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Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1

(retrovirus replication/transcription/latency)

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ABSTRACT **Human immunodeficiency virus type 1 (HIV-1) contains an open reading frame called *nef* at the 3′ end of its genome. The *nef* gene product has been reported to down-regulate viral growth by suppressing viral transcription through interaction with the long terminal repeat region. We have compared two isogenic HIV-1 (HIV-1-WI3) strains, one of which lacks *nef* expression, and found little difference between them in *in vitro* growth. We tested effects on viral entry, DNA synthesis, and RNA expression by measuring HIV-specific low molecular weight DNA and RNA after infection. The qualitative and quantitative aspects of DNA and RNA synthesis were comparable between the *nef*$^+$ and *nef*$^-$ strains. The effects on viral growth were also examined by following changes in reverse transcriptase activity during the course of infection. The presence of the *nef* gene product failed to slow viral growth in several different cell types tested, including the human T-lymphocyte cell lines H9 and CEM-SS, human primary T cells enriched for CD4$^+$ cells, and human monocytic cell lines U-937 and THP-1. On the contrary, the *nef*$^+$ strain grew more efficiently in some cell types than the *nef*$^-$ strain. The same results were obtained with *nef*$^+$ and *nef*$^-$ strains of a different virus, HIV-1-432, whose Nef had been reported to have a negative effect on viral growth. Our data suggest that the Nef protein does not act as a negative factor, at least in the experimental systems employed in our studies.**

Human immunodeficiency virus (HIV), along with the human T-cell leukemia viruses, shows unusual complexity in its genomic structure and gene regulation relative to other animal retroviruses. In the 9.2-kilobase (kb) genome, there are nine genes whose products have been serologically and/or genetically identified. These are *tat*, *rev*, *nef*, *vif*, *vpu*, *vpr*, and three typical retroviral genes, *gag*, *pol*, and *env*. Although complete understanding of the functions of *tat* and *rev* remains to be achieved, it is well established that they are trans-acting regulatory genes that are essential for viral growth. The interplay of the various regulators generates early and late transcriptional phases in the HIV life cycle; the earliest RNA is enriched in subgenomic species, and the genomic transcript appears at the later stage of infection (1, 2).

Another gene thought to be an important regulator is *nef* (formerly *3′-orf*, *E′*, *F*, *orfB*). The *nef* gene is located at the 3′ end of the viral genome, partially overlapping the U3 region (3–5). It encodes a 27-kDa protein (Nef) that is myristoylated at its N terminus (6). The *nef* gene has been of particular interest because of its claimed negative effect on viral growth and biochemical properties. Earlier studies showed that *nef* is not required for viral growth (7–11) and suggested that mutations in its coding region led to more efficient viral replication (7, 9–11). Based on these findings, this gene was considered a negative factor and named *nef* (standing for negative factor; ref. 12). It was reported that the Nef protein suppresses viral replication by down-regulating viral transcription through a nucleotide sequence in the long terminal repeat (LTR) region (10, 11). The precise location in the LTR that might interact with the Nef protein is still in dispute (10, 11). It was also reported that the Nef protein shares limited similarities with the Ras protein and that it has GTPase and GTP-binding activities and down-regulates CD4 expression (6). These genetic and biochemical features led to a hypothesis that *nef* plays a central role in the regulation of viral gene expression and that *nef* is somehow involved in the establishment or maintenance of a latent state during HIV infection (13, 14).

Because of these interesting features, we initiated a series of experiments designed to help understand the role(s) of *nef* in viral gene expression. However, during our initial investigations, reproducibly we found that there was little difference in growth between *nef*$^+$ and *nef*$^-$ strains. When there was a difference, the *nef*$^+$ strain generally grew more efficiently than the *nef*$^-$ strain. Because Nef has been implicated as a central regulator during viral infection, we have undertaken an extensive comparative analysis between *nef*$^+$ and *nef*$^-$ strains. In this study, we have used two different *nef*$^+$ strains and compared them to their homologous *nef*$^-$ strains. Our results suggest that Nef does not act as a negative factor, at least in the experimental systems we employed.

MATERIALS AND METHODS

Virus and Cell Cultures. Virus stocks were prepared by a shaking method as described (2, 15) except that supernatant of infected CEM-SS cultures was used as a viral source for HIV-1-432 and its derivative. The *nef*$^+$ strains were obtained by using the plasmids pWI3 for HIV-1-WI3 (2) and pNL432 for HIV-1-432 (10). The *nef*$^-$ strain of HIV-1-WI3 was prepared by transfection of HXB-Cla (provided by M. Reitz and H. G. Guo, National Institutes of Health). The plasmids pWI3 and HXB-Cla shared the identical viral genomic sequence except for the *nef* region. In HXB-Cla, the initiation codon of *nef* was removed by oligonucleotide-directed mutagenesis. The *nef*$^-$ strain of HIV-1-432 was prepared from the plasmid pNL432-Xho (10). The procedure of viral infection was described previously (2). The amounts of virus used for infection were standardized by reverse transcriptase activity. It was found that when the same amounts of reverse

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin.

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transcriptase were used for viral infection, the percentages of initially infected cells were comparable between two different viral strains as long as they were prepared simultaneously in the same host cell type. This indicated that the level of reverse transcriptase activity generally correlated with infectivity. Host cells used in this study included the T-cell lines and H9 (16) and CEM-SS (17), human primary T cells, and the monocytic cell lines THP-1 (18) and U-937 (19). All cells used in this study were grown in medium containing RPMI 1640 and 20% fetal bovine serum. Phytohemagglutinin (PHA) and interleukin 2 were also added when primary T cells were cultured. Isolation of primary T cells and growth conditions were described elsewhere (20).

Metabolic Labeling and Radioimmunoprecipitation. For metabolic labeling, 5 million cells were washed with and resuspended in 1.5 ml of selective medium lacking methionine and cysteine (Hazleton Biologic, Lenexa, KS), incubated for 2 hr at 37°C, and then labeled by addition of 400 μCi of [^{35}S]methionine and 400 μCi of [^{35}S]cysteine (DuPont/NEN; 1 μCi = 37 kBq) for 2–4 hr at 37°C. After labeling, cells were washed once with ice-cold phosphate-buffered saline, pelleted, and lysed in NTE buffer (100 mM NaCl/20 mM Tris·HCl, pH 7.5/10 mM EDTA) containing 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% SDS, and protease inhibitors (pepstatin A, 10 μg/ml; leupeptin, 10 μg/ml; aprotinin, 10 μg/ml; trypsin inhibitor, 10 μg/ml; and benzamidine, 10 mM). After 5 min on ice, the lysate was cleared by microcentrifugation for 10 min at 4°C. Lysate corresponding to 10^6 cells was incubated with an appropriate antibody for 4 hr at 4°C and then adsorbed onto protein A-Sepharose 6MB (Pharmacia) for 1 hr at 4°C. The Sepharose beads were washed three times in NTE buffer containing 1% Triton X-100, once in NTE buffer containing 0.05% SDS, and twice in NTE buffer and resuspended in a buffer containing SDS and 2-mercaptoethanol. Samples were boiled for 3 min and then analyzed by SDS/12.5% PAGE.

DNA and RNA Blot Analysis. Preparation of low molecular weight DNA and RNA and the probes used for hybridization analyses were as described by Kim *et al.* (2).

Analysis of Viral Growth. Viral growth was monitored by reverse transcriptase assay (21) and by indirect immunofluorescence assay using human serum (22).

RESULTS

Identification of the *nef* Gene Product. The plasmids pWI3 and HXB-Cla, used for the preparation of the *nef*$^+$ and *nef*$^-$ strains, respectively, share the same viral genomic sequence except for a lesion in the coding region of *nef* in the latter. To test whether the viral strains prepared from these plasmids indeed produce a full-length Nef protein, we analyzed lysates of infected cells by radioimmunoprecipitation and SDS/PAGE. H9 or CEM-SS cultures acutely infected with HIV were metabolically labeled with [^{35}S]methionine and [^{35}S]-cysteine for 2–4 hr. The cells were lysed and the clarified extracts were incubated with rabbit antibody specific for Nef and human serum that can recognize Nef. A 27-kDa protein was specifically recognized in the *nef*$^+$ strain by both rabbit (Fig. 1, lane 3) and human (lane 7) antibodies, while no such protein was detected in the *nef*$^-$ strain with either antibody (lanes 4 and 8). The human serum also recognized other structural proteins. This type of analysis was done during the exponential viral growth phase in the experiments described below to show that the Nef protein was indeed present.

Effects on Viral Entry and Reverse Transcription. As an indicator of viral entry and the early stages of the viral life cycle, we measured unintegrated HIV DNA shortly after infection of H9 or primary T cells. Primary T cells enriched for CD4$^+$ cells were stimulated with PHA and infected with the *nef*$^+$ or *nef*$^-$ strain, and low molecular weight DNA was prepared at various times after infection with a high concentration of virus (2). The 9.2-kb linear viral DNA was synthesized by 5 hr postinfection in the *nef*$^+$ strain and its amount gradually increased as viral infection progressed (Fig. 2, lanes 3–6). Circular DNA appeared at 12 hr postinfection. The *nef*$^-$ strain showed an indistinguishable pattern of viral DNA synthesis (Fig. 2, lanes 7–10). These results suggested that the absence or presence of the *nef* gene product did not significantly alter the process of viral entry or DNA synthesis during a single cycle of viral growth.

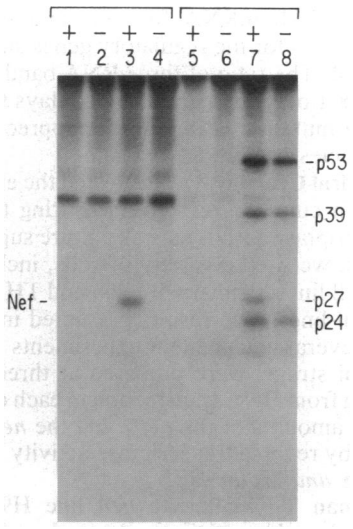

FIG. 1. Detection of the Nef protein by radioimmunoprecipitation and SDS/PAGE analysis. H9 cells infected with the *nef*$^+$ (+ lanes) and *nef*$^-$ (− lanes) strains of HIV-1-WI3 were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to radioimmunoprecipitation and SDS/PAGE analysis. Lanes 1 and 2, rabbit negative serum; lanes 3 and 4, rabbit antibody to the Nef protein; lanes 5 and 6, human negative serum; lanes 7 and 8, human serum recognizing the Nef protein as well as other, structural proteins.

Effects on RNA Expression. The *nef* gene product was reported (10, 11) to suppress viral replication by downregulating viral transcription through the LTR region. This has generally been tested by cotransfecting a plasmid containing the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the HIV LTR with others expressing *nef* and *tat*. We tested the effects of Nef on transcription by direct analysis of viral RNA levels during the course of infection. Total RNA was made from H9 or CEM-SS cells with *nef*$^+$ and *nef*$^-$ strains and subjected to Northern blot analysis. The standard three major RNA bands were observed; the 9.2-kb genomic RNA and mRNA for *gag*

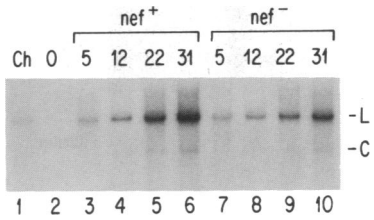

FIG. 2. Comparison of HIV DNA synthesis between the *nef*$^+$ and *nef*$^-$ strains. Primary T cells enriched for CD4$^+$ cells were infected with the *nef*$^+$ and *nef*$^-$ strains of HIV-1-WI3, and low molecular weight DNA was prepared by Hirt extraction (23) and subjected to Southern blot analysis (24). The DNA probe used for hybridization was the Sac I fragment of pWI3, which included most of the HIV genomic DNA. Numbers above lanes indicate hours postinfection. L and C are linear and circular DNA, respectively. Lane 1 (Ch) contained control linear DNA of HIV.

and *pol*, the 4.3-kb mRNA for *env*, and the 2-kb band containing mRNAs for the regulatory genes such as *tat*, *rev*, and *nef* (Fig. 3). The ratio of three RNA bands was approximately 2.5:0.8:1 over the period from 3 days to 6 days after infection. The mutation in *nef* did not appreciably alter the amount or species of viral RNA made.

Effects on Viral Growth. We also tested the effect of Nef on viral growth in cultured cells by measuring the amount of reverse transcriptase activity in cell culture supernatant over time. For this, we used a variety of cells, including H9 and CEM-SS T-cell lines, primary T cells, and THP-1 and U-937 monocytic cell lines. The results presented in Fig. 4 depict only one of several independent experiments with each cell line. The viral strains were prepared at three independent times, starting from DNA transfection in each case. For each infection, the amounts of the nef^+ and the nef^- virus were standardized by reverse transcriptase activity of viral stocks (see *Materials and Methods*).

In the human T-lymphocyte cell line H9—which was originally developed to continuously produce HIV (16), contains a relatively small amount of CD4, and shows almost no cytopathic effect after infection—nef^+ and nef^- strains grew at indistinguishable rates (Fig. 4*A*). There was ≈2-fold more nef^+ than nef^- virus produced at the time of peak production. The change in the percentage of infected cells, observed by indirect immunofluorescence, confirmed the lack of difference between the strains (data not shown).

The human T-lymphocyte cell line CEM-SS used here expressed a high concentration of CD4 molecules on its cell surface, relative to T-cell lines such as H9, and was readily fused and killed upon HIV infection (17). The changes in released reverse transcriptase activity after infection were comparable with nef^+ and nef^- virus except that, again, the nef^+ strain grew somewhat better than the nef^- strain (Fig. 4*B*).

To test cells that might be closer in properties to those the virus infects *in vivo*, we used primary T cells enriched for $CD4^+$ cells. We first used unstimulated primary T cells and found that both nef^+ and nef^- strains could not infect resting cells. Supernatant reverse transcriptase activity and immunofluorescence-positive cells were not detectable for more than 3 weeks. When we stimulated primary T cells with PHA and then infected them with either nef^+ or nef^- strains, the level of reverse transcriptase activity increased soon after infection, and many syncytia were formed (Fig. 4*C*). This rapid spread of virus in T cells is characteristic of HIV infection of the $CD4^+$ subpopulation of peripheral blood lymphocytes. In primary T cells, the nef^+ strain often grew more rapidly and to higher titers than the nef^- strain.

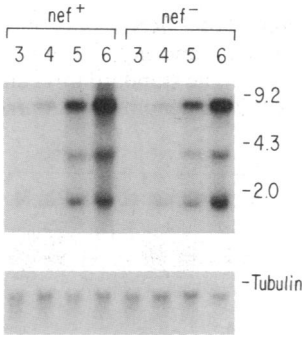

FIG. 3. Comparison of HIV RNA synthesis between the nef^+ and nef^- strains. Total RNA was prepared from H9 cells infected with the nef^+ and nef^- strains of HIV-1-WI3 and was subjected to RNA blot analysis. (*Upper*) The DNA probe used for hybridization was the 511-base-pair *Bgl* II fragment of pWI3, which includes the polyadenylylation signal sequence. Numbers above lanes indicate days postinfection, while those on the right side show the approximate sizes (kb) of HIV mRNA. (*Lower*) As a control for variation in amount of RNA loaded, the same filter was hybridized with the 1.7-kb *Pst* I fragment of tubulin cDNA (25).

We also tested the effects of the Nef protein on viral growth in the human monocytic cell lines U-937 (Fig. 4*D*) and THP-1 (Fig. 4*E*). U-937 and THP-1 cells phenotypically resemble mature monocytes and can be induced to differentiate to macrophage-like cells with phorbol 12-myristate 13-acetate. We infected these monocytic cells with the nef^+ and nef^- strains and then measured reverse transcriptase activity over time. As in the case of HIV-1-III_B, their parental strain, the nef^+ and nef^- strains grew much less efficiently in monocytic cell lines than in T-lymphoid cell lines. In U-937, both viral strains grew too slowly to compare the difference. In THP-1, the nef^+ strain grew a little more rapidly than the nef^- strain.

The nef^+ and nef^- strains used in the previous experiments were co-isogenic but the *nef* coding region was slightly different from that in other HIV strains. The amino acid sequence of Nef protein is variable among strains and the difference from strains used by others is small. But to be certain that strain difference did not account for the apparent discrepancy between our data and those of others, we obtained the strain (HIV-1-432) used in the previous report (10) in which it was claimed that *nef* acts as a negative factor at the level of transcription. When CEM-SS cultures were infected with the nef^+ and nef^- strains of this virus, the nef^+ strain grew slightly more efficiently than the nef^- strain (Fig. 4*F*), as in the case of HIV-1-WI3 (Fig. 4*B*).

DISCUSSION

We have investigated the effects of Nef on the various stages of viral growth by comparing properties of viral strains differing only in the *nef* gene. Two independently derived nef^+ and nef^- HIV-1 strains were compared. The presence or absence of the Nef protein in respective viral strains was confirmed by radioimmunoprecipitation. The pairs of viral strains were examined with respect to the initial kinetics of viral entry and reverse transcription, RNA expression, and virus production in a number of different cell lines. In contrast to the previous reports (7, 9–11), we failed to find that the nef^- strain grows more efficiently than the nef^+ strain. On the contrary, the nef^+ strain was often found to grow more efficiently than the nef^- strain. This positive effect of Nef is perhaps most prominent in primary T cells. At present, we have not identified a stage of the viral life cycle that is positively affected by Nef. Our results are in complete disagreement with previously published data showing that Nef plays a negative role in viral growth through interaction with the LTR region.

The use of different laboratory strains is not likely to have resulted in the observed discrepancy between our data and those of others. We have included experiments with the viral strains used in a previous report (10), and still could not reproduce the negative effects of Nef on viral growth. Also, in the accompanying paper, Hammes *et al.* (26) report that a Nef protein identical to the one used previously to claim that Nef is a transcriptional silencer (11) did not show any significant negative effect on the expression of a reporter gene driven by the HIV LTR. One can still argue that the Nef proteins used in these studies may not be functional ones. However, because the mutation rate of HIV is known to be high, it is difficult to define which virus isolate(s) bears the true wild-type *nef* gene. In this regard, it will be interesting to study the *nef* genes of virus isolated directly from patient materials without selection pressure.

It has also been reported that Nef suppresses viral transcription through interaction with the LTR region. To show this, HIV LTR–CAT gene constructs were cotransfected

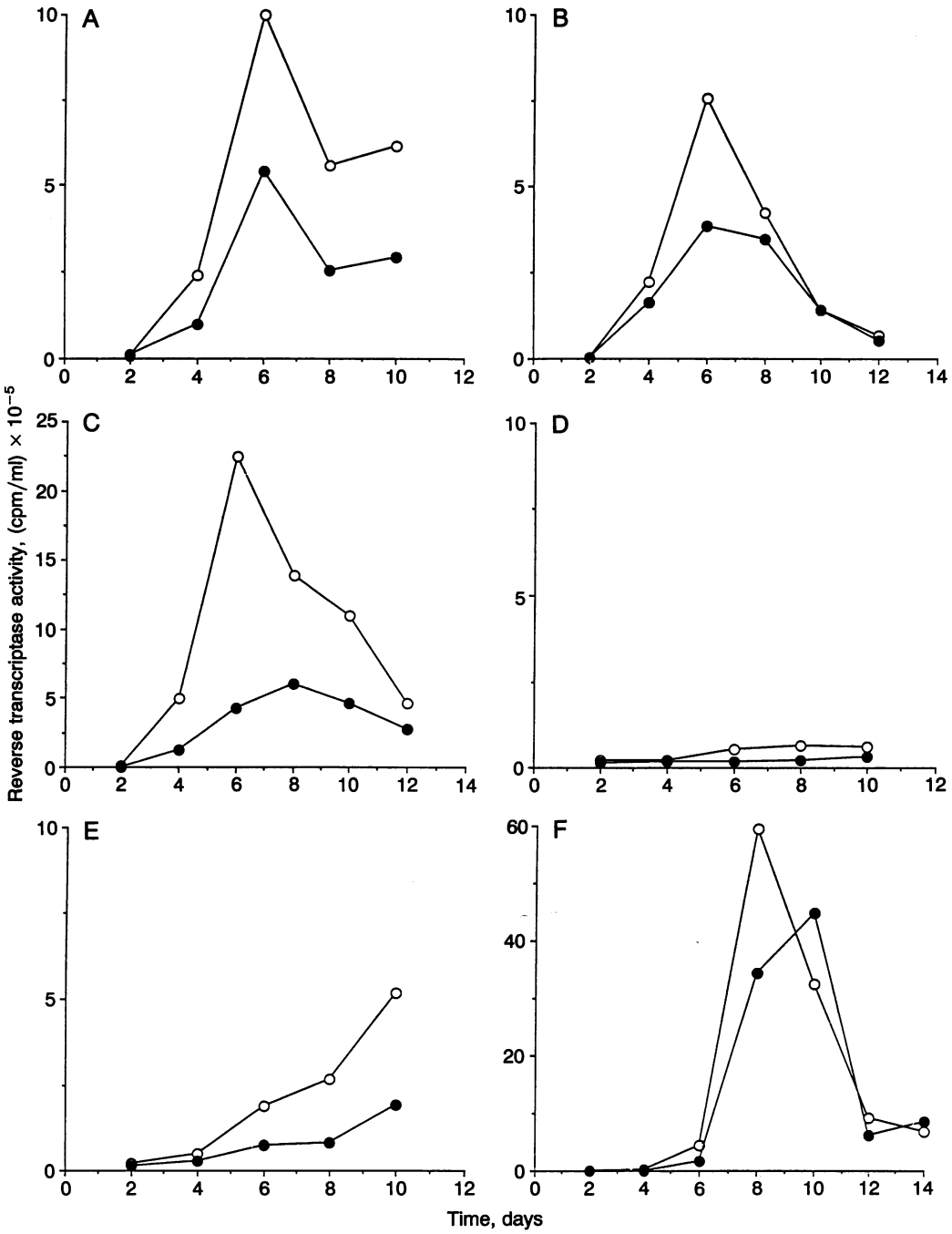

FIG. 4. Effect of Nef on viral growth. Cells were infected with *nef*$^+$ and *nef*$^-$ strains. In all infections, a low multiplicity of infection was used for infection to determine the rate of spread of virus from the change in reverse transcriptase activity in cell culture supernatant. In all cases, the percentage of initially infected cells, determined by indirect immunofluorescence assay, was <0.2%. For *A–E*, the *nef*$^+$ (○) and *nef*$^-$ (●) strains of HIV-1-WI3 were used. (*A*) H9 cells. (*B*) CEM-SS cells. (*C*) Primary T cells enriched for CD4$^+$ cells. (*D*) U-937 cells. (*E*) THP-1 cells. For *F*, CEM-SS cells were infected with the *nef*$^+$ (○) and *nef*$^-$ (●) strains of HIV-1-432.

with *nef* expression vectors, and the effects of Nef on expression driven by the HIV LTR were determined (10, 11). In this system, three to four different plasmids were simultaneously transfected into host cells. These plasmids included *tat* and *rev* expression vectors, depending on the assay system employed, as well as the HIV LTR–CAT construct and the *nef* expression vector. Two inherent problems that could make quantitative analysis of such experiments difficult are apparent with this approach. First, a relatively large amount of *nef* expression vector was used for transfection relative to other plasmids (11), and three or four different plasmids were transfected simultaneously, further multiplying the possibility of variation during the transfection (10, 11). Second, the *nef* expression vectors used in these studies also contained the entire 3′ LTR region, including the Tat-responsive element (TAR), which may compete with the LTR sequence upstream from the CAT gene for limiting transcription factors (10, 11). Indeed, this competition seems to be a significant factor. The accompanying report (26) shows that a *nef* expression vector lacking the untranslated region of the 3′ LTR does not exert a negative influence on expression driven by the HIV LTR. Further, when the untranslated 3′ LTR, including all the major transcriptional elements identified thus far, was placed under the control of the simian virus 40 early promoter, CAT gene expression driven by the HIV LTR in another plasmid was suppressed in

H9 and CEM cells at higher concentrations of the 3′ LTR plasmid (S.K. and D.B., unpublished data).

The aim of this report is to suggest to the HIV research community that Nef may not be a negative regulator as previously reported. Although there may be differences in Nef sequences or in cell lines used in different laboratories, the lack of negative activity reported in this study suggests that reevaluation of the widely accepted function of this protein may be in order. Because Nef has been proposed as a central regulator of viral gene expression and latency during pathogenesis (13, 14), it is vital to clarify at least whether Nef is indeed a negative factor. We hope that our report, together with the accompanying one (26), will initiate more rigorous analysis of Nef.

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