MOLECULAR AND CELLULAR BIOLOGY, Nov. 1988, p. 4858–4867
0270-7306/88/114858-10$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 8, No. 11

Regulation of Rous Sarcoma Virus RNA Splicing and Stability

SALVATORE ARRIGO† AND KAREN BEEMON*

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

Received 12 May 1988/Accepted 18 August 1988

Only a fraction of retroviral primary transcripts are spliced to subgenomic mRNAs; the unspliced transcripts are transported to the cytoplasm for packaging into virions and for translation of the *gag* and *pol* genes. We identified *cis*-acting sequences within the *gag* gene of Rous sarcoma virus (RSV) which negatively regulate splicing in vivo. Mutations were generated downstream of the splice donor (base 397) in the intron of a proviral clone of RSV. Deletion of bases 708 to 800 or 874 to 987 resulted in a large increase in the level of spliced RSV RNA relative to unspliced RSV RNA. This negative regulator of splicing (*nrs*) also inhibited splicing of a heterologous splice donor and acceptor pair when inserted into the intron. The *nrs* element did not affect the level of spliced RNA by increasing the rate of transport of the unspliced RNA to the cytoplasm but interfered more directly with splicing. To investigate the possible role of *gag* proteins in splicing, we studied constructs carrying frameshift mutations in the *gag* gene. While these mutations, which caused premature termination of *gag* translation, did not affect the level of spliced RSV RNA, they resulted in a large decrease in the accumulation of unspliced RNA in the cytoplasm.

Retroviral pre-mRNA splicing differs dramatically from that of cellular mRNAs. Nearly all eucaryotic transcripts either are spliced to completion, so that no unspliced RNA is detectable in the cytoplasm, or do not contain introns and so require no splicing (reviewed in references 14 and 27). Retroviruses, on the other hand, produce a single primary transcript which is utilized in both spliced and unspliced forms; unspliced RNAs serve as genomic RNA and also as mRNA for the *gag* and *pol* genes, while spliced mRNAs are translated to form *env* products (32, 36). In the case of the avian transforming retrovirus Rous sarcoma virus (RSV), alternative splicing of the primary transcript from a common splice donor also generates the *src* mRNA (32, 36). Retroviral RNA must be spliced to an intermediate degree rather than to completion, so that the proper balance of unspliced and spliced RNAs reaches the cytoplasm. Since retroviruses must use the same splicing machinery as the eucaryotic cells they infect, the retroviral transcripts need to be partially spliced by a machinery which seems geared to all-or-none splicing.

There may be regulatory functions within retroviruses which mediate incomplete splicing (reviewed in references 10 and 32). One possible mechanism is that the retroviral splice donors and acceptors may be recognized inefficiently by splicing factors. Alternatively, *cis*-acting sequences elsewhere in the viral genome may directly interfere with splicing processes or may facilitate transport of the unspliced precursor RNA away from the splicing machinery, indirectly resulting in a decrease in the level of spliced RNA. A third possibility is that viral proteins may be involved in regulating the level of splicing. A final possibility is that the regulation occurs at the level of RNA stability as well as of splicing.

Splicing of cellular mRNAs such as globin does not seem to require specific intron sequences but only splice donor, branch point, and splice acceptor sequences (28, 34, 37). Although there appears to be a minimal intron size, replacement of the intron sequences with pBR322 or polyomavirus fragments allows normal splicing to occur (28, 36). In contrast, there are indications from previous work that sequences within the intron may affect splicing of retroviral RNAs (1, 6, 8, 11, 15, 16, 18, 25, 26, 33).

We have identified *cis*-acting sequences within the intron of RSV RNA that act as a negative regulator of splicing (*nrs*). The *nrs* element was capable of exerting its effect upon a heterologous splice donor and acceptor pair. Since the *nrs* sequences do not function by increasing the rate of transport of the unspliced RNA to the cytoplasm, they presumably interfere more directly with the splicing process. Frameshift mutations did not affect the levels of spliced RNA; therefore, viral proteins do not appear to be necessary for the splicing inhibition. Surprisingly, frameshift mutations at several sites in the *gag* gene resulted in a large decrease in the steady-state level of unspliced RNA. These mutations, which cause premature termination of *gag* gene translation, appear to act by destabilizing the unspliced RNA in the cytoplasm.

MATERIALS AND METHODS

Construction of recombinant clones. pATV-8K (9), an infectious, proviral clone of the Prague-C (Pr-C) strain of RSV in which the pBR322 vector is inserted into a unique *Kpn*I site in the *pol* gene (Fig. 1), was obtained from R. Katz and A. M. Skalka. Deletion mutant TD was derived from pATV-8K by digestion with *Mst*II, filling in with the Klenow fragment, and religation. This resulted in the removal of bases 7155 to 8885 (29), consisting of most of the *src* gene. Clone SacII was derived from pATV-8K by digestion with *Sac*II and religation, removing bases 547 to 1809. SacII+1 was constructed by digestion of pATV-8K with *Sac*II, generation of blunt ends with T4 DNA polymerase, and religation, deleting bases 545 to 1809.

The following deletions were made initially in a p1Bam subclone (5) and subsequently reinserted into clone SacII after digestion with *Sac*II. This process reconstructed the deletions in the proviral clone, pATV-8K. Reinsertion of the wild-type (WT) *Sac*II fragment in the antisense orientation resulted in clone SacII−. XS was constructed by digestion with *Xho*I and *Sph*I, generation of blunt ends with T4 polymerase, and religation with a 10-mer *Bam*HI linker,

* Corresponding author.
† Present address: Department of Medicine, University of California at Los Angeles School of Medicine, Los Angeles, CA 90024.

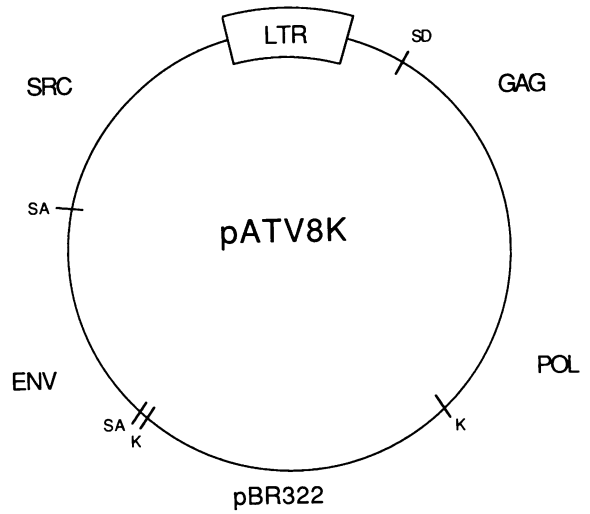

FIG. 1. Proviral clone of the Pr-C strain of RSV used to construct deletion mutants. A diagram of the genomic organization of pATV-8K is shown. pATV-8K contains a single long terminal repeat (LTR) and is permuted around the *Kpn*I site (K). The normal splice donor (SD) and splice acceptor (SA) sites for *env* and *src* mRNAs are shown.

removing bases 635 to 1010. Upon sequencing (35), it was determined that XS had lost a base from the *Bam*HI linker, resulting in a frameshift relative to the normal *gag* reading frame. NB was constructed by digestion with *Nru*I and *Bgl*II, filling in with the Klenow fragment, and religation with an 8-mer linker, removing bases 1354 to 1630. XNC was derived by digestion with *Xho*I and *Nar*I, filling in with the Klenow fragment, and religation with a 10-mer *Cla*I linker, removing bases 635 to 797. Construct NN was derived by digestion with *Nar*I and *Nru*I, filling in with the Klenow fragment, and religation, removing bases 799 to 1353. In-frame deletion mutant 189 (Δ799-987) and frameshift mutant 76 (Δ799-874) were gifts from K. Carlberg (5).

Frameshift mutant X+2 was derived by digestion with *Xho*I, filling in with the Klenow fragment, and religation with a 10-mer *Cla*I linker. This resulted in a 14-base insertion, which caused a frameshift at the *Xho*I site (nucleotide [nt] 634). N, N+1, and N+2 were derived by digestion with *Nar*I, followed by repair with the Klenow fragment and the addition of a 10-mer *Cla*I linker, an 8-mer *Kpn*I linker, or no linker, respectively. These insertions resulted in either a 10-base insertion (N+1) or a 2-base insertion (N+2), both of which caused frameshifts. The third insertion mutant at the *Nar*I site (nt 799) was a 12-base, in-frame linker insertion (N). Nr+1 and B+1 were constructed by digestion at an *Nru*I site (nt 1351) or at a *Bgl*II site (nt 1630). A 10-mer *Cla*I linker was inserted at the *Nru*I site, whereas the *Bgl*II site was repaired with the Klenow fragment and religated.

H+ and H− were constructed by insertion of an *Hpa*II fragment (nts 708 to 800), in both sense (H+) and antisense (H−) orientations, into the unique *Cla*I site of XNC. Neither H+ nor H− interrupts the normal reading frame for *gag* proteins.

The pRSVNeo-int constructs were derived by insertion of the respective *Sac*II fragments (nts 543 to 1806) from the pATV-8K constructs into the unique *Sac*II site within the c-*myc* intron of pRSVNeo-int (20) (see Fig. 5).

Cell culturing and DNA transfection. Secondary chicken embryo fibroblasts (CEFs) were cultured in medium 199 (GIBCO Laboratories) supplemented with 1% calf serum, 1% chicken serum, and 2% tryptose phosphate broth (GIBCO). Purified plasmid DNA (10 or 20 μg) in 3.0 ml of Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (GIBCO) was transfected onto a 9-cm plate in the presence of DEAE-dextran (200 μg/ml), followed 5 h later by a 1.5-min shock with 10% dimethyl sulfoxide, as described previously (21).

RNA isolation and mapping. At 48 h after transfection, the cells were scraped from the plate into TEN (0.04 M Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.15 M NaCl) and pelleted by low-speed centrifugation. Cells were lysed in guanidinium isothiocyanate buffer, and total RNA was pelleted through a 5.7 M CsCl cushion as described previously (7). Nuclear and cytoplasmic RNAs were prepared essentially as described previously (17), with the modifications that the lysis buffer contained 0.5% Nonidet P-40 and cells were Dounce homogenized three times.

The pATV-8K-specific riboprobe template was constructed by insertion of a 585-base-pair *Eco*RI-*Bam*HI fragment from pATV-8K into pGEM-2 (Promega Biotec). The pRSVNeo-int-specific riboprobe template was derived by insertion of a 793-base-pair *Sph*I-*Pst*I fragment from pRSVNeo-int (20) into pGEM-2. Prior to transcription, the pATV-8K-specific template was linearized with *Eco*RI to define a 626-base riboprobe, and the pRSVNeo-int-specific template was linearized with *Nco*I to define a 782-base riboprobe. Transcription of plasmids with T7 polymerase (Pharmacia, Inc.) and hybridizations to the resulting riboprobes were performed as previously described (23). The pATV-8K hybrids were digested with 5 μg of RNase A and 10 U of RNase T_1 per ml, whereas the pRSVNeo-int hybrids were digested with 10 U of RNase T_1 per ml alone. After ethanol precipitation, the protected fragments were denatured in 95% formamide for 5 min at 90°C and run on a 4 or 6% acrylamide–8 M urea sequencing gel (22). RNA levels were quantitated by densitometry of resultant autoradiograms.

RESULTS

Negative regulation of splicing by an element within the RSV *gag* gene. We wished to investigate both *cis* and *trans* functions which might be important in the regulation of the splicing and stability of retroviral RNA. Various deletions (Fig. 2) were made in a proviral clone of the Pr-C strain of RSV (Fig. 1). These constructs were transfected into CEFs, and total RNA was prepared 48 h posttransfection. The RNAs were subjected to an RNase protection assay with an antisense RNA probe complementary to the 5′ end of the RSV RNA. This probe spanned the only known splice donor site, allowing the simultaneous detection of unspliced RNAs and all RNAs spliced at this donor site (Fig. 3).

A number of large deletions in the viral genome had little effect on RNA levels or on splicing. RNA synthesized from deletion mutant TD (Δ7155-8885; nucleotide numbering according to Schwartz et al. [29]), which is lacking the *src* gene, appeared to be identical to WT RNA, both in the absolute amounts of RNA and in the ratios of spliced RNA to unspliced RNA (Fig. 3). Since the Pr-C strain of RSV has an unusually low level of spliced *src* mRNA but a normal level of *env* mRNA (33), deletion of *src* would not be expected to affect the total amount of spliced RNA significantly. RNA identical to WT RNA was also obtained from constructs bearing a large deletion in the *pol* gene (Δ2725-4268) (data not shown) or a deletion of part of the *gag* gene (mutant NB, Δ1354-1630) (Fig. 3). Approximately 5% of the

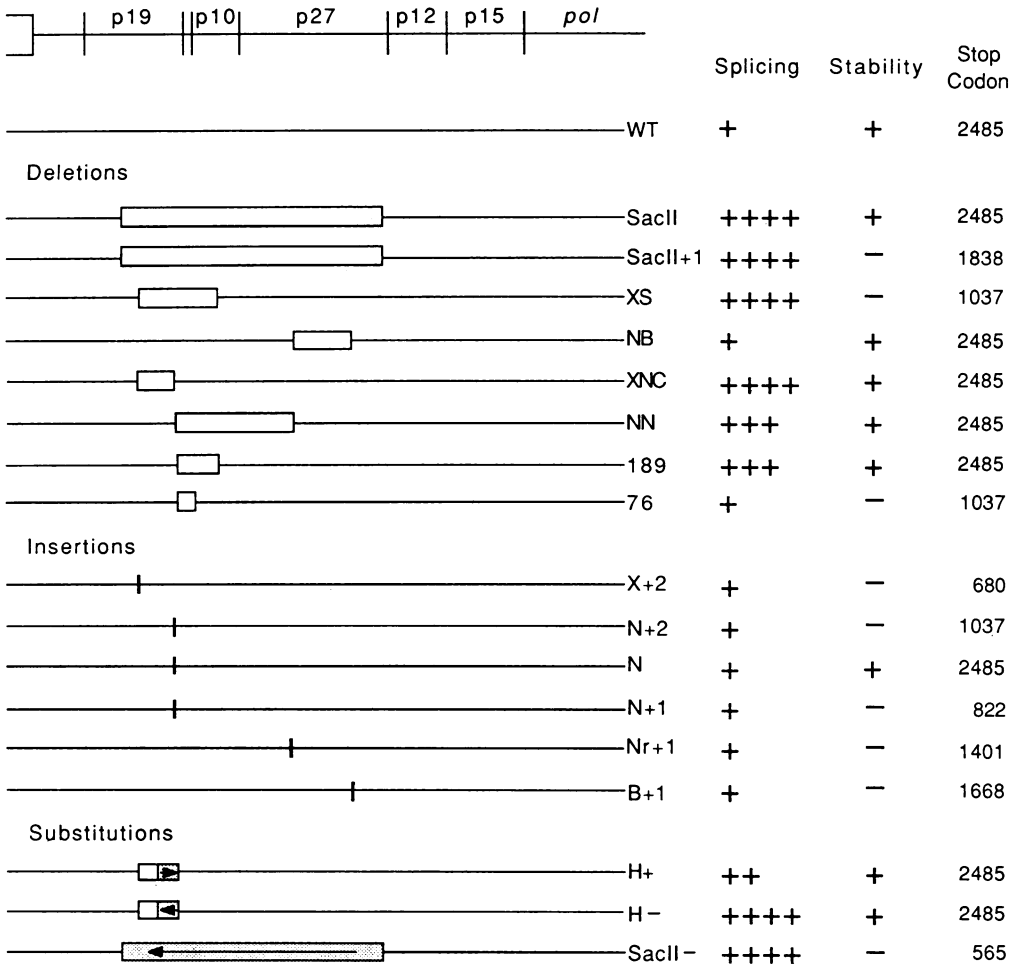

FIG. 2. Splicing and stability of proviral constructs with mutations in the *gag* gene of RSV. Schematic representation of mutants bearing deletions (empty boxes), insertions (vertical bars), and substitutions (shaded boxes). The steady-state level of spliced RNA for each of the mutants is represented by one to four plus signs: +, approximately 5% of the total RNA is spliced; ++, 10 to 15% is spliced; +++, 40 to 50% is spliced; and ++++, 50 to 70% is spliced. The stability of the unspliced RNA is also shown on the basis of steady-state levels of RNA: +, WT levels (or levels two- to threefold lower in the case of oversplicing mutants); −, levels at least 10-fold lower than WT levels. The *gag* gene translational termination codon is also shown for each construct.

viral RNA was spliced in all of these transfections. While this is a lower amount of spliced RNA than is seen in infected cells with this assay (K. Carlberg and K. Beemon, unpublished results), it is similar to the level of splicing observed by Meric and Spahr (24) in transient Northern (RNA) assays with a nonpermuted clone of Pr-C RSV.

In contrast, *gag* gene deletion mutants SacII (Δ547-1809) and XS (Δ635-1010) demonstrated a greater-than-10-fold increase in the amount of spliced RNA, with a concomitant decrease in the amount of unspliced RNA (Fig. 3). It appeared that in both of these deletion mutants a sequence from the RSV *gag* gene that acted as a negative regulator of splicing (*nrs*) was removed. The total amount of RNA synthesized from mutant SacII was comparable to that synthesized from the WT; however, mutant XS produced only about half that amount. While XS and SacII had similar levels of spliced RNA, XS had approximately 10-fold less unspliced RNA.

Localization of the negative regulator of splicing (*nrs*). To determine which sequences within *gag* were responsible for the inhibition of splicing, we made further deletions in the proviral clone (Fig. 2). These constructs were tested for their ability to inhibit splicing in the RNase protection assay (Fig. 3). Two representative assays are shown in Fig. 4A, and the results with all of the constructs tested are summarized in Fig. 2. Deletion mutant XNC (Δ635-797) demonstrated an increase in the percentage of spliced RNA to 50 to 70% spliced, a percentage which was identical to that seen with mutant SacII (Δ547-1809). The replacement of a fragment containing nts 708 to 800 into deletion mutant XNC restored most of the negative regulation of splicing, but only when this fragment was inserted in the sense orientation (mutant H+) and not in the antisense orientation (mutant H−) (Fig. 4A). The high percentage of spliced RNA observed with H− was identical to that seen with XNC. However, H+ resulted in an intermediate amount of spliced RNA (10 to 15% spliced), not quite fully restored to WT levels (5% spliced). Thus, sequences within nts 708 to 800 (upstream of the *Nar*I site) appear critical for the negative regulation of splicing, and this regulation is lost if these sequences are in the opposite orientation.

Deletion mutant 189 (Δ799-987, downstream of the *Nar*I site) demonstrated an increase in the percentage of spliced RNA to 40 to 50% of the total (Fig. 4A). This level was

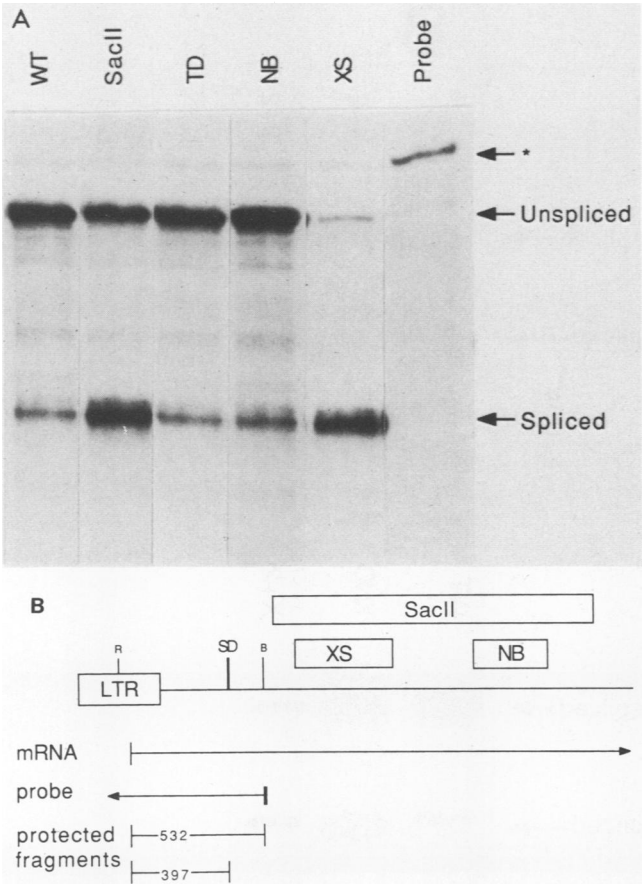

FIG. 3. Oversplicing of proviral clones which have deletions of *gag* sequences. (A) Results of an RNase protection assay with one-half of the total RNA from a 90-mm plate of CEFs transfected with 10 μg of the indicated deletion mutants. (B) Schematic representation of the RNase protection assay. The probe was generated by T7 polymerase transcription of an *Eco*RI (R)-*Bam*HI subclone of pATV-8K in pGEM-2. This 626-base antisense probe spans the splice donor (SD) site of RSV as well as the RNA cap site. This probe allows simultaneous detection of both spliced and unspliced RNAs from the normal splice donor site. The protected fragments for unspliced and spliced RNAs contain 532 and 397 bases, respectively. The location and extent of *gag* deletion mutants are shown. TD is a transformation-defective clone from which the *src* gene has been deleted. LTR, Long terminal repeat. The asterisk denotes the position of the full-length probe.

slightly lower than the level of splicing seen with deletion mutant XNC. A mutant with a larger deletion, NN (Δ799-1353), demonstrated a similar increase in the level of spliced RNA (to 40 to 50% spliced). Interestingly, a smaller deletion mutant with the same 5′ endpoint, mutant 76 (Δ799-874), demonstrated WT levels of spliced RNA. A similar result was obtained with mutant 82 (Δ799-880), which was a gift from K. Carlberg (data not shown).

Deletion mutants 76 and 82 have been shown to have deletions of sequences critical for the function of a transcriptional enhancer in the *gag* gene (5). Therefore, the *nrs* sequence and the *gag* enhancer (2) appear to be distinct *cis*-acting regulatory elements. These results imply that sequences upstream of base 799 as well as downstream of base 880 are important in negatively regulating splicing. We conclude from these experiments with deletion mutants that there are at least two noncontiguous regions within *gag* which constitute the *nrs* element and that both of these regions are necessary for full inhibition of splicing.

The proviral clone pATV-8K and all of the deletion mutants made from it contained pBR322 sequences interrupting the normal configuration of the virus (Fig. 1). We wished to determine whether the inhibition of splicing which was observed was dependent on the presence of vector sequences. To test this, DNAs from both the WT construct and an oversplicing mutant, XNC, were digested with *Kpn*I, purified away from pBR322 sequences on a sucrose gradient, and religated. DNAs lacking pBR322 sequences (Fig. 4C, −) were transfected into CEFs, and these transfections were compared with transfections of the supercoiled constructs which contained pBR322 (Fig. 4C, +). The effect of the *nrs* sequences on splicing was not dependent on the presence of pBR322 sequences (Fig. 4C). A similar experiment was performed with purified and religated XS and WT constructs. Both the 5′ riboprobe shown in Fig. 3 and an antisense riboprobe spanning the *src* splice acceptor site were used. The results demonstrated that the increase in splicing at the splice donor site with mutant XS was reflected in an increase in splicing at the *src* splice acceptor site (data not shown). We recently examined a SacII deletion mutant (Δ547-1809) generated from a nonpermuted proviral construct, pAPrC (24). Deletion of the *nrs* element from this construct also increased the ratio of spliced RNA to unspliced RNA (data not shown).

***cis*-Acting and orientation-dependent inhibition of splicing.** To examine the possibility that splicing inhibition required a viral protein, we constructed frameshift mutations at sites near the N terminus of the *gag* gene (Fig. 2). The results of RNase protection experiments with the resulting constructs are shown in Fig. 4B. Frameshift mutants X+2 and N+2 exhibited WT levels of spliced RNA. Since the mutations in these constructs prevented the synthesis of all *gag* proteins and also of a putative *gag*-encoded transactivator protein (4), we concluded that the negative regulation of splicing observed above was independent of *gag* protein production. These experiments suggest that the negative regulation of splicing is due to *cis*-acting *nrs* sequences within the *gag* gene.

When a portion of the *nrs* element (nts 708 to 800) was inverted to generate construct H− (Fig. 2), the negative regulation of splicing was eliminated (Fig. 4A). To determine whether the entire *nrs* element could function regardless of orientation, we reversed the orientation of the *Sac*II fragment to generate the proviral construct SacII− (Fig. 2). This construct exhibited a high level of spliced RNA and a low level of unspliced RNA that were identical to the levels in XS (data not shown). Thus, this change in orientation abolished the inhibitory effect of the *nrs* sequences. It appeared that the *cis*-acting *nrs* element could only exert its effect when present in the sense orientation.

Ability of the *nrs* element to exert its effect on a heterologous splice donor and acceptor pair. It was possible that the *nrs* element could only exert its negative effect on RNA splicing within its normal context in RSV RNA. To test the ability of the *nrs* element to inhibit splicing of a heterologous splice donor and acceptor pair, we inserted various *Sac*II fragments (nts 547 to 1809) from mutant and WT RSV proviral constructs into the center of the second intron of the chicken c-*myc* gene in pRSVNeo-int (20) (Fig. 5). The c-*myc* splice donor and acceptor are present in this plasmid, as is about 30 bases of flanking exon sequences. The RSV *Sac*II fragments were inserted into the c-*myc* intron at a unique *Sac*II site which is 360 bases downstream from the c-*myc* splice donor

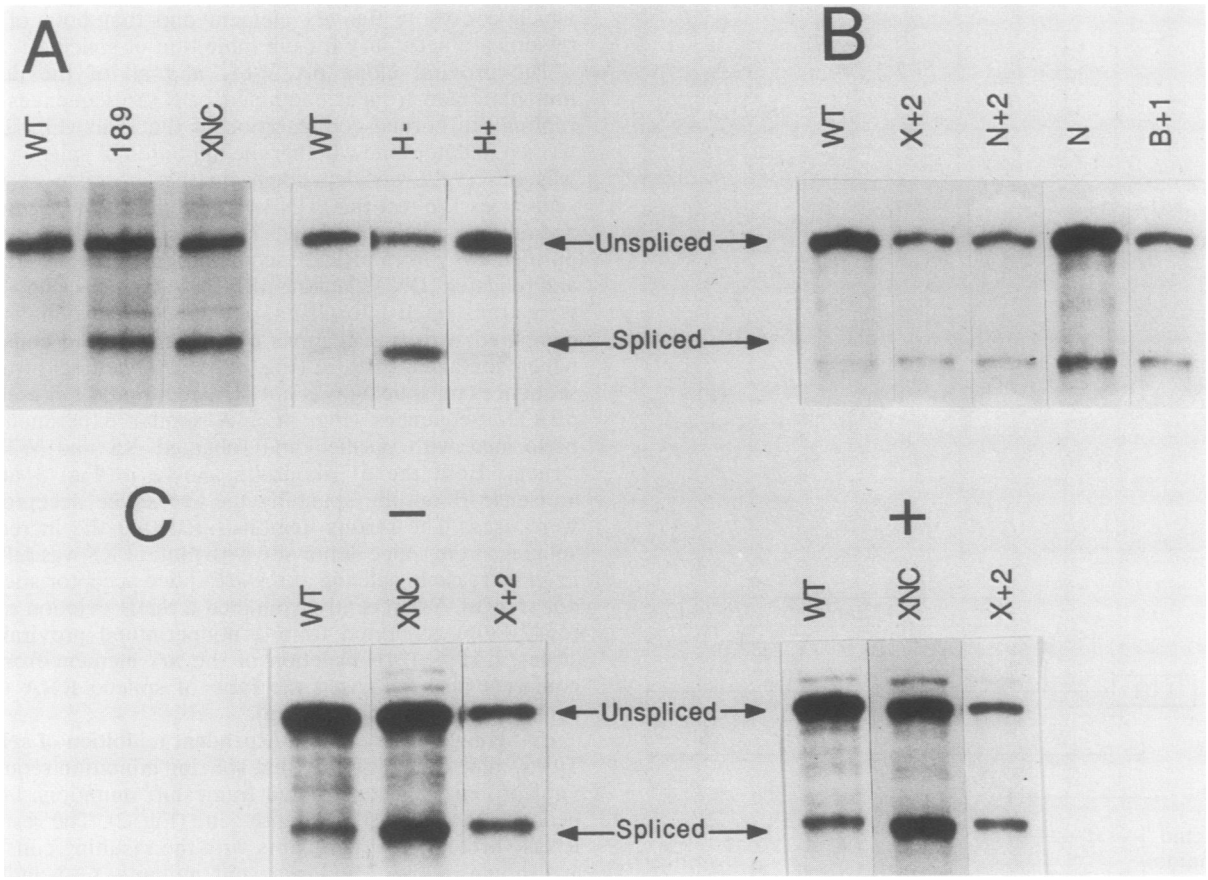

FIG. 4. Effects of RNA splicing and stability of mutations within the *gag* region of RSV. (A) Effects of deletion and substitution mutations. Results shown are from two RNase protection assays with one-third of the total RNA from a 90-mm plate of CEFs transfected with 10 μg of the indicated constructs, which are shown in Fig. 2. The probe used was the same as in Fig. 3. (B) Effects of insertion mutations. The RNase protection assay was carried out with the insertion mutants shown in Fig. 2. The overall increase in total RNA in N as compared with WT was not reproducible and most likely represents a higher transfection efficiency in this particular experiment. (C) Comparison of RNA from proviral constructs with and without pBR322. An RNase protection assay was carried out as described in panel A. The presence of pBR322 sequences is indicated by a plus sign. The minus sign indicates that the pBR322 sequences were removed from the plasmids by digestion with *Kpn*I, purification of proviral DNA on a sucrose gradient, and religation.

site (Fig. 5). The c-*myc* intron is located downstream of the termination site for translation of the *neo* gene. Constructs containing RSV *Sac*II fragments derived from the WT (inserted in sense and antisense orientations) as well as from H+ and H− mutants (Fig. 2) were analyzed by an RNase protection assay with an antisense riboprobe which spans the splice donor site of c-*myc* (Fig. 5).

We initially compared levels of spliced and unspliced total RNAs from CEFs transfected with these constructs (data not shown). Figure 6 shows a similar experiment in which cells were fractionated into nucleus and cytoplasm before RNA was prepared. The pRSVNeo-int construct containing no RSV insert yielded from 0 to 20% total unspliced RNA in different experiments. Insertion of the WT RSV *Sac*II fragment in the sense orientation led to an increase in the amount of unspliced RNA, so that approximately 50% of the total RNA molecules were unspliced. A construct containing an antisense RSV *Sac*II fragment (SacII−) showed no increase in the level of unspliced RNA. Insertion of the sense *Sac*II fragment from construct H+ resulted in an intermediate level of unspliced RNA, between that of the WT (sense) insert and a construct with no insert. Insertion of the sense *Sac*II fragment from construct H− appeared to have no effect on the level of unspliced RNA. Although the H+ and H− constructs differed only in the orientation of a 92-base segment of the 1.2-kilobase insert in the c-*myc* intron, only the H+ construct inhibited splicing (Fig. 6), as was seen above with the proviral constructs (Fig. 4A).

Consistent with previous experiments with pRSV*cat* constructs (2), we observed a two- to three-fold increase in the total level of RNA produced by those pRSVNeo-int constructs which contained an intact *gag* enhancer element (i.e., those with WT or H+ inserts). Since this enhancer is somewhat orientation dependent (2), the SacII− insert would not be expected to yield detectable enhancement. In addition, the H− insert disrupts a critical domain of the enhancer (5). This enhancer effect is distinct from the effect of the *nrs* element on splicing, as discussed above. The effect of this enhancer on the proviral clones was difficult to determine, owing to the effects on stability and splicing of most deletions which remove the *gag* enhancer.

These experiments showed an inhibitory effect on splicing from the c-*myc* splice donor when the *nrs* element was inserted into the intron in the proper orientation, showing that it can function in the absence of other viral sequences. Deletions within the *nrs* element which affected the level of splicing inhibition in the proviral constructs were reproduced qualitatively in this heterologous system. These re-

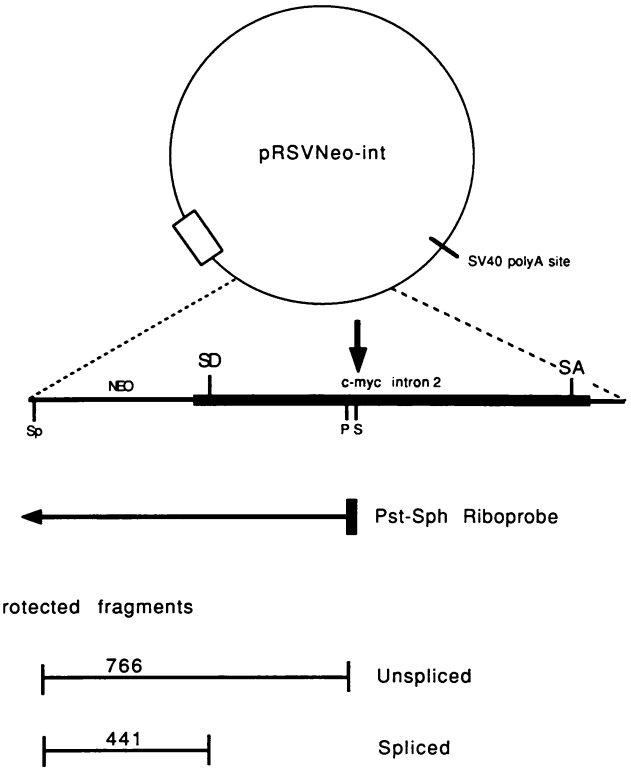

FIG. 5. Construction of pRSVNeo-int constructs containing the *nrs* element. Diagram of the pRSVNeo-int plasmid (20) into which various *Sac*II fragments (nts 543-1810) from the proviral clones shown in Fig. 2 were inserted. The unique *Sac*II site (S) which was used for these insertions is indicated by the vertical arrow. This plasmid contains as RSV long terminal repeat driving the transcription of the *neo* gene. The second intron of c-*myc*, as well as approximately 30 bases of adjacent exon sequences, is inserted between the coding region for *neo* and the simian virus 40 (SV40) polyadenylation signals. The splice donor (SD) and splice acceptor (SA) sites for c-*myc* are shown. A *Sph*I (Sp)-*Pst*I (P) fragment inserted into pGEM-1 was used as a template for T7 polymerase transcription to generate an antisense riboprobe. This probe allows the simultaneous detection of both spliced and unspliced RNAs from the c-*myc* splice donor. The protected fragments for unspliced and spliced RNAs contain 766 and 441 bases, respectively.

sults also confirm that a viral protein product is not necessary for this negative regulation of splicing.

The *nrs* element does not facilitate transport of RNA from the nucleus. It was possible that the decrease in the level of spliced RNA in the constructs containing the *nrs* element was a consequence of facilitated transport of the unspliced RNA from the nucleus to the cytoplasm. A rapid removal of unspliced RNA from the nucleus might result in a decrease in the pool of unspliced RNA available for splicing. To examine this possibility, we analyzed nuclear and cytoplasmic RNA fractions synthesized from the pRSVNeo-int constructs by using the RNase protection probe described in the legend to Fig. 5.

Most of the spliced RNA produced by all of the constructs was found in the cytoplasmic fraction (Fig. 6). The cytoplasmic fraction from transfections with all of the constructs contained only a trace amount of unspliced RNA. Apparently, the unspliced RNA either was unstable in the cytoplasm or was not efficiently transported out of the nucleus. If the *nrs* element were inhibiting splicing by facilitating transport of the unspliced RNA from the nucleus, we would have expected to observe a reduction in the level of unspliced RNA in the nuclear fraction with constructs containing the *nrs* element. Contrary to this hypothesis, inspection of the nuclear fraction revealed that the presence of the *nrs* element resulted in a large increase in the level of unspliced RNA within the nuclear fraction. We conclude that the *nrs* element exerts its effect on RNA by inhibiting splicing processes within the nucleus and not by facilitating transport of the unspliced RNA to the cytoplasm.

Decreased accumulation of unspliced RSV RNA caused by frameshift mutations within the *gag* gene. We have observed very low levels of unspliced RSV RNA synthesized from several proviral mutants having an alteration in the *gag* gene reading frame. These included deletion mutant XS (Δ635-1010) (Fig. 3) and insertion mutants X+2 and N+2, which shift frames at nts 634 and 799, respectively (Fig. 4B). To further investigate this effect, we generated several additional insertion mutations in the *gag* gene. Constructs N+1, Nr+1, and B+1 incurred frameshifts at nts 799, 1351, and 1630, respectively. All of these *gag* frameshift mutants exhibited a greater-than-10-fold decrease in the level of unspliced RNA relative to that in the WT (Fig. 4B). Significantly, the level of spliced RNA appeared to be the same as that in the WT. A relatively uniform decrease in the level of unspliced RNA was observed with all of these mutants (Fig. 4B), which prematurely terminated translation at sites ranging from nt 680 (X+2) to nt 1668 (B+1) (Fig. 2). Since translation of the *gag* gene is normally terminated at nt 2485 (29), more than 60% of the entire gene was translated by mutant B+1. Furthermore, construct SacII+1, which has an out-of-frame deletion of nts 545 to 1809, causing it to terminate translation at nt 1838, had much lower levels of unspliced RNA than did a companion in-frame deletion mutant, SacII (Fig. 7).

This decreased accumulation of unspliced RNA appeared to be restricted to mutants which cause premature translation termination in the *gag* gene. For example, insertion of a 12-base-pair in-frame linker at the *Nar*I site (nt 799), to generate construct N, resulted in wild-type levels of both unspliced and spliced RNAs (Fig. 4B). Similarly, construct H−, which has an in-frame, extended missense mutation involving 31 codons, had levels of unspliced RNA comparable to those in other constructs with in-frame *nrs* mutations (Fig. 4A). Although the *pol* gene is translated as a *gag-pol* readthrough product (36), frameshift mutations in the *pol* gene, unlike those in the *gag* gene, did not affect the levels of spliced or unspliced RNA (data not shown).

Since all of these constructs contained pBR322 sequences inserted within the RSV genome (Fig. 1), the effect of vector sequences on RNA accumulation was investigated. Figure 4C shows the result of an experiment in which pBR322 sequences were removed as described above from a proviral clone of frameshift mutant X+2. This mutant demonstrated a large decrease in the level of unspliced RNA relative to that in the WT, both with and without vector sequences. We have also seen this same effect with the B+1 frameshift mutation in nonpermuted proviral constructs (G. Barker and K. Beemon, unpublished results).

Difference in the cytoplasmic level of unspliced RNA caused by altered RNA stability. To determine where in the cell the difference in unspliced RNA levels could be detected, we assayed nuclear and cytoplasmic RNA fractions separately. Figure 7 shows the result of an experiment with fractionated CEFs transfected with WT, SacII, and SacII+1 proviral constructs. (SacII and SacII+1 are a pair of in-frame and out-of-frame deletion mutants [Fig. 2].) Both mutants have

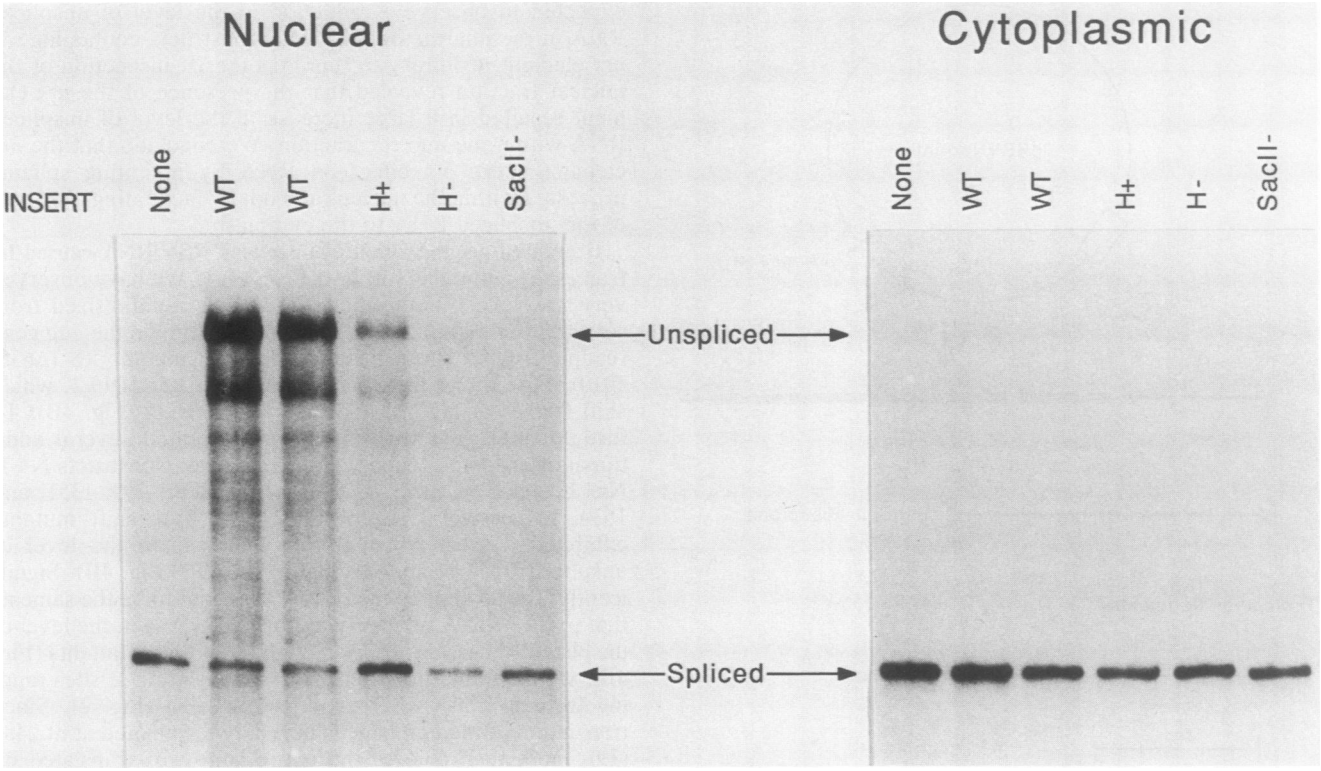

FIG. 6. Splicing inhibition but no increase in the level of unspliced RNA in the cytoplasm caused by insertion of RSV *gag* sequences into the c-*myc* intron of pRSVNeo-int. Results of an RNase protection assay with the riboprobe shown in Fig. 5 and RNAs from nuclear and cytoplasmic fractions are shown. Aliquots of the RNA from a 90-mm plate of CEFs transfected with 20 μg of pRSVNeo-int constructs were used for both nuclear and cytoplasmic assays. The two WT inserts represent two independent clones of the WT RSV *Sac*II fragment (nts 543 to 1310) inserted into pRSVNeo-int. The H+, H−, and SacII−, inserts are the mutated or inverted RSV *Sac*II fragments (Fig. 2).

deleted *nrs* sequences and showed elevated levels of spliced RNA (Fig. 7). However, SacII+1 had less unspliced RNA in the whole-cell assay than did SacII (Fig. 7, Total).

In the cytoplasmic fraction, SacII+1 had much less unspliced RNA than did SacII, whereas the level of spliced RNA appeared identical in the two mutants and was significantly higher than that in the WT (Fig. 7). In contrast, both SacII and SacII+1 mutants demonstrated essentially identical levels of unspliced and spliced RNAs in the nuclear fraction (Fig. 7). Thus, the decreased accumulation of unspliced RNA with the out-of-frame construct was only detected in the cytoplasm. The WT construct yielded significantly more unspliced RNA in the nucleus (about 50% of the total unspliced RNA) than did either mutant. Since none of the constructs showed an appreciable amount of spliced RNA in the nucleus, this experiment suggests that spliced RSV RNA may be transported more rapidly than unspliced RNA (Fig. 7).

Since the amounts of both RNA species in the nucleus and of the spliced RNA in the cytoplasm were identical for the two mutants (Fig. 7), differences in transcription or splicing seem unlikely to be responsible for the differences in unspliced RNA levels observed in the cytoplasmic fraction. A decrease in the rate of transport of the unspliced RNA to the cytoplasm with SacII+1 would be expected to result in its accumulation in the nuclear fraction; however, such accumulation was not seen. Similarly, if RNA from the out-of-frame constructs was preferentially degraded in the nucleus, we would have expected to see a difference in the levels of unspliced RNA there. The simplest explanation of this data is that frameshift mutations in the *gag* gene result in instability of the unspliced RNA in the cytoplasm.

When direct comparisons of the RNA stabilities of the WT and the B+1 frameshift mutant were carried out in the presence of actinomycin D, the unspliced RNA of the frameshift mutant was found to be considerably less stable than that of the WT (G. Barker and K. Beemon, unpublished results). This difference in stability could account for the observed differences in steady-state levels of unspliced RNA.

The in-frame SacII deletion mutant (Δ547-1809) had lost most of the coding sequences for the p19, p2, p10, and p27 *gag* proteins, yet its RNA appeared stable (Fig. 7). This result suggests that these proteins are not required to stabilize the unspliced RNA. Additional in-frame deletions in the remaining *gag* proteins, p12 (24) and p15 (S. Arrigo and K. Beemon, unpublished results), did not result in the severe alteration in RNA stability seen with the frameshift mutants. This result suggests that the stability of unspliced RNA does not require the synthesis of specific *gag* proteins, which could act in *trans*. However, translation through the *gag* region appears to be necessary for this RNA stabilization.

DISCUSSION

Using an RNase protection assay to measure levels of spliced and unspliced RSV RNAs transiently expressed, we identified an orientation-dependent sequence within the *gag* gene of RSV which negatively regulates the level of splicing from the normal splice donor. No viral proteins were re-

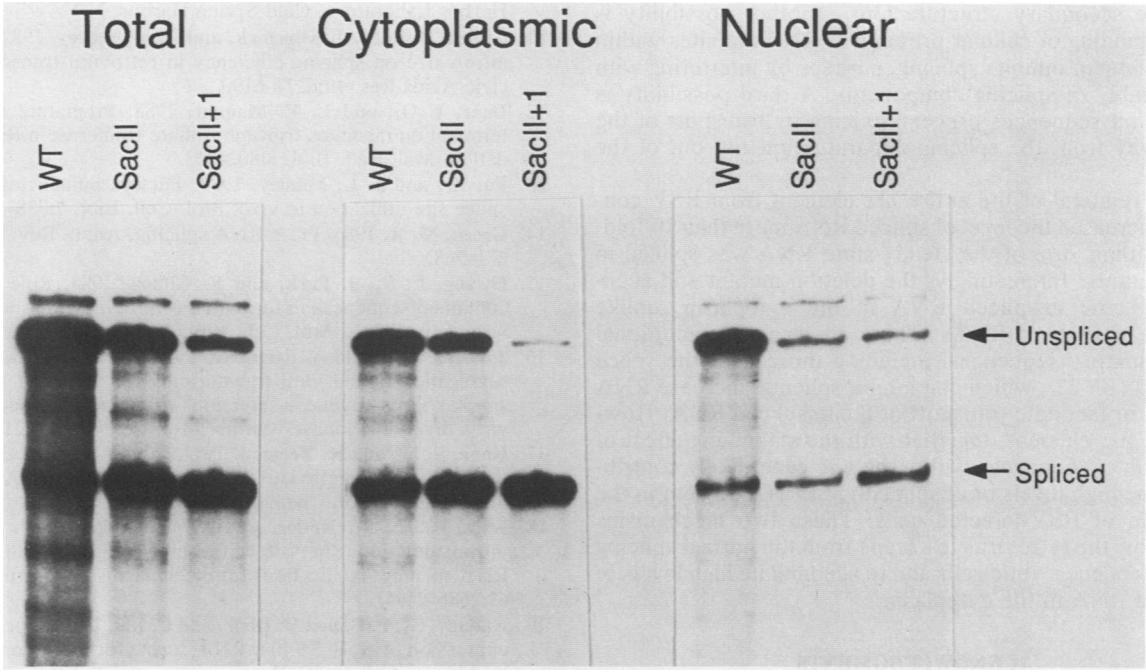

FIG. 7. Decreased level of unspliced RSV RNA in the cytoplasm in frameshift mutants. Results of an RNase protection assay (Fig. 3) with RNA from one-half of a plate of CEFs transfected with 10 μg of the indicated constructs (Fig. 2) and separated into nuclear and cytoplasmic fractions are shown. Total RNA was made from separate plates of identically transfected cells. SacII and SacII+1 are in-frame and out-of-frame RSV mutants, both of which have deleted *nrs* sequences.

quired for the inhibition of splicing by this *nrs* element. This fact was demonstrated by introducing frameshift mutations into the *gag* gene of RSV, as well as by moving the *nrs* element into a heterologous system. When inserted into a construct containing the second intron of c-*myc* (pRSVNeo-int), the intact *nrs* element and mutated forms of it exhibited an inhibition of splicing qualitatively similar to that seen with the proviral clones. In the pRSVNeo-int construct, the *nrs* element is located almost 200 bases further downstream from the splice donor site than in the proviral constructs. Apparently, the exact position of this element within the intron is not critical to its inhibitory effect on splicing. The *nrs* element may be useful for modulating the levels of spliced and unspliced products of retroviral vectors.

We have also found evidence that frameshift mutations, which cause premature termination of translation within the *gag* gene, result in a large decrease in the steady-state level of unspliced RNA in the cytoplasm. This effect, which does not require the production of *gag* proteins, appears to be due to a decrease in the stability of the unspliced RNA in such mutants. Negative effects of nonsense codons on mRNA accumulation were observed recently for β-globin (3) and triosephosphate isomerase (12) mRNAs.

Both positive and negative effects of intron mutations on gene expression from spliced retroviral mRNAs have been observed. Cullen et al. (11) reported that a large deletion (Δ534-4715) in the *env* mRNA intron of avian leukosis virus significantly increased *env* gene expression, as measured by a complementation assay. Interestingly, this deleted region includes the *nrs* element. Another deletion (Δ1631-4236), which is downstream of the *nrs* sequences, did not affect *env* gene expression (11). Temin and co-workers observed that insertion of viral or foreign DNA sequences into the intron of reticuloendotheliosis virus negatively affected the level of splicing (6, 25, 26). It is not clear whether this group identified a similar element; apparently, insertion of any foreign DNA sequences into the intron negatively regulates splicing in this system. Further, MuSVts110, which was generated by a 1,487-base deletion from the *gag* and *mos* genes of Moloney murine sarcoma virus, was spliced at sites which flank the deleted region and which are not used in the parental virus (8). In contrast, sequences residing in the *env* mRNA intron of Moloney murine leukemia virus were shown to be necessary for efficient splicing of murine leukemia virus-derived retroviral vectors containing the murine leukemia virus splice donor site and the *env* splice acceptor site (1, 15). Similarly, deletions and insertions within the *env* intron of Friend spleen focus-forming virus decreased the level of *env* protein production (16).

In addition to intron sequences, sequences near retroviral splice acceptors also appear to be important in the regulation of the level of splicing. Sequences near the RSV *src* splice acceptor site have been implicated by Stoltzfus et al. (33) in the regulation of the level of *src* mRNA splicing. Strain-specific differences in *src* mRNA levels were localized to sequences within a region extending from 100 bases upstream to 30 bases downstream of the splice acceptor site (33). Recently, a small insertion upstream of the *env* splice acceptor site in RSV was seen by Katz et al. (18) to result in marked oversplicing of the *env* mRNA. It will be interesting to investigate possible interactions between the *nrs* sequences and sequences near the splice sites.

We have shown that the *nrs* element exerts its effect on splicing within the nucleus and does not increase transport of the unspliced RNA to the cytoplasm. We envision three possible mechanisms for *nrs*-mediated inhibition of splicing. Since RNA secondary structure has been implicated in alternate splice site selection and utilization in simian virus 40 and adenovirus systems (13, 19, 30, 31), it may well play a similar role in retroviruses, which are thought to have

extensive secondary structure (36). Another possibility is that the binding of cellular proteins or RNAs to sites within the *nrs* element inhibits splicing, perhaps by interfering with the assembly of splicing components. A third possibility is that the *nrs* sequences prevent splicing by transport of the RNA away from the splicing apparatus but not out of the nucleus.

While removal of the entire *nrs* element from RSV constructs increased the level of spliced RNA more than 10-fold, no more than 70% of the steady-state RNA was spliced in these mutants. Interestingly, the deletion mutant still accumulated some unspliced RNA in the cytoplasm, unlike normal eucaryotic mRNAs. There are probably additional viral regulatory sequences, including those near the splice acceptors (18, 33), which can inhibit splicing, increase RNA stability, or facilitate transport of the unspliced RNA. However, the *nrs* element, together with the stabilizing effect of translation of sequences within the *gag* gene, likely contributes to the high levels of unspliced viral RNA present in the cytoplasm of RSV-infected cells. These two mechanisms could allow the retrovirus to escape from the normal splicing pathways of eucaryotic cells and to accumulate high levels of unspliced RNA in the cytoplasm.

ACKNOWLEDGMENTS

We thank Kristen Carlberg for helpful discussions, George Barker and Stacie Dalrymple for comments on the manuscript, Dorothy Regula for typing the manuscript, Richard Katz and Ann Skalka for pATV-8K, and Maxine Linial for pRSVNeo-int.

This work was supported by Public Health Service grant CA 33199 from the National Cancer Institute. S.A. was supported in part by a predoctoral training grant from the National Institutes of Health. K.B. was a recipient of a Faculty Research Award from the American Cancer Society.

LITERATURE CITED

1. **Armentano, D., S. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa.** 1987. Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. **61**:1647–1650.
2. **Arrigo, S., M. Yun, and K. Beemon.** 1987. *cis*-Acting regulatory elements within the *gag* genes of avian retroviruses. Mol. Cell. Biol. **7**:388–397.
3. **Baserga, S. J., and E. J. Benz, Jr.** 1988. Nonsense mutations in the human β-globin gene affect mRNA metabolism. Proc. Natl. Acad. Sci. USA **85**:2056–2060.
4. **Broome, S., and W. Gilbert.** 1985. Rous sarcoma virus encodes a transcriptional activator. Cell **40**:537–546.
5. **Carlberg, K., T. Ryden, and K. Beemon.** 1988. Localization and footprinting of an enhancer within the avian sarcoma virus genome. J. Virol. **62**:1617–1624.
6. **Chen, S. Y., and H. M. Temin.** 1982. Substitution of 5′ helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. Cell **31**:111–120.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
8. **Cizdziel, P. E., M. de Mars, and E. C. Murphy, Jr.** 1988. Exploitation of a thermosensitive splicing event to study pre-mRNA splicing in vivo. Mol. Cell. Biol. **8**:1558–1569.
9. **Cobrinik, D., R. Katz, R. Terry, A. M. Skalka, and J. Leis.** 1987. Avian sarcoma and leukosis virus *pol*-endonuclease recognition of the tandem long terminal repeat junction: minimum site required for cleavage is also required for viral growth. J. Virol. **61**:1999–2008.
10. **Coffin, J.** 1985. Genome structure, p. 17–73. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses: RNA tumor viruses, 2nd ed., vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Cullen, B. R., J. J. Kopchick, and D. W. Stacey.** 1982. Effect of intron size on splicing efficiency in retroviral transcripts. Nucleic Acids Res. **10**:6177–6190.
12. **Daar, I. O., and L. E. Maquat.** 1988. Premature translation termination mediates triosephosphate isomerase mRNA degradation. Mol. Cell. Biol. **8**:802–813.
13. **Fu, X., and J. L. Manley.** 1987. Factors influencing alternate splice site utilization in vivo. Mol. Cell. Biol. **7**:738–748.
14. **Green, M. R.** 1986. Pre-mRNA splicing. Annu. Rev. Genet. **20**: 671–708.
15. **Hwang, L. S., J. Park, and E. Gilboa.** 1984. Role of intron-contained sequences in formation of Moloney murine leukemia virus *env* mRNA. Mol. Cell. Biol. **4**:2289–2297.
16. **Joyner, A. L., and A. Bernstein.** 1983. Retrovirus transduction: segregation of the viral transforming function and the herpes simplex virus *tk* gene in infectious Friend spleen focus-forming virus thymidine kinase vectors. Mol. Cell. Biol. **3**:2191–2202.
17. **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.
18. **Katz, R. A., M. Kotler, and A. M. Skalka.** 1988. *cis*-Acting intron mutations that affect the efficiency of avian retroviral RNA splicing: implications for mechanisms of control. J. Virol. **62**:2686–2695.
19. **Khoury, G., P. Gruss, R. Dhar, and C. Lai.** 1979. Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. Cell **18**:85–92.
20. **Linial, M.** 1987. Creation of a processed pseudogene by retroviral infection. Cell **49**:93–102.
21. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyltransferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
22. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
23. **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.
24. **Meric, C., and P. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60**:450–459.
25. **Miller, C. K., J. E. Embretson, and H. M. Temin.** 1988. Transforming viruses spontaneously arise from nontransforming reticuloendotheliosis virus strain T-derived viruses as a result of increased accumulation of spliced viral RNA. J. Virol. **62**:1219–1226.
26. **Miller, C. K., and H. M. Temin.** 1986. Insertion of several different DNAs in reticuloendotheliosis virus strain T suppresses transformation by reducing the amount of subgenomic mRNA. J. Virol. **58**:75–80.
27. **Padgett, R. H., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55**:1119–1150.
28. **Ruskin, B., J. M. Greene, and M. R. Green.** 1985. Cryptic branch point activation allows accurate in vitro splicing of human β-globin intron mutants. Cell **41**:833–844.
29. **Schwartz, E. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
30. **Solnick, D.** 1985. Alternative splicing caused by RNA secondary structure. Cell **43**:667–676.
31. **Solnick, D., and S. I. Lee.** 1987. Amount of RNA secondary structure required to induce an alternative splice. Mol. Cell. Biol. **7**:3194–3198.
32. **Stoltzfus, C. M.** 1988. Synthesis and processing of avian sarcoma retrovirus RNA. Adv. Virus Res. **35**:1–37.
33. **Stoltzfus, C. M., S. K. Lorenzen, and S. L. Berberich.** 1987. Noncoding region between the *env* and *src* genes of Rous

sarcoma virus influences splicing efficiency at the *src* gene 3′ splice site. J. Virol. **61**:177–184.

34. **van Santen, V. L., and R. A. Spritz.** 1985. mRNA precursor splicing *in vivo*: sequence requirements determined by deletion analysis of an intervening sequence. Proc. Natl. Acad. Sci. USA **82**:2885–2889.
35. **Wallace, R. B., M. J. Johnson, S. V. Suggs, K. Miyoshi, R. Bhatt, and K. Itakura.** 1981. A set of oligodeoxyribonucleotide primers for DNA sequencing in the plasmid pBR322. Gene **16**: 21–26.
36. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. Molecular biology of tumor viruses: RNA tumor viruses, 2nd ed., vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Wieringa, B., E. Hofer, and C. Weissman.** 1984. A minimal intron length but no specific internal sequence is required for splicing of the large rabbit β-globin intron. Cell **37**:915–925.