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Human immunodeficiency virus type 1 negative factor is a transcriptional silencer

(latency/trans-acting regulation)

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ABSTRACT **The negative factor (nef) of human immunodeficiency virus (HIV) type 1 acts to down-regulate virus replication. To decipher the step in the virus life cycle affected by nef, functional proviral clones with (pHIV F$^-$) or without (pHIV F$^+$) a deletion mutation in the nef gene were constructed. In CD4$^+$ cells, 30- to 50-fold more virus was produced over the course of 18–20 days with cultures infected with F$^-$ compared to F$^+$ virus. In CD4$^-$ cell lines, 2- to 10-fold greater virus production was found from cultures transfected with pHIV F$^-$ than those transfected with pHIV F$^+$. The negative regulatory effects of nef on pHIV F$^-$ could be supplied in trans with a plasmid expressing only the nef gene product. Virus produced by COS-1 cells transfected with pHIV F$^-$ or pHIV F$^+$ showed similar binding, uptake, uncoating, and reverse transcription. Analysis of HIV-1 RNA and structural protein levels and rates of viral RNA synthesis in CD4$^-$ cells also showed 2- to 10-fold higher levels in cells transfected with pHIV F$^-$ compared to pHIV F$^+$. The activity of a HIV-1–chloramphenicol acetyltransferase (CAT) plasmid was also suppressed by nef, whereas other CAT plasmids were unaffected. These findings demonstrate that nef acts as a specific silencer of HIV-1 transcription. This activity may be critical for maintenance of HIV-1 latency *in vivo*.**

The human immunodeficiency virus type 1 (HIV-1) genome includes at least nine genes, two genes encoding structural proteins (*gag, env*), one gene encoding protease, reverse transcriptase, and integrase (*pol*), and six genes encoding regulatory proteins, virion infectivity factor (*vif* or *sor, orfA, -Q, -P'*), trans-activator (*tat*), regulator of expression of virion proteins (*rev* or *art, trs*), virion protein R (*vpr* or *R*), virion protein U (*vpu* or *U*), and negative factor (*nef* or *3'orf, F, orf B, E'*) (1–8). *vif, tat*, and *rev* are positive effectors of virus replication (9–12). *vpr* does not affect the level of virus replication (L.R. and D. Dedera, unpublished data). The effects of *vpu* remain to be characterized. In contrast, nef down-regulates HIV-1 replication (13, 14).

The nef gene product is a 27-kDa protein (15), which is myristoylated and phosphorylated. Antibodies to nef are found in many HIV-1-infected individuals. Purified nef, produced in bacteria, shows GTP-binding, GTPase, and autophosphorylation activities (16). Sequence similarities of nef with other guanine nucleotide binding proteins, including ras, have also been described (16, 17).

To determine the mechanism by which HIV-1 replication can be down-regulated, we sought to identify the step in the virus life cycle that is primarily affected by nef.

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MATERIALS AND METHODS

Cell Lines. T-lymphoid cell lines Jurkat, Molt 3, CEM, and H9 were kindly provided by J. Hoxie and R. C. Gallo. CD4$^-$ cell lines COS-1, HeLa, and HOS, and the weakly CD4$^+$ SW480 colon carcinoma cell line were obtained from American Type Culture Collection.

DNA Clones. pHIV F$^+$ was previously designated pHXB2/3gpt (18). It was constructed by ligation of the *Xho* I/*Xba* I fragment of pHXB3, which includes nucleotides 8474–9213 and 3' flanking cellular sequences, into the corresponding positions of the clone HXB2gpt2. Plasmid pSP is a clone that lacks HIV-1 viral sequences and was formerly designated SP65HPgpt (10). Clone HXF was constructed from HXB2gpt2 by digestion with *Bss*HII and *Bam*HI, treatment with *Escherichia coli* DNA polymerase I Klenow fragment, and T4 DNA ligase, thus deleting nucleotides 259–8052. Clone pSVF was constructed by digestion of pHIV F$^+$ with *Bam*HI, treatment with *E. coli* DNA polymerase I Klenow fragment, ligation of an *Xba* I linker, digestion with *Xba* I, and ligation of the resultant 1.5-kilobase (kb) fragment (nucleotides 8053–9213 and 3' flanking cellular sequences) in the correct orientation into pSVL (Pharmacia), which includes a simian virus 40 enhancer, promoter, splice, and polyadenylylation signals. Clone pHXB5' includes nucleotides 222–5580 of the HIV-1 genome. It was derived from plasmid HXB2gpt2 (18) by deleting out the other two *Sac* I fragments (nucleotides 34–221 and 5581–9213). The mitochondrial DNA probe was kindly provided by R. Low (19). The actin cDNA clone was kindly provided by J. Milbrandt (20). The *Alu* probe is derived from a c-*sis* genomic clone, pL33M (21).

Transfection Method. Cells were transfected by the calcium phosphate precipitation method as described (22).

Nuclear Run-Off Assays. Cells were lysed in 10 mM Hepes, pH 8.0/1.5 mM magnesium chloride/10 mM potassium chloride with douncing. Nuclei were pelleted at 2000 rpm for 10 min at 4°C and resuspended in 20 mM Tris·HCl, pH 8.0/6 mM magnesium acetate/84 mM potassium chloride/10 mM ammonium chloride/0.3 mM EDTA/1 mM dithiothreitol/10% glycerol (vol/vol) at 200 × 10^6 nuclei per ml. For transcription, 250 μl of nuclei was added to 0.8 mM dithiothreitol/creatinine phosphokinase (0.8 μg/ml)/creatine phosphate (2.0 mg/ml)/0.2 mM each GTP, CTP, and ATP/25 μl of [α-^{32}P]UTP (ICN; >3000 Ci/mmol; 10 mCi/ml; 1 Ci = 37 GBq) and incubated at 30°C for 30 min. Incorporation was linear over this time period. Samples were treated with guanidine isothiocyanate-sodium acetate, and extracted once

Abbreviations: HIV-1, human immunodeficiency virus type 1; nef, negative factor; RT, reverse transcriptase; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat.

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with phenol, twice with chloroform/isoamyl alcohol (49:1), and precipitated twice with alcohol. Equivalent amounts of labeled RNAs were hybridized with nitrocellulose filters with 0.5 μg of plasmid HXB2 (18), pHXB5′, or actin. Filters were exposed for 1–6 days and analyzed by densitometry.

Chloramphenicol Acetyltransferase (CAT) Assays. CAT vectors and assays have been described (22).

RESULTS

nef Down-Regulates HIV-1 Replication in CD4$^+$ Lymphoid Cells. A functional proviral clone with an intact *nef* gene, pHIV F$^+$, was used to construct a deletion mutant, pHIV F$^-$, by *Xho* I digestion and BAL-31 deletion of 45 nucleotides (nucleotides 8472–8516). The predicted protein product is truncated from 206 to 191 amino acids, with the loss of amino acid residues 34–48.

COS-1 cells were transfected with pHIV F$^+$ or pHIV F$^-$, followed by cocultivation with Jurkat T-lymphoid cells. The F$^-$-infected cells produced significant amounts of virus, as demonstrated by reverse transcriptase (RT) assay, whereas no virus was detected from the F$^+$-infected cells (Fig. 1). However, low levels of virus could be detected in F$^+$-infected Jurkat cells when a p24 antigen assay was used (data not shown). The rate of syncytia formation and cell killing in F$^+$- and F$^-$-infected cells paralleled the results of virus production (data not shown). In experiments with additional T-lymphoid cell lines, CEM, Molt 3, and H9, virus production by F$^-$-infected cells over a period of 20 days was at least 30- to 50-fold greater than that of F$^+$-infected cells (data not shown). Experiments using clones with deletions in nef produced results qualitatively similar to those described here for pHIV F$^-$ (refs. 10 and 18; L.R., W. Hu, S. J. Lee, A. Fisher, F. Wong-Staal, and R. C. Gallo, unpublished data).

Transfection of pHIV F$^+$ and pHIV F$^-$ together resulted in rates and levels of virus production from Jurkat cells comparable to that found when pHIV F$^-$ was transfected alone (Fig. 1).

Analysis of cell-surface CD4 antigen expression, by flow cytometry with the OKT4 antibody, at day 9 of infection of Jurkat cells (Fig. 1) showed 95% of the F$^+$-infected cells expressed the CD4 antigen, whereas no CD4 antigen could be detected on the F$^-$-infected cells.

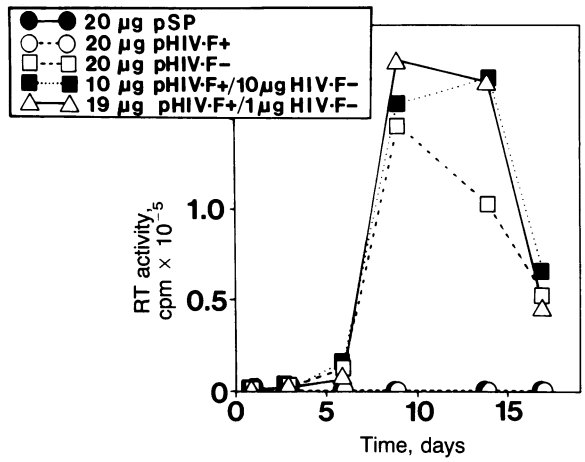

FIG. 1. The HIV-1 *nef* gene is a potent negative regulator of virus replication that does not function as a secreted product. The indicated amounts of HIV-1 functional proviral clones with (pHIV F$^+$) or without (pHIV F$^-$) an intact *nef* gene were transfected onto 50% confluent 75-cm^2 flasks of COS-1 cells. After 2 days, 10 × 10^6 Jurkat cells were added and cocultivated with the COS-1 cells for 2 days. The Jurkat cells were then removed and grown separately, and this time is designated day 0. RT activity was measured by a modification of published methods (23) using [α-^{32}P]TTP, 8-fold concentrated samples of media, and an 18-hr incubation.

nef Depresses Virus Production from CD4$^-$ Cell Lines. To evaluate whether nef affects a step in the latter half of the virus life cycle, pHIV F$^-$ and pHIV F$^+$ were transfected into CD4$^-$ cell lines without cocultivation with lymphoid cells. Under these conditions, viral RNA, protein, and particle production occurs, but reinfection is prevented by the absence of viral receptors (24). Transfection of COS-1 cells with pHIV F$^-$ generated 2- to 10-fold higher levels of virus than cells transfected with pHIV F$^+$, as determined by p24 antigen measurements (Fig. 2*a*) and RT assay (data not shown). Similar results were obtained in other CD4$^-$ cell lines, including HeLa (Fig. 2*b*) and HOS (data not shown), as well as the SW480 colon carcinoma cell line (data not shown), which expresses only very low amounts of CD4 (25).

COS-1 cells cotransfected with pHIV F$^-$ and an excess of a plasmid expressing only the *nef* gene product (HXF) reduced virus production as determined by p24 antigen assays to a level equivalent to that of COS-1 cells transfected with pHIV F$^+$ alone (Fig. 2*a*). Suppression of virus production, although less dramatic, was also obtained by cotransfection of HeLa cells with pHIV F$^-$ and HXF (Fig. 2*b*). Thus, nef can act in trans to down-regulate virus production.

Infectivity Is Not Affected by nef. Equivalent quantities of F$^-$ and F$^+$ virus derived from the COS-1 cells were then used to infect H9 cells (Fig. 3). After digestion with *Sac* I and *Xho* I, the HIV-1 probe hybridized to DNA fragments from F$^-$-infected cells of 5.5 and 3.5 kb and DNA fragments from F$^+$-infected cells of 5.5 and 2.8 kb (Fig. 3*a*). The difference in sizes of the smaller DNA fragments is a reflection of the loss of the *Xho* I site of pHIV F$^-$ due to the deletion mutation (Fig. 3*c*). Similar amounts of viral DNA were found in both F$^+$- and F$^-$-infected cells (Fig. 3*a*). Hybridization to a mitochondrial DNA probe is shown to demonstrate that equivalent amounts of DNA from each experiment were analyzed (Fig. 3*b*). The hybridizing species are sensitive to DNase I but not RNase A treatment, thus demonstrating that viral DNA but not RNA was measured. These results demonstrate a lack of effect of nef on the early steps in the

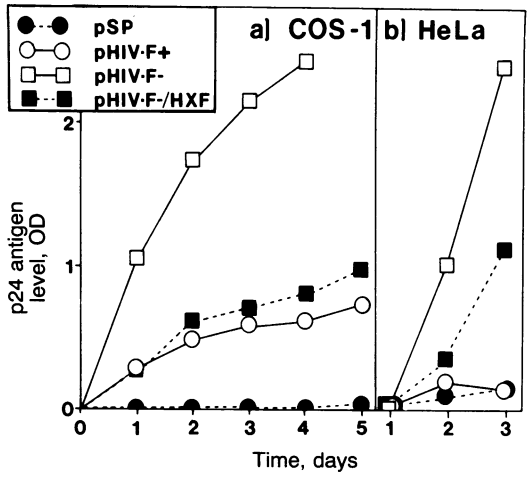

FIG. 2. The HIV-1 *nef* gene product is a trans-acting repressor of HIV-1 production in CD4$^-$ cells. Ten micrograms of pHIV F$^-$ or pHIV F$^+$ was cotransfected with 30 μg of pSP (lacking HIV-1 sequences) or 30μg of HXF (a *nef* expression clone), or 40μg of pSP was transfected alone onto 50% confluent 100-mm plates of COS-1 cells (*a*) or HeLa cells (*b*). Samples of cell culture media were tested for p24 antigen by an ELISA (DuPont). A sample of p24 (1 ng/ml) had an OD value of 0.82, and OD values were linear with protein concentration within the range of values reported. This experiment was repeated five times in COS-1 cells and twice each in HeLa, HOS, and SW480 cells with similar results.

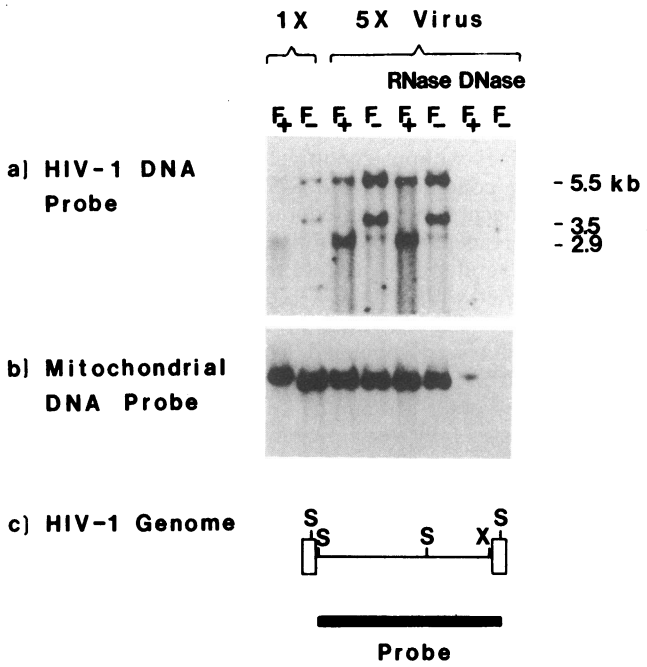

FIG. 3. The HIV-1 *nef* gene product does not affect early steps in the virus life cycle. Virus obtained 4 days after transfection of COS-1 cells (as described in Fig. 1) with pHIV F^- (F^- virus) or pHIV F^+ (F^+ virus) was quantitated by p24 antigen and RT assays. Equivalent amounts of each virus preparation (5× virus) or 20% of that amount (1× virus) were added to 10×10^6 H9 cells. Four hours later, Hirt supernatant DNAs were prepared (26) and 5 μg of each DNA was digested with *Sac* I and *Xho* I. The indicated samples were also digested with RNase A (10 μg/ml) for 30 min at 37°C or DNase I (10 μg/ml) for 30 min at 37°C prior to Southern blot transfer and hybridization with a full-length HIV-1 probe (*a*) or a mitochondrial DNA probe (*b*). The HIV-1 probe was derived from an equimolar mixture of 5.5- and 3.5-kb *Sac* I digestion products of clone pHXB2 (18). (*c*) Scheme shows the relative positions of the *Sac* I (S) and *Xho* I (X) sites in the HIV-1 genome and the sequences present in the HIV-1 probe. This experiment has been repeated five times with similar results.

virus life cycle—i.e., binding, uptake, uncoating, or reverse transcription.

nef Specifically Suppresses HIV-1 Transcription. The effects of nef on individual steps in the second half of the virus life cycle were examined next. Intracellular HIV-1 *gag* p24 and *env* gp120 and gp41 production were measured by radioimmunoprecipitation of [^{35}S]methionine and cysteine-labeled proteins with antisera from an HIV-1-infected patient. COS-1 cells transfected with pHIV F^- showed 2- to 10-fold higher levels of structural proteins than cells transfected with pHIV F^+ (data not shown).

The levels of viral RNAs were also 2- to 10-fold higher in pHIV F^-- as compared to pHIV F^+-transfected COS-1 and HeLa cells (Fig. 4 *a* and *b*) and SW480 cells (data not shown). However, the relative distribution of individual viral RNA species was unchanged (Fig. 4*b*). In the complementation experiments using cotransfection with HXF, an additional band for a 3.5-kb RNA species was seen, which represents the transcript from HXF. Depression of HIV-1 RNA levels by HXF was variable depending on the cell type used, the ratio of HXF to proviral DNA, and the transfection efficiency. In all cases, the level of HIV-1 RNAs correlated closely with the amount of virus produced as determined by p24 antigen measurements. Hybridization to either *Alu* I (Fig. 4*a*) or actin sequences (Fig. 4*b*) was used in each case to allow corrections for differences in efficiency of RNA extraction. Preparations of unintegrated DNA from these cells demonstrated equivalent transfection efficiency of pHIV F^- and pHIV F^+ (data not shown).

The effects of nef on viral RNA levels occurred at the level of RNA synthesis. COS-1 and SW480 cells were transfected with pHIV F^+ or pHIV F^-, nuclei were isolated and permeabilized, and labeled RNAs were prepared *in vitro* from preinitiated transcripts. The ratio of hybridization to HIV-1 sequences compared to actin sequences of labeled RNA from F^- nuclei was 2- to 6-fold greater than that of labeled RNA from F^+ nuclei (Fig. 4*c*).

This transcriptional effect was also examined by using a clone expressing the *nef* gene product from a simian virus 40 enhancer and promoter, pSVF, that was cotransfected with the HIV-1–CAT construct (Fig. 5). CAT activity was depressed in a dose-dependent manner by pSVF. Nucleotides −453 to −156 relative to the RNA initiation site in HIV-1–CAT were not required for nef responsiveness (data not shown). The specificity of this effect was demonstrated by the lack of an effect of pSVF on CAT activity directed by HTLV-I–CAT utilizing the human T-lymphotropic virus type I long terminal repeat (LTR), MPMV–CAT utilizing the Mason–Pfizer monkey virus LTR, RSV–CAT utilizing the Rous sarcoma virus LTR, or pSV2–CAT utilizing the simian virus 40 enhancer and promoter.

DISCUSSION

The data presented here demonstrate that nef is a negative regulator of HIV-1 replication. In experiments in lymphoid cell lines followed over 20 days, nef decreases virus replication by at least a factor of 30–50. The more profound depression of HIV-1 replication by nef noted in these experiments compared to those of other investigators (13, 14) is likely due to the choice of the isolate-dependent nef that is used in these experiments. The sequence of nef is highly variable, and in several cases a truncated protein is synthesized. Thus, it is likely that nef is partially or completely attenuated in many HIV-1 strains.

Lymphoid cells infected with the F^- HIV-1 strain showed a profound depression of surface expression of CD4 antigen. Such down-regulation of CD4 has been demonstrated previously and likely occurs at a posttranslational step (28–30). The relatively slight decrease in CD4 antigen on F^+-infected cells under conditions in which HIV-1 replication is profoundly depressed argues against the hypothesis that receptor modulation by nef mediates the marked repression of HIV-1 virus replication as suggested by Guy and coworkers (16). However, it should be noted that in these experiments the level of surface CD4 antigen was not correlated with the level of HIV-1 envelope protein expression, and thus subtle alterations of CD4 expression by nef cannot be excluded.

When cultures of Jurkat cells were coinfected with F^+ and F^- viruses under conditions in which few cells are infected with both viruses (Fig. 1), the phenotype of the F^- virus was dominant. These data suggest that nef did not down-regulate virus production via a secreted product. However, this experiment does not exclude that high levels of *nef* expression may act at least partially through a secreted product.

To determine the step in virus replication affected by nef, two experimental systems were used. To assess effects of nef on early steps in the HIV-1 life cycle, unintegrated DNA levels were measured 4 hr after infection of lymphoid cells. Virus derived from pHIV F^- and pHIV F^+ showed similar infectivity by this assay. The effects of nef on the latter half of a single cycle of virus replication were measured by analysis of the amount of virus produced from $CD4^-$ cells transfected with the proviral clones pHIV F^- or pHIV F^+. These studies demonstrated a 2- to 10-fold increased rate of virus particle production, viral protein accumulation, and viral RNA synthesis and accumulation in pHIV F^--

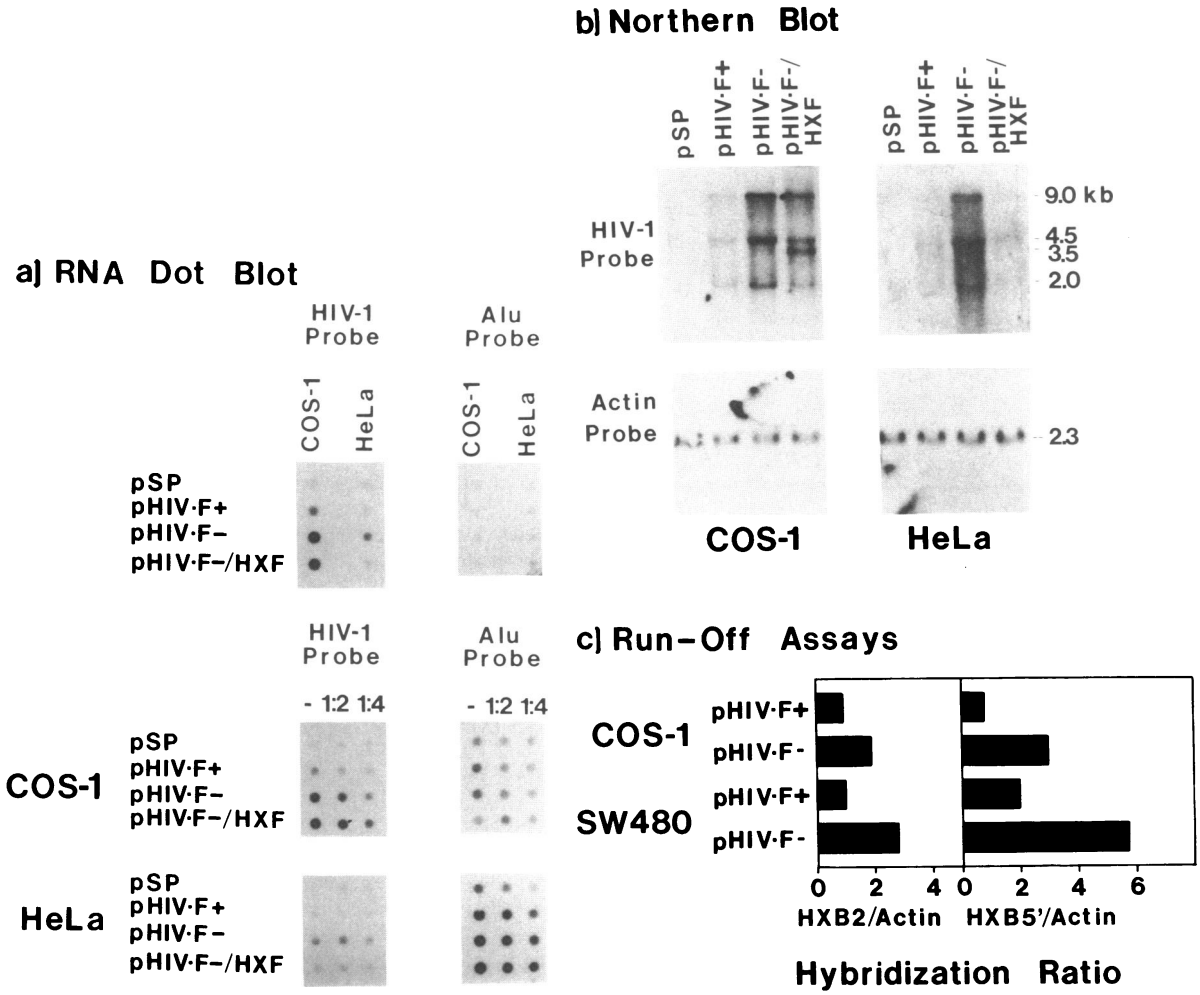

FIG. 4. The HIV-1 nef product down-regulates the level and rate of synthesis of HIV-1 RNAs. Plates (150 mm) of COS-1, HeLa, or SW480 cells were transfected with 10μg of pHIV F$^-$, pHIV F$^+$, or pSP alone (*c*) or together with an additional 30μg of pSP or HXF (*a* and *b*) as described in Fig. 2 legend. After 3 days, the cells were scraped and RNA was isolated (27) and 10 (undiluted), 5 (1:2 dilution), or 2.5 (1:4 dilution) μg of RNA was applied to an Optiblot filter with a dot-blot apparatus (*a*) (Biorad) or 35μg was electrophoresed and blotted onto Optiblot (*b*) (IBI). Filters were hybridized with the full-length HIV-1 genomic probe described in Fig. 3 legend, *Alu* sequences derived from the fifth intron of the c-*sis* gene, or an actin cDNA. Assays of p24 antigen (OD) in the supernatant at the time of RNA preparation were as follows for COS-1 cells transfected with each plasmid: pSP, 0.019; pHIV F$^+$, 0.232; pHIV F$^-$, 1.988; pHIV F$^-$/HXF, 0.271; for HeLa cells: pSP, 0.013; pHIV F$^+$, 0.071; pHIV F$^-$, 1.132; pHIV F$^-$/HXF, 0.058. This experiment was repeated three times in COS-1 cells as well as twice in HeLa and SW480 cells with similar results. (*c*) Products of nuclear run-off assays were hybridized to 0.5-μg aliquots of pHXB2 (ref. 18; HIV-1 nucleotides 1–9213), pHXB5′ (HIV-1 nucleotides 222–5580), or actin. The ratio of hybridization to HIV-1 sequences compared to actin is shown.

transfected cells compared to those transfected with pHIV F$^+$. The magnitude of the nef effect in lymphoid cells is consistent with 2- to 10-fold down-regulation of virus production per cycle demonstrated in CD4$^-$ cell lines. These results indicate that the primary effect of nef is by down-regulation of transcriptional initiation. However, subtle effects of nef on other steps in virus replication cannot be excluded.

Studies with the HIV-1 LTR fused to the CAT reporter gene also demonstrate a dose-dependent suppression of activity by the nef product. Furthermore, specificity of nef for the HIV-1 LTR was demonstrated by the lack of an effect on other eukaryotic promoters derived from simian virus 40 or the LTRs of HTLV-I, MPMV, or Rous sarcoma virus.

nef is a membrane protein (31) most likely anchored to the inner surface of the plasma membrane by an amino-terminal myristic acid modification. nef has sequence similarity to other nucleotide binding proteins, including p21ras, p60src, cAMP-dependent protein kinase, and epidermal growth factor and insulin receptors (17). Sequence similarity is also noted to the phosphorylation domain of the interleukin 2 receptor. GTP binding and GTP cleaving activities of nef have also been demonstrated (16). Lastly, nef has been shown to serve as a substrate for both autophosphorylation and phosphorylation by protein kinase C. Thus, nef may act as a signal transducer, and its effects as a transcriptional silencer may be mediated by another cellular gene product. It is possible that nef phosphorylates a cellular protein that may directly or indirectly activate a factor(s) that interacts with the HIV-1 LTR. Other examples of phosphorylation-induced binding and transcriptional activation have been described (32). The relationship of the posttranslational modifications and nucleotide binding properties of nef to its ability to down-regulate HIV-1 transcription remain to be determined.

The physiologic role of nef *in vivo* also remains to be determined. A function for nef in maintaining HIV-1 latency must by considered. Mutations that inactivate nef or factors that repress its expression or action may be critical in activating HIV-1 from a latent to an actively replicating stage. Studies of nef expression at different stages of disease in HIV-1-infected individuals will be crucial for testing this hypothesis.

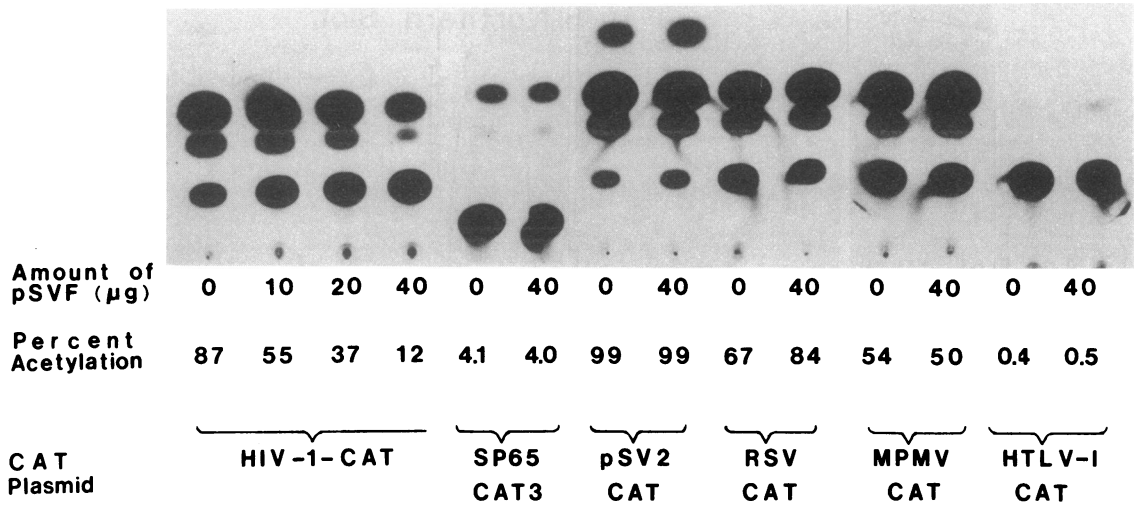

FIG. 5. The HIV-1 *nef* gene product specifically down-regulates HIV-1-CAT activity in a dose-dependent manner. Indicated amounts of pSVF or pSVL to a total of 40μg were cotransfected with 10μg of HIV-1–CAT, formerly designated C15–CAT (9); SP65–CAT3 which lacks a eukaryotic promoter; human T-lymphotropic virus (HTLV)-I–CAT; MPMV–CAT; Rous sarcoma virus (RSV)–CAT; or pSV2–CAT. CAT assays were performed as described with 2-hr incubations for cells transfected with HIV-1–CAT or SP65–CAT or 20-min incubations for cells transfected with the other plasmids. Unacetylated and acetylated forms of chloramphenicol were quantitated with a liquid scintillation counter. With 2μl of extracts from cells transfected with pSV2–CAT and pSVF or pSVL, with 5-min incubations, there was 7.7% or 10.5% acetylation, respectively, and with 15-min incubations, there was 29% or 35% acetylation, respectively. CAT assays performed with extracts from cells transfected with SP65–CAT3 were analyzed on a separate thin-layer chromatography plate under conditions that provided increased separation of unacetylated and monoacetylated chloramphenicol as compared to the other assays. This experiment was repeated in COS-1 and HeLa cells with similar results.

Note. After submission of this manuscript, a publication appeared that confirms the transcriptional silencer effects of nef shown here (33). However, a difference in the location of the cis-acting sequences responsive to nef are reported by these two studies.

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