Jpn. J. Cancer Res. 81, 1124–1131, November 1990

Repressive Effect of the *nef* cDNA of Human Immunodeficiency Virus Type 1 on the Promoter Activity of the Viral Long Terminal Repeat

Shigehisa Mori, Ritsuko Takada, Kunitada Shimotohno and Takashi Okamoto

Virology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104

The *nef* gene product of human immunodeficiency virus type 1 (HIV-1) has been implicated as a negative factor for viral replication and is suspected to play an important role in the maintenance of viral latency. However, there seems to be evidence both for and against the negative effect of *nef* gene product. In the present report, we reevaluated the function of the *nef* gene by means of transient CAT assays with two human T cell lines. In most of the experiments, carefully controlled triplicate studies were carried out. We observed that not only the *nef*-expression plasmid, but also an effector plasmid containing the *nef* cDNA sequence in a reverse orientation, not expressing the Nef protein, showed a similar extent of repression of the HIV-1 promoter activity. We also examined the repressive effect of the *nef* cDNA with deletion mutants of HIV-1 long terminal repeat and heterologous promoters. The results led us to conclude that the apparent "repressor"-like action of the *nef* cDNA itself could be explained by competition for certain transcription factors required for HIV-1 gene expression by identical sequences also present in the *nef* cDNA.

Key words: Acquired immunodeficiency syndrome — Gene expression — Repression — Transient CAT assay — Viral latency

Human immunodeficiency virus (HIV) is a causative agent of the acquired immunodeficiency syndrome (AIDS). However, a long latency period, usually five to ten years, exists prior to the clinical development of AIDS.[1] During the latency period between the viral infection and onset of the disease, both viral and host factors have been considered to be involved in maintaining the latency by counteracting the various extracellular stimuli such as antigenic stimulation, cytokines and infection of other viruses.

Among the viral regulatory proteins, HIV-1-encoded negative factor gene (*nef*)[2,3] product (Nef) has been suspected to play an important role in maintaining the viral latency by repressing the viral gene expression. Nef is a 27 kDa protein consisting of 206 amino acids, myristilated at its N-terminus, and it has been shown to be located inside the cytoplasmic membrane.[4] Because of its biochemical ability to bind and hydrolyze GTP and the presence of localized amino acid sequence homologies with some cellular signal-transducing molecules such as c-*src* and c-Ha-*ras* gene products,[5,6] Nef protein has been suspected to be involved in one of the cellular signal transduction pathways.

It has been reported that the HIV-1 clone lacking the *nef* open reading frame exhibits higher efficiency of viral replication in T cell cultures[7–9] and that the *nef*-expressing plasmid when co-transfected with CAT gene expression plasmid directed by HIV-1 long terminal repeat (LTR) causes marked repression of HIV-1 gene expression in transient CAT assay.[10,11] However, the possible action of Nef as a specific repressor of the HIV-1 gene expression is still controversial.[12–15] Using a similar strategy to that employed in the previous report, Kim *et al.* examined the effect of *nef* gene disruption and found that the presence or absence of its open reading frame did not have any significant difference on the viral replication efficiency in cell culture.[12] Hammes *et al.* presented evidence against the possible role of Nef as a transcriptional repressor, using transient CAT assay.[13] They claimed that the apparent "repressor"-like activity of the *nef* gene could be explained by competition for transcription factors interacting with the *cis*-regulatory elements within the viral 5′ LTR by identical sequences within the cDNA of the *nef* gene.

In the present study, we undertook to determine the effect of the cDNA itself on the HIV-1 gene expression by creating various constructs and examining their activities with the transient CAT assay.

MATERIALS AND METHODS

Cell lines The human T lymphocyte cell lines, Jurkat and MOLT-4, were grown in RPMI1640 medium containing 10% fetal bovine serum, 2 m*M* glutamine, and 67 μg/ml kanamycin. COS-1 cells, a simian kidney-derived fibroblast-like cell line transformed by SV40, were grown in modified D-MEM medium containing 10% fetal bovine serum, 2 m*M* glutamine, and 67 μg/ml kanamycin. These

Abbreviations: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; CAT, chloramphenicol acetyl-transferase; LTR, long terminal repeat; CMV, cytomegalovirus.

cell lines were maintained carefully to keep the cell viability greater than 95%.

Plasmids Construction of CD12, a plasmid expressing CAT gene under the control of HIV-1 LTR, and its deletion mutants (Fig. 1A) has been described elsewhere.[16, 17] Other plasmids expressing CAT gene under the control of immediate early promoter of human cytomegalovirus (CMV CAT)[18] or promoter from chicken β-actin gene (β-actin CAT) were utilized.[19] These CAT-expressing plasmids are called the "reporter gene" in this manuscript.

For expression of HIV-1 regulatory proteins, Nef and Tat, pCV3nef and pCV1tat were used, respectively.[3, 20] The Tat is a virus-specific *trans*-activator and pCV1tat was utilized in the present transfection experiments to augment the level of the HIV-1 gene expression. These constructs were kindly provided by Drs. Suresh K. Arya and Flossie Wong-Staal, National Institutes of Health, USA. pCV3nefR was created by inserting the cDNA segment containing the *nef* gene into the original eukaryotic expression vector, pCV0, at its *Pst* I site in an anti-sense orientation. Similarly, pTZ18U-nef was created by inserting the *nef* cDNA into the *Pst* I site of a plasmid pTZ18U (purchased from United States Biochemical Corporation, Ohio). These plasmids are collectively called the "effector gene." The structures of these constructs are diagrammatically shown in Fig. 1B.

Transfection and CAT enzyme assay Cells were transfected with plasmid by the electroporation procedure or by the calcium phosphate precipitation method. A commercially available pulse generator, Gene Pulser (BioRad, Calif.) was utilized for electroporation. The cells were taken from a logarithmically growing culture and resuspended at a density of 1×10^7 cells/ml in RPMI1640 medium containing 1% fetal bovine serum. Plasmid DNA, 8 to 25 μg, was added to 200 μl of the cell suspension (containing 2×10^6 cells) and these cells were kept on ice for 10 min. This cell-DNA mixture was then transferred to a Gene Pulser cuvette (purchased from BioRad) and subjected to a single pulse of 960 μF at 200 V (with an electric field of 500 V/cm). After electroporation, cells were incubated on ice for 10 min, transferred to a tissue culture dish containing 4 ml of the complete RPMI1640 medium, and incubated in 5% CO_2 and 100% humidity at 37°C for 48 h. Cells were then harvested, and cell extracts were prepared for CAT enzyme assay. Enzyme activity was assayed as described

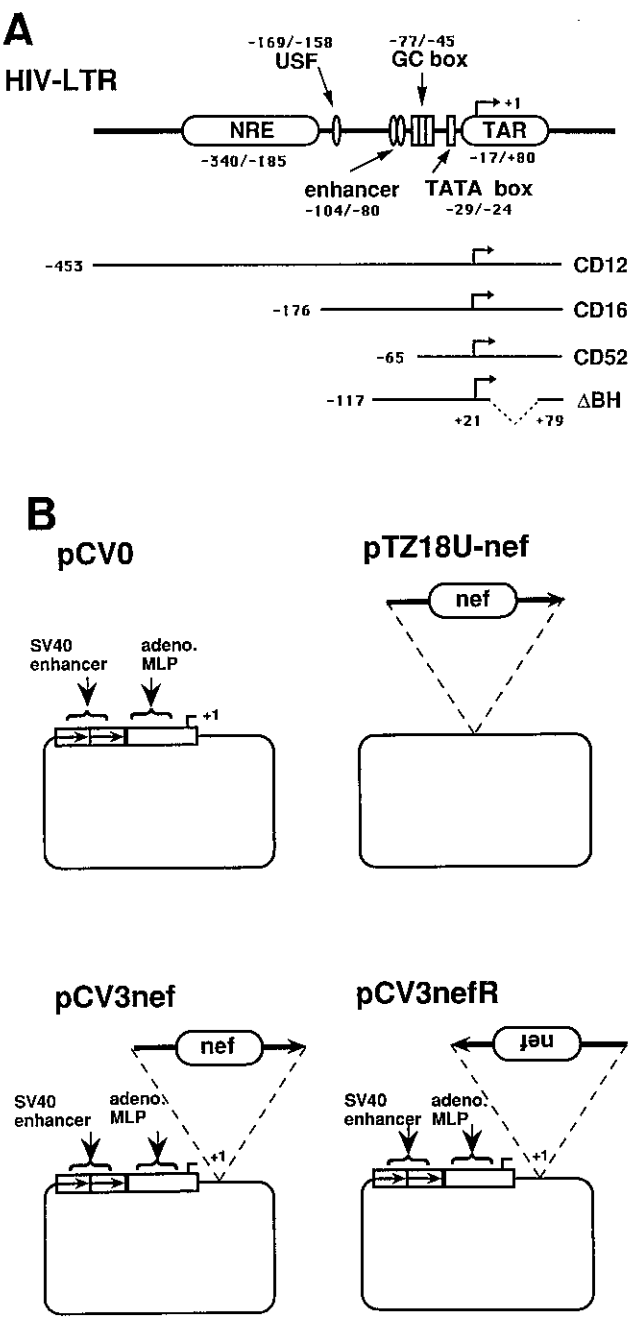


Fig. 1. Constructions of plasmids. A, *cis*-Regulatory elements of the HIV-1 LTR. CD12 is a reporter gene expressing CAT gene under the control of HIV-1 LTR. CD16, CD52, and ΔBH are deletion mutants originated from the CD12 clone. The details of plasmid construction have been described previously.[16, 17] NRE, negative regulatory element; USF, upstream stimulatory factor-binding site; TAR, *trans*-acting responsive element. B, Schematic representation of the effector genes. pCV0 is an eukaryotic expression vector using adenovirus major late promoter and SV40 enhancer. pCV3nef is a derivative of pCV0 and expresses Nef protein of HIV-1 (from the HTLV-IIIB strain). pCV3nefR is constructed from pCV3nef containing the *nef* cDNA in a reversed orientation. pTZ18U-nef was constructed from pTZ18U by inserting the *nef* cDNA at the *Pst* I site.

previously with a fixed protein amount of the cell lysate.[21] Protein concentration of each lysate was determined in triplicate using Bradford's method (Protein assay; BioRad). CAT activities were quantitated by densitometric scanning of autoradiograms using a chromatoscanner CS9000 (Shimadzu, Kyoto). For standardization of the experiments, we performed these transfection experiments mostly in triplicate or at least in duplicate. The intra-assay variation of CAT enzyme activity,[22] as expressed by the percent conversion of ^{14}C-chloramphenicol into its acetylated forms, estimated from the triplicate study was within the range of 3 to 10%.

Immunoblotting COS-1 cells, seeded at 5×10^5 cells/10 cm plate the day before, were transfected with 20 μg of pCV0, pCV3nefR, or pCV3nef by the calcium phosphate precipitation method.[23] After 48 h, the cells were harvested, lysed in SDS gel sample buffer, and heated for 10 min at 100°C. The cell lysates were sonicated and a fixed amount of protein was applied to a 15% SDS-polyacrylamide gel. After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride membrane (Immobilon PVDF; Millipore, Calif.). The membrane was soaked in 5% skim milk, exposed to anti-Nef mouse monoclonal antibody (American BioTechnologies, Mass.) and reacted with biotinylated horse anti-mouse IgG antibody, avidin DH, and biotinylated horseradish peroxidase (VECTASTAIN ABC kit; Vector, Mass.). Antibody binding was detected by developing with N-3,3′-diaminobenzidine and hydrogen peroxide.

RESULTS

Repression of HIV-1 gene expression by the plasmids containing the *nef* cDNA The reporter plasmid, HIV-1 LTR CAT (CD12), was transfected into Jurkat cells with the Nef-expressing plasmid, pCV3nef, as well as the Tat-expressing plasmid, pCV1tat. The pCV1tat was co-transfected, because of the relatively low efficiency of transfection with a suspension cell culture, to augment the level of the HIV-1 gene expression throughout the experiments. Fig. 2A illustrates a typical result of CAT enzyme assay with the lysate from the plasmid-transfected cells. In the experiments the amount of total plasmid DNA was adjusted by addition of the vector plasmid, pCV0. Approximately 2×10^6 Jurkat cells were transfected with 1.4 μg of CD12, 1.4 μg of pCV1tat, and the effector plamids at the indicated ratio (gene dose) to the reporter gene. As shown in Fig. 2B, co-transfection of pCV3nef led to repression of CAT gene expression from the HIV-1 LTR in a dose-dependent manner. However, pCV3nefR, containing the *nef* cDNA in a reversed orientation, also showed a similar extent of repression.

This observation was not restricted to this cell line. Another human T cell line, MOLT-4, was transfected

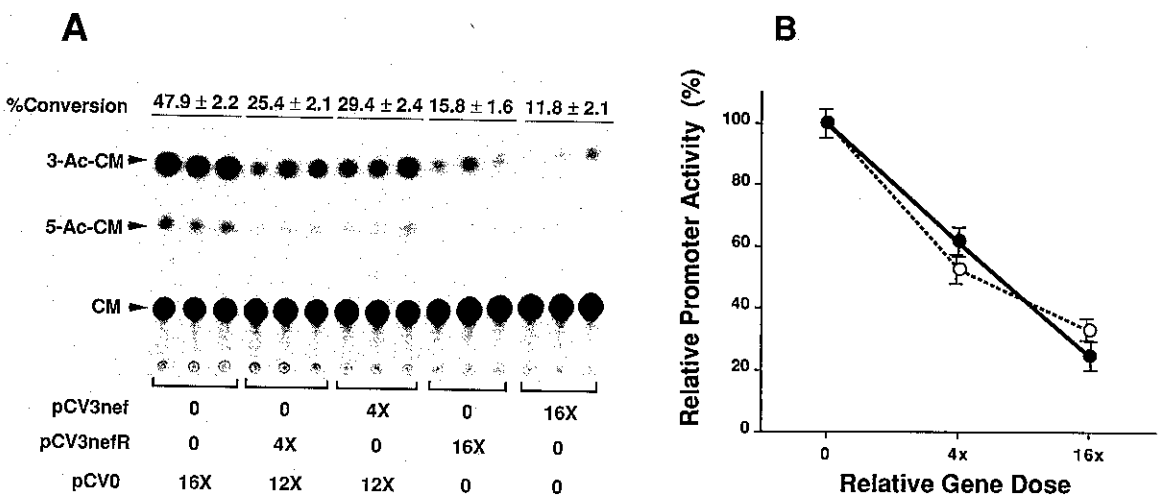
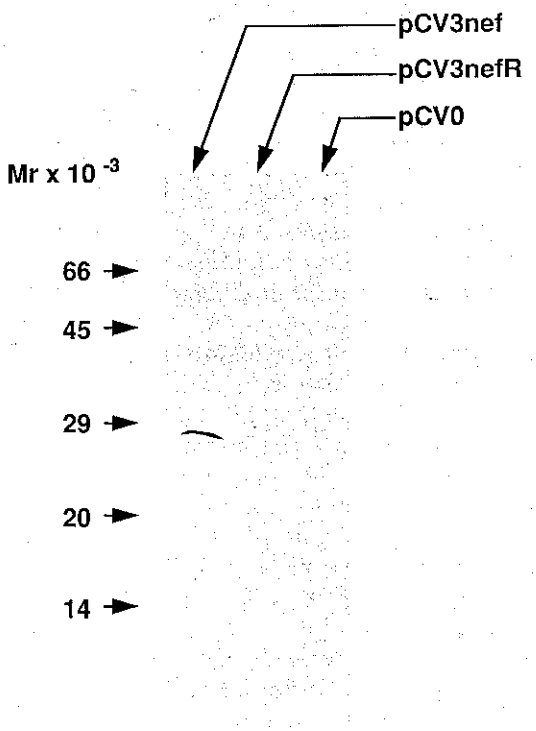


Fig. 2. Repression of the HIV-1 gene expression by the *nef* constructs. A, Autoradiogram of CAT enzyme assay. Approximately 2×10^6 Jurkat cells were transfected by electroporation with 1.4 μg of CD12, 1.4 μg of pCV1tat, and the effector genes at the gene dose ratio to the reporter gene as indicated. The amount of total plasmid DNA content for each transfection was adjusted by supplementing with pCV0. The CAT enzyme activities were quantitated by densitometric scanning of the autoradiogram and indicated by the percent conversion of the substrate, ^{14}C-chloramphenicol, to its acetylated forms. Experiments were performed in triplicate, and the average and the standard error of the CAT enzyme activities are indicated at the top of the autoradiogram. B, Diagrammatic representation of the data in Fig. 2A. The relative promoter activities of the HIV-1 LTR were plotted with regard to the gene dose of the co-transfected effector genes. (●), pCV3nef; (○), pCV3nefR.



with the same combination of plasmids as in Fig. 2. The results of triplicate experiments also revealed that co-transfection of pCV3nefR demonstrated as much repressive effect on HIV-1 as pCV3nef (data not shown).

Confirmation of the plasmid containing the *nef* cDNA without expressing Nef protein Since pCV3nefR also showed the repressive effect on the promoter of HIV-1 LTR, we tried to determine whether this repression of HIV-1 gene expression was caused by the Nef protein improperly produced in the cells transfected with pCV3nefR. We first examined the Jurkat cells transfected with the effector plasmids by immunoblotting, but we could not detect production of the Nef protein in the cells transfected with either pCV3nef or pCV3nefR. We then utilized COS-1 cells in which a Nef-expressing plasmid had been previously shown to produce the Nef protein

Fig. 3. Confirmation of the plasmid containing the *nef* cDNA without expression of Nef protein. COS-1 cells were transfected with pCV0, pCV3nefR, or pCV3nef by the calcium phosphate precipitation method. Forty-eight hours after transfection, cells were harvested, sonicated and the cell lysates were prepared. The proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane. The membrane was probed with the anti-Nef mouse monoclonal antibody. Arrows on the left indicate the positions of protein molecular weight markers.

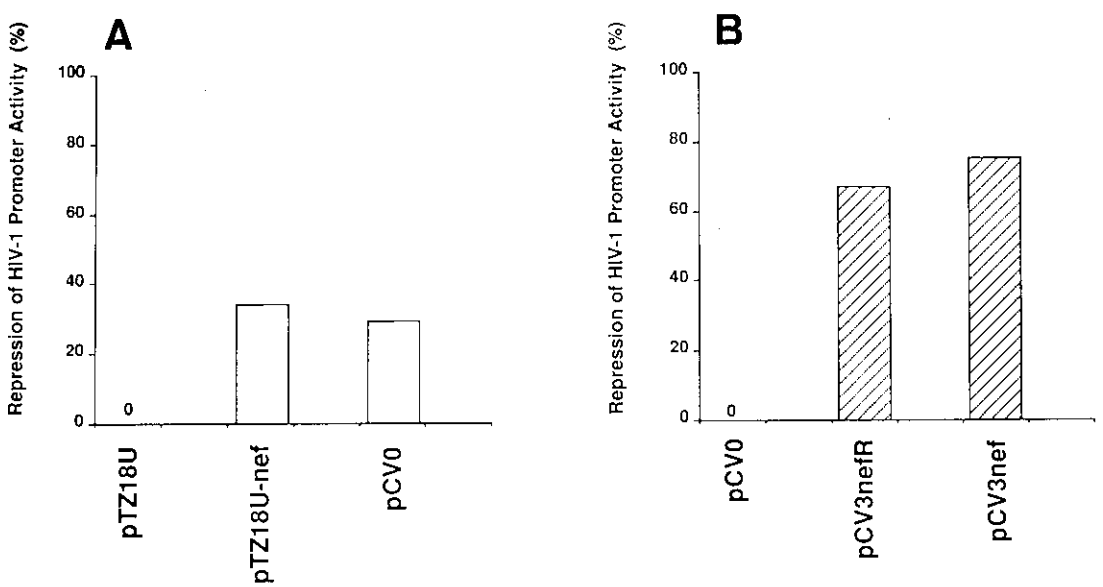


Fig. 4. Effect of the *nef* cDNA as a competitor for HIV-1 LTR. A, Comparison of the extents of repression of the HIV-1 LTR activity by pTZ18U-nef, pCV0, or pTZ18U. These plasmids were co-transfected into Jurkat cells at a 16:1 ratio to HIV-1 LTR CAT with pTZ18U as a control. B, Comparison of the extents of repression of the HIV-1 LTR activity by pCV3nef or pCV3nefR when pCV0 was used as a control. Data were taken from the results in Fig. 2. The values of the percent repression were obtained by subtracting the relative promoter activity (see the legend to Fig. 2) from 100 (%).

because of the higher transfection efficiency.[13] A protein band of approximately 27 kDa, identified specifically in the cells transfected with pCV3nef, was not detected in cells transfected with pCV3nefR by immunoblotting (Fig. 3). These results led us to conclude that the repression observed in Fig. 2 was caused by the *nef* cDNA itself.

Competition with the HIV-1 LTR for transcription factors by the *nef* cDNA We next addressed the question of whether the *nef* cDNA could act as a competitor for factors participating in the transcriptional regulation of HIV-1 LTR since the genetic location of the *nef* gene largely overlaps the U3 region of the 3′ LTR. Fig. 4A shows a comparison of the extents of HIV-1 repression by the pTZ18U-nef (containing only the *nef* cDNA) and pCV0 (containing a strong eukaryotic promoter taken from adenovirus major late promoter supplemented with SV40 enhancer). It should be noted that these two plasmids exhibited some repressive activity on the HIV-1 gene expression when pTZ18U was used as a control. Either pCV0 alone or the *nef* cDNA alone possessed HIV-1-repressing activity without producing any protein. Fig. 4B shows the extents of HIV-1 repression by pCV3nef (in a sense orientation) or pCV3nefR (in an anti-sense orientation) when pCV0 was used as a control. The extent of repression by pCV3nefR (67.0%) or pCV3nef (75.4%) in Fig. 4B is much greater than that by pTZ18U-nef (34.0%) in Fig. 4A.

In the experiment illustrated in Fig. 5, in order to examine if the amount of the transcription factors involved in the competition by the *nef* cDNA was much smaller than the amount of the competitor, the amount of the reporter gene was reduced from 1.4 μg to 0.5 (Fig. 5B) or 0.1 μg (Fig. 5A) leaving the effector-to-reporter ratio (gene dose) unchanged. Since there was no significant difference in repression by plasmid containing the *nef* cDNA between Fig. 5A and Fig. 5B, we concluded that the amount of the transcription factors involved in the competition by the *nef* cDNA might be so limited that the repression was still observable even at the decreased (one-fourteenth) amount of the *nef* constructs. We could not further reduce the gene dose of the reporter gene below 0.1 μg because of the limitation of sensitivity of this assay system.

Effect of deleting the *cis*-regulatory elements within the HIV-1 LTR Since promoter activities are controlled by the *cis*-regulatory elements located usually upstream and sometimes downstream of the transcription initiation site (denoted as "+1" in Fig. 1A), we next looked at the effect of deleting these *cis*-elements in the HIV-1 LTR. As shown in Fig. 6, repression of gene expression by co-transfection with pCV3nef or pCV3nefR was significantly diminished by deleting the upstream sequence from nucleotide position −176 containing the NRE (negative regulatory element). The repression, though to a lesser extent, was still observable even when the further downstream sequences, such as NF-κB binding sites, GC boxes, and TAR (*trans*-acting responsive element), were deleted.

Studies with heterologous promoters Similarly, CAT-expressing plasmids under the control of immediate early promoter of human cytomegalovirus (CMV CAT) or chicken β-actin promoter (β-actin CAT) were repressed

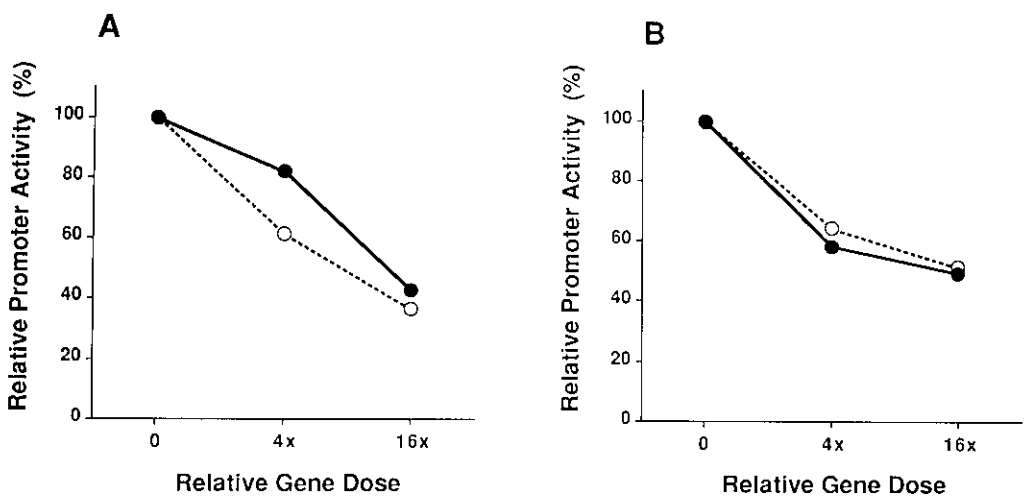


Fig. 5. Effect of decreased gene dose of the reporter and the effector genes on the repression. The amount of the reporter gene, pCD12, was reduced to 0.1 (A) or 0.5 (B) μg, leaving the effector-to-reporter ratio unchanged. Jurkat cells were used as in Fig. 2. The amount of total plasmid DNA content was adjusted by addition of pTZ18U. The graph was plotted as in Fig. 2B. (●), pCV3nef; (○), pCV3nefR.

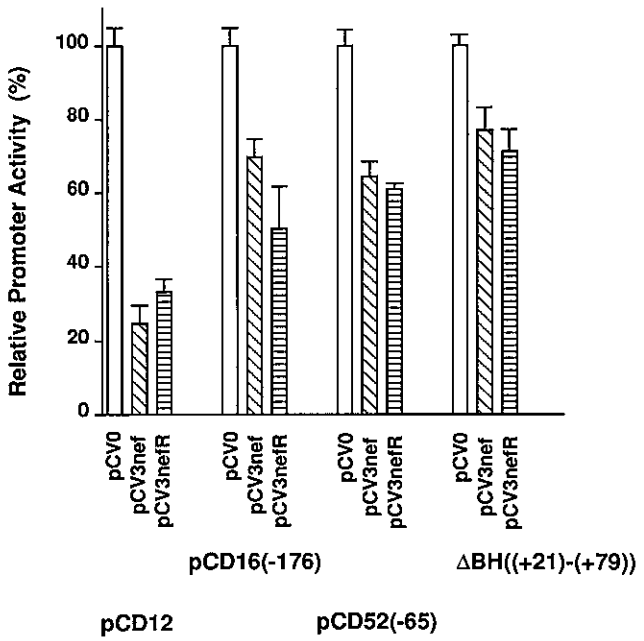


Fig. 6. Effect of deletion of the *cis*-regulatory elements within the HIV-1 LTR on the responsiveness to the repression by the *nef* constructs. The location of the *cis*-elements in HIV-1 LTR and the extents of deletion are shown in Fig. 1A.

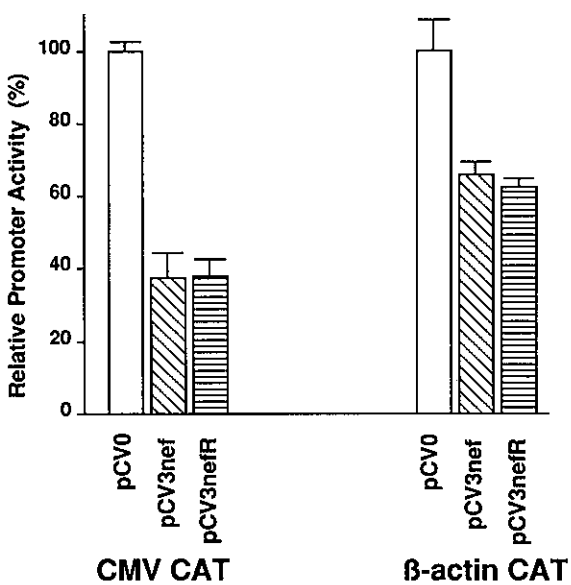


Fig. 7. Effect of co-transfection of the *nef* constructs on gene expression from the heterologous promoters. The CAT-expressing plasmids under the control of cytomegalovirus immediate early promoter (CMV CAT) or chicken β-actin promoter (β-actin CAT) were co-transfected with pCV0, pCV3nef or pCV3nefR into Jurkat cells. The ratio of the effector to the reporter gene dose was 16:1.

by pCV3nef or pCV3nefR. Fig. 7 shows a typical example of the results obtained when the plasmids were co-transfected at a reporter:effector ratio of 1:16. The extents of suppression by pCV3nef or pCV3nefR were almost identical for both reporter plasmids. However, the CMV promoter was repressed more strongly than β-actin promoter.

DISCUSSION

We observed a significant level of repressive action of the *nef* cDNA sequence on the HIV-1 promoter without expression of the Nef protein. To investigate the mechanism of this repressive effect of the *nef* cDNA, we performed transient CAT assay using various effector plasmids as well as LTR deletion mutants of HIV-1 and heterologous promoters including CMV and chicken β-actin promoters.

The repressive action of the *nef* cDNA sequence alone could be explained by the competition for transcription factors required for gene expression from the HIV-1 LTR (Figs. 4, 6 and 7). The *nef* cDNA and the HIV-1 LTR, namely U3 and R regions, overlapped as shown in Fig. 8. Repression of the promoter activity of HIV-1 LTR by even the vector, pCV0, alone was noted (Fig. 4). Since there is no marked similarity between the *cis*-regulatory elements within the HIV-1 LTR and those within the promoter region of the pCV0 except for a TATA box, it is supposed that some general factors for transcription not directly involved in the specific DNA sequence recognition might be competed out by the pres-

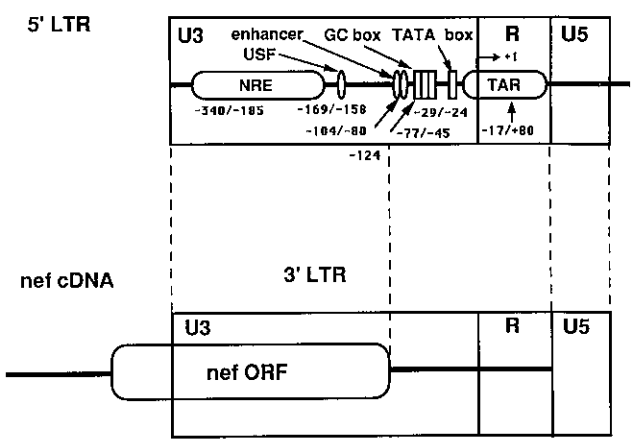


Fig. 8. Overlapping feature of the HIV-1 LTR and the *nef* cDNA sequences. The *cis*-elements of the HIV-1 LTR are shown in the upper figure. The *nef* cDNA contains the entire sequences of the U3 and R regions of LTR as well as some viral sequences upstream from the 3'LTR.

ence of a large dose of pCV0 plasmid. The repressive effect of the *nef* cDNA sequence inserted into the vector with a eukaryotic promoter was greater than that of the *nef* cDNA sequence inserted into the vector without a eukaryotic promoter. It is suggested that the combination of the vector with eukaryotic promoter and the *nef* cDNA sequences created a more-than-additive effect on the repression of the HIV-1 LTR (Fig. 4).

The repression of HIV-1 gene expression was partially dependent on the *cis*-elements within the target promoter, which was concordant with the finding of Hammes *et al.*[13] However, our results argue against Hammes *et al.*[13] in that in our study the HIV-1 LTR containing the upstream sequences from −176 was the most susceptible to competition by the *nef* cDNA and the HIV-1 LTR containing only the downstream sequence was repressed to a lesser extent (Fig. 6). Additionally, this repressive effect on HIV-1 gene expression by the *nef* cDNA was evident even with heterologous promoters such as immediate early promoter of human CMV and chicken β-actin promoter (Fig. 7). The CMV promoter was repressed more strongly, probably because it shared more *cis*-elements with the HIV-1 LTR than the chicken β-actin promoter: among the *cis*-elements within the HIV-1 LTR, the CMV promoter has NF-κB binding sites[24] in addition to GC boxes and the TATA box, which are also present in the chicken β-actin promoter.[25]

These results led us to conclude that the repressive effect of the *nef* cDNA itself might be caused by competition for certain factors required for HIV-1 transcription by the identical sequence also present in the *nef* cDNA. These factors include those interacting with the *cis*-regulatory elements within the viral 5′-LTR and those not directly involved in the recognition of specific DNA sequences. We cannot discuss the repressive effect of Nef protein itself on the basis of our present study. Moreover, currently we cannot explain why different results about the Nef protein have been reported by different investigators. Considering the possible role of *nef* gene and its product in the maintenance of viral latency in individuals carrying HIV-1, further studies are necessary to understand the viral program leading toward the clinical progression to AIDS, and to develop a new strategy to prevent the disease process.

ACKNOWLEDGMENTS

We thank Drs. F. Wong-Staal and S. K. Arya of National Institutes of Health, USA, for plasmids, pCV0 and pCV3nef, and Dr. S. Kato of the Department of Microbiology, Keio University, for providing the cell lysate used as the positive control in immunoblotting. We also thank Dr. T. Akagi and Mr. H. Ono of the Virology Division, National Cancer Center Research Institute for their help in electroporation and in cell culture, respectively. This work was supported by Grants-in-Aids from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan. S.M. and R.T. are awardees of Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

(Received August 9, 1990/Accepted August 31, 1990)

REFERENCES

1) Curran, J. W., Morgan, W. M., Hardy, A. M., Jaffe, H. W., Darrow, W. W. and Dowdle, W. R. The epidemiology of AIDS: current status and future prospects. *Science*, **229**, 1352–1357 (1985).
2) Allan, J. S., Coligan, J. E., Lee, T-H., McLane, M. F., Kanki, P. J., Groopman, J. E. and Essex, M. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. *Science*, **230**, 810–813 (1985).
3) Arya, S. K. and Gallo, R. C. Three novel genes of human T-lymphotropic virus type III: immune reactivity of their products with sera from acquired immune deficiency syndrome patients. *Proc. Natl. Acad. Sci. USA*, **83**, 2209–2213 (1986).
4) Franchini, G., Robert-Guroff, M., Ghrayeb, J., Chang, N. T. and Wong-Staal, F. Cytoplasmic localization of the HTLV-III 3′*orf* protein in cultured T cells. *Virology*, **155**, 593–599 (1986).
5) Guy, B., Kieny, M. P., Rivière, Y., Peuch, C. L., Dott, K., Girard, M., Montagnier, L. and Lecocq, J-P. HIV F/3′ *orf* encodes a phosphorylated GTP-binding protein resembling an oncogene product. *Nature*, **330**, 266–269 (1987).
6) Guy, B. Rivière, Y., Dott, K., Regnault, A. and Kieny, M. P. Mutational analysis of the HIV nef protein. *Virology*, **176**, 413–425 (1990).
7) Fisher, A. G., Ratner, L., Mitsuya, H., Marselle, L. M., Harper, M. E., Broder, S., Gallo, R. C. and Wong-Staal, F. Infectious mutants of HTLV-III with changes in the 3′ region and markedly reduced cytopathic effects. *Science*, **233**, 655–659 (1986).
8) Terwillinger, E., Sodroski, J. G., Rosen, C. A. and Haseltine, W. A. Effects of mutations within the 3′*orf* open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity. *J. Virol.*, **60**, 754–760 (1986).
9) Luciw, P. A., Cheng-Mayer, C. and Levy, J. A. Mutational analysis of the human immunodeficiency virus: the *orf*-B region down-regulates virus replication. *Proc. Natl. Acad. Sci. USA*, **84**, 1434–1438 (1987).
10) Ahmad, N. and Venkatesan, S. *Nef* protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science*, **241**, 1481–1485 (1988).
11) Niederman, T. M. J., Thielan, B. J. and Ratner, L.

Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. *Proc. Natl. Acad. Sci. USA,* **86,** 1128–1132 (1989).

12) Kim, S., Ikeuchi, K., Byrn, R., Groopman, J. and Baltimore, D. Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA,* **86,** 9544–9548 (1989).
13) Hammes, S. R., Dixon, E. P., Malim, M. H., Cullen, B. R. and Greene, W. C. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. *Proc. Natl. Acad. Sci. USA,* **86,** 9549–9553 (1989).
14) Cheng-Mayer, C., Iannello, P., Shaw, K., Luciw, P. A. and Levy, J. A. Differential effects of *nef* on HIV replication: implications for viral pathogenesis in the host. *Science,* **246,** 1629–1632 (1989).
15) Bachelerie, F., Alcami, J., Hazan, U., Israël, N., Goud, B., Arenzana-Seisdedos, F. and Virelizier, J. L. Constitutive expression of human immunodeficiency virus (HIV) *nef* protein in human astrocytes does not influence basal or induced HIV long terminal repeat activity. *J. Virol.,* **64,** 3059–3062 (1990).
16) Okamoto, T. and Wong-Staal, F. Demonstration of virus-specific transcriptional activator(s) in cells infected with HTLV-III by an *in vitro* cell-free system. *Cell,* **47,** 29–35 (1986).
17) Okamoto, T., Benter, T., Josephs, S. F., Sadaie, R. and Wong-Staal, F. Transcriptional activation from the long terminal repeat of human immunodeficiency virus *in vitro*. *Virology,* **177,** 606–614 (1990).
18) Foecking, M. K. and Hofstetter, H. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene,* **45,** 101–105 (1986).
19) Schmidt, A., Setoyama, C. and Crombrugghe, B. D. Regulation of a collagen gene promoter by the product of viral *mos* oncogene. *Nature,* **314,** 286–289 (1985).
20) Arya, S. K., Guo, C., Josephs, S. F. and Wong-Staal, F. *Trans*-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science,* **229,** 69–73 (1985).
21) Okamoto, T., Matsuyama, T., Mori, S., Hamamoto, Y., Kobayashi, N., Yamamoto, N., Josephs, S. F., Wong-Staal, F. and Shimotohno, K. Augmentation of human immunodeficiency virus type 1 gene expression by tumor necrosis factor α. *AIDS Res. Hum. Retroviruses,* **5,** 131–138 (1989).
22) Hollon, T. and Yoshimura, F. K. Variation in enzymatic transient gene expression assays. *Anal. Biochem.,* **182,** 411–418 (1989).
23) Chen, C. and Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.,* **7,** 2745–2752 (1987).
24) Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B. and Schaffner, W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell,* **41,** 521–530 (1985).
25) Seiler-Tuyns, A., Eldridge, J. D. and Paterson, B. M. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. *Proc. Natl. Acad. Sci. USA,* **81,** 2980–2984 (1984).