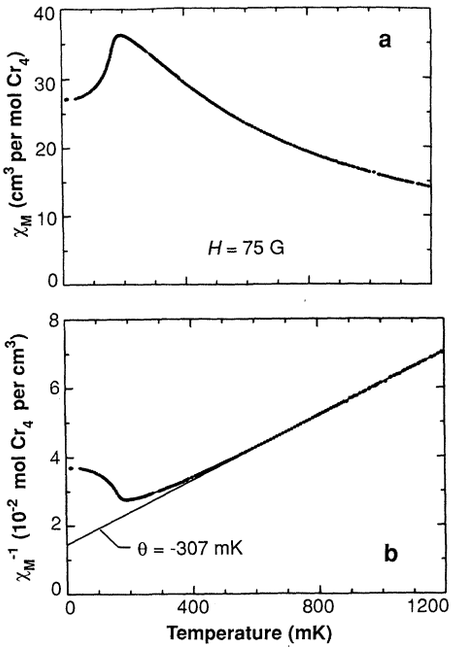

Fig. 4. The dc molar magnetic susceptibility (**a**) and its inverse (**b**) versus temperature for $[Cr_4S(O_2CCH_3)_8(H_2O)_4](BF_4)_2 \cdot H_2O$ between 13 mK and 1.2 K. The data have been normalized to the data in Fig. 2 above 1 K.

G_S = 4, 9, 11, 10, 6, 3, and 1 for S = 0, 1, . . .,6, respectively. An excellent single-parameter fit of Eq. 2 to the data in Fig. 2 was obtained for $J/k_B = +14.5 \pm 0.3$ K (ferromagnetic intracluster interactions) and $g = 2$, as shown by the solid curve through the data in Fig. 2. This agreement strongly supports the original assumptions that the strengths of the Cr-Cr intracluster exchange interactions are all about the same and are Heisenberg-like.

The electron paramagnetic resonance spectrum at 15 K of **I** dissolved and frozen in methanol shows a single broad peak with $g = 1.983$ and peak-to-peak width of 160 G. The g value is approximately that found from the magnetic studies on the solid compound.

The 12 unpaired electrons in the relatively small $[Cr_4S(O_2CCH_3)_8(H_2O)_4]^{2+}$ ion suggest many potential uses. The complex cation could be used as a contrasting agent for nuclear magnetic resonance (NMR) imaging or as a zero-shift relaxation reagent to aid the accumulation of ^{13}C NMR data. The water ligands on **I** are potentially labile, making it possible to bind this cation to a ligand on a protein or other macromolecule. As such a spin label, its magnetic effect on neighboring nuclei would reveal its location. Attaching the tetranuclear Cr_4S unit to a polymer would allow its strong paramagnetism to be exploited by using a magnetic field to orient the cluster and the attached polymer. The cluster could also be useful as a selective microwave absorber that could, in a magnetic field, facilitate localized heating in a particular organelle, organ, organism, or inanimate substructure. Moreover, the cation could be used as part of a thermometer for measurements above 0.2 K.

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11. The $[Cr_4S(O_2CCH_3)_8(H_2O)_4](BF_4)_2 \cdot H_2O$ was prepared as follows: Cr metal (−100 mesh powder, 0.5 g, 9.6 mmol) and elemental S (0.6 g, 18.8 mmol) were added to 50 ml of a 1:1 mixture of acetic acid:acetic anhydride in a 100-ml flask equipped with a condenser. The flask was placed in an oil bath that had been preheated to 147° ± 1°C, and the mixture was stirred vigorously for 3.5 hours. The resulting blue-green solution was poured (while still hot) into 200 ml of cold water and mixed thoroughly. After standing for 3 hours the white precipitate was separated by filtration through a bed of kieselgühr on a medium-porosity sintered glass funnel. The filtrate was adsorbed on a Dowex 50W-X2 cation-exchange column (15 by 45 mm). After the precipitate was washed with several volumes of water, elution with 0.1*M* HBF_4 solution removed a green band of $[Cr_3O(O_2CCH_3)_6(H_2O)_3]^+$. The blue band of $[Cr_4S(O_2CCH_3)_8(H_2O)_4]^{2+}$ was then removed with 0.5*M* HBF_4, and the blue solution was placed in a desiccator for evaporation. When the volume was reduced to about 1 ml, the mixture of blue crystals and white powder was filtered and washed with ether. The solid mixture in the filter was extracted three times with methanol (4 ml each time). Evaporation of the combined dark blue methanol extracts gave 472 mg of $[Cr_4S(O_2CCH_3)_8(H_2O)_4](BF_4)_2 \cdot H_2O$ as well-formed dark blue crystals (yield: 20% based on Cr metal). The ultraviolet-visible spectrum of **I** in H_2O yields the following data in the form $\lambda(\epsilon)$, where λ is wavelength in nanometers and ϵ is the molar extinction coefficient in mol^{-1} cm^{-1}: 705(76)sh, 605(540), 490(54)sh, 442(147), 325(1000)sh (sh, shoulder). Elemental analysis calculated for $Cr_4SF_8O_{21}C_{16}B_2H_{34}$ (percent by weight): C, 19.67; H, 3.48; S, 3.28; Cr, 21.31; F, 15.57. Composition found: C, 18.93; H, 3.46; S, 3.50; Cr, 20.48; F, 15.39.
12. Complex **I** crystallizes in space group *Pnma* (number 62) with a = 21.006(4) Å, b = 19.086(3) Å, and c = 9.323(1) Å (numbers in parentheses are standard deviations in the last digit). The structure was solved by a combination of direct methods and difference Fourier syntheses, and refined to a final R factor of 0.048. We acknowledge the contribution of C. Day, Crystalytics Company, in the solution of the structure. The atomic coordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, England. Any request should be accompanied by the full literature citation for this communication.
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Nef Protein of HIV-1 Is a Transcriptional Repressor of HIV-1 LTR

NAFEES AHMAD AND SUNDARARAJAN VENKATESAN*

In studies of the genetics of human immunodeficiency virus type 1 (HIV-1), the product of the *nef* gene, formerly known as F, 3′-*orf*, or B-ORF, was a negative regulator of HIV-1 replication. Proviruses with mutations in the *nef* gene replicated better than their standard counterparts during transient expression, and the mutant virus maintained its enhanced replication even after serial passages in T lymphocytes. The *nef* protein trans-suppressed, in a dose-dependent manner, the replication of wild-type and *nef* mutant proviruses and the expression of reporter genes linked to the HIV-1 long terminal repeat (LTR). The repression induced by the *nef* protein was mediated by inhibition of transcription from the HIV-1 LTR, which contains a far upstream cis element (previously recognized to be a negative regulatory element) between 340 and 156 nucleotides upstream of the RNA initiation site.

THE BASAL TRANSCRIPTION OF HIV-1 is governed by the interactions of several cis-acting elements in the HIV-1 LTR with cellular transcriptional factors such as TATAA, SP1, and NFκB factors (*1*). Replication of the virus is dependent on the functional expression of certain small virus-coded regulatory proteins, such as the *tat* gene product (Tat) of 86 amino

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.

acids (*2*) and the *rev* gene (formerly known as the *art/trs*) product of 116 residues (*3*). Tat is required for viral replication and the expression of genes linked to the HIV-1 LTR (*4*) and the *rev* protein (Rev) is essential for the expression of the HIV *gag* and *env* gene products (*3*). Tat and Rev are encoded by two overlapping open reading frames within two exons of a heterogeneous class of multiply spliced 2.0-kb transcripts that also encode (*5*) Nef, the viral product of the *nef* gene (also termed F, 3′-*orf*, or B-ORF). A 27-kD membrane-associated protein corresponding to Nef was reported to contain a blocked NH_2-terminus, ascribed to NH_2-terminal myristoylation (*6*). The function of this protein was not defined, however, because, in early studies of the role of Tat and Rev in viral replication, the proviral DNA carried a prematurely terminated *nef* (*3, 7*). Since this mutant replicated efficiently, Nef was considered dispensable for viral replication. More recently, replication of proviruses with overlapping deletions in *nef* was found to be unaltered or enhanced (*8, 9*) over that of proviruses with intact *nef*. It was suggested that Nef might repress viral replication, depending on the host cell, but no mechanisms were suggested. Other investigators have pointed out a structural analogy between Nef and the cytoplasmic G proteins, notably the p21 *ras* gene product, and suggested a somewhat novel role for Nef in cellular regulation (*10, 11*). Here we show that frameshift or premature termination mutation in the *nef* gene endows HIV-1 with enhanced viral replication and that, conversely, Nef trans-suppresses viral replication in a dose-dependent manner. By use of reporter genes linked to the HIV-1 LTR we also show that Nef is a down-regulator of transcription from HIV-1 LTR containing a far upstream sequence previously designated (*12*) as a negative regulatory element (NRE).

To study the Nef-mediated regulation of HIV-1 expression, we constructed two different *nef* mutant proviruses. An infectious proviral DNA, pNL432, which expresses all the known HIV-1 proteins (*13, 14*), was used to generate pNLXho. In this plasmid, *nef* was shifted beyond the 35th residue to the +1 frame, resulting in a fusion protein of 45 residues. To eliminate even this vestige of Nef expression, a second mutant provirus (pNL6673) carrying termination codons at positions 4 and 24 of *nef* was also constructed (Fig. 1A). Nef was supplied in trans, by cotransfection of a cDNA plasmid, pNLV102, which contained *nef* under the control of HIV-1 LTR or by pCMV B-ORF where *nef* was downstream of the core enhancer of cytomegalovirus (CMV) immediate early promoter (Fig. 1B). The ability of the standard provirus and the cDNA plasmids to express Nef was assessed by cell-free translation of in vitro synthesized *nef* transcripts and DNA transfection of *nef* plasmids. Two polypeptides of 27 and 25 kD (at 10:1 molar abundance) were translated from all in vitro transcripts except for the one corresponding to *nef* in pNLXho. Both

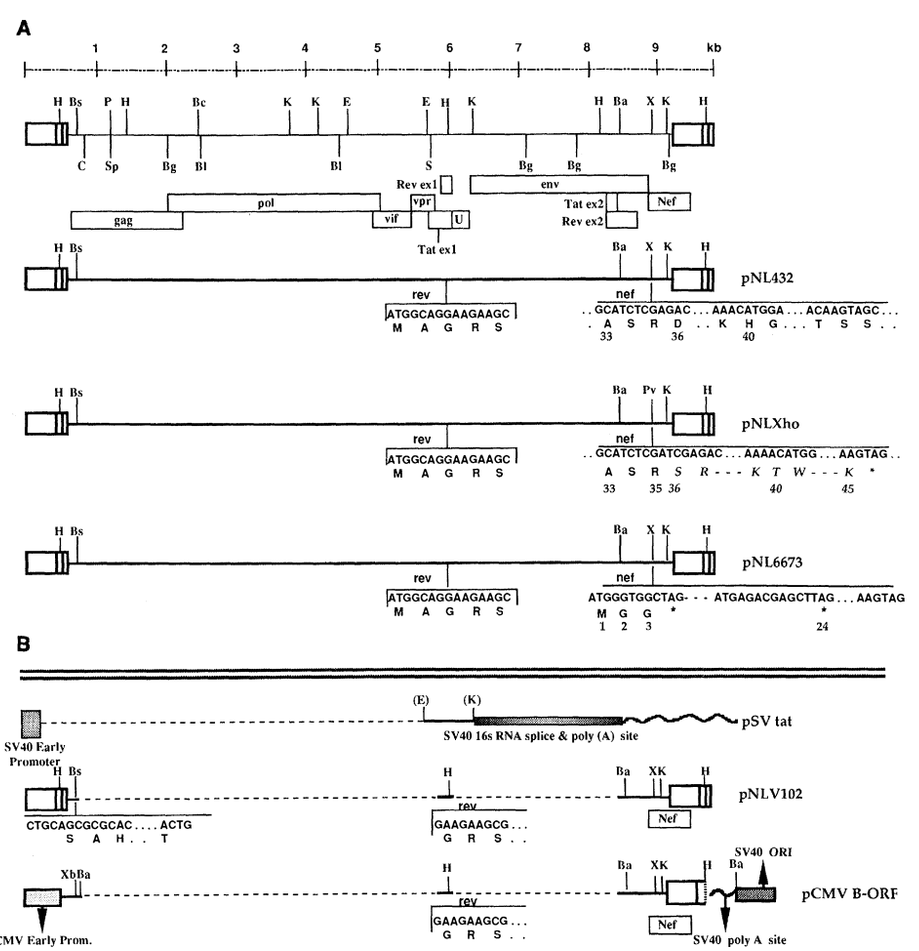

Fig. 1. Schematic diagram of HIV-1 provirus plasmids and of expression plasmids encoding HIV-1 Tat and Nef (**A**). Proviral configuration of HIV-1 is drawn to scale at the top with ORFs indicated by open rectangles and restriction sites denoted by letters. An infectious proviral DNA, pNL432 (*13*), that expresses all the known HIV-1 proteins was cleaved at the lone Xho I site in *nef*, blunt-ended with T4 DNA polymerase, and self-ligated to yield pNLXho. The Bam HI–Hind III (8522–9663) fragment of this recombinant was sequenced to confirm that the *nef* ORF was shifted beyond the 35th residue to the +1 frame resulting in a fusion protein of 45 residues containing 9 residues in the +1 frame. In addition, a single signature Pvu I site had replaced the original Xho I site. A second mutant provirus (pNL6673) carrying termination codon substitutions at positions 4 and 24 of *nef* was constructed by exchanging the Bam HI–Xho I fragment (8522–8944) in the standard provirus with a corresponding fragment that had been subjected to M13-oligonucleotide–directed mutagenesis (*14*). The relevant amino acid residues of Nef denoted by single-letter amino acid codes are numbered to indicate their position within the *nef*. (**B**) Structures of the HIV-1 *tat* and *nef* expression plasmids. In pSVtat, the first exon of the HIV-1 *tat* (between the Eco RI and the Kpn I site in the provirus) is linked to the SV40 early promoter. pNLV102 was constructed from an expressible Okayama-Berg cDNA plasmid, pV102 corresponding to a 1574-nt HIV-1 mRNA. The cDNA insert had fully preserved 5′ and 3′ ends of the mRNA and had a 69-bp central exon fused to the 1352 bp of the 3′ terminal exon. The HIV-1 insert was recovered by partial cleavage at the flanking Hind III sites in the 5′ and 3′ LTR. The Hind III fragment encompassing all the HIV-1 coding sequence and the 5′ and 3′ sequences was transferred to pNL432 between the Hind III sites at the 5′ and 3′ LTRs, yielding pNLV102. The coding potential of the subgenomic plasmids was evaluated as described (*21*). *nef* was also cloned in two opposite orientations downstream of the core enhancer in the CMV immediate early promoter (pCMV B-ORF) by using a Nar I–Hind III fragment from the original cDNA plasmid, pV102, encompassing the HIV-1 cDNA sequences between the Nar I site at 639 and the Hind III at 9663.

the p27 and p25 were also synthesized during virus infection and the smaller of these two proteins corresponded to initiation from the Met at position 20 of Nef (*15*).

Transient virus production from the standard and *nef* mutant proviruses was evaluated by extracellular particle-associated reverse transcriptase (RT) assay after the DNA transfections. pNLXho was 1.8 to 3.0 times more efficient in virus production than the standard pNL432 in SW480 cells (Fig 2A). Similar enhancement was also observed with the double termination mutant pNL6673. Accelerated replication of pNLXho was observed in CV-1, COS-1, HeLa, and Vero cell monolayers or in A3.01 T lymphocytes transfected by the DEAE-dextran procedure (Fig. 2B). The virus released from each transfectant in Fig. 2B was diluted to a constant number of RT units and used to infect $CD4^+$ T lymphocytes, and virus production was monitored by RT assay. Figure 2C shows representative results of one such experiment with A3.01 transfectants. Compared with an equivalent inoculum from a standard provirus transfectant, the amount of *nef* mutant virus released from the transfectant generated 1.5 to 2 times more progeny virus particles during infection of fresh A3.01 $CD4^+$ lymphocytes. In other experiments, the *nef* mutant viruses grew 100 to 380% better than wild-type virus. The enhanced replication phenotype was also maintained on serial passages and correlated with aborted Nef expression, readily distinguishable by the presence of the signature Pvu I site in the intracellular mutant proviral DNA.

The effect of the Nef protein on viral replication was assessed by measuring the virus produced by SW480 cells cotransfected with pNL432 and a Nef expression plasmid, pCMV B-ORF. As shown in Fig. 2D, virus production from pNL432 was suppressed in a dose-dependent manner only when the *nef* gene in the sense orientation with respect to CMV promoter was cotransfected. The decrease in the virus production correlated with the expression of Nef from the cotransfected cDNA. When *nef* in the antisense orientation was transfected, no effect on virus production was noticed, although the antisense *nef* RNA in this case might have been expected to inhibit the expression of the endogenous Nef from the standard provirus. It is possible that the expression of endogenous Nef from pNL432 is not affected by the antisense RNA in these experiments. Suppression of the enhanced replication of the *nef* mutant pNLXho was also effected by cotransfection of pCMV B-ORF.

To examine the possibility that Nef reduced virus production by inhibition of viral structural protein synthesis, we evaluated the transient expression of HIV-1 envelope protein from HIV-1 LTR as a function of Nef coexpression. Since HIV-1 *env* protein synthesis depends on the expression of Rev (*3*), a Rev expression plasmid, pNLV102A/X, containing HIV-1 LTR-linked *rev* cDNA was cotransfected with pHIVenv. To obtain optimal expression of Rev, HIV-1 Tat would also be required. Tat was therefore supplied in the form of pSVtat, containing the first (functional) exon of HIV-1 *tat* fused to the SV40 early promoter. When this combination of pHIVenv, pNLV102A/X, and pSVtat was transfected, substantial amounts of HIV-1 envelope gp160, gp120, and gp41 proteins were synthesized; they were detected by immunoblotting with the use of either rabbit antiserum to recombinant gp120 or pooled AIDS patient sera (Fig. 3A). With increasing amounts of cotransfected pCMV B-ORF, there was a proportionate decrease in the level of *env* protein expression. The decrease in the expression of *env* proteins coincided with increasing Nef expression and approached a maximum inhibition of 90%. When *nef* was placed in the wrong orientation in pCMV B-ORF, *env* protein expression was unaffected (Fig. 3A). Nef also induced a similar inhibition of HIV-1 p55 *gag* expression from the HIV-1 LTR.

The relative effects of Nef on the expression of reporter genes linked to the HIV-1 LTR or a heterologous promoter were then examined. For this purpose, expression of the bacterial chloramphenicol acetyltransferase (CAT) activity in cells transfected with the CAT gene linked to HIV-1 LTR (pHIV CAT) or the core enhancer of CMV immediate early promoter (pCMV CAT) was studied as a function of Nef expression. Initial efforts to assess the effect of Nef on basal levels of CAT expression from the HIV-1 LTR were unreliable owing to the high degree of variability in the basal transcription. Therefore, we measured Nef-induced inhibition of pHIV CAT expression during transactivation by the HIV-1 tat protein (Fig. 3B). The percentage of ^{14}C-labeled chloramphenicol that was acetylated by extracts of individual transfectants revealed a predictable decrease in the CAT activity in the SW480 or CV-1 transfectants, commensurate with increasing pCMV B-ORF expression. Plasmids containing *nef* in the wrong orientation produced no effect, and CAT expression from the CMV pro-

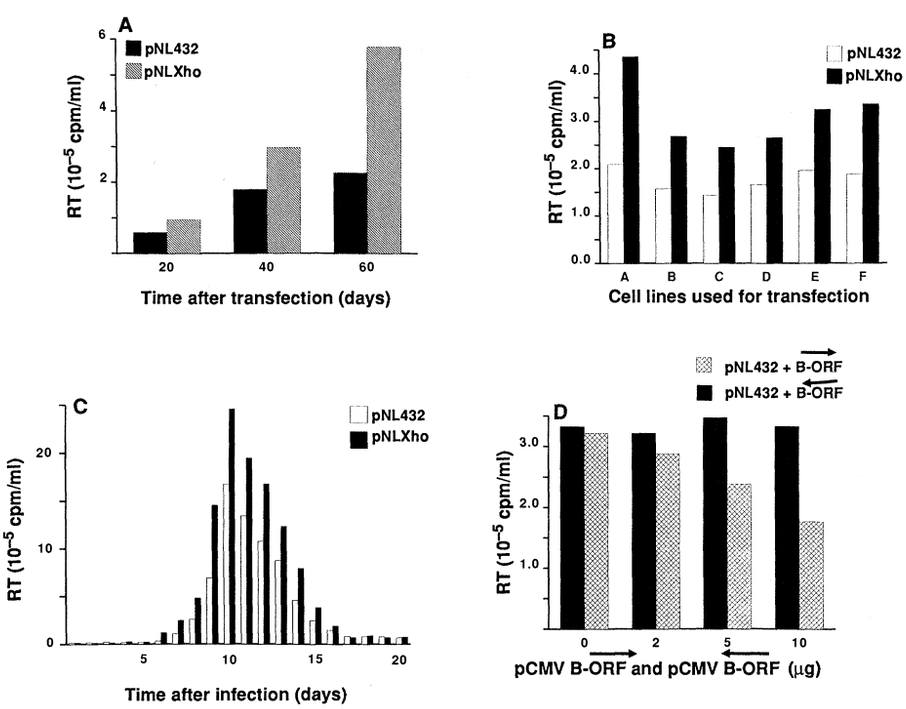

Fig. 2. Transient expression of standard or *nef* mutant proviruses and the effect of Nef on viral replication (*22*). (**A**) The time course of virus released in the medium was measured by the particle-associated RT assay in SW480 cells transfected with 5 μg each of pNL432 and pNLXho. (**B**) Transient virus production by standard and mutant proviruses after transfection of SW480 (A), CV-1 (B), COS-1 (C), Vero (D), HeLa S3 (E), and A3.01 (F) cells. In each case, released virus was diluted to constant RT units and used to infect A3.01 T4 lymphocytes. (**C**) Extracellular virus released by the A3.01 transfectants (B) was filtered through a 0.45-μm Millipore filter, and equivalent infectious units from each transfectant were used to infect A3.01 lymphocytes. (**D**) Transient virus production by pNL432 transfectants was monitored as a function of cotransfection of increasing amounts of pCMV B-ORF in the sense or antisense orientation, respectively (indicated by the right and left arrows).

moter (pCMV CAT) was unaffected by cotransfection of the Nef expression plasmid.

We then inquired whether the Nef-induced inhibition of HIV-1 LTR-linked CAT expression was mediated by cis-acting elements in the LTR. CAT expression from pHIV CAT that was linearized by cleaving (in the vector) near the 5′ boundary of the U3 was still responsive to the repressive influence of Nef. To further delineate the 5′ boundaries of the Nef-responsive cis element in the LTR, pHIV CAT derivatives containing progressive 5′ deletions of the LTR were cotransfected with pSVtat and pCMV B-ORF. Two such 5′ deletions extending up to positions −400 and −340 in the HIV-1 LTR continued to be repressed by Nef. In other experiments, the Tat-responsive CAT expression of a pHIV CAT derivative containing only the TATAA element and the TAR region of the LTR (−35/+80) was not inhibited by Nef. CAT expression from pHIVCAT, linearized at the single Ava I restriction site at −156, was also unresponsive to Nef coexpression. Therefore, both the CAT and the HIV-1 *env* were placed downstream of an Ava I–Hind III (−156/+80) fragment of the LTR and cotransfected with pSVtat and pCMV B-ORF in the sense or antisense orientation. With this Ava I–Hind III version of the LTR, the expression of CAT was totally unresponsive to Nef coexpression (Fig. 3B). In a similar manner, *env* gene expression from the LTR that lacked sequences upstream of the Ava I site continued unabated in the presence of pCMV B-ORF.

We then used S1 analysis of CAT mRNA to determine whether the effects of Nef on CAT expression reflected transcriptional suppression (Fig. 4). The levels of detectable CAT mRNA transcribed from the pHIV-CAT decreased proportionately with increasing pCMV B-ORF of right polarity (Fig. 4A). With 10 μg of pCMV B-ORF, there was a 70 to 90% decrease in these transcripts in independent experiments. Because the nuclease S1 assay only measured the RNAs containing the CAT gene sequences, the reduction of RNA levels might have been due to either increased degradation of RNA mediated by the TAR sequence or repressed initiations. However, the CAT mRNA levels remained unresponsive to pCMV B-ORF expression in cells transfected with pΔNRE HIV CAT (Fig. 4B), although the CAT transcripts in this case included the TAR sequence. Therefore, the Nef-mediated reduction of CAT transcripts from pHIV CAT was probably due to reduced initiations.

From these results, we concluded that the cis acting element responsive to Nef was located between 156 and 340 nucleotides (nt) upstream of the RNA start site. Although we could not clearly define the boundaries of the cis element, when the EcoRV-Ava I (−338/−156) fragment was replaced upstream of the pΔNRE HIV CAT in the normal orientation, CAT expression became susceptible to Nef-induced inhibition. The region responsive to Nef roughly corresponds to the domain for the NRE that was initially recognized for its possible cis-acting transcriptional inhibitory effect (*12*). Recent reports indicate that the NRE might be a target for interactions with many cellular transcriptional factors. For instance, Garcia *et al.* (*1*) have identified a putative protein-binding region near the extreme 3′ end of the NRE (−173/−159). Farther upstream, between positions −357 and −278, three sequence motifs with similarity to the Ap1 binding site were also identified (*16*). The NRE is also presumed to contain two elements with partial homology to direct

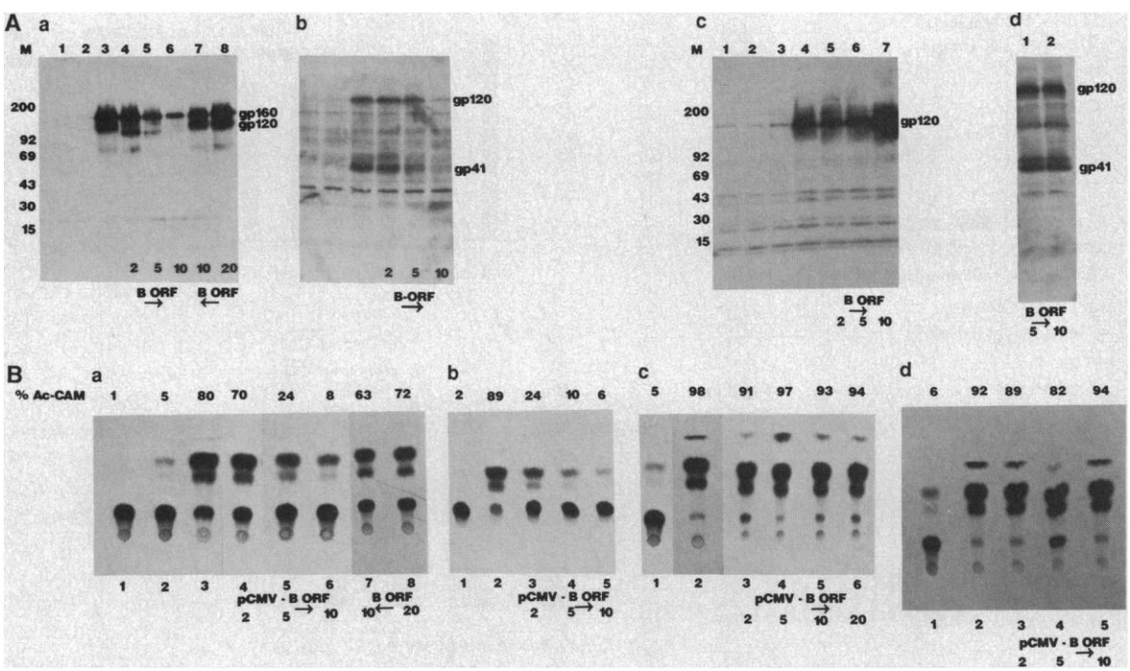

Fig. 3. Influence of Nef on the expression of HIV-1 *env* and CAT gene linked to HIV-1 LTR or CMV early promoter. Two reporter genes, namely, the HIV-1 *env* and the bacterial CAT gene placed under the control of complete HIV-1 LTR, HIV-1 LTR lacking the NRE, or CMV core enhancer were used (*23*). (**A**) Expression of *env* linked (a and b) to the HIV-1 LTR and (c and d) to the LTR lacking NRE. Expression of *env* proteins was monitored in SW480 transfectants by immunoblotting using rabbit hyperimmune serum against recombinant HIV *env* gp120 (a and c) or pooled AIDS patients' serum (b and d) and ^{125}I-labeled protein A. Results obtained with cells transfected with pHIVenv (lane 1); pHIVenv and pSVtat (lane 2); pHIVenv, pSVtat, pNL A/X (lane 3); and increasing amounts (in micrograms of DNA) of pCMV B-ORF in the sense (right arrow, sense orientation, lanes 4 to 6) or antisense orientation (lanes 7 and 8) are shown in (a) and (b). Lane 1 in (c) shows transfection with calf thymus DNA. Lanes 2 to 7 in (c) correspond to lanes 1 to 6 in (a) except that pΔNREenv was substituted for pHIVenv. Lanes 1 and 2 in (d) correspond to lanes 6 and 7 in (c). (**B**) Bacterial CAT expression was monitored by CAT assays (*23*) of cells transfected with pHIV CAT in SW480 cells (a) or CV-1 cells (b). CAT assays of SW480 cells transfected with pCMV CAT (c) or pΔNRE HIV CAT (d) are also shown. Results obtained with carrier DNA (lane 1 in a to c) and with pHIV CAT (a, lane 2) pCMV CAT (c, lane 2) or pΔNRE HIV CAT (d, lane 1) alone are also shown. Otherwise, CAT expression from pHIV CAT or pΔNRE HIV CAT was measured in the presence of optimal amounts of pSV tat (lanes 3 to 8 in a, and lanes 2 to 5 in b and d). pCMV CAT transfectants (c) did not contain pSVtat. Increasing amounts of *nef* in the sense (lanes 4 to 6 in a, 3 to 5 in b and d, and 3 to 6 in c) or antisense orientation (lanes 7 to 8 in a) were cotransfected in the indicated lanes.

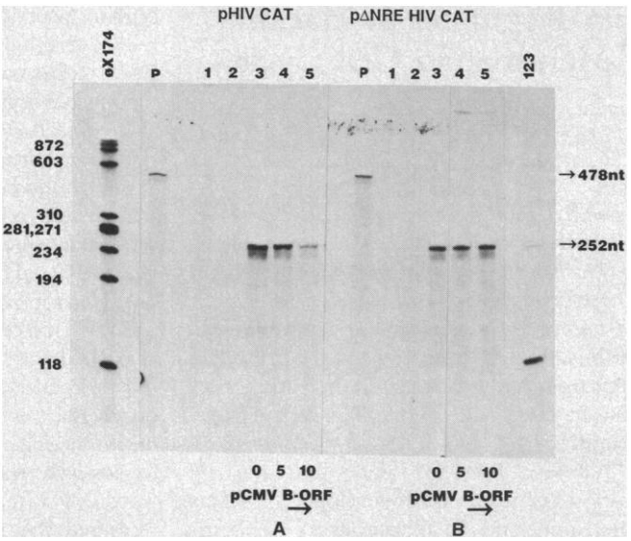

Fig. 4. Quantitative S1 analysis of CAT mRNA synthesized in the various HIV CAT plasmid transfectants. Whole cell RNA (20 μg) (*24*) was annealed at 42°C for 16 hours with a uniformly ^{32}P-labeled 478-nt antimessenger sense DNA probe synthesized on a M13mp9 CAT gene recombinant (*25*), with the M13 sequencing primer. The 478-nt fragment isolated by Hinc II cleavage and gel purification contained 252 nt corresponding to the 5′ end of CAT gene. Nuclease S1-resistant hybrids from the annealing reaction were resolved by electrophoresis on 6% acrylamide and urea gels (*25*). (**A**) Lanes 1 to 5, results obtained with RNA from cells transfected with calf thymus DNA; pHIV CAT alone; pHIV CAT and pSVtat; pHIV CAT, pSVtat, and 5 μg of pCMV B-ORF; and pHIV CAT, pSVtat, and 10 μg of pCMV B-ORF, respectively. (**B**) Lanes 1 to 5, similar reactions except that pΔNRE HIV CAT was used in place of pHIV CAT. The position of the S1-resistant band of 252 nt is indicated. The 252-nt band in each case was cut out and the radioactivity determined by liquid scintillation spectrometry. Lane P in both of the panels denotes the electrophoretic profile of the purified 478-nt probe. End-labeld Hae III fragments of ΦX174 RF DNA and end-labeled 123-bp DNA ladders served as molecular size markers.

repeats in the promoters for the human interleukin-2 and interleukin-2 receptor genes (*16*). Therefore, the NRE may contain multiple internal domains, each differentially regulated by Nef.

The ability of Nef to repress HIV-1 LTR transcription in a dose-dependent manner introduces yet another level of complexity in the mechanism of HIV-1 gene regulation. The repressive effect of Nef would be desirable as a buffer to moderate the effects of Tat early in infection, perhaps negating stray activation of latent virus during subtle changes in the physiological environment. Evidence that the effects of Nef may be mediated by a complex cytoplasmic signaling pathway comes from the known sequence homologies of Nef with guanosine triphosphate (GTP) binding and guanosine triphosphatase (GTPase) domains of known cytoplasmic G proteins, notably, *ras* p21 (*10, 11*), and the recent demonstration of GTP binding and GTPase activities associated with the *Escherichia coli*–expressed Nef (*10*). Recently, Imler *et al.* (*18*) proposed that the *ras* oncogene activated a far upstream cis element (the *ras*-responsive element, RRE), presumably by enhanced DNA binding of a transcriptional factor related to Ap1 or c-*jun*. On the basis of this analogy, Nef might modulate HIV-1 transcription by altering the binding of Ap1-like factors to the NRE.

Many of the features that would qualify Nef as a "membrane-associated G protein" (*6, 10*) have been assumed to reside in a single polypeptide of 206 residues. However, we have evidence that a second *nef* product initiates at a Met codon at position 20. There appears to be no functional role for this 5′ truncated version of Nef, and we do not know whether it is cytoplasmic. However, since both the bovine papilloma virus E2 ORF (*19*) and the adenovirus E1A region (*20*) encode two mutually antagonistic regulatory proteins sharing their respective COOH-terminal sequences, perhaps HIV-1 has evolved an analogous functional mechanism for Nef.

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21. Bam HI–Hind III inserts of the *nef* expression plasmids and the corresponding Bam HI–Hind III fragments (8522 to 9663) from standard and mutant proviruses were also cloned downstream of the SP6 or T7 polymerase promoters in the commercial vectors pGEM3 and pGEM4 (Promega Biochemicals). T7 transcripts of these inserts were synthesized in vitro and translated in a rabbit reticulocyte system to verify the translatability of *nef*.
22. Subconfluent monolayers in T25 flasks were transfected 12 hours after seeding with 20 μg of total DNA (adjusted with carrier calf thymus DNA) by the modified calcium phosphate transfection technique (*14*). For provirus transfections, between 5 and 10 μg of DNA sufficed to saturate the monolayer system. For transfecting A3.01 lymphocytes, the DEAE-dextran procedure (*3*) was used. For the analytical transfections we used 2×10^7 cells and 2 to 5 μg of test DNA with carrier DNA added up to a maximum of 10 μg. The virus production was monitored by RT assay (*14*) of the culture fluid every 12 hours. All values represent the averages of three or four independent experiments that varied by no more than 4 to 10%.
23. For studying CAT expression, a CAT gene bearing Hind III–Bam HI fragment from pSV2CAT (*13*) was placed downstream of the Hind III site at position +80 of the HIV-1 LTR. The resulting plasmid, pHIV CAT, contained the entire U3 and the R region up to the Hind III site. pΔNRE HIV CAT was constructed by transferring the CAT-bearing Hind III–Bam HI fragment from pSV2CAT to a plasmid containing the Ava I–Hind III (−156/+80) fragment of HIV-1 LTR downstream of the Hind III site. In pCMV CAT, the CAT gene was downstream of the CMV core enhancer. For each set of CAT plasmids, the values were calculated from three independent transfections. The conversion rates (%) were determined from the linear portion of the assay when the monomethylated derivative was the most predominant product. To generate pHIVenv, HIV-1 *env* ORF-bearing Eco RV fragment was obtained from a recombinant M13mp18 [mpPE-env (*27*)] RF DNA and blunt-end ligated between the Hind III and Hpa I sites of pHIV CAT (thus exchanging the CAT gene with HIV *env*). pΔNRE HIVenv was constructed by transferring the *env*-bearing Sac I fragment to the Sac I site in the HIV-1 LTR of a plasmid containing the Ava I–Hind III (−156/+80) fragment of the LTR. This construct had a continuous LTR sequence between −156/+80 upstream of the *env*-coding sequence.
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