

## *cis*-Acting RNA Packaging Locus in the 115-Nucleotide Direct Repeat of Rous Sarcoma Virus†

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The *v-src* gene of Schmidt-Ruppin strain A of Rous sarcoma virus is flanked by a 115-nucleotide direct repeat. Mutants that lack either the upstream or downstream copy replicate normally. However, mutants that lack both copies do not replicate. Cloned viral DNA lacking both copies of the 115-nucleotide sequence is capable of directing the transcription of viral RNA posttransfection. This viral RNA is polyadenylated, spliced, exported from the nucleus, and translated into protein normally. However, virions isolated from the culture medium 48 h posttransfection lack viral RNA. When mutant DNA is cotransfected with wild-type DNA, the virions produced 48 h later contain wild-type RNA but not mutant RNA, even though both RNAs are present in the cytoplasm. We propose that the 115-nucleotide element of Rous sarcoma-avian leukosis virus encodes a *cis*-acting sequence that is necessary for the proper incorporation of viral RNA into virions.

The genome of Rous sarcoma virus (RSV) contains two 115-nucleotide sequences that have approximately 80% nucleotide homology (1, 17, 22). These sequences are in the same genomic orientation, forming a direct repeat that flanks the transforming gene, *v-src*, in the 3' end of the genome. Many transformation-defective variants of RSV have lost the complete *v-src* sequence and retain a single hybrid copy of the 115-nucleotide sequence (15, 25). Moreover, the 3' ends of the Rous-associated virus-O (5), Y73 virus (7), and Fujinami virus (19) genomes contain a sequence that is highly homologous to the 115-nucleotide sequence of RSV. Because no Rous sarcoma-avian leukosis virus genome has yet been identified that lacks both copies of the 115-nucleotide sequence, the possibility that this sequence performs a necessary function was investigated.

The RNA sequences involved in the recognition of viral RNA by viral proteins during virion assembly have not been defined for RSV. A naturally occurring mutant of RSV, SE21Q1b, does not export its own RNA, nor is its RNA exported by a superinfecting helper virus (10). Although the 5' end of the SE21Q1b genome bears a 150-nucleotide deletion (18), the relationship between the deletion and the phenotype has not been fully characterized. The regions upstream from the *gag* gene of spleen necrosis virus (23) and Moloney murine leukemia virus (12) have been shown to contain a *cis*-acting

locus that is necessary for incorporation of viral RNA into virions. Although we have found a similar locus within the 5' portion of the *gag* gene of RSV (unpublished data), we describe here a second *cis*-acting locus that is needed for proper incorporation of RSV RNA into virus particles. This locus is found within the 115-nucleotide element near the 3' end of the genome. An RSV viral RNA genome must contain both the 5' portion of *gag* and at least a portion of a single 115-nucleotide element to be packaged properly.

### MATERIALS AND METHODS

**Chemicals and enzymes.** DNA polymerase and restriction enzymes were purchased from New England Biolabs; T4 polynucleotide kinase was purchased from P-L Biochemicals, Inc.; agarose and S1 nuclease were purchased from Sigma Chemical Co.

**Preparation of cloned retrovirus DNA.** The large circular form of the DNA of Schmidt-Ruppin strain A of RSV, cloned as a permuted linear molecule into  $\lambda$ gtWES, was a gift from W. DeLorbe (2). Viral DNA was digested with restriction enzymes *Pvu*II and *Sac*I, and the long terminal repeat (LTR)-containing fragment (from the *Pvu*II site near the 3' end of *v-src* to the *Sac*I site 150 nucleotides downstream from *U<sub>5</sub>*) was subcloned into a pBR322 derivative containing single *Pvu*II and *Sac*I sites. The plasmid was digested with *Pvu*II (the site is shown in Fig. 1) and then with exonuclease *Bal*31 (12). The DNA was then modified with the large fragment of *Escherichia coli* DNA polymerase (12), and this was followed by ligation to restriction endonuclease *Cla*I linkers (CATCGATG). *E. coli* HB101 was transformed with the DNA (12), and individual colonies were screened by restriction

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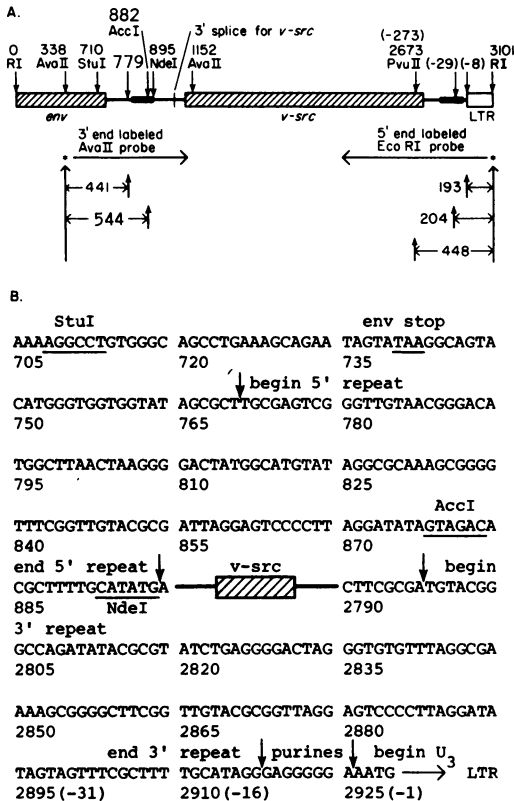


FIG. 1. Restriction map and nucleotide sequence of RSV *v-src*-containing *EcoRI* fragment. (A) Map of *v-src* and surrounding region. *env* is a portion of the envelope gene. The solid boxes represent the 115-nucleotide direct repeats. The positive numbers above the map represent the numbers of nucleotides downstream from the *EcoRI* (RI) site in the *env* gene. The negative numbers represent the numbers of nucleotides upstream from the left terminus of the LTR shown. The arrows and numbers below the map aid in interpreting the data presented in Fig. 3. (B) Nucleotide sequence of the regions flanking the *v-src* gene.

mapping for the extent of deletion downstream from *v-src*. A plasmid was isolated that retained only eight of the nucleotides upstream from the LTR, and this plasmid was digested with *SacI* (which cleaves in the 5' viral leader sequence); this DNA was treated with exonuclease *Bal31* and the large fragment of *E. coli* DNA polymerase and ligated to *Clal* linkers. Screening, as described above, yielded a plasmid that contained a second *Clal* site in U<sub>5</sub> near the primer-binding site, such that digestion with *Clal* liberated essentially a single LTR. This LTR fragment was modified with the large fragment of *E. coli* DNA polymerase and blunt-end-ligated to a pBR322 ampicillin-resistant derivative (pBR322 sequences 2,050 to 4,350). In summary, the manipulations described above created a plasmid that contained a single RSV LTR with minimal viral flanking sequences.

The wild-type RSV genome shown in Fig. 2 was

constructed by inserting the remainder of the RSV genome into a unique *EcoRI* site within the LTR region of the LTR-containing plasmid. This was performed by coligating several purified subgenomic fragments with the LTR plasmid which had been cut with *EcoRI* and treated with alkaline phosphatase (12). The *v-src* gene was then deleted from the provirus-containing plasmid from the *AccI* site at position 882 (Fig. 1A) to the *PvuII* site at position 2,673 (Fig. 1A). A single *Clal* linker was inserted in place of the *v-src* region. This mutant was called pr882/-273 (position 2,673 in Fig. 1A is 273 nucleotides upstream from the downstream LTR). Exonuclease *Bal31* was then used to expand the deleted region by digesting the molecule (in either direction) from the unique *Clal* site. *Clal* linkers were ligated to the *Bal31*-digested DNA in order to reestablish a restriction endonuclease *Clal* site in each deletion mutant. The extent of each deletion was determined by sequencing the DNA flanking the newly established *Clal* site (14). The deletion within provirus pr779/-29 extended from nucleotide 779 (Fig. 1A) to nucleotide 2,897 (or to nucleotide -29). The other deletion mutants were named in an analogous manner by using upstream and downstream endpoint nucleotide numbers.

Deletion mutant pr779/-273/P#3 (See Fig. 7) was constructed by digesting pr779/-273 DNA to completion with restriction enzymes *Clal* and *PvuII* (the *PvuII* site was within the *gag* gene near its 5' end), modifying the DNA with the large fragment of *E. coli* DNA polymerase, and ligating the DNA to *Clal* linkers. We chose a recombinant that had incorporated a *Clal* linker in the gap that was left by deletion of *env*, *pol*, and most of *gag*. Proviruses pr779/-29/E#7 and pr779/-29/E#11 (see Fig. 7) were constructed by digesting pr779/-29 with *Clal* and ligating the DNA to the *StuI*-to-*NdeI* fragment (Fig. 1A), which had been modified with terminal *Clal* linkers. The pr779/-29/E#11 recombinant contained the 115-nucleotide element in the same transcriptional orientation as in the wild type; the pr779/-29/E#7 recombinant contained the element in the backward orientation.

All of the plasmids were amplified with chloramphenicol and purified by two cesium chloride bandings after Triton X-100 lysis (12). The plasmid concentrations were determined by measuring UV absorbance at 260 nm. Portions of the preparations were electrophoresed on agarose gels and stained with ethidium bromide. In no case did *E. coli* chromosomal DNA represent more than 10% of the DNA.

**Cells and transfections.** *c/o* chicken embryo fibroblasts (SPAFAS, Inc.) were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum in the presence of penicillin and streptomycin. The cells were plated onto 10-cm dishes at 30% confluency. Standard transfection techniques were used (24), with the following modifications. A 15- $\mu$ g portion of plasmid DNA was applied to each plate without carrier DNA. At 4 h after transfection, the medium was removed, and the cells were exposed to 2 ml of 15% (vol/vol) glycerol in medium for 2 min and then placed in fresh medium. Growth was assayed by determining the reverse transcriptase activity in the culture medium 14 days posttransfection (16). A polyribocytidylic acid template, an oligodeoxyguanydic acid primer, and [ $\alpha$ -<sup>32</sup>P]dGTP at a concentration of 10  $\mu$ M and a specific activity of 1 Ci/mmol were used. The assay

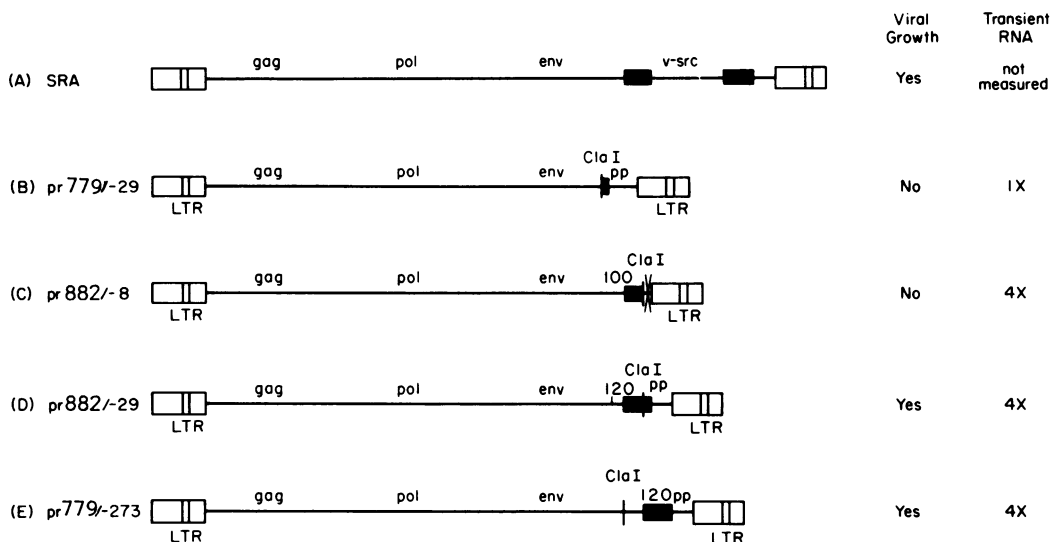


FIG. 2. Representation of the wild-type genome (SRA) and four mutants used in this study. The solid boxes represent the 115-nucleotide direct repeats. pp, Functional polypurine tract (only mutant pr882/-8 had a nonfunctional polypurine tract); *Cla*I, insertion of a *Cla*I linker. Mutant pr882/-8 contained only the 5'-most 94 nucleotides of the upstream repeat. Viral growth and transient RNA levels were assayed as described in the text.

was considered positive when more than  $5 \times 10^5$  cpm was incorporated into DNA; uninfected cells never yielded more than  $1.5 \times 10^4$  cpm incorporated.

**Isolation of RNA and S1 nuclease analysis.** Labeling of DNA fragments and DNA sequencing were performed by the procedures of Maxam and Gilbert (14). The procedures used for RNA isolation and S1 nuclease analysis have been described previously (21). The 814-nucleotide *Ava*II fragment (Fig. 1A) was 3'-end-labeled and used to detect deletions of the RSV RNA genome upstream from *v-src*. The 3,100-nucleotide *Eco*RI fragment (Fig. 1A) was 5'-end-labeled and used to detect deletions downstream from *v-src*. To determine the amount of splicing that occurred at the *gag* 5' splice site, the *Hin*fl-to-*Bam*HI fragment (see Fig. 5) was subcloned into a nonviral plasmid containing other *Hin*fl sites. All *Hin*fl sites were 3'-end-labeled, and the *gag*-containing fragment was purified by preparative electrophoresis and used as a probe for S1 nuclease analysis.

**Labeling of viral proteins.** Infected fibroblasts in 60-mm plates were placed in methionine-free medium for 10 min at 42 h after DNA transfection. The medium was changed to 0.5 ml of methionine-free medium supplemented with 300  $\mu$ Ci of [ $^{35}$ S]methionine (>800 Ci/mmol). The cells were incubated at 37°C for 1 h, and then 4 ml of methionine-rich medium was added. The preparations were incubated at 37°C for another 4 h. The medium was then centrifuged at 4500 rpm for 15 min, and the supernatant was layered over 5 ml of a solution containing 25% (wt/vol) sucrose, 20 mM Tris-chloride (pH 7.5), and 1 mM EDTA (pH 7.5) in a Beckman SW41 tube and centrifuged at 35,000 rpm for 90 min. The pellets were suspended in 20  $\mu$ l of sample buffer (8), boiled for 3 min, and electrophoresed on a 12.5% acrylamide-sodium dodecyl sulfate slab gel.

## RESULTS

The cloned RSV genome was manipulated such that the *v-src* gene and defined lengths of flanking region (Fig. 1) were deleted, as described above. Figure 2 shows four deletion mutants that were constructed for this study and a map of the wild-type genome. Mutant pr779/-29 lost the *v-src* region, lost the entire upstream copy of the 115-nucleotide direct repeat, and lost all but the 21 most-downstream nucleotides of the downstream copy of the 115-nucleotide direct repeat, yet retained all major translational reading frames, as well as a functional polypurine tract (20). Mutant pr882/-8 retained, in addition, 94 nucleotides of the upstream 115-nucleotide direct repeat, but lost a functional polypurine tract and lacked any of the downstream direct repeat. Mutant pr882/-29 was similar to pr882/-8, except that a functional polypurine tract was restored, as well as 21 of the most-downstream nucleotides of the 115-nucleotide direct repeat. Mutant pr779/-273 was similar to pr779/-29, except that the entire downstream 115-nucleotide direct repeat was restored.

Cloned mutant DNA was transfected into permissive chicken fibroblasts, and infectivity was assessed by measuring reverse transcriptase levels in the culture medium 2 weeks later. As summarized in Fig. 2, mutants pr779/-29 and pr882/-8 did not lead to chronic infections, whereas mutants pr882/-29 and pr779/-273 did.

Intracellular viral RNA levels were measured

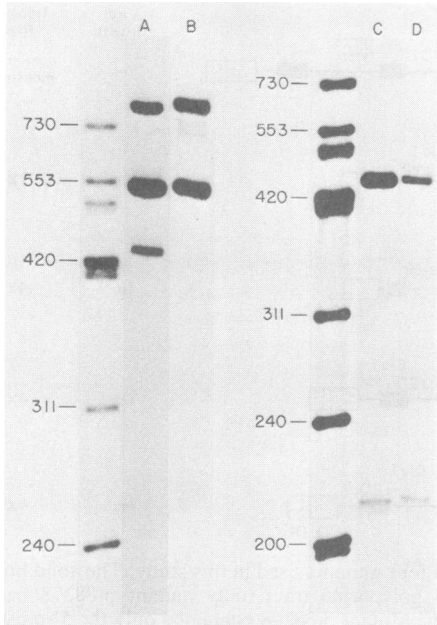


FIG. 3. S1 nuclease analysis of total RNAs from cells transfected with mutant plasmids. The bands represent the S1 nuclease digestion products after hybridization. For lane A the 814-nucleotide 3'-end-labeled *Ava*II probe shown in Fig. 1A was hybridized to RNA from cells cotransfected 28 h earlier with equal amounts of pr882/-29 and pr779/-29 DNAs. For lane B the same probe was hybridized to RNA from a plate of cells co-transfected 1 week earlier with equal amounts of pr882/-29 and pr779/-29 DNAs (done in parallel with lane A). The 814-nucleotide bands in both lane A and lane B represent the self-hybridizing DNA probe. For lane C the 3,100-nucleotide 5'-end-labeled *Eco*RI probe (Fig. 1A) was hybridized to RNA from cells cotransfected 28 h earlier with a 2:1 mixture of pr779/-273 and pr779/-29 DNAs. For lane D the *Eco*RI probe was hybridized to RNA from cells cotransfected 28 h earlier with a 2:1 mixture of pr779/-273 and pr882/-29 DNAs.

either 28 h, 48 h, or 7 days posttransfection. Figure 3, lane A, shows the S1 nuclease digestion products after hybridization of a 3'-end-labeled 814-nucleotide *Ava*II restriction fragment (Fig. 1A) to the total RNA from cells that had been cotransfected 28 h earlier with equal amounts of plasmids pr882/-29 and pr779/-29. As shown in Fig. 1, mutant pr779/-29 should have yielded a 441-nucleotide band, and mutant pr882/-29 should have yielded a 544-nucleotide band. Figure 3, lane A, shows these bands in a ratio (confirmed by scintillation counting of gel fragments) of approximately 4:1 favoring mutant pr882/-29. When the RNA from a parallel plate of cells from the same cotransfection was analyzed after 7 days of culture, only mutant pr882/-29 was detected (Fig. 3, lane B).

A 5'-end-labeled 3,100-nucleotide *Eco*RI re-

striction fragment (Fig. 1A) was used as a probe to distinguish mutants pr779/-29 and pr779/-273 after cotransfection. As shown in Fig. 1, mutant pr779/-29 should have yielded a 204-nucleotide band, and mutant pr779/-273 should have yielded a 448-nucleotide band. Figure 3, lane C, shows that 28 h after cotransfection with a 2:1 mixture of pr779/-273 and pr779/-29 DNAs the concentration of mutant pr779/-273 RNA was approximately eight times the concentration of mutant pr779/-29 RNA. Figure 3, lane D, shows the S1 nuclease digestion products after hybridization to cellular RNA harvested 28 h after cotransfection with a 2:1 mixture of pr779/-273 and pr882/-29 DNAs. The bands were present in a ratio of approximately 2.5:1 favoring the larger band. Figure 3, lane D, demonstrates that the upstream and downstream 115-nucleotide direct repeats had approximately the same activity in this assay.

To assess the effect of the 115-nucleotide sequence on the levels of viral RNA exported within virions, both intracellular RNA and virion RNA from the same plate of cells were measured 48 h posttransfection. Figure 4, lane A, shows the S1 nuclease digestion products after hybridization of the 3'-end-labeled 814-nucleotide *Ava*II fragment (Fig. 1A) to the total

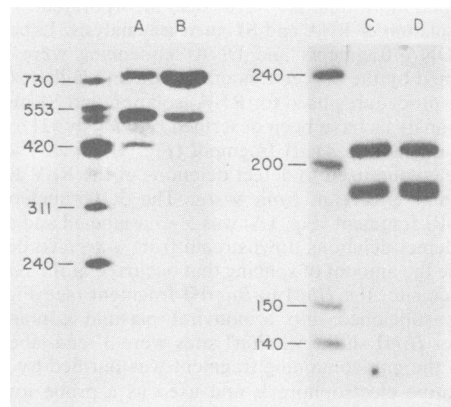


FIG. 4. S1 nuclease analysis of total cellular and virion RNAs from cells transfected with mutant plasmids. The bands represent S1 nuclease digestion products after hybridization. For lane A the 814-nucleotide 3'-end-labeled *Ava*II probe (Fig. 1A) was hybridized to RNA from cells co-transfected 48 h earlier with a 2:1 mixture of pr779/-29 and pr882/-29 DNAs. For lane B the same *Ava*II probe was hybridized to virion RNA purified from culture media from the same plate of cells used for lane A 48 h posttransfection. For lane C the 3,100 nucleotide 5'-end-labeled *Eco*RI probe (Fig. 1A) was hybridized to RNA from cells cotransfected 48 h earlier with equal amounts of pr882/-29 and pr882/-8 DNAs. For lane D the same *Eco*RI probe was hybridized to virion RNA from the same plate of cells used for lane C 48 h posttransfection.

RNA from cells cotransfected 48 h earlier with a 2:1 mixture of pr779/-29 and pr882/-29 DNAs. Figure 4, lane B, shows the S1 nuclease digestion products after hybridization of the same probe to virion RNA harvested 48 h posttransfection from the culture medium of the same plate of cells used in the analysis shown in Fig. 4, lane A. The ratio of pr779/-29 RNA to pr882/-29 RNA within the virions was markedly less than the ratio within the cells. To rule out the possibility that intracellular viral RNA levels must reach a certain threshold before being packaged, pr779/-29 and pr882/-29 DNAs were co-transfected in a 15:1 ratio; the respective RNAs were present intracellularly in a ratio of 2:1, but again only pr882/-29 RNA could be detected within the virions (data not shown). The level of RNA within the virions produced by mutant pr779/-273, measured by the same technique, was equivalent to the level in pr882/-29 (data not shown). Since mutant pr779/-273 contained the downstream 115-nucleotide sequence and mutant pr882/-29 contained the upstream 115-nucleotide sequence, we concluded that the information necessary for this effect on virion RNA levels was encoded within each 115-nucleotide sequence.

We tested the effect of deleting a portion of the polypurine region (a *cis*-lethal mutation [20]) on the intracellular and virion RNA levels posttransfection. Figure 4, lane C, shows the S1 nuclease digestion products after hybridization of the 3,100-nucleotide 5'-end-labeled *Eco*RI fragment (Fig. 1A) to the total intracellular RNA from cells cotransfected 48 h earlier with equal amounts of pr882/-29 and pr882/-8 DNAs. Figure 4, lane D, shows the S1 nuclease digestion products after hybridization of the same probe to virion RNA harvested 48 h posttransfection from the culture medium of the same plate of cells used in the analysis shown in Fig. 4, lane C. These data show that deletion of a necessary portion of the polypurine region did not affect the levels of intracellular or virion RNA 48 h posttransfection. They also demonstrate that reinfection was not necessary to produce detectable levels of RNA within virions since pr882/-8 was incapable of reinfection. Figure 4, lanes C and D, also show that cellular and virion RNA levels were not affected by deletion of the 21 most-downstream nucleotides in the 115-nucleotide direct repeat, thus separating the polypurine locus from the locus responsible for the effect shown in Fig. 4, lanes A and B.

The studies described above demonstrated that the viral RNA genomes lacking both 115-nucleotide elements were blocked somewhere along the normal pathway from cell nucleus to virus particle. In an effort to determine better at which step in this pathway the block occurred,

the following experiments were done. The cytoplasmic RNA from cells transfected with a mutant or wild-type genome was fractionated by using oligodeoxythymidylic acid-cellulose. Polyadenylic acid-containing RNA was then analyzed by S1 nuclease mapping (Fig. 5). The DNA probe for the S1 nuclease analysis was obtained by subcloning the *Hinf*I-to-*Bam*HI fragment (Fig. 5) into a nonviral plasmid and then 3'-end-labeling the DNA after *Hinf*I digestion. When this probe was hybridized to viral RNA and the hybrids were digested with S1 nuclease, a 332-nucleotide fragment was created if the probe had hybridized to viral RNA that

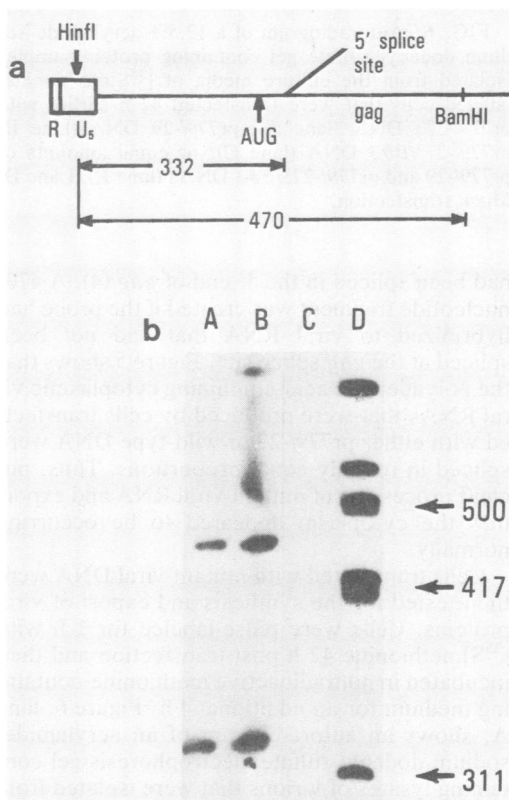


FIG. 5. (a) Schematic diagram of the 5' end of the viral RNA. R, Terminal redundancy; U<sub>5</sub>, unique 5' sequence; AUG, first codon of the *gag* gene. The numbers below the diagram represent the distances (in nucleotides) from the *Hinf*I site in U<sub>5</sub> to either the 5' splice site in *gag* or the *Bam*HI site in *gag*. (b) Autoradiogram. The bands represent the S1 nuclease digestion products after hybridization of the *Hinf*I 3' probe described in the text to cytoplasmic polyadenylic acid-containing RNA from cells transfected 28 h earlier with pr779/-29 (lane A), cytoplasmic polyadenylic acid-containing RNA from cells transfected 28 h earlier with wild-type DNA (lane B), cytoplasmic polyadenylic acid-containing RNA from chicken fibroblasts (lane C), and  $\phi$ X174 *Hinf*I markers (lane D).

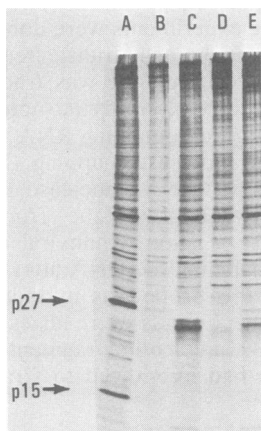


FIG. 6. Autoradiogram of a 12.5% acrylamide-sodium dodecyl sulfate gel containing protein samples isolated from the culture media of [ $^{35}$ S]methionine-labeled cells that were transfected 42 h earlier with pr779/-273 DNA (lane A), pr779/-29 DNA (lane B) pr779/-273/P#3 DNA (lane C), or equal amounts of pr779/-29 and pr779/-273/P#3 DNAs (lane E). Lane D, Mock transfection.

had been spliced in the 5' end of *gag* (4). A 470-nucleotide fragment was created if the probe had hybridized to viral RNA that had not been spliced at the *gag* splice site. Figure 5 shows that the polyadenylic acid-containing cytoplasmic viral RNAs that were produced by cells transfected with either pr779/-29 or wild-type DNA were spliced in roughly equal proportions. Thus, nuclear processing of mutant viral RNA and export into the cytoplasm appeared to be occurring normally.

Cells transfected with mutant viral DNA were then tested for the synthesis and export of viral proteins. Cells were pulse-labeled for 1 h with [ $^{35}$ S]methionine 42 h post-transfection and then incubated in nonradioactive methionine-containing medium for an additional 4 h. Figure 6, lane A, shows an autoradiogram of an acrylamide-sodium dodecyl sulfate electrophoresis gel containing lysates of virions that were isolated from the culture medium of cells transfected with pr779/-273 DNA 48 h earlier. Processed *gag* proteins p27 and p15 were clearly present. Figure 6, lane B, shows a similar preparation from the culture medium of cells transfected 48 h earlier with pr779/-29 DNA; although less intense, the p27 and p15 bands were present (compare with lane D [mock-transfected fibroblasts]). Thus, the mutant RNA lacking both 115-nucleotide sequences was translated, and the *gag* polypeptide was processed and exported. However, the levels of p27 and p15 in the culture medium were consistently lower after transfection of pr779/-29 DNA than after trans-

fection of any of the other mutant DNAs. To determine whether this was due to a fourfold-lower level of pr779/-29 RNA (Fig. 3) or due to some block at the level of virion export, the following experiment was done. A DNA mutant, pr779/-273/P#3, was constructed, in which all of the *env* and *pol* coding regions and most of the *gag* coding region were deleted (Fig. 7), but the mutant retained the downstream 115-nucleotide sequence, both LTRs, the 5' leader region, and 470 nucleotides of the 5' portion of the *gag* coding region. When pr779/-273/P#3 DNA was transfected into cells, wild-type levels of viral RNA were produced transiently; and when pr779/-273/P#3 DNA was cotransfected with wild-type DNA, both RNA species were packaged into virions and exported normally (data not shown). Thus, pr779/-273/P#3 RNA contained all of the *cis*-acting sequences needed for normal RNA packaging into virions. Figure 6, lane C, shows the  $^{35}$ S-labeled proteins found in the culture medium 48 h after transfection of pr779/-273/P#3 DNA. A doublet at approximately 21,000 daltons was present; this doublet represented a fusion peptide consisting of most of the p19 *gag* product fused to a normally noncoding segment in the 3' flanking region of *v-src*. (This observation suggests that p19 *gag* alone contains the sequences needed for its export into the culture medium.) Figure 6, lane E, shows a similar preparation 48 h after cotransfection of pr779/-29 and pr779/-273/P#3 DNAs. The p27 and p15 bands were present, as was the 21,000-dalton doublet. The presence of a packagable RNA (pr779/-273/P#3) in these cells

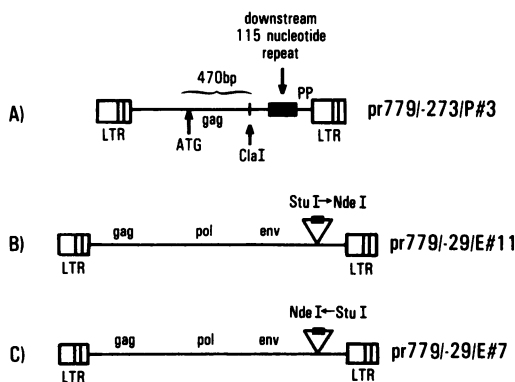


FIG. 7. Schematic diagram of pr779/-273/P#3 DNA cloned as a provirus in an ampicillin-resistant plasmid (not shown). ATG, First codon of *gag* gene. A *Cla*I linker joins nucleotide 470 of *gag* to the extreme 3' terminus of *v-src* and downstream sequences. PP, Functional polypurine tract. pr779/-29/E#7 and pr779/-29/E#1 show that the *Stu*I-to-*Nde*I fragment (Fig. 1A) was inserted into the *Cla*I site of pr779/-29 in either orientation. bp, Base Pair.

did not markedly influence the relative amounts of p27 and p15 found in the virions. Moreover, the intensities of the p27 and p15 bands, compared with the 21,000-dalton band, roughly paralleled the ratio of pr779/-29 RNA to pr779/-273/P#3 RNA found intracellularly (data not shown). Thus, it appeared that the lower levels of pr779/-29 translation products found in virions simply reflected the lower levels of this RNA intracellularly posttransfection and were not due to a lack of a packagable viral RNA within the cells.

To guarantee that the phenotype observed in mutant pr779/-29 was actually due to the lack of a 115-nucleotide element and not due to an unanticipated mutation in some other region of the genome accidentally introduced during cloning in *E. coli*, a single copy of the upstream 115-nucleotide element (*StuI*-to *NdeI* fragment [Fig. 1]) was introduced in either orientation into the *ClaI* site of plasmid pr779/-29 (Fig. 7). When the element was inserted in the normal orientation, the DNA was infectious upon transfection (high levels of reverse transcriptase after 2 weeks). However, when the element was inserted backwards, the DNA was not infectious, and the RNA carried a *cis*-acting defect. Thus, proper insertion of the 115-nucleotide element back into pr779/-29 repaired the defect, discounting any possibility of a relevant defect in another region of the pr779/-29 genome.

### DISCUSSION

The measurement of reverse transcriptase levels 2 weeks after transfection demonstrated that at least one copy of the 115-nucleotide direct repeat of RSV was necessary for normal viral growth after transfection of viral DNA (Fig. 2). In an attempt to determine the function(s) of the 115-nucleotide sequence, viral RNA transcripts were analyzed soon after transfection. Figure 3 shows that inclusion of either copy of the 115-nucleotide sequence within the viral genome increased the intracellular viral RNA level (measured 28 h posttransfection) approximately fourfold. The 115-nucleotide sequence clearly acted in *cis* to elevate the viral RNA levels. The fourfold difference in RNA levels was not due to reinfection by the infectious virus since the *cis*-lethal mutant pr882/-8 (20) was able to produce wild-type levels of RNA 28 h posttransfection (Fig. 4).

Figure 4 shows that transfection of viral DNA lacking both copies of the 115-nucleotide element led to the production of viral RNA intracellularly, but that the viral RNA was not detectable within virions. The experiment shown in Fig. 5 was done to rule out the possibility of a gross defect in RNA processing or export from the nucleus. The experiment shown in Fig. 6

demonstrated that mutant viral transcripts were translated normally and that the *gag* translation product appeared to be exported and cleaved normally (although at lower levels due to lower levels of mutant viral mRNA).

There are two ways to explain the observation that pr779/-29 intracellular RNA is present at lower levels than the intracellular RNA produced by a genome containing a 115-nucleotide element (Fig. 3). The 115-nucleotide element influences either the amount of transcription or the stability of the RNA (or both). The observation that insertion of one copy of the 115-nucleotide element back into mutant pr779/-29 repaired the defect when it was placed in the normal orientation but not when it was placed in the backward orientation diminishes the possibility that this repeated element functioned solely as a viral enhancer element (enhancer elements are known to increase transcription during transient expression experiments when they are linked in either orientation to genes in *cis* [6]). However, it has been reported recently that approximately 40 nucleotides adjacent to the polypurine tract of the downstream 115-nucleotide element are part of an enhancing element which extends downstream about 90 nucleotides into U<sub>3</sub> (11). Deletion of the region within the 115-nucleotide element decreases the frequency of stable biochemical transformation (another test for enhancing elements [6]) sevenfold (11). Our transient expression data (Fig. 3 and 4) are consistent with the hypothesis that the 115-nucleotide element contains a portion of an overlapping enhancer locus. However, a decreased transcription rate alone cannot account for the differential distribution of mutant RNA between virions and cytoplasm. Some other *cis*-acting mechanism must be involved.

Since the 115-nucleotide element lies within the viral RNA transcript, differential RNA stability must be considered. If the 115-nucleotide element did affect stability, it would do so by interacting with or preventing the interaction with a cellular or viral molecule (since the rate of spontaneous RNA degradation at neutral pH is negligible). One possibility is that the pool of full-length viral RNA bound to viral proteins (and thus hypothetically stabilized) is large, such that the lack of this interaction (pool) would decrease the amount of total intracellular RNA. Although this is an attractive hypothesis, one would expect the ratio of unspliced viral RNA to spliced viral mRNA to decrease markedly in a mutant RNA that was unable to bind to stabilizing viral proteins. However, Fig. 5 shows that there was little change in the ratio of unspliced viral RNA to spliced viral RNA in pr779/-29-transfected cells, certainly not enough to account for fourfold-lower total RNA levels. An-

other possibility is that the 115-nucleotide element somehow facilitates ribosome binding and increases the pool of viral RNA bound to ribosomes. If so, one would again expect a shift in the ratio of unspliced viral RNA to spliced viral RNA (this time an increase) in pr779/-29-transfected cells. Figure 5 again makes this possibility seem unlikely, and Fig. 6 shows that pr779/-29 RNA is translated in direct proportion to its lower total concentration. Although it is formally possible that the 115-nucleotide element protects viral RNA from a cellular RNase, there is no a priori reason to suspect it. Although we retain RNase sensitivity as a formal possibility and realize that this type of RNA instability could be invoked as a mechanism to explain the lowered levels of virion RNA, we do not favor this explanation since one would expect to see a gradually decreasing concentration of genomic RNA en route to the periphery of the cell and thus a lowered ratio of full-length viral RNA to spliced viral RNA in pr779/-29-transfected cells.

Therefore, we favor the hypothesis that pr779/-29 RNA is present in lower levels because it is synthesized in lower amounts (presumably due to deletion of a portion of the overlapping enhancer element) and that pr779/-29 RNA is as stable as wild-type RNA. Thus, the decreased ratio of pr779/-29 RNA to pr882/-29 RNA in virions must be explained by the ability of some mechanism in the periphery of the cell to distinguish pr882/-29 RNA from pr779/-29 RNA. The most likely mechanism is the mechanism which is involved in the packaging of viral RNA into virions.

The murine retroviruses have very few nucleotides separating the 3' end of the envelope gene from the polypurine tract, and it has been shown that deleted spleen necrosis virus genomes are packaged normally even when they retain only 40 nucleotides upstream from U<sub>3</sub> (in addition to U<sub>5</sub> and the upstream packaging locus [23]). Therefore, neither of these viral genomes appears to have an equivalent *cis*-acting locus upstream from the polypurine tract. However, it is known that the enhancer elements of murine retroviruses are contained entirely within U<sub>3</sub> 120 to 200 nucleotides downstream from the polypurine-U<sub>3</sub> junction. This downstream skewing of the enhancer element (relative to RSV) leads us to suspect that a locus equivalent to the one contained within the 115-nucleotide element of RSV may be present in the U<sub>3</sub> region of murine retroviruses and, perhaps, spleen necrosis virus. Although it has been shown that Harvey sarcoma virus genomes lacking U<sub>3</sub> can be packaged, the event occurs at such a low frequency that it does not disprove the possibility of a packaging locus in U<sub>3</sub> (3). An alternative argument is that RSV is unique in its arrangement of *cis*-acting

packaging loci and that no equivalent to the 115-nucleotide element exists in other viruses.

Although the genomes shown in Fig. 7B and C establish without a doubt that the 115-nucleotide element is required for proper viral replication, it is not obvious why the virus evolved in this way. If the exclusion of subgenomic mRNAs from virions is controlled by a packaging locus within the normal viral intron, why should the genome contain a similar locus in its exonic sequences? One possibility is that the locus lies within the intron of an alternative spliced RNA species. Another possibility is that two packaging loci must interact to fold the RNA into a conformation that is suitable for packaging.

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