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Efficient Encapsidation of Human Immunodeficiency Virus Type 1 Vectors and Further Characterization of *cis* Elements Required for Encapsidation

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To determine whether there is a *cis*-acting effect of translational expression of *gag* on RNA encapsidation, we compared the encapsidation of wild-type RNA with that of a mutant in which the translation of *gag* was ablated. This comparison indicated that there is not such a *cis* effect. To determine what is necessary and sufficient for encapsidation, we measured the relative encapsidation efficiencies of human immunodeficiency virus type 1 vector RNAs containing mutations in domains proximal to the canonical encapsidation signal or containing large deletions in the remainder of the genome. These data indicate that TAR and two additional regions are required for encapsidation and that the 5′ end of the genome is sufficient for encapsidation. The Rev-responsive element is required mainly for efficient RNA transport from the nucleus to the cytoplasm. A foreign sequence was found to have a negative effect on encapsidation upon placement within the parental vector. Interestingly, this negative effect was compounded by multiple copies of the sequence.

The sequence within the 5′ untranslated leader region of retroviral RNA contains important *cis* elements for many steps in viral replication. For human immunodeficiency virus type 1 (HIV-1), the terminal repeat (r), the unique 5′ sequence (u5), and the primer binding site (pbs) have been shown to be important in the initiation and translocation steps of reverse transcription (7, 26, 31, 63–65). Following reverse transcription, sequences originally derived from u5 are important for integration of the viral DNA (25, 58, 62). After transcription, the major splice donor (5′ss), which is used to generate the subgenomic mRNAs, is required for efficient expression of most of the internal genes of HIV-1. The TAR stem-loop within r is important for maximal transcription from the viral promoter (17, 21, 27, 32, 47, 51), and sequences within r and u5 are important for the polyadenylation of transcripts (12). Sequences throughout the 5′ untranslated region have been implicated in the process of dimerization (4, 18, 57, 60). In particular, a stem-loop called SL1, located between the pbs and the 5′ss, has been shown to be important for initiation of the dimerization process in vitro (16, 36, 44, 48, 52, 59).

Encapsidation is the process whereby genomic RNA is incorporated into the assembling virus particle. Subgenomic RNAs and nonviral RNAs are largely excluded from encapsidation (40, 45, 46). The process of encapsidation involves the recognition of *cis* elements within genomic RNA (E or Ψ) by the product of the *gag* gene, the *trans* factor shown to be required for particle formation and encapsidation (33). The most well characterized of the E elements are those that are present downstream of the pbs and that extend into the 5′ end of the *gag* gene. The secondary structure of this region consists of four hairpins, SL1, SL2, SL3, and SL4 (5, 15, 28). Three of these hairpins, SL1, SL3, and SL4, function in encapsidation (45, 46), but they are not sufficient for encapsidation in vivo.

Within the 5′ untranslated leader region, sequences upstream of SL1 are also required for encapsidation (35, 45). Since these sequences are upstream of the 5′ss, they are present in both genomic and subgenomic RNAs. In addition, sequences within *gag* and downstream of SL4 augment encapsidation (40, 53). Since these sequences are present only in genomic RNAs, they are likely to contribute to the selective encapsidation of genomic RNAs. The exact nature of all of the *cis* elements upstream and downstream of SL1 through SL4 and the functions that they serve in the process of encapsidation remain to be fully elucidated. Finally, some data have indicated that sequences within *env* are required for encapsidation (34, 55). Some of these sequences within *env* encompass the Rev-responsive element (rre). However, the rre is required for efficient transport of genomic RNA from the nucleus to the cytoplasm, and therefore it is unclear whether these sequences function directly or indirectly in encapsidation.

Retroviral vectors contain *cis* elements sufficient for viral replication but usually lack essential *trans* factors. Retroviral vectors can be used to transduce foreign genes into cells provided that essential *trans* factors can be supplied by helper cells, helper viruses, or helper plasmids. Encapsidation of HIV-1 vectors by helper plasmids and helper cell lines can be attained, but the encapsidation efficiency of the vectors is low (10, 40, 53). This relatively low encapsidation efficiency may be due to the absence of a full complement of the *cis* elements required for encapsidation or to the inadvertent disruption of encapsidation elements by mutations designed to ablate translation of the *gag* gene. Alternatively, E may not have been able to function within the context in which it was expressed because the presence of a foreign sequence may have impinged upon the capability of E to function.

To gain a better understanding of the requirements for efficient HIV-1 vector RNA encapsidation and to identify regions of the viral genome outside the canonical E region (SL1 to SL4) that contribute to encapsidation, we examined the encapsidation efficiencies of mutants that lack discrete seg-

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ments of the viral genome. Our results indicate that ablation of any of three regions upstream of E results in substantial reduction in encapsidation. These three regions are TAR, a contiguous region that likely contains a hairpin structure termed the r-u5 stem-loop, and a third region of undefined secondary structure that surrounds the pbs. In contrast, the rre and the adjacent region do not contribute substantially to encapsidation in a direct way. Finally, introduction of a foreign gene reduced encapsidation efficiency marginally. However, the introduction of multiple copies of the gene had a synergistic negative effect on encapsidation. These data help to define what is sufficient in *cis* for encapsidation and have implications for successful HIV-1 vector design.

MATERIALS AND METHODS

Construction of plasmid DNAs. Standard techniques were used for molecular cloning (43). The creation of pMSMBA has been described previously (45). Unless otherwise noted, nucleotide designations refer to the DNA sequence of pNL4-3 (1). RNA nucleotide (nt) 1 corresponds to DNA nt 455. pCT is derived from pMSMBA and contains a stop codon within the capsid domain of the *gag* gene. pCT was created in several steps. First, two fragments obtained by PCR amplification of pMSMBA with two sets of primers, the sense mismatch primer complementary to the sequence of nt 687 to 715 (sense mismatch primer 687-715 *Eco*RI) (45) with the antisense mismatch primer complementary to the sequence of nt 1483 to 1460 (antisense mismatch primer 1483-1460 *Xba*I) (5′-TTGGTTC TCTCTAGAGGCCTGGTG) and the sense mismatch primer 1460-1483 *Xba*I (5′-CACCAGGCCTCTAGAGAGAACCAA) with the antisense mismatch primer 1999-1976 *Xba*I (5′-TTTTGGCTATCTAGACTTCTTTGC), were digested with *Xba*I and ligated. Next, the ligation mixture was PCR amplified with the sense primer 687-715 *Eco*RI and the antisense primer 1535-1511 (45). The resulting 848-bp fragment was digested with *Bss*HII and *Spe*I and ligated into pMSMBA digested with the same two enzymes.

pdTAR was created by a similar protocol. Two fragments obtained by PCR amplification of pCT with two sets of primers, the sense primer −25-−8 (5′-A AGCTTATGCATGCGGCC) with the antisense mismatch primer 464-443 *Xba*I (5′-CCTCTAGACCCAGTACAGGCAA) and the sense mismatch primer 504-528 *Xba*I (5′-GGTCTAGACACTGCTTAAGCCTCAA) with the antisense mismatch primer 774-743 *Age*I (45), were digested with *Xba*I and ligated. This ligation mixture was PCR amplified with the sense primer −25-−8 and the antisense primer 774-743 *Age*I. The resulting 799-bp fragment was digested with *Not*I and *Bss*HII and ligated into pCT that had been digested with the same two enzymes. A similar protocol was used to create pdR/U5, pR/U5, pR/U5*, pdR/L, and pdPBS with an appropriate set of internal primers, the sense mismatch primer 551-575 *Xba*I (5′-GGTCTAGATCTGCCCGTCTGTTGTG) with the antisense mismatch primer 519-495 *Xba*I (5′-CCTCTAGACCTTCCCTAGTTA GCCA), the sense mismatch primer 510-533 *Xba*I (5′-CCTCTAGATTAAGCC TCAATAAAG) with the antisense mismatch primer 519-495 *Xba*I (see sequence above), the sense mismatch primer 544-567 (5′-GTGCTCAAATTTGGGTGT GCCCGT) with the antisense mismatch primer 567-544 (5′-ACGGGCACACC CAAATTTGAGCAC), the sense mismatch primer 675-698 *Xba*I (5′-GAGGA GATCTCTAGACGCAGGACT) with the antisense mismatch primer 519-495, and the sense mismatch primer 624-635/654-665 *Mfe*I (5′-AATCTCTAGCAAT TGAAAGCGAAA) with the antisense mismatch primer 665-654/635-624 *Mfe*I (5′-TTTCGCTTTCAATTGCTAGAGAT), respectively.

pdU5/L was created by PCR amplification of pCT by using the sense primer −25-−8 with the antisense primer 720-697/564-556 (5′-CCGTGCGCGCTTCA GCAAGCCGAGGGCACACAC). The resulting 616-bp fragment was digested with *Not*I and *Bss*HII and ligated into pCT digested with the same two enzymes.

pdRRE was created in steps. First, two fragments obtained by PCR amplification of pMSMBA with two sets of primers, the sense primer 6321-6343 (5′-T GTGGGTCACAGTCTATTATGGGG) with the antisense mismatch primer 7773-7750 *Mlu*I (5′-AGGAACAAAACGCGTATTCCCACT) and the sense mismatch primer 7991-8014 *Mlu*I (5′-CTGGGGATTACGCGTTGCTCTGGA) with the antisense primer 8927-8903 (5′-ATTGCTACTTGTGATTGCTCCAT G), were digested with *Mlu*I and ligated. Next, the ligation mixture was PCR amplified with the sense primer 6321-6343 and the antisense primer 8927-8903. The resulting 1,477-bp fragment was digested with *Nhe*I and *Xho*I and ligated into pMSMBA digested with the same two enzymes.

pd(X-N) was created by digesting pCT with *Xba*I and *Nhe*I followed by ligating the larger fragment to generate a plasmid containing a deletion between these two sites. pd(N-H) was created by digesting pCT with *Nhe*I and *Hpa*I, treating the DNA with the Klenow fragment of DNA polymerase, and ligating the larger fragment. pB(SVpA) was created by digesting pMSM50 (45) with *Bam*HI and *Nae*I and ligating a 264-bp fragment containing the simian virus 40 (SV40) poly(A) site into pCT that had been digested with *Bam*HI and *Sma*I. pd(X-N)B(SVpA) was created by digesting pB(SVpA) with *Xba*I and *Nhe*I and recircularizing the larger fragment through ligation. pd(X-X) was created by digesting pCT with *Xba*I and *Xho*I, treating the DNA with the Klenow fragment of DNA polymerase, and ligating. pBSSK(−)CTE was created by digesting pKB504CTE, containing the Mason-Pfizer monkey virus (MPMV) sequence from nt 8039 to 8184 (kindly provided by Kathy Boris-Lawrie), with *Bam*HI and *Pst*I and ligating a fragment into pBSSK(−) (Stratagene) digested with the same two enzymes. pd(X-X)CTE was created by digesting pBSSK(−)CTE with *Xba*I and *Xho*I and ligating a fragment containing the MPMV constitutive transport element (cte) into pCT that had been digested with *Xba*I and *Xho*I.

pH-1 was created by digesting GB108 (14) with *Xba*I and *Nhe*I and ligating a fragment containing the hygromycin B resistance gene into pCT that had been digested with the same two enzymes. pH-2 was created by digesting pH-1 with *Xba*I and *Nhe*I and ligating the fragment containing the hygromycin B resistance gene into pH-1 digested with *Nhe*I. pH-4 was created by digesting pH-2 with *Xba*I and *Nhe*I and ligating the fragment containing two copies of the hygromycin B resistance gene into pH-2 digested with *Nhe*I.

pGEM(1247-1523) and pGEM(1247-1523TAG) were created by PCR amplification of pMSMBA and pCT, respectively, with the sense primer 687-715 *Eco*RI (45) and the antisense mismatch primer 1535-1512 *Bam*HI (5′-CATCC TATTGGATCCTGAAGGGTA), respectively. The resulting 848-bp fragments were digested with *Bam*HI and *Nsi*I and ligated into pGEM11zf(−) (Promega) digested with the same two enzymes.

Transfections. Twenty-four hours before transfection, 293 cells were seeded at a density of 1.0×10^7 cells per 150-mm-diameter plate in 30 ml of Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and were incubated at 37°C in 5% CO_2. Transfections were carried out with 15 μg of each of the two plasmid DNAs per 150-mm-diameter plate for a total of 30 μg of DNA. The calcium phosphate precipitation method was used as described previously (3).

RNA isolation. After 48 to 72 h, the media and cytoplasmic RNA were concurrently collected. The cytoplasmic RNA, nuclear RNA, and total cell RNA were harvested as previously described (43) and the concentrations were determined by spectrophotometric absorption at a wavelength of 260 nm. Virus was harvested from the media by first removing cellular debris by centrifugation at 4,000 rpm in a Beckman AccuspinFR tabletop centrifuge. The virus was then pelleted from the clarified media by centrifugation through a 20% sucrose cushion for 2.5 h at 25,000 rpm in an SW28 rotor. The viral pellet was then resuspended in 500 μl of TNE buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA). The physical virus titer was determined by using an antigen-capture assay to quantitate p24 (Coulter Cytometry). To isolate RNA, virions were disrupted by the addition of sodium dodecyl sulfate to 1% and by incubation in a boiling water bath for 5 min. The sample was then treated with proteinase K (500 μg/ml) at 37°C for 30 min followed by phenol (pH 4.5)-chloroform extraction, chloroform extraction, and ethanol precipitation in the presence of 20 μg of poly(C) RNA. The nucleic acids isolated both from the cytoplasm of the transfected cells and from the virus were treated with DNase I by resuspending the isolated nucleic acids in 50 μl of a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 40 U of RNasin (Promega), 1 mM dithiothreitol, and 10 U of RNase-free DNase I (Boehringer-Mannheim). The nucleic acids were incubated at 37°C for 30 min. To terminate the reaction, the samples were again treated with proteinase K, phenol-chloroform extracted, chloroform extracted, and precipitated with ethanol.

RNase protection. Antisense probe ($\sim 1.0 \times 10^8$ cpm/μg) was synthesized by linearizing pGEM(1247-1523) or pGEM(1247-1523TAG) with *Hin*dIII. T7 RNA polymerase (Promega) was then used to transcribe the template in the presence of [α-^{32}P]CTP (43). Either 25 μg of cytoplasmic RNA or one-fifth of the virion RNA preparation supplemented with 25 μg of poly(C) RNA was mixed with 10^6 Cerenkov counts of ^{32}P-labeled antisense RNA (~200 fmol) and was precipitated with ethanol. Samples were washed with 70% ethanol and resuspended in 20 μl of hybridization buffer (100 mM sodium citrate [pH 6.4], 300 mM sodium acetate [pH 6.4], 1 mM EDTA in 80% formamide), heated at 95°C for 3 min, and hybridized at 42°C for 16 h. Then, 200 μl of RNase digestion mixture (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 2 μg of RNase T_1 per ml, 5 μg of RNase A per ml), was added for a 30-min incubation at 37°C. Sodium dodecyl sulfate was added to 1% and proteinase K was added to 0.5 mg/ml. Samples were incubated for 30 min at 37°C, phenol-chloroform extracted, chloroform extracted, and precipitated with ethanol in the presence of 25 μg of poly(C) RNA carrier. Pellets were dissolved in 10 μl of formamide-loading buffer (2 mM EDTA [pH 8.0] in 80% formamide), heated at 95°C for 3 min, and subjected to polyacrylamide gel electrophoresis (6% acrylamide, ratio of bisacrylamide to acrylamide, 19:1; 8 M urea). To create size markers on denaturing polyacrylamide gels, we labeled pGEM11zf(−) (Promega) digested with *Hpa*II by treatment with the Klenow fragment in the presence of [α-^{32}P]dCTP (43). The quantitation of the various protected RNA species was determined by PhosphorImager analysis (Molecular Dynamics).

RESULTS

Translation of *gag* does not increase RNA encapsidation in *cis*. Retroviral vectors contain essential *cis* elements for viral replication but lack essential *trans* factors. However, at least some HIV-1 vectors exhibit relatively low encapsidation effi-

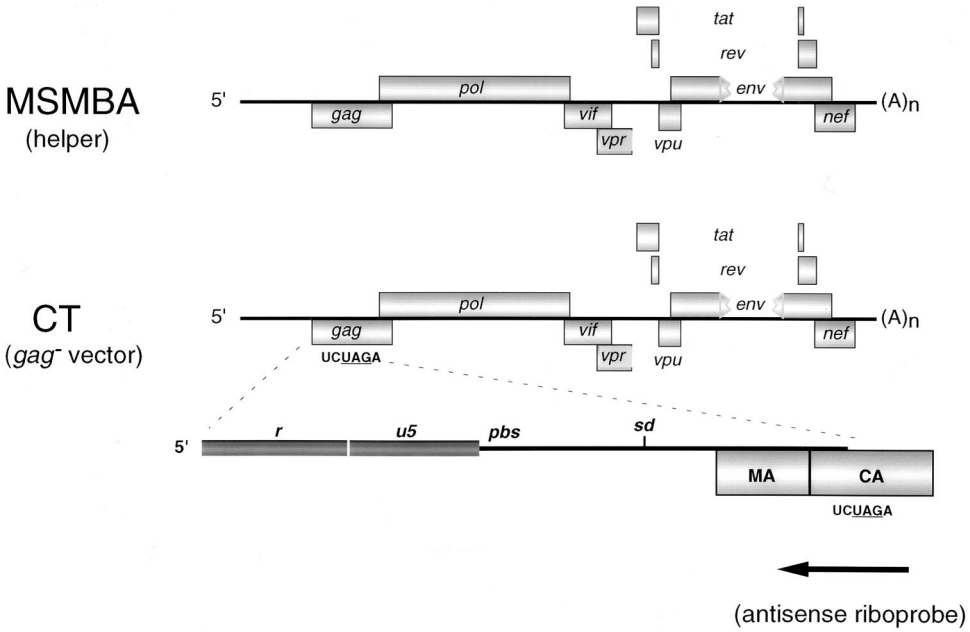

FIG. 1. Schematic representation of MSMBA RNA (helper) and CT RNA (vector). pMSMBA contains a stop codon at DNA nt 6359 within *env* (RNA nt 5905) and a deletion from DNA nt 6365 to 7252 (RNA nt 5911 to 6798) (40). pCT is a derivative of pMSMBA that contains a stop codon at DNA nt 1472 (RNA nt 1018) within the capsid domain of *gag*. The antisense riboprobe used in the experiments described in the text hybridizes to a portion of the *gag* gene in pMSMBA or pCT as noted. sd, splice donor, 5′ss.

ciencies (40), presumably because the mutations introduced into these vectors, which had been created to disrupt translation of Gag mRNA, had the inadvertent effect of disrupting *cis* elements essential for encapsidation. To systematically examine the criteria required for efficient vector encapsidation, we first created an HIV-1 vector by disrupting translation of Gag mRNA at a location distal to *cis* elements known to be essential for encapsidation (53). pCT is a derivative of the proviral clone pMSMBA carrying the mutant *env* gene (45) and contains an *Xba*I site at nt 1469 and a stop codon at nt 1472 within the capsid domain (CA or p24) of the *gag* gene (Fig. 1). pCT efficiently expresses genomic RNA at a level similar to that of pMSMBA (Fig. 2). However, the truncated Gag derivative would not be expected to be proficient for particle assembly. As expected, the truncated version of Pr55 could not be detected in the media of pCT-transfected cells as evidenced by Western blot analysis (data not shown).

To determine the relative encapsidation efficiency of CT RNA, we cotransfected 293 cells with pCT and pMSMBA and performed an RNase protection assay on RNA isolated from the cytoplasm of the transfected cells and on RNA isolated from virions (Fig. 2). In this experiment, pMSMBA served as the source of Gag protein for encapsidation of both MSMBA RNA and CT RNA. The probe used in the RNase protection analysis was capable of detecting and distinguishing CT RNA and MSMBA RNA. Thus, it was possible to determine the relative encapsidation efficiencies of these two RNAs by determining the ratio of CT to MSMBA RNA within the virion and dividing by the ratio of these two RNAs within the cytoplasm. The CT genomic RNA was encapsidated at an efficiency of 0.93 relative to the MSMBA RNA. As expected, we did not detect genomic RNA within the media of cells transfected with pCT alone (Fig. 2). Since CT RNA was efficiently encapsidated, the stop codon that was created within pCT does not disrupt any *cis* elements necessary for encapsidation. Moreover, since CT RNA is encapsidated nearly as well as MSMBA RNA, the *trans* factors necessary for particle formation and encapsidation do not exhibit a *cis* effect; there is not preferential encapsidation of the mRNA that functions as mRNA for the translation of *gag*.

Requirement of sequences at the 5′ end of HIV-1 RNA for encapsidation. We previously showed that two stem-loops, SL1 and SL3, and potentially a third, SL4, are important in encapsidation (45). However, our results and the results of others suggest that sequences upstream of SL1, SL3, and SL4, are also required for encapsidation (35, 45). Using a model of the 5′ end of HIV-1 RNA (56) (Fig. 3A), we made derivatives of pCT containing precise deletions of regions containing known or suspected secondary structures at the 5′ end of the RNA and determined the effect of these mutations on encapsidation. The 5′ end of the HIV-1 genome is composed of TAR and two regions that are likely to contain ordered structure. TAR is the primary *cis*-acting element that is recognized by the viral Tat protein. Based on a progressive comparison of probable RNA secondary structure among diverse replication-competent HIV-1 strains (Mfold-Phylo program; University of Wisconsin, Genetics Computer Group), it is likely that the nucleotides immediately distal to TAR form a structure termed the r-u5 stem-loop. The disposition of the nucleotides distal to the r-u5 stem-loop is unclear. However, based on a comparison of the corresponding region in type C retroviruses, Aiyar et al. (2) have argued that there is likely to be conservation of a structure that helps to define a region called the u5-leader (u5-1) stem-loop.

pdTAR, pdR/U5, and pdU5/L contain deletions of the TAR stem-loop, the putative r-u5 stem-loop, and the putative u5-l stem-loop, respectively (Fig. 3B to D). pdR/L contains an exact deletion of both the putative r-u5 stem-loop and the u5-1 stem-loop (Fig. 3E). Deletion of TAR resulted in an approximately fourfold decrease in the steady-state level of cytoplasmic pdTAR RNA (Fig. 2). All other mutants expressed cytoplasmic RNA at a level similar to that of pCT. The relative

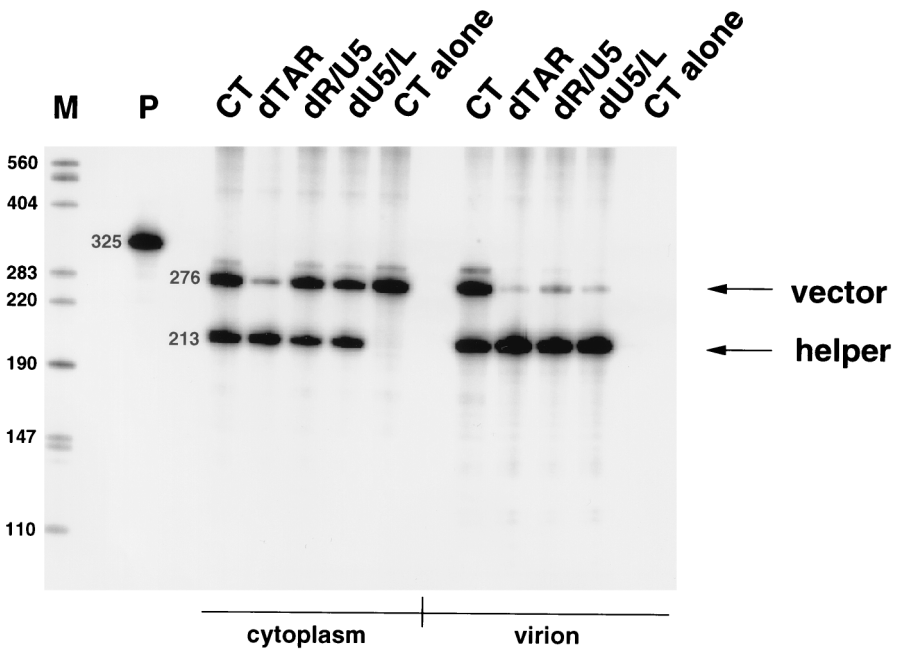

FIG. 2. The 5′ end of HIV-1 RNA is required for encapsidation. pCT or derivatives containing mutations throughout the 5′ end of the viral RNA were cotransfected into 293 cells along with pMSMBA. The cytoplasmic RNA from the transfected cells and the virion RNA were subjected to RNase protection analysis. The marker (M) was generated as described in Materials and Methods. The probe (P) used was generated from pGEM(1247-1523TAG). This probe is complementary to a portion of the *gag* gene from pCT and is capable of distinguishing vector and helper RNAs. The amount of probe loaded on the gel is 1/50 of the amount of probe used in the experiment. The diagnostic bands for vector RNA and helper RNA are 276 and 213 nt, respectively. pCT was also transfected alone to verify the dependence of pCT on pMSMBA for particle production.

encapsidation efficiency of each of these mutants was determined as described above and was found to be reduced (Table 1). We think it unlikely that the fourfold reduction in cytoplasmic pdTAR RNA accounts for the decrease in RNA encapsidation. An examination of other HIV-1 vectors that are reduced in cytoplasmic accumulation to levels similar to that of pdTAR but that retain an intact E region indicates that these RNAs are still encapsidated efficiently (data not shown). Thus, reduction of intracellular viral RNA does not by itself diminish the encapsidation efficiency of that RNA.

The pbs is the sequence present on genomic RNA to which the tRNA primer anneals and is the initiation site of reverse transcription. Upon the annealing of the tRNA primer to genomic RNA, the secondary structure of genomic RNA changes in vitro (31). Since the deletion within encapsidation-defective mutant pdU5/L would remove the pbs, we decided to see whether the pbs was required for encapsidation. We created a mutant, pdPBS, in which the pbs was deleted and an *Mfe*I site was created (Fig. 3F). The relative encapsidation efficiency of this mutant was substantially higher than that of pdU5/L (Table 1). Thus, it is unlikely that the dramatic decrease in the encapsidation of pdU5/L is due to the absence of the pbs. Moreover, it is unlikely that the stable association of the tRNA primer with the viral RNA is required in *cis* for encapsidation.

To determine whether disruption of the r-u5 stem-loop affects encapsidation, we created a mutant (pR/U5) which contains a set of base substitutions designed to disrupt base pairing within the putative r-u5 stem-loop, and a mutant (pR/U5*) which contains these same mutations in conjunction with second site mutations designed to restore base pairing (Fig. 4). We measured the relative encapsidation efficiencies of these two mutants and found that both were similarly low (Table 1). Since the second site mutations within pR/U5* do not function as compensatory mutations, it is unclear whether the r-u5 stem-loop forms and whether this is an encapsidation element. However, these data show that the sequence within the putative r-u5 stem-loop is critical for encapsidation.

The untranslated leader region and the 5′ end of *gag* are sufficient for encapsidation. Some data indicate that the 5′ end of HIV-1 RNA may not be sufficient for encapsidation (10) and that sequences within *env* may be necessary for encapsidation (34, 55). We attempted to assess whether regions outside the 5′ end of the RNA contribute to encapsidation by determining whether deletions and substitutions throughout the genome affect encapsidation. Several of these mutations are shown in Fig. 5A and B. pd(X-N) contains a deletion between the *Xba*I site of pCT at nt 1469 in *gag* and an *Nhe*I site at nt 7250 in *env*. pB(SVpA) contains a replacement of sequences downstream of the *Bam*HI site at nt 8465 in *env* with sequences containing the SV40 polyadenylation signal. pd(X-N)B(SVpA) has both the deletion present in pd(X-N) and the replacement present in pB(SVpA). pd(N-H) contains a deletion spanning the rre between the *Nhe*I site and the *Hpa*I site at nt 8648 in *env*. The relative encapsidation efficiencies of each of these mutants were determined by an RNase protection assay. We found that all HIV-1 RNA sequences downstream of the *Xba*I site were dispensable for encapsidation with the exception of the sequence between nt 7254 and 8651 within the *env* gene. pd(X-N)B(SVpA) RNA was encapsidated efficiently, while pd(N-H) RNA was not encapsidated efficiently (Fig. 6, Table 2).

The rre is required in most cell types for efficient transport of genomic viral RNA from the nucleus to the cytoplasm (20, 42). Since, the deletion in pd(N-H) removes the rre, we attempted to determine whether the encapsidation defect of pd(N-H) RNA could be attributed solely to the absence of the rre. We created rre-negative mutant pdRRE (Fig. 5B), which

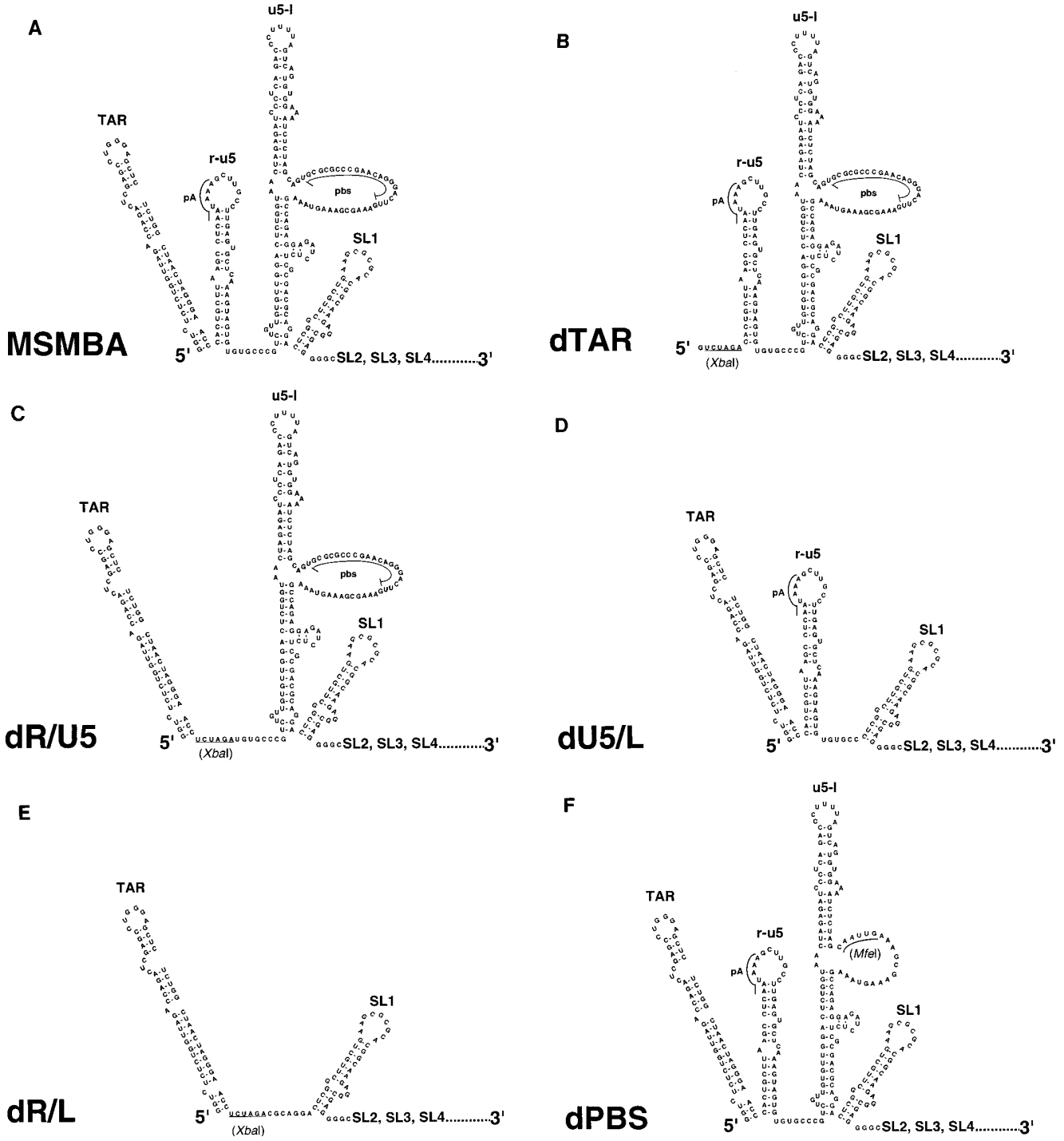

FIG. 3. Organization of the 5′ end of HIV-1 RNA. (A) MSMBA (wild type). The secondary structure shown was generated by using the Mfold-Phylo program available from the University of Wisconsin Genetics Computer Group. Since the pbs ultimately is annealed with the 3′ end of $tRNA_1^{Lys}$, we specified that the pbs remain not base paired in generating the possible secondary structure. The TAR stem-loop, putative r-u5 stem-loop, and putative u5-1 stem-loop are shown. In addition, the pbs, stem-loop 1 (SL1), and the polyadenylation signal (pA) in the 5′ r sequence are shown. While the polyadenylation signal is present in both the 5′ and 3′ r sequences, only the 3′ polyadenylation signal is efficiently utilized (12). (B) dTAR contains an *Xba*I sequence in place of the TAR stem-loop from DNA nt 456 to 511 (RNA nt 2 to 57). (C) dR/U5 contains an *Xba*I sequence in place of the putative r-u5 stem-loop from DNA nt 512 to 558 (RNA nt 58 to 104). (D) The putative u5-1 stem-loop is deleted in dU5/L from DNA nt 565 to 696 (RNA nt 111 to 242). (E) dR/L contains an *Xba*I sequence in place of the putative r-u5 and u5-l stem-loops from DNA nt 512 to 689 (RNA nt 58 to 235). (F) dPBS contains an *Mfe*I sequence in place of the pbs from DNA nt 633 to 656 (RNA nt 179 to 202).

contains a deletion of nt 7762 to 8002 spanning the rre in *env*. In addition, we generated an rre-negative mutant which contains a deletion between the *Xba*I site in *gag* and the *Xho*I site at nt 8887 in *nef* designated pd(X-X) and a derivative of pd(X-X), which contains the *cis*-transport element (CTE) from MPMV located between the same *Xba*I and *Xho*I sites. The CTE can function in place of Rev and the rre to facilitate RNA transport from the nucleus to the cytoplasm (13), but the RNA

TABLE 1. Relative encapsidation efficiencies of vectors containing mutations within the 5′ end of HIV-1 RNA

Vector[a]	Rel. Enc. Eff.[b]
pCT	0.93 ± 0.16
pdTAR	0.26 ± 0.05
pdR/U5	0.11 ± 0.01
pdU5/L	0.04 ± 0.02
pdR/L	0.02 ± 0.01
pdPBS	0.53 ± 0.06
pR/U5	0.10 ± 0.01
pR/U5*	0.11 ± 0.02

[a] All vectors are derivatives of the parental vector, pCT.

[b] The relative encapsidation efficiency (Rel. Enc. Eff.) of each vector was calculated by dividing the ratio of vector RNA to helper RNA in the virion by the ratio of vector RNA to helper RNA in the cytoplasm as determined by RNase protection analysis (Fig. 2). The results represent at least three independent experiments.

secondary structures of the rre and the CTE are not similar (30, 42, 61). The relative encapsidation efficiencies of pdRRE and pd(X-X) were low, while the CTE significantly restored encapsidation (Fig. 7, Table 2). The fact that the CTE restored encapsidation in the absence of the rre indicates that the encapsidation defect of RNA lacking the rre is mainly due to inefficient or aberrant transport of this RNA from the nucleus to the cytoplasm. Moreover, these data indicate that the 5′ segment of the viral genome is sufficient for encapsidation provided that the RNA is transported efficiently and properly to the cytoplasm.

Effect of a foreign sequence on HIV-1 RNA encapsidation. Retroviral vectors are used as tools to transduce foreign sequences into cells. We attempted to determine how the presence of a foreign sequence, the hygromycin B resistance gene, would affect encapsidation of HIV-1 RNA. Starting with a vector, pd(X-N), that lacks most of the viral genome but is encapsidated efficiently (Table 2), we created derivatives of pd(X-N) containing one, two, or four inserts of the hygromycin B resistance gene (pH-1, pH-2, and pH-4, respectively) (Fig. 8). Each of these vectors was cotransfected along with the helper plasmid pMSMBA, and the relative encapsidation efficiency of each of these vectors was determined by RNase protection analysis of cytoplasmic and virion-associated RNA. The presence of one copy of the hygromycin B resistance gene (pH-1) reduced relative encapsidation efficiency moderately (Table 3). However, the presence of two (pH-2) or four (pH-4) copies of the hygromycin B resistance gene reduced relative encapsidation efficiency more profoundly. Since all of these vectors express RNAs that are shorter than genomic RNA, simple exclusion of these RNAs for encapsidation is unlikely.

DISCUSSION

Our data indicate that the 5′ end of HIV-1 RNA extending from the transcription start site to position 1017 within the genomic RNA is sufficient for encapsidation provided that the rre is present to effect efficient nuclear transport. In addition to the region generally viewed as E, the upstream region containing TAR, the r-u5 stem-loop, and the u5-1 stem-loop function in encapsidation. Moreover, the magnitude of the effect of mutations in this upstream region is similar to that of mutations in the SL1 to SL4 region. Thus, for HIV-1, it is probably appropriate to consider the entire 5′ untranslated leader region and the 5′ end of *gag* as E. The exact function of TAR and the two putative stem-loops, r-u5 and u5-1, in encapsidation remains to be determined. Some have shown that this region of HIV-1 RNA is preferentially bound by Gag in vitro (24), but others have shown that Gag preferentially binds a region containing SL1, SL2, SL3, SL4, and sequences within *gag* (8, 9, 15, 39, 57). The TAR stem-loop appears to be a multifunctional element. In addition to its roles in transcription and encapsidation, TAR has recently been shown to be required for reverse transcription (26). To see whether the role of TAR in encapsidation was Tat dependent, we introduced a nonsense mutation at the ninth codon of the *tat* gene. This *tat* mutation was then introduced in parallel into MSMBA and dTAR, each of which also contained a human cytomegalovirus promoter-enhancer in place of the U3 portion of the HIV long terminal repeat. Measurement of the relative encapsidation efficiencies of the RNAs expressed from these constructs indicated that TAR plays a role in encapsidation that is independent of Tat.

The exact portion of *gag* that is required for encapsidation and the nature of the signals within *gag* have not been determined. Luban and Goff found that sequences from nt 335 to 503 within genomic RNA were required for encapsidation (40). Our laboratory found that sequences from nt 389 to 1053 within the genomic RNA enhanced the ability of vectors to be propagated (14). Parolin et al. observed no enhancement in the ability of vectors to be propagated when sequences past RNA nt 990 were included (53). In fact, they found that the ability of these vectors to be propagated was reduced upon the addition of *gag* sequence downstream of RNA nt 990. From their work, it appears that the 3′ end of the HIV-1 encapsidation signal lies between RNA nt 797 and 990 within *gag*.

We observed efficient encapsidation of an HIV-1 vector when the proteins essential for particle formation were provided in *trans*. Other HIV-1 vectors have exhibited inefficient encapsidation. For instance, Luban and Goff created an HIV-1 vector by mutating the Kozak consensus sequence upstream of the *gag* start codon such that translation was inefficient (40). When transfection was performed with a helper plasmid, the

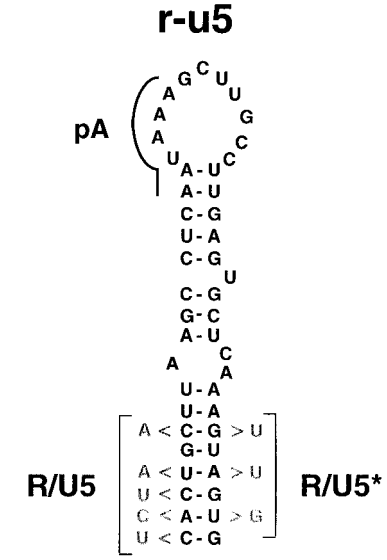

FIG. 4. Base substitutions within the putative r-u5 stem. pR/U5 contains a group of five mutations, three of which disrupt the base pairing within the stem at RNA nt 59, 61, and 63. These mutations correspond to DNA nt 513, 515, and 517, respectively. Base pairing would still occur in the presence of the mutations at RNA nt 58 and 60. pR/U5* contains the same mutations present in pR/U5 in conjunction with a group of three mutations designed to restore base pairing within the stem at RNA nt 99, 101, and 103. These latter mutations correspond to DNA nt 553, 555, and 557, respectively. pA, polyadenylation signal.

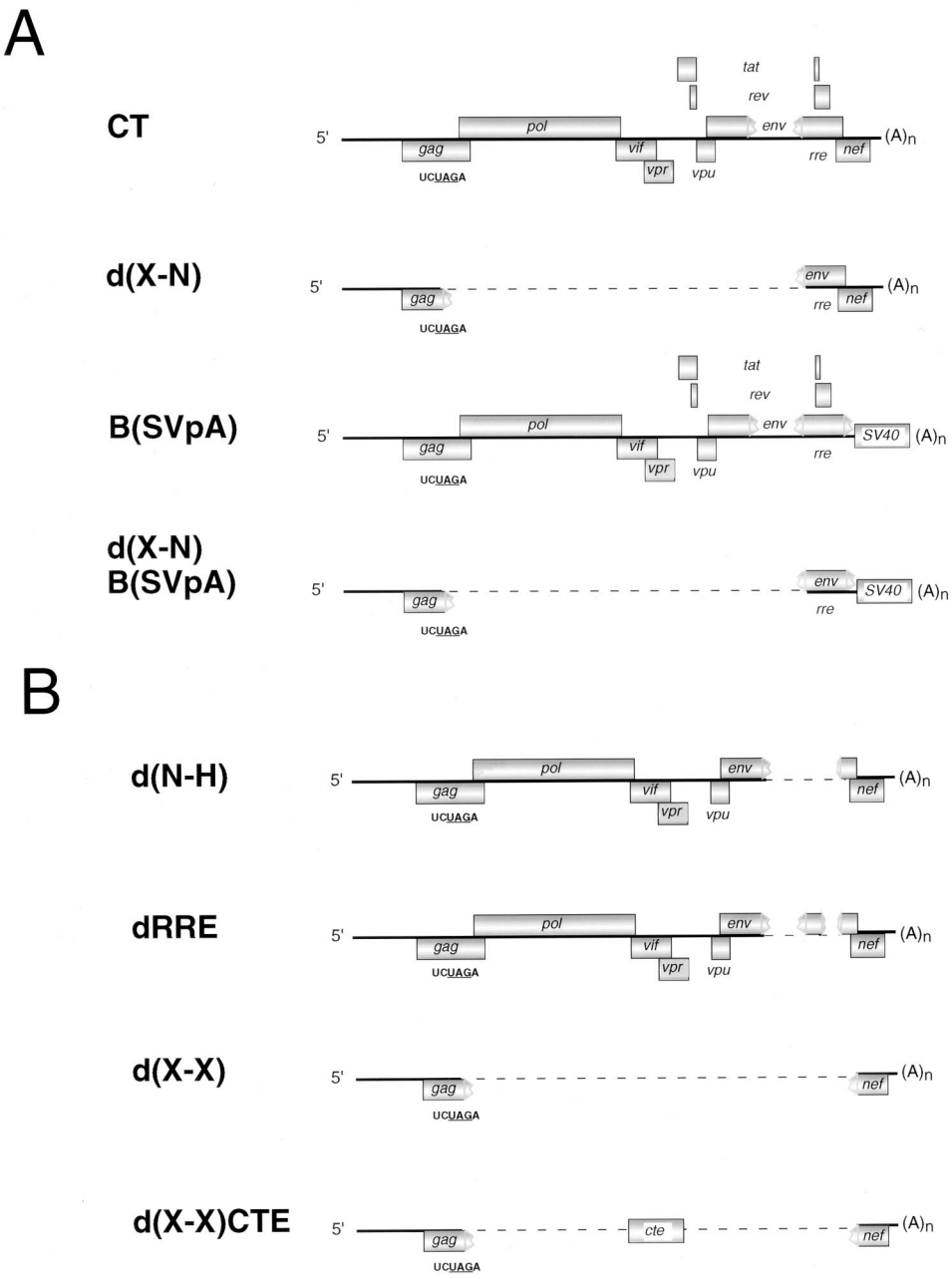

FIG. 5. Mutants containing deletions that eliminate substantial segments of the HIV-1 genome. (A) pd(X-N) has a deletion from the *Xba*I site at DNA nt 1469 (RNA nt 1015) in *gag* to the *Nhe*I site at DNA nt 7250 (RNA nt 6796) in *env*. pB(SVpA) has a replacement of sequences downstream of the *Bam*HI site at DNA nt 8465 (RNA nt 8011) in *env* with sequences spanning the SV40 polyadenylation signal. pd(X-N)B(SVpA) has both the deletion from the *Xba*I site to the *Nhe*I site and the replacement sequences downstream of the *Bam*HI site. (B) Derivatives of pCT lacking the rre. pd(N-H) has a deletion from the *Nhe*I site in *env* to the *Hpa*I site at DNA nt 8648 (RNA nt 8194) in *env*. pdRRE has a deletion from DNA nt 7762 to 8002 (RNA nt 7308 to 7548) spanning the rre. pd(X-X) has a deletion from the *Xba*I site in *gag* to the *Xho*I site at DNA nt 8887 (RNA nt 8433) in *nef*. pd(X-X)CTE contains the MPMV CTE cloned between the *Xba*I site in *gag* and the *Xho*I site in *nef*.

relative encapsidation efficiency of this HIV-1 vector was found to be low, presumably because the mutations created to ablate translation inadvertently affected E. Berkowitz et al. (10) created an HIV-1 vector that expresses most of the sequence that we have defined here as being sufficient for encapsidation. However, that vector lacks 19 nt at the 5′ end of the RNA and is not encapsidated efficiently when expressed in a helper cell line. Our results indicate that the inability of that vector to be encapsidated can probably be attributed to at least two defects. First, since the 19 nt at the 5′ end of the RNA are not present, the vector does not contain the entire TAR stem-loop. We observed that the relative encapsidation efficiency of a vector lacking TAR is approximately 0.26. Second, that vector expresses a foreign gene. We observed that the presence of the hygromycin B resistance gene reduced the relative encapsidation efficiencies of pH-1, pH-2, and pH-4 (Table 3), and it is possible that a similar reduction in the relative encapsidation efficiency may result from the presence of other foreign se-

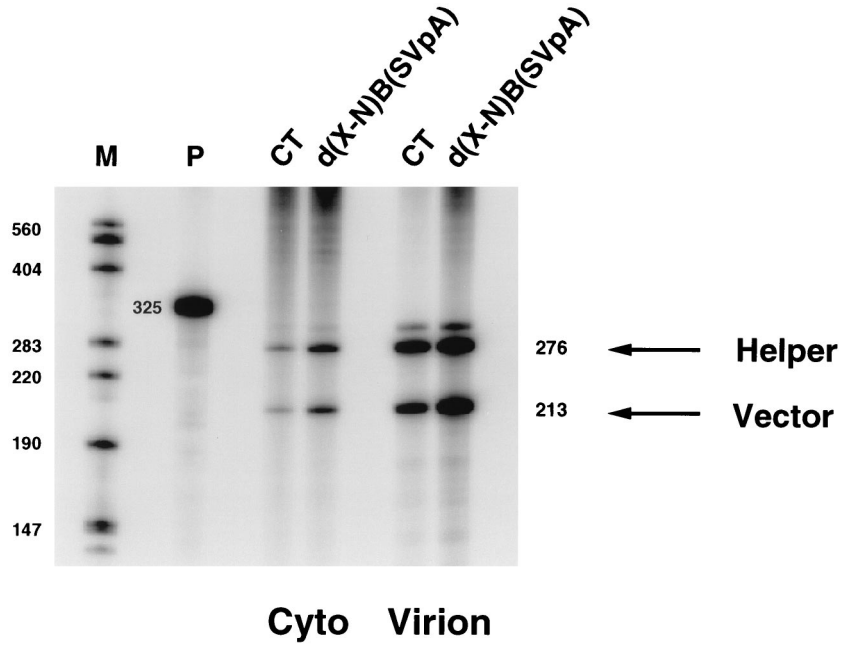

FIG. 6. Sequences within *env* in conjunction with sequences at the 5′ end of HIV-1 RNA are sufficient for encapsidation. 293 cells were cotransfected with pMSMBA and pCT or pd(X-N)B(SVpA). RNA isolated from the cytoplasm (Cyto) of the transfected cells and from virions (Virion) was subjected to RNase protection analysis as described in the legend for Fig. 2 except that the probe used here was generated from pGEM(1247-1523), which contains a portion of *gag* from pMSMBA. The diagnostic bands for helper RNA (276-nt band) and for vector RNA (213-nt band) are reversed from those of Fig. 2. The amount of probe loaded on the gel is 1/50 of the amount of probe used in the experiment.

quences. Interestingly, the helper cell line used by Berkowitz et al. (10) expresses RNA that is not encapsidated, so competition for encapsidation between vector RNA and helper RNA does not occur. The fact that they did not observe encapsidation of their vector implies that *cis* elements, TAR in particular, are required for encapsidation even in the absence of competition by encapsidation-competent RNA.

Although we were unable to genetically demonstrate that the secondary structure of the r-u5 stem-loop is required for encapsidation, its conservation among the primate lentiviruses coupled with the low level of sequence identity throughout this region suggests that the secondary structure is real (6). Among the primate lentiviruses, the only widely conserved sequences throughout the r-u5 stem-loop are the polyadenylation signal and a GCUU tetranucleotide sequence located from RNA nt 62 to 65 within the stem (6). Our pR/U5 and pR/U5* mutants, which are substantially reduced in encapsidation efficiency, contain groups of base substitutions that change the GCUU tetranucleotide sequence. Interestingly, the r-u5 hairpin loop contains a palindromic sequence reminiscent of SL1. A palindromic sequence is also present in the loops of the putative r-u5 stem-loops of strain cpz of simian immunodeficiency virus (SIVcpz) and SIVsyk (6). Recent electron microscopic data and computer modeling indicate that the r-u5 stem-loop may have an important role in RNA-RNA association within the dimer (29).

In contrast to the situation for the r-u5 stem-loop, there is

TABLE 2. Relative encapsidation efficiencies of vectors lacking substantial segments of the HIV-1 genome

Vector[a]	Rel. Enc. Eff.[b]
pCT	0.93 ± 0.16
pd(X-N)	1.15 ± 0.15
pB(SVpA)	0.90 ± 0.08
pd(X-N)B(SVpA)	1.52 ± 0.08
pd(N-H)	0.03 ± 0.01
pdRRE	0.04 ± 0.02
pd(X-X)	0.03 ± 0.02
pd(X-X)CTE	0.51 ± 0.11

[a] All vectors are derivatives of the parental vector, pCT.

[b] The relative encapsidation efficiency (Rel. Enc. Eff.) of each vector was calculated as described for Table 1. The results represent at least three independent experiments.

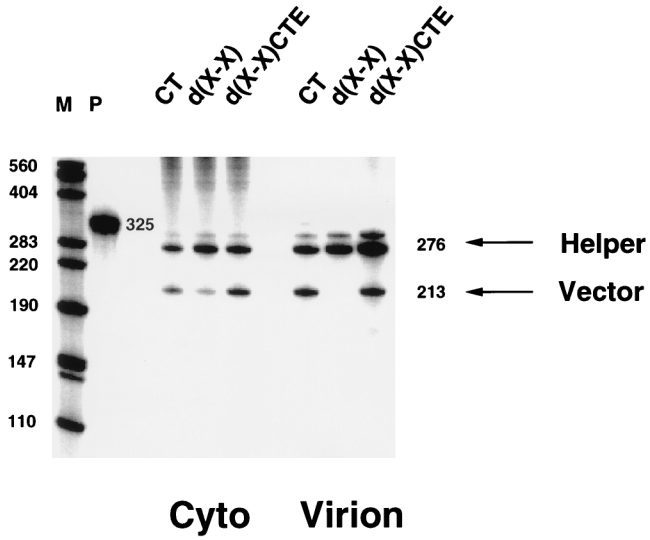

FIG. 7. The MPMV CTE restores encapsidation in the absence of the rre. 293 cells were cotransfected with pMSMBA and pCT, pd(X-X) or pd(X-X)CTE. Cytoplasmic RNA (Cyto) and virion RNA (Virion) were subjected to RNase protection analysis as described in the legend for Fig. 6.

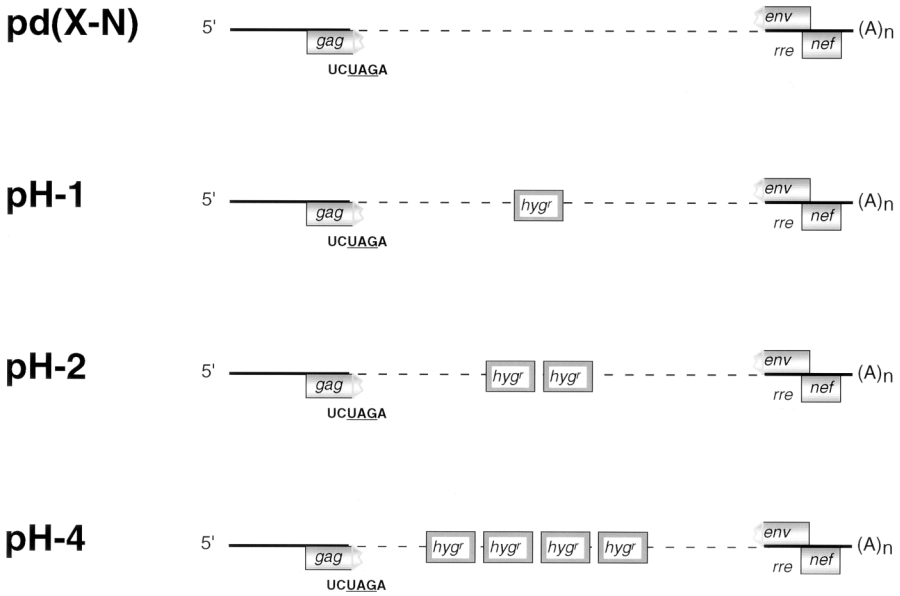

FIG. 8. Derivatives of pd(X-N) containing one, two, or four copies of the hygromycin B resistance gene.

not wide agreement on the secondary structure surrounding the pbs that we have presented here as the u5-1 stem-loop (5, 56). However, at the top of the u5-1 stem-loop there is a subregion called the u5-IR stem-loop, and the secondary structure of this stem-loop appears to be generally conserved among different retroviruses (2). Similarly, sequences within u5 are important for encapsidation of murine leukemia and spleen necrosis virus RNAs (19, 49).

In addition to the pbs, the u5-1 stem-loop contains other *cis* elements that are important for the annealing of the $tRNA_3^{Lys}$ and the initiation of reverse transcription (63, 65). Although the secondary structure of genomic RNA changes upon the annealing of the $tRNA_3^{Lys}$ to genomic RNA in vitro (31), this change in secondary structure may not be relevant to encapsidation. First, the processes of tRNA incorporation into the assembling virus particle and genomic RNA encapsidation may be completely separate, since genomic RNA encapsidation is not required for selective incorporation of tRNAs into the assembling virus particle in murine leukemia or mouse mammary tumor viruses (37, 54). Additionally, the reverse transcriptase domain of Gag-Pol is required for selective incorporation of $tRNA_3^{Lys}$ into the virus particle and annealing (38, 41), but reverse transcriptase is not required for genomic RNA encapsidation (33). Accordingly, we find that relative encapsidation efficiency is only modestly reduced in the absence of the pbs in agreement with a previous report (50). However, it will be interesting to determine if the sequences flanking the pbs that are important in annealing and initiation of reverse transcription are important for genomic RNA encapsidation.

TABLE 3. Relative encapsidation efficiencies of vectors containing the hygromycin B resistance gene

Vector[a]	Rel. Enc. Eff.[b]
pd(X-N)	1.15 ± 0.15
pH-1	0.53 ± 0.15
pH-2	0.24 ± 0.06
pH-4	0.06 ± 0.02

[a] All vectors are derivatives of pd(X-N).

[b] The relative encapsidation efficiency (Rel. Enc. Eff.) of each vector was calculated as described for Table 1. The results represent at least three independent experiments.

Others have suggested that sequences within *env* and spanning the rre may be directly required for encapsidation (34, 55). Our results indicate that the role of these sequences in encapsidation is mainly indirect; they function to enhance the transport of genomic RNA from the nucleus to the cytoplasm where encapsidation occurs. Our rre-negative, CTE-containing RNA could be encapsidated at an efficiency approximately half that of the parental vector RNA, so the rre has a direct effect on encapsidation of twofold at most. It is possible that genomic RNA may have to be transported from the nucleus to the cytoplasm by a specific pathway for optimal encapsidation. Interestingly, genomic and subgenomic RNAs are localized to different subregions within the nucleus (11), and the Rev/rre transport pathway is likely to be different from the mRNA transport pathway (22, 23).

We observed that the presence of a foreign sequence, the hygromycin B resistance gene, has a negative effect on the ability of HIV-1 RNA to be encapsidated. The negative effect of the hygromycin B resistance gene on encapsidation may not be specific but rather may reflect a general reduction in encapsidation of HIV-1 RNA in the presence of any foreign sequence. It will be interesting to determine whether other heterologous sequences have a similar negative effect on encapsidation and whether placing direct repeats within a vector decreases encapsidation efficiency. Since the 5′ end of HIV-1 RNA is sufficient for encapsidation, it is interesting that replacing a nonessential viral sequence with a foreign sequence can have a negative effect on encapsidation. Obviously, the foreign sequence does not behave in a neutral way; it may be that the foreign sequence influences the secondary structure of the encapsidation signal at the 5′ end of the RNA or impinges on other viral processes that occur prior to encapsidation.

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