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Constitutive Expression of Human Immunodeficiency Virus (HIV) *nef* Protein in Human Astrocytes Does Not Influence Basal or Induced HIV Long Terminal Repeat Activity

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Since human immunodeficiency virus (HIV) *nef* has been suggested to exert regulatory effects on HIV long terminal repeat (LTR) activity, we transiently transfected HIV LTR chloramphenicol acetyltransferase or luciferase expression vectors into a human astrocytoma clone (U-373nef) that constitutively expresses the HIV *nef* gene. In these cells, basal HIV LTR activity, as well as tumor necrosis factor-induced or *tat*-driven activity, was similar to that in control cells. Lack of any detectable effect of HIV *nef* on LTR activity was not the result of mutations in integrated *nef* DNA, as was shown by polymerase chain reaction. These data suggest that the role of *nef* in HIV genome transcription does not necessarily involve a direct influence on HIV LTR activity.

Conservation of the *nef* gene in human immunodeficiency virus (HIV) and simian immunodeficiency virus is compatible with the concept that the *nef* protein provides a biological advantage in the evolution and life cycle of these pathogens. The *nef* gene codes for a protein of 27 kilodaltons which is myristylated (p25 form) (2) and shares structural homology with members of the proto-oncogene family such as $p21^{ras}$ and $p60^{src}$ (14, 27). A comparison between $p21^{ras}$, a membrane signal transducer (17, 28), and HIV *nef* is relevant here, since we have recently reported that $p21^{ras}$ can induce transactivation of the HIV type 1 (HIV-1) regulatory region, the long terminal repeat (LTR) (3).

The *nef* gene product is not obligatory for HIV-1 replication (30), but deletion of the *nef* gene from an HIV-1 infectious provirus reportedly enhances replication in human lymphoblastoid cells (1, 21, 25). This observation led to the concept that the *nef* gene product regulates HIV production negatively. It was suggested that the target sequence(s) responsible for the suppressive effect of *nef* on LTR activity may be situated between −340 and −156 upstream of the RNA initiation site in the LTR (1), i.e., within the region containing negative regulatory elements (NREs) (13). Recent reports disagree with these conclusions. Kim et al. (20) suggest that *nef* gene expression does not influence HIV-1 replication negatively. Hammes et al. (16) observed no negative effects of *nef* on HIV-1 LTR-directed transcription in human lymphoblastoid and myelomonocytic cells.

Our approach to analyzing functional effects of HIV *nef* on HIV LTR activity was to study the influence of constitutive *nef* protein expression in human cells known for their low permissiveness to HIV replication (9). We therefore established a line of astrocytoma cells that constitutively express *nef* (U-373nef) by using a cytomegalovirus (CMV) *nef* expression vector. By using this line, we analyzed the expression of LTR reporter gene vectors in transient transfection assays. The system used avoids shortcomings inherent to transient cotransfection assays where massive introduction of exogenous DNA may compete nonspecifically for transcription factors (5). By using this method, we did not observe a detectable influence of HIV-1 *nef* protein on HIV-1 LTR activity.

The pCMV-nef vector used for stable transfection consisted of the HIV-1 (LAV-1-Bru strain) *Bam*HI-*Xmn*I fragment (nucleotides 8068 to 384) fused to the human CMV immediate-early enhancer-promoter (−522 to +97 *Eco*RI-*Hin*dIII fragment of pCMV-CAT) (11). This vector was introduced by electroporation (single pulse, 180 V, 1,010 μF) into a human CD4-negative astrocytoma cell line (U-373MG). A neomycin resistance gene, pSVtk-neoβ (24), was cotransfected at a 1/5 ratio, and cells were maintained in selective Dulbecco modified Eagle medium containing G418 (400 μg/ml) (6, 18). Control astrocytoma cells were stably transfected with a pCMV-CAT expression vector (11) by the same procedures. Addition of G418 was stopped after 10 months of culturing. Results of the stable U-373nef and control U-373CAT clones reported below were obtained in the absence of G418 and after confirmation that cells were mycoplasma free.

Figure 1A shows Southern blot analysis of DNA from the U-373nef clone. As expected, the probe hybridized to a 1.14-kilobase band of a *Bam*HI-*Hin*dIII digest of cell DNA, corresponding to an internal *nef* fragment. The number of integrated copies of the *nef* gene, determined by dot blot analysis (8), was four copies per cell genome. Northern (RNA) blot analysis, as described previously (4), showed that *nef*-specific RNA (approximately 1.15 ± 0.1 kilobases) was detected in cytoplasmic lysates (Fig. 1B).

For in situ hybridization (15), the *nef* insert (fragment *Bam*HI-*Taq*I, nucleotides 8068 to 9013) from HIV (LAV-1-Bru strain) was cloned into a pSP72 vector which provides SP6/T7 promoters (Promega-Biotec, Madison, Wis.). RNA probes of both linearized template orientations were generated with either Sp6 (sense) or T7 (antisense) RNA polymerase. When U-373nef cells were hybridized with the *nef* antisense probe, more than 70% of the cells were positive with 50 or more grains per cell (Fig. 2B). U-373nef cells hybridized with a sense RNA probe revealed less that 15 dispersed grains per cell (Fig. 2A), as did control U-373 cells hybridized with sense and antisense *nef* RNA probes. Indirect immunofluorescence studies were performed on cells of the U-373nef clone fixed with 4% paraformaldehyde, incu-

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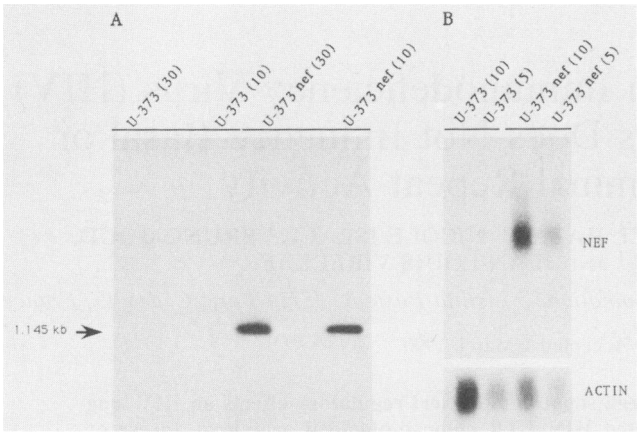

FIG. 1. Identification of *nef* DNA (A) and RNA (B) in a U-373nef clone by Southern and Northern blot analysis, respectively. (A) Genomic DNA (10 and 30 μg) from U-373nef and control U-373 cells was digested to completion with *Bam*HI and *Hin*dIII restriction enzymes, submitted to electrophoresis in 1.5% agarose, and hybridized to an internal *nef* gene DNA probe (*Bam*HI-*Taq*I fragment; nucleotides 8068 to 9013 of HIV(LAV-1-Bru strain). kb, Kilobases. (B) The same probe was used for detection of *nef* mRNA by RNA blot analysis using 10 or 5 μg of RNA, as indicated in parentheses. The mRNA levels from U-373nef and U-373 control cell lysates were compared with a β-actin probe.

bated with bovine serum albumin and saponin, and stained with an anti-*nef* monoclonal antibody (E12; Transgene, Strasbourg, France) and rhodamine-labeled sheep antibody to mouse immunoglobulin G. Figure 2D shows intense *nef* protein expression in the U-373nef clone. Staining occurred only in the cytoplasm, confirming results reported previously in HIV-infected cells (12), and was particularly pronounced around the nucleus. Reinforcement of peripheral fluorescence suggested a concentration of the protein at the plasma membrane of some cells. Immunofluorescence with a control antibody specific for the endoplasmic reticulum showed that the organization of the intracytoplasmic organelles was not modified in the U-373nef cells compared with that of control U-373 cells (data not shown). Neither the growth nor the behavior of the U-373nef line differed significantly from that of the control line.

In U-373nef cells, the functional effects of constitutively expressed *nef* protein on the homologous LAV-1-Bru LTR were analyzed by transient expression of CAT or luciferase reporter genes under the control of the HIV LTR and compared with their activity in control cells. In cells transfected by a standard calcium phosphate coprecipitation technique, CAT activity was determined by standard procedures (23), and luciferase was assayed as described elsewhere (29a). The levels of CAT expression were comparable in U-373nef and control U-373 cells (Fig. 3A), irrespective of the concentration of the LTR CAT vector (*Bgl*II-*Hin*dIII

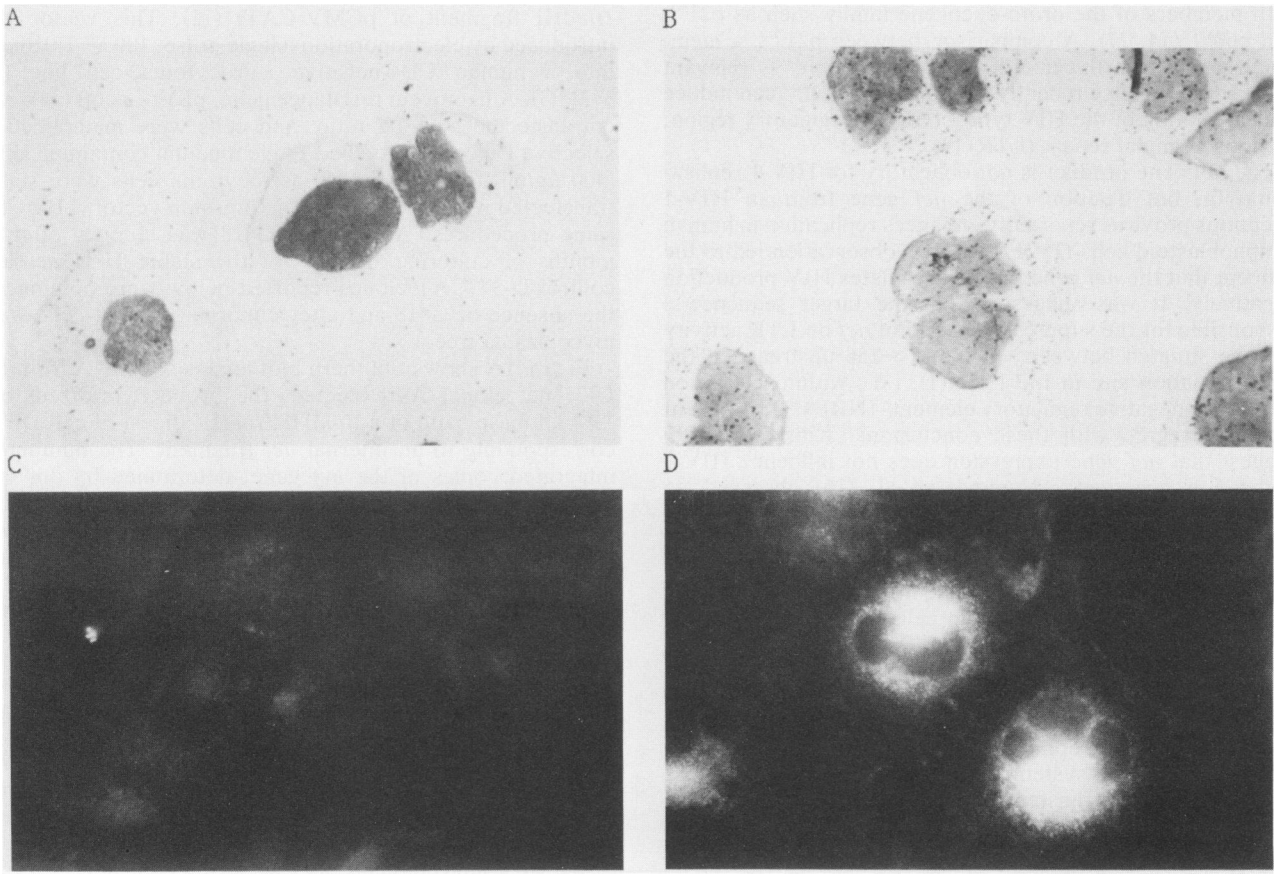

FIG. 2. Analysis of *nef* mRNA expression in U-373nef cells by in situ hybridization (A and B). U-373nef cells were spun onto slides in a Shannon Elliott Cytospin and hybridized with a ^{35}S-labeled antisense RNA probe specific for *nef* (B) or a control sense *nef* probe (A). Immunofluorescent staining of control U-373 cells (C) or U-373nef cells (D) with anti-*nef* antibody. Scale, 1 = 17 μm.

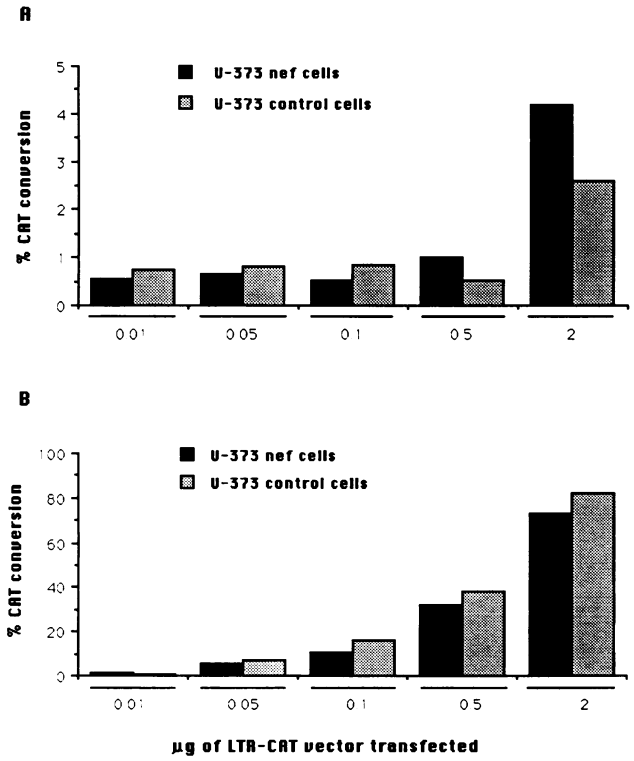

FIG. 3. CAT activity observed after transient transfection of various concentrations (10 ng to 2 μg) of HIV LTR CAT vector into either U-373nef or control U-373 cells (2×10^6). Measurement of CAT activity was performed on 10^6 cells and expressed as the percent conversion of [^{14}C]chloramphenicol to its acetylated products. (A) Transfection of LTR CAT exclusively. (B) Cotransfection of LTR CAT and a CMV *tat* expression vector (100 ng). Results shown are from one representative experiment out of four with different plasmid preparations.

fragment; nucleotides −489 to +79 linked to the CAT gene) used. CAT activity did not differ in U-373nef cells compared with that in controls (Fig. 3B) when cotransfected with a vector permitting expression of homologous *tat* protein, pCMV-tat (*tat* cDNA inserted downstream of the human CMV immediate-early enhancer-promoter region [−522 to +97]). Tumor necrosis factor, a potent transcription activator of the HIV-1 enhancer (10, 19, 26), induced the same level of transcriptional stimulation of HIV LTR CAT in both cell lines. The same was true when an HIV enhancer CAT-driven expression vector was employed (Table 1). This vector carries three copies of a synthetic oligonucleotide containing the exact sequence of the HIV-1 enhancer (10-base-pair repeat located between −108 and −75 in the HIV-1 LTR) upstream of a truncated herpes simplex thymidine kinase promoter (19). Aside from some variability observed in individual experiments between both cell lines, no consistent increase or decrease in LTR expression was detected in more than 15 experiments with either the LTR CAT or LTR luciferase vector.

The above results suggested that constitutive *nef* expression did not influence HIV LTR (U3+R) activity or that of the HIV enhancer element. Despite the presence of the NRE region in the *Bgl*II-*Hin*dIII HIV LTR vector, no influence of constitutively expressed *nef* protein was observed. Deletion of the NRE region (upstream of the *Sca*I site at −142) did not modify promoter activity in U-373nef compared with that in

TABLE 1. Response to exogenous tumor necrosis factor of HIV LTR CAT or HIV enhancer CAT expression vector transfected into U-373nef and U-373 control cells[a]

Plasmid used	Cell line[b]	% CAT activity with:		Fold amplification[c]
		No stimulus	rTNF	
HIV LTR CAT	Control	11.3	20.1	1.8
	nef+	9.2	27.7	3
HIV enhancer CAT	Control	9	25.1	2.8
	nef+	10.5	30.1	2.9

[a] Cells were transfected with 5 μg of plasmid vectors. They were stimulated with recombinant tumor necrosis factor (rTNF) (50 μg/ml) for 14 h before preparation of lysates. Results are expressed as means of percent CAT conversion of six experiments.

[b] nef+ cells correspond to the stable U-373nef clone. Control cells were the parental type U-373 astrocytoma line.

[c] Fold amplification represents the ratio of percent CAT conversion observed in lysates from tumor necrosis factor-induced compared with that in lysates from unstimulated cultures.

control U-373CAT cells (Table 2). In agreement with a recent report showing five- to 10-fold enhancement of ΔNRE HIV provirus replication (22), we found that basal activity of the ΔNRE LTR vector was usually superior to that of the whole LTR, as is seen in the representative experiment shown in Table 2.

Altogether, our results indicated that constitutive expression of HIV *nef* protein in human astrocytoma cells does not significantly modify HIV LTR activity. This conclusion agrees with data recently reported by some investigators (7, 16) but appears to contradict other data obtained in transient cotransfection assays (1, 25). At the high concentrations needed for transient transfection assays, we observed that a control vector, containing the CMV enhancer-promoter region without HIV *nef* DNA, interferes nonspecifically with HIV LTR activity (data not shown), presumably through competition for transcription factors (5).

The lack of negative effects of *nef* on HIV LTR activity in our system was not due to mutation in any potentially functional domains (14) of the *nef* DNA integrated into the U-373nef clone, since perfect homology to the sequence of the HIV (LAV-1-Bru strain) *nef* gene originally transfected was found by polymerase chain reaction analysis (data not shown). The *nef* protein constitutively expressed in our

TABLE 2. Luciferase activity induced by wild-type or ΔNRE HIV LTR expression vector

Plasmid used[a]	Cell line[b]	Luciferase activity[c]		Fold amplification[d]
		Basal	*tat* Induced	
HIV LTR LUC	Control	818	99600	122
	nef+	454	32900	73
ΔNRE LTR LUC	Control	2980	106800	36
	nef+	2100	45600	22

[a] Cells (2×10^4) were microtransfected with 50 ng of either the HIV LTR (−644 to +78) or the ΔNRE LTR (−142 to +78) luciferase expression vector. *tat* was introduced by cotransfection of 10 ng of a CMV *tat* expression vector.

[b] nef+ cells correspond to the stable U-373nef clone. Control cells were the U-373CAT clone.

[c] Results are expressed as means of the luciferase (LUC) activity of quadruplicate wells, standardized as a function of the number of live cells (29 and references within).

[d] Fold amplification represents the ratio of *tat*-induced versus basal LTR luciferase vector.

system appears to interact functionally with the astrocytoma cell environment, since preliminary results in our laboratory indicate that the U-373nef cells exhibit enhanced binding protein(s) to a region of the HIV LTR NRE.

Despite many efforts, the exact role(s) of the HIV *nef* gene has not yet been elucidated. Much remains to be learned about the cellular or viral (or both) target(s) of this highly conserved nonstructural protein.

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