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Enhancement of human immunodeficiency virus type 1 infectivity by Nef is producer cell-dependent

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The growth kinetics of wild-type and *nef* mutant viruses of human immunodeficiency virus type 1 were comparatively analysed in several human CD4 cell lines. Delayed replication of *nef* mutant virus was observed in all cell lines examined. To determine the stage in the virus replication cycle that is affected by Nef, a single-round replication assay was performed. Initially, the expression of marker genes in transfected cells was examined in order to study the role of Nef in the late phase of infection. The results obtained indicated that Nef is dispensable during the transcription to virion production stage. Next, the effect of Nef on the early phase was investigated with a single-round infection. It was demonstrated that Nef is required in the early phase of the virus replication cycle, from virion adsorption to integration. Finally, the infectivity of virus stocks prepared from four cell lines was determined. The relative infectivity of the *nef* mutant from the four cell lines differed. Taken together, we conclude that Nef acts via modulation of viral particles to enhance virus infectivity in a cell-dependent manner.

Introduction

One of the accessory genes of human immunodeficiency virus type 1 (HIV-1), designated *nef*, encodes a 25 or 27 kDa protein. In early studies, it was suggested that the gene product, Nef, acted as a negative factor in virus replication (Terwilliger *et al*., 1986) by repressing transcription from the long terminal repeat (LTR) promoter (Ahmad & Venkatesan, 1988; Niederman *et al*., 1989). However, these findings were not confirmed in a subsequent report (Hammes *et al*., 1989). Several studies have reported that Nef could moderately facilitate the virus replication rate (de Ronde *et al*., 1992; Kim *et al*., 1989; Terwilliger *et al*., 1991; Zazopoulos & Haseltine, 1992, 1993). However, a drastic effect of Nef as a positive factor has been observed in experimental infections *in vivo*, which demonstrated that Nef of simian immunodeficiency virus SIVmac is necessary for maintaining high virus loads and for disease progression of AIDS in rhesus monkeys (Kestler *et al*., 1991). This result was supported by Jamieson *et al*. (1994) using HIV-1-infected severe combined immunodeficient mice which had been transplanted with foetal human thymus and liver tissues.

Subsequently, this positive effect was clearly revealed in *in vitro* infection experiments using quiescent primary T-lymphocytes (Miller *et al*., 1994; Spina *et al*., 1994) and was further observed in $CD4^+$ cell lines infected with low virus inputs (Chowers *et al*., 1994). In the latter observation, it was also suggested that Nef enhances viral particle infectivity (Chowers *et al*., 1994). Miller *et al*. (1995) have recently shown that the increased infectivity by Nef is independent of virus entry, and is manifested at the stage after entry but prior to or coincident with viral gene expression. Schwartz *et al*. (1995) have indicated that Nef acts at an early stage of the virus replication cycle, but not when the virus binds to the receptor and before the completion of reverse transcription. Moreover, Aiken & Trono (1995) have suggested that Nef functions at the stage of particle formation, leading to the efficient completion of proviral DNA synthesis, not to enhanced virus internalization. This study was confirmed by Chowers *et al*. (1995).

In the present study, we firstly confirmed the delayed replication kinetics of *nef* mutant virus in several cell lines. To determine the replication stage influenced by Nef, we used the single-round replication assay (Helseth *et al*., 1989; Sakai *et al*., 1995). The results obtained showed that Nef is dispensable in

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the stage from transcription to virion production but is required in the early phase of the virus replication cycle, from virion adsorption to integration. These findings are in agreement with previous reports (Aiken & Trono, 1995; Chowers *et al*., 1995; Miller *et al*., 1995; Schwartz *et al*., 1995). We finally examined the infectivity of the *nef* mutant virus produced in several cell lines. The relative infectivity of the mutant differed depending on the cells in which virus was produced.

Methods

Cell culture and transfection. A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated foetal calf serum (FCS). $CD4^{+}$ human leukaemia cell lines H9 (Popovic *et al*., 1988), CEMx174 (Salter *et al*., 1985), A3.01 (Folks *et al*., 1985), and M8166 (Shibata *et al*., 1991) were maintained in RPMI 1640 medium containing 10% FCS.

For transfection, uncleaved plasmid DNA was introduced into SW480 cells by the calcium phosphate co-precipitation method (Graham & Van der Eb, 1973; Wigler *et al*., 1979) and into H9, CEMx174, M8166 and A3.01 cells by the modified DEAE-dextran method (Takai & Ohmori, 1990).

Infection. Culture supernatant was harvested, filtered through a 0·45 μm pore filter (Millipore), and assayed for reverse transcriptase (RT) activity. The virus stocks were stored at −80 °C. H9, CEMx174, A3.01 and M8166 cells were infected by incubating 10^{6} cells with virus supernatants, adjusted for identical RT activity or p24 concentration (determined by HIV-1 p24 antigen ELISA; Cellular Products), in the presence of 2 μg polybrene.

RT assay. Virion-associated RT activity was measured as described previously (Willey *et al*., 1988). For quantification, spots on DE81 paper were cut out and RT activity was determined by scintillation counting.

CAT assay. The chloramphenicol acetyltransferase (CAT) assay has been previously described (Gorman *et al*., 1982). CAT levels were assayed in equivalent amounts of cell lysates from transfected SW480 or CEMx174 cells, and from infected H9, CEMx174 or M8166 cells.

DNA constructs. An infectious proviral clone of HIV-1, pNL432 [pNL-wild-type (wt)] and its mutants, designated pNL-Xh (*nef* mutant) and pNL-Ss (*vpu* mutant), have been described previously (Adachi *et al*., 1986, 1991). pNL-SsXh (*nef-vpu* double mutant) was constructed from pNL-Ss by digestion with restriction enzyme *Xho*I, blunt-ending with T4 DNA polymerase, and resealing with T4 DNA ligase. To make pNLenCAT, the CAT coding sequence of pSV2CAT (784 bp *Hin*dIII–*Sau*3AI fragment) (Sakai *et al*., 1990) was inserted into the *Ssp*I site (at nucleotide 6153) in the *vpu* gene and the *Dra*I site (at nucleotide 6591) in the *env* gene of pNL432. pNLenCAT-Xh was constructed from pNLenCAT by digestion with *Xho*I, blunt-ending with T4 DNA polymerase, and resealing with T4 DNA ligase. An *env* expression vector pNLnΔBS was generated from pNL ΔBS (Shibata *et al*., 1995) by introducing the mutation into the *Xho*I site as described above, and pNLnΔBS-Nh, used as an *env* negative control, was generated from pNLnΔBS by blunt-end ligation of the *Nhe*I site.

Results

Growth kinetics of *nef* mutant virus in various CD4 cell lines

In preliminary experiments, the growth ability of wt and *nef* mutant viruses prepared from SW480 cells transfected with pNL-wt or pNL-Xh was examined in H9, CEMx174, A3.01 and M8166 cell lines. Although the effect of the *nef* mutation was small, the *nef* mutant virus always showed delayed growth kinetics relative to those of wt virus (data not shown), confirming reports that Nef is a positive factor in virus replication (Chowers *et al*., 1994; Kim *et al*., 1989; Terwilliger *et al*., 1991; Zazopoulos & Haseltine, 1992, 1993). To substantiate the minor difference in growth kinetics between wt and *nef* mutant viruses, we introduced an additional *vpu* mutation, which abrogates efficient virion production (Gott-

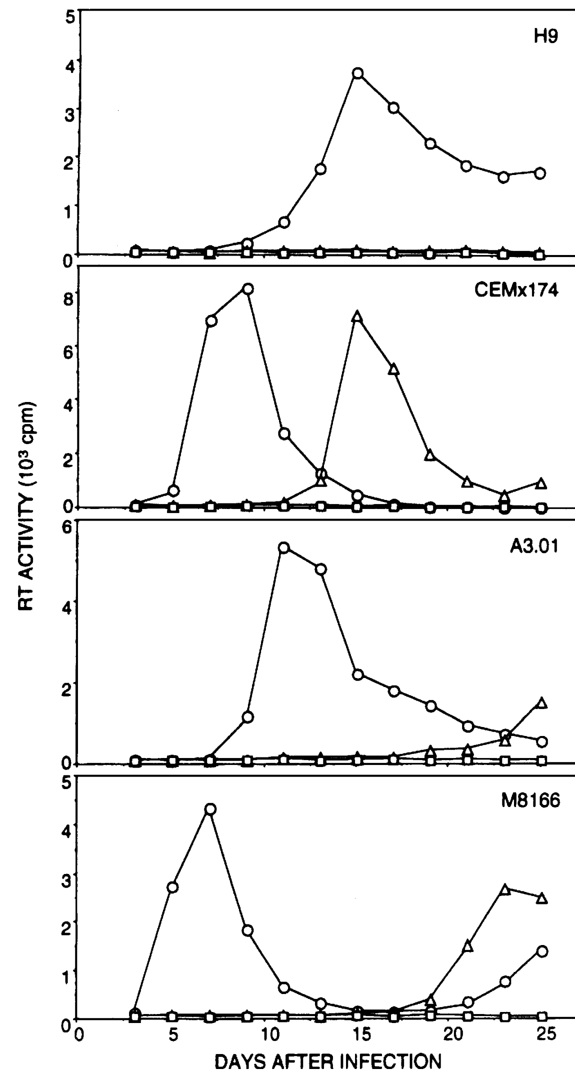

Fig. 1. Growth potential of *vpu* mutant and *vpu-nef* double mutant viruses of HIV-1 in various cell lines. Virus stocks used were prepared from culture supernatants of SW480 cells transfected with *vpu* mutant pNL-Ss (Adachi *et al*., 1991) or *vpu-nef* double mutant pNL-SsXh. H9, CEMx174, A3.01 and M8166 cells (10^{6}) were infected with 10^{6} RT units of cell-free virus, and monitored for RT production in the culture supernatants at intervals. ○, NL-Ss (*vpu* mutant virus); △, NL-SsXh (*vpu-nef* double mutant virus); □, mock-infection.

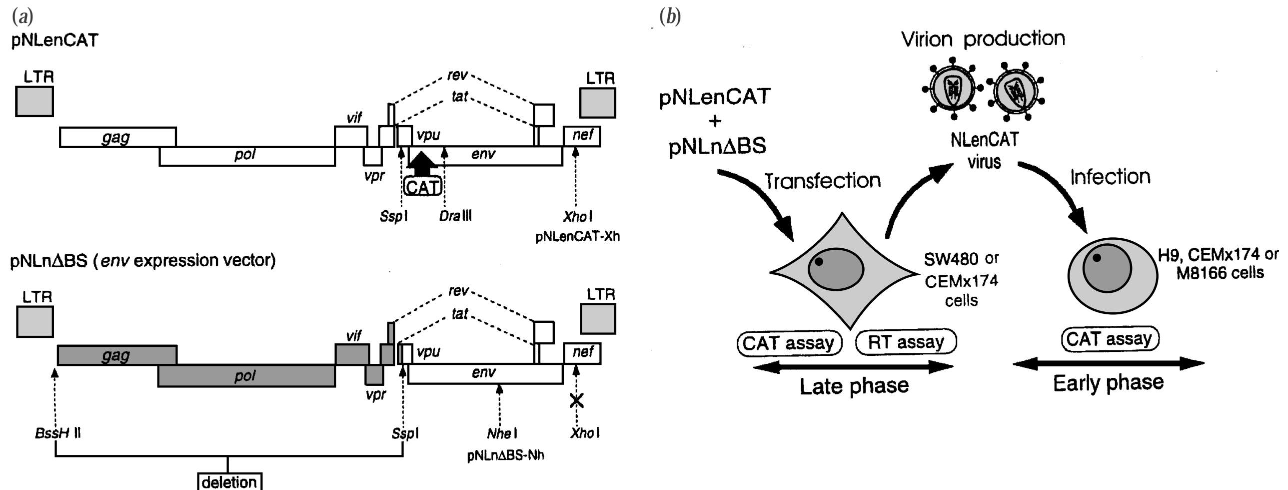

Fig. 2. Single-round replication assay. (*a*) Env-deficient pNLenCAT and env expression vector pNLnΔBS constructs used for a single-round replication assay. (*b*) Schematic representation of a single-round replication assay. CAT and RT production in transfected cells is indicative of a normal late replication phase (from transcription to virion release). CAT production in cells infected with NLenCAT virus is indicative of the progression of the early phase of the virus replication cycle (from attachment to integration).

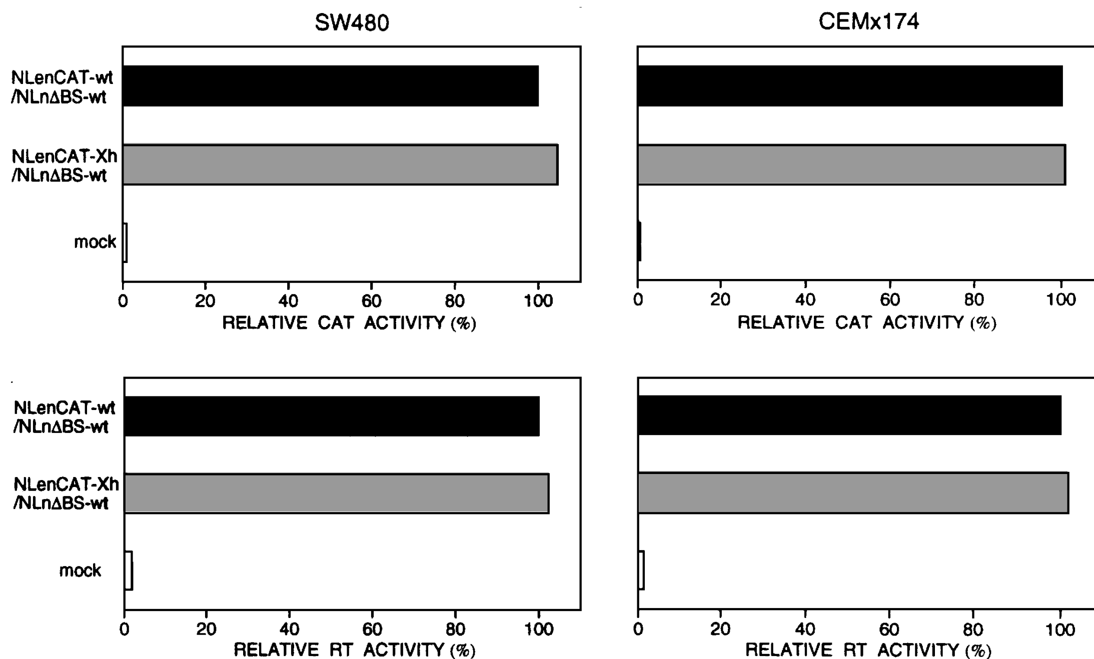

Fig. 3. Marker gene expressions in two cell lines transfected with pNLenCAT-wt plus pNLnΔBS-wt or with pNLenCAT-Xh (*nef* mutant) plus pNLnΔBS-wt. SW480 and CEMx174 cells were co-transfected with 20 μg pNLenCAT-wt or pNLenCAT-Xh and 10 μg pNLnΔBS-wt; 48 h later, CAT activity in the lysates prepared from transfected cells and RT activity in the culture supernatants were determined.

linger *et al.*, 1993; Klimkait *et al.* 1990; Sakai *et al.*, 1995), into the *nef* mutant and monitored the replication of the *nef-vpu* and *vpu* mutant viruses. The four $CD4^+$ cell lines were infected with the mutant viruses obtained from SW480 cells transfected with pNL-Ss or pNL-SsXh. As clearly observed in Fig. 1, the *nef-vpu* double mutant grew much less than the *vpu* mutant in all cell lines examined. These results provide strong evidence that Nef plays a positive role in virus replication.

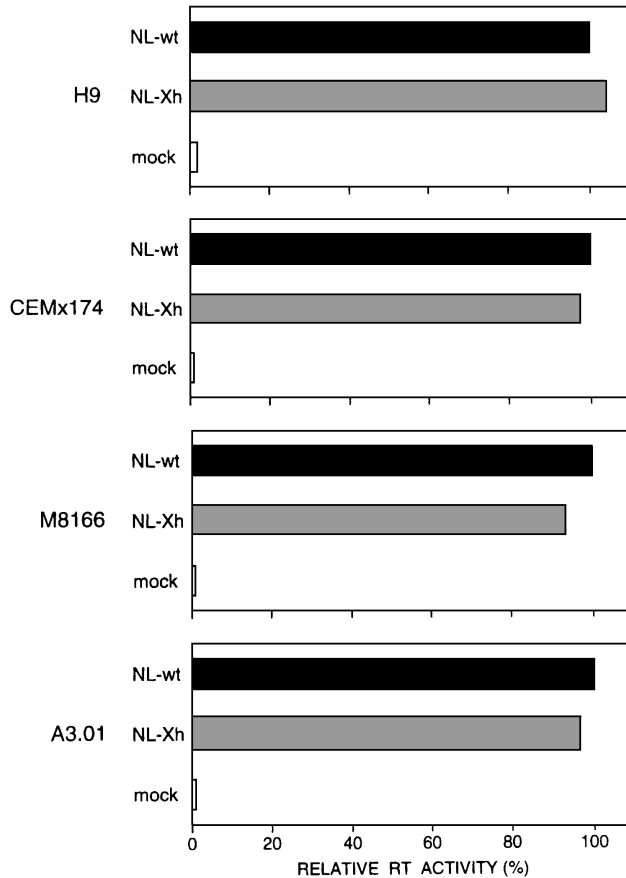

Fig. 4. Virion production in various cell lines transfected with pNL-wt or pNL-Xh (*nef* mutant). H9, CEMx174, A3.01 and M8166 cells were transfected with 20 µg pNL-wt or pNL-Xh; 48 h later, RT activity in the culture supernatants was determined.

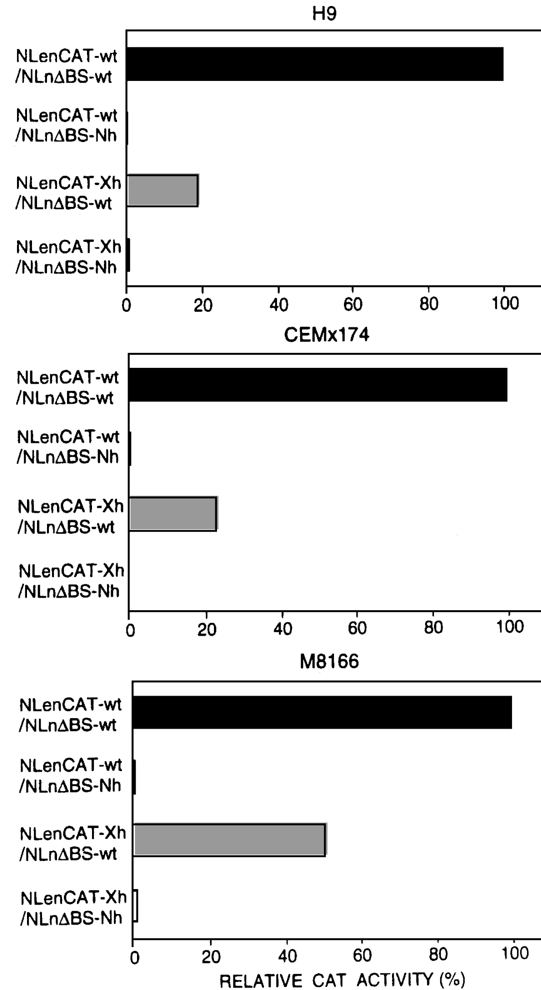

Fig. 5. Analysis of the early phase of the virus replication cycle. Virus samples (40 ng p24) obtained from SW480 cells co-transfected with pNLenCAT and pNLnΔBS were inoculated into H9, CEMx174 and M8166 cells; 48 h later, CAT activity in the cell lysates was monitored.

Determination of the virus replication stage affected by Nef

To determine the stage in the virus replication cycle when Nef is required, we performed a single-round infection assay, which is similar to that described previously (Helseth *et al*., 1989; Sakai *et al*., 1995). To do this, *env-vpu*-deficient proviral clones carrying a marker CAT gene with or without intact *nef* (pNLenCAT and pNLenCAT-Xh, respectively), an *env* expression vector without *nef* (pNLnΔBS), and its *env*-deficient mutant (pNLnΔBS-Nh) were constructed (Fig. 2*a*). In this system, when Env is supplied from pNLnΔBS in *trans*, pNLenCAT is able to produce infectious particles carrying CAT and undergo one round of replication (Fig. 2*b*). Efficiencies of transcription/translation and virion production in the late virus replication phase can be determined by monitoring CAT activity in transfected cells and RT activity in supernatants from these cells, respectively. CAT activity in cells infected with the CAT-carrying virus represents the efficiency with which early replication phase (from virus entry to integration) proceeds.

As shown in Fig. 3, upon co-transfection into SW480 cells, pNLenCAT-Xh (*nef* mutant) and pNLnΔBS-wt expressed CAT activity similar to that of the wt clone. The amount of mutant progeny released into the culture medium, as monitored by RT activity, was also comparable to that of the wt clone. The same experiment was carried out in a lymphocytic cell line (CEMx174) with similar results (Fig. 3). Furthermore, in the other transfection experiments, pNL-wt and pNL-Xh, the parental clones, behaved exactly like pNLenCAT-wt and pNLenCAT-Xh in H9, CEMx174, A3.01 and M8166 cells (Fig. 4). These results indicated that Nef is dispensable for the stage from transcription to virion production in the late phase of the virus replication cycle.

To determine whether Nef is required in the early stage of virus replication, progeny CAT-carrying virions obtained from transfections (Fig. 2 *b*) were inoculated into H9, CEMx174 and M8166 cells, and CAT activity was monitored. As shown in

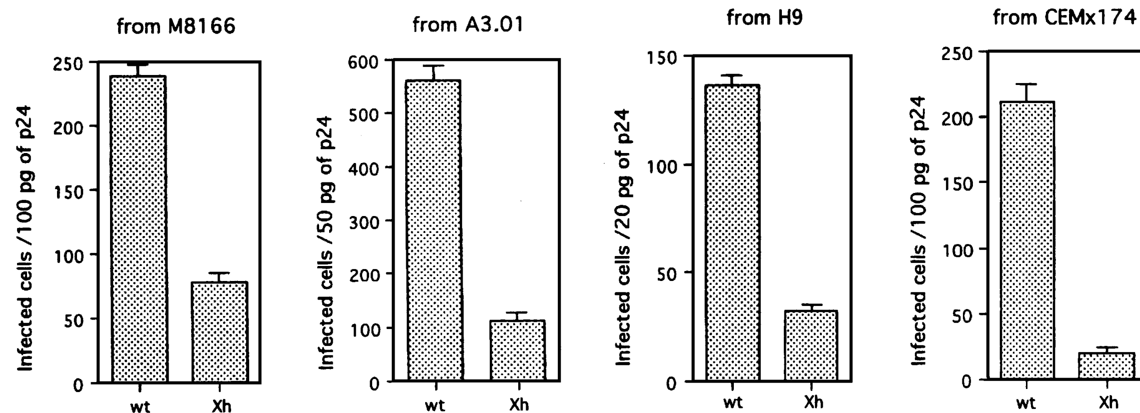

Fig. 6. Infectivity of *nef* mutant virions produced in various cell lines. Infectivity of cell-free virus samples prepared from transfected cells indicated was assessed by infecting MAGI cells and counting blue foci 48 h later (Kimpton & Emerman, 1992). Each infection was performed in triplicate; the average infectivity and standard deviation is shown. Xh, *nef* mutant virus.

Fig. 5, infection of H9 and CEMx174 cells with virus from pNLenCAT-Xh plus pNLnΔBS-wt resulted in approximately 5-fold less CAT activity than that in cells infected by virus from pNLenCAT-wt plus pNLnΔBS-wt. In M8166 cells, which are highly sensitive to HIV-1 infection, virus from pNLenCAT-Xh plus pNLnΔBS-wt expressed 2-fold less CAT activity. These data were reproducibly obtained in repeated experiments and clearly indicate that Nef up-regulates some process in the early phase of virus replication (from adsorption to integration).

The infectivity of virions produced in various cell lines

We then examined how the early defect in the *nef* mutant virions is reflected in infectivity. Virions were prepared from various cells transfected with wt or *nef* mutant clones, and their infectivity was determined by a single-cycle infection assay (MAGI assay; Kimpton & Emerman, 1992). As shown in Fig. 6, the infectivity was expressed as the number of infected cells per 20–100 pg p24 antigen. Although the *nef* mutant virus was always less infectious, the magnitude of the defect varied. In these experiments, a 3- to 5-fold greater infectivity was consistently observed for wt viruses prepared from M8166, H9 and A3.01 cells. Of note, an approximately 11-fold reduction in infectivity was observed for the *nef* mutant virus produced in CEMx174 cells.

Discussion

In recent studies, Nef has been reported to be a positive rather than a negative factor in virus replication (Chowers *et al*., 1994; de Ronde *et al*., 1992; Kim *et al*., 1989; Miller *et al*., 1994; Spina *et al*., 1994; Terwilliger *et al*., 1991; Zazopoulos & Haseltine, 1992, 1993). In this regard, our data in the present work are perfectly consistent with these reports. Our results show that the growth of *nef* mutant virus was clearly delayed relative to that of parental virus (Fig. 1).

We detected no difference in the levels of CAT and RT production of wt and *nef* mutant viruses in the several cell lines transfected with each proviral DNA clone (Figs 3 and 4). These results indicate that Nef plays no obvious role at the late stage of virus replication. It was also demonstrated that Nef plays an important role in the early phase of replication, from adsorption to integration, as indicated by CAT production in cells infected with wt or *nef* mutant virus carrying the CAT gene (Fig. 5). This result is in agreement with previous reports (Aiken & Trono, 1995; Chowers *et al*., 1995; Miller *et al*., 1995; Schwartz *et al*., 1995), in terms of the replication phase affected by Nef. In the present work, the infectivity of the *nef* mutant prepared from several producer cell lines was also monitored. Considerable differences in the relative infectivity of the mutant were noted in the different cell lines (Fig. 6).

The conclusion of this report that Nef acts at the very late stage of virus replication, such as assembly, release, and maturation, to enhance the efficiency of the early replication process in a producer cell-dependent way raises the obvious possibility that Nef may interact with the virion structural proteins. Thus far, we have detected no significant difference in virion proteins between wt and *nef* mutant virus as judged by immunoprecipitation (our unpublished results), which is in agreement with the recent report by Schwartz *et al*. (1995). A more detailed analysis of structural components of virions, produced in the presence and the absence of Nef, and in various cell lines, will allow a better understanding of the mechanism by which Nef influences virus infectivity.

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References

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A. & Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. *Journal of Virology* **59**, 284–291.

Adachi, A., Ono, N., Sakai, H., Ogawa, K., Shibata, R., Kiyomasu, T., Masuike, H. & Ueda, S. (1991). Generation and characterization of the human immunodeficiency virus type 1 mutants. *Archives of Virology* **117**, 45–58.

Ahmad, N. & Venkatesan, S. (1988). Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science* **241**, 1481–1485.

Aiken, C. & Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *Journal of Virology* **69**, 5048–5056.

Chowers, M. Y., Spina, C. A., Kwoh, T. J., Fitch, N. J. S., Richman, D. D. & Guatelli, J. C. (1994). Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact *nef* gene. *Journal of Virology* **68**, 2906–2914.

Chowers, M. Y., Pandori, M. W., Spina, C. A., Richman, D. D. & Guatelli, J. C. (1995). The growth advantage conferred by HIV-1 *nef* is determined at the level of viral DNA formation and is independent of CD4 downregulation. *Virology* **212**, 451–457.

de Ronde, A., Klaver, B., Keulen, W., Smit, L. & Goudsmit, J. (1992). Natural HIV-1 Nef accelerates virus replication in primary human lymphocytes. *Virology* **188**, 391–395.

Folks, T., Benn, S., Rabson, A., Theodore, T., Hoggan, M. D., Martin, M., Lightfoote, M. & Sell, K. (1985). Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immune deficiency syndrome (AIDS)-associated retrovirus. *Proceedings of the National Academy of Sciences, USA* **82**, 4539–4543.

Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

Gottlinger, H. G., Dorfman, T., Cohen, E. A. & Haseltine, W. A. (1993). Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by *gag* gene constructs of widely divergent retroviruses. *Proceedings of the National Academy of Sciences, USA* **90**, 7381–7385.

Graham, F. L. & Van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467.

Hammes, S. R., Dixon, E. P., Malim, M. H., Cullen, B. R. & Greene, W. C. (1989). Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. *Proceedings of the National Academy of Sciences, USA* **86**, 9549–9553.

Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W. & Sodroski, J. (1989). Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *Journal of Virology* **64**, 2416–2420.

Jamieson, B. D., Aldrovandi, G. M., Planelles, V., Jowett, J. B. M., Gao, L., Bloch, L. M., Chen, I. S. M. & Zack, J. A. (1994). Requirement of human immunodeficiency virus type 1 *nef* for in vivo replication and pathogenicity. *Journal of Virology* **68**, 3478–3485.

Kestler, H. W., III, Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D. & Desrosiers, R. C. (1991). Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**, 651–662.

Kim, S., Ikeuchi, K., Byrn, R., Groopman, J. & Baltimore, D. (1989). Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences, USA* **86**, 9544–9548.

Kimpton, J. & Emerman, M. (1992). Detection of replication-competent and pseudotype human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated-galactosidase gene. *Journal of Virology* **66**, 2232–2239.

Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A. & Orenstein, J. M. (1990). The human immunodedeficiency virus type 1-specific protein Vpu is required for efficient virus maturation and release. *Journal of Virology* **64**, 621–629.

Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C. & Feinberg, M. B. (1994). The human immunodeficiency virus type 1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *Journal of Experimental Medicine* **179**, 101–113.

Miller, M. D., Warmerdam, M. T., Page, K. A., Feinberg, M. B. & Greene, W. C. (1995). Expression of the human immunodeficiency virus type 1 (HIV-1) *nef* gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. *Journal of Virology* **69**, 579–584.

Niederman, T. M. J., Thielan, B. J. & Ratner, L. (1989). Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. *Proceedings of the National Academy of Sciences, USA* **86**, 1128–1132.

Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. (1988). Detection, isolation and continuous production of cytopathic retroviruses from patients with AIDS and pre-AIDS. *Science* **224**, 497–500.

Sakai, H., Siomi, H., Shida, H., Shibata, R., Kiyomasu, T. & Adachi, A. (1990). Functional comparison of transactivation by human retrovirus rev and rex genes. *Journal of Virology* **64**, 5833–5839.

Sakai, H., Tokunaga, K., Kawamura, M. & Adachi, A. (1995). Function of human immunodeficiency virus type 1 Vpu protein in various cell types. *Journal of General Virology* **76**, 2717–2722.

Salter, R. D., Howell, D. N. & Cresswell, P. (1985). Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* **21**, 235–246.

Schwartz, O., Marechal, V., Danos, O. & Heard, J.-M. (1995). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cells. *Journal of Virology* **69**, 4053–4059.

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A. & Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *Journal of Virology* **65**, 3514–3520.

Shibata, R., Sakai, H., Kawamura, M., Tokunaga, K. & Adachi, A. (1995). Early replication block of human immunodeficiency virus type 1 in monkey cells. *Journal of General Virology* **76**, 2723–2730.

Spina, C. A., Kwoh, T. J., Chowers, M. Y., Guatelli, J. C. & Richman, D. D. (1994). The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *Journal of Experimental Medicine* **179**, 115–123.

Takai, H. & Ohmori, H. (1990). DNA transfection of mouse lymphoid cells by the combination of DEAE-dextran-mediated DNA uptake and osmotic shock procedure. *Biochimca et Biophysica Acta* **1048**, 105–109.

Terwilliger, E., Sodroski, J. G., Rosen, C. & Haseltine, W. A. (1986). 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. *Journal of Virology* **60**, 754–760.

Terwilliger, E. F., Langhoff, E., Gabuzda, D., Azopoulos, E. & Haseltine, W. A. (1991). Allelic variation in the effects of the *nef* gene on replication of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences, USA* **88**, 10971–10975.

Wigler, M., Pellecer, A., Silverstein, S., Axel, R., Urlaub, G. & Chasin, L. (1979). DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proceedings of the National Academy of Sciences, USA* **76**, 1372–1376.

Willey, R. L., Smith, D. H., Lasky, L. A., Theodore, T. S., Earl, P. L., Moss, B., Capon, D. J. & Martin, M. A. (1988). In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is for critical for infectivity. *Journal of Virology* **62**, 139–147.

Zazopoulos, E. & Haseltine, W. A. (1992). Mutational analysis of the human immunodeficiency virus type 1 Eli Nef function. *Proceedings of the National Academy of Sciences, USA* **89**, 6634–6638.

Zazopoulos, E. & Haseltine, W. A. (1993). Effect of *nef* alleles on replication of human immunodeficiency virus type 1. *Virology* **194**, 20–27.

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