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Construction and Characterization of Replication-Competent Simian Immunodeficiency Virus Vectors That Express Gamma Interferon

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We report the construction and characterization of several replication-competent simian immunodeficiency virus (SIV) vectors with a deletion in the viral *nef* gene ($SIV_{\Delta nef}$) that express gamma interferon (IFN-γ). The expression of the cytokine gene was controlled either by the simian virus 40 early promoter or by the SIV 5′ long terminal repeat regulatory sequences, utilizing the *nef* gene splice signals. To enhance the expression of IFN-γ, the two in-frame *nef* start codons were mutated without altering the Env amino acid sequence (SIV_{HyIFN}). Plasmids containing full-length proviral genomes were used to obtain high-titer stocks of each recombinant virus in cell cultures. Expression of IFN-γ by SIV_{HyIFN} reached levels as high as 10^6 U/ml after 11 days in culture. The IFN-γ gene was unstable and sustained deletions after serial passage of $SIV_{\Delta nef}$ vectors in CEM-X-174 cells. The degree of instability appears to depend on size and orientation of the insert and the expression of IFN-γ. Only one virus, SIV_{HyIFN}, expressed detectable levels of IFN-γ up to the sixth passage. Prospects for the use of IFN-γ and other lymphokines to enhance the safety and efficacy of live attenuated vaccines are discussed.

Simian immunodeficiency virus (SIV) infection of macaques is a model for human immunodeficiency virus (HIV) infection (11). In light of the immunity-enhancing (2, 17, 31) and attenuating (12, 15, 18, 20, 27) activities of gamma interferon (IFN-γ), we believe that prospects are good for enhancing the safety and efficacy of live attenuated HIV vaccines by expressing this and other lymphokines.

Retroviral vectors efficiently transfer gene sequences into cells and promote their stable expression. Some simple retroviruses that have been engineered are replication competent; others need helper packaging cells. Recently, more complex retroviruses, such as HIV, have been used as vectors for the expression of reporter genes such as those encoding luciferase (4, 5), chloramphenicol acetyltransferase (10), and alkaline phosphatase (16). Other recombinant HIVs have been used to study the functions of viral genes (7, 25, 29) or even antiviral compounds (24). We have applied similar methods in using another complex retrovirus, SIV, as an expression vector. We have constructed recombinant SIVs that have deletions in the *nef* gene ($SIV_{\Delta nef}$) and that express the lymphokine IFN-γ. We also report their abilities to grow in different cell types and their genetic stabilities after serial passage in cell cultures.

MATERIALS AND METHODS

Cells and viruses. CEM-X-174 cells and rhesus peripheral blood mononuclear cells (PBMCs) were used for SIV isolation and propagation; these cells were maintained in RPMI supplemented with 10% fetal bovine serum. Human A549 cells (American Type Culture Collection, Rockville, Md.) were propagated in Dulbecco's modified Eagle's (DMEM) supplemented with 10% fetal bovine serum and antibiotics. SIV_{mac239} and derivatives of the virus were propagated in either CEM-X-174 cells or rhesus PBMCs. Encephalomyocarditis virus (EMCV) used for the antiviral assay of human IFN-γ (HuIFN-γ) was propagated in A549 cells.

Construction of $pSIV_{239\Delta nef}$. The first step in the construction of a recombinant SIV_{mac239} that expresses HuIFN-γ was the deletion of a portion of the *nef* coding sequences and incorporation of a unique *Sal*I cloning site at the position of the deletion. The cloning strategy involved PCR amplification of specific sequences with the following four oligonucleotide primers: sense primers A (5′GTACCATGGCCAAATGCAAG3′, *Nco*I, nucleotide [nt] 8720) and C (5′ATAGACATGTCGACTTTTAT3′, *Sal*I, nt 9681) and antisense primers B (5′ATTGTCGACCCTCACAAGAG3′, *Sal*I, nt 9491) and D (5′TGCTAGGAATTCTCCTGCTT3′, *Eco*RI, nt 10530) (nucleotides numbers for SIV_{mac239} are as in GenBank accession number M33262; restriction sites are underlined, and mutations appear in boldface). Plasmid pVP-2, containing the 3′ half of SIV_{mac239} provirus, was the template for all PCR amplifications.

The region on the SIV genome encompassing the 3′ half of the *env* coding sequence was amplified with PCR primers A and B. Primer B incorporated two stop codons and a *Sal*I site into the *nef* translation frame. The ends of the amplified DNA fragment were repaired with T4 DNA polymerase, and the DNA was cloned into the *Hin*dII site of plasmid pBluescript (Promega, Madison, Wis.) to generate pA+B. We selected the plasmid with the closest proximity of the new *Sal*I and the vector *Eco*RI sites. In a separate PCR, primers C and D were used to generate a DNA fragment that included the 3′ long terminal repeat (LTR) of SIV_{mac239} with *Sal*I and *Eco*RI sites at the 5′ and 3′ ends, respectively. This fragment was then digested with *Sal*I and *Eco*RI and cloned into plasmid pA+B to generate pAD.

A fragment extending from the *Sac*I site in the *env* gene (nt 9487) to a *Sac*I site in the cellular DNA sequence in pVP-2 was removed by *Sac*I digestion. A similar *Sac*I fragment was isolated from pAD, which contained the same *Sac*I site in *env* to another *Sac*I site in the polylinker region of pBluescript. The latter *Sac*I fragment was cloned into pVP-2 generate pVP-2/Δnef.

Plasmid pMA239 (provided by A. Adachi, Kyoto University, Kyoto, Japan) contains the complete SIV_{mac239} proviral genome; a *Hin*dIII site is located in flanking cellular DNA at the 5′ end of the provirus, and an *Eco*RI site is present in the 3′ cellular DNA flanking sequences. Both pMA239 and pVP-2/Δnef were digested with *Sph*I (nt 6707) and *Eco*RI, and the 3′ halves were interchanged to produce $pSIV_{239\Delta nef}$. This plasmid contains the SIV_{mac239} provirus with a 186-base deletion in the *nef* gene and a unique *Sal*I site between the end of *env* and the beginning of the 3′ LTR.

Construction of SIV vectors expressing IFN-γ. Plasmid pSV7b, containing the simian virus 40 (SV40) early promoter, was modified to eliminate 300 bp of SV40 DNA sequences not involved in transcription regulation. pSV7b was digested with *Sal*I and *Pvu*II, treated with Klenow enzyme, and religated to produce pSVΔ with a *Sal*I site in proximity to the SV40 early promoter sequences. The HuIFN-γ gene was obtained as a *Sma*I cassette from pHuIFN-γ (12) and cloned into the *Sma*I site of pSVΔ. The SV40 early promoter and the HuIFN-γ coding sequences were released as a *Sal*I cassette and inserted in the *Sal*I site of $pSIV_{239\Delta nef}$; because the cloning of this cassette was not directional, both orientations were obtained. Plasmid $pSIV_{239\Delta nef}$/SV-γ(s) has the SV40 promoter and IFN-γ gene sequences in the same orientation as the SIV reading frame, whereas $pSIV_{239\Delta nef}$/SV-γ(as) has the gene cassette in the opposite orientation.

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A second set of SIV vectors was engineered to express the IFN-γ gene under the control of SIV regulatory sequences, utilizing the *nef* gene splice signals. $pSIV_{239\Delta nef}$ was digested with *Sal*I, and its ends were blunted in a reaction with the Klenow fragment of DNA polymerase. The HuIFN-γ, obtained as a *Sma*I cassette from pHuIFN-γ, was cloned into $pSIV_{239\Delta nef}$, and two plasmids with HuIFN-γ in each orientation were obtained. Plasmid $pSIV_{239\Delta nef}/\gamma(s)$ contains the HuIFN-γ gene in the same direction as the SIV genes, whereas $pSIV_{239\Delta nef}/\gamma(as)$ has the HuIFN-γ gene in the opposite orientation.

Finally, to preclude translation initiation, a recombinant SIV was generated with mutations in the two ATG codons at the beginning of the *nef* translation frame. T residues (positions 9334 and 9352) in each ATG codon at the beginning of *nef* (codons 1 and 10, respectively) were each mutated to C residues (underlined) by site-directed mutagenesis using the synthetic mutant oligonucleotide 5′CTGACC TACCTACAATA**C**GGGTGGAGCTATTTCCA**C**GAGGCGGTCCAGGCC3′. The mutated plasmid pTZ-SIV-MI was digested with *Nhe*I (position 8999) and *Sac*I (position 9487), and the 488-bp DNA fragment was purified. Similarly, plasmid p239Δnef/IFN was digested with *Nhe*I and partially with *Sac*I, and the 12,600-bp DNA fragment was purified. Both DNA fragments were finally ligated to produce p239ΔnefATG/γ. Nucleic acid sequencing was performed to confirm that no unintended changes had been introduced in SIV sequences during PCR amplification.

Preparation of infectious SIV vectors. All plasmids were grown in *Escherichia coli* DH5α cells. To reduce the instability of plasmid DNA containing retroviral sequences, bacteria containing these plasmids had to be grown at 30°C with low oxygenation and gentle agitation. Plasmids containing full-length retroviral genomes were used in electroporation of CEM-X-174 cells. Briefly, cells in the exponential phase of growth were resuspended in electroporation medium (10 mM dextrose and 0.1 mM dithiothreitol in RPMI 1640) at a concentration of 1.3×10^7 cells per ml. A volume of 0.3 ml of cell suspension (4×10^6 cells) was mixed with 5 μg (100 μl) of plasmid DNA. The DNA-cell mixture was kept on ice at electroporation conditions of 960 μF and 200 V. After pulsing, cells were removed from the electroporation chamber and resuspended in 5 ml of 10% fetal calf serum–RPMI. Transfected cells were kept in the exponential growth phase; cultures were maintained for no more than 14 days and monitored daily to score cytopathic effects. Viral replication was measured by an enzyme-linked immunosorbent assay (ELISA) specific for the SIV p27 antigen (Coulter Corp., Hialeah, Fla.).

Rhesus macaque PBMCs were obtained by centrifugation in Ficoll gradients and stimulated with 0.5 μg of *Staphylococcus* enterotoxin A per ml for 48 h. Cells were then electroporated with proviral DNA as described for CEM-X-174 cells and cultured in medium containing 50 U of recombinant human interleukin-2 (Cetus Corp., Emeryville, Calif.) per ml.

Antiviral activity of IFN-γ. The antiviral activity of IFN-γ was determined by measuring inhibition of the cytopathic effects of EMCV on A549 cells (12). Supernatants from CEM-X-174 cells infected with SIV vectors were diluted threefold in DMEM for assay of IFN-γ titers. Aliquots of 50 μl of these dilutions were placed in 96-well plates, and 10^4 A549 cells in 100 μl of DMEM with 10% fetal calf serum were added to each well. After 24 h of incubation, the cells were challenged with the minimum dose of EMCV (10^4 PFU per well) that gave 100% cytopathic effect in cells not treated with IFN-γ. Units of IFN-γ are expressed as the reciprocal of the dilution of sample giving 50% protection against challenge virus.

Stability of $SIV_{\Delta nef}$ vectors after serial passage in cell culture. CEM-X-174 cells (10^6) were infected with 10 ng of virus (p27), incubated for 1 h at 37°C, washed twice in RPMI 1640, and maintained in culture for 7 days. Progeny virus was quantitated by SIV *gag*-specific ELISA (Coulter Corp.), and 10 ng of virus was then used to infect fresh CEM-X-174 cells. Proviral DNA was isolated from infected cells after each passage and subjected to analysis by PCR; primers for PCR amplification were A (sense primer; described above) and E (5′AAATC CCTTCCAGTCCCCCC3′, antisense, nt 9710). PCR conditions were denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min; the number of cycles was 30. The IFN-γ production for each virus passage in cell culture was determined as described above.

RESULTS

Replication of $SIV_{\Delta nef}$ vectors expressing IFN-γ. Several $SIV_{\Delta nef}$ vectors were engineered and tested for infectivity, IFN-γ expression, and genetic stability (Fig. 1). Initially, we incorporated the early SV40 promoter to achieve a high level of IFN-γ expression ($SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$). Next, we constructed $SIV_{\Delta nef}$ vectors expressing IFN-γ under the control of the 5′ LTR (SIV_{IFN} and SIV_{NFI}). Finally, we mutated the two in-frame *nef* start codons without altering the Env amino acid sequence (SIV_{HyIFN}).

Plasmids containing proviral forms of $SIV_{\Delta nef}$ vectors were used to produce infectious viruses by transfection of different types of cells. Several of the SIV vectors replicated rapidly and reached peak titers by 6 days after transfection of CEM-X-174 cells. After 9 days, the amounts of SIV p27 in the medium were similar for all cultures with SIV vectors (Fig. 2A). In addition, extensive cytopathologic effects in CEM-X-174 cells, characterized by multinucleate syncytia, were similar in cultures transfected with all SIV vectors and SIV_{mac239} (data not shown). In macaque PBMCs, however, $SIV_{\Delta nef}$ replicated faster and to higher titers than any of the $SIV_{\Delta nef}$ vectors containing IFN-γ sequences (Fig. 2B).

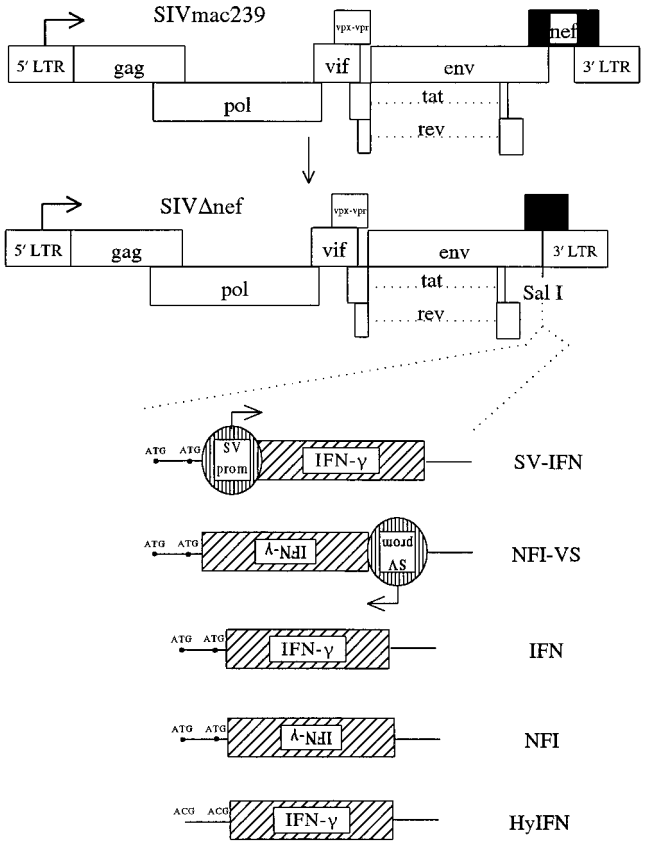

FIG. 1. Strategy for the generation of $SIV_{\Delta nef}$ and recombinant $SIV_{\Delta nef}$ expressing IFN-γ. A 186-base fragment of the SIV_{mac239} *nef* coding sequence (between the end of *env* and the beginning of the 3′ LTR) was deleted, and a unique *Sal*I cloning site was incorporated for the insertion of the human IFN-γ gene. The SV40 early promoter and the HuIFN-γ coding sequences were cloned in both orientations to produce the $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$ proviral genomes. Additionally, the HuIFN-γ coding sequences were inserted, without the SV40 promoter, to generate SIV_{IFN} and SIV_{NFI}. Finally, SIV_{HyIFN} was generated by mutating T residues at positions 9334 and 9352 in the ATG codons at the beginning of *nef* (codons 1 and 10) to C residues to preclude translation.

Expression of lymphokine by $SIV_{\Delta nef}$ vectors. IFN-γ produced by SIV vectors was measured by a standard antiviral assay (Fig. 3). Expression of IFN-γ by SIV_{HyIFN} was detected by 2 days after transfection, at a time when SIV p27 was barely detectable. Levels of IFN-γ for this recombinant virus were the highest of all the vectors tested; after 11 days in culture, this vector produced as much as 10^6 U/ml. Cells transfected with $SIV_{SV\text{-}IFN}$ expressed readily detectable amounts of HuIFN-γ, although the maximum level was about 2 orders of magnitude lower than that of SIV_{HyIFN}. In contrast, the expression of IFN-γ by SIV_{IFN} was significantly lower. $SIV_{NFI\text{-}VS}$ had very low IFN-γ activity by 11 days (20 U/ml), and SIV_{NFI} did not express detectable quantities of the lymphokine.

Stability of SIV vectors. All $SIV_{\Delta nef}$ recombinants were an-

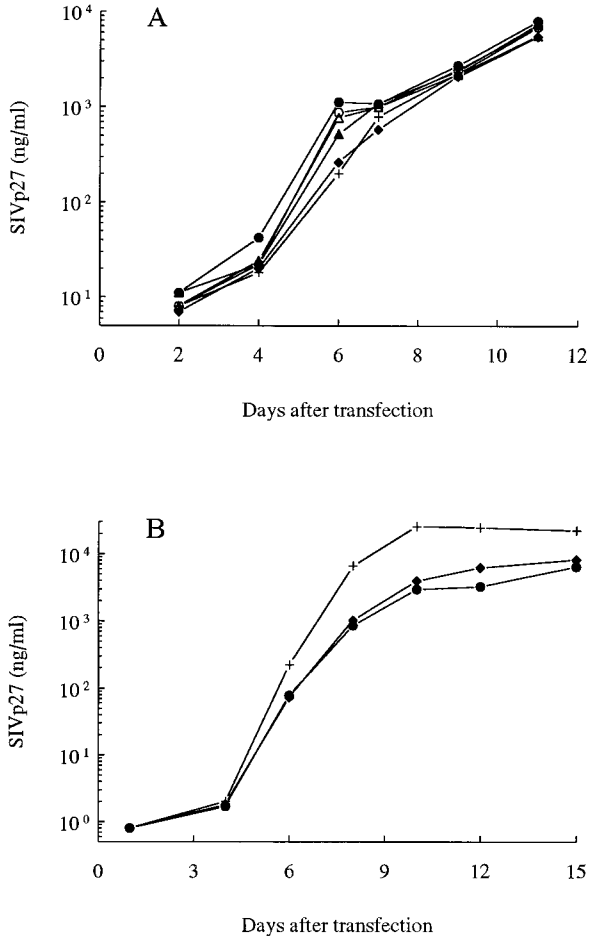

FIG. 2. Characterization of the in vitro replication of $SIV_{\Delta nef}$ recombinants. (A) Replication in CEM-X-174 cells. Proviral DNA of $SIV_{\Delta nef}$ (+), $SIV_{SV\text{-}IFN}$ (△), $SIV_{NFI\text{-}VS}$ (▲), SIV_{IFN} (○), SIV_{NFI} (●), and SIV_{HyIFN} (◆) was used to generate infectious virus in CEM-X-174 cells by electroporation as described in Materials and Methods. (B) Replication in rhesus macaque PBMCs. $SIV_{\Delta nef}$ (+), SIV_{NFI} (●), and SIV_{HyIFN} (◆) proviral DNA was electroporated in stimulated PBMCs as described in Materials and Methods. Transfected cells were kept in the exponential growth phase with daily monitoring to score cytopathic effects. Viral replication was measured by an ELISA specific for SIV p27 antigen (Coulter Corp.).

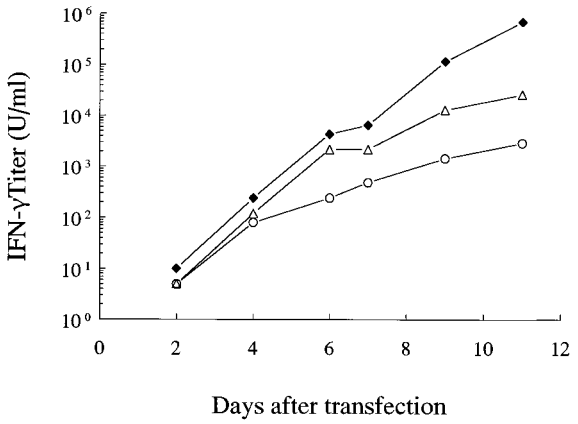

FIG. 3. Kinetics of IFN-γ expression by $SIV_{\Delta nef}$ recombinants. Accumulation of human IFN-γ in the supernatant of CEM-X-174 cells transfected with $SIV_{SV\text{-}IFN}$ (△), SIV_{IFN} (○), and SIV_{HyIFN} (◆) proviral DNA. The antiviral activity of human IFN-γ was measured by prevention of the cytopathic effect of EMCV in human A549 cells as described in Materials and Methods.

alyzed for stability after serial passage in cell culture by PCR amplification of the 3′ end of each viral vector, using primers that encompassed the IFN-γ sequences (Fig. 4A). The degree of instability of the SIV vectors appeared to depend on the size and the orientation of the insert. $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$, which contain the 800-bp SV40 promoter/IFN-γ insert, deleted part of these heterologous sequences rapidly; for $SIV_{SV\text{-}IFN}$, deletion fragments were detected as early as the first passage. For SIV_{IFN} and SIV_{HyIFN}, instability was first observed after the third passage; by the sixth passage, PCR amplification analysis failed to detect intact IFN-γ sequences in either of these vectors. In contrast, SIV_{NFI}, which contains the same 500-bp IFN-γ insert but in the opposite orientation, still retained most of the insert after six passages.

Supernatants from each passage were assayed for IFN-γ production by the various SIV vectors (Fig. 4B). Expression of IFN-γ by $SIV_{SV\text{-}IFN}$ was undetectable by the third passage; this observation is consistent with the rapid deletion of IFN-γ coding sequences from the viral genome. SIV_{IFN} and SIV_{HyIFN}, both more stable than $SIV_{SV\text{-}IFN}$, continued to produce IFN-γ by the fifth passage. Only SIV_{HyIFN}, which expressed the highest amount of the lymphokine for every passage, still produced detectable IFN-γ by the sixth passage, despite the fact that the PCR amplification analysis of this vector revealed extensive loss of IFN-γ sequences.

DISCUSSION

Our long-term goal is to develop a system for the generation of safer and more efficacious live attenuated vaccines for infectious diseases and in particular for AIDS. Inactivated SIV or recombinant subunit vaccines have provided limited or no protection against infection with SIV (1, 14, 23, 28). Macaques vaccinated with live $SIV_{\Delta nef}$ did not develop disease (19) and resisted challenge with virulent SIV_{mac251} (6). However, $SIV_{\Delta nef}$ persists indefinitely in macaques, provides partial or no protection until a year or more after immunization (6, 30), and is pathogenic to neonatal macaques (3), limiting its use as a vaccine (8).

One approach toward the development of effective vaccines that we and others have taken is to exploit the immunity-enhancing (2, 31) and attenuating activities of lymphokines such as IFN-γ and interleukin-2 (9, 12, 20, 26). Vaccinia virus recombinants expressing interleukin-2 or IFN-γ have clearly been demonstrated to be attenuated for nude mice by a 10^6-fold. The mechanism of attenuation for the most part remains an enigma. Here we describe the construction and characterization of replication-competent $SIV_{\Delta nef}$ vectors expressing human IFN-γ as a step toward development of a safer and more efficacious attenuated live vaccine for AIDS. Human IFN-γ is fully active in monkey cells, induces an antiviral state in Vero cells, and enhances major histocompatibility complex class II expression in macaque PBMCs (21, 22).

In our initial attempts, we incorporated a strong heterologous promoter (early SV40 promoter) to achieve a high level of IFN-γ expression. Of the two SIV vectors developed with this promoter, only $SIV_{SV\text{-}IFN}$ expressed high levels of IFN-γ; the vector carrying the opposite orientation, $SIV_{NFI\text{-}VS}$, manifested minimal activity after several days in culture. We believe that the polyadenylation signals located in the 3′ LTR of SIV are important for increasing the stability of IFN-γ mRNAs

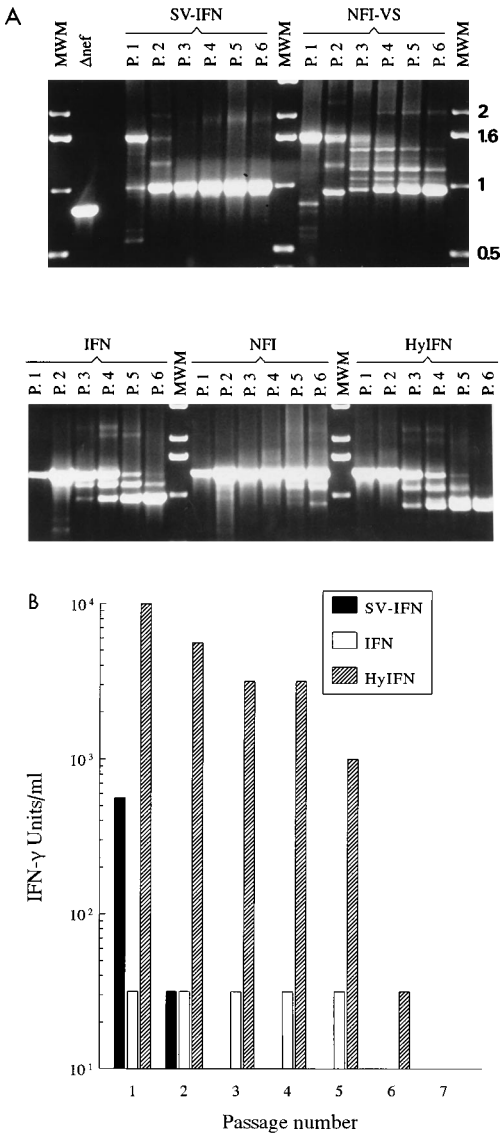

FIG. 4. Genetic stability of $SIV_{\Delta nef}$ vectors after six serial passages in cell culture. CEM-X-174 cells were infected with 10 ng of virus (p27) and maintained in culture for 7 days. Progeny virus was quantitated by SIV *gag*-specific ELISA (Coulter Corp.), and 10 ng was then used to infect fresh CEM-X-174 cells. (A) PCR analysis of the 3′-end regions of $SIV_{\Delta nef}$ vectors. Proviral DNA was isolated from infected cells after each passage and subjected to analysis by PCR with primers A (nt 8720) and E (nt 9710) as described in Materials and Methods. Molecular weight markers (MWM) are indicated in kilobases. The full-length PCR fragments are 1,600 bp for $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$, 1,300 bp for SIV_{IFN}, SIV_{NFI}, and SIV_{HyIFN}, and 800 bp for $SIV_{\Delta nef}$. (B) IFN-γ production for each virus passage in cell culture. The concentration of IFN-γ was determined as described for Fig. 3. The species specificity was confirmed by a human-specific IFN-γ ELISA kit (Intertest-γ; Genzyme Corp., Cambridge, Mass.).

initiated by the SV40 promoter, and $SIV_{NFI\text{-}VS}$ lacks signals for efficient polyadenylation.

Because $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$ were highly unstable, we designed a vector to express IFN-γ by using the same SIV signals that produce *nef* mRNA. We believe that rapid deletion of the insert is due to the limited capacity of retroviruses to accommodate extra genomic material as well as their tendency to delete sequences not providing a selective advantage for replication. We speculated that by reducing the size of the insert (i.e., removing the SV40 early promoter) in the SIV vector, stability of the vector would be enhanced. Indeed, SIV_{IFN} and SIV_{NFI} are more stable vectors than $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$. However, the IFN-γ expression capabilities of SIV_{IFN} were far more limited than those of $SIV_{SV\text{-}IFN}$, confirming the importance of a strong promoter; the antisense SIV_{NFI} had no detectable IFN-γ expression.

In order to use the SIV transcription machinery in full, the two in-frame *nef* start codons that were still present in our $SIV_{\Delta nef}$ vector were eliminated, leaving unaltered the Env amino acid sequence. The two ATGs were thought to interfere with the translation of the IFN-γ mRNA. As expected, the new vector, SIV_{HyIFN}, expressed high levels of IFN-γ. As was true of $SIV_{SV\text{-}IFN}$ and $SIV_{NFI\text{-}VS}$, the genetic stability of the vectors carrying the IFN-γ gene with the sense orientation (SIV_{HyIFN} and SIV_{IFN}) was lower than that of SIV_{NFI}. Although we have not sequenced the deletion products, we believe that the particular arrangement of IFN-γ and 3′ LTR sequences and the expression of IFN-γ may contribute to gene instability.

We hypothesize that the establishment of persistent infection by $SIV_{\Delta nef}$ can be reduced or eliminated by the expression of IFN-γ, a cytokine with potent antiviral activity on retroviruses (8a). In addition, the expression of IFN-γ is expected not only to attenuate $SIV_{\Delta nef}$ but also to enhance the protective immune responses of macaques to challenge with pathogenic uncloned SIV_{mac251}. Moreover, the deletion of IFN-γ after the several passages has an advantage: IFN-γ will be present only during the early phase of the immune response to SIV, eliminating the possibility of any untoward effects with the continuous expression of the lymphokine. These predictions have proved correct in studies with macaques vaccinated with SIV_{HyIFN} and challenged with SIV_{mac251} (13). Therefore, the incorporation of IFN-γ or other lymphokines is a methodological advance in vaccinology that promises to lead to the development of safer and more efficacious vaccines for AIDS.

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