPrC) is not directly comparable as it contains the *src* gene and its 3′ splice site, in addition to both DRs. Data presented in Table 1 are the average of four independent experiments.

Analysis of packaged virion RNA is shown in Fig. 6A. The pPrCΔDR construct (lane 2) exhibited a greater than 25-fold reduction in packaged viral RNA relative to that of the single DR constructs (lane 3 and 4). Insertion of either DR1 or DR2 restored high levels of packaged virion RNA; in fact, we were

TABLE 1. Quantitation of RSV unspliced and spliced RNA in fractionated CEFs[a]

Type of RNA ratio	Viral construct	Ratio		
		Total	Cytoplasm	Nucleus
Unspliced/spliced	pPrCΔDR	0.3 ± 0.1	0.5 ± 0.2	1.2 ± 0.6
	pPrC-DR1	1.4 ± 0.5	1.8 ± 0.2	1.9 ± 0.8
	pPrC-DR2	1.5 ± 0.6	2.0 ± 0.2	2.1 ± 1.4
	pPrC	4.4 ± 1.2	4.2 ± 1.1	3.4 ± 2.3
Unspliced cytoplasm/ unspliced nucleus[b]	pPrCΔDR	0.16 ± 0.08		
	pPrC-DR1	1.40 ± 0.50		
	pPrC-DR2	1.80 ± 0.50		
	pPrC	1.30 ± 1.60		

[a] Values are the means ± standard deviations of four separate transfection and fractionation experiments.
[b] The relative levels of unspliced RNA are normalized to pmyc 23 transfection control RNA.

surprised to observe higher-than-wild-type levels (lane 1) of virion RNA with these constructs. Since the pPrCΔDR construct is defective in production of virion proteins (Fig. 5B), additional experiments with virion proteins supplied in *trans* will be necessary to address the role of the DR sequence in packaging of virion RNA. It appears that the level of packaged RNA observed in Fig. 6A may reflect limiting levels of viral proteins.

Next, RNase protection assays examined the relative steady-state levels of RNAs expressed in whole-cell preparations. Total intracellular viral RNA was probed with a riboprobe spanning the viral 5′ splice site (Fig. 6B), and spliced and unspliced RNA levels were quantitated separately. While the level of spliced RNA for the construct lacking DR sequences (lane 2) was similar to that with the single DR constructs (lanes 3 and 4), the level of unspliced RNA was reduced approximately fivefold in the construct lacking the DR sequences (Fig. 6B).

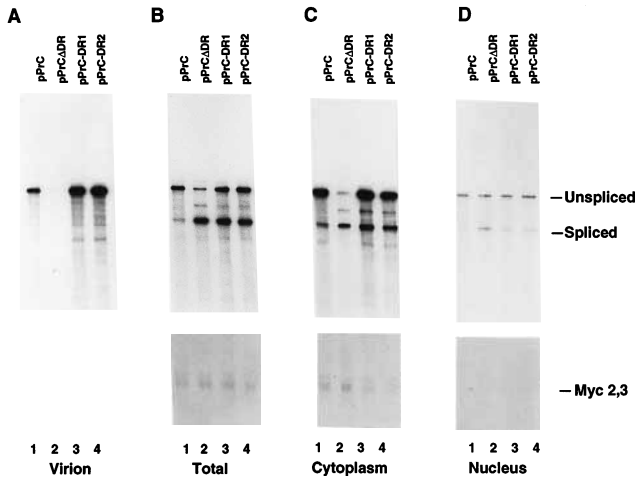

FIG. 6. Analysis of viral RNA levels in viral particles and total, nuclear, and cytoplasmic fractions derived from transfected CEFs. Cells were transfected with the viral constructs shown in Fig. 3, and RNA was harvested 48 h later. RNase protection assays were carried out with a riboprobe spanning the RSV 5′ splice site. Results are depicted for viral particles (A), total cellular RNA (B), cytoplasmic RNA (C), and nuclear RNA (D). The pMyc23 construct was cotransfected with the viral constructs as a control for RNA recovery and cell fractionation and assayed in the same hybridization reaction with the viral probe. Lanes 1, wild-type pPrC; lanes 2, deletion mutant pPrCΔDR; lanes 3, pPrC-DR1; lanes 4, pPrC-DR2.

This resulted in a fivefold decrease in the ratio of unspliced to spliced RNA for pPrCΔDR compared with either virus containing one copy of either DR1 or DR2 (Table 1). The imbalance in relative amounts of spliced and unspliced RNA observed with pPrCΔDR may contribute to the replication defect of this construct. Previous studies of RSV mutants with improved *env* or *src* 3′ splice site sequences, resulting in a majority of spliced mRNA species, showed that these are defective for replication (33, 64).

Since the reduction in Gag protein synthesis from pPrCΔDR relative to pPrC-DR1 or pPrC-DR2 constructs (Fig. 5) appeared greater than the reduction in the whole-cell level of unspliced viral RNA (potential *gag* mRNA) (Fig. 6B), we asked whether transport of the unspliced RNA from the nucleus to the cytoplasm might be restricted in the absence of both DR sequences, making this RNA unavailable for translation. To examine this possibility, cells were separated into nuclear and cytoplasmic fractions, and RNA in both fractions was probed by the same RNase protection assay. As a control, we assayed a cotransfected pMyc23 construct in the same hybridization reaction. This construct contains portions of cellular *myc* exons 2 and 3 and the intervening intron, which is spliced efficiently (40). In the fractionated cells, an average of greater than 90% of the spliced *myc* transcripts were observed in the cytoplasmic fractions (Fig. 6C and D). We observed by Northern (RNA) analysis that 80% of U1 small nuclear RNA was retained in the nuclear fractions (data not shown). These controls demonstrated that neither cytoplasmic nor nuclear fractions were significantly contaminated.

Figure 6C depicts the cytoplasmic RNA levels of unspliced and spliced viral RNA, and Table 1 shows quantitation of viral RNA species. The viral mutant lacking both DR1 and DR2 showed an average ninefold reduction in unspliced, cytoplasmic RNA levels relative to those of constructs with one DR (Fig. 6C). This value is an underestimate of the difference in total unspliced RNA levels between these constructs since virion RNA was not considered in these calculations. When the constructs depicted in Fig. 3 were made packaging defective by introduction of a deletion in the NC protein (mutant 10.8 described in reference 42), we observed 30-fold less cytoplasmic RNA with the construct lacking DR sequences in comparison with those with a single DR sequence (data not shown). Quantitation of spliced RNA levels in the cytoplasm (Fig. 6C) revealed a twofold decrease for pPrCΔDR compared with pPrC-DR1 and pPrC-DR2. This resulted in a net fourfold reduction in the ratio of unspliced to spliced RNA levels in the cytoplasm for the pPrCΔDR construct (Table 1).

Nuclear RNA levels were also measured (Fig. 6D). In contrast to the cytoplasmic results, all of the constructs manifested similar levels of unspliced and spliced RNA in the nucleus. Thus, the ratio of cytoplasmic to nuclear unspliced RNA was decreased about 10-fold for the construct lacking both DR elements compared with the average cytoplasmic/nuclear unspliced RNA ratio for pPrC-DR1 and pPrC-DR2 constructs (Table 1). Similar experiments with packaging-defective constructs showed a 30-fold decrease in the ratio of cytoplasmic to nuclear unspliced RNA levels (data not shown). These experiments demonstrated a significant decrease in levels of unspliced cytoplasmic RNA and in the ratio of unspliced to spliced RNA levels in the cytoplasm.

Unspliced viral RNAs lacking DR sequences are unstable. The reduced levels of cytoplasmic unspliced RNA observed with the construct lacking DRs (Fig. 6; Table 1) suggested that the RNA was less stable than RNA from constructs with a single DR. To more directly measure the effect of the DR sequences on the stability of viral RNAs, CEFs were trans-

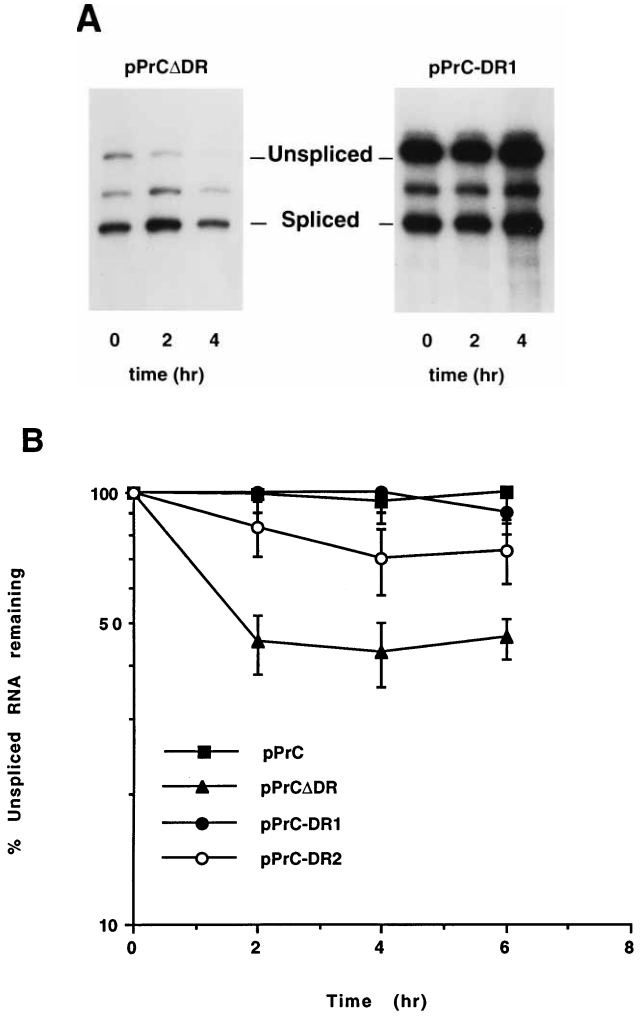

FIG. 7. Effect of the DR element on RNA stability. Transfected CEFs were incubated with actinomycin D for each designated time period followed by RNA isolation and RNase protection analysis to determine viral RNA levels. (A) Depiction of a representative RNase protection assay of pPrCΔDR and pPrC-DR1 constructs with a probe spanning the 5′ splice site. (B) Graph of the levels of unspliced RNA at various times after addition of actinomycin D, presented as a percentage of the RNA level at the time of addition of actinomycin D. All of the unspliced RNA levels have been normalized to spliced RNA levels, which are relatively constant during actinomycin D treatment (6, 7). The data shown represent the average of four separate transfection experiments with the viral constructs shown in Fig. 3.

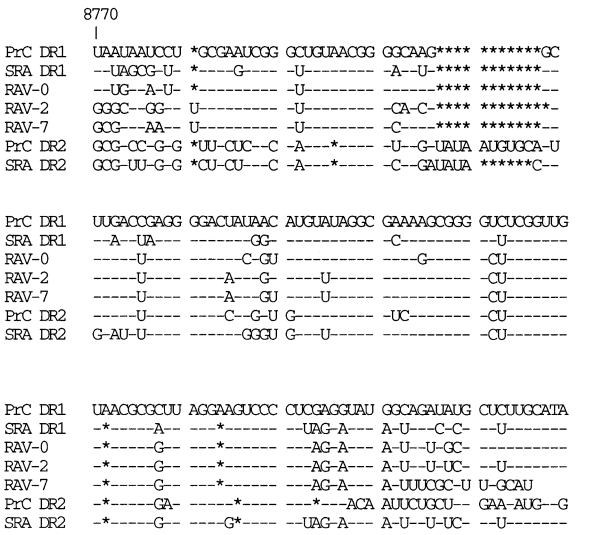

FIG. 8. Conservation of the DR elements between avian retroviruses. The DR sequences and 5′ flanking regions for DR1 (upstream of *src*) and DR2 (downstream of *src*) from the RSV genomes of the PrC (52) and SR-A (18, 61) strains were aligned with the single DR sequence and 5′ flanking sequences of RAV-0 (62), RAV-2 (9), and RAV-7 (46). For this comparison, numbering corresponds to that established for PrC DR2 (52).

fected with the RSV constructs shown in Fig. 3. Forty-eight hours after transfection, cells were incubated with 1 μg of actinomycin D per ml, and RNA was harvested at 2-h intervals and subjected to RNase protection assays (Fig. 7A). For each time point, the level of unspliced RNA was normalized relative to that of spliced RNA to adjust for differences in RNA recovery. We found that spliced RNA was relatively stable with all of the constructs compared with RNA of the endogenous cellular glyceraldehyde phosphate dehydrogenase gene assayed in the same hybridization reaction as the viral probe (data not shown).

As previously observed (6, 7, 59), the wild-type pPrC RSV RNA was very stable, showing little detectable change in RNA levels over the 6-h incubation period (Fig. 7B). A high degree of stability was also observed with the pPrC-DR1 construct (Fig. 7A and B). The pPrC-DR2 construct was slightly less stable than either the wild-type or pPrC-DR1 construct (Fig. 7B). The relative levels of unspliced pPrC-DR2 RNA declined to about 70% of the initial level after 4 h of incubation with actinomycin D. The pPrCΔDR RNAs appeared to be less stable than any of the other transcripts; the unspliced RNA levels fell to approximately 50% of the initial level during the first 2 h of actinomycin D treatment and then leveled off (Fig. 7A and B). We have not determined the intracellular compartment in which this increased RNA turnover is occurring. Unspliced RNA degradation may be occurring in the nucleus, possibly as a consequence of a block to export; alternatively, it may be occurring in the cytoplasm.

Sequence of RSV DR element is conserved among avian retroviruses. Since the DR sequence appears to be critical for replication of RSV, we examined the conservation of this element between avian retroviruses. We observed a high amount of sequence similarity between the avian sarcoma viruses (PrC [52] and SR-A [18, 61] strains of RSV), which have two copies of the DR element, and avian viruses with a single DR element such as Rous-associated virus-2 (RAV-2) (9), RAV-7 (46), and the endogenous virus RAV-0 (62). The upstream PrC DR1 sequence has considerable homology to RAV-0, RAV-7, RAV-2, and DR-1 of SR-A RSV (Fig. 8). The downstream DR2 of both PrC and SR-A strains of RSV shows more variation from the other DR sequences. In particular, the PrC DR2 sequence has an insertion of 11 nt (at nt 8804) not present in either the DR1 sequence of PrC RSV or the single DR of RAV-7 or RAV-0. SR-A DR2 also contains an insertion at the same site, but it consists of only 5 nt.

A highly variable region between these different DR sequences was observed between nt 8832 and 8838 (PrC RSV numbering system [52]). This variable region formed the 5′ half of a loop in computer-predicted, RNA secondary structures, which appear to be highly conserved among all the avian retroviral DR sequences shown in Fig. 8 (data not shown). There was reduced similarity between sequences at the 3′ end of DR1 and DR2 (Fig. 8); however, at least part of these sequences were necessary for activation in the CAT-IR-1 con-

structs (Fig. 2). The DR sequences were initially identified as 115 (DR1) and 123 (DR2) nt in SR-A RSV (18, 61) and 93 (DR1) and 101 (DR2) nt in PrC RSV (52) because of this incomplete homology. For these comparisons, we have used the 123-nt designation for the DR sequence (Fig. 8). It is possible that the sequence differences between DR1 and DR2 account for the slight difference in stability of unspliced RNA noted above (Fig. 7); however, this was not reflected in the viral replication rates or unspliced viral RNA and Gag protein levels.

DISCUSSION

The results presented here suggest that at least one copy of the RSV *cis*-acting RNA element found in the 3′ UTR is essential for viral replication. This conserved element was necessary for expression of RSV Gag proteins from unspliced viral transcripts. Further, RSV constructs lacking both DRs showed a significant decrease in cytoplasmic levels of unspliced viral RNA. RNA stability studies suggested that the unspliced RNA of the construct lacking both copies of the DR element was less stable than that of the constructs with a single copy of the DR element. We have not determined whether the unspliced viral RNA is degraded in the nucleus or the cytoplasm in the absence of this *cis*-acting RNA element. If degradation is occurring in the nucleus, it could result from a block in nucleocytoplasmic export of unspliced RNA. Replacement of either single DR element into the deleted construct restored viral replication, Gag protein synthesis, and high levels of unspliced RNA in the cytoplasm.

Our results confirm earlier studies with SR-A RSV constructs, which show that one copy of the DR sequence is essential for viral replication (56). In agreement with previous studies (2, 56), we observed much less packaged virion RNA when the DR element was absent. However, in contrast to the study of Sorge et al. (56), we also saw greatly reduced levels of Gag proteins and unspliced viral RNA in the cytoplasm when the DR sequence was absent. Since stable export of unspliced RNA to the cytoplasm and Gag protein synthesis are both prerequisites for packaging, we think that the previously observed packaging defect is actually the result of a defect occurring at an earlier step in viral RNA metabolism, that of cytoplasmic accumulation of unspliced RNA.

The RSV *cis*-acting RNA element was also found to promote Rev-independent expression of HIV-1 Gag proteins in CEFs, suggesting that it may function in a similar manner as the HIV-1 Rev-RRE regulatory complex. Bray et al. (11) obtained similar results upon insertion of the MPMV CTE into HIV-1 constructs; thus, it appears that *cis*-acting sequences involved in posttranscriptional regulation may be common to all simple retroviruses. Both the MPMV CTE and the avian retroviral DR element are located in the 3′ UTR of the viral RNAs. While no significant sequence similarity was observed between the MPMV and RSV elements, computer-predicted RNA secondary structures are similar for both elements, both having a long stem structure (data not shown). While both the MPMV CTE and the RSV 3′ UTR element promote cytoplasmic expression of unspliced viral RNA, we have not established that their mechanisms of action are identical. Comparisons between the two elements are difficult since we have not yet found a cell type in which both the MPMV and RSV DR elements are active. The MPMV CTE was proposed to facilitate nucleocytoplasmic transport of HIV unspliced RNA on the basis of experiments in COS cells (11). The RSV DR element is not active in COS cells, and our experiments with CEFs suggest that it may be promoting cytoplasmic accumulation of unspliced RNA by stabilizing the RNA. If the instability of the unspliced RSV RNA in the absence of the DR sequences is a consequence of a block in export, its stabilization may be coupled to transport, and the RSV DR element may be a CTE. Additional experiments are necessary to resolve this issue.

Rev has been observed to play a role in nearly all aspects of posttranscriptional regulation of expression of HIV-1 unspliced and incompletely spliced, RRE-containing transcripts. Rev has been observed to facilitate RNA transport from the nucleus to the cytoplasm (20, 23, 25, 38), to stabilize RNA in both the nucleus and cytoplasm (23, 37), to inhibit its splicing (21, 22, 34), and to promote its loading onto polysomes (4, 14, 19). Since RNA degradation rates vary in different cell types, the same element can appear to be carrying out different functions in different cells. Malim and Cullen have observed that differences in degradation rates of HIV-1 mRNAs between COS and human T cells can affect the ratio of unspliced to spliced viral RNAs in the cell in the absence of Rev (37). The distribution of RSV RNA lacking the DR sequences in CEFs appears analogous to that of HIV-1 RNA generated in Rev-defective stably transformed human T cells (37).

PrC RSV replication is restricted to avian cells. We have preliminary evidence that the activity of its DR element is also restricted to avian cells, and this may contribute to the replication defect of the virus elsewhere. Interestingly, the decreased ratio of unspliced to spliced RNAs observed with the RSV construct lacking the DR sequences is very similar to that observed when RSV is transfected into mammalian cells (48). It is possible that this is a result of instability of the unspliced RNA rather than increased splicing efficiency as previously proposed. Consistent with this, a recent study shows that the Rev-RRE system increases the production of avian retroviral Gag proteins and allows viral particle production in mammalian cells, apparently acting to increase cytoplasmic levels of unspliced viral RNA (44).

Rev is capable of overcoming the poorly understood repression of HIV-1 protein expression due to CRS elements (14, 36, 51, 53), as well as that due to spliceosomal retention of RNA with suboptimal splice sites (12). It can also facilitate nuclear export of RRE-containing RNAs which lack CRS elements and splice sites (25). Rev mediates an alternative path for mRNA export without removal of introns by splicing as is normally required for cellular mRNAs (25). However, suboptimal splice sites also appear to be necessary for maintenance of incompletely spliced RNAs (12, 47). It is interesting that RSV, which has suboptimal 3′ splice sites as does HIV (47), does not have CRS elements (53). However, the RSV DR element is capable of promoting low-level expression with HIV-1 constructs containing CRS elements in the absence of Rev.

Since RSV does not encode Rev-like regulatory proteins, we think it is likely that the RSV DR element functions in conjunction with cellular factors, which are likely to be involved in stability and nuclear export of RNA. The HIV-1 Rev protein can shuttle between the nucleus and the cytoplasm (5, 43, 50), probably acting as a chaperone for transport of intron-containing viral RNAs. The activation domain of Rev has recently been observed to bind nucleoporin-like proteins, suggesting that it may interact with the nuclear pore complex (10, 26, 60). In contrast, a herpes simplex virus thymidine kinase *cis*-acting RNA element, which promotes cytoplasmic accumulation of RNA transcribed from β-globin cDNA, was recently found to bind heterogeneous nuclear ribonucleoprotein particle L (35). It will be interesting to see whether the RSV DR element interacts with these or with novel cellular proteins.

Further work will be necessary to determine whether the alteration in the ratio of unspliced to spliced viral RNA seen with the construct lacking DR sequences is entirely due to instability of the unspliced RNA. If the DR element facilitates transport of the unspliced RNA out of the nucleus and away from the splicing apparatus, this could also contribute to a higher unspliced/spliced RNA ratio. The defect in replication observed with pPrCΔDR may in part be due to this imbalance in the ratio of unspliced to spliced RNAs. Previously studied RSV mutants with improved *env* or *src* 3′ splice site sequences that result in a majority of spliced mRNA species have been defective for replication (33, 64).

ACKNOWLEDGMENTS

We thank Denis Lazarev for construction of pDL2 and Paula Pitha-Rowe for comments on the manuscript. We thank Lou Hammarskjold for stimulating discussions and for HIV-1 expression plasmids, Mark McNally for the CAT-IR-1 plasmid, John Cammarata for pPrC, Tom Hope for the β-galactosidase plasmid, D. P. Bolognesi for anti-p19^{gag} serum, and the NIH AIDS Research and Reference Reagent Program for IgG from HIV-positive donors and for recombinant HIV-1 p24.

This work was supported by Public Health Service grants CA48746 and GM48327. R.A.O. was supported by NIH Predoctoral Training grant 52T32G07231.

REFERENCES

1. **Amendt, B. A., D. Hesslein, L.-J. Chang, and C. M. Stoltzfus.** 1994. Presence of negative and positive *cis*-acting elements within and flanking the first *tat* coding exon of human immunodeficiency virus type 1. Mol. Cell. Biol. **14:** 3960–3970.
2. **Aronoff, R., and M. Linial.** 1991. Specificity of retroviral RNA packaging. J. Virol. **65:**71–80.
3. **Arrigo, S., and K. Beemon.** 1988. Regulation of Rous sarcoma virus RNA splicing and stability. Mol. Cell. Biol. **8:**4858–4867.
4. **Arrigo, S. J., and I. S. Y. Chen.** 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 *vif*, *vpr*, and *env*/*vpu* 2 RNAs. Genes Dev. **5:**808–819.
5. **Arrigo, S. J., S. Heaphy, and J. K. Haines.** 1992. In vivo binding of wild-type and mutant human immunodeficiency virus type 1 Rev proteins: implications for function. J. Virol. **66:**5569–5575.
6. **Barker, G. F., and K. Beemon.** 1991. Nonsense codons within the Rous sarcoma virus *gag* gene decrease the stability of unspliced viral RNA. Mol. Cell. Biol. **11:**2760–2768.
7. **Barker, G. F., and K. Beemon.** 1994. Rous sarcoma virus RNA stability requires an open reading frame in the *gag* gene and sequences downstream of the *gag-pol* junction. Mol. Cell. Biol. **14:**1986–1996.
8. **Birnie, G.** 1978. Isolation of nuclei from animal cells in culture. Methods Cell Biol. **XVII:**13–26.
9. **Bizub, D., R. A. Katz, and A. M. Skalka.** 1984. Nucleotide sequence of noncoding regions in Rous-associated virus-2: comparisons delineate conserved regions important in replication and oncogenesis. J. Virol. **49:**557–565.
10. **Bogerd, H. P., R. A. Fridell, S. Madore, and B. R. Cullen.** 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell **82:**485–494.
11. **Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K. Jeang, D. Rekosh, and M. L. Hammarskjold.** 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. Proc. Natl. Acad. Sci. USA **91:**1256–1260.
12. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789–795.
13. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
14. **Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen.** 1991. Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. J. Virol. **65:**5305–5313.
15. **Coffin, J. M.** 1990. Retroviridae and their replication, p. 1437–1500. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
16. **Cullen, B. M.** 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiol. Rev. **56:**375–394.
17. **Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischer, and H. M. Goodman.** 1980. Nucleotide sequence of an avian sarcoma virus oncogene (*src*) and proposed amino acid sequence for gene product. Nature (London) **287:**198–203.
18. **Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischer, and H. M. Goodman.** 1983. Corrections to the nucleotide sequence of the *src* gene of Rous sarcoma virus. Nature (London) **301:**736–738.
19. **D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis.** 1992. The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of *gag*/*pol* and *vpu*/*env* mRNAs. Mol. Cell. Biol. **12:**1375–1386.
20. **Emerman, M., R. Vazeux, and K. Peden.** 1989. The *rev* gene product of human immunodeficiency virus affects envelope specific RNA localization. Cell **57:**1155–1165.
21. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46:**807–817.
22. **Felber, B. K., C. M. Drysdale, and G. N. Pavlakis.** 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J. Virol. **64:**3734–3741.
23. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86:**1496–1499.
24. **Felber, B. K., and G. N. Pavlakis.** 1993. Molecular biology of HIV-1: positive and negative regulatory elements important for virus expression. AIDS **7**(Suppl.)**:**S51–S62.
25. **Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Luhrmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475–483.
26. **Fritz, C. C., M. L. Zapp, and M. R. Green.** 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. Nature (London) **376:**530–533.
27. **Fu, X.-D., R. A. Katz, A. M. Skalka, and T. Maniatis.** 1991. The role of branchpoint and 3′ exon sequences in the control of balanced splicing of avian retrovirus RNA. Genes Dev. **5:**211–220.
28. **Gontarek, R. R., M. T. McNally, and K. Beemon.** 1993. Mutation of an RSV intronic element abolishes both U11/U12 snRNP binding and negative regulation of splicing. Genes Dev. **7:**1926–1936.
29. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
30. **Higuchi, R.** 1990. Using PCR to engineer DNA, p. 61–70. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
31. **Huang, Z.-M., and T. S. B. Yen.** 1994. Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts in the cytoplasm. J. Virol. **68:**3193–3199.
32. **Kane, S. E., and K. Beemon.** 1987. Inhibition of methylation at two internal N^6-methyladenosine sites caused by GAC to GAU mutations. J. Biol. Chem. **262:**3422–3427.
33. **Katz, R. A., and A. M. Skalka.** 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. Mol. Cell. Biol. **10:** 696–704.
34. **Kjems, J., and P. A. Sharp.** 1993. The basic domain of Rev from human immunodeficiency virus type 1 specifically blocks the entry of U4/U6 · U5 small nuclear ribonucleoproteins in spliceosome assembly. J. Virol. **67:**4769–4776.
35. **Liu, X., and J. E. Mertz.** 1995. HnRNP L binds a *cis*-acting RNA sequence element that enables intron-independent gene expression. Genes Dev. **9:**1766–1780.
36. **Maldarelli, F., M. A. Martin, and K. Strebel.** 1991. Identification of post-transcriptionally active inhibitory sequences in human immunodeficiency virus type 1 RNA: novel level of gene regulation. J. Virol. **65:**5732–5743.
37. **Malim, M. H., and B. R. Cullen.** 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. Mol. Cell. Biol. **13:**6180–6189.
38. **Malim, M. H., J. Hauber, S. V. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338:**254–275.
39. **McNally, M., and K. Beemon.** 1992. Intronic sequence and 3′ splice sites control Rous sarcoma virus RNA splicing. J. Virol. **66:**6–11.
40. **McNally, M., R. Gontarek, and K. Beemon.** 1991. Characterization of Rous sarcoma virus intronic sequences that negatively regulate splicing. Virology **185:**99–108.
41. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
42. **Meric, C., and P. F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S dimer formation and packaging. J. Virol. **60:**450–459.
43. **Meyer, B. E., and M. H. Malim.** 1994. The HIV-1 Rev *trans*-activator shuttles between the nucleus and the cytoplasm. Genes Dev. **8:**1538–1547.
44. **Nasioulas, G., S. H. Hughes, B. K. Felber, and J. M. Whitcomb.** 1995. Production of avian leukosis virus particles in mammalian cells can be me-

diated by the interaction of the human immunodeficiency virus protein Rev and the Rev-responsive element. Proc. Natl. Acad. Sci. USA **92:**11940–11944.

45. **Ogert, R. A., M. K. Gentry, E. C. Richardson, C. D. Deal, S. N. Abramson, C. R. Alving, P. Taylor, and B. P. Doctor.** 1990. Studies on the topography of the catalytic site of acetylcholinesterase using polyclonal and monoclonal antibodies. J. Neurochem. **55:**756–763.
46. **Onuki, Y., A. Ohshima, Y. Kawarabayasi, and T. Takeya.** 1987. Comparison of the structural organizations in the 3′-terminal regions of five avian retrovirus strains: RAV7, RAV50, B77, PR-B, and SR-B. Virology **156:**163–166.
47. **O'Reilly, M. M., M. T. McNally, and K. L. Beemon.** 1995. Two strong 5′ splice sites and competing, suboptimal 3′ splice sites involved in alternative splicing of human immunodeficiency virus type-1 RNA. Virology **213:**373–385.
48. **Quintrell, N., S. H. Hughes, H. E. Varmus, and J. M. Bishop.** 1980. Structure of viral DNA and RNA in mammalian cells infected with avian sarcoma virus. J. Mol. Biol. **143:**363–393.
49. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1986. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
50. **Richard, N., S. Iacampo, and A. Cochrane.** 1994. HIV-1 Rev is capable of shuttling between the nucleus and cytoplasm. Virology **204:**123–131.
51. **Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic *cis*-acting art gene-responsive sequences of the human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **85:**2071–2075.
52. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32:**853–869.
53. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Distinct RNA sequences in the *gag* region of human immunodeficiency virus type 1 decrease RNA stability and inhibit expression in the absence of Rev protein. J. Virol. **66:**150–159.
54. **Smith, A. J., M. L. Cho, M. L. Hammarskjold, and D. Rekosh.** 1990. Human immunodeficiency virus type 1 $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. **64:**2743–2750.
55. **Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine.** 1986. A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature (London) **321:**412–417.
56. **Sorge, J., W. Ricci, and S. H. Hughes.** 1983. *cis*-acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. J. Virol. **48:**667–675.
57. **Staffa, A., and A. Cochrane.** 1994. The *tat*/*rev* intron of human immunodeficiency virus type 1 is inefficiently spliced because of suboptimal signals in the 3′ splice sites. J. Virol. **68:**3071–3079.
58. **Stoltzfus, C. M.** 1988. Synthesis and processing of avian sarcoma retrovirus RNA. Adv. Virus Res. **35:**1–37.
59. **Stoltzfus, C. M., K. Dimock, S. Horikami, and T. A. Ficht.** 1983. Stabilities of avian sarcoma virus RNAs: comparison of subgenomic and genomic species with cellular mRNAs. J. Gen. Virol. **64:**2191–2202.
60. **Stutz, F., M. Neville, and M. Rosbash.** 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. Cell **82:**495–506.
61. **Takeya, T., R. A. Feldman, and H. Hanafusa.** 1982. DNA sequences of the viral and cellular *src* gene of chickens. J. Virol. **44:**1–11.
62. **Tsichlis, P. N., L. Donehower, G. Hager, N. Zeller, R. Malavarca, S. M. Astrin, and A. M. Skalka.** 1982. Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its nononcogenic parent: genetic regions that control growth rate and oncogenic potential. Mol. Cell. Biol. **2:**1331–1338.
63. **Wong-Staal, F.** 1990. Human immunodeficiency viruses and their replication, p. 1529–1544. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
64. **Zhang, L., and C. M. Stoltzfus.** 1995. A suboptimal *src* 3′ splice site is necessary for efficient replication of Rous sarcoma virus. Virology **206:**1099–1107.
65. **Zhang, X., and K. L. Beemon.** 1996. Unpublished results.
66. **Zolotukhin, A. S., A. Valentin, G. N. Pavlakis, and B. K. Felber.** 1994. Continuous propagation of RRE(−) and Rev(−)RRE(−) human immunodeficiency virus type 1 molecular clones containing a *cis*-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. J. Virol. **68:**7944–7952.