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Distinct RNA Sequences in the *gag* Region of Human Immunodeficiency Virus Type 1 Decrease RNA Stability and Inhibit Expression in the Absence of Rev Protein

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The expression of Gag, Pol, Vif, Vpr, Vpu, and Env proteins from unspliced and partially spliced human immunodeficiency virus type 1 (HIV-1) mRNAs depends on the viral protein Rev, while the production of Tat, Rev, and Nef from multiply spliced mRNAs does not require Rev. To investigate the difference between *gag* and *tat* mRNAs, we generated plasmids expressing *tat-gag* hybrid mRNAs. Insertion of the *gag* gene downstream of the *tat* open reading frame in the *tat* cDNA resulted in the inhibition of Tat production. This inhibition was caused, at least in part, by a decrease in the stability of the produced mRNA. Deletions in *gag* defined a 218-nucleotide inhibitory sequence named INS-1 and located at the 5′ end of the *gag* gene. Further experiments indicated the presence of more than one inhibitory sequence in the *gag*-protease gene region of the viral genome. The inhibitory effect of INS-1 was counteracted by the positive effect mediated by the Rev–Rev-responsive element interaction, indicating that this sequence is important for Rev-regulated *gag* expression. The INS-1 sequence did not contain any known HIV-1 splice sites and acted independently of splicing. It was found to have an unusually high AU content (61.5% AU), a common feature among cellular mRNAs with short half-lives. These results suggest that HIV-1 and possibly other lentiviruses have evolved to express unstable mRNAs which require additional regulatory factors for their expression. This strategy may offer the virus several advantages, including the ability to enter a state of low or latent expression in the host.

The genome of human immunodeficiency virus type 1 (HIV-1) contains the *gag*, *pol*, and *env* genes common to all retroviruses. In addition to these genes, the HIV-1 genome contains several open reading frames (ORFs) encoding the accessory and regulatory proteins Tat, Rev, Nef, Vpu, Vif, and Vpr. The expression of all of these ORFs from one promoter is accomplished primarily by the generation of alternatively spliced mRNAs grouped into three size classes: full length (9 kb), intermediate (4 to 5 kb), and small, multiply spliced (2 kb). The full-length and intermediate mRNAs encoding Gag, Pol, Env, Vpu, Vif, Vpr, and Tat-1 (21, 38, 51, 52) contain the *cis*-acting Rev-responsive element (RRE) (15, 16, 19, 25, 26, 36, 47). The expression of the full-length and intermediate mRNAs depends on the viral Rev protein (15, 17, 21, 25, 51, 52, 57). In the absence of Rev, most of the full-length and intermediate mRNAs are further spliced, generating the class of small, multiply spliced mRNAs encoding Tat, Rev, and Nef (15, 17, 18, 25, 51, 52). These mRNAs lack the RRE and are the only HIV-1 mRNAs that can be expressed efficiently in the absence of Rev.

The Rev protein acts posttranscriptionally to increase the transport and utilization of all viral mRNAs that contain the RRE (4, 16, 19, 25, 26, 36). Rev binds to RRE (5, 12–14, 27, 35, 41, 66). Several lines of evidence from in vivo experiments indicate that the Rev-RRE interaction, although necessary, is not sufficient for the activation of RRE-containing mRNAs and that additional cellular factors interacting with Rev are necessary for Rev function (1, 5). The mechanism of Rev function has been the subject of intense study. It has been shown that Rev increases the half-life of RRE-containing HIV-1 mRNAs (19), promotes their transport from the nucleus to the cytoplasm (16, 19, 26, 36), and promotes their efficient translation (2, 4, 5, 25, 57).

Experiments with constructs expressing mutant *gag* and *env* mRNAs lacking splice sites showed that only low levels of these mRNAs accumulate in the absence of Rev and that their expression is Rev dependent (19, 31, 36). These results led to the conclusion that Rev acts independently of splicing (19, 36). The inability of *gag* and *env* mRNAs to be efficiently expressed in the absence of Rev led to the prediction that they contain sequences other than splice sites which inhibit their expression (16, 19, 25, 47). It was shown that *env* sequences lower the levels of chloramphenicol acetyltransferase (CAT) produced by CAT-HIV hybrid mRNAs (47). It was proposed that this inhibitory effect is mediated by sequences named *cis*-acting repressive sequences within the *env* region. The nature and mode of action of these sequences have remained unclear. The presence of inhibitory sequences that affect the accumulation and utilization of *gag* and *env* mRNAs was also proposed on the basis of measurements of viral mRNAs and proteins (17, 19, 25, 42, 57). Although it was suggested that these sequences affect the stability and utilization of viral mRNAs, the mechanisms by which they mediate the decreased levels of *gag* and *env* expression are not understood.

Examination of the effects of Rev on the localization and splicing of hybrid globin-RRE mRNAs led to the conclusion that splice sites are necessary for Rev regulation (9). It was proposed that regulation by Rev involves the dissociation of splicing components and pre-mRNA. It was also suggested that complex formation between U1 small nuclear RNA and *env* mRNA is necessary for the formation of unspliced

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mRNA that is subject to regulation by Rev (34). In view of these results, the question arises as to whether the postulated inhibitory sequences contain elements recognized by the splicing machinery or whether additional, splicing-independent mechanisms inhibit viral mRNA transport and stability.

We designed experiments to identify inhibitory sequences within the HIV-1 genome and to study their function in detail. Here we report the identification and characterization of an inhibitory sequence in the HIV-1 *gag* gene. On the basis of the characterization, we have named this sequence INS-1. The relationship of INS-1 to *cis*-acting repressive sequences is not clear and will require additional characterization. The INS-1 element does not contain any functional splice sites and acts in *cis* by lowering steady-state mRNA levels. Thus, INS-1 appears to function, at least in part, at the level of mRNA stability. This effect could be overcome by the Rev-RRE interaction. Therefore, sequences other than splice sites are important for regulation by the HIV-1 Rev protein. Our experiments suggest that mechanisms affecting mRNA stability are important for the regulation of HIV-1 and possibly other complex retroviruses.

MATERIALS AND METHODS

Recombinant plasmids. The following plasmids have been described previously: pNL1.4.7 (50) (see Fig. 1B), pHCMVs-rev (5), and pHCMV-SEAP (6). To generate RRE- and *gag*-containing *tat* cDNAs, we cloned the HIV-1 RRE upstream of the HIV-1 *gag* gene in the polylinker of pBluescript KS(−) (Stratagene). The 330-bp RRE fragment from p330 (58) was cloned into the *Eco*RI and *Bam*HI sites in pBluescript KS(−). The resulting plasmid was opened at the *Bam*HI site and ligated to the *Bss*HII-*Apa*I *gag* fragment from pMcgag (19), which was derived from the HIV-1 molecular clone HXB2. The *gag* fragment has a deletion between nucleotides (nt) 257 and 330 which removes the major 5′ splice site at nt 289 and the proposed packaging signal upstream of the 5′ splice site (10, 33). The resulting plasmid contained the 330-bp RRE upstream of *gag* in the pBluescript KS(−) polylinker and was used to generate the various RRE-*gag* fragments that were cloned into the *tat* cDNA expression plasmid pNL1.4.7. RRE-*gag* fragments were generated by restriction enzyme digestion or by polymerase chain reaction (PCR) (48, 49) with one oligonucleotide primer at the 5′ end of the RRE and the other at various locations in the *gag* gene. The various RRE-*gag* fragments were cloned into the *Bam*HI site of pNL1.4.7 (50), resulting in pTRG(330-1552), pTRG(330-1397), pTRG(330-1174), pTRG (330-1053), pTRG(330-961), pTRG(330-691), pTRG(330-631), pTRG(330-480), pTRG(330-413), and pTRG(330-354). The numbering of the revised HXB2 sequence starts at the first nucleotide of the R region in the 5′ long terminal repeat (LTR) (39, 45, 46, 58). The numbers in parentheses indicate the 5′ and 3′ positions of the *gag* fragment contained in each plasmid. To generate deletions in the 5′ end of the *gag* fragment, we digested plasmid pTRG(330-691) with *Bam*HI and religated it. This procedure removed the *gag* fragment and resulted in pTR, which contained a unique *Bam*HI site immediately downstream of the RRE. The various 5′ *gag* deletions of the *gag* fragment from nt 330 to 691 were generated by PCR and subcloned directly into the *Bam*HI site of pTR. This procedure resulted in pTRG(355-631), pTRG(414-631), and pTRG(481-631). pTG(330-1552) was generated by subcloning of the *Bss*HII-*Apa*I *gag* fragment from pMcgag (19) into the *Bam*HI site of pNL1.4.7 (50). pTG(961-1552) was constructed by excising the 5′ end of *gag* in pTG(330-1552) with *Bss*HII and *Pst*I. To construct pNL17R, we PCR amplified the $p17^{gag}$ coding sequence from pMcgag and subsequently cloned it into the *Eco*RV site of the pBluescript KS(−) vector. A premature stop codon was introduced immediately after the last codon of $p17^{gag}$ through one of the PCR primers. The *gag* fragment does not contain the major HIV-1 splice donor. The 330-nt RRE fragment described above was subcloned into the *Hin*dIII site downstream of $p17^{gag}$, resulting in pBS17R. $p17^{gag}$ and RRE were excised as one fragment by digestion of pBS17R with *Bss*HII and *Bam*HI. The *Bss*HII-*Bam*HI fragment from pBS17R was subcloned into *Bss*HII-*Bam*HI-digested plasmid pNL1.4.7, resulting in the eucaryotic expression plasmid pNL17R (see Fig. 6A).

Cells and transfections. HL3T1 cells contain silent copies of the HIV-1 LTR promoter linked to the CAT gene (65). HLtat cells constitutively express the truncated, but functional, Tat-1 protein (50). The various amounts of plasmids used for transfection were adjusted to 17 μg/0.5 ml of precipitate per 60-mm plate (approximately 10^6 cells) with pUC19 carrier DNA and were transfected by the calcium phosphate coprecipitation technique (23) as described previously (19, 20). One microgram of *tat-gag* plasmid was used for each transfection unless indicated otherwise. This amount usually resulted in 20 to 40% transfected cells, as determined by immunofluorescence. Cells were harvested 20 h posttransfection. To quantitate Tat production from all Tat-expressing plasmids, we determined the amount of plasmid that resulted in a CAT response in the linear range of the assay. All Tat-expressing plasmids were serially diluted and transfected into HL3T1 cells to determine the amount of plasmid that resulted in a CAT response in the linear range of the assay. The CAT values were normalized to the amount of plasmid used for transfection. Cotransfection with a plasmid (pHCMV-SEAP) producing secreted human placental alkaline phosphatase (SEAP) (6) was used as a control for transfection efficiency. Comparable levels of SEAP were produced in the different transfections.

RNA extractions and Northern (RNA) blotting. Total RNA was extracted 1 day posttransfection by the heparin-DNase procedure (32). Nuclear and cytoplasmic fractions were separated as described by Greenberg and Ziff (24), and the RNA was prepared by the heparin-DNase procedure (32). The filters were hybridized to a gel-purified, nick-translated, PCR-amplified DNA fragment spanning nt 8304 to 9008 at the 3′ end of the HXB2 genome. Measurements of RNA stability were performed by the addition of actinomycin D (final concentration, 10 μg/ml) to the culture medium of transfected HL3T1 cells 20 h posttransfection. Total RNA was harvested at different times after actinomycin D treatment. The RNA blots were quantitated with the AMBIS radioanalytic imaging system.

Immunoprecipitations, Western immunoblotting, and CAT assays. At day 1 posttransfection, HLtat cells (50) were metabolically labeled with [^{35}S]cysteine for 3 h. The cells were lysed in 0.5× RIPA buffer, immunoprecipitated with anti-Tat antiserum, and analyzed by electrophoresis on 15% denaturing polyacrylamide gels as described previously (18). For Western immunoblotting, transfected cells were lysed in 0.5× RIPA buffer and subjected to electrophoresis on 15% denaturing polyacrylamide gels as described previously (25). The blots were hybridized to HIV-1 patient serum, and the proteins were visualized with the enhanced chemiluminescence Western blotting system (Amersham). CAT assays were performed as described previously (22).

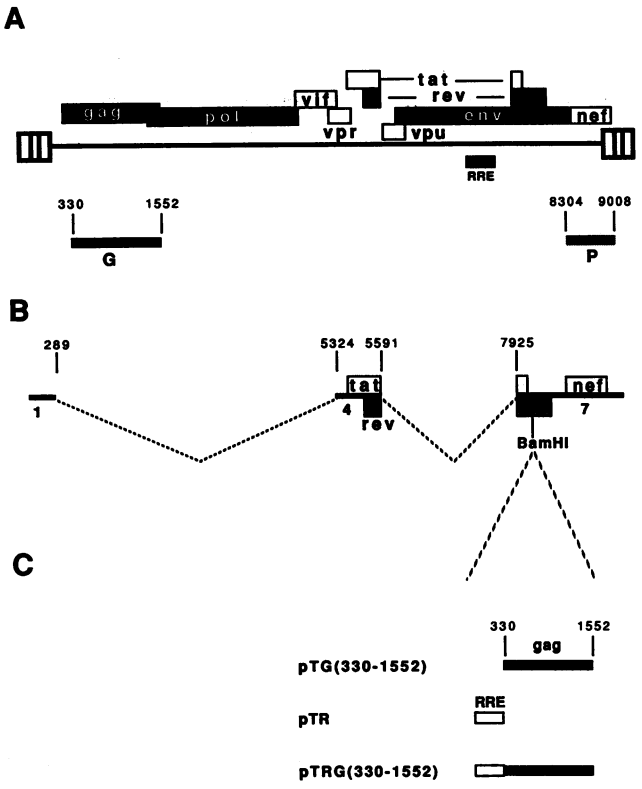


FIG. 1. (A) Structure of the HIV-1 genome. Open, shaded, or black bars indicate ORFs. The RRE is indicated. The black bar marked G indicates the *gag* fragment that is present in pTG(330-1552). The shaded bar marked P indicates the probe used for the Northern blot analysis. (B) Structure of HIV-1 *tat* mRNA 1.4.7. The nucleotide positions of the exons in *tat* mRNA are indicated. The numbering of the revised sequence of the HIV-1 molecular clone HXB2 used in our experiments starts at the first nucleotide of the R region in the 5′ LTR (39, 45, 46, 58). The exons are named as described by Muesing et al. (38) and Schwartz et al. (50, 51). The coding regions for *tat*, *rev*, and *nef* are indicated. The location of the *Bam*HI restriction site into which the various RRE, *gag*, or RRE-*gag* fragments were subcloned is indicated. (C) Fragments that were cloned into *tat* cDNA pNL1.4.7 to generate plasmids pTR, pTG (330-1552), and pTRG(330-1552). Open bars indicate the 330-nt *Sty*I fragment containing the RRE (58), and black bars indicate the *gag* fragment spanning nt 330 to 1552.

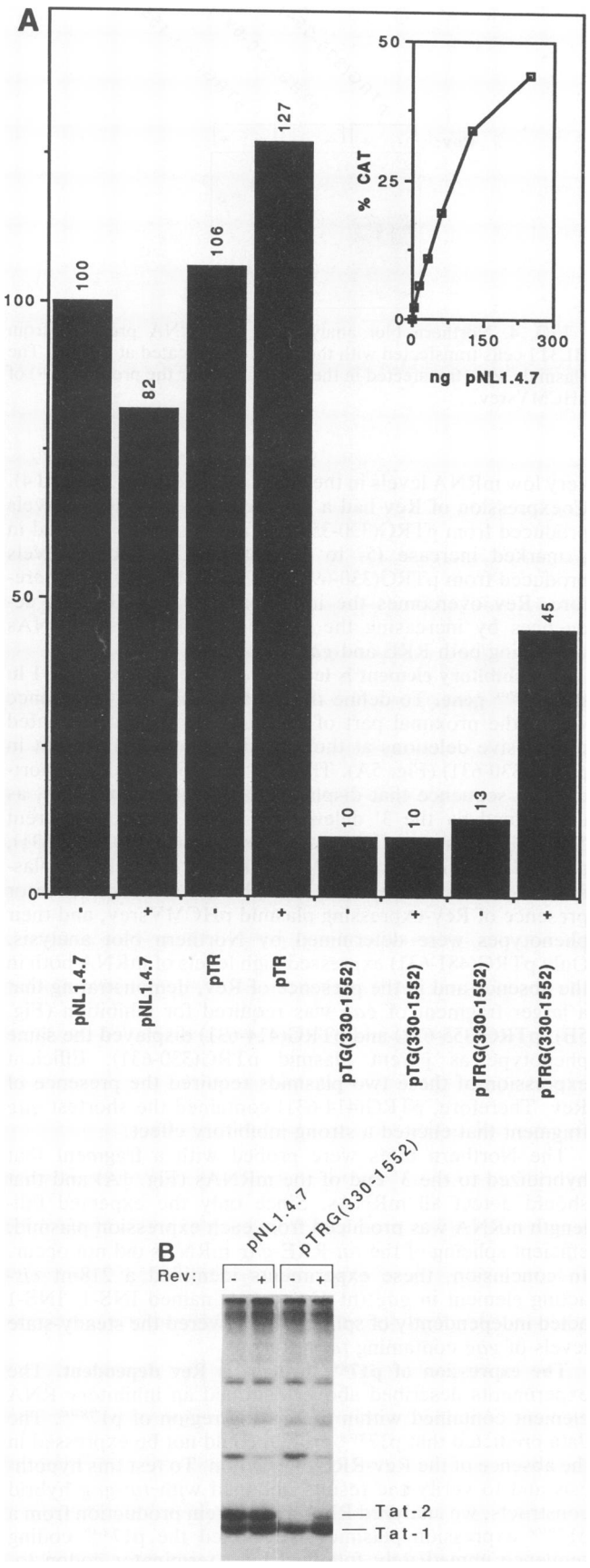
RESULTS

Insertion of *gag* sequences in *tat* mRNA inhibits the production of Tat. Studies of the expression of HIV-1 *gag* have shown that *gag* cannot be expressed in the absence of Rev (17, 19, 25, 57). In contrast, multiply spliced mRNAs, such as *tat* mRNA 1.4.7 (Fig. 1), are expressed efficiently in the absence of Rev. To determine whether *gag* sequences have a dominant inhibitory effect on the expression of *tat* mRNA, we generated plasmids expressing *tat-gag* and *tat*-RRE-*gag* hybrid mRNAs. Three different derivatives of *tat* cDNA pNL1.4.7 were constructed and contained *gag*, RRE, or both RRE and *gag* sequences inserted at the *Bam*HI site downstream of the *tat* ORF. These plasmids were named pTG(330-1552), pTR, and pTRG(330-1552), respectively, and are shown in Fig. 1. The numbers in parentheses indicate the *gag* sequences inserted in the construct. The *gag* fragment was inserted downstream of the *tat* ORF in these plasmids. The *gag* ORF cannot be translated, since the *tat* AUG has a strong consensus sequence for translational initiation which efficiently blocks the expression of downstream ORFs (50). The RRE was inserted upstream of the *gag* fragment. The exact position of these elements in relation to each other was not found to be important (see also Fig. 6, pNL17R). Several investigators have shown that the RRE retains activity when placed at different locations within the transcription unit (19, 36). Tat production from these plasmids was analyzed and quantitated by transfection into HL3T1 cells, which contain silent copies of the HIV-1 LTR promoter linked to the CAT gene. Upon transfection with a Tat-producing plasmid, such as *tat* cDNA pNL1.4.7, CAT expression was activated in a dose-dependent manner (Fig. 2A, inset). Therefore, under these conditions, the levels of CAT activity reflect the amounts of Tat protein.

To assess Tat production from pTG(330-1552), pTR, and pTRG(330-1552), we transfected these plasmids into HL3T1 cells in the absence or presence of Rev-expressing plasmid pHCMVsrev (5) and determined the levels of CAT activation (Fig. 2A). Tat production was compared with that obtained from wild-type *tat* cDNA pNL1.4.7. This plasmid expressed high levels of Tat and was not affected by the presence of Rev. In the experiment shown in Fig. 2A, pTR expressed Tat at almost the same levels as pNL1.4.7, although a small inhibitory effect of the RRE could be observed in several other experiments. Coexpression of Rev increased Tat production from pTR slightly, further indicating that the RRE exerted a small inhibitory effect on Tat expression under these conditions. Interestingly, Tat expression from pTG(330-1552) was 10-fold lower than from pNL1.4.7, indicating that the presence of *gag* sequences substantially inhibited Tat expression. Rev did not affect Tat expression from the former plasmid, since it did not contain the RRE. A similar inhibitory effect on Tat production was observed when pTRG(330-1552), containing both *gag* and RRE sequences, was transfected into HL3T1 cells. Tat expression from this plasmid was elevated in the presence of Rev, demonstrating that the inhibitory effect exerted by the *gag* sequences could be at least partially overcome by Rev.

To verify that CAT protein levels reflected the levels of Tat protein, we also analyzed the production of Tat from pNL1.4.7 and pTRG(330-1552) directly by immunoprecipitation of Tat. pNL1.4.7 and pTRG(330-1552) were transfected into HLtat cells, a HeLa-derived cell line constitutively producing the functional truncated one-exon Tat protein Tat-1. This 14-kDa protein could be distinguished in size from the complete 16-kDa Tat protein (Tat-2) produced by *tat* cDNA pNL1.4.7 and its derivatives. Radioimmunoprecipitation with anti-Tat antiserum demonstrated that pNL1.4.7 expressed high levels of Tat both in the absence and in the presence of Rev (Fig. 2B). Tat production from pTRG(330-1552) was undetectable in the absence of Rev, while cotransfection with Rev-producing pHCMVsrev resulted in high levels of Tat. The results of these experiments were in agreement with the CAT measurements (Fig. 2A), verifying that the expression of Tat from pTRG(330-1552) could be substantially increased by the Rev-RRE interaction.

Deletion mutant construction and analysis. To define the location of inhibitory sequences in *gag* more precisely, we generated a series of progressive deletions in the 3′ end of the *gag* fragment in pTRG(330-1552) (Fig. 3A). Tat production from each mutant was assessed following transfection into HL3T1 cells. Cotransfection with a plasmid (pHCMV-SEAP) producing SEAP (6) was used as a control for transfection efficiency. The results of the CAT measure-



ments are shown in Fig. 3B. The plasmid containing the smallest *gag* fragment that resulted in a significant inhibitory effect was pTRG(330-631). There was a gradual decrease in inhibition when smaller *gag* fragments were used (see below and Fig. 3C). These results defined the 3′ border of the inhibitory sequence of *gag* as nt 631.

***gag* sequences inhibit Tat expression by decreasing the steady-state levels of *tat* mRNAs.** To investigate the mechanism of function of the *gag* inhibitory sequences, we also studied the expression of the various plasmids at the RNA level. Northern blot analysis demonstrated that *gag*-containing *tat* cDNAs that expressed low levels of Tat protein also expressed low levels of mRNA (Fig. 3C). Quantitation of the mRNA levels revealed that low Tat production could be explained to a large extent by low mRNA levels. The RNA results also defined the 3′ border of the inhibitory sequence as nt 631, in agreement with the results of the protein measurements presented in Fig. 3B.

To investigate whether other inhibitory sequences were present in the 3′ end of the *gag* gene, we deleted the sequences from nt 330 to 961 in pTG(330-1552), resulting in pTG(961-1552) (Fig. 3A). Analysis of the expression of protein and RNA from pTG(961-1552) revealed that higher protein and RNA levels were detected with this plasmid than with pTRG(330-1552) (Fig. 3B and C), demonstrating that some of the inhibitory sequences had been deleted. However, pTG(961-1552) produced lower protein and mRNA levels than did *tat* cDNA pNL1.4.7 (Fig. 3B and C), indicating that additional sequences in the 3′ end of *gag* also affected mRNA levels. Taken together, these results demonstrated that a strong inhibitory sequence was located between nt 330 and 631 and that an independent but weaker inhibitory sequence was present in the 3′ end of *gag*, between nt 961 and 1552.

To determine whether the inhibitory effect of *gag* was dependent on the HIV-1 promoter, we inserted the *tat-gag* DNA fragment TRG(330-691) 3′ to the human cytomegalovirus promoter. The resulting plasmids produced only very low levels of *tat-gag* mRNA in the absence of Rev, while cotransfection with Rev resulted in an increase in the mRNA levels (data not shown), demonstrating that the INS-1 element acted independently of the promoter.

We next investigated the effect of Rev on the levels of RRE- and *gag*-containing *tat* mRNAs. RNA production from three plasmids, pTRG(330-354), pTRG(330-691), and pTRG(330-1552), which contain *gag* fragments of different lengths, was quantitated as described in Materials and Methods in the absence or presence of Rev-expressing plasmid pHCMVsrev (Fig. 4). pTRG(330-354) produced high levels of RNA while pTRG(330-691) and pTRG(330-1552) produced

FIG. 2. (A) CAT activity measured after transfections of HL3T1 cells with the various plasmids indicated at the bottom of the histogram. Each plasmid was transfected in the absence (−) or the presence (+) of Rev-expressing plasmid pHCMVsrev. CAT activity measured after transfection with pNL1.4.7 was set at 100%. (Inset) Titration experiments verified that the CAT response was proportional to the level of Tat-producing plasmid. Cells were transfected with the indicated concentrations of pNL1.4.7, and the produced CAT protein was quantitated. (B) Immunoprecipitations of Tat protein from cells transfected with pNL1.4.7 or pTRG(330-1552) in the absence (−) or the presence (+) of Rev. Tat-2 is the complete 16-kDa Tat protein produced from pNL1.4.7 and pTRG(330-1552). Tat-1 is the truncated 14-kDa Tat protein constitutively produced by HLtat cells.

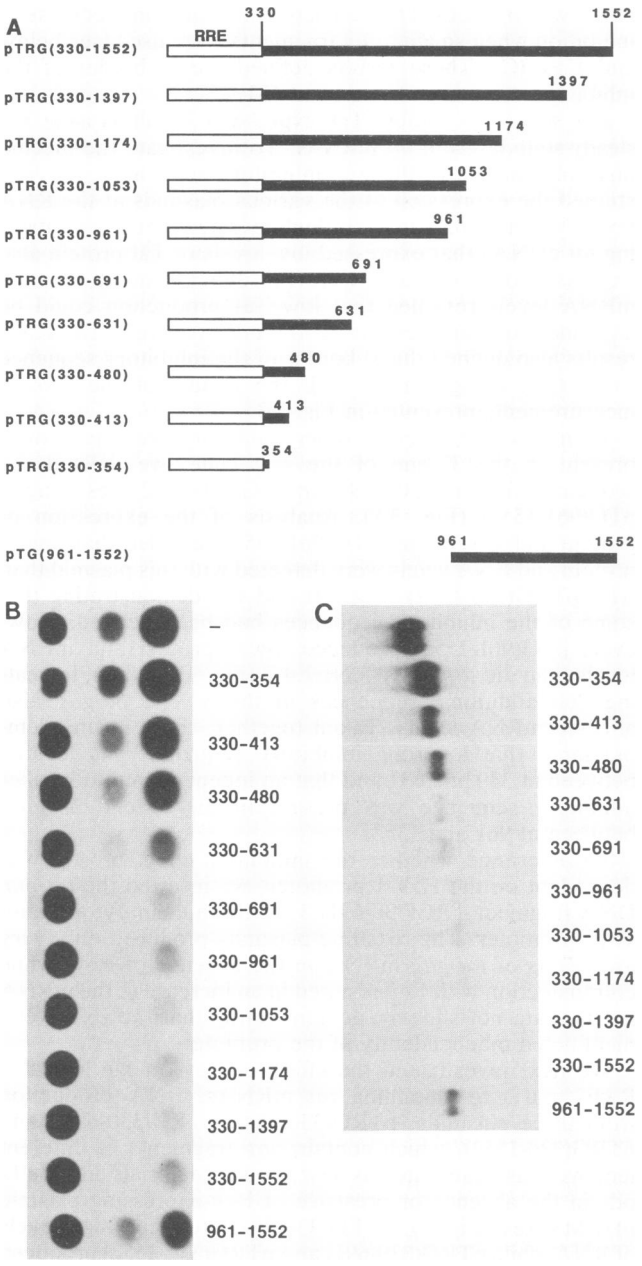


FIG. 3. (A) Schematic structures of the RRE-*gag* fragments containing different deletions at the 3′ end of *gag*. The names of the plasmids are shown to the left. Open bars indicate the 330-nt *Sty*I fragment containing the RRE, and black bars indicate the *gag* fragments. The nucleotide positions of the 3′ ends of the *gag* fragments are indicated. (B) CAT activity in extracts of HL3T1 cells transfected with the different plasmids indicated in panel A. The numbers indicate the *gag* sequences contained in each transfected plasmid. The minus sign indicates transfection with *tat* cDNA pNL1.4.7, which did not contain any *gag* sequences. (C) Northern blot analysis of total RNA prepared from HL3T1 cells transfected with the different plasmids shown in panel A. The numbers indicate the *gag* sequences contained in each transfected plasmid. The minus sign indicates transfection with *tat* cDNA pNL1.4.7, which did not contain any *gag* sequences.

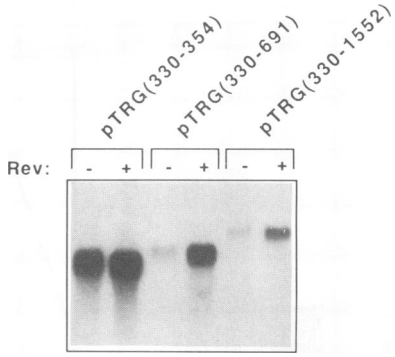


FIG. 4. Northern blot analysis of total RNA prepared from HL3T1 cells transfected with the plasmids indicated at the top. The plasmids were transfected in the absence (−) or the presence (+) of pHCMVsrev.

very low mRNA levels in the absence of Rev (Fig. 3C and 4). Coexpression of Rev had a small effect on the RNA levels produced from pTRG(330-354). In contrast, Rev resulted in a marked increase (5- to 10-fold) in the mRNA levels produced from pTRG(330-691) and pTRG(330-1552). Therefore, Rev overcomes the inhibitory effect of the *gag* sequences by increasing the steady-state levels of mRNAs containing both RRE and *gag* sequences.

An inhibitory element is located between nt 414 and 631 in the p17^{gag} gene. To define the shortest inhibitory sequence within the proximal part of the *gag* gene, we constructed progressive deletions at the 5′ end of the *gag* fragment in pTRG(330-631) (Fig. 5A). This plasmid contained the shortest *gag* sequence that displayed a full inhibitory effect, as determined by the 3′ deletions (Fig. 3C). Three different 5′-end deletions were generated, resulting in pTRG(355-631), pTRG(414-631), and pTRG(481-631) (Fig. 5A). These plasmids were transfected into HL3T1 cells in the absence or presence of Rev-expressing plasmid pHCMVsrev, and their phenotypes were determined by Northern blot analysis. Only pTRG(481-631) expressed high levels of mRNA both in the absence and in the presence of Rev, demonstrating that a larger fragment of *gag* was required for inhibition (Fig. 5B). pTRG(355-631) and pTRG(414-631) displayed the same phenotype as parent plasmid pTRG(330-631). Efficient expression of these two plasmids required the presence of Rev. Therefore, pTRG(414-631) contained the shortest *gag* fragment that elicited a strong inhibitory effect.

The Northern blots were probed with a fragment that hybridized to the 3′ end of the mRNAs (Fig. 1A) and that should detect all mRNAs. Since only the expected full-length mRNA was produced from each expression plasmid, efficient splicing of the *tat*-RRE-*gag* mRNAs did not occur. In conclusion, these experiments identified a 218-nt *cis*-acting element in *gag* (nt 414 to 631) named INS-1. INS-1 acted independently of splicing and lowered the steady-state levels of *gag*-containing *tat* mRNAs.

The expression of p17^{gag} protein is Rev dependent. The experiments described above identified an inhibitory RNA element contained within the coding region of p17^{gag}. The data predicted that p17^{gag} protein could not be expressed in the absence of the Rev-RRE interaction. To test this hypothesis and to verify the results obtained with *tat-gag* hybrid constructs, we analyzed RNA and protein production from a p17^{gag} expression plasmid. We cloned the p17^{gag} coding sequence immediately followed by a terminator codon to-

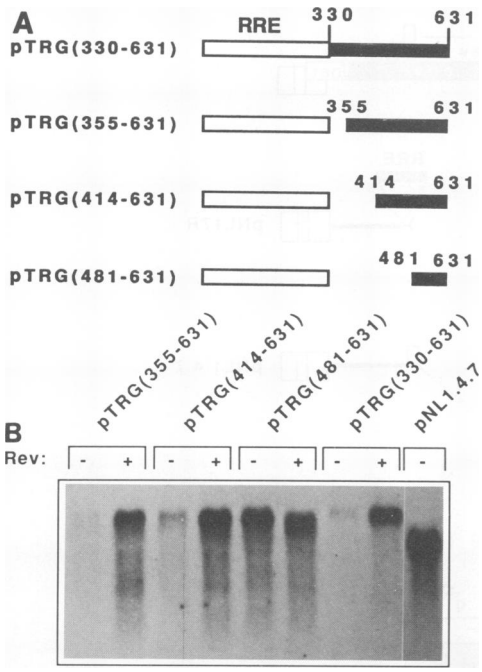


FIG. 5. (A) Structures of the RRE-*gag* fragments containing different deletions at the 5′ end of the *gag* fragment present in plasmid pTRG(330-631). The names of the plasmids are shown to the left. Open bars indicate the 330-nt RRE fragment, and black bars indicate *gag* sequences. The *gag* region nucleotides included in each plasmid are indicated. (B) Northern blot analysis of total RNA prepared from HL3T1 cells transfected with the plasmids indicated at the top. The *tat-gag* plasmids containing the various *gag* deletions shown in panel A were transfected into HL3T1 cells in the absence (−) or the presence (+) of Rev.

gether with a 330-nt RRE fragment downstream of the HIV-1 LTR promoter. This procedure resulted in plasmid pNL17R (Fig. 6A). This plasmid was transfected into HLtat cells in the absence or presence of plasmid pHCMVsrev. RNA analysis of the transfected cells revealed that very low levels of $p17^{gag}$ mRNA were produced in the absence of Rev, while high mRNA levels were detected in the presence of Rev (Fig. 6B). The levels of $p17^{gag}$ mRNA produced in the presence of Rev were comparable to those produced by *tat* cDNA pNL1.4.7. To assess the production of $p17^{gag}$ from pNL17R, we subjected extracts of transfected cells to Western blot analysis. Very low levels of $p17^{gag}$ were detected in the absence of Rev, while cotransfection with pHCMVsrev resulted in dramatically increased levels of $p17^{gag}$ (Fig. 6C). Deletion of RRE from pNL17R did not alter the expression in the absence of Rev, demonstrating that the RRE did not negatively affect $p17^{gag}$ expression (data not shown).

These results verified that an inhibitory element is located in the coding region of $p17^{gag}$ and that this element acts by decreasing the steady-state levels of mRNA. The negative effect on *gag* expression exerted by INS-1 could be overcome by the Rev-RRE interaction, demonstrating that the expression of $p17^{gag}$ is Rev dependent.

Inhibitory element INS-1 affects the RNA levels in the nucleus. The experiments described above indicated that the decrease in Tat protein expression caused by INS-1 could be accounted for by the low mRNA levels. We next investigated whether the differences between total *tat-gag* and *tat* mRNA levels reflected differences in the nuclear pools of transfected cells. Nuclear and cytoplasmic RNA fractions were prepared from HL3T1 cells cotransfected with pNL1.4.7 and pTRG(330-961) and analyzed by Northern blotting. *tat-gag* mRNAs were found in lower levels than *tat* mRNA, both in the nucleus and in the cytoplasm of transfected cells (Fig. 7). The low levels of mRNA TRG(330-961) in the nucleus could only be detected by longer exposures of the blots. These results indicated that the INS-1 element decreased the levels of RNA already in the nucleus.

The INS-1 element decreases the half-lives of *gag*-containing RNAs. To further study the effect of the INS-1 element on RNA levels, we measured the stability of *gag*-containing *tat* mRNAs. HL3T1 cells cotransfected with pNL1.4.7 and pTRG(330-961) were treated with actinomycin D to block transcription, and total RNA was analyzed at various times. This allowed simultaneous comparison of the stabilities of the two different *tat* mRNAs in the same cells under the same experimental conditions. *gag*-containing mRNAs were less stable than *tat* mRNA 1.4.7 (Fig. 8A). We next determined the half-lives of *tat* mRNA 1.4.7 and *gag*-containing mRNA TRG(330-961). To accurately quantitate the low mRNA levels produced by *gag*-containing mRNA TRG(330-961), we cotransfected 3 μg of pTRG(330-961) and 1 μg of pNL1.4.7. Quantitation of one of three independent experiments is shown in Fig. 8B. *tat* mRNA had a half-life of 4 h, and the half-life of *tat-gag* mRNA was approximately 1 h (Fig. 8B). These results indicated that the INS-1 element contained sequences that decreased mRNA stability. The differences in RNA stability measured here may not account for the whole inhibitory effect exerted by the INS-1 element. Since the stability measurements were performed on total RNA, of which the major component is cytoplasmic RNA, the stability of the nuclear RNA pool was not measured directly. The stabilities of *tat* mRNA and *tat-gag* mRNAs may be different in the nucleus and in the cytoplasm. Further experiments are necessary to determine whether the decrease in mRNA stability is the only mechanism of action of INS-1.

Inspection of the INS-1 sequence revealed that it is AU rich (61.5%) compared with the complete HIV-1 genome. Figure 9 shows a plot of the AU content within INS-1. Two regions of very high AU content are apparent. Deletion of one of these AU-rich regions in mutant pTRG(481-631) abolished the inhibitory effect of INS-1, suggesting that the instability of mRNA caused by INS-1 may be associated with the AU-rich subregions. Interestingly, either of the two AU subregions does not act alone, as demonstrated by mutants pTRG(330-480) and pTRG(481-631). This result suggests that redundant sequence elements or, alternatively, a complex structure of the complete 218-nt sequence is required for inhibition.

DISCUSSION

We have identified and characterized an inhibitory RNA element named INS-1 and located in the HIV-1 $p17^{gag}$ coding sequence. Evidence also exists for the presence of more than one INS element in the *gag*-protease gene region of the virus. The inhibitory effect of INS-1 could be overcome, at least partially, by the Rev-RRE interaction, demonstrating that this sequence is important for Rev-regulated viral expression.

Since the *gag* element decreased the stability of the mRNAs, we asked whether any homology existed between *gag* and cellular mRNAs with short half-lives. Regulation of mRNA stability has been known to be an important regula-

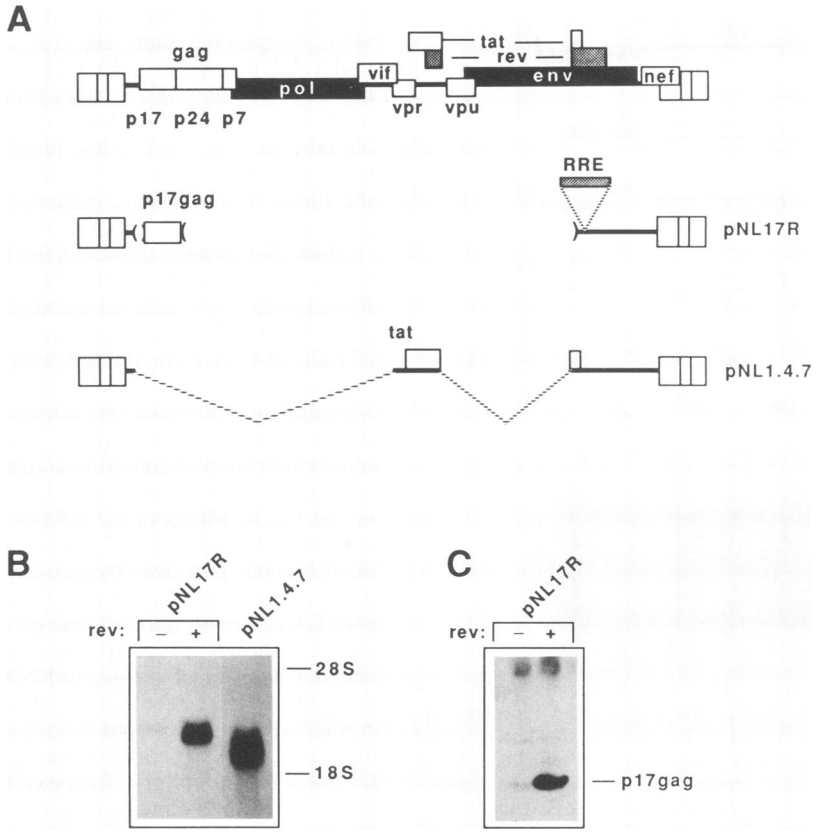


FIG. 6. (A) Structure of the HIV-1 genome and of expression plasmids pNL17R and pNL1.4.7. Open, shaded, or black bars indicate ORFs. The RRE is indicated. (B) Northern blot analysis of total RNA from HLtat cells transfected with pNL17R in the presence (+) or absence (−) of pHCMVsrev or with *tat* cDNA pNL1.4.7. (C) Western blot analysis of extracts from HLtat cells transfected with pNL17R in the presence (+) or absence (−) of pHCMVsrev.

tory step in gene expression (for reviews see references 8 and 11 and references therein). In the mRNAs studied to date, it appears that there exist regions controlling instability rather than stability and that more than one instability region may be present in the same mRNA. Among the mRNAs for which instability is important in regulating expression are the mRNAs encoding products involved in growth control, such as c-*fos*, c-*myc*, lymphokines, and cytokines. Histone and transferrin receptor mRNAs are also regulated by mRNA stability.

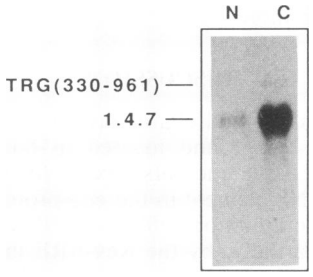


FIG. 7. Northern blot analysis of nuclear (N) and cytoplasmic (C) RNAs prepared from HL3T1 cells cotransfected with both *tat* cDNA pNL1.4.7 and pTRG(330-961). Transfections were performed as described in Materials and Methods. Nuclear and cytoplasmic RNAs were separated as described by Greenberg and Ziff (24). The positions of the RNAs are indicated on the left.

Sequences specifying RNA instability have been mapped to AU-rich regions in the 3′ untranslated regions in cellular mRNAs encoding granulocyte-macrophage colony-stimulating factor (54), c-*myc* (28, 44), and c-*fos* (56, 61, 63). Recent reports have also described elements which are located in the coding regions of c-*myc* and c-*fos* mRNAs and which affect the stability of these mRNAs (55, 64). The *gag* INS-1 sequence has an AU content of 61.5%, a content which is high compared with that of *tat* mRNA and most cellular mRNAs, which have AU contents of about 50%. The pentanucleotide AUUUA, which has been suggested to be an important determinant of RNA instability in cellular mRNAs (54), was not present in INS-1, indicating that the *gag* sequence contains a novel regulatory RNA element. Further mutagenesis experiments and comparisons with other elements are required to define the instability determinants in more detail. In this context, it is interesting that nine copies of the sequence AUUUA exist within the *gag-pol* region of HIV-1. These elements may be associated with the additional instability regions suggested by our data. Sequence analysis has shown that all the studied lentiviruses contain AU-rich regions (for a review, see reference 61a), suggesting that these viruses may have similar regulatory circuits.

Rev protein acts on RRE-containing mRNAs by promoting nuclear export and mRNA utilization (4, 16, 19, 26, 36). It has been reported that splice sites are required for Rev regulation and that they act by retaining the unspliced

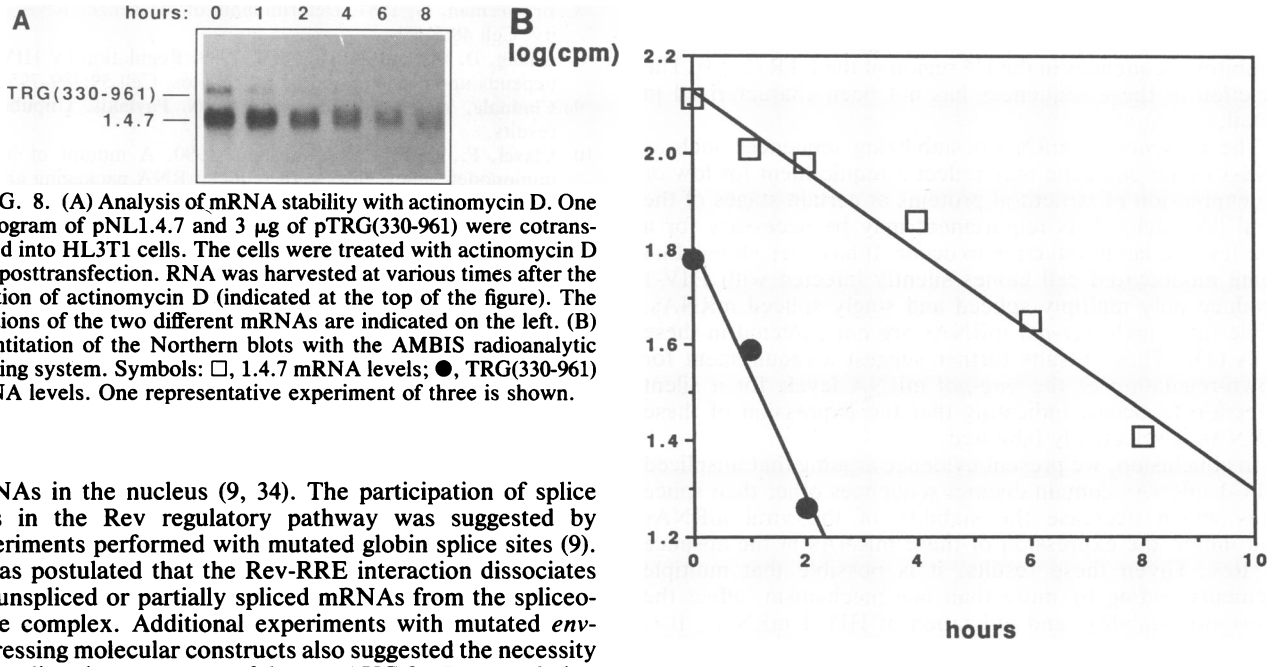


FIG. 8. (A) Analysis of mRNA stability with actinomycin D. One microgram of pNL1.4.7 and 3 μg of pTRG(330-961) were cotransfected into HL3T1 cells. The cells were treated with actinomycin D 20 h posttransfection. RNA was harvested at various times after the addition of actinomycin D (indicated at the top of the figure). The positions of the two different mRNAs are indicated on the left. (B) Quantitation of the Northern blots with the AMBIS radioanalytic imaging system. Symbols: □, 1.4.7 mRNA levels; ●, TRG(330-961) mRNA levels. One representative experiment of three is shown.

mRNAs in the nucleus (9, 34). The participation of splice sites in the Rev regulatory pathway was suggested by experiments performed with mutated globin splice sites (9). It was postulated that the Rev-RRE interaction dissociates the unspliced or partially spliced mRNAs from the spliceosome complex. Additional experiments with mutated *env*-expressing molecular constructs also suggested the necessity of 5′ splice sites upstream of the *env* AUG for Rev regulation (34). The requirement for splice sites does not appear to be general, since *gag*-containing plasmids are dependent on regulation by Rev in the absence of any functional 5′ splice sites (19). Furthermore, *tat* mRNA contains three functional 3′ splice sites that can be activated by the introduction of an upstream 5′ splice site (9a). However, the presence of these 3′ splice sites on *tat* mRNA does not inhibit Tat expression (3, 18, 38, 50), indicating that not all viral splice sites can inhibit the expression of viral mRNAs in the absence of Rev.

All retroviruses express Gag and Gag-Pol proteins from unspliced mRNAs that contain unused splice sites. The retroviral splice sites are inefficient by design (29, 30, 59, 60), and a mix of unspliced and spliced mRNAs is therefore generated to produce the correct proportion of structural viral proteins. Thus, if unused splice sites led to nuclear retention or RNA degradation, we would expect that all retroviral *gag-pol* mRNAs would be defective. The existing evidence suggests that they are not. For example, Rous sarcoma virus Gag and Pol proteins are expressed in mammalian cells in the absence of other viral proteins (37, 62). In contrast, study of the expression of Gag and Env proteins of complex retroviruses, even in the absence of any functional splice sites, strongly suggests that the corresponding mRNAs are defective in expression unless viral Rev (or Rex) protein is present. This evidence suggests that these viruses may contain specific inhibitory RNA sequences which are not present in the less complex retroviruses.

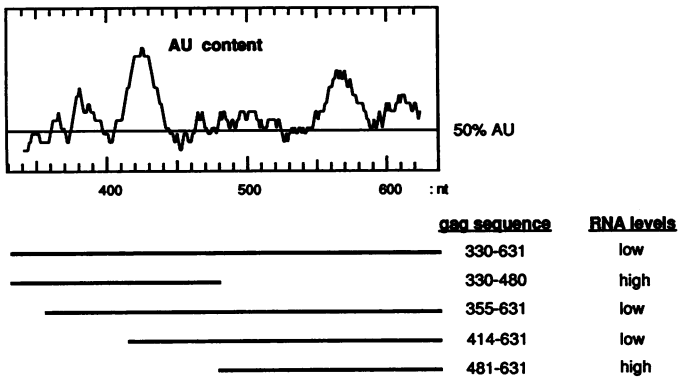


FIG. 9. Graphic representation of the AU content within the INS-1 region. Two subregions with very high AU contents exist within INS-1. The deletions affecting these subregions and their phenotypes are shown at the bottom.

The INS-1 element affects the abundance of *gag*-containing mRNAs. This effect may be different from that of the splice sites in HIV-1 *env*, which have been shown to inhibit the transport of RNAs from the nucleus to the cytoplasm (9, 34). An alternative explanation for the low levels of *gag*-containing RNAs may be that these mRNAs are processed differently from most cellular mRNAs or by an alternative pathway that requires Rev. These proposals are not mutually exclusive, and the results of different laboratories suggest that the viral transcripts are defective for more than one reason. To explain the different observations, we have proposed that Rev acts as a chaperone to guide RRE-containing mRNAs through a specific transport and utilization pathway which is initiated immediately after transcription and leads to efficient translation (4). This pathway must provide partial protection from splicing and degradation within the nucleus. This hypothesis is consistent with all the experimental results and explains the stabilization, transport, and translational effects. According to this view, the Rev-RRE interaction is a positive interaction needed to correct a preexisting defect on the viral mRNAs caused by several INS elements. The splice sites in *env* (9, 34) and the INS-1 sequence in the *gag* gene may act at different stages of posttranscriptional processing of the HIV-1 mRNAs. Sequences similar in function to INS-1 may also be present in the *env* region. Regions in the *env* gene have been shown to down-regulate CAT expression when linked to the CAT gene (47) and to inhibit the expression of Tat from a hybrid *tat-env* cDNA (40). Similarly, we have evidence that instability sequences also exist in the *gag* gene of other

complex retroviruses (58a). It has also been shown that human T-cell lymphotropic virus types I and II contain inhibitory sequences in the U5 region of the LTR (7, 53). The function of these sequences has not been characterized in detail.

The presence of mRNA-destabilizing sequences such as INS-1 in the *gag* gene may reflect a requirement for low or no expression of structural proteins at certain stages of the viral life cycle. This requirement may be necessary for a low-level or latent infection to occur. It has been shown that some monocytoid cell clones silently infected with HIV-1 produce only multiply spliced and singly spliced mRNAs, while full-length *gag-pol* mRNAs are not detected in these cells (43). These results further suggest a requirement for down-regulation of the *gag-pol* mRNA levels for a silent infection to occur, indicating that the expression of these mRNAs is selectively inhibited.

In conclusion, we present evidence arguing that unspliced HIV-1 mRNAs contain distinct sequences other than splice sites which decrease the stability of the viral mRNAs and inhibit the expression of these mRNAs in the absence of Rev. Given these results, it is possible that multiple elements, acting by more than one mechanism, affect the transport, stability, and utilization of HIV-1 mRNAs. It is reasonable to hypothesize that these inhibitory sequences, not previously found in other retroviruses, have an important function in the life cycle of HIV-1 and other lentiviruses.

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