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Complementation of the *rev* Gene Mutation among Human and Simian Lentiviruses

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The functional exchangeability of the *rev* gene was assessed in transient transfection experiments by using in vitro-constructed *rev* and *gag* mutants of the following three primate lentiviruses: human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus from the African green monkey (SIV_{AGM}). Cotransfection into SW480 cells of the *rev* and *gag* mutants derived from the DNA of each infectious virus resulted in the generation of progeny particles as determined by reverse transcriptase assay. *rev* gene mutants of HIV-2 and SIV_{AGM} were also complemented by all *gag* mutants derived from the three viruses. In contrast, no evidence of complementation was obtained following cotransfection of the HIV-1 *rev* mutant and the *gag* mutant of HIV-2 or SIV_{AGM}.

The genome of human immunodeficiency virus type 1 (HIV-1) contains at least six additional genes other than *gag*, *pol*, and *env*, which are common to all retroviruses (10). Of these accessory genes, *tat* and *rev* are essential for the infectivity of HIV-1 (4, 7, 21, 26). The product of *tat* has been shown to drastically augment overall viral gene expression (3, 17, 22, 23), and *rev* encodes a protein that selectively enhances the expression of *gag* and *env* (5, 21).

We have recently shown that *tat* and *rev* are also indispensable for the infectivity of HIV-2 (19) and simian immunodeficiency virus from the African green monkey (SIV_{AGM}) (20). The phenotypes of *tat* and *rev* mutants derived from HIV-2 and SIV_{AGM} suggest that the underlying mechanisms of this effect are the same for HIV-1. In fact, the *tat* gene product from each virus could transactivate the expression of nonself long terminal repeats to various degrees (19).

For a better understanding of *rev* gene function, complementation experiments were initiated by using in vitro-generated mutants derived from the infectious DNA clones of HIV-1 (1), HIV-2 (19), and SIV_{AGM} (20). Various *rev* and *gag* mutants were cotransfected into SW480 cells, which are very sensitive to transfection analysis (1), and transient reverse transcriptase (RT) production and *gag* expression were monitored. We demonstrate here that the *rev* gene defect of HIV-1 was complemented by HIV-1 *rev* but not by HIV-2 *rev* or SIV_{AGM} *rev*.

MATERIALS AND METHODS

Cell culture and DNA transfection. A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. For transfection, uncleaved plasmid DNA was introduced into SW480 cells by the calcium coprecipitation method (1, 11, 28).

RT assays. RT activity was measured as described previously (29). For quantitation, spots on DE81 papers were cut out and RT activity was determined by scintillation counting.

Western immunoblotting. Lysates of transfected SW480 cells were prepared as described previously (29), and proteins were resolved on sodium dodecyl sulfate–12% polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. The membranes were incubated overnight at room temperature with a serum sample from an individual infected with virus and with ^{125}I-labeled protein A for 3 h and were washed and visualized by autoradiography (29).

Viral DNA constructs. The infectious clones, pNL432 (HIV-1) (1), pGH123 (HIV-2) (19), and pSA212 (SIV_{AGM}) (20), were used as wild-type DNAs. To construct mutants, infectious DNA clones or subclones were cleaved with restriction enzymes, end modified by T4 DNA polymerase or by linker insertion if needed, and resealed by T4 DNA ligase. Mutated subclones were recloned into the infectious clones. Restriction enzymes used to introduce mutations into selected portions of the genomes are described in Fig. 1, 2, and 5. HIV-1 mutant designations and their mutations are as follows: pNL-Ac (*gag* mutant; 2-base-pair [bp] insertion), pNL-Kp (*env*; 4-bp deletion), pNL-St (*env*; 8-bp insertion), pNL-ΔBg (*env*; 507-bp deletion), pNL-Hi (*env*; 4-bp insertion), pNL-Av (*env*, *tat*, *rev*; 4-bp insertion), pNL-Ba (*env*, *rev*; 4-bp insertion), and pNL-Hp (*env*; 8-bp insertion). Other mutants constructed were pSEΔRRE (HIV-1) and pPXΔRRE (HIV-2), which contained a large deletion affecting multiple genes in the genome (see Fig. 5). The construction and characterization of HIV-2 and SIV_{AGM} mutants designated pGH-Hi (HIV-2 *gag* mutant), pGH-Ps (HIV-2 *rev* mutant), pSA-EIII (SIV_{AGM} *gag* mutant), and pSA-ΔH (SIV_{AGM} *rev* mutant) were as described elsewhere (19, 20).

Prediction of secondary structure of RRE. Putative *rev* responsive elements (RREs) within the *env* genes of HIV-2 and SIV_{AGM} were selected by homology to the mutationally defined RRE of HIV-1 (15). The prediction of stable secondary structures of these sequences was performed by computer analysis (8) and modified as previously described (15).

RESULTS

Complementation among mutants. Mutant clones used in the complementation analysis are shown in Fig. 1. All *gag*

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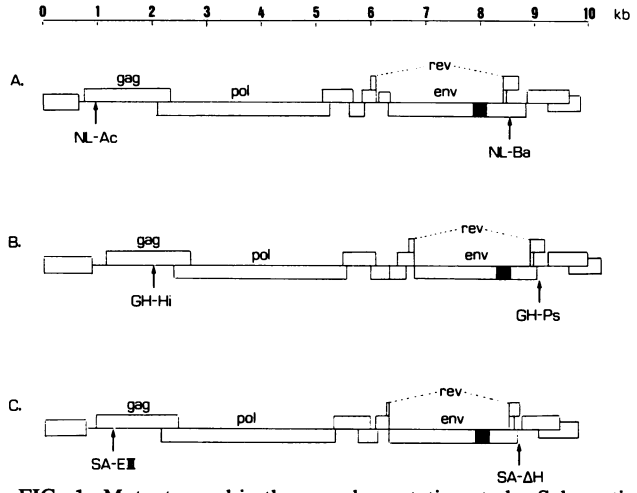

FIG. 1. Mutants used in the complementation study. Schematic representations of genome organization (flanked by long terminal repeats) of HIV-1 (pNL432) (A), HIV-2 (pGH123) (B), and SIV_{AGM} (pSA212) (C) are shown. Open reading frames other than *gag*, *pol*, *env*, and *rev* are indicated by open boxes. Closed boxes represent RRE. Mutant designations, enzymes used to make mutants, and location of mutation (arrows) are indicated. Abbreviations: Ac, *Acc*I; Ba, *Bam*HI; Hi, *Hin*dIII; Ps, *Pst*I; EIII, *Eco*47III; ΔH, *Hin*cII and *Hin*dIII; kb, kilobases.

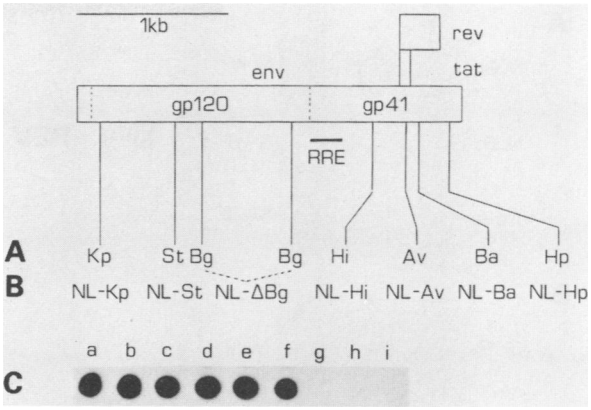

FIG. 2. Construction and characterization of HIV-1 *env* mutants. Schematic representation of HIV-1 *env* gene is at the top. Coding exons 2 of *rev* and *tat* genes are also shown. The position of RRE is underlined. (A) Enzymes used to construct mutants. Abbreviations: Kp, *Kpn*I; St, *Stu*I; Bg, *Bgl*II; Hi, *Hin*dIII; Av, *Ava*I; Ba, *Bam*HI; Hp, *Hpa*I. (B) Mutant designations. (C) RT production in SW480 cells 48 h after transfection. Cells were transfected with: pNL432 (a), pNL-Kp (b), pNL-St (c), pNL-ΔBg (d), pNL-Hi (e), pNL-Hp (f), pNL-Av (g), pNL-Ba (h), and pUC19 (i). kb, Kilobases.

and *rev* mutants except pNL-Ba (HIV-1) were constructed so as not to affect other coding sequences besides those of target genes. We previously demonstrated that *gag* mutants of HIV-2 and SIV_{AGM} are defective in their expression of *gag* proteins and RT because of the premature termination codon in the *gag* gene and that *rev* mutants also do not produce *gag* proteins or RT (19, 20). The HIV-1 *gag* mutant pNL-Ac contained a premature termination codon in the p17 (*gag*)-coding sequence and was expected not to express authentic *gag* proteins and RT. The HIV-1 *rev* mutant pNL-Ba also has a frameshift mutation in the 3′ portion of the gp41-coding region. To know the effect of *env* expression on RT production, several *env* mutants of HIV-1 were constructed (Fig. 2) and analyzed by transfection. All the *env* mutants except those with alterations in coding exon 2 of *tat* and *rev* could produce RT comparable to that of wild-type DNA (Fig. 2C). The result shows that the lack of RT production by pNL-Ba was due to a mutation in the *rev*-coding sequence.

Complementation was judged by RT production in transfected SW480 cells. SW480 cells were cotransfected with *gag* and *rev* mutants, and RT production in the culture fluids was monitored 48 h later (Fig. 3). In the transfected cells, functional *rev* protein and RT were supplied by *gag* mutants and *rev* mutants, respectively. In single DNA transfectants, no RT production was observed, as expected (Fig. 3, lanes R and G). Cotransfection of homologous pairs of mutated DNAs resulted in RT production (Fig. 3, lane X). However, cotransfection of pairs of HIV-1 *rev* and HIV-2 or SIV_{AGM} *gag* mutants generated no detectable RT (Fig. 3A, lane X). RT activity was still not detectable in the reaction mixtures containing 50-fold-concentrated virus samples (prepared by centrifugation) (results not shown). In contrast to this result, HIV-2 and SIV_{AGM} *rev* mutants, particularly HIV-2 mutants, could be complemented for RT production by heterologous *gag* mutants quite efficiently (Fig. 3B and C). Quantitative data from the complementation analysis is presented in Table 1. Cotransfection of HIV-1 *rev* and *gag* mutants and of HIV-2 *rev* and *gag* mutants resulted in RT production comparable to that of wild-type DNA, whereas the combination of both SIV_{AGM} mutants yielded approximately 40% RT activity relative to that of wild-type DNA. Complementation efficiency between mutants derived from two viruses seemed to be variable depending on the pairs used. However, relative RT activity was quite constant and reproducible. Western blot analysis was also performed to determine whether the absence of RT production by the pairs of HIV-1 *rev* mutants and HIV-2 or SIV_{AGM} *gag* mutants was due to the lack of viral protein synthesis. Figure 4 shows *gag*-related proteins produced in the transfected SW480 cells. As expected, neither the HIV-1 *gag* mutant (Fig. 4, lane 3) nor the *rev* mutant (Fig. 4, lane 4) could express *gag* proteins (p55, p24, and p17) which were present in the cells transfected with wild-type DNA (Fig. 4, lane 2). Among cotransfectants (Fig. 4, lanes 5 through 7), only the pair of HIV-1 *rev* and *gag* mutants produced *gag* proteins (Fig. 4, lane 5).

To confirm that the structure of the *rev* supplier did not affect the complementation data described above, other mutants which contain deletions encompassing *gag*-, *pol*-, *vif*-, and *rev*-responsive elements (see below) in *env* were constructed (Fig. 5A) and cotransfection experiments were carried out. Essentially the same result as that shown in Fig. 3 was obtained (Fig. 5B). The HIV-2 *rev* mutant was complemented by both deletion mutants, whereas the HIV-1 *rev* mutant was complemented only by the deletion mutant derived from HIV-1.

Comparison of predicted structure of *rev* protein and RREs among three viruses. Recently, several groups have proposed the mechanisms underlying regulation by HIV-1 *rev*, that is, the activation of nuclear export of unspliced viral mRNA (12, 13, 15), and the mechanisms which increase the stability and transport of the mRNA (6). A *cis*-acting element designated RRE within the *env* region of HIV-1 is required for *rev* function (6, 12, 13, 15, 18). We were interested in analyzing

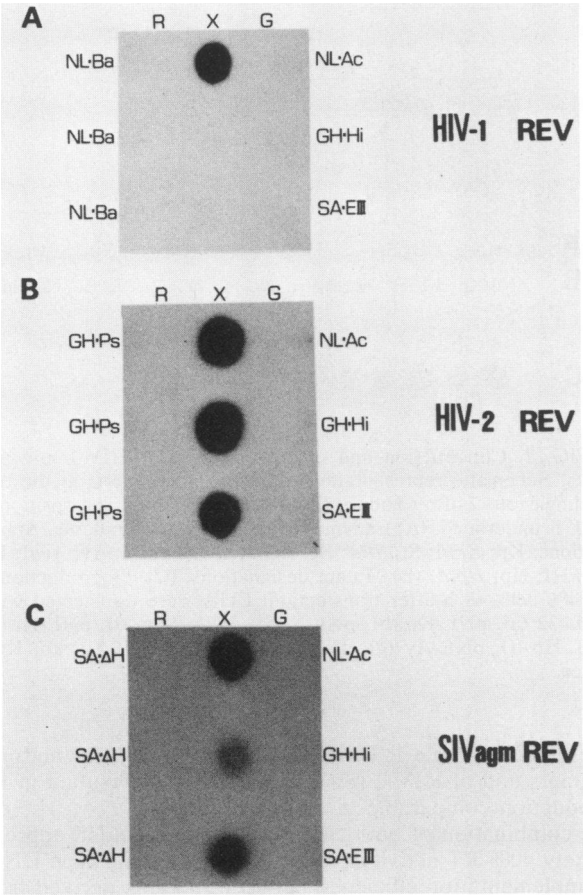

FIG. 3. Complementation between *gag* and *rev* mutants. SW480 cells were transfected with both *gag* and *rev* mutant DNAs, and RT production 48 h after transfection was determined. Lanes R and G show the results of single transfections, whereas lanes X show those of double transfections. Mutants used for transfection are indicated on both sides of the autoradiogram. See the legend to Fig. 1 for abbreviations.

RRE as well as the *rev* gene product itself. Figure 6A shows amino acid sequences of the *rev* proteins of HIV-1, HIV-2, and SIV_{AGM} deduced from nucleotide sequences. Although overall homology was low (30 to 35%), an arginine-rich region was quite conserved and located at similar positions in the three *rev* proteins. Our three *rev* mutants contain alterations in the downstream region of this conserved sequence. The *rev* protein of HIV-1 was not uniquely different among the three viruses, as far as primary amino acid sequence was concerned. Figure 6B illustrates stable predicted RNA stem-loop structures, which fully encompass the minimal RRE defined by mutational analysis (HIV-1) (15) and by nucleotide homology to HIV-1 RRE (HIV-2 and SIV_{AGM}) (about 65% homology). The location in the genome of putative RREs of HIV-2 and SIV_{AGM} (Fig. 1, 2, and 5) and their deduced secondary structures are very similar to those of HIV-1. The free energies (8) for the predicted structure of HIV-2 and SIV_{AGM} are −91.3 and −104.5 kcal (1 cal = 4.184 J)/mol, respectively, comparable to that of HIV-1 (−88.4 kcal/mol), indicating that the secondary structure of RRE RNAs of the three viruses would be equally stable. The structural analysis presented in this section suggests that the *rev* protein of HIV-1, HIV-2, and SIV_{AGM} exerts its function through a similar mechanism. However, no striking difference to explain the distinct patterns of positive complementation among mutants from these three viruses was observed.

TABLE 1. Complementation between *rev* and *gag* mutants[a]

Complementation pairs	Relative RT production in[b]:		
	Expt. 1	Expt. 2	Expt. 3
NL-432 alone	100	100	100
NL-Ba × NL-Ac	96	115	81
NL-Ba × GH-Hi	ND	ND	ND
NL-Ba × SA-EIII	ND	ND	ND
GH123 alone	100	100	100
GH-Ps × NL-Ac	90	149	110
GH-Ps × GH-Hi	111	120	123
GH-Ps × SA-EIII	42	68	51
SA212 alone	100	100	100
SA-ΔH × NL-Ac	69	64	51
SA-ΔH × GH-Hi	10	14	11
SA-ΔH × SA-EIII	23	58	39

[a] SW480 cells were transfected with DNAs indicated, and RT production in the culture fluids 48 h later was determined.

[b] Data for experiment 1 was taken from Fig. 3. RT activity expressed in cotransfectants is relative to that produced by wild-type DNA. ND, No detectable RT activity.

DISCUSSION

A major finding of our experiments involving the cotransfection of *rev* and *gag* mutants into SW480 cells is that HIV-1 *rev* cannot be substituted by HIV-2 and SIV_{AGM} *rev* (Fig. 3 and 4) (Table 1). Because of the nature of our system (monitoring RT production and *gag* expression in the cells transfected with mutant proviral clones), there is a possibil-

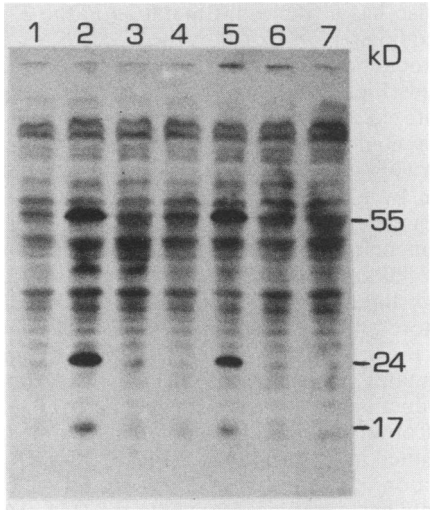

FIG. 4. Detection of *gag* proteins produced in transfected SW480 cells by Western blotting by using a serum specimen from an infected individual. Cell lysates were prepared from cells transfected with: pUC19, lane 1; pNL432 (HIV-1 wild type), lane 2; pNL-Ac (HIV-1 *gag* mutant), lane 3; pNL-Ba (HIV-1 *rev* mutant), lane 4; pNL-Ba and pNL-Ac, lane 5; pNL-Ba and pGH-Hi (HIV-2 *gag* mutant), lane 6; and pNL-Ba and pSA-EIII (SIV_{AGM} *gag* mutant), lane 7. kD, Kilodaltons.

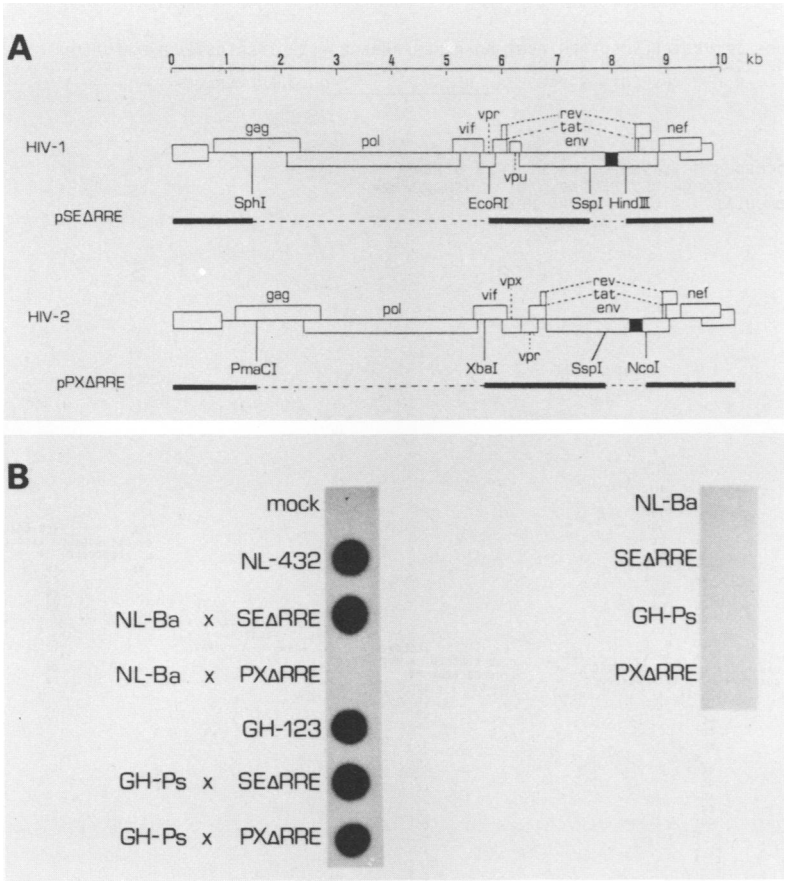

FIG. 5. Complementation between *rev* and large deletion mutants. (A) Structure of deletion mutants of HIV-1 (pSEΔRRE) and HIV-2 (pPXΔRRE) together with schema of genome organization are shown. The location of deletion (horizontal dashed lines) and restriction sites used to introduce deletions are indicated. Closed boxes represent RREs. (B) RT production of SW480 cells 48 h following transfection. DNAs used for transfection are indicated to the left of the autoradiogram. See the legend to Fig. 1 for abbreviations.

ity that gene products other than the *rev* protein, particularly *vpu* (unique to HIV-1), might affect the results. However, we (16) and others (25) have already demonstrated that a *vpu* mutation has no effect on RT production in transient transfection assays. In addition, the expression of HIV-1 *vif*, *vpr*, *env* (Fig. 2), and *nef* is not required at all for transient RT production (2, 14, 16, 22, 25). We also showed that *tat* can be exchanged among HIV-1, HIV-2, and SIV_{AGM} (19). The results of Western blotting analysis (Fig. 4) parallel the RT data (Fig. 3) (Table 1). This fact indicates that the lack of RT production in some cotransfectants was not ascribed to a gene function required for the late stage of virus replication (such as virion assembly and maturation). On the basis of these data, we conclude that failure of complementation of the HIV-1 *rev* mutant by HIV-2 and SIV_{AGM} can be ascribed, at least in part, to *rev* function itself. The results of complementation experiments between large deletion mutants and *rev* mutants (Fig. 5) also support this conclusion.

Cotransfection of the *rev* mutant of SIV_{AGM} and *gag* mutants resulted in RT production to various degrees (Fig. 3C) (Table 1). This is in contrast to the data for the HIV-2 *rev* mutant (Fig. 3B) (Table 1). Because functional RT came from the same *rev* mutant, the observed heterogeneity with respect to RT activity would be explained by the function of a gene or sequence other than the *pol* gene. *rev* and RRE may be involved in this variation. We also noticed that a small amount of RT was produced even in the cotransfectant of SIV_{AGM} *rev* and *gag* mutants relative to that of the positive control (Fig. 3C) (Table 1). This might reflect that *rev* activity of SIV_{AGM} is weak and that relatively more *rev* protein is necessary for the full augmentation of virus replication.

At present, we do not know the molecular basis of the complementation data presented above. Particularly, the observed noncomplementability of the HIV-1 *rev* mutant by other viruses is important. One possible explanation is that some of the mutants are readily corrected by recombination with the other genome and others are not, giving the results reported above. However, this is unlikely, because the amounts of RT produced were quite high relative to those from positive controls (Table 1). Furthermore, the large deletion mutants (which lack the *pol* gene and RRE) designed to assess recombinational complementation behaved exactly like the *gag* mutants in the cotransfection experiments (Fig. 5). Four other possibilities to explain the lack of complementation of the HIV-1 *rev* mutant by other virus DNAs can be considered. (i) HIV-1 *rev* protein is distinct from others. The *rev* protein may have stronger *rev* activity than others. (ii) HIV-1 RRE is unique. The RRE may be recognized only by HIV-1 *rev* protein. (iii) There is an unknown signal sequence(s) which may contribute to some mechanism(s) for activation or repression of HIV-1. (iv) Any combination of the above possibilities can be considered. Comparative analysis of structures associated with *rev* func-

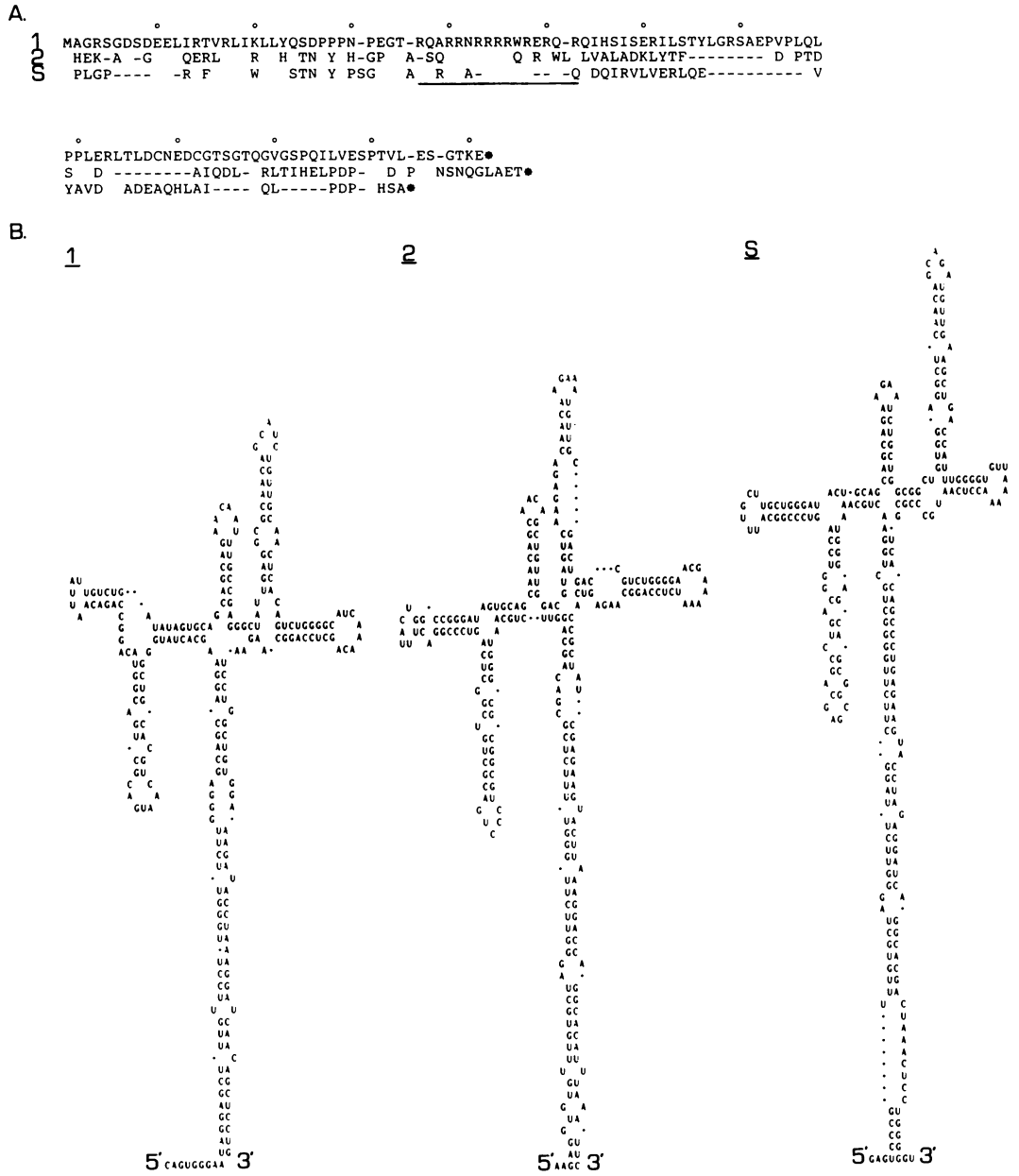

FIG. 6. Predicted amino acid sequence of *rev* protein (A) and secondary structure of RRE (B). 1, 2, and S represent HIV-1, HIV-2, and SIV_{AGM}, respectively. Nucleotide sequence data of HIV-1 432 strain Los Alamos data bank), HIV-2 GH-1 strain (13a), and SIV_{AGM} TYO-1 strain (9) were used. (A) Conserved arginine-rich region (underlined) and end of *rev* protein (asterisk) are shown. Blank spaces indicate the identical amino acid with that of HIV-1. Dashes represent the deletion of the amino acid. (B) Putative RREs of HIV-2 (nucleotides 7760 to 8010) and SIV_{AGM} (7363 to 7633) were determined by homology match with HIV-1 RRE (7749 to 7993), and a stable structure of RNA was predicted (15). The free energy for each predicted secondary structure is −88.4 kcal/mol (HIV-1), −91.3 kcal/mol (HIV-2), and −104.5 kcal/mol (SIV_{AGM}) (8). The 5′ and 3′ ends are indicated.

tion has revealed no essential differences among the three viruses (Fig. 6). However, the data presented here do not exclude any explanations. Detailed functional studies are required to elucidate the mechanism underlying *rev* complementation.

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