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Context-Dependent Role of Human Immunodeficiency Virus Type 1 Auxiliary Genes in the Establishment of Chronic Virus Producers

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Two molecularly cloned viruses, human immunodeficiency virus type 1 (HIV-1)-NL4-3 (NL4-3) and HIV-1-HXB-2 (HXB-2), have been used to study the role of HIV-1 auxiliary genes in the establishment of chronic virus producers. NL4-3 encodes all known HIV-1 proteins, whereas HXB-2 is defective for three auxiliary genes: *vpr*, *vpu*, and *nef*. Studies were done in H9 cells, a T-cell line unusually permissive for the establishment of chronic virus producers. NL4-3 and HXB-2 undergo lytic phases of infection in H9 cultures with HXB-2, but not NL4-3, supporting the efficient establishment of chronic virus producers. Tests of mutant NL4-3 genomes containing various combinations of defective auxiliary genes revealed that both *vpr* and *nef* limited the ability of NL4-3 to establish chronic virus producers. Tests of a series of recombinants between NL4-3 and HXB-2 revealed that 5′ internal sequences as well as fragments containing defective auxiliary genes affected the establishment of chronic virus producers. Viral envelope sequences and levels of virus production did not correlate with the ability to establish chronic virus producers. These results suggest that complex interactions of viral auxiliary and nonauxiliary gene functions with the host cell determine the ability to establish chronic virus producers.

One of the poorly understood phenomena associated with human immunodeficiency virus type 1 (HIV-1) infections is the ability to establish chronic virus producers. Spreading infections in T cells are almost invariably cytopathic. However, cultures of long-term virus-producing cells can be established in certain T-cell lines (6, 10, 13, 34, 37). In this study, two molecularly cloned viruses, HIV-1-NL4-3 (NL4-3) and HIV-1-HXB-2 (HXB-2), have been used to study the role of HIV-1 sequences in chronic virus production. The two viruses differed both in the ability to establish chronic virus producers and in the expression of functional auxiliary genes. One, NL4-3, had a low ability to generate chronic virus producers and encodes all known HIV-1 gene products (1). The other, HXB-2, had a high ability to establish chronic producers and is defective for three nonessential auxiliary genes: *vpr*, *vpu*, and *nef* (12).

Prior to the undertaking of studies on the genetic basis of chronic virus production, four T-cell lines were compared for the ability to establish chronic virus producers. Infections of C8166, H9, A3.01, and Jurkat cells were initiated with NL4-3 or HIV-1-IIIb. C8166 cells did not establish chronic virus producers, as these cells did not survive the lytic phase of infection. In H9, A3.01, and Jurkat cultures, the lytic phase of infection was followed by the appearance of surviving populations of cells. In A3.01 cultures, the survivors did not express virus. In H9 and Jurkat cultures, surviving populations contained both virus-expressing and non-virus-expressing cells. The levels of chronically expressing cells were much higher in H9 than in Jurkat cultures (data not shown and reference 29). On the basis of this screen, further studies were conducted in H9 cells.

NL4-3 and HXB-2 have distinct abilities to establish chronic virus producers. As a first step to studies on the role of HIV-1 sequences in chronic virus production, molecular clones of the parental viruses were compared for the ability to establish chronic virus producers. Infections were initiated by transfecting 1 μg of plasmid DNA per one million H9 cells with DEAE-dextran (Pharmacia, Uppsala, Sweden). Infections were monitored for virus-expressing cells (Fig. 1A), cell growth (Fig. 1B), and cell death (Fig. 1C) (30).

The pNL4-3- and pHXB-2-transfected cultures exhibited distinctive profiles for the establishment of chronic virus producers (Fig. 1). NL4-3 established low levels of chronic virus producers (15 to 20%), whereas HXB-2 established high levels (>80%) (Fig. 1A). In keeping with this trend, HXB-2-transfected cultures had normal growth characteristics similar to those of uninfected H9 cells (Fig. 1B). In contrast, NL4-3-transfected cultures exhibited both poorer cell growth and more cell death (Fig. 1B and C). NL4-3 and HXB-2 transfections also differed in that HXB-2-transfected cells exhibited an ~10-day lag behind NL4-3 transfections for the appearance of infected cells (Fig. 1A). This lag was not observed if virus was used to initiate infections. The lag was consistently observed with different preparations of HXB-2 DNA.

The infection and growth patterns of the parental genomes were highly reproducible, with similar results being obtained in independent growth tests. For studies on the temporal expression of mutant viruses, simultaneous growth patterns of the parental genomes served as standards.

Tests for the effects of *vpr*, *vpu*, and *nef* on the ability of NL4-3 to establish chronic virus producers. To test for the effects of auxiliary gene differences between NL4-3 and HXB-2 on the establishment of chronic virus producers, mutants of NL4-3 containing all possible combinations of defective *vpr* (NLΔR), *vpu* (NLΔU), and *nef* (NLΔN) genes were constructed (Table 1). Tests for the abilities of the mutant

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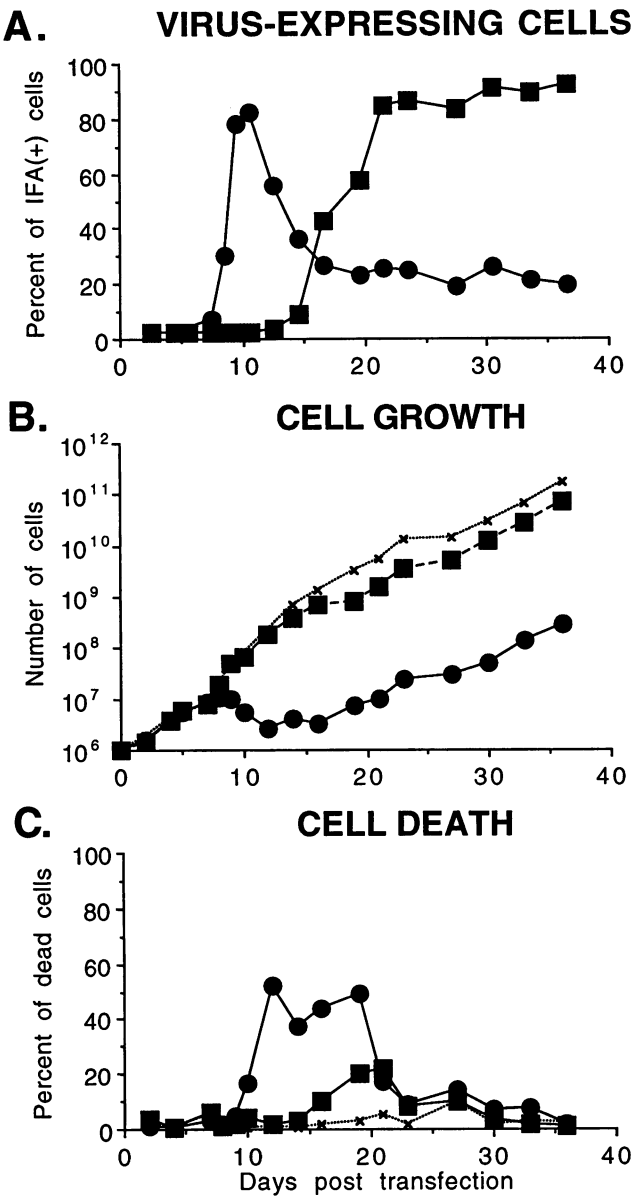

FIG. 1. Temporal analysis of pNL4-3- and pHXB-2-transfected H9 cultures (● and ■, respectively) and uninfected H9 cells (×). (A) Temporal appearance of virus-expressing cells; (B) growth of cultures; (C) temporal presence of dead cells. IFA, indirect immunofluorescence assay. The cell numbers shown for the growth curves were extrapolated from the growth rates obtained for each time point.

genomes to establish chronic virus producers were initiated by transfections. Transfections with parental DNAs were carried out in parallel to allow direct comparison of the ability of mutant and parental genomes to establish chronic virus producers. Transfected cultures were monitored over time for virus-expressing cells, cell growth, and cell death.

The NL4-3 auxiliary gene mutants varied in their abilities to establish chronic virus producers. Overall, three patterns were observed: inefficient establishment of chronic virus producers (Fig. 2A), intermediate establishment of chronic virus producers (Fig. 2B), and efficient establishment of chronic virus producers (Fig. 2C). Two mutants, NLΔU and NLΔUΔN, had low abilities to generate chronic virus producers (Fig. 2A). The efficiency with which these mutants generated chronic virus producers was similar to that of the NL4-3 parent. Three mutants, NLΔR, NLΔN, and NLΔRΔU, showed intermediate abilities to establish chronic virus producers (Fig. 2B). Cultures infected with these viruses exhibited transient high levels of virus-expressing cells (days 5 to 15 of culture). Two viruses, the double mutant, NLΔRΔN, and the triple mutant, NLΔRΔUΔN, had high abilities to generate chronic virus producers (Fig. 2C). There was no difference in the relative abilities of these two mutants to generate chronic virus producers. This suggests that defects in both *vpr* and *nef* are sufficient for NL4-3 to establish high levels of chronic virus producers and that mutations in *vpu* do not further enhance this ability.

In general, the ability to establish chronic virus producers correlated inversely with the amount of cell death in cultures. This can be seen most clearly in Table 2 in which mutants are listed from decreasing to increasing ability to establish chronic virus producers. The two NL4-3 mutants that had low abilities to establish chronic virus producers (NLΔU and NLΔUΔN) exhibited high levels of cell death during the acute phase of infection. By contrast, the two mutants that were efficient at generating chronic virus producers, NLΔRΔN and NLΔRΔUΔN, caused only low levels of cell death. In keeping with this trend, cultures infected with two of the three viruses with intermediate abilities to generate chronic virus producers, NLΔR and NLΔN, had intermediate levels of cell death. However, the culture infected with the third virus with an intermediate ability to establish chronic virus producers, NLΔRΔU, had low levels of dead cells. This exception suggests that low cytopathicity does not ensure the efficient outgrowth of chronic virus producers.

The efficient establishment of chronic virus producers did not necessarily correlate with cultures having a normal growth potential. The growth potential of cultures was determined both by the amount of cell death during the acute phase of infection and by the doubling times of the surviving cells. Following the acute phase of infection, the doubling times for NLΔRΔN- and NLΔRΔUΔN-expressing cultures were longer than for uninfected or HXB-2-expressing cultures. These cultures had only intermediate growth characteristics and did not exhibit the normal growth characteristic of uninfected or HXB-2-expressing H9 cells (Fig. 2C).

Use of NL4-3-HXB-2 recombinants to map sequences that affect the establishment of chronic virus producers. To test for effects of nonauxiliary as well as auxiliary gene sequences on the generation of chronic virus producers, reciprocal recombinants between NL4-3 and HXB-2 were constructed and tested for the ability to establish chronic virus producers. The construction and expression of *vpr*, *vpu*, and *nef* by these recombinants is illustrated in Fig. 3. As with the NL4-3 mutants, results of growth tests are summarized by grouping constructs that encoded low (Fig. 4A), intermediate (Fig. 4B), and high (Fig. 4C) abilities to establish chronic virus producers.

Temporal analysis of H9 cells transfected with the NL4-3-HXB-2 recombinants revealed effects of 5′ internal (non-long terminal repeat) sequences as well as effects of fragments encoding defective auxiliary genes on the ability to establish chronic virus producers (Fig. 3 and 4). In the presence of the 5′ *Spe*I-to-*Eco*RI fragment of NL4-3 (bp 1507 to 5743 of NL4-3 proviral DNA), HXB-2 fragments encoding defective *vpr*, *vpu*, and *nef* or defective *vpr* and *vpu* did not confer high ability to establish chronic virus producers (see NLHX-4 and NLHX-2, Fig. 3 and 4B). However, when 5′ internal sequences were from HXB-2, 3′ HXB-2 fragments encoding defective *vpr* and

TABLE 1. Comparison of *vpr*, *vpu*, and *nef* genes of NL4-3, NL4-3 mutants, and HXB-2

Protein	Description for virus(es)		
	NL4-3	NL4-3 mutants[a]	HXB-2
Vpr	96-amino-acid protein	Fill-in of *Eco*RI site (nt 5743) creating a frameshift at amino acid 63 and a truncation 16 amino acids later	Mutation creating a frameshift at amino acid 71 and a truncation 7 amino acids later
Vpu	81-amino-acid protein	Addition of an *Xho*I linker at *Ssp*I site (nt 6153) creating a frameshift at amino acid 32 and a truncation 3 amino acids later (38)	Loss of initiator methionine
Nef	206-amino-acid protein	Fill-in of the *Xho*I site (nt 8887) creating a frameshift at amino acid 35 and a truncation 11 amino acids later (2)	Mutation generating a stop codon after amino acid 123

[a] Mutants with multiple gene defects were constructed by interchanging appropriate restriction endonuclease fragments. nt, nucleotide.

vpu (NLHX-3) or defective *nef* (NLHX-5) were able to confer high ability to establish chronic virus producers (Fig. 3 and 4C). Thus, in the recombinants, the ability to establish chronic virus producers required 5′ internal HXB-2 sequences as well as 3′ fragments containing defective *vpr* and *vpu* or defective *nef* genes.

As with the NL4-3 mutants (Table 2), the overall ability to establish chronic virus producers correlated with low cytopathic potential (Fig. 3). For example, recombinants NLHX-3 and NLHX-5, with high abilities to establish chronic virus producers, were the least cytopathic for cultures. The recombinant NLHX-1, with the lowest ability to establish chronic virus producers, showed high cytopathic potential for cells in culture. Two of the three recombinants with intermediate abilities to establish chronic virus producers, NLHX-4 and NLHX-6, had intermediate cytopathic potential for cells in culture. One exception to this overall trend was observed for NLHX-2. In NLHX-2-transfected cultures, low cell death did not correlate with the efficient establishment of chronic virus producers (Fig. 3 and 4B).

In contrast to the NL4-3 mutants, the NL4-3-HXB-2 recombinants were able to establish chronic virus producers that exhibited normal cell growth (Fig. 4). This is shown by tests of NLHX-3 and NLHX-5, the two recombinants with the highest abilities to establish chronic producers (Fig. 4C). Both NLHX-3- and NLHX-5-transfected cultures had doubling times similar to those of the uninfected or HXB-2-expressing H9 cultures.

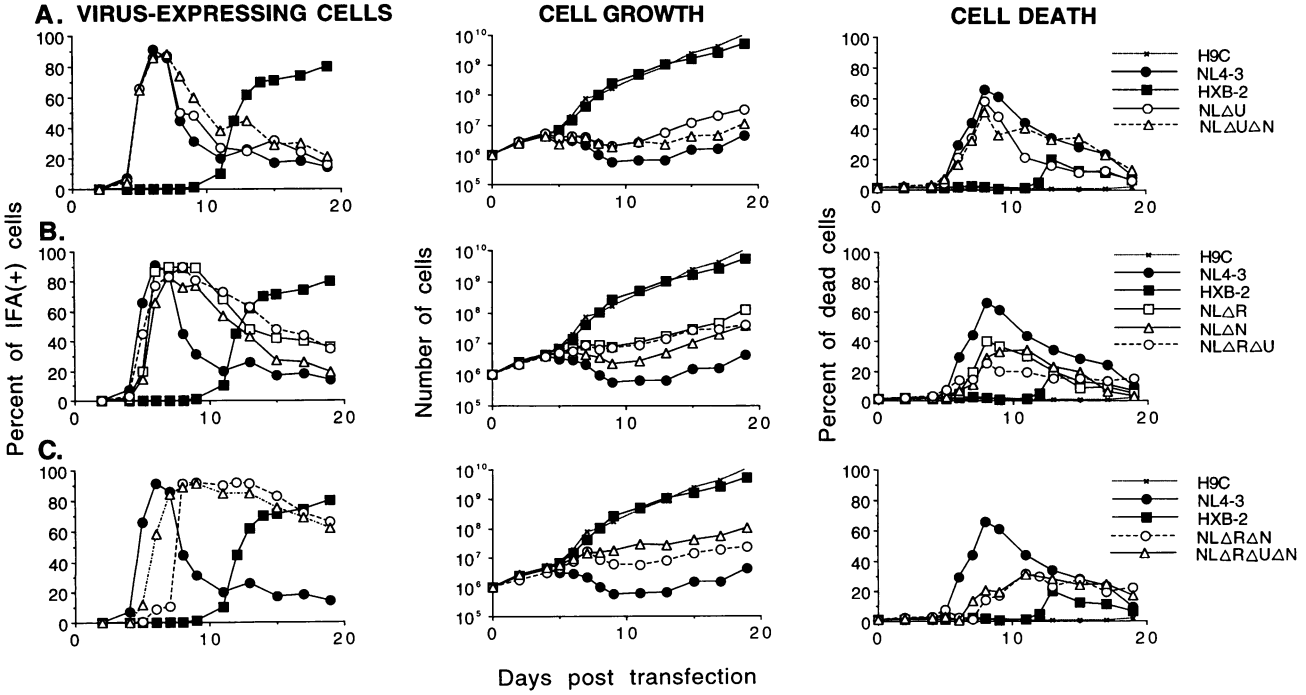

FIG. 2. Temporal analysis of H9 cultures transfected with pNL4-3, pHXB-2, and NL4-3 mutants. (A) Mutants that were inefficient at the establishment of chronic virus producers; (B) mutants with intermediate abilities to establish chronic virus producers; (C) mutants that were efficient at establishing chronic virus producers. IFA, indirect immunofluorescence assay; H9C, uninfected H9 cells. The open symbols present data for the mutants. The solid symbols present data for NL4-3 and HXB-2.

TABLE 2. Summary of data on the NL4-3 mutants

Test virus	Expression of auxiliary gene[a]:			Extent of tested phenotype[b]		
	vpr	*vpu*	*nef*	Chronic producers	Growth of culture	Cell death
NL4-3	+	+	+	+	+	+++++
NLΔUΔN	+	−	−	+	+	++++
NLΔU	+	−	+	+	++	++++
NLΔR	−	+	+	++	++	+++
NLΔN	+	+	−	++	++	+++
NLΔRΔU	−	−	+	++	++	++
NLΔRΔN	−	+	−	++++	++	++
NLΔRΔUΔN	−	−	−	++++	+++	++
HXB-2	−	−	−	+++++	+++++	+

[a] + and −, presence and absence of auxiliary gene expression, respectively.
[b] Symbols are on a scale of + for least effect to +++++ for maximum effect.

Virus production does not determine the ability to establish chronic virus producers. To test for effects of virus production on the ability to establish chronic virus producers, temporal virus production was compared for the parental viruses and NLHX-3, the recombinant with the best ability to establish chronic virus producers (Fig. 5). The amount of viral antigen in supernatants of the infected cultures was quantitated with an antigen capture enzyme-linked immunosorbent assay (ELISA) (11). Virus production by the three tested viruses was quite similar. Each of the infections produced higher levels of virus during the acute phase of infection than during the chronic phase, with the actual levels of virus production not reflecting the efficiency of the establishment of chronic producers. Thus, virus production did not play a determining role in the establishment of chronic virus producers.

Complex context-dependent requirements for HIV-1 sequences in the establishment of chronic virus producers. We have demonstrated that multiple regions of the HIV-1 genome determine the ability to generate chronic virus producers. For NL4-3 infections, defective *vpr* and *nef* genes were sufficient to change the efficiency of the generation of chronic virus producers from low to high (Fig. 2 and Table 2). For infections with NL4-3-HXB-2 recombinants, 5′ internal HXB-2 sequences, as well as 3′ fragments containing defective auxiliary genes, were required for the efficient establishment of chronic virus producers (Fig. 3 and 4). These 3′ fragments could encode either defective *vpr* and *vpu* or defective *nef* genes. Thus, the effects of defective auxiliary gene sequences were context dependent.

***env* sequences and levels of virus production do not determine the ability to establish chronic virus producers.** *env* is a frequent determinant of the cytopathic potential of HIV-1 isolates (5, 9, 21, 22, 23, 33, 37, 39). The ability of the NL4-3-HXB-2 recombinants to generate chronic virus producers did not correlate with the presence of HXB-2 *env* sequences (Fig. 3). The *env* sequences of NL4-3 and HXB-2 are both derived from the HIV-1 LAI family of viruses (1, 4, 12, 14, 38a). Env proteins of both viruses cause syncytia during the early and lytic phases of infection. However, once the lytic phase of infection had passed, the presence of syncytium-inducing Env glycoproteins did not prevent the efficient establishment of chronic virus producers.

High levels of viral expression have also been suggested to be associated with the cytopathic effects of HIV-1 infections (25, 35). Temporal levels of virus production were quite similar for the two parental viruses as well as a recombinant with high ability to establish chronic virus producers (Fig. 5). Thus, virus load, as measured by virus production, did not appear to be a determinant of the ability to establish chronic virus producers.

Involvement of multiple regions may reflect the involvement of multiple phenomena. Studies presented here reveal the complex relationship of sequences in the HIV-1 genome to the establishment of chronic virus producers. The fact that a minimum of two defective auxiliary genes, or two regions of the HXB-2 genome, were required for NL4-3 to efficiently

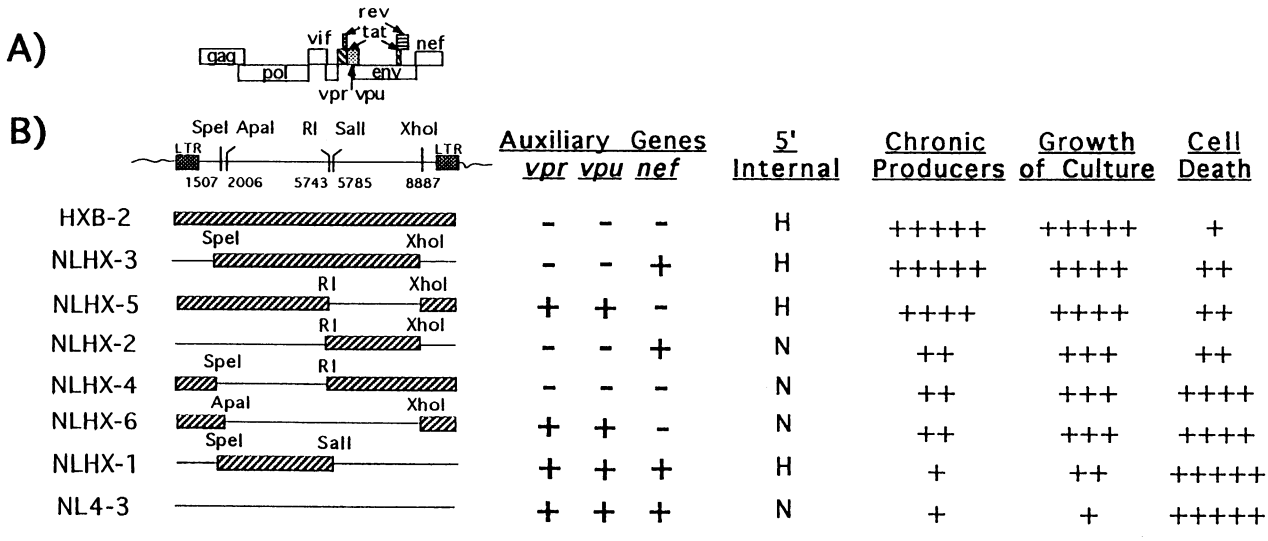

FIG. 3. Summary of data on the NL4-3-HXB-2 recombinants. (A) Representation of the HIV-1 genome; (B) restriction endonuclease sites used in construction of the recombinants, recombinant genomes, and growth characteristics of cultures transfected with the recombinants. ▨, HXB-2 sequences; ——, NL4-3 sequences; RI, *Eco*RI; + and −, presence and absence of auxiliary genes, respectively; H, HXB-2 5′ internal sequences; N, NL4-3 5′ internal sequences. The last three columns represent the extent to which various growth profiles were observed with + indicating least effect and +++++ indicating maximum effect.

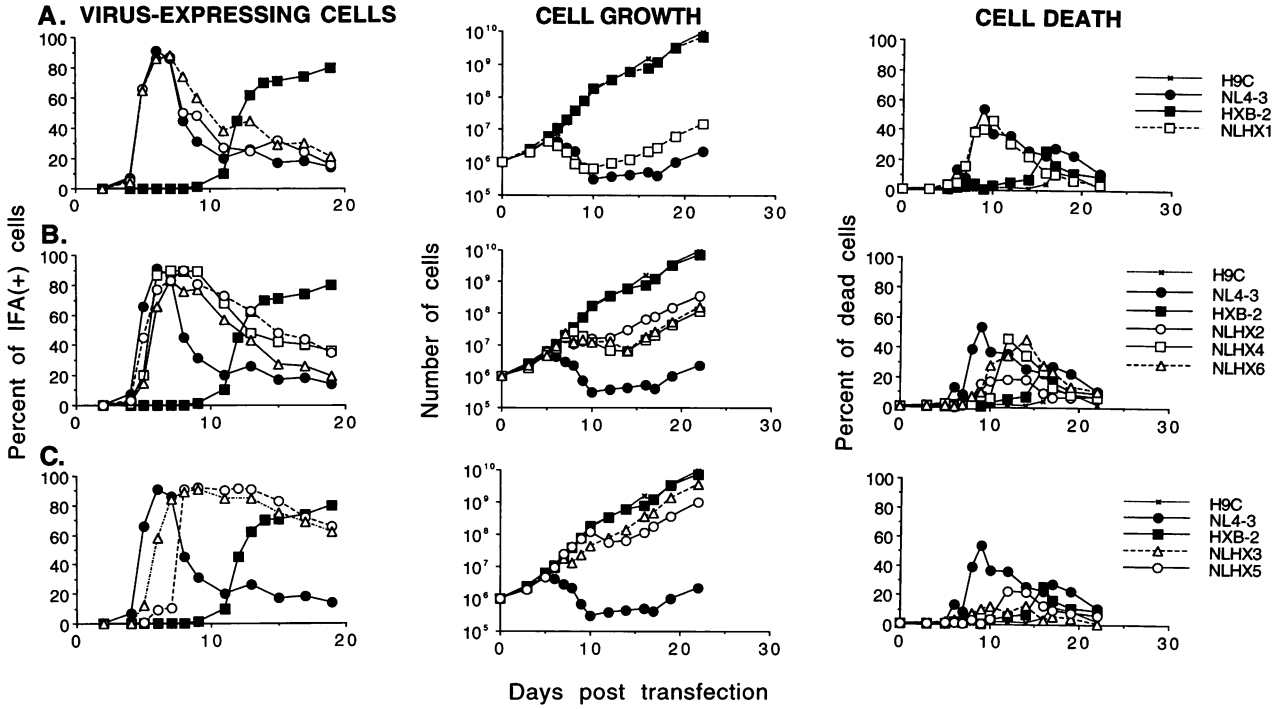

FIG. 4. Temporal analysis of H9 cells transfected with the NL4-3-HXB-2 recombinants. (A) Recombinants that were inefficient at the establishment of chronic virus producers; (B) recombinants with intermediate abilities to establish chronic virus producers; (C) recombinants that were efficient at establishing chronic virus producers. IFA, indirect immunofluorescence assay; H9C, uninfected H9 cells. The open symbols present data for the recombinants. The solid symbols present data for NL4-3 and HXB-2. The cell numbers shown for the growth curves were extrapolated from the growth rates obtained for each time point.

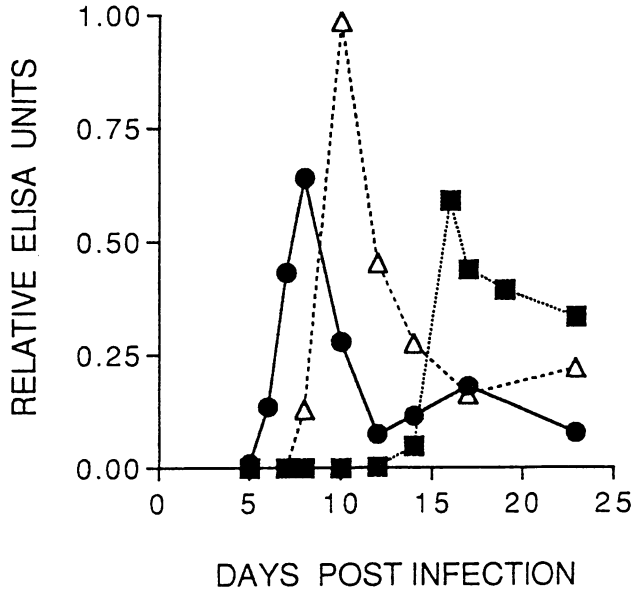

FIG. 5. Temporal analysis of virus production in H9 cultures transfected with the recombinant NLHX-3 (△) and the parental viruses NL4-3 (●) and HXB-2 (■). ELISA units were normalized with a standard NL4-3 stock.

establish chronic virus producers suggests that at least two virus-host interactions may be important for chronic virus production. We suggest that these may include (i) cytopathic interactions with vital cell functions and (ii) noncytopathic interactions with signalling pathways that control cell growth. As for cytopathic interactions, prior studies would be consistent with the activities of *vpr* (7, 8, 18, 24) and sequences in the 5′ internal region (13, 19, 31, 32, 36) potentially being cytopathic for vital cell functions. As for effects on signalling pathways, reported activities of Nef on the expression of cell surface molecules such as CD4 (3, 15–17, 20, 32a), lymphokines such as interleukin 2 (26), and transcriptional factors such as NF-κB and AP-1 (27, 28) could potentially affect the growth of virus-expressing cells. A potential ability of Nef to limit growth would be consistent with the two viruses having low cytopathic potentials but only intermediate abilities to establish chronic producers (NLΔRΔU and NLHX-2) both expressing functional Nef-encoding genes.

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