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Inactivation of the Human Immunodeficiency Virus Type 1 Inhibitory Elements Allows Rev-Independent Expression of Gag and Gag/Protease and Particle Formation

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The expression of *gag*, *pol*, and *env* of human immunodeficiency virus type 1 (HIV-1) depends on the presence of the viral Rev protein. This dependence is, at least in part, due to the presence of negatively acting sequences (inhibitory or instability elements [INS]) located within unspliced and partially spliced mRNAs. The positive interaction of Rev with the Rev-responsive element in these mRNAs counteracts the negative effects of the inhibitory sequences. Here, we demonstrate that in addition to the previously identified INS1 within $p17^{gag}$, several other INS elements exist within the *gag*/*pol* region of HIV-1. These elements act independently of each other and were eliminated by mutagenesis after the introduction of multiple point mutations not affecting the coding region, leading to constitutive high levels of Gag expression. Expression vectors containing an intact or nearly intact $p55^{gag}$ region allowed the production of immature viral particles in mammalian cells in the absence of any other HIV proteins. The introduction of additional mutations in the *protease* region allowed efficient production of Gag/protease, which resulted in processing of the $Pr55^{gag}$ precursor and production of mature Gag particles with a lentivirus-like conical-core structure. The elimination of a newly identified INS element within *pol* and the previously identified CRS located within *int* was accomplished by the same methodology. Sequence comparisons of the identified inhibitory elements revealed no apparent homologies and demonstrated that these sequences are not splice sites. These results demonstrate that the elimination of INS elements leads to efficient expression of HIV-1 mRNAs in the absence of Rev or any posttranscriptional activating mechanisms.

The Rev protein is essential for expression of the subsets of unspliced and partially spliced viral mRNAs of human immunodeficiency virus type 1 (HIV-1), which encode Gag, Pol, Env, Vif, Vpr, and Vpu (for recent reviews, see references 8, 24, 25, and 34). The interaction of Rev with the highly structured Rev-responsive element (RRE) located within the *env* region is crucial for regulated expression of these mRNAs. Rev shuttles between the nucleus and the cytoplasm and promotes the export of the subset of RRE-containing mRNAs. Rev-RRE interaction also results in increased stabilities of these mRNAs and their efficient polysomal loading. Since RRE-containing mRNAs are not expressed in the absence of Rev, it was postulated that they are defective due to the presence of inhibitory sequences, INS, IN, or CRS, that prevent their expression. Such sequences have been identified previously within $p17^{MA}$ (INS1) (27, 29), *gag*/*pol* (IN) (20), *pol* (CRS) (7), *env* (23), and RRE (5, 23). We previously demonstrated that mutagenesis of the INS1 that altered the nucleotide sequence but not the encoded protein eliminated its negative effect and resulted in constitutive high and Rev-independent $p17^{gag}$ expression (27).

Unspliced HIV-1 mRNA produces the Gag precursor protein ($Pr55^{gag}$) and the Gag-Pol fusion protein ($Pr160^{gag\text{-}pol}$) (for reviews, see references 25 and 34). These proteins are targeted to the cell membrane, where particle formation occurs, via their N-terminal myristoyl group. After budding, the virion undergoes morphological maturation upon cleavage of the precursor polyproteins by the retroviral protease. The mature Gag products are matrix ($p17^{MA}$), capsid ($p24^{CA}$), $p2^{gag}$, nucleocapsid ($p7^{NC}$), $p1^{gag}$, and $p6^{gag}$. The matrix protein is responsible for membrane association of Gag, while the capsid forms the core viral particle. $p7^{NC}$ contains two conserved Cys-His motifs which are necessary for the packaging of viral mRNA into the virion. $p6^{gag}$ interacts directly with the accessory viral protein Vpr. Particle formation and maturation require high levels of Gag and Gag-Pol expression, which occur only in the presence of Rev protein.

Here, we identify and characterize additional down-regulatory RNA elements within *gag*, *protease*, and *pol*. Similar to the approach used to inactivate INS1 (27), multiple clustered point mutations that did not alter the amino acid sequence of the overlapping open reading frames (ORFs) were introduced into these inhibitory sequences. Inactivation of the INS elements in *gag* resulted in high levels of $Pr55^{gag}$ expression and formation of immature particles in the absence of the Rev protein. The elimination of the INS element in the *protease* ORF permitted the production of mature particles in the absence of Rev. Similarly, the previously described CRS element located within the *int* ORF (7) was eliminated by clustered point mutations. Additional INS elements were identified within *int* and *RT*. Taken together, these analyses showed the presence of multiple independently acting INS elements within *gag*/*pol*. The introduction of multiple point mutations was successful in each

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case in eliminating the down-regulatory effect of the elements. Therefore, this approach leads to high-level HIV-1 mRNA expression independent of any posttranscriptional regulation.

MATERIALS AND METHODS

Recombinant plasmids. Mutagenesis was performed with uracil-containing single-stranded DNA and was verified by sequence analysis. The plasmids contain the pBlueScript KS$^-$ vector backbone and different parts of the HIV-1 *gag/pol* ORF lacking the major HIV-1 splice donor site as previously described (29). The nucleotide numbering is that of the HXB2R sequence (GenBank accession no. K03455 and M38432), where +1 is the start of transcription (22). ll expression vectors contain the HIV-1 5′ long terminal repeat (LTR) promoter and parts of *gag/pol*, followed by a terminator of translation, multiple unique restriction sites (*Bam*HI, *Xba*I, *Xho*I, *Apa*I, and *Asp*718), and the HIV-1 3′ LTR, providing the polyadenylation signal (nucleotides [nt] 8561 to 9265). The 5′ and 3′ LTRs and human flanking regions from pNL4-3 (1) were cloned as a *Pst*I fragment into the *Pst*I site of pBlueScript KS$^-$. The pBlueScript vector lacks portions of the polylinker sequences flanking the insert from *Kpn*I to *Pst*I and from *Sma*I to *Xba*I. The *gag/pol* sequences originated from HXB2 (12).

p37 consists of the $p17^{MA}$ and $p24^{CA}$ portions of the *gag* gene, followed by a terminator of translation, and encodes the $p37^{gag}$ protein, which can be measured by $p24^{gag}$ antigen capture assay.

p37M1-4 (previously termed p37M1234) is similar to p37 but contains the mutated INS1 element (27).

p17Stop24M1-4 contains the $p24^{CA}$ gene cloned after the mutated $p17^{MA}$ gene in p17M1-4 (27). First, the $p24^{CA}$ gene was PCR amplified with primers 7425 (nt 732 to 753) and 7424 (nt 1418 to 1402) from pMcgag (11) and cloned as a blunt-end fragment into the *Eco*RV site of pBlueScript KS$^-$. The $p24^{CA}$ gene was then cloned as an *Eco*RI-*Asp*718I fragment directionally into p17M1-4, generating p17Stop24M1-4. Due to the translational stop codon at the end of $p17^{MA}$, this plasmid expresses only $p17^{MA}$ protein.

p37M1-10 contains the $p37^{gag}$ gene with INS1 and INS2 eliminated. To generate the RRE-containing derivative p37M1-4R, the *Cla*I-*Not*I fragment of pKS-RRE was inserted into the *Cla*I-*Xho*I sites of p37M1-4 after the *Not*I and *Xho*I sites were filled in. pKS-RRE contains the 330-nt *Sty*I fragment of HXB2 (nt 7266 to 7595) encompassing the RRE from pMVgagSty330 (33) cloned as an *Eco*RI-*Bam*HI fragment directionally into pBlueScript KS$^-$.

p37M1-10R contains the RRE which was PCR amplified and inserted as a *Bam*HI-*Xba*I fragment into the polylinker located downstream of the $p37^{gag}$ gene of p37M1-10.

p55B contains the *gag* ORF and part of the *pol* ORF to the *Bal*I site (nt 2165), which includes the complete *protease* ORF, and was generated by replacing the *Bss*HII-*Asp*718I fragment of p17 (27) with the *Bss*HII-*Asp*718I fragment of pNLcgag. pNLcgag is similar to the previously published pMcgag (11) and contains the *gag/pol* gene of HXB2 without the major splice donor inserted into a vector containing the 5′ and 3′ LTRs, flanking regions, and plasmid backbone of pNL4-3.

In p55BM1-4, the *Bss*HII-*Sph*I portion of p55B has been replaced by a similar fragment from p37M1-4 with INS1 eliminated. To eliminate INS2, p55BM1-4 was mutagenized with oligonucleotides M6, M7, M8, and M10 (Fig. 1A), generating p55BM1-10.

p55M1-10 is a derivative of p55AM1-10 (see below) that contains the $p55^{gag}$ gene with INS1 and INS2 eliminated but lacks *protease* sequences. *gag* sequences were PCR amplified from p55AM1-10 with oligonucleotides 4702 (nt 355 to 375) and 10951 (nt 871 to 1844). Oligonucleotide 10951 also contains an introduced *Eco*47III restriction site. The resulting PCR fragment was digested with *Sph*I and *Eco*47III and cloned into the *Sph*I-*Asp*718-digested p55BM1-10 after the *Asp*718 site was filled in, generating p55M1-10.

pCMV55M1-10 was generated from p55M1-10 by replacing the HIV-1 LTR with the cytomegalovirus major late promoter. The cytomegalovirus promoter (nt 174401 to 173655 [GenBank accession no. X17403]) was PCR amplified from pHCMV-CAT (12a) with oligonucleotides which introduce *Stu*I and *Bss*HII restriction sites and was used to replace the HIV-1 LTR.

p37Stop55M1-10 was generated after mutagenesis of p55M1-10 with oligonucleotide 11357 (5′-ggcaagagttttgTAGgaagcaatgag-3′), which introduces the translational stop codon UAG at the C terminus of $p37^{gag}$.

p55M1-13 and p55M1-PO are similar to p55M1-10 but contain additional mutations, introduced by oligonucleotides M11, M12, and M13 (see Fig. 6A), in the $p15^{gag}$ gene. p55M1-PO contains additional mutations in the overlapping *gag/pol*, introduced by oligonucleotide P0.

The p46 constructs produce a shorter Gag protein, migrating to 46 kDa, and differ from the p55 plasmids only by the deletion of a single nucleotide (nt 1550), which causes a frameshift mutation in *gag*. $p46^{gag}$ has 17 additional amino acids (GlyProProGlyArgArgAlaValGlyAsnValGluArgLysAspThrLys) encoded by the +1 reading frame at the C terminus. The mutation was initially introduced into p55AM1-P2 during mutagenesis, and shorter variants p46M1-13 and p46BM1-13P2 were generated as described above for the corresponding p55 constructs.

p55BM13P3 and p55BM13P5 include *pol* sequences up to the *Msc*I site and were generated from their respective longer p55A counterparts by deletion of the *Msc*I-*Asp*718I fragment. Both contain *gag* mutations M1 to M13 and *pol* mutations P0, P1, and P2, but only p55BM13P5 contains additional changes in the P1A and P2A regions. The expressed ORF includes $Pr55^{gag}$, protease, and 24 residues of reverse transcriptase, followed by 133 C-terminal residues of Nef.

p55AM1-10 was generated by replacing the *Apa*I-*Asp*718 fragment from p55BM1-10 with the *Apa*I-*Asp*718 fragment from HXB2. This plasmid contains a complete *gag*, including mutations M1 to M10, and extends into *pol* up to the *Asp*718 site (nt 3372). p55AM1-10 was used to introduce mutations (oligonucleotides noted parenthetically) in P0 (9585), P1 (9339), P1A (12056), P2 (9340), P2A (12057), P3.2 (9586), P4 (9342), P5 (9343), P6 (9344), and P7 (9345), generating p55AM1-R5.

Shorter portions of gag/*pol* were generated by different deletions from p55AM1-R5. p55EM1-10 was generated by replacing the *Apa*I-*Asp*718 fragment of p55BM1-10 with the *Apa*I-*Eco*RI fragment from pHXB2. This plasmid extends the *gag/pol* region to the *Eco*RI site (nt 4141). This region differs from the published HXB2 sequence by a single nucleotide at position 4155 (G-to-A transition).

p55AAM1-R5 was extended to the second *Asp*718I site (nt 3701) of HXB2 by insertion of the *Asp*718I fragment from p55EM1-10 into the *Asp*718I site of P55AM1-R5.

p37M1-4CRS was generated by insertion of the PCR-amplified CRS (nt 3700 to 4194) in sense orientation into the blunt-ended *Asp*718 site downstream of the $p37^{gag}$ coding region in p37M1-4. Oligonucleotides P9 (9347), P9.2 (9588), P10 (9348), P11 (9589), and P12 (9590) were used to mutagenize CRS, generating p37M1-4CRSP10 (oligonucleotide P10), p37M1-4CRSP12 (oligonucleotide P12), p37M1-4CRSP10P12 (oligonucleotides P10 and P12), and p37M1-4CRSP9-12, containing mutagenized P9, P9.2, P10, P11, and P12 regions. In p37M1-4CRSP9-12, nt 3823 is also deleted.

To add missing *int* sequences, *Eco*RI-*Hpa*I-*Nde*I restriction sites were introduced 3′ of CRS in p37M1-4CRSP9-12 with oligonucleotide 12612, generating p37M1-4CRSP9-12EHN. The 474-bp *Eco*RI-*Nde*I fragment (nt 4194 to 4668) of pHXB2 was cloned into the *Eco*RI-*Nde*I sites of p37M1-4CRSP9-12EHN, generating p37M1-4intP9-12. p37M1-4CRSintNNP9-12 is an extension of p37M1-4intP9-12 to the *Nde*I site (nt 5949), generated after insertion of the *Nde*I fragment from pHXB2, which results in the restoration of the ORF. The *gag* and *protease* sequences generated by mutagenic oligonucleotides are shown in Fig. 1A (see Fig. 6A also) or are listed below. Lowercase letters in oligonucleotide sequences indicate the HXB2 sequence, and uppercase letters indicate mutations generated during mutagenesis. The following mutated sequences (with oligonucleotides noted parenthetically) were generated: P4 (9342 [nt 2462 to 2417]), gacttcaggaaAtatacGgcGttCaccatCccGa gCatCaacaaCgagacaccagg; P5 (9343 [nt 2873 to 2921]), ttagtggggaaGGtgaaCtg ggcGagCcagatCtacccGgggattaaag; P6 (9344 [nt 3098 to 3150]), ggccaatggacGta CcaGatCtaCcaGgagccGttCaaGaaCctgaaaacagg; 7 (9345 [nt 3242 to 3290]), tgg ggaaagacGccGaaGttCaaGctgcccatCcaGaaggaGacatggg; P9 (9347 [nt 3749 to 3800]), gtcagtgctggGatcCggaaGgtGctattCCtGgaCggGatCgataaggccc; P9.2 (9588 [nt 3806 to 3863]), gaacatgagaaGtaCcacTCCaaCtggCgCgcTatggcCagCgaCttCaa cctgccac; P10 (9348 [nt 3950 to 4001]), ggaatatggcaGctGgaCtgCacGcaCCtGga GggGaaGgtGatcctggtag; P11 (9589 [nt 4031 to 4096]), gcagaagttatCccTgcTgaa acTgggaggaaacagcatattttcttttaaaattagcaggaaga; and P12 (9590 [nt 4097 to 4151]), tggccagtgaagacgatccaCacAgacaaCggAagcaaCttcacTggtgctacgg.

Analyses of mRNA and protein produced from transfected cells. QIAGEN column-purified plasmid DNAs were used for all experiments. To study the effect of Rev, transfection mixtures contained 1 μg of Rev-expressing plasmid pCMVsRev (4). HLtat is a HeLa-derived cell line that constitutively expresses HIV-1 Tat (28). Cells were transfected by a previously described calcium phosphate coprecipitation technique (11, 14). HLtat cells were incubated with precipitates for 4 h. HPB-ALL, Jurkat, and U937 cell lines (5×10^6 cells each) were transfected by the DEAE-dextran technique with 5 μg of pCMVp37M1-10 for 30 (HPB-ALL and U937 cells) or 50 (Jurkat cells) min. Cells were washed, cultured for 2 days, and analyzed for Gag production. Proteins were analyzed after 1 day from HLtat cells and after 2 days from HPB-ALL, Jurkat, and U937 cells. Gag production was analyzed by Western immunoblotting with HIV-1 patient serum (Scripps Laboratory, Inc.) after separation on 12.5% denaturing polyacrylamide gels as previously described (15) and was quantitated by $p24^{gag}$ antigen capture assay (Cellular Products Inc.). To control for equal transfection efficiencies, transfection mixtures contained the chloramphenicol acetyltransferase (CAT) expression vector pHCMV-CAT (12a) or the luciferase expression vector pRS-Vluc (10). Subsequent measurements of CAT levels in cell lysates were performed with a commercial CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer). Luciferase was measured as previously described (10, 33). Similar amounts of CAT or luciferase were produced on different plates within each experiment.

RNA was isolated from duplicate plates on day 1 posttransfection (9). Twenty micrograms of cytoplasmic RNA was subjected to Northern blot analysis as previously described (15). HIV-1 mRNAs were detected by a probe generated after PCR amplification with oligonucleotides 3555 and 2313, spanning nt 8572 to 9183 of molecular clone HXB2.

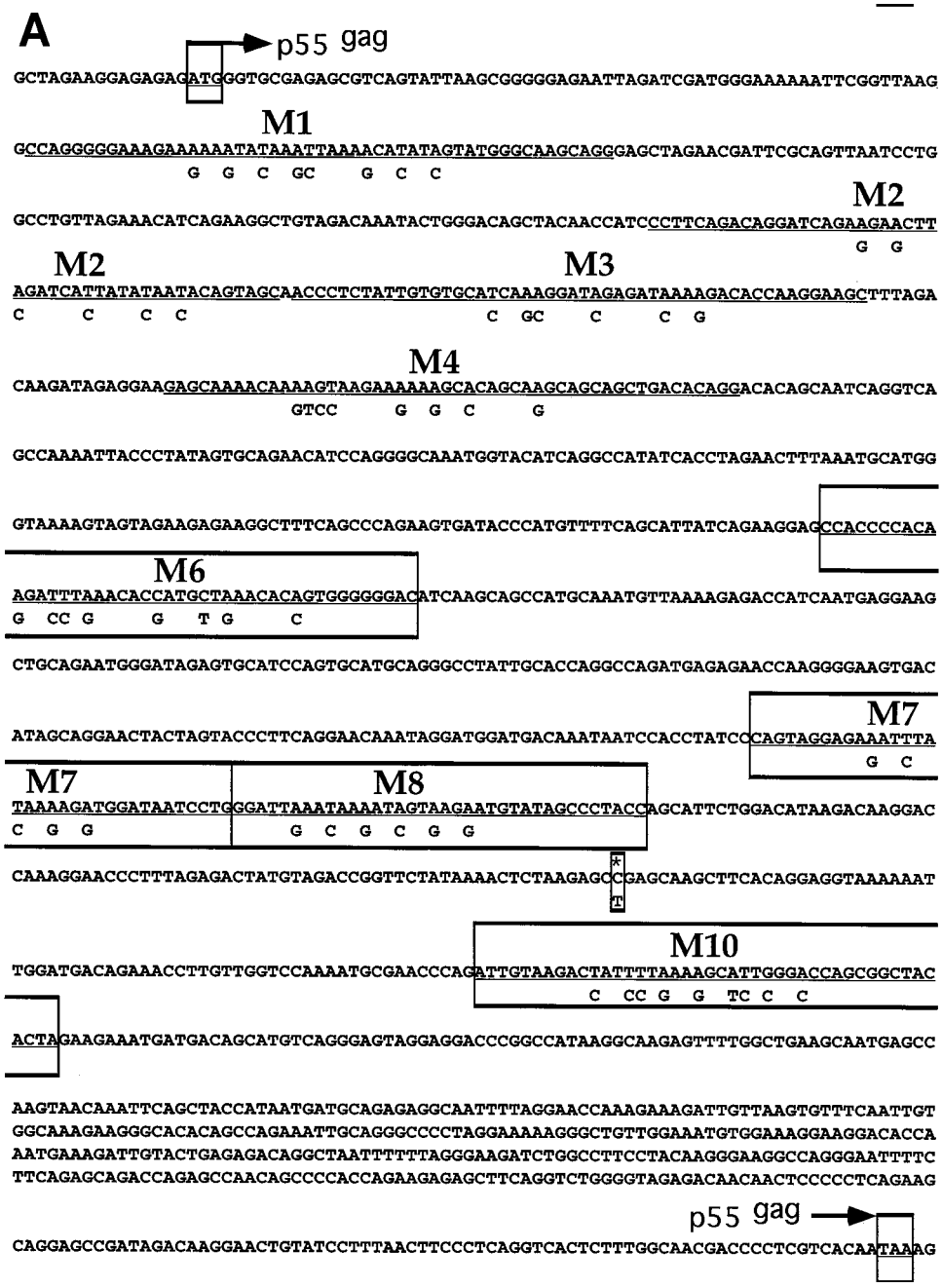


FIG. 1. Identification and elimination of the inhibitory sequence, INS2, located in the $p24^{CA}$ coding region. (A) Nucleotide sequence of the $p55^{gag}$ region. The locations of the oligonucleotides (M1 to M10) used to generate mutants are underlined. Changes introduced within the $p24^{CA}$ region are boxed. Nucleotide changes introduced by mutagenic oligonucleotides are indicated below the wild-type $p55^{gag}$ coding sequence. An asterisk marks the additional silent point mutation. (B) Gag production by different $p37^{gag}$ expression vectors. The constructs are shown on the left. Tripartite boxes indicate the HIV-1 5′ and 3′ LTRs. The $p17^{MA}$, $p24^{CA}$, and $p15^{gag}$ genes and RRE are also indicated. The introduced translational stop codon between $p24^{CA}$ and $p15^{gag}$ is also shown. Shaded areas mark the positions of INS1 and INS2. Mutations eliminating the INS effect, which are indicated by the name of the plasmid (M1-4 or M1-10), are indicated by X's. Parallel plates of HLtat cells were transfected with the indicated constructs, and Gag production was quantitated by an antigen capture assay (indicated on the right). Every construct (two individual clones) was transfected in duplicate, and the means of the obtained $p24^{gag}$ values are shown. Cotransfection with a *rev*-expressing plasmid (+ rev) is indicated. Measurements of luciferase activities from cotransfected pRSVluc revealed differences of less than twofold among plates within an experiment.

Electron microscopy. Six 60-mm-diameter plates (about 5×10^6 HLtat cells) were harvested at 48 h posttransfection by scraping in phosphate-buffered saline. Cells were pelleted at 1,000 rpm for 5 min at room temperature, washed once with phosphate-buffered saline, and pelleted again. Cells were fixed in 1.5% glutaraldehyde (5 ml of fresh glutaraldehyde for 5×10^6 to 10×10^6 cells) for at least 2 h at 4°C and without dissolving the pellet (19).

RESULTS

Identification and elimination of an additional inhibitory sequence, INS2, located within the $p24^{gag}$ coding region. We previously identified a negative element within the $p17^{MA}$ cod-

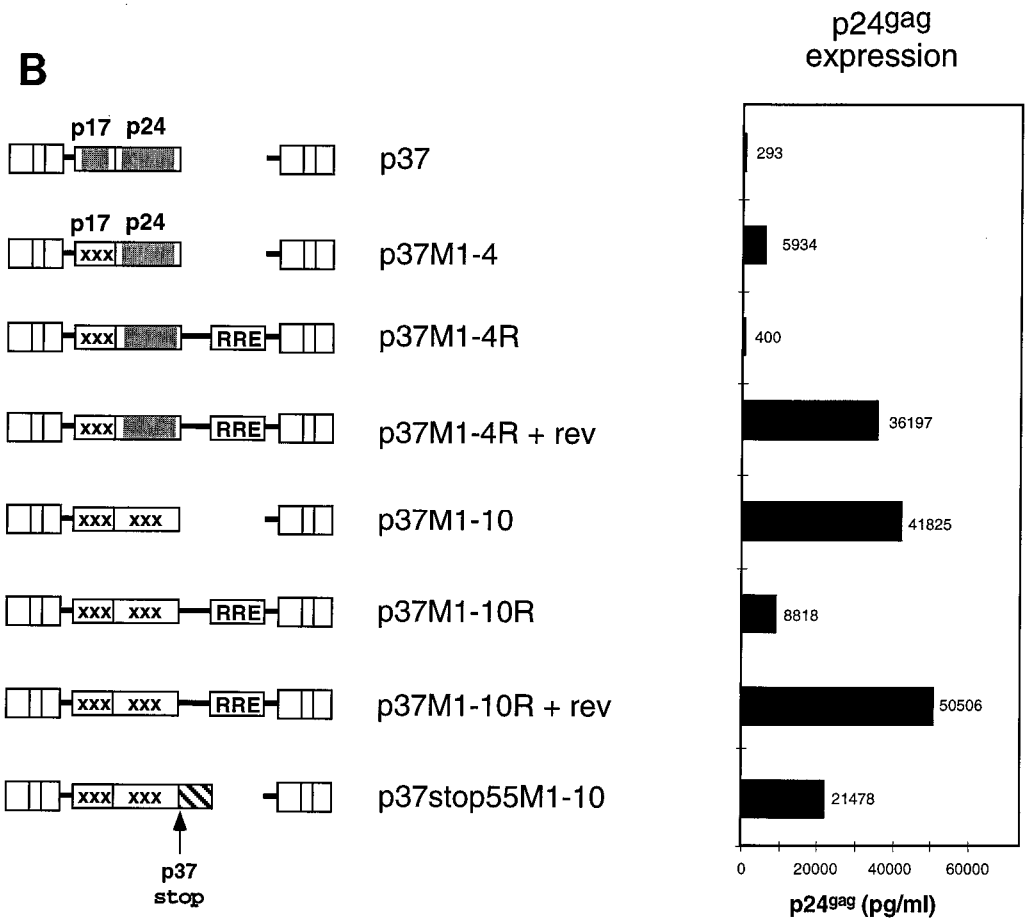


FIG. 1—*Continued.*

ing region of HIV-1 which inhibited *gag* expression as well as the expression of other mRNAs when present in *cis* (27, 29). Clustered point mutations within the $p17^{MA}$ coding region (with oligonucleotides M1 through M4 [Fig. 1A]) resulted in a markedly elevated level of $p17^{MA}$ production (vector p17M1-4 [27]). INS1 is not the only negative element within *gag*, since expression of the complete $p55^{gag}$ from vector p55M1-4, which contains the same mutations in the p17 region, is very low (27), indicating that additional negative elements exist.

To identify and eliminate the remaining INS elements in *gag*, we constructed and tested a series of expression vectors (Fig. 1B). Since the results of preliminary experiments indicated that additional INS elements were present in the $p24^{CA}$ region, a stop codon was introduced after the last amino acid of the $p24^{CA}$ protein to generate vector p37, which expressed the $p17^{MA}$ and $p24^{CA}$ regions of *gag* ($p37^{gag}$). The expression of Gag from this vector was very low, as expected. The introduction of clustered mutations, M1 to M4, that had successfully eliminated INS1 in p17M1-4 increased expression substantially, as determined by $p24^{gag}$ measurements. To determine whether expression of this construct is affected by Rev, the 330-nt RRE region was inserted into p37M1-4, resulting in plasmid p37M1-4R. This plasmid showed very low expression, which is in agreement with previous conclusions from our group and others that the RRE itself has an inhibitory effect (5, 23). The presence of Rev dramatically increased (about 100-fold) the Gag production of RRE-containing p37M1-4R. These levels are substantially higher (sixfold) than those produced by p37M1-4, suggesting the presence of an additional INS element within the $p24^{CA}$ coding sequence. Since this putative element acts independently of INS1 in p37M1-4, it was named INS2.

An inspection of the $p24^{CA}$ region revealed regions of unusually high AU content (Fig. 1A), as previously noted for INS1 (27), and two AUUUA pentanucleotides (nt 883 to 887 and 1116 to 1120, respectively), which have been implicated in the rapid degradation of cellular mRNAs (30). We used the same strategy to eliminate the inhibitory effect of the INS2 element as that previously described (27). Therefore, we designed four oligonucleotides (M6, M7, M8, and M10) spanning different AU-rich sequences (nt 881 to 1349), including the two AUUUA sequences (nt 883 to 887 and 1116 to 1120) within the $p24^{CA}$ coding region (Fig. 1A). The oligonucleotides were designed to introduce point mutations that eliminate the AUUUA elements and decrease the AU content but do not alter the coding capacity of *gag*. To introduce many point mutations simultaneously, we included all four mutant oligonucleotides in a single mutagenesis reaction. Several clones containing different combinations of mutagenized regions were identified. Their phenotypes were evaluated by the transfection of mutant plasmids into HLtat cells and subsequent analysis of Gag production by Western immunoblotting (data not shown). The plasmid that expressed the highest levels of Gag, p55BM1-10, included all 28 nucleotide changes of oligonucle-

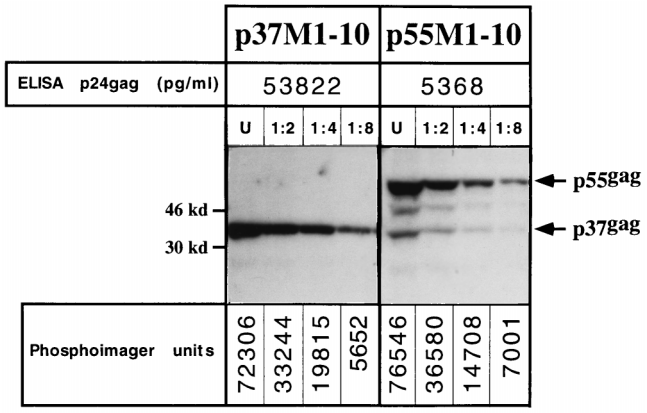


FIG. 2. Detection of p37^{gag} and p55^{gag} by ELISA and Western immunoblotting. HLtat cells, transiently transfected with p37M1-10 and p55M1-10, were harvested 1 day after transfection. The expressed proteins were analyzed by an antigen capture assay (Cellular Products Inc.), and the results are shown at the top. Serial dilutions of the same extracts were analyzed by Western immunoblotting, and the results (in arbitrary units with a PhosphorImager) are shown at the bottom. U, undiluted cell extracts; 1:2, 1:4, and 1:8, serially diluted cell extracts. kd, kilodaltons.

otides M6, M7, M8, and M10 and an additional point mutation, changing nt 1253 from C to T, which did not affect the amino acid sequence of Gag. Thus, a total of 29 point mutations within a region of 468 nt were created in a single step of mutagenesis.

To check whether these mutations eliminated the effect of INS2, we first generated the p37^{gag} expression vector, p37M1-10, that contains the mutations in both INS1 and INS2 described above. To test for constitutive *gag* expression, this construct was transfected into HLtat cells and Gag production was analyzed by p24^{gag} antigen capture assay. p37M1-10 produced approximately sixfold-higher levels of Gag than did p37M1-4, indicating that the additional mutations in the p24^{CA} region eliminated at least part of the effect of INS2 (Fig. 1B).

As a measure of successful INS2 elimination, we explored whether the presence of RRE in *cis* and Rev protein in *trans* was able to increase Gag production from p37M1-10. We inserted RRE into p37M1-10, generating p37M1-10R, and studied its Gag protein expression. The presence of RRE lowered Gag production by approximately fivefold, while the presence of Rev restored expression to levels similar to those of p37M1-10 (Fig. 1B). Therefore, we concluded that the introduced mutations eliminated the effect of INS within the p24^{CA} region completely.

The expression of p37M1-10 was also analyzed in other human cell lines, such as HPB-ALL, Jurkat, and U937, upon transient transfection. All these cell lines produced high levels of Gag in a Rev-independent fashion (data not shown), indicating that the effect of INS elimination is a general phenomenon in several human cell lines. In addition, p37M1-10 was introduced stably into HLtat cells and high-level-producer cell lines (i.e., 340 pg of Gag per μg of total cellular protein) were generated.

Rev-independent expression of Pr55^{gag} precursor. We next compared the expression of p37^{gag} to the expression of Gag precursor Pr55^{gag} produced by p55M1-10, which contains in addition to p17^{MA} and p24^{CA} the C-terminal portion of the *gag* ORF, encoding p15^{gag}. First, we checked whether the p15^{gag} region has any down-regulatory effect on p37M1-10 expression. We generated p37Stop55M1-10 by inserting a translation terminator into plasmid p55M1-10 after the last amino acid of p37^{gag} (Fig. 1B). The levels of Gag produced by p37Stop55M1-10 were at most twofold lower than those produced by p37M1-10. These data suggested that no major inhibitory sequence is located within the p15^{gag} sequence.

TABLE 1. Comparison of detection efficiencies of p37^{gag} and p55^{gag} with p24^{gag} antigen capture kits from different manufacturers[a]

Vector	p24^{gag} (pg/ml)		
	Cellular Products kit	DuPont kit	Coulter kit
p37M1-10	60,771	91,774	441,352
p55M1-10	6,572	7,160	38,025

[a] The same extracts were measured with all three kits.

To express Pr55^{gag}, the translational stop codon at the end of p37^{gag} in p37Stop55M1-10 was removed, generating p55M1-10. The Gag expression levels of p37M1-10 and p55M1-10 were analyzed and quantitated by Western immunoblotting and antigen capture assay after transfection in parallel plates of HLtat cells. Serial dilutions of the two samples were analyzed by Western immunoblotting, which revealed that both constructs synthesized similar amounts of Gag (Fig. 2). This conclusion was further supported by quantitation of the signals with a PhosphorImager. The same samples were also evaluated by a commercially available HIV-1 p24^{gag} antigen capture assay. Unexpectedly, these measurements revealed a dramatic difference (6- to 10-fold) in the amounts of p24^{gag} in these two samples. This finding supports the idea that the efficiency of p24^{gag} detection within the p37^{gag} context is higher than that within the Pr55^{gag} context. One reason for the differences in detection efficiency could be differences in protein conformation in the two assays used. In conclusion, these data demonstrate that inactivation of INS1 and INS2 is necessary and sufficient to allow high levels of Pr55^{gag} production in a Rev-independent manner.

Differences in detection sensitivity of p37^{gag} versus Pr55^{gag} in antigen capture assays. We then asked whether the monoclonal antibody in the antigen capture kit used could be responsible for the difference in the detectability of p24^{CA}. To test this hypothesis, we analyzed identical cell extracts with three commercially available antigen capture kits (Coulter, DuPont, and Cellular Products). As shown in Table 1, all three assays showed 9- to 12-fold-higher values for p24^{gag} produced

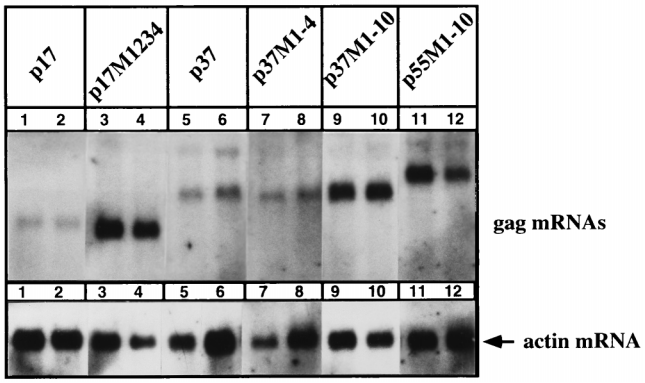


FIG. 3. Northern blot analysis of cytoplasmic mRNAs isolated from HLtat cells transfected with the indicated constructs. Two individual colonies of every construct were analyzed. Blots were hybridized with a probe detecting the 3′ portion of RNAs (top) and were subsequently rehybridized with an actin-specific probe (bottom).

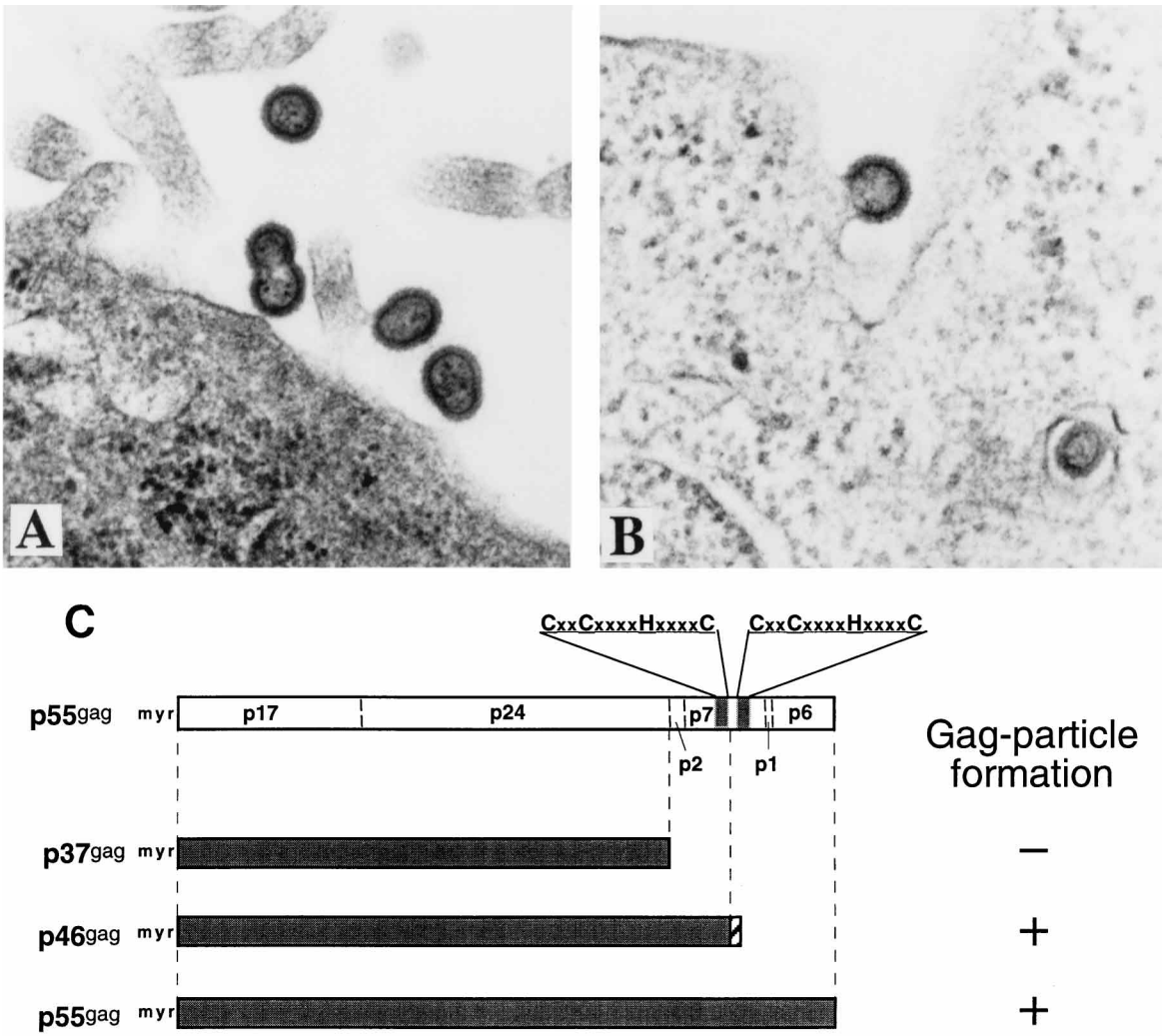


FIG. 4. Rev-independent production of p46^{gag} particles in human cells. (A and B) HLtat cells transfected by p46M1-10 were harvested and analyzed by electron microscopy 2 days after transfection. Immature particles containing p46^{gag} (A) and a budding particle (B) are shown. (C) Gag precursor protein organization. The p37^{gag}, p46^{gag}, and p55^{gag} proteins were expressed from the Rev-independent plasmids p37M1-10, p46M1-10, and p55M1-10, respectively. CxxCxxxxHxxxxC, CCHC box or zinc finger motif involved in RNA encapsidation. +, formation; −, no formation.

by p37M1-10 compared to those produced by p55M1-10. Western immunoblot analysis confirmed that the Gag levels in the samples tested differed at most by twofold (not shown). These data show that the observed difference in p24^{gag} detection was evident with all the kits tested and reflect different efficiencies in detecting p24^{CA} within different precursor Gag proteins. Furthermore, this comparative analysis also showed that two kits (DuPont and Cellular Products) yield very similar results, whereas the Coulter kit yields significantly (five to six times) higher p24^{gag} readings.

The presence of INS2 affects steady-state levels of *gag* mRNA. To test whether the effect of INS2 is exerted upon translation of this sequence, as has been shown for some other negative elements (3, 16, 26, 36), we generated p17^{gag} expression vector p17M1-4Stop24, which contains a translation stop codon inserted between the p17^{MA} and p24^{CA} coding sequences of p37M1-4, preventing translation of the p24^{CA} gene. The Gag production of p17M1-4Stop24 was compared to that of p17M1-4, lacking the p24^{gag} sequence, by Western immunoblot analysis (data not shown). Since the addition of the p24^{CA} region led to undetectable levels of p17^{MA} production, these data suggested that the INS2 element acts at the RNA level at a step preceding and independent of translation, as previously demonstrated for the INS1 region.

To examine the effect of INS2 on RNA accumulation, cytoplasmic mRNAs from cells transfected with different *gag* expression plasmids, p17, p17M1-4, p37, p37M1-4, p37M1-10, and p55M1-10, were analyzed on Northern blots (Fig. 3). As expected (27), the elimination of INS1 led to increased p17M1-4 mRNA levels compared to p17 mRNA levels. It also resulted in slightly increased levels of p37M1-4 mRNA compared to mRNA levels produced from p37. The elimination of INS2 in p37M1-10 resulted in greatly increased accumulation of *gag* mRNA compared to mRNAs produced by p37 and p37M1-4. These results further indicate that the presence of INS2 within p24 of p37M1-4 is responsible for the low steady-state levels of *gag* mRNA which result in low-level production of Gag protein, as previously shown for INS1. As expected from the protein data, there is not significant difference in RNA accumulation between p55M1-10 and p37M1-10. The differences in cytoplasmic RNA levels expressed from different constructs were also reflected in their nuclear levels (data not

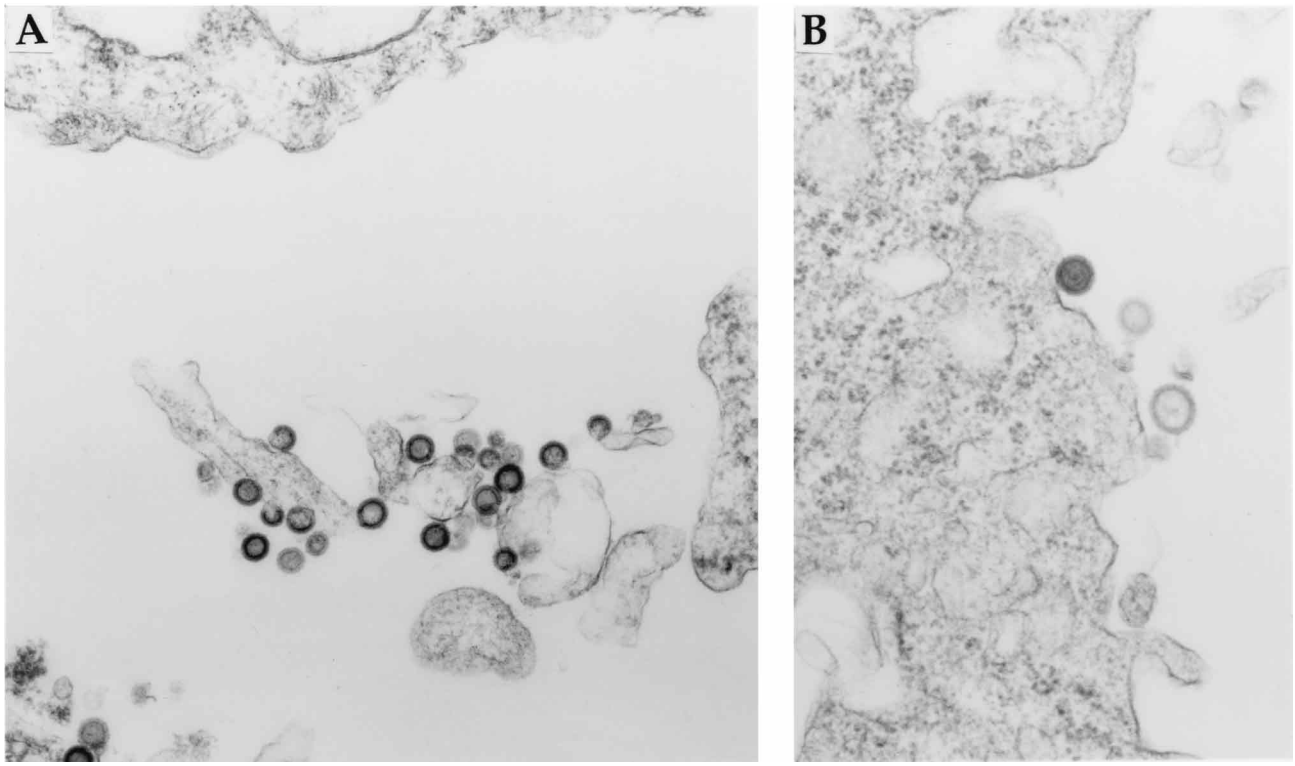


FIG. 5. Rev-independent production of p55^{gag} particles in human cells. HLtat cells transfected by p46M1-10 were harvested and analyzed by electron microscopy 2 days after transfection. Immature particles containing PR55^{gag} particles (A and B) are shown.

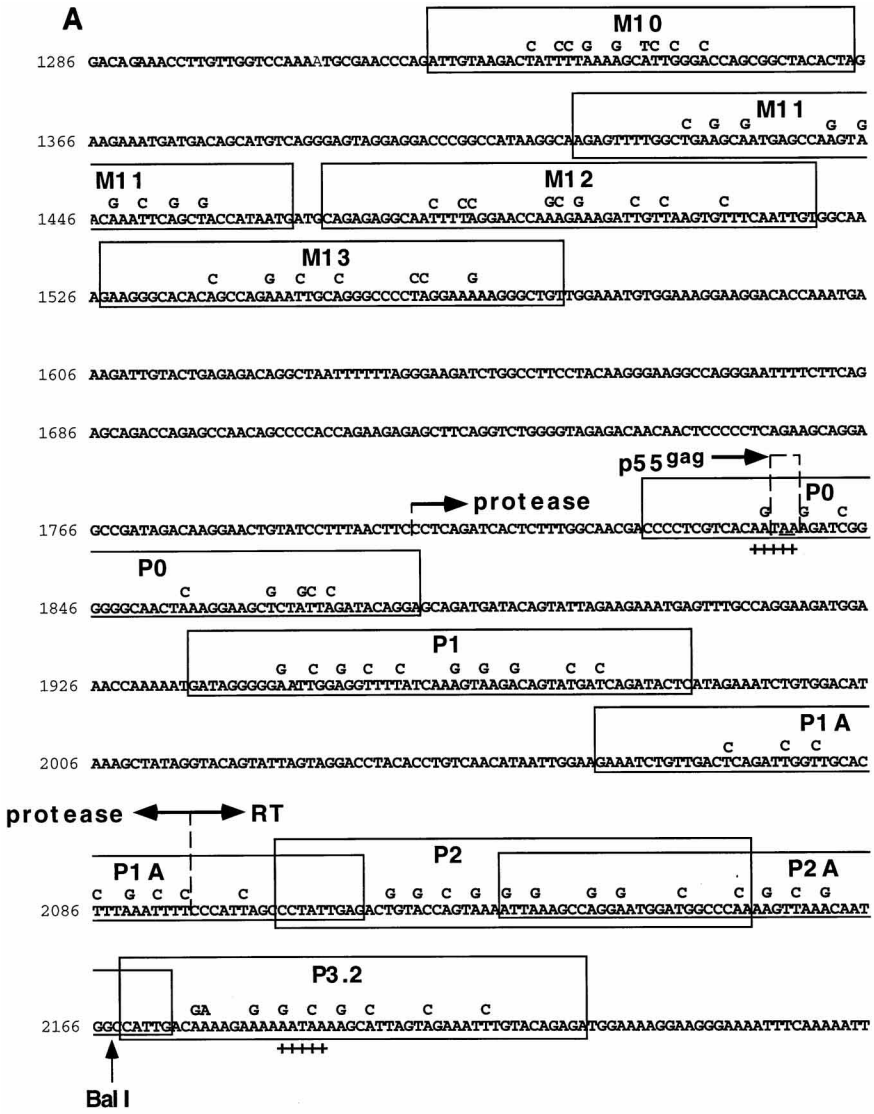
shown). These data support the model that INS-containing mRNAs do not accumulate in the nuclear compartment substantially but have shorter half-lives.

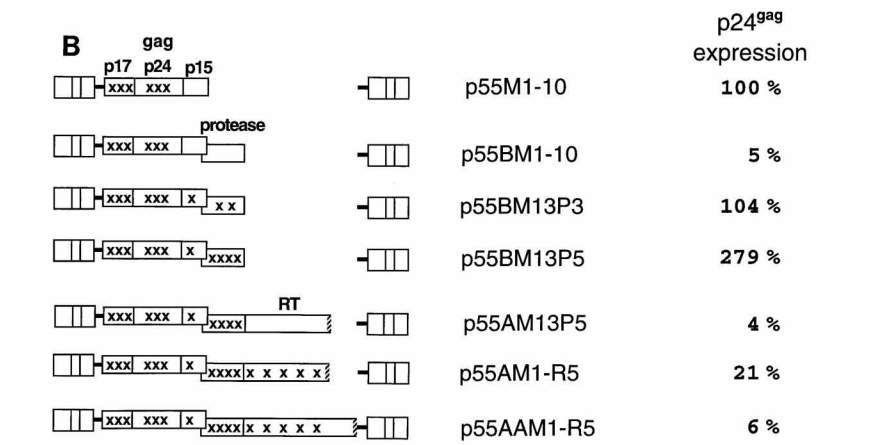
Rev-independent Pr55^{gag} expression results in the formation of Gag particles in human cells. To investigate Gag particle formation in mammalian cells in the absence of Rev, HLtat cells were transfected with p37M1-10, p46M1-10, and p55M1-10 (Fig. 4C). p46M1-10 expresses a Gag protein containing the amino-terminal part of p7^{gag} (p7^{NC}) protein, including one of the two zinc finger domains responsible for RNA interaction. p46^{gag} lacks part of Pr55^{gag} due to premature termination of translation in p15^{gag} and thus does not contain the 95 carboxy-terminal amino acids with the second zinc finger motif. All three constructs produced similar proportions of extracellular Gag. Two days after transfection, cells were fixed and inspected by electron microscopy for HIV particles (Fig. 4 and 5). No particles were produced from cells transfected by p37M1-10, supporting previous findings (13, 21, 35) and indicating that sequences within the p15^{gag} region are necessary for particle formation and budding. Interestingly, cells transfected with p46M1-10 produced virions (Fig. 4A and B), demonstrating that the protein sequence within p46^{gag} is sufficient for particle formation. These results support previous findings that the formation of infectious particles depends on the presence of at least part of the p15^{gag} protein (13, 17, 35). The expression of p55M1-10 demonstrated the release of immature particles from transfected cells into the medium (Fig. 5A) and characteristic retroviral budding particles (Fig. 5B).

Identification and elimination of INS3 located within *protease*. To achieve Rev-independent production of mature virions, we attempted expression of the *gag-protease* region in similar expression vectors, which revealed additional INS elements and required their elimination (Fig. 6A). Sequences to the *Bal*I site (nt 2164) located within *RT* were added 3′ of p55^{gag} in p55M1-10, generating p55BM1-10 (Fig. 6B). Upon transfection in HLtat cells, p55BM1-10 produced 20-fold less Gag compared to that of parental plasmid p55M1-10 (Fig. 6B). This observation led to the conclusion that an additional inhibitory sequence(s) was located within *protease*/*RT* and was active even after the elimination of INS1 and INS2.

To exclude any effect of the AU-rich regions present in the p15^{gag} sequence, we generated derivatives of p55M1-10 which contain 34 and 42 additional point mutations within p15^{gag}, generating p55M1-13 and p55M1-13PO, respectively (three of the mutations in p55M1-13PO are located downstream of the translational stop codon). Similarly high levels of Gag were produced from these constructs compared to that of p55M1-10, indicating that the altered sequences had no effect (data not shown). This finding supports our analysis of p37Stop55M1-10, indicating that no other major INS-like sequence is present (see above).

To increase *gag* expression in the presence of the *protease*/*RT* region, we introduced clustered point mutations, as described above, which affected the RNA sequence without altering the amino acid sequence (Fig. 6A). p55BM13P3 has all the mutations within the p17^{gag}, p24^{gag}, and p15^{gag} genes described above and contains 26 additional point mutations (regions P0, P1, and P2) in *protease*/*RT*. Of these mutations, 18 are located in *protease* and 8 are located in *RT*. Figure 6B demonstrates that the mutations in p55BM13P3 resulted in a dramatic increase in Gag production, to levels similar to those produced by p55M1-10. These data confirmed that additional inhibitory sequences similar to INS1 and INS2 were present in this region and that the introduced mutations eliminated these elements. Western immunoblotting was employed to visualize the processing of Pr55^{gag} (Fig. 6C). Unexpectedly, no process-





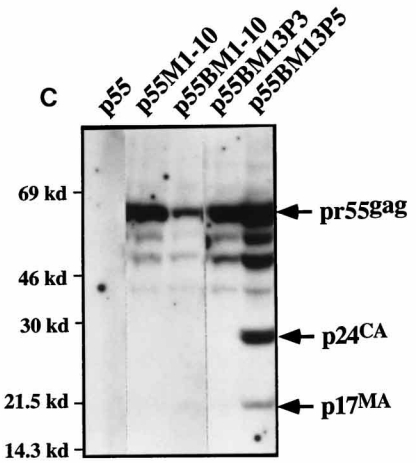


FIG. 6. Identification and elimination of INS3 in the *protease* region. (A) Nucleotide sequence of a portion of the *gag*/*pol* region. The beginnings and ends of coding regions ($p55^{gag}$, *protease*, and RT) are indicated by arrows. The names and locations of the oligonucleotides used to generate all the mutations that resulted in p55BM13P5 are boxed. Nucleotide changes are indicated above the wild-type sequence. The sequence shown includes $p15^{gag}$ and *protease*. AATAAA sequences resembling polyadenylation signals are indicated by plus signs. (B) Gag expression of several plasmids carrying clustered point mutations within the *protease* and *RT* 'regions. The constructs are shown on the left. X's indicate the approximate locations of introduced mutations. HLtat cells were transfected by the indicated plasmids and harvested 1 day later. Gag expression was quantitated by an antigen capture assay. Gag production from p55M1-10 was normalized to 100%. Data are the averages of three experiments. (C) Analysis of extracts of cells transfected by the indicated expression plasmids by Western immunoblotting. The immunoblot was incubated with HIV-1 patient serum, followed by ^{125}I-protein A, and the bands were visualized after autoradiography. The serum used does not recognize $p15^{gag}$ or its processing products. kd, kilodaltons.

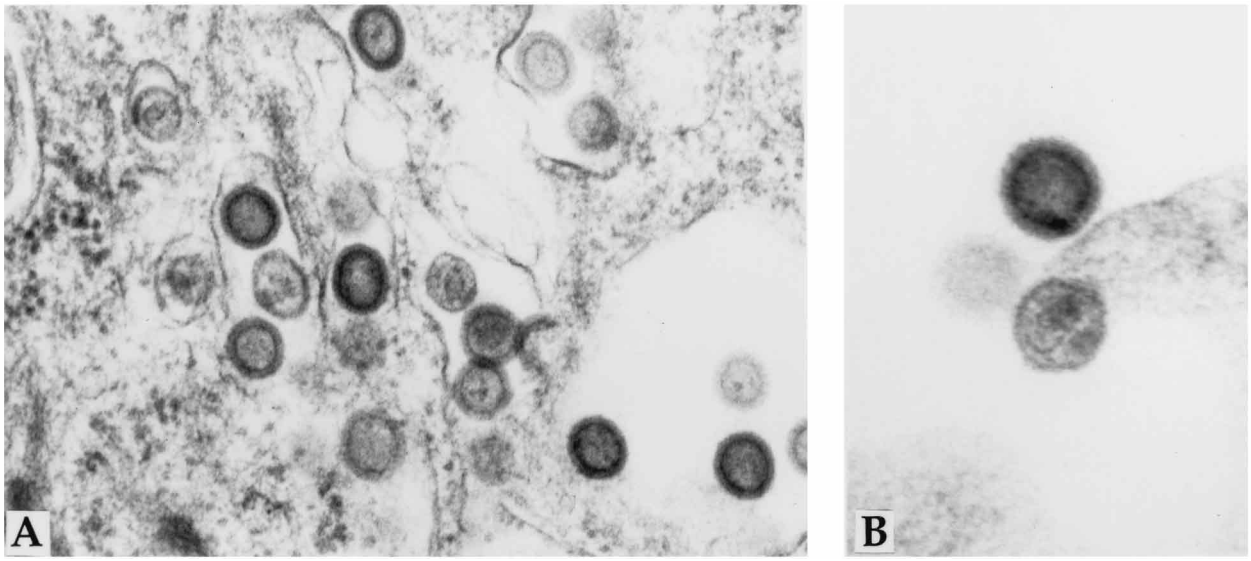


FIG. 7. Production of mature viral particles from a Rev-independent Gag-*protease* expression vector. HLtat cells were transfected by p55BM13P5 and analyzed by electron microscopy. (A) Immature and mature viral particles; (B) higher magnification of a mature particle.

ing of the polyprotein produced by p55BM13P3 was detected; nevertheless, this vector has the coding capacity for a Gag-protease fusion protein. We hypothesized that this could be due to a nonfunctional protease as a result of the altered C terminus or due to improper folding of the Gag-protease precursor protein because of missing sequences in the *RT*/*int* region. Alternatively, protease was not produced in sufficient amounts to allow processing.

Since there were still two AU-rich regions present in *protease*/*RT*, additional mutations were introduced into regions P1A and P2A, generating p55BM13P5 (Fig. 6B). p55BM13P5 contains 13 additional mutations, of which 7 are located in *protease* and 6 are located in *RT*. As shown in Fig. 6B, these mutations led to a further threefold increase in Gag production. An analysis of cell extracts by Western immunoblotting confirmed increased Gag production and demonstrated efficient processing of $Pr55^{gag}$ to $p24^{CA}$ and $p17^{MA}$ (Fig. 6C). This finding clearly shows that elevated expression levels of the precursor protein result in sufficient amounts of protease to allow processing. As described above for other INS elements, the introduction of multiple nucleotide changes within a large region was also necessary to eliminate INS3.

Rev-independent production of mature Gag particles. To verify the formation of mature Gag particles, HLtat cells transfected by p55BM13P5 were subjected to analysis by electron microscopy (Fig. 7). Two types of viral particles, immature particles similar to those produced from p55M1-10 and particles with a more mature lentivirus-like capsid structure in the form of an electron-dense, cone-shaped inner core, were observed. This maturation is clearly the result of proteolytic processing of the Gag precursor protein by the retroviral protease.

Presence of additional INS elements in *RT*. To express additional portions of *pol* downstream of the *Bal*I site, p55BM13P5 was extended to include sequences up to an *Asp*718 site (nt 3371), generating p55AM1-P5 (Fig. 6B). After transient transfection of this construct into HLtat cells, *gag* expression decreased by about 70-fold. Additional point mutations were introduced to destroy the AU-rich sequences as well as several AUUUA motifs, generating p55AM1-R5. Figure 6B shows that Gag production from this plasmid was increased by about fivefold. However, the addition of further sequences extending to the second *Asp*718 site (nt 3701) in p55AAM1-R5 led to a loss in Gag production (Fig. 6B). These data indicate that other INS elements are located within different regions of the *RT* region. The elimination of all such elements is apparently required for Rev-independent expression of the polyprotein.

The *int* coding sequence contains INS elements in addition to CRS. In order to express *int* Rev independently, it was necessary to eliminate another previously identified inhibitory sequence element, CRS (7), located between nt 3793 and 4052 within the *int* ORF. To facilitate monitoring the elimination of the inhibitory effect, a larger fragment (nt 3700 to 4194) spanning CRS was cloned 3′ to the $p37^{gag}$ gene in p37M1-4 (Fig. 8), generating p37M1-4CRS. The presence of CRS reduced *gag* expression by about eightfold. This reduction was seen only when CRS was cloned in the sense orientation (data not shown). To destroy the inhibitory effect of CRS, the single AUUUA element, as well as other AU-rich regions, was targeted for mutagenesis with oligonucleotides P9, P9.2, P10, P11, and P12. Changes introduced into region P10 (p37M1-4CRSP10), P12 (p37M1-4CRSP12), or both (p37M1-4CRSP10P12) had no effect (Fig. 8). The introduction of additional mutations in regions P9, P9.2, and P11 (p37M1-4CRSP9-12) restored Gag production levels. The addition of the *Eco*RI-*Nde*I fragment spanning the remaining portion of *int* in p37M1-4intP9-12 reduced the levels of *gag* expression dramatically, even in the presence of a mutated CRS element (Fig. 8). This finding suggests that additional INS elements are located within *int.*

DISCUSSION

Here, we have demonstrated the presence of multiple negatively acting sequences within *gag*/*pol* which are responsible for the Rev dependence of unspliced HIV-1 mRNA. These elements act independently of each other, but their effects are additive in several cases. The introduction of clustered point mutations has been shown to reproducibly overcome the down-regulatory effect of such elements. The presence of multiple negative elements indicates that tight regulation might be beneficiary for the virus. The presence of more than one down-

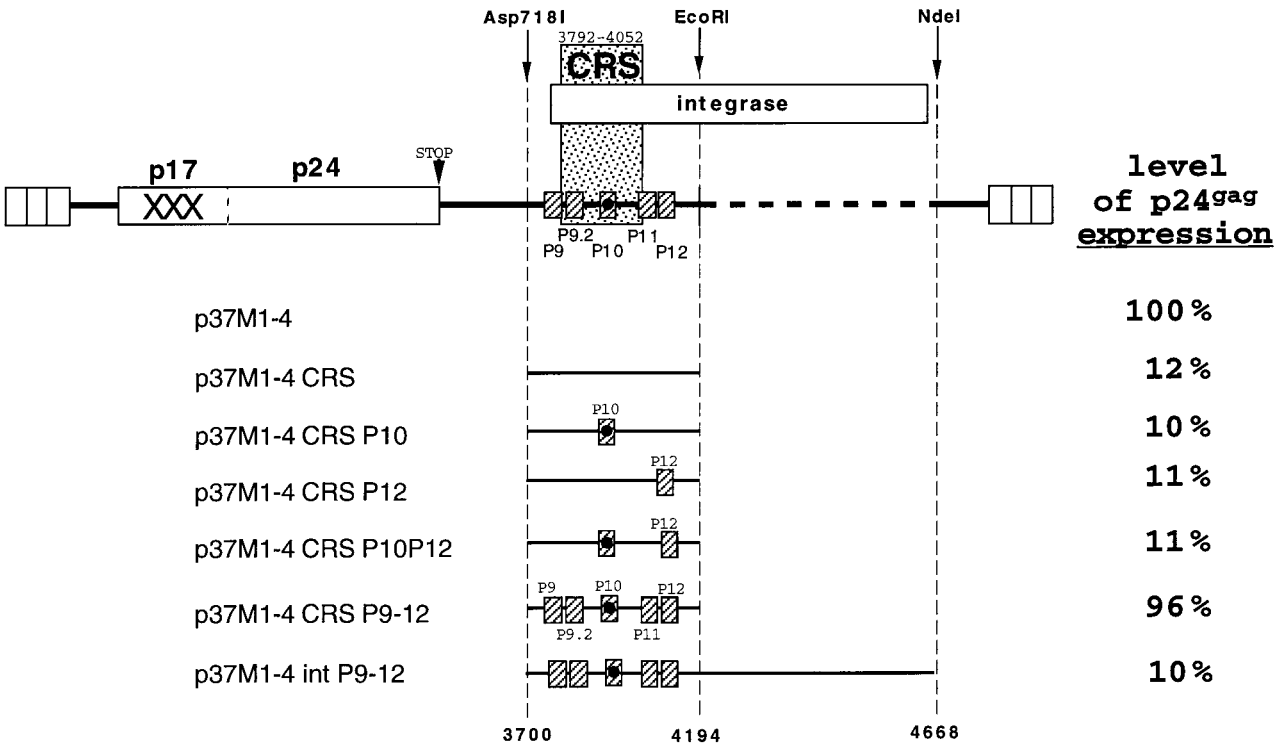


FIG. 8. Elimination of CRS and identification of additional INS elements in the *int* ORF. Insertions of various *int* fragments into p37M1-4 were used to monitor Gag production and inhibitory effects of the *int* region. The *gag* and *int* ORFs are indicated. The position of CRS is indicated by a dotted box, and the oligonucleotides used for mutagenesis of CRS are indicated by small hatched boxes, with the names given. A dot within a box indicates an AUUUA sequence in pNL4-3.

regulatory element is not unique to HIV, since it has been demonstrated that two independently acting instability elements are present within c-*fos* and c-*myc* mRNAs, which are involved in tight and rapid regulation of these RNAs (6, 31).

The presence of many down-regulatory elements raises the question of their mode of function. We have observed that several regions linked to instability have high AU contents and that mutagenesis which alleviates inhibition also lowers the AU content. Interestingly, while all Rev-dependent mRNAs have unusually high AU contents, the AU content of the multiply spliced RNA species is much lower. Comparisons of the four mutated sequences within INS1 revealed AU contents of 68 to 95%. Similarly, the AU contents of the other INS regions vary between 46 and 92%. After the introduction of multiple point mutations, the AU contents of these elements were lowered by about 40%. The average cellular mRNA usually has an AU content of around 50%, whereas it has been observed that particularly unstable mRNAs, like c-*myc*, c-*fos*, c-*myb*, granulocyte-macrophage colony-stimulating factor, alpha interferon (IFN-α), IFN-β, INF-γ, tumor necrosis factor alpha, interleukin-1, and interleukin-3 mRNAs (16), share unusually high AU contents, which are believed to be involved in the instability and rapid degradation of these mRNAs. In addition to the AU content, some of the viral INS elements contain the AUUUA pentanucleotide, which has been shown to be involved in determining the instabilities of cellular mRNAs, like c-*fos*, cytokine tumor necrosis factor alpha (16, 31, 32), and granulocyte-macrophage colony-stimulating factor (30). There is no apparent sequence homology among HIV INS elements. In addition, some reports indicate that not all HIV INS elements are AU rich. In the *env* region, elements contributing to mRNA instability and low expression include RRE (5, 23), indicating the presence of elements acting by a different mechanism.

A current hypothesis about the function of INS elements is that they are binding sites for cellular factors which contribute to mRNA instability. Interestingly, results from our laboratory indicate preferential binding of the poly(A)-binding protein PABP1 to the p17 region INS1 (2). In this case, inappropriate binding of PABP1 may confer instability by misfolding or missassembly of the appropriate ribonucleoprotein particle containing the mRNA or by transporting the ribonucleoprotein to the wrong cellular compartment. Alternatively, the phenotype associated with INS elimination may be the result of translational activation. In humans, codon usage has been proposed to influence translatability and several mRNAs were shown to be translated more efficiently when the codon usage was changed to reflect that found in stable and well translated human mRNAs. This codon preference is biased for G or C at the third position.

Our results indicate that in the case of HIV mRNAs, it is the RNA sequence, not translatability, that is responsible for the observed effects. First, the mere presence of Rev is able to increase protein expression by 2 logs; it is difficult to explain this function of Rev by a translation activation mechanism. Second, we have shown that INS sequences act similarly whether or not they are translated (see above) (27, 29). In addition, the phenotype associated with INS elimination (increased stability, increased nucleocytoplasmic transport, and increased translatability) is not easily explained by increased translation.

The work presented here was performed with *gag* expression vectors that do not contain the major 5′ splice site of HIV-1 and do not splice. This allowed us to study the effects of INS

elimination in the absence of splicing and to conclude that HIV mRNAs are poorly expressed even in the absence of any active splicing (see above) (11, 23, 27). To study the effects of INS elimination in the presence of splicing, *gag* expression vectors containing an intact 5′ splice site of HIV were generated (34a). These vectors produced high levels of Gag, indicating that INS elimination also improved *gag* expression in the presence of splicing. Quantitative analysis showed that splice site-containing vectors expressed approximately three times less mRNA and protein compared to that expressed by splice site-deleted vectors. These results show that the observed INS effects are independent of splicing, whereas splicing and other mechanisms can decrease mRNA expression.

The failure of $p37^{gag}$ to produce viral particles is interesting, since recent in vitro studies have shown that sequences within the capsid protein are responsible for virus assembly (18, 35). The lack of $p6^{gag}$ sequences, which has been shown to be involved in the release of assembled particles, does not explain the complete lack of viral particle formation by $p37^{gag}$. Viral particles are formed and released by the $p46^{gag}$ construct, in which the complete $p6^{gag}$ sequence is not present due to the frameshift mutation. These experiments also define p46 as the minimal region of Gag necessary for efficient particle formation. Since p46 lacks one of the two Cys-His zinc finger motifs associated with RNA binding, it will be of interest to determine whether interactions with RNA play a role in particle formation.

Our data show that the available $p24^{gag}$ ELISA kits are able to recognize $p24^{gag}$ protein within unprocessed $p37^{gag}$ and $p55^{gag}$ proteins, but the recognition efficiency of $p24^{gag}$ epitopes is influenced by the processing status. Therefore, these kits are reliable for the quantitation of Gag proteins when the processing status of these proteins is the same. When Gag proteins are not efficiently processed, $p24^{gag}$ antigen capture ELISAs may give erroneous results. This is important in cases where the effects of HIV protease inhibitors are studied, since these drugs inhibit Gag processing.

In our system, only minimal protease processing can be detected intracellularly. Intracellular processing of the Gag precursor protein has been described previously when *protease* was overexpressed in vitro and seemed to interfere with particle release in that cell culture system (19). The preservation of the frameshifting area in our *gag*/*pol* constructs probably results in protease levels that are optimal for timely processing of the Gag precursor.

Rev-independent expression vectors allow the efficient expression of Gag proteins in many cell lines (data not shown) that are not able to support efficient Rev-RRE-dependent rescue of these RNAs. The availability of HIV genomic regions able to be expressed at high levels in the absence of any posttranscriptional control may increase expression of simple *gag*/*pol* and *env* vectors in mice and primates. *gag*/*pol* expression vectors may be important for vaccination approaches against HIV-1, since the *gag*/*pol* region is more conserved than is the *env* region and may be important for an effective immune response against HIV and for protection against infection. Attenuated simian immunodeficiency viruses (SIV) with deletions in the *nef* gene were able to generate a strongly protective immune response in macaques. Such vaccines have prevented infection by chimeric SIV-HIV, which shares core proteins with the attenuated vaccine but has an entirely different envelope. In addition, an attenuated SIV-HIV carrying the HIV_{IIIB} *env* protected against intrarectal challenge with pathogenic SIV_{sm} (25a). Thus, immunity was produced by some mechanism other than envelope-specific neutralizing antibody. The contribution of the *gag*/*pol* region in the protective response in such cases should be further assessed. The expression of only this region in nonreplicating vectors similar to those described in this work may provide further insights into the correlates of protective immunity against HIV-1.

Efficient HIV gene expression in many cells is also of interest for gene transfer experiments. The ability to express the HIV-1 Gag protein independent of any regulatory factors makes the generation of simpler lentiviral vectors possible. Since HIV and the other lentiviruses are able to infect quiescent cells, these new retroviral vectors could be used for gene transfer in nondividing or slowly dividing cells.

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