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Novel Design Architecture for Genetic Stability of Recombinant Poliovirus: the Manipulation of G/C Contents and Their Distribution Patterns Increases the Genetic Stability of Inserts in a Poliovirus-Based RPS-Vax Vector System

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Poliovirus has been studied as a live recombinant vaccine vector because of its attractive characteristics. The genetic instability, however, has hampered recombinant polioviruses (PVs) from being developed as an appropriate vaccine. A variety of different foreign inserts were cloned directly into our poliovirus Sabin 1-based RPS-Vax vector system, resulting in the production of recombinant PVs. The genetic stability of each recombinant PV was examined during 12 rounds of consecutive passage. It was found that the genetic stability of the recombinants was not well correlated with their insert size. Instead, elevated stability was frequently observed in recombinants with inserts of high G/C contents. Furthermore, a comparative study using different constructs of the human immunodeficiency virus *env* gene revealed that the internal deletion of the unstable insert was seemingly caused by the presence of the adjacent A/T-rich region. The instability of these inserts was completely remedied by (i) increasing the G/C contents and (ii) replacing the local A/T-rich region with the G/C-rich codon without a change of the amino acid. This means that stability is closely associated with the G/C content and the G/C distribution pattern. To see whether these findings can be applied to the design of genetically stable recombinant PV, we have reconstructed the heteromultimeric insert based on our design architecture, including the above-mentioned G/C rules and the template/ligation-free PCR protocol. The heteromultimeric insert was very unstable, as expected, but the manipulated insert with the same amino acid sequence showed complete genetic stability, not only in vitro, but also in vivo. Even though this guideline was established with our RPS-Vax vector system, to some extent, it can also be applied to other live viral vaccine vectors.

Many attempts have been made to manipulate poliovirus (PV) as a favorable vaccine vector because of its attractive characteristics of safe use, low cost, convenient administration, and long-lasting protective immunity in both mucosal and systemic immune responses, which have been established for decades. Four different strategies have been employed to date in the development of PV-based versatile vaccine vectors. Early efforts were devoted to attaching small epitopes to one of the neutralizing domains in the capsid protein (9, 10, 14, 17, 36). However, widespread application of this hybrid virion strategy was limited by the size of the epitope (smaller than 25 amino acids) that could be inserted. The second strategy was to replace one of the structural viral genes with the protein insert (8, 9, 12, 30, 34, 35). Using this method, desired foreign antigens could be incorporated without size restriction; however, the recombinant virus is replication defective and requires a helper virus or capsid protein-expressing system for each cycle of propagation (12, 30, 34, 35). The propagation-defective viral vector is attractive for its safety feature, even in the neuronal delivery of cytokine (8), but may also pose obstacles for inducing vigorous immune responses.

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The third strategy focused on construction of the dicistronic genome by placing a foreign insert together with the 5' noncoding internal ribosome entry site (1, 2, 24, 27, 45), but this vector strategy was flawed by frequent observation of the gradual deletion of the foreign gene in the early stages of serial passage. The cause of the genetic instability was not determined, but it was apparently related to the spatial restriction of the PV capsids. The last strategy, called the polyprotein fusion strategy, was directed at fusing the foreign insert to either the N terminus or the junction between the capsid proteins and the nonstructural proteins in the PV polyprotein with an artificial cleavage site produced by endogenous proteases (5, 26). Accordingly, the foreign insert is cleaved by one of the proteases and remains as a free form in the cytoplasm after being translated together with the viral polyproteins.

Several foreign inserts were integrated and expressed successfully from the poliovirus Mahoney-origin vector (5, 13, 44) or poliovirus Sabin 3-based vector (26). Exploiting the polyprotein fusion strategy (5, 26), we have constructed a recombinant poliovirus Sabin 1-based vaccine vector, referred to as RPS-Vax (18), which has multiple cloning sites and an artificial 3C recognition site at the N-terminal end of the Sabin 1 polyprotein. A number of foreign inserts were incorporated into the RPS-Vax vector system, followed by the production of many recombinant PVs.

However, one of the most important obstacles for the

polyprotein fusion strategy seems to be the genetic instability of the recombinant PVs. Many of our recombinant PVs were genetically unstable, as described previously (29, 39). Previous reports have suggested that the genetic instability of the recombinant PV would be associated with the insert size limitation and/or genetic recombination within (39) or between sequences during minus-strand synthesis (29, 42). Nevertheless, a clear molecular mechanism controlling insert stability has not been well established.

The stability of the constructs was predicted with difficulty from the sequence and had to be determined empirically. It was anticipated, consequently, that the application of the recombinant vaccine would be greatly expedited if the stability of the given insert could be assessed prior to experimental onset. For this reason, we have examined potential factors governing the genetic stability of foreign inserts by constructing and exploring many different recombinant PVs, which contain a series of different original or adjusted sequences of foreign inserts, and then concluded that (i) the insert genetic stability is strongly associated with the G/C contents and their distribution patterns within the size limitation and (ii) the insert genetic stability could be markedly enhanced by manipulating the insert on the basis of our insert-designing architecture described in this paper.

MATERIALS AND METHODS

Cells and viruses. HeLa cells (HeLa S3; American Type Culture Collection [ATCC]) were used for transfection experiments and poliovirus propagation. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (Gibco-BRL), 1× penicillin-streptomycin (Gibco-BRL), and 50 μg of gentamicin per ml. Mahoney (wild-type neurovirulent strain), Sabin 1 (attenuated strain of Mahoney), and recombinant PVs were produced by transfection of HeLa cell monolayers with viral RNAs, which were transcribed from reconstructed or original cDNAs. Recombinant cDNAs and their RNA transcripts are described below. Wild-type PVs Lancing and Leon were obtained from the ATCC.

Mice. Human poliovirus receptor-transgenic (ICR-PVR-Tg21) mice (21) were kindly provided by A. Nomoto, University of Tokyo, Tokyo, Japan. These mice were bred and maintained in the Animal Resources Center at the Korea Research Institute of Bioscience and Bioengineering under specific-pathogen-free conditions.

Materials. All enzymes used in this experiment were purchased from New England Biolabs (NEB; Beverly, Mass.), Gibco-BRL (Gaithersburg, Md.), Bio-Rad (Hercules, Calif.), Boehringer Mannheim Biochemicals (Mannheim, Germany), and Bioneer (Seoul, Korea). Cell culture media and serum were obtained from Gibco-BRL. *Escherichia coli* JM109 (Promega, Madison, Wis.) and XL1-Blue (Stratagene, La Jolla, Calif.) were used for transformation and propagation of plasmids. CJ236 and MV1190 for site-specific insertion and mutagenesis and were purchased from Bio-Rad (Hercules, Calif.).

Recombinant plasmid. The RPS-Vax system, developed in our laboratory, consists of pTZ-18/R (Pharmacia Biotech, Uppsala, Sweden) as a backbone and full-length Sabin 1 recombinant cDNA, containing multiple cloning sites and a viral protease recognition site at the N-terminal end of the Sabin 1 polyprotein (18). This system was mainly used for cloning and production of recombinant PV. The Sabin 1 cDNA, pVS(1)IC-0(T), was kindly provided by A. Nomoto (Institute of Medical Science, The University of Tokyo, Minato-ku, Japan). The Mahoney cDNAs, pEV104 and pT7PV1–5, were kindly provided by E. Wimmer (State University of New York, Stony Brook). The HIV-1 (pHXB2 and pSHIV$_{89.6}$-3') and the simian immunodeficiency virus (SIV) (pSIVmac239) cDNA clones were supplied by the NIH AIDS Research and Reference Reagent Program (Rockville, Md.). The cDNA clone (adw) of the hepatitis B virus (HBV) was obtained from the ATCC. The DNA clone of hepatitis C virus (HCV) was kindly provided by Y. C. Sung (Pohang University, Pohang, Korea). The synthetic oligonucleotides were supplied by Bio-Synthesis, Inc. (Lewisville, Tex.) and Universal DNA, Inc. (Tigard, Oreg.), Genotech Co. (Daejeon, Korea), and Bio Basic, Inc.

Construction of recombinant PV cDNAs. The DNA sequences coding for the various antigenic epitopes was amplified by PCR (11) with primers designed to have *Sst*II and *Eag*I restriction sites at the 5' and 3' ends, respectively. The PCR products were purified and digested with *Sst*II and *Eag*I (NEB) and subcloned into the corresponding sites of the RPS-Vax as illustrated in Fig. 1A. In the case of the multimeric concatemer epitope, we designed the insert DNA by using the proper codon as specified in the general rules described in this paper. All of these plasmid constructs were tested for their capacity to produce progeny viruses by transfection experiments.

In vitro transcription and transfection of HeLa cells. Plasmid DNAs were linearized with *Sal*I (NEB) and purified by extraction three times with phenol-chloroform, followed by ethanol precipitation to minimize the contamination of RNase. One microgram of linearized plasmid DNA was transcribed in vitro with 5 U of T7 RNA polymerase (NEB) per ml in the reaction buffer (40 mM Tris-HCl [pH 8.0], 8 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl, 5 mM dithiothreitol, 1 U of RNasin per ml, 2 mM deoxynucleoside triphosphate mix) for 30 min at 37°C. Monolayers of HeLa cells (3×10^5) were grown in 60-mm dishes. Less than 1 μg of RNAs was transfected into the cells by a DEAE-dextran procedure (7, 20). Cells were incubated until a full cytopathic effect was observed, and a second passage of the supernatants was performed at this stage. Titers of viruses in the supernatant of these transfected cultures were determined by endpoint dilution, such as 50% tissue culture infective dose ($TCID_{50}$), or a plaque assay on HeLa cell monolayers.

Virus infection and one-step growth curve. HeLa cell monolayers grown in 60-mm plates were infected with wild-type or recombinant polioviruses at a multiplicity of infection (MOI) of 10. The virus was allowed to adsorb to the cells for 1 h at 37°C. Unbound viruses were removed by washing twice with phosphate-buffered saline (PBS), and 3 ml of warm DMEM containing 10% fetal bovine serum was added. The supernatants were collected every 3 h and then titrated for the amounts of progeny virus at each time after infection. The virus titers were determined by a $TCID_{50}$ assay.

Serial passages, viral RNA extraction, and RT-PCR. Each recombinant virus generated from transfection of the HeLa cells with recombinant viral RNA transcript as described above was consecutively introduced into HeLa cells. In each passage, HeLa cell monolayers were infected with the recombinant virus harvested from the previous infection at an MOI of 10 as described above and then cultured for 18 to 24 h. Supernatants were harvested as a virus source for each passage when full cytopathic effect appeared. They were mixed with 4% polyethylene glycol and 0.5 M NaCl (final concentrations) and allowed to stand for 10 min at room temperature and then precipitated by centrifugation for 10 min at 15,000 rpm. Viral RNA was extracted from the pellet with phenol-chloroform followed by ethanol precipitation. Reverse transcription (RT)-PCR was performed for each viral RNA sample with Sabin 1 primers (680–697/sense, 5'-CAT TGA GTG TGT TTA CTC-3', and 797–814/antisense, 5'-GGT AGA ACC ACC ATA CGC-3') using a Pre-Mix RT-PCR kit (Bioneer Inc.) by following the instructions given in the vendor's manual. PCR was performed for 25 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s. Amplified cDNA fragments were analyzed in agarose gels.

Western blot analysis. HeLa cells were infected with wild-type or recombinant PVs at an MOI of 10 at each passage. Cells were harvested 18 h after infection, washed and resuspended in PBS, and then mixed with the same volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.03% bromophenol blue, 0.01 mg/ml xylene cyanol). After being boiled for 10 min, samples were applied to an SDS–12% polyacrylamide gel and then transblotted to a nitrocellulose membrane using a semidry gel transfer system (Bio-Rad). Blotted membranes were screened with monkey anti-SIV serum (kindly provided by G. Hunsmann, German Primate Center, Gottingen, Germany) or mouse antiserum obtained from a mouse immunized with specially designed recombinant proteins (bovine serum albumin-conjugated PV2,3-specific epitope peptides) for this experiment (see Fig. 6C). An ECL chemiluminescence detection kit (Amersham) or alkaline phosphatase-conjugated secondary antibody and nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) were used to detect the specific bands.

Rapid synthesis of the long concatemeric DNA by template/ligation-free PCR. In order to make a heteromultimeric epitope-concatemer that contains several multiple silent mutations, we established a primer-annealing ligation-free PCR method by modification of previous protocols (19, 25, 41). In principle, a long insert DNA can be synthesized by simple PCR without an original template DNA and an extra ligation step. The entire sequences of the designed PVm-150 and PVm-150/M were divided into eight segments with seven different complementary regions (CR), so that one segment was used as the template while being primed by the other. Each CR consists of 15 bases and more than 50% G/C content.

Our strategy consisted of two consecutive procedures, maturation and amplification (schematically illustrated in Fig. 1B). As a maturation procedure, five

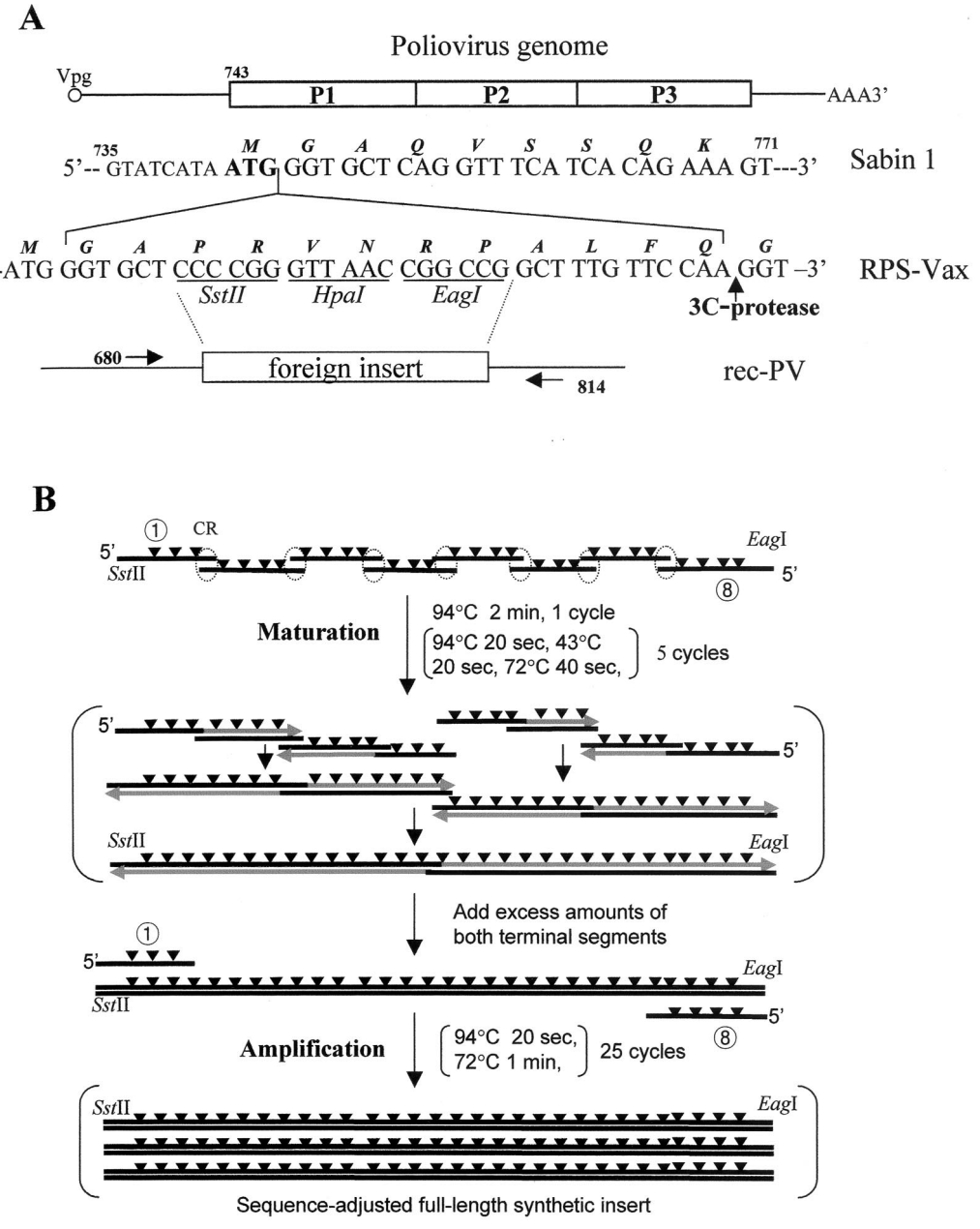

FIG. 1. (A) Schematic diagram of Sabin 1-derived RPS-Vax viral genome and RPS-Vax-derived recombinant PVs. The RPS-Vax genome contains a multiple cloning site (MCS) and 3C protease cutting site at the N-terminal end of the long polyprotein. A foreign insert integrated into the MCS can easily be detected by RT-PCR with the primer set indicated by arrows. (B) Schematic illustration of the template- and ligation-free PCR procedures that was used for the synthesis of long heteromultimeric concatemers or heavily sequence-adjusted inserts without template DNA. CR, complementary region. The solid triangles represent the mutation sites on the synthetic DNA. The circled numbers 1 and 8 represent long synthetic primers of between 60 and 100 bases in length.

rounds of denaturation (at 94°C for 20 s), annealing (at 43°C for 20 s), and polymerization (at 72°C for 40 s) steps were performed to anneal eight DNA fragments (7.5 pmol of each in 100 μl of reaction buffer) into a full-length DNA. PCR amplification followed the reaction for 25 cycles at the conventional PCR conditions (94°C for 20 s and 72°C for 1 min) in the presence of 45 pmol of both terminal segments as a primer set for preparation of full-length synthetic inserts. PVm-137/M and PVm-132/M inserts were also synthesized by following this protocol.

Recovery of recombinant PV from the inoculated Tg-PVR mice. Transgenic PV receptor (Tg-PVR) ICR mice 6 to 8 weeks old were inoculated intracerebrally with 10^7 PFU of recombinant poliovirus using a microsyringe and specially designed 26- to 30-gauge needles. Mice were sacrificed daily after inoculation. The spleen was separated from each mouse and homogenized using a Dounce homogenizer (15 strokes). The homogenates were centrifuged at 3,000 rpm for 20 min, and the supernatants were transferred into HeLa cell monolayers to recover the virus passed in vivo. They were tested for genomic integrity by RT-PCR, as described above.

RESULTS

Construction of various chimeric viruses using the RPS-Vax system. We have constructed a number of PV recombinants by cloning foreign genes into the multiple cloning sites of the

TABLE 1. Genetic stability and other biochemical characteristics of RPS-Vax-derived recombinant PVS[a]

Foreign insert	Insert size[b] (bp)	Hydrophobicity index[c]	G/C content[d] (%)	ΔG[e] (kcal/mole)	Virus produced[f]	No. of stable passages[g]
Monomers						
SIV gag-100	300	−0.53	45.0	−101.7	+	>12
SIV gag-100/M¶	**300**	**−0.53**	**34.0**	**−84.3**	**+**	**4***
SIV gag-114	342	−0.45	44.7	−105.6	+	>12
SIV p27-167	501	−0.55	43.7	−92.6	+	5†
SIV p27-150	450	−0.48	43.8	−102.3	+	>12
SIV env-108	324	−0.89	35.4	−82.8	+	5*
SIV env-108/M¶	**324**	**−0.89**	**50.3**	**−112.7**	**+**	**>12**
HIV-1 env-98	294	−0.53	30.6	−56.2	+	2*
HIV-1 env-98/M¶	**294**	**−0.53**	**34.7**	**−65.0**	**+**	**>12**
HIV-1 env-83	249	−0.60	32.5	−48.9	+	>12
HIV-1 env-71	213	−0.51	30.0	−36.5	+	4*
PV 2-127	381	−0.28	47.5	−113.1	+	>12
PV 2-118	354	−0.27	44.6	−103.9	+	>12
PV 3-110	330	−0.44	50.0	−110.4	+	>12
HCV core-160	480	−0.84	62.3	−196.3	+	3†
HCV core-100	300	−1.24	60.3	−172.5	+	>12
HBVsAg-100	300	+0.89	49.3	−118.5	−	N/A
HBVsAg-76	228	+0.36	50.2	−82.8	−	N/A
Heterodimers						
PV2.3-131	393	−0.23	48.0	−106.2	+	7
PV2.3–112	336	−0.13	43.8	−110.2	+	>12
HBVcs	306	+0.02	46.4	105.2	+	>12
Concatenate multimers‡						
HIV-1 mV3	360	−0.79	33.9	−58.1	+	3*
HIV-1 PND8	240	+0.19	43.3	−76.8	+	9
Designed multimers§						
PVm-150/M¶	**450**	**−0.51**	**57.3**	**−138.9**	**+**	**>12**
PVm-137/M¶	**411**	**−0.64**	**58.4**	**−132.9**	**+**	**>12**
PVm-132/M¶	**396**	**−0.46**	**58.8**	**−147.6**	**+**	**>12**

[a] Symbols: †, insert DNA longer than acceptable size (>450 bp); *, insert DNA containing A/T-rich region (<25% in about 30 bp); ‡, insert DNA having repeated sequences as a concatamer; §, insert DNA having heteromultimeric epitope which was synthesized on the basis of our design architecture; and ¶, descriptions in bold reveal the mutant clones whose sequences were adjusted on the basis of our design architecture without a change of amino acid.
[b] All inserts also have an additional 27 bp that codes for the multiple cloning site and 3C protease site in RPS-Vax (not included in the table).
[c] Mean hydrophobicity index of coding inserts that was calculated by assigning each amino acid a numerical value.
[d] Average G/C contents of inserts counted by DNASIS program (window size set at 9).
[e] ΔG indicates the free energy of the insert RNA in secondary structure predicted by the DNASIS program (sets maximum bulge and interior loop size at 30).
[f] Chimeric virus produced (+) or not (−) by transfection of HeLa cells with recombinant RNA transcript.
[g] Maximum number of passages still showing the genetic stability of the insert, which was determined by RT-PCR. N/A, not applicable.

Sabin 1-derived RPS-Vax vector (18). Foreign genes were derived from major antigenic determinants in the structural proteins of other pathogenic viruses, including HIV, SIV, and hepatitis B and C viruses, as well as other PV strains. The cDNA fragments of the antigen coding region were individually synthesized or PCR amplified and then ligated into the multiple cloning sites of the RPS-Vax vector to produce a recombinant PV cDNA clone. Each clone was in vitro transcribed into recombinant viral RNA and then transfected into HeLa cells as described elsewhere (7), resulting in the production of recombinant PVs.

The replication-competent recombinant viruses, as judged by the cytopathic effect of the transfected HeLa cells, and their biochemical characteristics are summarized in Table 1. Some of the recombinants were designed to have homo- or heteromultimeric antigenic determinants to enhance the epitope-specific immune response or to elicit a dual or multi-immunogenicity by a single administration. We have constrained the overall size of the insert to less than 500 bases because our repeated experiments revealed that the genetic stability of the RPS-Vax-based recombinant viruses decreases markedly with inserts larger than this.

The kinetics of RNA synthesis of replication-competent PV recombinants was almost similar to that of the control Sabin 1 (data not shown). However, the replication capacity of the recombinant viruses was a maximum of one log lower than that of the control Sabin 1 in the one-step growth experiments (data not shown), as described previously (18). Stable expression of the foreign antigens during the replication of the recombinant PVs in HeLa cells was confirmed by Western blotting and radioimmunoprecipitation (RIP) analyses, using patient sera as well as either polyclonal or monoclonal antibodies against individual antigen. (Parts of the data are described in the following section.) In most cases, the immunoblotting analyses showed a single prominent band of a correctly processed polypeptide accompanied by a number of minor bands at higher molecular weights. These are likely due to the incomplete processing of fusion protein.

Each recombinant PV showed different genetic stability depending on its foreign insert. The genetic stability of each recombinant PV was examined by RT-PCR (and sequencing, if necessary,) and Western blotting for the foreign insert during consecutive passages. We have extended the number of passage cycles to 12 because the lower number (<6) of passage cycles generally used in previous studies (29, 39) was considered insufficient to draw any decisive conclusion about its sta-

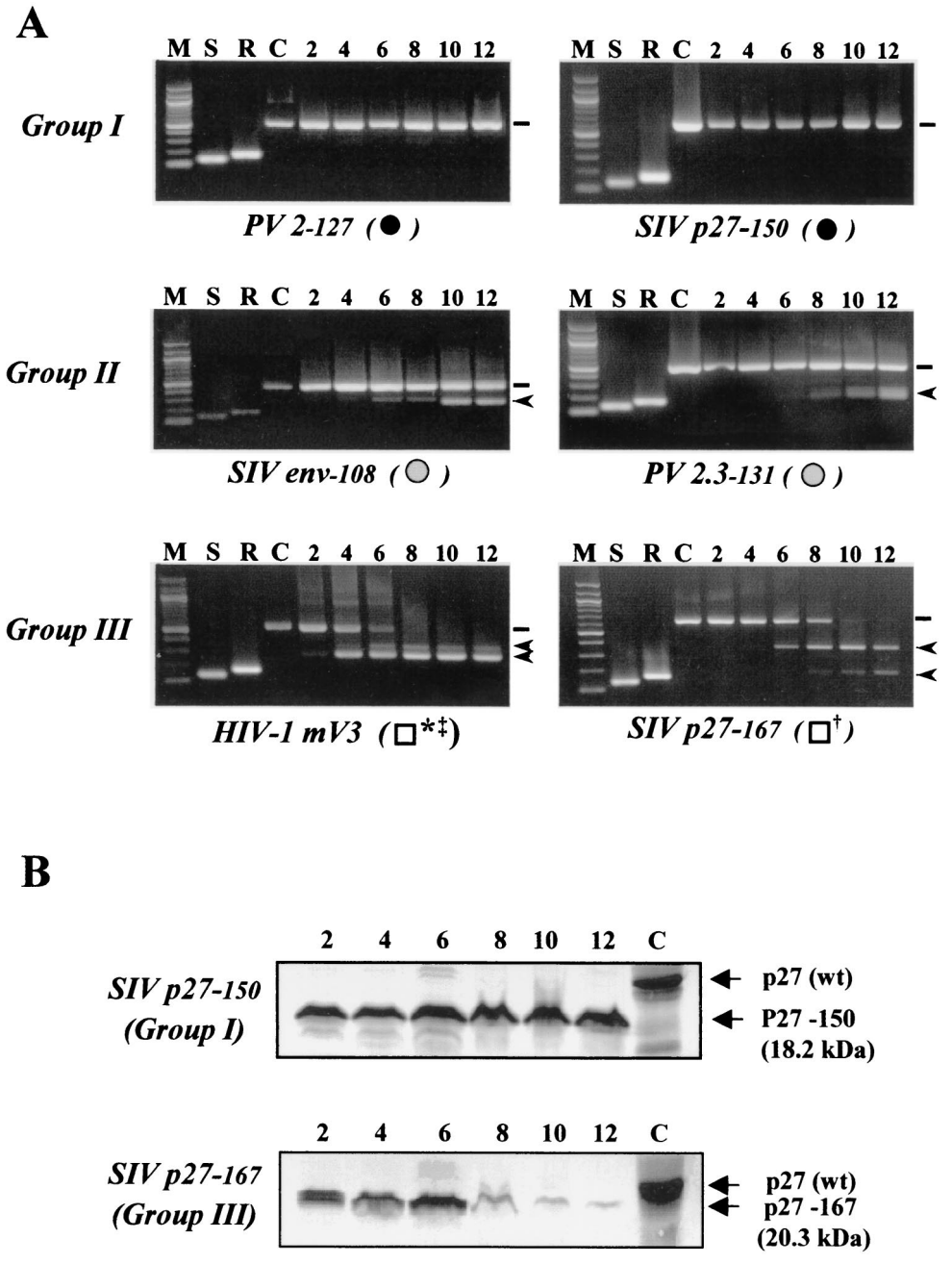

FIG. 2. Genetic stability of recombinant PVs. Each recombinant PV, containing a different foreign insert, was tested for genetic stability by RT-PCR and Western blot during consecutive passages in HeLa cell monolayers. (A) RT-PCR patterns of recombinant PVs during consecutive passages. Recombinant PVs were categorized into three groups based on the patterns of insert stability described in the text. The symbol in parentheses next to the name of each insert represents the group to which it belongs (described in Fig. 3) and the insert specificity, described in Table 1. Lanes: M, 100-bp ladder size markers; S, poliovirus Sabin 1; R, RPS-Vax vector-derived virus; C, insert-containing recombinant plasmid. The numbers indicate the passage cycle of each recombinant PV. The bar and arrowhead indicate the original and truncated forms of the inserts, respectively. (B) Passage stability of the recombinant PVs examined for insert expression by Western blot. The numbers indicate the passage cycle of the recombinant PV to be infected into HeLa cells. Recombinant PV-infected HeLa cell lysates and control SIV (C) were screened by Western blotting with monkey anti-SIV serum. Arrows indicate the wild-type (wt) p27 of SIV and recombinant p27 expressed from recombinant PV.

bility. In each passage, the recombinant PV was harvested at full cytopathic effect, extracted by phenol-chloroform, and then subjected to RT-PCR with Sabin 1 PCR primer sets, as shown in Fig. 1A. The genetic stability of each recombinant was deduced from the gel pattern of PCR products in each of the passages. Based on its apparent genetic stability, we have categorized the recombinant PVs into three classes: stable (group I), metastable (group II), and unstable (group III) recombinants (Fig. 2).

Group I is the class that retains the intact insert without any

truncated form of insert during the entire period of 12 consecutive passages. Recombinant PVs carrying foreign genes, such as PV2–127 and SIV p27–150 (Fig. 2A), typically belong to this group. This pattern of stability was observed in a total of 15 out of 24 different recombinants (63%).

On the other hand, group II is defined as the class that displayed the intact insert throughout the 12 passages, but the truncated form also appeared in the later stages. This pattern of stability was observed in three recombinants, containing an insert of SIV env-108 (monomeric), PV2,3–131 (heterodimeric), or HIV-1 PND8 (concatemeric). As shown in the middle panels of Fig. 2A, the genomic integrity of the antigens, SIV env-108 and PV2.3–131, was stably maintained in the recombinant viruses during the early passages, but was slowly attenuated in the later cycles, as shown by the gradual accumulation of a shortened band (marked by an arrowhead in Fig. 2A). However, a major portion of the virus population still retained the intact insert during the entire 12 passages.

Group III, on the contrary, is characterized as the class showing genetic instability during the 12 passages. In this class, the instability of the gene was so severe that an intact insert band disappeared rapidly in the later stages. This was shown in the recombinant containing HIV-1 mV3, tandem repeated V3 epitope, or SIV p27–167 (bottom panels of Fig. 2A). Three additional monomeric clones (HIV-1 env-98, HIV-1 env-71, and HCV core-160) belong to this group. The genetic deletions of unstable clones were site specific and had progressed moderately.

Each recombinant PV expresses the foreign insert during its replication in the infected cells. Therefore, the genomic stability should also be repeated in protein expression. We examined the correlation between genomic stability and expression stability by Western blotting using two different recombinant PVs, RPS-Vax /SIV p27–150 and RPS-Vax /SIV p27–167 . RPS-Vax /SIV p27 -150 was genetically stable during the entire 12 passages, but the RPS-Vax /SIV p27–167, belonging to group III in genomic stability, was not (Fig. 2A). Consistently, both recombinants showed similar patterns in expression stability, in the Western blot experiments with monkey anti-$SIV_{mac}239$ serum (Fig. 2B), suggesting that the RT-PCR analysis would be enough to determine the genetic stability for each recombinant PV. Small protein bands which might have been expressed from the truncated inserts detected in RT-PCR (Fig. 2A) did not appear in the Western blot at the corresponding sites (Fig. 2B).

Genetic stability of the insert associated with G/C content. In order to determine the effect of the insert size on stability, we examined the size of the foreign insert of each class of recombinants. We found, surprisingly, that stability was not well correlated to the linear size of the insert in the range of 200 to 400 nucleotides (Table 1). This finding led us to reconsider other potential factors influencing the genetic stability of the insert. Given the genetic properties of PV and the deletion pattern of the recombinants, it is very likely that genetic stability is determined mainly by the encapsidation efficiency of the recombinant genome. It was presumed that the content of G/C provides a primitive guideline to measure the spatial compactness of the insert RNA. The G/C content may also be important for the flexibility of the tertiary structure of RNA.

We investigated the G/C content of each RNA insert and then plotted each recombinant on the field of G/C content and insert size to relate it to the apparent stability. As summarized in Fig. 3A, most of the stable inserts (group I), except HIV-1env-83, were found to have a G/C content higher than 40% and a size smaller than 400 bases. On the other hand, the inserts with a G/C content less than 30% seemed to be genetically unstable regardless of the insert size (HIV-1 env-71, 213-bp insert). These results suggest that the stable inserts would form a compact RNA conformation and readily be encapsidated into a rigid viral capsid.

To elucidate the correlation between the genetic stability of the recombinant PV and G/C content of the insert, we adjusted 44 nucleotides on the sequence of the genetically unstable insert SIV env-108 to make it have a higher G/C content (SIV env-108/M) without any change in the amino acid sequence. As shown in Fig. 3B, the sequence-adjusted SIV env-108/M insert having a higher G/C content (50.3%) completely recovered its genetic stability, while its original clone SIV env-108 (35.4%) was genetically unstable during the passages. These results were similarly repeated in the recombinant PVs expressing HIV-1 p24 or Nef (data not shown). On the other hand, to confirm the correlation between G/C content and genetic stability in another way, we reduced the G/C content of the stable insert and tested the genetic stability of the modified recombinant PV. SIV gag-100 was genetically stable, and its G/C content was 45% (Table 1). However, when the G/C content of the insert was reduced to 34% by replacing the 34 G or C sites with A or T on the entire nucleotide sequence (300 bp) without a change of amino acid sequence, the clone SIV gag-100/M lost its genetic stability, as shown in Fig. 3C. These results strongly support our hypothesis that the genetic stability of the recombinant PVs is strongly associated with the G/C content of the insert.

Inserts larger than 450 bp were also unstable, even though they had a G/C content of up to 62.3% (HCV core-160). This means that foreign inserts larger than 450 bp are not acceptable to the RPS-Vax vector system when producing genetically stable recombinant PVs. Certainly, this delimitation of the insert can be maximally introduced in our strategy and is somewhat larger than the size limitation (10 kDa) in the Mahoney vector system, as addressed in the previous report (29).

Even distribution of G/C content is also important for overall insert stability. Of particular interest was a result obtained from the comparative stability study of three inserts derived from an HIV-1 *env* gene. The three different inserts denoted by env-98, -83, and -71 were prepared to include the principal neutralizing domain of the *env* gene (Fig. 4A). Only the env-83 insert displayed complete stability, while the other two inserts, env-98 and -71, showed prominent genetic instability with multiple discrete bands of truncated fragments (Fig. 4B) even though they had very similar G/C contents (30 to 32.5%) (Table 1 and Fig. 4A).

We purified the major truncated fragment in RT-PCR, indicated by an arrow in Fig. 4B, from env-98 and env-71 and then subjected it to DNA sequencing to determine whether this deletion took place in a sequence-specific manner. We identified that regions 165 to 261 and 142 to 264 (in bases) were deleted from env-98 and env-71, respectively, implying that the region between 165 and 261 was a common deletion site (Fig. 4A). Nevertheless, we could not find any short repeated sequences around the deletion site which might have

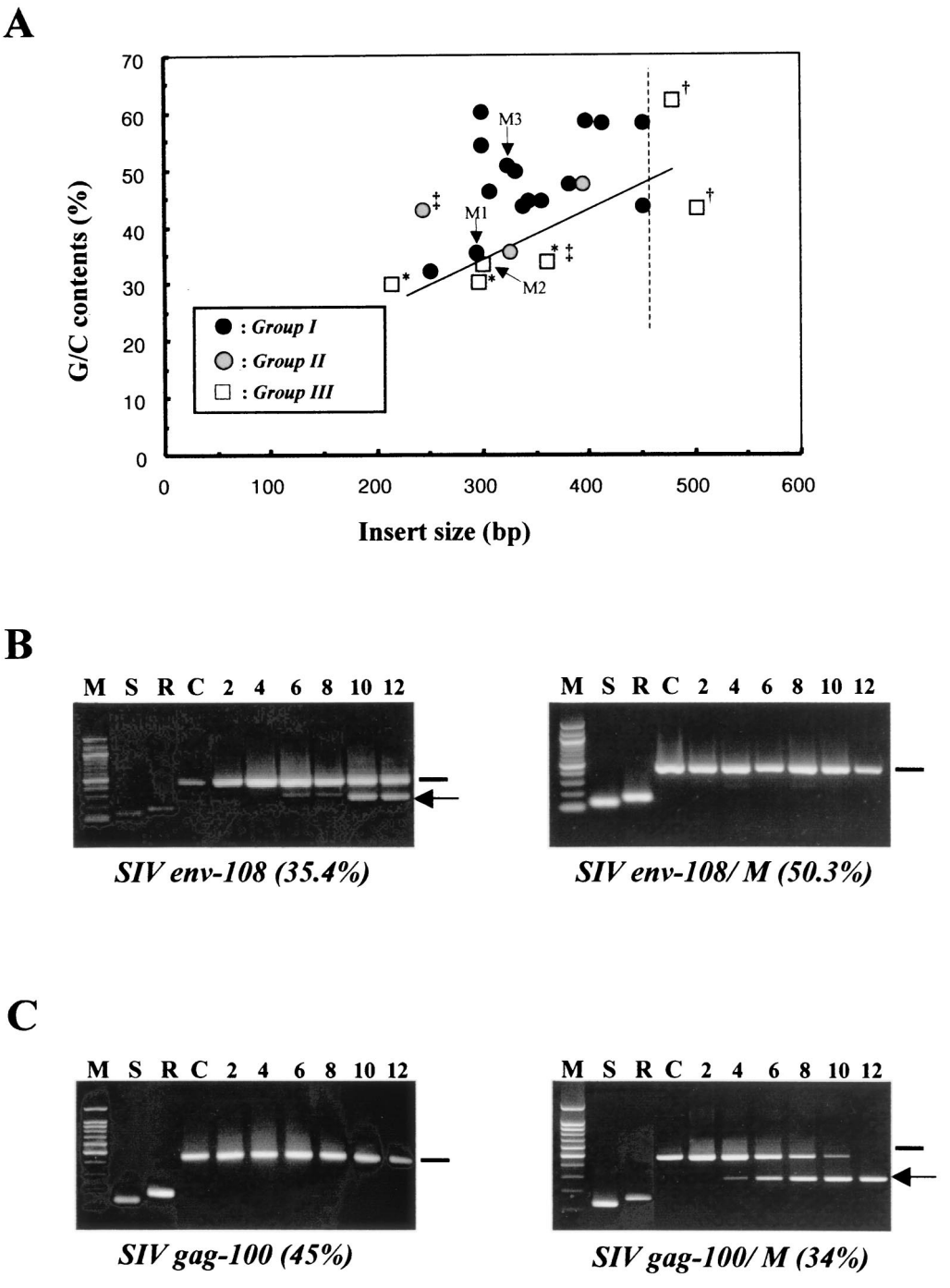

FIG. 3. (A) Genetic stability of the recombinant PVs in association with the insert size and G/C contents of introduced foreign genes. The genetic stability of each recombinant PV, determined by RT-PCR, is illustrated in the diagram in association with insert size and G/C contents. Each line represents the postulated limitations of acceptable insert size (dashed line) and G/C contents (solid line) for stable passages of each recombinant PV. *, A/T-rich region-containing insert. ‡, insert containing multiple-epitope concatemer repeats. †, insert larger than the acceptable size. M1, HIV-1 env-98/M. M2, SIVgag-100/M. M3, SIV env-108/M. (B) Genetic stability of recombinant PV SIV env-108 and its sequence-adjusted form, SIV env-108/M. The values in parentheses represent the G/C content of the insert. (C) Genetic stability of recombinant PV SIV gag-100 and its sequence-adjusted form, SIV gag-100/M. The values in parentheses represent the G/C content of the insert.

caused internal deletion via nonhomologous RNA recombination mechanisms, as suggested in the previous report (30). Particularly noteworthy, however, is the fact that the terminal sequence commonly present at the 3' end of env-98 and env-71 but not env-83 is extremely A/T-rich, and the local G/C content is only 20% (Fig. 5A). We speculated that the local A/T-rich sequence would be a potential cause for the marked genetic instability of the env-98 and env-71 inserts.

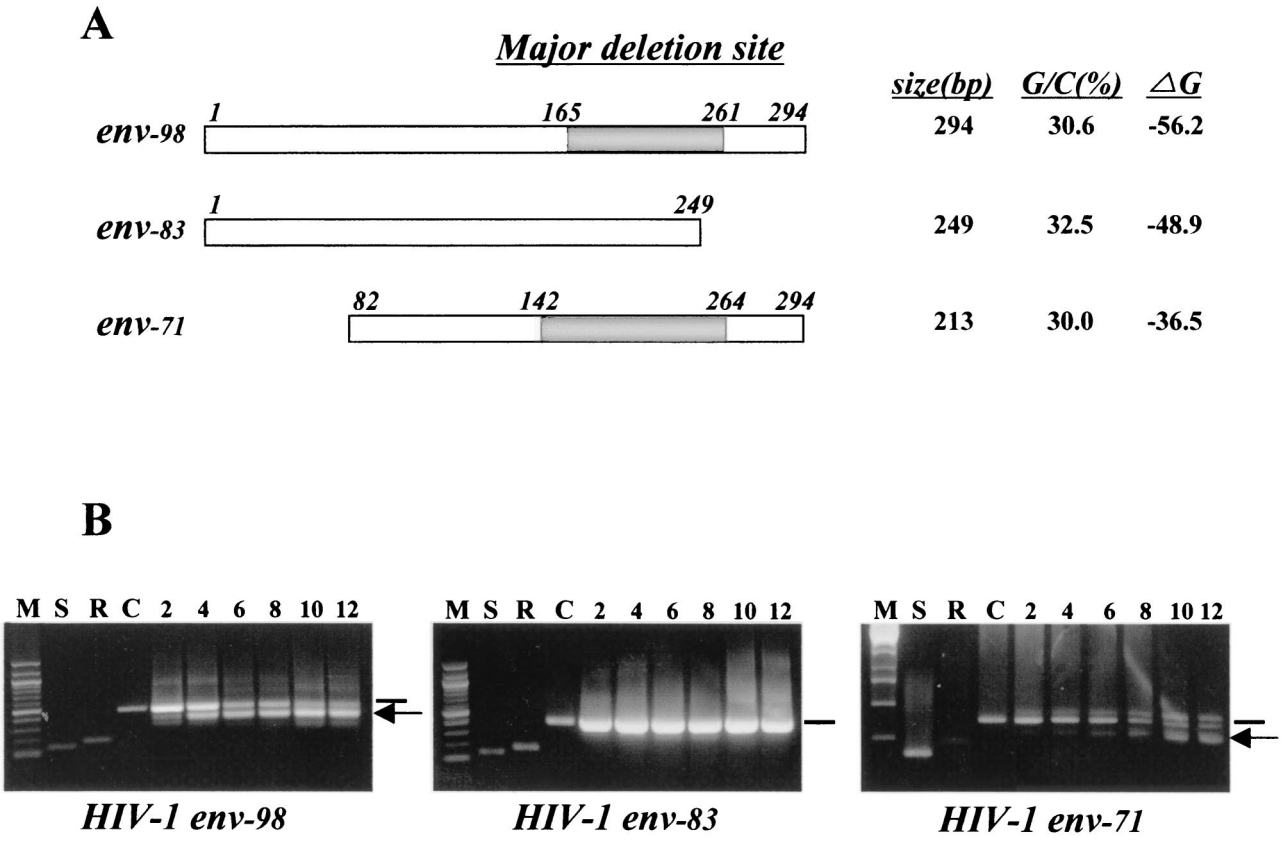

FIG. 4. Genetic stability of recombinant PVs expressing partial fragments of the HIV-1 (HXBc2) *env* gene. Partial fragments of the HIV-1 *env* gene were cloned into the RPS-Vax vector, resulting in production of recombinant PVs. (A) Coverage, G/C contents, and free energy of each *env* gene fragment are schematically illustrated. The solid box in the diagram indicates the major deletion site during the passages of the recombinant PV. The numbering of the 294 bp corresponds to the sequence from 787 to 1080 of the HIV-1 envelope (*env*). (B) RT-PCR patterns of partial *env* fragment-containing recombinant PVs during consecutive passages. Lanes: M, 100-bp size markers; S, poliovirus Sabin 1; R, RPS-Vax vector-derived virus; C, insert-containing recombinant plasmid. The numbers represent the passage cycle of each recombinant PV. The bar and arrow indicate the original and truncated forms of the insert, respectively.

To verify the hypothesis, multiple silent mutations were introduced into the A/T-rich region of the env-98 recombinant by replacing A/T with G/C at a total of 13 different positions around the 3' end and measured the genetic stability of the mutant, called env-98/M (Fig. 5). These substitutions increased the regional G/C content up to 46.7% from 20% (Fig. 5A and 5B). To our surprise, the HIV-1 env-98/M showed complete genetic stability throughout the passage (Fig. 5C). This result was also repeated in the HIV-1 env-71/M integrated recombinant PV (data not shown). These remarkable elevations of the recombinant PV stability by sequence substitutions strongly suggest that the local A/T-rich sequence destabilizes the overall RNA structure and promotes the site-specific deletion of the neighboring region. It also demonstrates that the genetic stability can be manipulated by adjusting the global G/C content of the RNA insert.

Increasing the G/C contents and adjusting the G/C distribution patterns dramatically improved the genetic stability of the recombinant PV containing heteromultimeric inserts. In our experiments, we found that the G/C contents and their distribution patterns are important for the genetic stability of foreign inserts of less than 450 bp in the RPS-Vax-derived recombinant PVs.

In order to verify our findings and to see whether they are applicable for generation of a genetically stable recombinant PV, we constructed three different heteromultimeric repeated inserts (PVm-150, PVm-137, and PVm-132) and their sequence-adjusted forms (PVm-150/M, PVm-137/M, and PVm-132/M, respectively) by ligation-free PCR without a template, as described in Materials and Methods and illustrated in Fig. 1B. Sequence adjustment was performed without a change of amino acid sequences. Among these synthetic inserts, PVm-150 comprises three repeats of the VP1 neutralizing epitopes (12 amino acids) of poliovirus type 2 (Lancing), two repeats (10 amino acids) of poliovirus type 3 (Leon), and two repeats of 5 amino acids (Fig. 6A). PVm-150/M was synthesized by adjusting the sequence of PVm-150 on the basis of our G/C rules (Fig. 6A). The sequence substitution increases the G/C contents and free energy of the PVm-150/M up to 58.4% and −138.9 kcal, respectively (Table 1).

These synthetic genes were cloned into the RPS-Vax system, followed by production of recombinant PVs. RPS-Vax/PVm-150 was genetically unstable (left panel of Fig. 6B), but its sequence-adjusted clone, RPS-Vax/PVm-150/M, having a high G/C content and even G/C distribution without a change in the amino acid sequence, showed perfect genetic stability (right

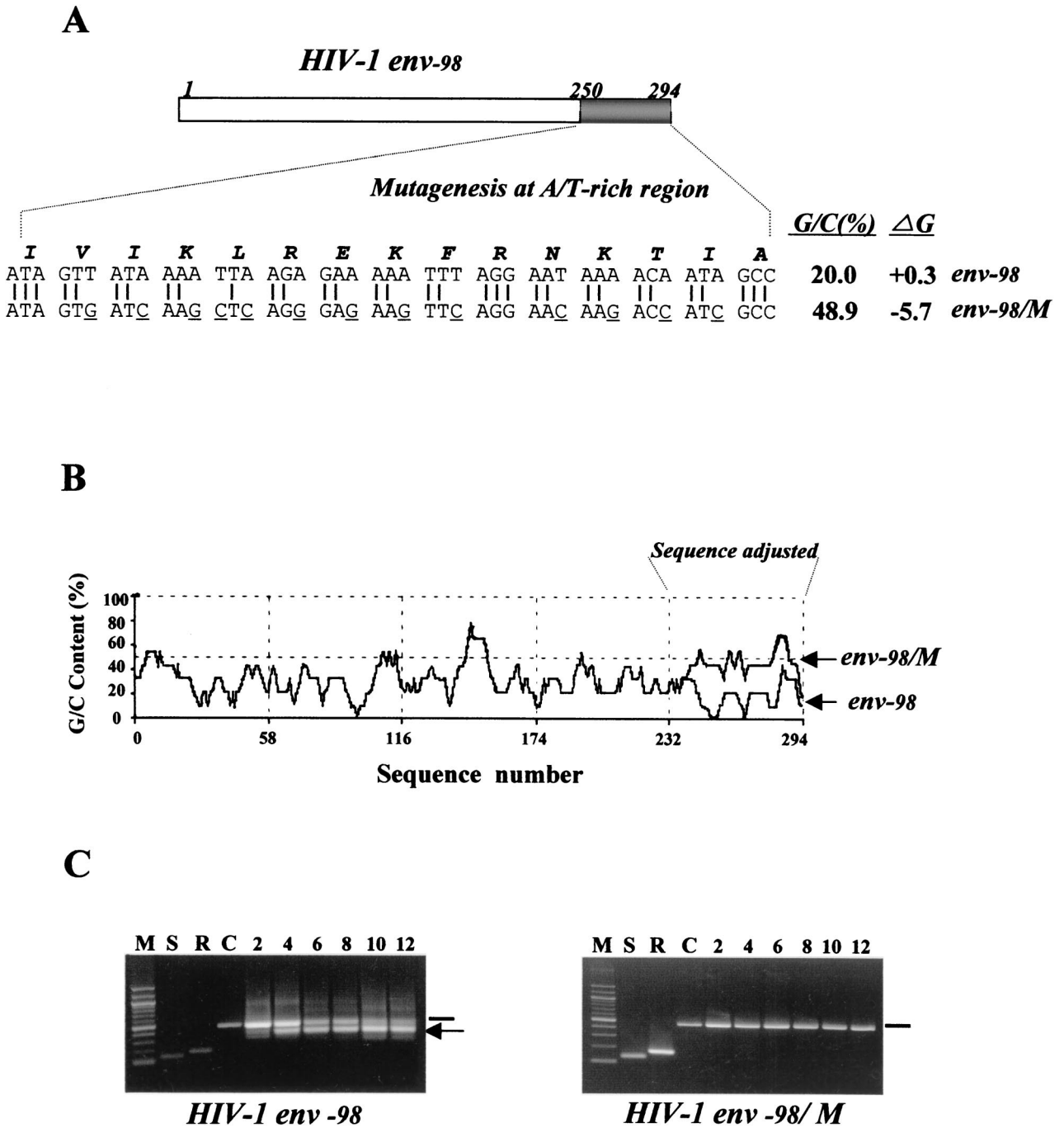

FIG. 5. Genetic instability of recombinant PVs is markedly remedied by increasing the G/C contents at the A/T-rich region of the insert. (A) The genetically unstable insert HIV-1 env-98 was sequence adjusted at the A/T-rich region marked by the solid box in the diagram. Thirteen A/T sites were replaced with G/C by mutagenesis without any change in the amino acid sequence. (B) The G/C contents of the inserts before and after sequence substitution were analyzed by the DNASIS program at a window size of 9 and expressed as a histogram. Sequence substitution increased the local G/C contents of the insert. (C) The genetic stability of the original (HIV-1 env-98) and the sequence-adjusted (HIV-1 env-98/M) recombinant PVs during consecutive passage cycles are represented by RT-PCR. Lanes: M, 100-bp size markers; S, poliovirus Sabin 1; R, RPS-Vax vector-derived virus; C, insert-containing recombinant plasmid. The numbers represent the passage cycle of each recombinant PV. The bar and arrow indicate the original and truncated forms of the insert, respectively.

panel of Fig. 6B). The Rec-PV-containing inserts, PVm-137 and PVm-132, were also genetically unstable, as shown by the RPS-Vax/PVm-150 (data not shown), but their sequence-adjusted constructs were quite stable during consecutive passages (Table 1). Genetic stability of RPS-Vax/PVm-150/M, examined by RT-PCR, was also confirmed by Western blot experiments (Fig. 6C). These results clearly demonstrate that our findings are applicable to explaining increases in the genetic

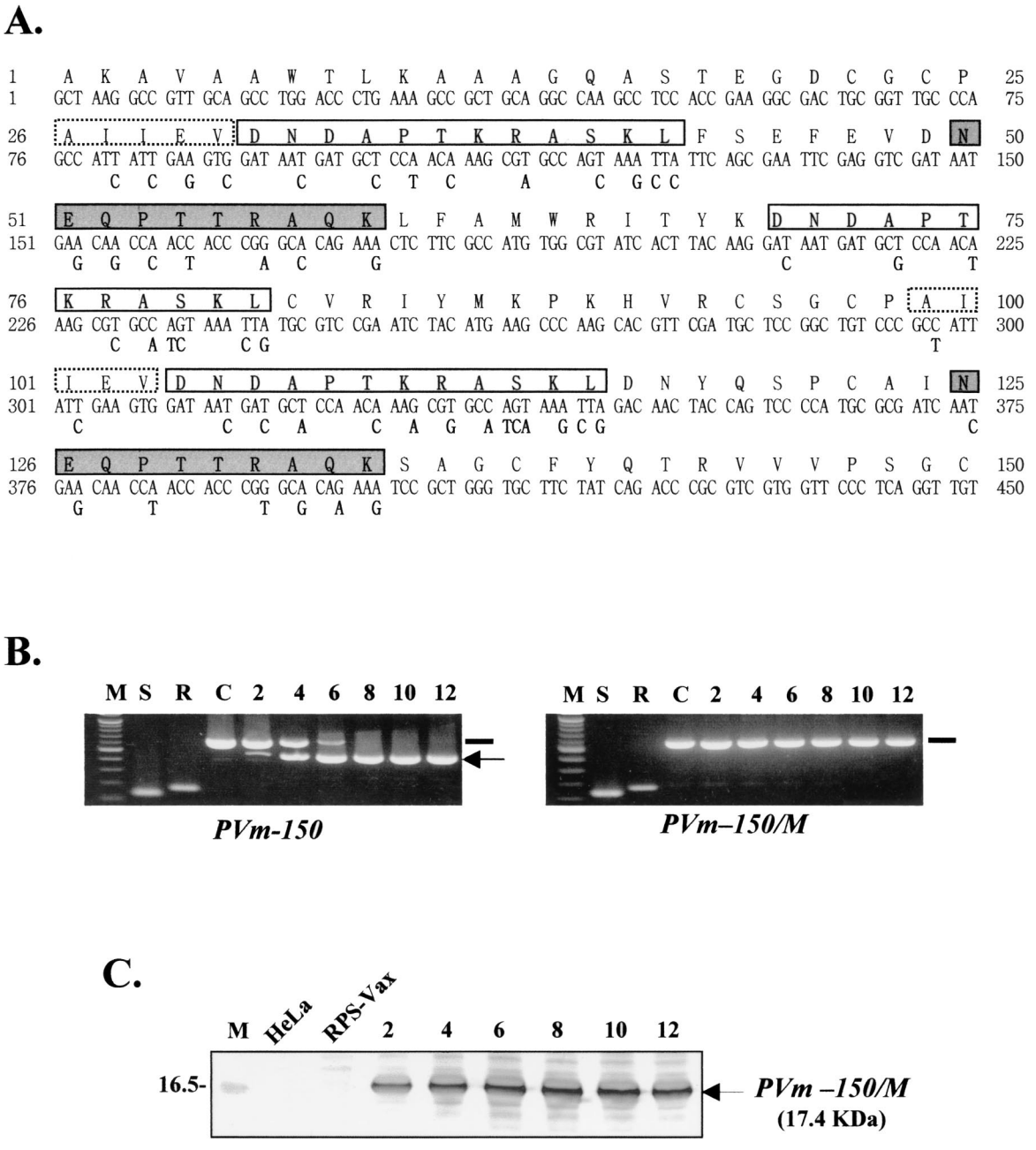

FIG. 6. Synthetic inserts containing heteromultimeric epitopes were tested for genetic stability. (A) The genome sequence and composition of the recombinant PV inserts PVm-150 and its sequence-adjusted clone (PVm-150/M), synthesized by ligation-free PCR, are illustrated. The white and gray boxes with solid lines indicate the amino acid repeats of the VP1 epitope of poliovirus type 2 and type 3, respectively. The box with the dotted line indicates the 5-amino-acid repeats. The backbone is the nucleotide and the derived amino acid sequences of PVm-150. The bases in bold letters below the nucleotide backbone indicate the nucleotide substitutions for PVm-150/M. (B) The passage stability of the recombinant PVs is represented by RT-PCR. Lanes: M, 100-bp size markers; S, poliovirus Sabin 1; R, RPS-Vax vector-derived virus; C, insert-containing recombinant plasmid. The numbers represent the passage cycle of each recombinant PV. The bar and arrow indicate the original and truncated forms of the insert, respectively. (C) The genomic integrity of the sequence-adjusted recombinant PV (RPS-Vax/PVm-150/M) was examined during the passages by Western blot. The numbers indicate the passage cycle of the recombinant PV to be infected into HeLa cells. Uninfected, RPS-Vax-infected, and recombinant PV-infected HeLa cell lysates were screened by Western blot with peptide-specific antiserum.

stability of the RPS-Vax-derived recombinant PVs even though they have repeated epitope-containing foreign inserts.

Recovered genetic stability of recombinant PVs in HeLa cell culture was also maintained in vivo. Until now, the genetic stability of the recombinant PVs was examined by serial passage in HeLa cell culture. To investigate whether the genetic stability of the recombinant PVs determined in cell cultures was also repeated in vivo, two recombinant viruses, RPS-Vax/PVm-150 and RPS-Vax/PVm-150/M, were inoculated intracerebrally into Tg-PVR mice. The viruses were recovered daily

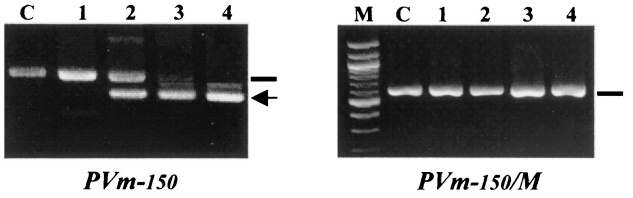

FIG. 7. Genetic stability of recombinant PVs in vivo. PVR-transgenic mice were inoculated intracerebrally with recombinant PV containing the original (PVm-150) and the sequence-adjusted (PVm-150/M) heteromultimeric insert. Every day, viruses were recovered from the spleen of each inoculated mouse and followed by a single round of amplification in HeLa cells. Viruses recovered from the passages in vivo were examined for their genetic integrity by RT-PCR. C denotes the insert-containing recombinant plasmid. The number indicates the day after infection on which the recombinant PV was recovered. The bar and arrow indicate the intact and the truncated forms of the inserts, respectively, which were generated during the replication of recombinant PV in vivo.

from the spleen of each mouse for 4 days after the intracerebral injections and tested for genetic stability by RT-PCR.

As shown in Fig. 7, each recombinant virus recovered from the inoculated mice demonstrated patterns of genetic stability very similar to those shown in HeLa cell cultures. RPS-Vax/PVm-150 showed serious internal deletion even after 2 days, and no intact bands longer than 3 days after the inoculation (left panel in Fig. 7), suggesting that the recombinant PVs having heteromultimeric repeated sequences are very unstable during their replication, not only in vitro but also in vivo, whereas RPS-Vax/PVm-150/M, having sequences adjusted by our G/C rule without any amino acid changes in the insert, revealed complete genetic integrity without showing any insert deletion pattern during the same period of replication, even in vivo (right panel in Fig. 7). These results imply that the RPS-Vax-derived recombinant virus maintains its own genetic stability consistently during its replication, not only in vitro but also in vivo.

DISCUSSION

The greatest obstacle in the construction of recombinant PVs as vaccine candidates is the inability to predict the genetic stability of an incorporated genome during viral proliferation. Our work was initiated to establish general guidelines in designing the foreign antigens which could be stably incorporated into the versatile poliovirus-derived vaccine vector based on the polyprotein processing strategy. In this paper, we would like to address our design architecture, which is applicable for designing a foreign insert which can produce a genetically stable recombinant PV with our RPS-Vax vaccine vector.

We have constructed a number of recombinant PVs by cloning several foreign inserts into the Sabin 1-derived RPS-Vax system. However, the HBV surface antigen-coding inserts (HBVsAg-100 and HBVsAg-76) were not properly incorporated into the recombinant PVs (Table 1). We do not have a clear understanding of the reasons for this, but we thought that the failure in producing recombinant PVs with these inserts seemed to be associated with the inserts' hydrophobicity. The peptide sequences of these antigens were markedly hydrophobic (+0.89 and +0.35) in their biochemical characteristics compared to other antigens (Table 1). This finding led us to suggest that one should avoid the strong hydrophobic region when designing a foreign insert to be incorporated into the RPS-Vax system.

Even though the group II recombinant PVs were genetically unstable, a substantial proportion of the virus population still retained the intact insert, as judged from the intensity of the major band (Fig. 2A). Interestingly, the minor band in groups II and III appears to contain a unique deletion fragment of a distinct size rather than a variety of random deletion products. This indicates that the deletion event occurs specifically through a single favored site, although the molecular basis for this specificity is not well understood. Moreover, this specific partial deletion process occurs within the foreign gene, as judged by the sequencing. In the later stages of the passages, the size of the truncated fragment remains the same, implying that a partial fragment of the original insert, once established, was steadily passed along with the parental vector without further modifications. In group III, apparent bands of truncated fragments emerged progressively, and the intact band disappeared in the later stage, suggesting that the intermediate by-product of the recombinant PV, containing a shortened fragment, was much more efficient than the one having an intact insert in its replication capacity. Nevertheless, no truncated forms of foreign proteins were detected in the Western blot analysis (Fig. 2B). This can be explained in two ways. First, the intracellular proteolytic machinery might have degraded the truncated antigens because of their inappropriate folding. Second, even when they were folded stably in the cytoplasm, they might have lost the original conformational epitope detectable by the antibody. In either case, they could not have been detected by the Western blot.

We learned from the repeated Western blot experiments that the independent structure of the foreign antigens seemed to be important, not only for their immunogenicity but also for their protein stability. This is because even when antigens are correctly cleaved from the polyprotein by viral protease, they will either associate together or be degraded by the intracellular proteolytic machinery in the cytoplasm if they remain unfolded or in a state of inappropriate folding. In either case, they would be unable to induce the immunity expected when the recombinant PV was inoculated. However, it is difficult to deduce the folded structure of the protein from the given amino acid sequence due to our limited understanding of protein folding.

Based on our experience with these repeated results, we propose to exploit hydropathic screening of the coded polypeptide. This provides a crude insight into the existence of a hydrophobic core within the protein antigen that would be helpful in explaining appropriate folding. To do this, one should conduct a hands-on examination of the overall quantity and distribution of hydrophobic and polar residues. However, it would be highly recommended to discard any foreign insert found in the entire translate (such as the HBVs shown in Table 1) which showed extreme hydropathy.

In analyzing the recombinant PVs for their genetic stability, we could not find any strong correlation between genetic stability and insert size when the latter was less than 450 bases (Table 1). The mechanism of RNA recombination is not fully understood, but it could be deduced that the genetic instability

of recombinant PVs containing dimeric or concatenated multimeric foreign inserts, such as PV2,3–131, HIV-1 mV3, and HIV-1 PND8, was due to internal deletion by homologous recombinations of the repeated sequences, as mentioned previously (16). However, the instability of the recombinant PVs having monomeric inserts smaller than the above (such as SIV env-108) suggests that some factor other than size is involved in the genetic stability of the RPS-Vax-derived recombinant PVs.

Considering the stringency of picornavirus packaging, if the recombinant RNA, in three-dimensional structure, is too voluminous to be encased in the rigid capsid, packaging pressure may cause a deletion of the nonessential foreign insert. The three-dimensional conformation of the insert RNA is associated, at least in part, with the number of hydrogen bonds in canonically base-paired regions and sequence-dependent free energy (4, 6, 15, 31). Based on this rationale, we have studied the correlation between genetic stability and the G/C contents of each insert and found that most of the stable inserts showed a high G/C content of over 40% (Table 1 and Fig. 3A). Our findings were confirmed by two consecutive experimental results in which (i) increasing the G/C content of an unstable insert augmented the genetic stability of its recombinant PV (Fig. 3B) and (ii) reducing the G/C content of a stable insert made the recombinant PV lose its genetic stability during the passages (Fig. 3C). This suggests that a high G/C content might facilitate the packaging of recombinant viral RNA. The detailed mechanisms are still unknown, but the fact that the guanine base is able to pair with the uracil in addition to the normal G/C pairing within a single-stranded RNA (15) might give more dynamic flexibility to the insert structure in the recombinant viral RNA, which results in an effective encapsidation, followed by the production of genetically stable recombinant PVs.

According to the results shown in Fig. 3A, the solid border indicates that small inserts do not require a high G/C content to achieve their genetic stability. Actually, env-83 had only a32.5% G/C content but was genetically stable up to 12 passages (Fig. 4B), whereas env-98 and -71 were not stable, even though they were similar in size and G/C content (Fig. 4A). From the perspective of a molecular basis, it can be postulated that the deletion would be promoted by short repeated sequences around the deletion site, so that nonhomologous recombination between progeny RNAs could take place, as suggested in the previous report (29).

In the sequencing experiment, we found that the deletion took place in a sequence-specific manner and was not associated with the repeated sequence. We also found that env-98 and -71 had a common high A/T-rich region right behind the deletion site on the 3' end (Fig. 5A). Surprisingly, the sequence substitution in this region with high G/C bases (Fig. 5B) caused the recombinant PV (env-98/M) to acquire complete genetic stability during the serial passages, as shown in Fig. 5C. This strongly suggests that (i) the local A/T-rich region in the insert seems detrimental to the genetic stability of recombinant PV and (ii) acceptable amounts of G/C should be evenly distributed throughout the insert to stabilize the recombinant PV.

Recently, growing demand for immunofocusing in vaccine development necessitates the construction of a multimeric (3, 22, 40, 43) or concatenated epitope (29, 32) to enhance immunogenicity against a specific epitope or to generate multivalent vaccines. However, the repeated sequence is very detrimental to the genetic stability of the recombinant virus, probably due to the homologous recombination-mediated internal deletion during virus replication, as reported previously (16, 23, 33). Actually, our recombinant PV, containing original repeats of concatenated epitopes (Fig. 6A), was genetically unstable, as shown in Fig 6B. To determine whether our design architecture is also applicable to overcoming this limitation of genetic instability in the concatenated epitope, we reconstructed concatenated epitope-containing long foreign inserts, based on our G/C rules, by exploiting template- and ligation-free PCR protocols, as schematically illustrated in Fig, 1B and described in Materials and Methods.

As shown in Fig 6A, one of the synthetic inserts, PV-150/M, had numerous silent mutations, which not only increased the G/C contents (evenly distributed), but also removed the internal repeated sequence. The recombinant PVs containing sequence-adjusted inserts showed complete recovery of genetic stability at the genomic and protein levels (Fig. 6B and 6C). Tang et al. (39) have also reported the enhancement of the genetic stability of recombinant viruses by a silent mutation which reduces the homology of the direct repeats. However, in these cases, only 30 to 50% of the recombinant viruses retained the original insert, even after the third passage. In any sequence substitutions, the genetic stability of recombinant viruses was not fully recovered in their experimental results. On the contrary, the recombinant PV constructed according to our G/C rules showed complete genetic stability for at least 12 rounds of passage. These results substantiate the feasibility of our novel architectural design for the construction of genetically stable recombinant PVs, even though they contain concatenated epitopes as foreign inserts.

Finally, we evaluated the genetic stability of the sequence-adjusted recombinant PV in vivo. In the previous experiment with recombinant coxsackievirus B3 (38), while the insert was retained through passage 4 in tissue culture, it was almost lost in vivo in an organ-specific manner. Actually, the recombinant PV showing genetic instability in a HeLa cell culture was much more unstable in infected mice (left panel in Fig. 7), whereas the sequence-adjusted recombinant PV RPS-Vax/PV23–150/M showed complete genetic stability, even in vivo, during the same period (right panel in Fig. 7).

The genetic stability test was performed in vivo for 4 days because we could not detect live viruses in the infected mice after that period. Our present results suggest that the loss of the insert would be accelerated in vivo, and the genetically stable recombinant PVs are preferentially selected much more radically in vivo than in vitro, probably as a result of immunological pressure. In some ways, they were in good agreement with the previous reports showing that recombinant viruses of higher genetic stability were more effective in inducing immune responses in mice (39).

In conclusion, our work demonstrates (i) that the genetic stability of recombinant PV is strongly associated with the G/C contents and G/C distribution patterns in foreign inserts and (ii) that the genetic instability of foreign inserts can be promoted by increasing the G/C contents and/or replacing the local A/T-rich region with a G/C-rich codon. Based on the present results, we have established an insert design architec-

ture which includes G/C rules and a template- and ligation-free PCR protocol. Our G/C rules are as follows: first, adopt a host-specific codon usage; second, use the high G/C content codon from the available codons; third, distribute the G/C evenly; and fourth, minimize the local repeats throughout the insert.

The feasibility of our architectural design was confirmed by construction of a heteromultimeric insert showing complete genetic stability, not only in vitro but also in vivo. However, the architectural design is not applicable to inserts longer than 450 bp. These findings strongly suggest that the genetic stability of the recombinant PVs is closely related to the tertiary conformation of the insert RNA, which is determined mainly by its nucleotide composition. The suitability of the compact conformation of RNA in the encapsidation process may account for its preference for the high G/C content and the even distribution of the G/C sequence in ranges of less than 450 bp for stable recombinant PVs. Even though these guidelines were established with a poliovirus-derived RPS-Vax vector system, to some extent they would be applicable not only for the construction of recombinant RNA viruses, but also for the development of other live-vector-based vaccines.

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