A

C

B

Fig. 4. Deletion analysis of HIV-1 Vif. (A) Steady-state expression of Vif in 293T cells transfected with various proviral mutant clones. 293T cells were transfected with 20 µg of various pNL clones indicated, and cell lysates were prepared at 48 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. ΔVif, pNL–Nd [34]; WT, pNL432 [21,34]. (B) Growth kinetics in H9 cells of various proviral mutant clones. H9 cells were electroporated with 10 µg of various pNL clones indicated, and RT production in the culture supernatants was determined at intervals. WT, pNL432 [21,34]; ΔVif, pNL–Nd [34]. (C) Phenotype of various mutant clones. The results in (A) and (B) are summarized. Characteristics of E88del, W89del, R90del, K91del, K92del, and R93del mutants have been previously described [22] and are included in this summary.

3.3. Rapid and extensive degradation is unique to Vif among accessory proteins

We finally asked whether Vif is unique among the four HIV-1 accessory proteins with respect to its high sensitivity to proteasome degradation. To address this question, we performed pulse/chase analyses using wt full-length proviral clone pNL432. Transfected 293T cells were pulse-labeled and chased as described above, and the degradation kinetics of Vif, Vpr, Vpu and Nef were monitored. Gag-p24 was used as a control. As shown in Fig. 6A, Vif was unstable and decayed much more rapidly and extensively than Vpr, Vpu and Nef. The blocking effect of the proteasome inhibitor MG-132 on the degradation of accessory proteins was then examined (Fig. 6B). Transfected 293T cells were pulse-labeled for 5 min and chased for 30 min in the presence or absence of MG-132, and the ratio of expression level of accessory proteins (soluble fraction) was determined. As clearly seen in Fig. 6B, the effect was the greatest for Vif.

4. Discussion

We have recently shown that rapid turn-over of Vif in cells may be virologically important [20]. Excess amounts of Vif

exert harmful effects on virus infectivity, and therefore HIV-1 has apparently evolved to control the intracellular expression level of Vif. In this study, we report for the first time on the mechanism, the cell-type independency, and the region in Vif critical for the rapid intracellular degradation. We also show that this unstable nature of Vif is unique among HIV-1 accessory proteins.

Our results here strongly suggest that the mechanism of Vif degradation involves the cellular proteasome pathway. The proteasome inhibitors MG-132 and clasto-lactacystin β-lactone specifically blocked the degradation (Figs. 1, 5, and 6). We have detected large amounts of polyubiquitinated derivatives of Vif that are greatly increased by the proteasome inhibitor MG-132 (Fig. 3). These results are quite consistent with those on the presence of APOBEC3G recently reported by Mehle et al. [14]. In the absence of APOBEC3G, however, they found that only a minor fraction of Vif is monoubiquitinated, and that Vif is a relatively stable protein with a half-life of approximately 90 min [14]. These observations were very different from our results presented here. In contrast to the soluble cytosolic Vif, which is rapidly degraded, the insoluble cytoskeleton-associated Vif was quite stable and accumulated in cells (Figs. 1 and 2). This phenomenon was observed in permissive 293T and HeLa cells, and in non-permissive H9 cells (Figs. 1 and 2). Whether
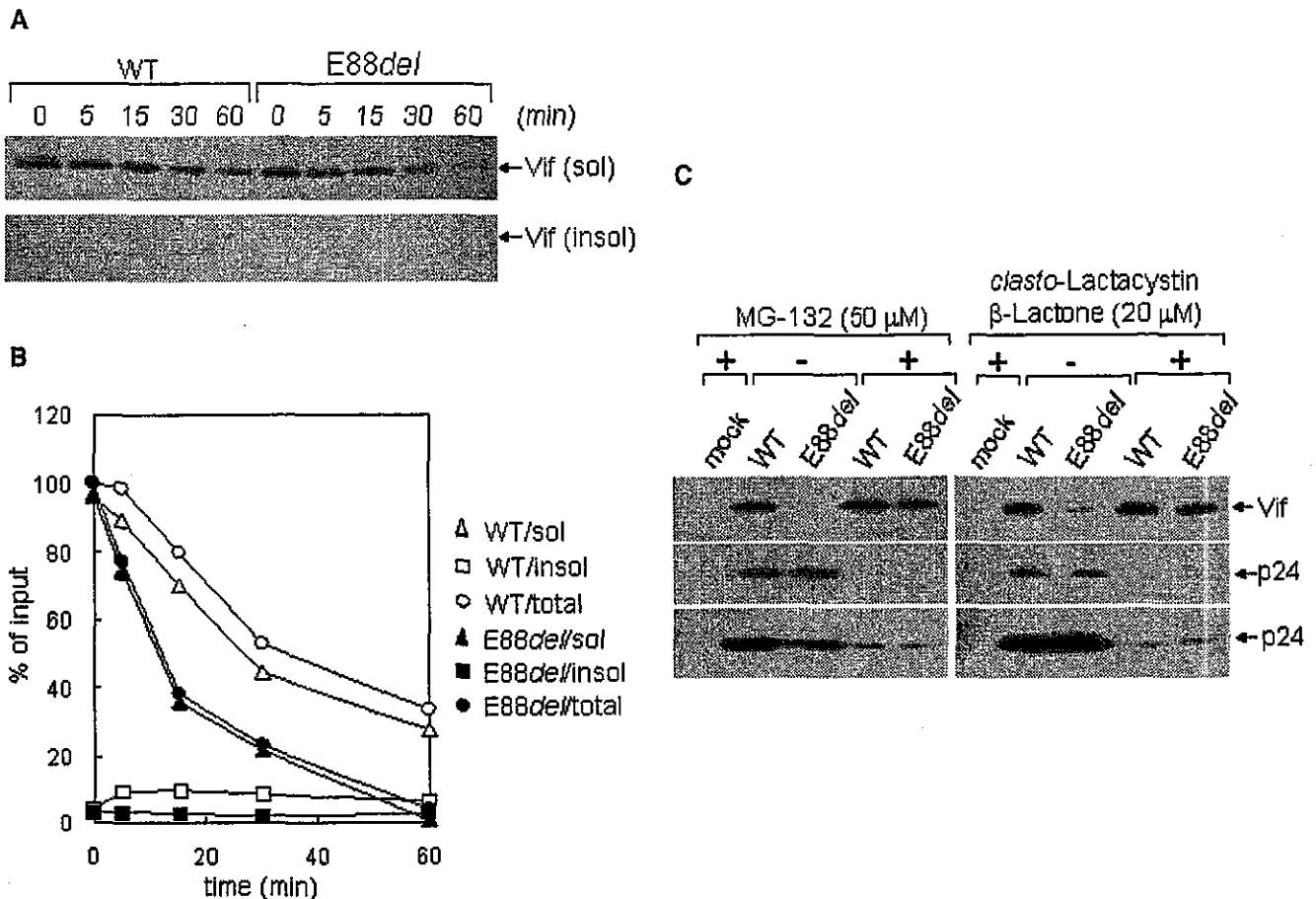
Fig. 5. Comparative analysis of wt and E88del mutant Vif proteins. (A) Stability of wt and mutant Vif proteins. 293T cells were transfected with 5 μg of pNL-A1 (WT) or pNLA1-E88del. Cells were collected 24 h after transfection, labeled for 5 min with [35S]-methionine and [35S]-cysteine, and chased for up to 60 min, as indicated above the lanes. Cell lysates were prepared as described in Section 2 and precipitated with a Vif-specific polyclonal antibody Vif93 [29]. Vif proteins were identified by SDS-PAGE followed by fluorography; sol, soluble fraction; insol, insoluble fraction. (B) Degradation kinetics of wt and mutant Vif proteins. Vif-specific bands in (A) were quantified by scanning, and relative values were plotted as a function of time. Total, sol plus insol. (C) Steady-state expression of wt and mutant Vif proteins in the presence or absence of proteasome inhibitors. The 293T cells were transfected with 3 μg of wt pNL432 or pNL-fE88del and cultured in the presence (+) or absence (–) of proteasome inhibitors MG-132 and clasto-lactacystin β-lactone. Cells were harvested at 36 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. Appropriate adjustments in sample volumes were made to give bands of similar intensity (wt Vif). Also, results of two different blots for Gag-p24 are presented to show that each lane for comparison contained approximately similar amounts. Expression level of viral proteins in cells in the presence of the inhibitors was low relative to that in the absence of the inhibitors.

the cytoskeletal Vif represents a population refractory to ubiquitination and has a pivotal role for Vif function remains to be determined. It would be vitally important to generate degradation-resistant Vif mutants and characterize them biochemically and biologically.

Deletion analysis of Vif in this study has demonstrated that there are some amino acids that confer stability on Vif. We have recently reported that mutations affecting residues 88 and 89 of NL432 Vif, located within a β-strand structure, as predicted by the PredictProtein, resulted in a reduced level of Vif expression [22]. Based on this observation, we performed mutational analysis of the area. Deletion of amino acids in the predicted β-strand structures (residues 63–70 and 86–89 in NL432 Vif), but not those located outside of the regions, diminished Vif expression to a negligible level and abolished viral infectivity (Fig. 4). Apparently, formation of the β-strand structures is important for stable expression of Vif. In addition, we showed that the E88del mutant Vif is

degraded more rapidly than wt Vif, and that this degradation is proteasome-dependent (Fig. 5). Together with the results in our recent work [20], we can conclude that there is an optimal level of Vif expression in cells for viral replication.

Recent work proposed that Vif induces proteasome-dependent degradation of APOBEC3G [13–16], but the precise molecular mechanism for this activity of Vif is still unclear. We have already shown by pulse/chase analyses that there is no significant difference in the stability of APOBEC3G in the presence or absence of Vif [17] and that there is no significant difference in the stability of Vif in the presence or absence of APOBEC3G [20]. Furthermore, we found that the half-life of Vif in non-permissive H9 cells (Fig. 2) is very different from that of APOBEC3G in the presence of Vif, which was recently reported [13,15,16, 18,19]. Based on these findings, it seems unlikely that Vif and APOBEC3G form a complex that leads to subsequent simultaneous degradation of both proteins by the proteasome path-
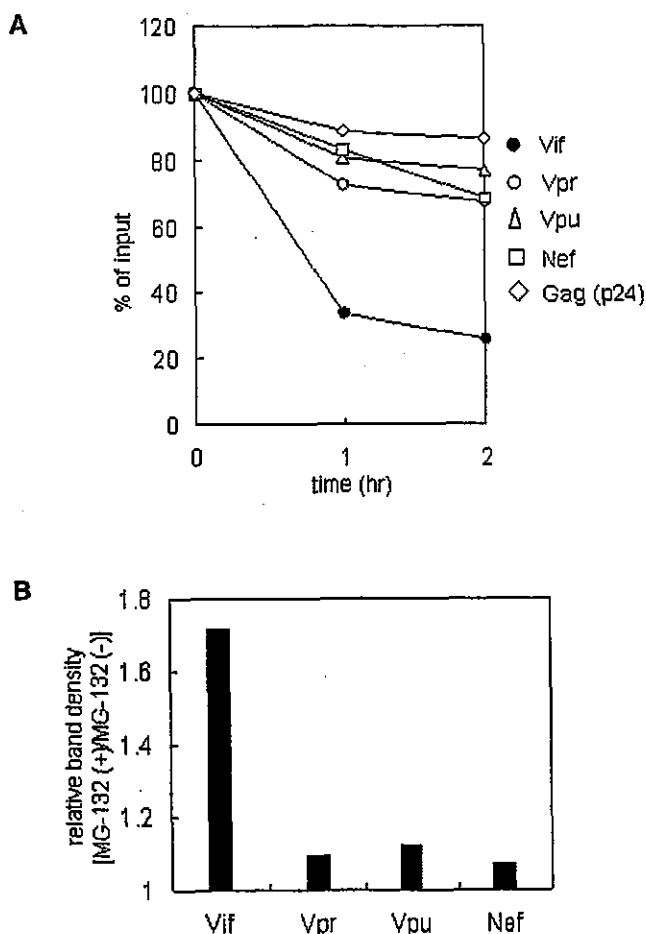
A

B

Fig. 6. Comparative analysis of HIV-1 accessory proteins. (A) Degradation kinetics of the HIV-1 accessory proteins. The 293T cells were transfected with 5 µg of pNL432 or a control Gag-p24 expression vector pSG-Gag (p24) cFLAG. Cells were collected 24 h after transfection, labeled for 5 min with [³⁵S]-methionine and [³⁵S]-cysteine, and chased for up to 2 h as indicated. Cell lysates (soluble fraction) were prepared and precipitated with an antibody against each viral protein (anti-Vif, Vif93; anti-Vpr, raised against full-length synthetic Vpr peptide (Strebel et al., unpublished); anti-Vpu, NIH catalog no. 969; anti-Nef, NIH catalog no. 2949; anti-Gag-p24, NIH catalog no. 6521). Viral proteins were identified by SDS-PAGE followed by fluorography. Specific bands of viral proteins were quantified by scanning, and relative values were plotted as a function of time. (B) Blocking effect of proteasome inhibitor MG-132 on the degradation of accessory proteins. 293T cells were transfected with various clones, as described in (A). Cells were collected 24 h after transfection, labeled for 5 min with [³⁵S]-methionine and [³⁵S]-cysteine, and chased for 30 min. Part of cells were incubated in the presence of MG132 (50 µM) during the labeling and chasing time, as indicated. Cell lysates were prepared and precipitated with an antibody against each accessory protein as above. Accessory proteins were identified by SDS-PAGE followed by fluorography. Specific bands of accessory proteins were quantified by scanning, and the ratio of values for each accessory protein is shown.

way. We and others have reported that Vif affects APOBEC3G translation [17–19]. However, the reduction of intracellular APOBEC3G in the presence of Vif does not fully account for the Vif-induced reduction of virus-associated APOBEC3G [17]. Further investigations are required to elucidate the molecular mechanism by which Vif counteracts the activity of APOBEC3G.

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Microbes and Infection

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Original article

Functional analysis of HIV-1 *vif* genes derived from Japanese long-term nonprogressors and progressors for AIDS

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Abstract

We analyzed the function of human immunodeficiency virus type 1 (HIV-1) *vif* gene from Japanese long-term nonprogressors (LTNPRs) and progressors (PRs) for acquired immunodeficiency syndrome (AIDS). We constructed a basic HIV-1 infectious clone, which facilitated the incorporation and evaluation of *vif* from infected individuals. Proviral reporter clones carrying *vif* from six Japanese LTNPRs and seven PRs were then generated and their in vitro growth kinetics were analyzed. The *vif* clones, which could confer infectivity on reporter viruses, were considered active, and the ratio of the active clones to the number of clones examined per individual was determined. For the majority of LTNPRs, there was no correlation between presence or absence of functional *vif* with long-term nonprogression for AIDS. There was one exception in which all the clones examined had inactive *vif*, suggesting a probable association of inactive *vif* with the nonprogression. All PRs with high viral load had a high ratio of active *vif* clones. Our results suggest that the presence of functional *vif* would influence HIV-1 infectivity and disease progression in infected individuals.
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Keywords: HIV-1; AIDS; *Vif*; Long-term nonprogressors

1. Introduction

The time between developing acquired immunodeficiency syndrome (AIDS) and first exposure to human immunodeficiency virus type 1 (HIV-1) varies among individuals. Some individuals, termed long-term nonprogressors (LTNPRs), remain free from AIDS related complex (ARC) or AIDS for more than 10 years after infection without any antiretroviral therapy. Viral factors, host factors or both may contribute to this long-term nonprogression for AIDS [1]. HIV-1 *nef* gene has been suggested as one of the viral factors associated with the status of disease progression, as many LTNPRs specifically had gross deletions in *nef* [2–6]. Recently we reported by molecular virological analysis that *nef* is important for AIDS development [7]. In that study, we examined various

activities of HIV-1 *nef*; (1) enhancement of viral infectivity, (2) down-regulation of CD4, (3) down-regulation of MHC-I, (4) binding ability with Hck [7]. The results obtained have demonstrated that augmentation of viral infectivity by *nef* is critically associated with AIDS [7].

Earlier studies on simian immunodeficiency virus (SIV) also show the importance of *nef* for simian AIDS [8,9]. In those reports, another accessory gene, *vif*, was found to be responsible for AIDS [8,10]. As for the potential role of HIV-1 *vif* for AIDS induction, few studies have been reported so far [11,12]. In the tissue culture system, Vif is known to confer infectivity on progeny virions in a producer cell-dependent manner, and producer cells are, therefore, divided into permissive and nonpermissive [13–16]. Viruses lacking *vif* fail to replicate in nonpermissive cells such as peripheral blood mononuclear cells (PBMCs), macrophages, and H9 cells. We have reported that various mutant *vif* clones show distinct growth phenotypes in H9 and semi-permissive A3.01

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cells [17,18]. Hassaine et al. [11] examined the sequence of *vif* from LTNPRs and found that the amino acid at position 132 of Vif is associated with the viral load in LTNPRs. A recent report by Alexander et al. [12] described that a two-amino-acid insertion in Vif may contribute to the nonprogression of mother and child for AIDS. In this study, proviral reporter clones carrying *vif* from six LTNPRs or seven progressors (PRs) were generated, and their growth kinetics in nonpermissive and semi-permissive cells were examined. The results described here suggest that HIV-1 *vif* is important for the induction of AIDS.

2. Materials and methods

2.1. Study subjects

Samples from six Japanese LTNPRs (p1–p6) and seven PRs (p12–p18) were examined in the present study. The details of all study subjects have been described previously [19,20].

2.2. Cells

Lymphocytic cell lines H9 (ATCC HTB-176) and A3.01 [21] were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum as previously described [21,23]. A monolayer cell line 293T [22] was maintained in Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described [23].

2.3. Transfection and reverse transcription (RT) assay

For transfection of uncleaved plasmid DNA into lymphocytic H9 and A3.01 cells, the electroporation method was used as previously described [23]. For transfection of adherent 293T cells, the calcium–phosphate coprecipitation technique was used [23]. After electroporation, virus production in the culture supernatants was monitored at intervals by virion-associated reverse transcriptase (RT) activity as previously described [24].

2.4. Western immunoblotting

Cell lysates were prepared as described before [24], and proteins were resolved on sodium dodecyl sulfate–12.5% polyacrylamide gels, followed by electrophoretic transfer to polyvinylidene fluoride membranes (Immobilon-P, Millipore Co., Bedford, MA, USA). The membranes were treated with anti-Vif (NIH AIDS Research and References Reagent Program, catalog no. 2746) and anti-p24 [25] antibodies and visualized using an ECL plus Western blotting detection system (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

2.5. Construction of basic and pilot clones

The wild-type (wt) infectious molecular clone of HIV-1, designated pNL432 (GenBank Accession no. AF324493),

has been described previously [23]. For construction of basic clone pNL-SX, shown in Fig. 1, an appropriate DNA fragment of pNL432 was subcloned into pBluescipt SK(+) (Stratagene, La Jolla, CA, USA), and the mutations were introduced using QuikChange site-directed mutagenesis kit (Stratagene) as follows. *Sma*I and *Xba*I sites were generated at 5′ and 3′ ends of *vif*, respectively. Four methionine codons were changed to one valine and three threonine codons so as not to act as initiation codon. The mutated DNA fragment was cloned back into pNL432 to construct pNL-SX. To construct pilot clones carrying NLVif, NLΔ19Vif and NLΔVif, shown in Fig. 1 (pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif, respectively), wt and mutant *vif* sequences were amplified by polymerase chain reaction (PCR) with *Sma*I at the 5′ and *Xba*I at the 3′ ends. As templates, pNL432 was used for NLVif and NLΔ19Vif, and its frame-shift mutant pNL-Nd [26,27] was used for NLΔVif. Two oligonucleotide primer pairs used were as follows: 5′-TCCCCCGGGATGGAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAGACTAGTGTCCATTCATTGTATG-3′ (antisense) for NLVif and NLΔVif, 5′-TCCCCCGGGATG-GAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAG-ACTATCTGTCCTCTGTCAGTTTC-3′ (antisense) for NLΔ19Vif. The PCR conditions were as follows: 94 °C for 45 s, 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR-amplified DNA fragments were inserted into pNL-SX to construct various pilot clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif.

2.6. Proviral reporter constructs

Template DNAs from individuals for *vif* amplification in this study have been previously described [20]. Oligonucleotide primers and cycling conditions are essentially the same as described above for NLVif. The amplified DNA fragments were inserted into pNL-SX to obtain various reporter constructs.

3. Results and discussion

3.1. Establishment of an assay system for evaluating vif function

In this study, we analyzed the function of *vif* from individuals infected with HIV-1. As the ability to confer infectivity on virions is the most important function assigned to *vif* so far [13–16], we established a virological assay system based on a molecular clone modified from pNL432 [23]. As shown in Fig. 1A, we constructed a basic clone, designated pNL-SX, carrying *Sma*I and *Xba*I sites downstream of *pol* and upstream of *vpr*. We then amplified, by PCR, the wt *vif* sequence of pNL432 (NLVif), its shorter version of *vif* lacking 3′-57 bp (NLΔ19Vif), and its frame-shift *vif* mutant, which does not express most amino acid sequences of Vif

A

pNL-SX

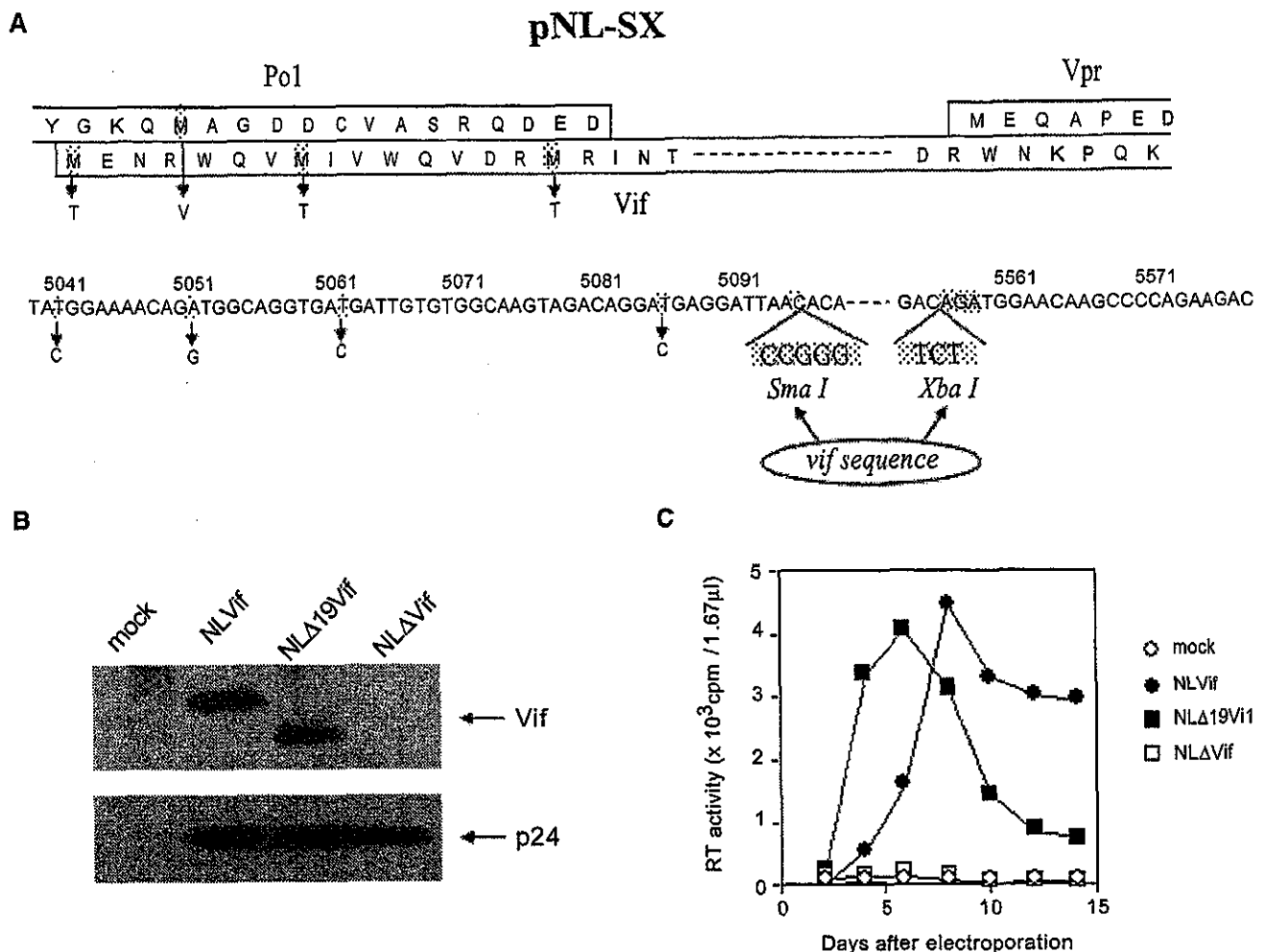**B**   **C**

Fig. 1. Construction and characterization of the clones to determine activity of *vif* genes from individuals infected with HIV-1. (A) Schematic representation of the pNL-SX clone used in this study. To construct clone pNL-SX, four methionine (in *pol* and *vif* genes) codons of pNL432 (GenBank Accession no. AF324493) [23] were changed to one valine and three threonine codons so as not to act as initiation codon, and *Sma*I and *Xba*I sites were introduced as indicated. Various *vif* sequences with *Sma*I at 5′ and *Xba*I at 3′ ends were amplified by PCR using pNL432, its *vif* mutants, and DNAs from the individuals as templates. The amplified DNAs were inserted into pNL-SX to generate pilot and reporter clones. Amino acid (Pol, Vif and Vpr) and nucleotide sequences of pNL432 (nucleotide nos. 5040–5579) are shown. (B) Monitoring of Vif expression by Western blotting. 293T cells were transfected with the pilot proviral clones, as previously described [23], and cell lysates were prepared on day 2. Cell lysates were analyzed for the expression of Vif and Gag-p24 proteins by Western immunoblotting using appropriate antibodies. The three sequences inserted into pNL-SX were indicated at the top. NLVif, full-length *vif* sequences of pNL432 [23]; NLΔ19Vif, mutant *vif* sequences of NL432 lacking C-terminal 19 amino acids [28]; NLΔVif, mutant *vif* sequences of pNL432 carrying a frame-shift mutation at the *Nde*I site, which can encode only 28 amino acids of Vif sequence [26]. Mock, pUC19. (C) Growth kinetics in H9 cells of the pilot proviral clones. Cells were electroporated with 10 µg of the proviral clones [23], and RT production in the culture supernatants was determined, as previously described [24]. The same clones were used in (B) and (C).

(NLΔVif), and inserted these sequences into pNL-SX to construct pilot proviral clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif. We and others have already reported that the C-terminal 19 amino acids of Vif (174–192) are dispensable without altering its functionality [28,29]. To confirm that pNL-SX is valid for virological assessment, the ability of these pilot clones to express Vif and to confer infectivity on virions was determined. These clones were transfected into 293T cells, and the expression of wt (NLVif) and the short version (NLΔ19Vif) of Vif was monitored by Western blotting. As can be clearly seen in Fig. 1B, wt and short Vif were readily detected. These clones were then electroporated into nonpermissive H9 cells, and RT production in the culture supernatants was monitored to

determine their infectivity. As shown in Fig. 1C, viruses carrying wt (NL-SX/NLVif) and short type *vif* (NL-SX/NLΔ19Vif) could efficiently replicate in H9 cells, as expected, in contrast to the virus lacking functional *vif* (NL-SX/NLΔVif). These results indicated that pNL-SX can be used for evaluating the functional ability of *vif* from individuals infected with HIV-1.

3.2. Analysis of vif activity from LTNPRs and PRs

The pNL-SX system described above was used to evaluate the functionality of *vif* derived from six Japanese LTNPRs, who were free from ARC or AIDS for more than 10 years after infection, and from seven PRs [19,20]. *Vif* sequences in

Table 1
Activity of HIV-1 *nef* and *vif* genes derived from LTNPRs and PRs of AIDS

Individual[a]	nef[b] MAGI infectivity	vif Clone[c]	Growth[d] in H9	A3.01
p1 LTNPR (<1.0)	m,m	10	–	–
		12-a	+	+
		12-b	+	+
		12-c	+	+
		12-d	+	+
		13	+	+
		14	+	+
			0.86	0.86
p2 LTNPR (<1.0)	nd	1	+	+
		4	+	+
		5-a	+	+
		5-b	+	+
			1.00	1.00
p3 LTNPR (<1.0)	m,–	1	–	–
		3	+*	+*
		4	–	–
		5	+	+
		6	+	+
			0.60	0.60
p4 LTNPR (<1.0)	m,m	1	–	–
		2	–	–
		3	+	+
		4	+	+
		5	+	+*
			0.60	0.60
p5 LTNPR (<1.0)	nd	1	–	–
		3	–	–
		4	–	–
		9	–	–
			0.00	0.00
p6 LTNPR (<1.0)	m,m	c-a	–	–
		c-b	–	–
		g	+	+
		h	+	+
		i	–	–
			0.40	0.40
p12 PR (2.2)	nd	7	+	+
		8-a	–	–
		8-b	–	–
		9	–	–
		10	–	–
			0.20	0.20
p13 PR (2.4)	+	1	+	+
		2-a	+	+
		2-b	+	+
		7	+	+
			1.00	1.00
p14 PR (20)	+,+	3	–	+*
		5	+	+*
		6	+	+
		7	–	–
			0.50	0.75

Individual[a]	nef[b] MAGI infectivity	vif Clone[c]	Growth[d] in H9	A3.01
p15 PR (88)	+,+	1	+	+
		2	+	+
		4	+	+
		5	–	+
		6	+	+
			0.80	1.00
p16 PR (29)	+,+	1	+	+
		2	+	+
		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p17 PR (21)	+	1	+	+
		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p18 PR (3.8)	+	1	+	+
		2	+	+
		4	+	+
			1.00	1.00

[a] Identification numbers of individuals, stage of disease progression, and viral RNA level in the plasma ($\times 10^4$ copies per ml), in parentheses, are indicated [19].

[b] Infectivity of the clones based on NL432 carrying *nef* sequences derived from the individuals (one or two clones per person) was determined by MAGI assay [7]. Infectivity of the clones relative to that of the clone carrying NL432 *nef* is shown. +, >60%; –, <30%; m, 30–60%; nd, not done.

[c] (The same number or the same alphabet)-(alphabet) indicates that the clones carry the same *vif* sequences. For example, three and four different *vif* sequences have been identified in p2 and p6, respectively.

[d] Growth ability in H9 and A3.01 cells of viral clones derived from pNL-SX (Fig. 1A) carrying *vif* genes from the individuals. Growth phenotype of the clones similar to that of NL-SX/NLVif and that of NL-SX/NLΔVif (Fig. 2A) is indicated by + and –, respectively. Growth phenotype of the viruses which grew more poorly than NL-SX/NLVif but better than NL-SX/NLΔVif is indicated by +* (Fig. 2A). The ratio of the number of clones with active (+ and +*) *vif* to that of clones examined is indicated on the last line.

these individuals were amplified by PCR and inserted into pNL-SX to construct 53 reporter clones (Table 1). Nonpermissive H9 and semi-permissive A3.01 cells were used to examine the growth ability of all clones generated, as mutant *vif* clones show distinct growth phenotypes in these two cell lines [17,18]. Representative growth kinetics of the clones are shown in Fig. 2A. In these experiments, clones carrying wt (NLVif) and Δ*vif* (NLΔVif) served as positive and negative controls, respectively. Based on the growth phenotypes, various *vif* sequences were considered to be active or inactive. Clones p1-12, p3-3 and p13-1 were thought to be active, while clones p1-10, p3-4, p14-3 and p14-7 were inactive as judged by virus replication in H9 cells (Fig. 2A). In A3.01 cells, clones p1-12, p3-3, p13-1 and p14-3 were active, while p1-10, p3-4, and p14-7 were inactive (Fig. 2A). The functionality of all *vif* sequences from LTNPRs and PRs is summarized in Table 1. Two clones, p14-3 and p15-5, showed
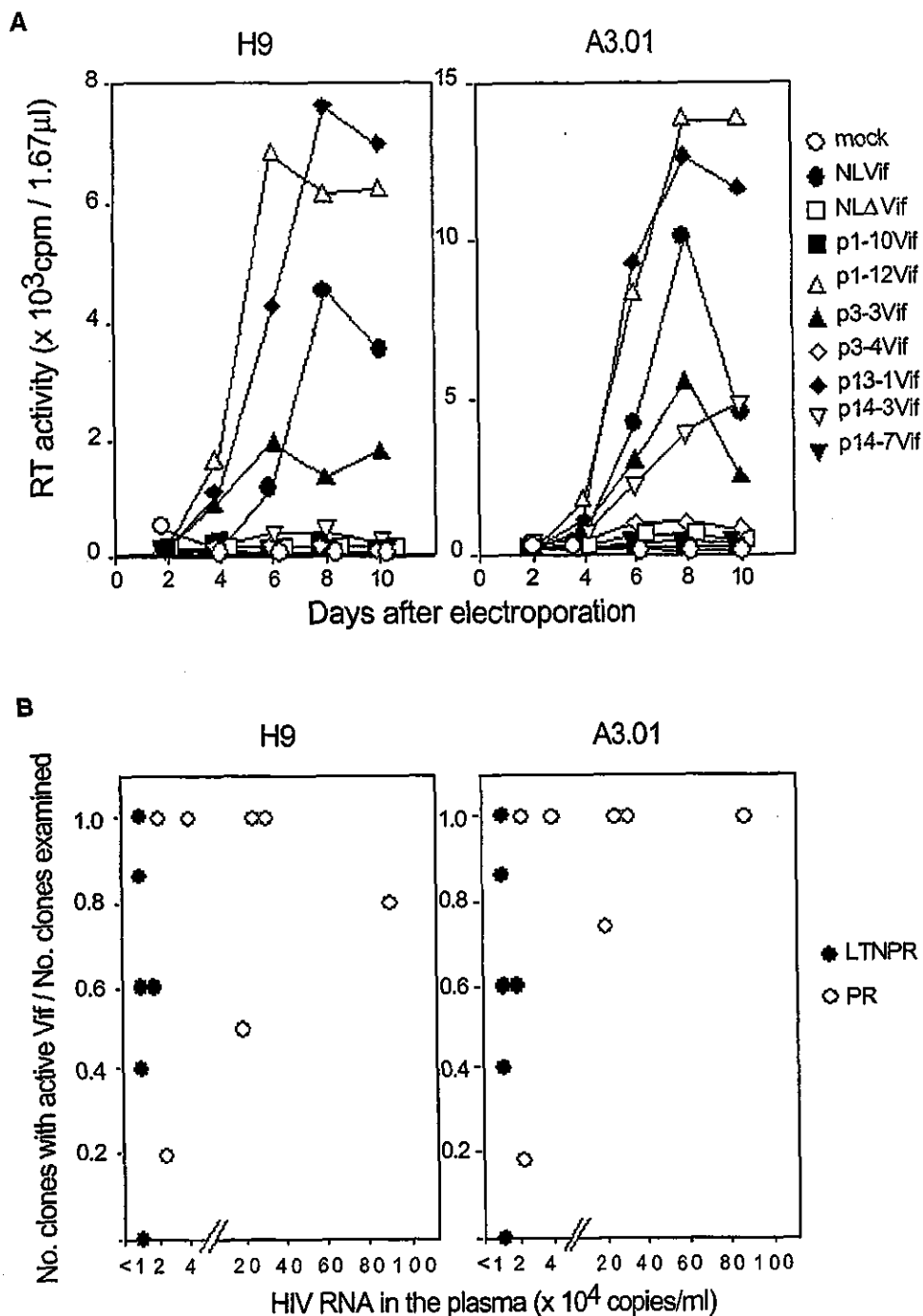
Fig. 2. Activity of HIV-1 *vif* genes derived from LTNPRs and PRs for AIDS. (A) Growth kinetics in H9 and A3.01 cells of various clones carrying *vif* sequence from the individuals. Cells were electrophorated, as previously described [23], with 10 µg of proviral clones carrying the *vif* sequences indicated on the right, and RT production in the culture supernatants at intervals was determined, as previously described [24]. Selected samples are shown in the figure for clarity (all the data are presented in Table 1). The clones were constructed by insertion of the *vif* sequences derived from the individuals into *SmaI* and *XbaI* sites of pNL-SX (Fig. 1A). The *vif* sequences from the individuals are indicated by the individual no.-clone no. Sequences NLVif and NLΔVif (for details, see the legend to Fig. 1) were used as positive and negative controls, respectively. Mock, pUC19. (B) Ratio of active and inactive *vif* clones in LTNPRs and PRs for AIDS. The ratio of clones with active *vif* in each person versus viral RNA level in his/her plasma (10⁴ copies per ml) [19] is plotted for all the clones examined (Table 1).

distinct growth phenotypes in H9 and A3.01 cells (Fig. 2A, Table 1).

The ratio of active *vif* clones (in both cell lines) to the total number of clones screened per individual is also shown in Table 1. When the ratio versus the viral load in plasma

(Table 1) was plotted, results as shown in Fig. 2B were obtained. LTNPRs enrolled in the present study had varied ratios of active *vif* clones, ranging from 0 to 1.0. The results from the majority of LTNPRs indicate no apparent relationship between presence or absence of functional *vif* and long-

term nonprogression of infection. Subject p5 was an exception and had inactive *vif* in all the clones screened. This finding suggested a possibility that a defect of *vif* function in p5 could be responsible for the long-term nonprogression. On the other hand, all PRs with high viral load (HIV RNA >20 × 10^4 copies per ml) had high ratios (0.5 or >0.5) of active *vif* clones. We and others have reported that primary cultured PBMCs and macrophages are non or less permissive for HIV virions lacking *vif* [30–35]. Recently, molecular biological demonstration has shown that *vif* confers infectivity on virions in nonpermissive cells. Vif counteracts an endogenous cellular factor, APOBEC3G, that inhibits HIV-1 replication [36,37]. It is well anticipated that the activity of *vif* modulates HIV-1 replication in individuals infected with virus.

3.3. Conclusion

Table 1 summarizes the major data in our two papers (reference [7] and this article). In these two reports, *nef* and *vif* from the same individuals were systematically analyzed for their potential roles in the development of AIDS. The *nef* genes from LTNPRs were found to have lower activity to enhance viral infectivity than those from PRs. The results of our *nef* and *vif* studies have shown that all PRs with a high viral load have a high ratio of active genes, strongly suggesting active *vif* and *nef* are required for HIV-1 replication in individuals. The *vif* and *nef* genes, therefore, would influence the progression and outcome of the disease in infected individuals. This is consistent with the previous reports analyzing *vif* sequences in disease progression [11,12]. On the other hand, the active/inactive ratio of *vif* varied in the samples from LTNPRs (from 0 to 1.0), different from that of *nef*. Without active *nef*, infected individuals appeared not to develop ARC or AIDS. In tissue cultures, *nef* is critical for HIV replication in resting PBMC [38,39] but not in stimulated PBMC or macrophages [32,35]. The *vif* is known to be indispensable for virus replication in these cells [30–35]. Because *vif* is more essential in virus replication than *nef*, it is reasonable to assume that *vif*-inactive viruses cannot or hardly persist in individuals. Further study is necessary to draw a clear conclusion regarding the functional importance of *vif* for disease progression.

Acknowledgments

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Role of Us3 gene of herpes simplex virus type 1 for resistance to interferon

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Abstract. The sensitivity of a Us3-deletion mutant virus of herpes simplex virus type 1 (HSV-1) to consensus interferon (IFN) was compared with that of parental wild-type (wt) and the repaired viruses. Although one-step growth of the Us3-deficient virus in the IFN-treated HEp-2 cells was not markedly affected at a high multiplicity of infection (MOI), both the progeny virus yield and cytopathic effect were suppressed in a significantly higher degree in the mutant virus-infected cells than those in wt or the repaired virus-infected cells. This enhanced IFN-sensitivity of the mutant virus was more clearly demonstrated by the infection at a low MOI. In addition, both the size and number of plaques of the Us3-deficient virus in Vero cells were remarkably reduced with increasing concentrations of IFN, compared to those of wt or the repaired virus. These results indicate that the deletion of Us3 gene makes HSV-1 more sensitive to IFN.

Introduction

Us3 genes of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are dispensable for the virus growth in cell cultures (1,2), but are conserved among the α-herpesviruses (3,4). The Us3 gene encodes serine/threonine protein kinase which phosphorylates both viral and cellular proteins although not all substrates of the Us3 kinase are thought to be known (5-7). Previously we and others reported that the Us3-deficient mutants of HSV-1 and HSV-2 induce massive apoptosis of the infected cells (8,9), indicating that Us3 gene is one of the antiapoptotic genes of HSV. In addition, the Us3-deficient mutants of both HSV-1 and HSV-2 showed weaker pathogenicity than the parental wild-type (wt) viruses when the viruses were inoculated to mice (8,10,11), although the mechanism for the observed loss of pathogenicity is not yet clear.

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During the course of the studies on the effects of pro-inflammatory cytokines on the induction of apoptosis by virus infection (12), we noticed that the cytopathic effect (CPE) by the Us3-deletion mutant virus was often suppressed by interferon (IFN) at the concentrations where the CPE by wt virus was not. IFN is a major factor in the innate immunity against virus infection (for review see ref. 13). Considering the possibility that the weak pathogenicity of the Us3-deficient virus may result from the enhanced sensitivity to IFN, we examined here the virus yield, cytopathic effects, growth curve and plaque formation of the Us3-deletion mutant in the presence of IFN in comparison with those of wt and the repaired viruses.

Materials and methods

Cells and viruses. HEp-2 and Vero cells were grown in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum (FCS). HSV-1 strain F, the Us3-deletion mutant virus, R7041 (1), and the repaired virus, R7306 (1), were kindly provided by Bernard Roizman (University of Chicago) and used throughout the experiments. These viruses were propagated in Vero cells in MEM supplemented with 0.5% FCS and stored at -80°C until use as described previously (14,15). The amount of virus was measured by the plaque assay method.

Treatment with IFN. The purified IFN preparation of Amgen's consensus IFN, characterized in a previous study (16), was used throughout the experiments. This IFN has been genetically engineered and has amino acid sequence comprising the most frequently observed residue at each position among the sub-species of human IFN-α molecules (17). The purified IFN preparation was mixed with a stock bovine serum albumin (BSA) solution used as a carrier protein and lyophilized. After being dissolved in MEM containing 0.1% gelatin, the IFN solution was divided into small aliquots and stored at -80°C until use.

To examine the effects of IFN on the virus growth, mono-layered HEp-2 cells in 35 mm-dishes were incubated with fresh MEM containing 0.1% BSA and varying concentrations of IFN for overnight (pretreatment). The IFN-treated cells were then infected with virus at an indicated multiplicity of infection (MOI). The infected cells were further incubated at
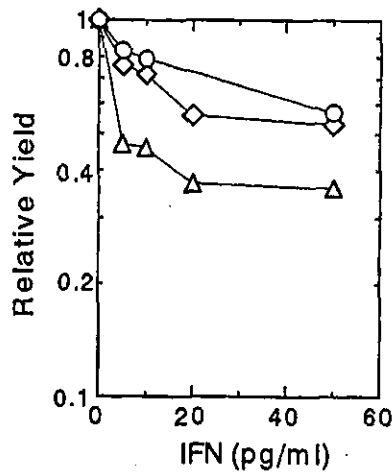
Figure 1. Effect of IFN on the yield of progeny virus. Subconfluent mono-layers of HEp-2 cells in 35 mm-dish were incubated in MEM containing 0.1% BSA and the indicated concentrations of IFN for overnight (16 h). The IFN-treated cells were infected with wt virus (○), Us3-deletion mutant R7041 (△) or the repaired virus R7306 (◇) at an MOI of 25. The infected cells were incubated at 37°C in the fresh medium with the same constituents as that for the pretreatment. At 26 h p.i., the infected cells along with the culture medium were harvested and the amount of progeny virus was determined.

37°C for the indicated period in the MEM containing 0.1% BSA with the addition of the same concentrations of IFN as in the pretreatment of the culture. The amounts of total progeny virus in the infected cells were determined at the indicated time after three cycles of freezing and thawing of

the infected cells along with the culture media. For the determination of the cell-free virus, the culture fluid was harvested.

Results and Discussion

HSV-1 is known to be insensitive to IFN (16). To examine the effect of the Us3 gene deletion on the sensitivity of HSV-1 to IFN, we compared the progeny virus production of HSV-1 F (parental wt virus), R7041 (the Us3-deletion mutant virus) and R7306 (the repaired virus) in the IFN-treated cells. When HEp-2 cells were pretreated with various concentrations of IFN and were infected with wt virus, R7041 or R7306, the relative yields of three viruses at 26-h post infection (h p.i.) decreased with increasing concentrations of IFN (Fig. 1). Among these viruses, the Us3-deletion mutant virus, R7041, showed the most notable decrease in the yield in the IFN-treated cells, while both wt and the repaired viruses showed moderate sensitivity to the IFN-treatment.

In agreement with the observed notable decrease, cytopathic effect (CPE) of R7041 was suppressed by IFN even at low concentration (10 pg IFN/ml), as shown in Fig. 2. Because these HEp-2 cells were incubated in the serum-free medium over 38 h in this experiment, a significant amount of mono-layered cells rounded up without virus-infection (Fig. 2A) as a result of non-specific degeneration. The treatment with IFN did not affect this non-specific CPE (Fig. 2E). The infection with wt virus suppressed the observed non-specific CPE in HEp-2 cells (Fig. 2B). Because wt virus induced only a marginal CPE at 16 h p.i., it was not possible to determine
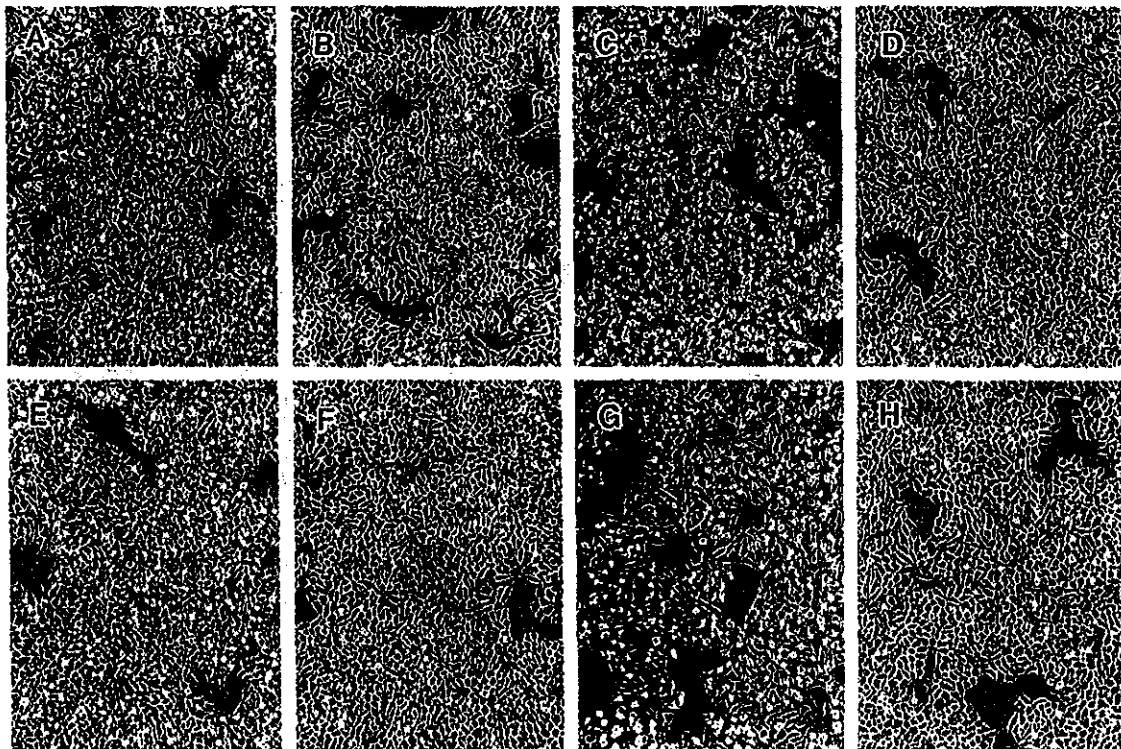

Figure 2. Cytopathic effect of the viruses in the presence of IFN. Subconfluent monolayers of HEp-2 cells in 35 mm-dish were incubated for overnight (22 h) in the MEM containing 0.1% BSA and mock (A-D) or 10 pg/ml of IFN (E-H). The treated cells were mock-infected (A and E) or infected with wt virus (B and F), Us3-deletion mutant R7041 (C and G) or the repaired virus R7306 (D and H) at an MOI of 17 and were incubated in the fresh medium with the same constituents as that for the pretreatment. At 16 h p.i., the infected cells were observed under phase-contrast microscope.
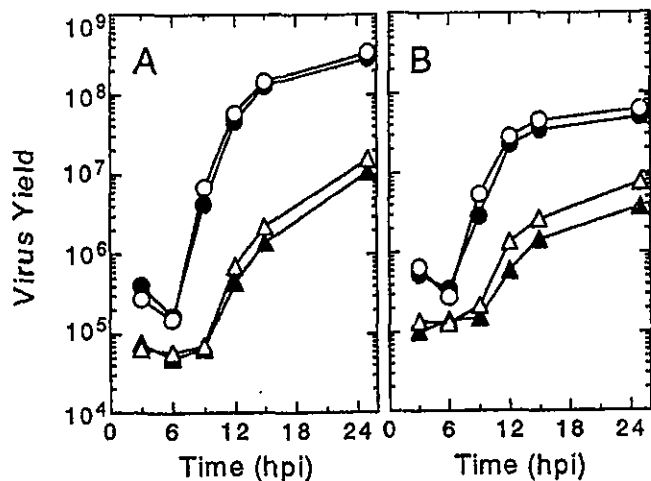
Figure 3. One-step growth curves of the viruses in the presence of IFN. Subconfluent monolayers of HEp-2 cells in 35 mm-dish were incubated in MEM containing 0.1% BSA and mock (o, Δ) or 10 pg/ml of IFN (●, ▲) for 11 h. The treated cells were infected with wt virus (A) or Us3-deletion mutant R7041 (B) at an MOI of 17 and were incubated in the fresh medium with the same constituents as that for the pretreatment. At the indicated time after infection, the amounts of the total progeny viruses (o, ●) and those of the cell-free viruses (Δ, ▲) were determined.

whether the CPE by the parental virus can be suppressed by this IFN-treatment (Fig. 2F). In contrast, the Us3-deficient mutant virus was apoptogenic and induced massive CPE even at the middle stage of virus multiplication, in agreement with our previous observation (9). At 16 h p.i., the mutant virus-infected cells showed severe shrinkage and cell-rounding (Fig. 2C), which was notably suppressed by the treatment

with IFN (Fig. 2G). The cells infected with the repaired virus showed the similar morphology with those infected with wt virus (Fig. 2D and H).

Fig. 3 shows one-step growth curves of wt virus and its Us3-deletion mutant, R7041, in HEp-2 cells in the presence or absence of the IFN-treatment (10 pg IFN/ml). When the cells were infected with either ·rus, the progeny virus appeared in the infected cells at 6 h p.i., increased with time and reached a plateau at approximately 18 h p.i. Cell-free virus appeared in the medium at 10 h p.i. and apparently kept increasing even at 25 h p.i. The IFN-treatment affected little both the multiplication and the release of the parental wt virus (Fig. 3A). Under these conditions, the final yield of the total progeny and the cell-free virus in the IFN-treated cells were 85% and 69% of those in the untreated cells, respectively. As to the Us3-deletion mutant virus (Fig. 3B), the IFN-treatment again affected little the virus multiplication, but significantly affected the release of cell-free virus. The final yield of the total progeny and the cell-free virus in the treated cells were 62% and 49% of those in the untreated cells, respectively. In the experiments described in Figs. 1, 2 and 3, the MOI used were 25, 17 and 17, respectively. Even at such artificially high MOI, all the results in these experiments agreed with the conclusion that R7041, the Us3-deletion mutant virus, is significantly more sensitive to IFN than the parental virus.

Considering that IFN plays an important role in early stages of virus infection in vivo and that the virus concentration at these stages must be extremely low, we examined the effects of IFN on the virus infection at low MOI. First, we characterized the formation of plaques of these viruses in Vero cells in the presence of various concentrations of IFN (Fig. 4). Both the parental and repaired viruses showed large clear plaques with
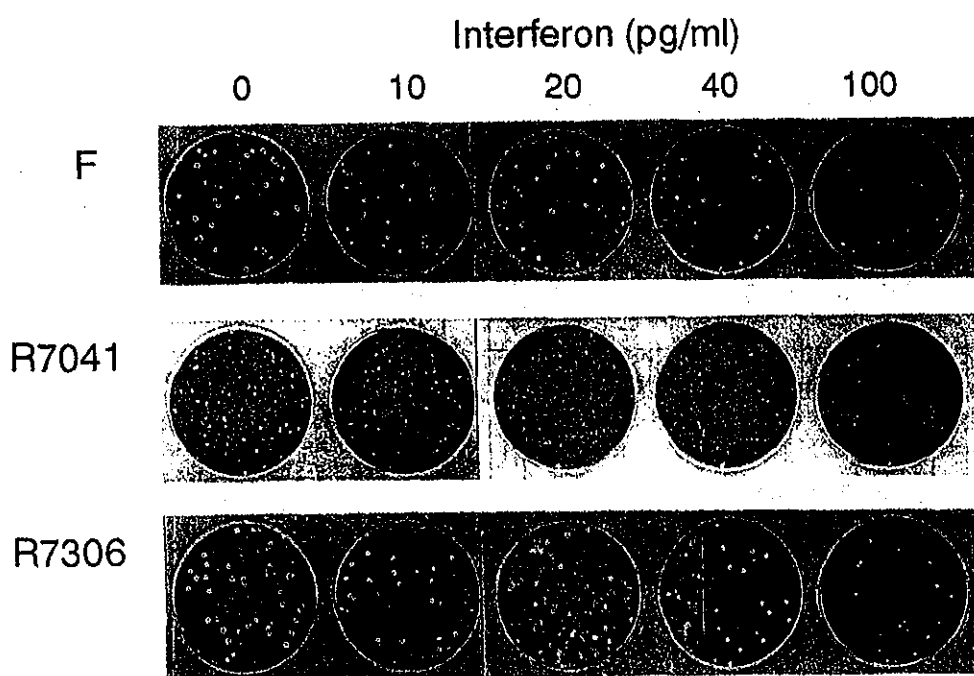

Figure 4. Plaque morphology of the viruses in the presence of various concentrations of IFN. Subconfluent monolayers of Vero cells were incubated in MEM containing 0.1% BSA and the indicated concentrations of IFN for overnight (18 h). The treated monolayers were allowed to be adsorbed with an aliquot of virus preparations of wt virus, Us3-deletion mutant R7041 or the repaired virus R7306. The infected cell monolayers were incubated in the medium containing 0.5% FCS, 0.6% methylcellulose and the indicated concentrations of IFN. After 3 days of incubation, the cell monolayers were fixed and stained with 10% formaline solution containing 0.5% crystal violet.
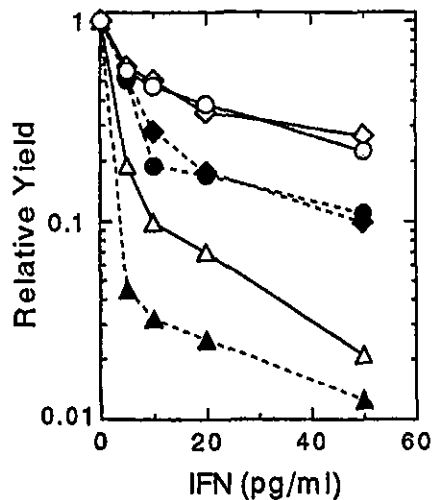
Figure 5. Effect of IFN on the yield of progeny virus at low MOI. Sub-confluent monolayers of HEp-2 cells in 35 mm-dish were incubated in MEM containing 0.1% BSA and the indicated concentrations of IFN for 16 h. The IFN-treated cells were infected with wt virus (o, ●), Us3-deletion mutant R7041 (Δ, ▲) or the repaired virus R7306 (◊, ◆) at an MOI of 0.09 and were incubated in the fresh medium with the same constituents as that for the pretreatment. At 48 h p.i., the amounts of the total progeny viruses (o, Δ, ◊) and those of the cell-free viruses (●, ▲, ◆) were determined.

the size decreasing at 40 pg IFN/ml. In contrast, R7041, the Us3-deficient mutant virus, formed much smaller plaques even in the untreated Vero cells with the size decreasing even at 10 pg IFN/ml. The number of plaques (plating efficiency) of the mutant virus was also decreased more remarkably in the presence of IFN than wt virus. For example, in the presence of 20 pg of IFN/ml, the decrease in the plaque number of wt virus was approximately 20% while that of the mutant virus was about 45%. These results confirmed the enhanced IFN-sensitivity of the mutant virus. The results in Fig. 4 also indicate that the observed enhancement is not cell-type specific, because the enhanced sensitivity of R7041 was also observed in HEp-2 cells (Figs. 1-3). Second, as shown in Fig. 5, we examined the effect of IFN on the relative virus yields after the infection with low virus concentration (MOI = 0.09). When the infected cells were incubated in the presence of various concentrations of IFN, all the viruses showed enhanced sensitivity to IFN at this MOI; the slope of the decrease in yield of total progeny virus in Fig. 5 was greater than the slope at high MOI (Fig. 1). In addition, R7041 showed marked decrease in the virus yield with increasing concentrations of IFN, clearly demonstrating the enhanced IFN-sensitivity of the Us3-deletion mutant virus. Fig. 5 also shows the effect of IFN on the amount of cell-free virus. The release of cell-free virus was more sensitive to IFN than the production of total progeny virus. R7041 again showed more extensive decrease by IFN than wt or the repaired virus, supporting the notion that sensitivity of Us3-deficient virus to IFN is enhanced.

The key finding of the present study is that the Us3-deletion mutant of HSV-1 shows the enhanced sensitivity to IFN at any level of MOI: even by the infection at high MOI, the progeny virus yields of the mutant virus decreased with increasing concentrations of IFN. The enhanced sensitivity was readily and clearly demonstrated by the infection at

low MOI, at which the conditions are more similar to that in nature. The repaired virus, R7306, always showed the similar IFN-sensitivity to the parental virus, HSV-1 F, confirming that the deletion of Us3 gene is responsible for the observed enhancement of R7041 virus in the sensitivity to IFN.

The results described here indicate that the lack of viral Us3 protein kinase makes HSV-1 susceptible to IFN. Although it has not yet been clear how HSV-1 overcomes antiviral action of IFN, the virus is considered to possess multiple pathways which interrupt IFN-signaling pathway, because many viral gene products, such as ICP0, UL13, UL41 and so on (19-23), are known to be involved in the IFN-sensitivity of HSV. In addition, there is accumulating evidence that various viral proteins which can interfere with cellular factors involved in IFN-signaling pathway (for review see refs. 13,24). Present study raises the possibility that the Us3 protein kinase interacts some steps in this signaling pathway directly or indirectly. Further characterization of the substrates of Us3 kinase will reveal the mechanism underlying the enhanced sensitivity of the Us3-deficient mutant virus to IFN.

Acknowledgements

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Determination of HIV-1 infectivity by lymphocytic cell lines with integrated luciferase gene

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Abstract. We have established lymphocytic cell lines H9 and M8166 that contain integrated copy of luciferase gene under the control of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). While H9 is known to be non-permissive for or insensitive to some particular mutant strains of HIV/simian immunodeficiency virus (SIV), M8166 is one of the most susceptible lines to various HIV/SIVs. The luciferase gene driven by HIV-1 LTR was transfected into H9 and M8166 cells with the *neo* gene, and cell lines were selected by G418. The indicator cell lines thus obtained were designated H9/H1*luc* and M8166/H1*luc*, and monitored for their susceptibility to various HIV clones including *in vitro*-constructed mutants. Both cell lines, particularly M8166/H1*luc*, were found to be exquisitely sensitive to HIV-1 and HIV-2. Furthermore, they exhibited the response to infections by various viral clones exactly as expected from the characteristics of the original cell lines. These results indicated that our new indicator cell lines H9/H1*luc* and M8166/H1*luc* are eminently useful for a variety of molecular virological studies on HIV/SIV.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a member of lentiviral subfamily of retroviruses. It grows slowly and slightly, and sometimes persists in tissue culture system. Standard assays for quantitative titers of HIV-1 are thus time-consuming and difficult to determine (1-3). Because quantitative monitoring of viral infectivity is essential for the biological and molecular biological study of HIV-1, a number of new systems using reporter gene have been established quite recently (4-10). The marker gene used in these systems were those for chloramphenicol acetyltransferase (CAT) (4-6), ß-galactosidase (ß-gal) (7,10), green fluorescent protein (GFP) (8), and luciferase (9,10). The parental cell lines for indicator cells were those of adherent non-lymphoid cells (6,7,10) and of lymphocytic or monocytic cell lineage (4,5,8,9).

Although various reporter systems described above have been successfully used and contributed much to the progress of HIV-1 research, they appear to have their own weak points with respect to the tedious performance, relatively low sensitivity (4-6) and non-primary lymphoid nature of indicator cells (6-10). In this study, to improve these issues, we have established two indicator lymphocytic cell lines for HIV-1 replication using luciferase gene as reporter. One line was derived from H9 and another from M8166. It is known that H9 is non-permissive for or insensitive to some variants of HIV/simian immunodeficiency virus (SIV) like primary lymphocytes and monocyte-derived macrophages, and that M8166 is one of the most susceptible cell lines to various HIV/SIVs (11,12). We demonstrate here that the two new indicator cell lines are useful for monitoring the replication of various wild-type (wt) and mutant clones of HIV-1 and HIV-2.

Materials and methods

Cells. A monolayer cell line 293T (13) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described (14). Lymphocytic cell lines H9 (15) and M8166 (16) were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum as previously described (14). Stable indicator cell lines of H9 and M8166 were selected by cultivation with G418 (1.2 mg/ml) for a few weeks.

Transfection. The 293T and H9 cells were transfected by the calcium-phosphate co-precipitation and electroporation methods, respectively, as previously reported (14). M8166 cells were transfected by the Nucleofector™ system (Amaxa Inc., Gaithersburg, MD, USA).

Infection. Indicator cells containing reporter luciferase gene were infected with cell-free viruses prepared from transfected 293T cells as previously described (14).

Reverse transcriptase (RT) and luciferase assays. RT assay using ^{32}P-dTTP has been previously described (17). Luciferase activity was determined by the Luciferase Assay System (Promega Corp., Madison, WI, USA).

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Table I. Indicator cell lines for HIV-1 infectivity.

Indicator cell lines	Parental cell lines	Reporter genes	References
BF24	THP-1	CAT	(4)
CEM-GFP	CEM	GFP	(8)
H9/H1*luc*	H9	Luciferase	This study
H938	H9	CAT	(5)
HLCD4-CAT	HeLa T4	CAT	(6)
JC53-BL	HeLa T4	Luciferase, ß-gal	(10)
LuSIV	CEMx174	Luciferase	(9)
MAGI	HeLa T4	ß-gal	(7)
M311	Molt-4	CAT	(4)
M8166/H1*luc*	M8166	Luciferase	This study
U38	U-937	CAT	(5)

DNA constructs. Infectious DNA clones of HIV-1 and HIV-2 designated pNL432 (14) and pGL-AN (18,19), respectively, have been previously described. Mutants of pNL432 designated pNL-Nd (*vif* frame-shift mutant) (20), pNL-Ss (*vpu* mutant) (21), pNL-Xh (*nef* mutant) (22), and pNL-SsXh (*vpu-nef* double mutant) (22) have also been described. Expression vector of the *neo* gene designated pRVSVneo has been reported previously (23). Reporter DNA clone carrying HIV-1 long terminal repeat (LTR) and luciferase designated pH1*luc* was constructed by blunt-end ligation of the *Pst*I fragment of pH1-CAT (24,25) containing HIV-1 LTR and the *Hind*III-*Sal*I fragment of pGL3-Basic Vector (Promega Corp., Madison, WI, USA) containing *luc* gene. Reporter DNA clone carrying HIV-1 LTR, luciferase gene, and *neo* gene designated pH1*luc-neo* was constructed by joining the *Nde*I-*Xba*I fragment of pH1*luc* containing HIV-1 LTR and *luc* gene, and the *Nde*I-*Xba*I fragment of pRVSVneo containing *neo* gene.

Results and Discussion

HIV infectivity can be determined by plaque assay (1), cytotoxicity assay (2), and focal immunoassay (3), which rely on virus spread or production of viral structural proteins for detection. However, now, a method of titrating HIV based on activation of marker gene driven by viral LTR is widely-used (Table I) mainly because it is easy, rapid, sensitive, quantitative, and reproducible. We initiated the present study to obtain new indicator cell lines that are potentially more useful for characterization of a variety of variants and mutants of HIV than those previously reported (Table I).

In order to develop cell lines that accommodate our purpose, we first constructed reporter DNA clones designated pH1*luc* and pH1*luc-neo* as described in Materials and methods. Upon co-transfection with pNL432 into 293T cells, these clones were activated to 15- to 30-fold as judged by the expression of luciferase in cells (data not shown). Indicator H9 cell lines were selected by co-transfection of pH1*luc* and pRVSVneo (approximately 10:1 molar ratio) followed by G418 selection. Of 11 clones obtained, the best cell line was chosen by its response to the NL432 virus and designated H9/H1*luc* (data not shown). Because we failed to obtain any indicator M8166 cell lines using this strategy, transfection of pH1*luc-neo* by the Nucleofector system was carried out. Linearized pH1*luc-neo* was transfected into M8166 cells, and cells were cultured in the presence of G418. A single cell line (M8166/H1*luc*) was obtained with this method, and found to respond well to the NL432 virus (data not shown).

We next determined the kinetics of luciferase production in H9/H1*luc* and M8166/H1*luc* cell lines after infection with HIV-1 NL432 virus. A cell-free virus sample was prepared from 293T cells transfected with pNL432, and inoculated into the two indicator cell lines. As shown in Fig. 1, within 48 h following virus infection, luciferase activity was readily detected and reached a high level in both indicator cell lines. It was noted that M8166/H1*luc* is much more susceptible to
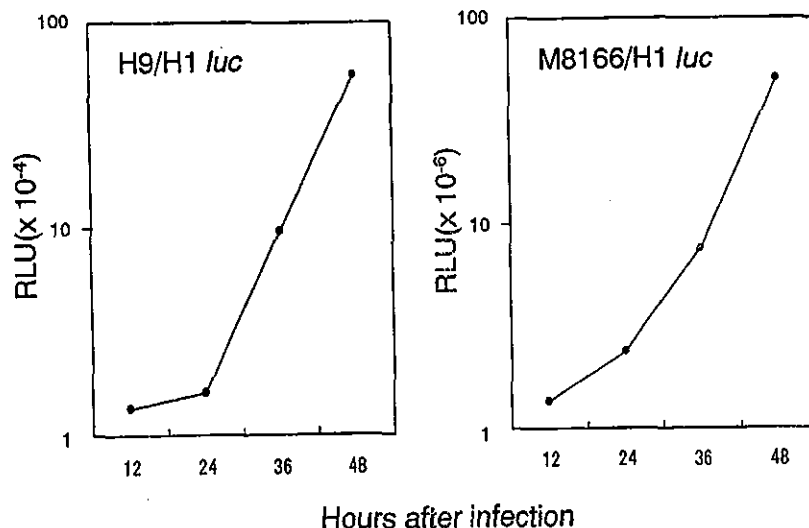

Figure 1. Kinetics of luciferase production in indicator H9/H1*luc* and M8166/H1*luc* cells. A cell-free virus sample was prepared from 293T cells transfected with pNL432 (HIV-1 wt), and inoculated into H9/H1*luc* and M8166/H1*luc* cells. Cell lysates were prepared at the indicated intervals, and monitored for luciferase activity. Level of luciferase production was calculated by subtraction of that by a negative control (mock-infected cells). Representative results are shown here. RLU, relative light unit.
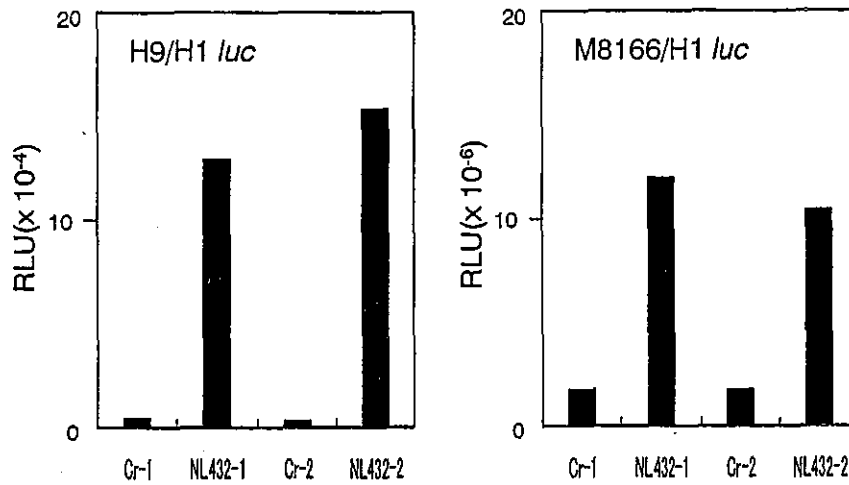
Figure 2. Stability of luciferase expression in indicator H9/H1*luc* and M8166/H1*luc* cells in response to HIV-1 infection. Cell-free virus sample was prepared from 293T cells transfected with pNL432 (HIV-1 wt), and inoculated into H9/H1*luc* and M8166/H1*luc* cells. Cell lysates were prepared at 48 h post-infection, and monitored for luciferase activity. Target indicator cells used for results 1 and 2 were those of early and late passages (3 months after the early passage). Representative results are shown here. RLU, relative light unit.
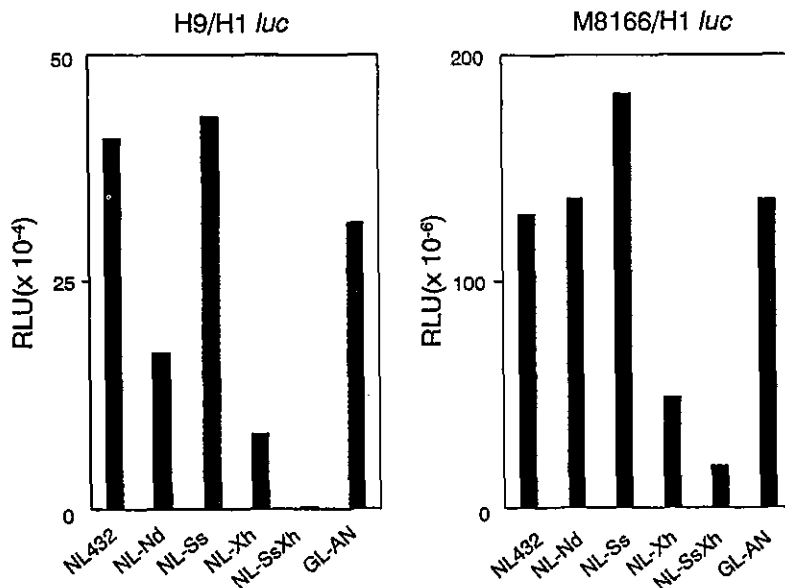

Figure 3. Susceptibility of indicator H9/H1*luc* and M8166/H1*luc* cells to various HIV clones. Cell-free virus samples were prepared from 293T cells transfected with various proviral clones, and inoculated into H9/H1*luc* and M8166/H1*luc* cells. Cell lysates were prepared at 48 h post-infection, and monitored for luciferase activity. The level of luciferase production was calculated by subtraction of that by a negative control (mock-infected cells). Representative results are shown here. Proviral clones used here: NL432, HIV-1 wt; NL-Nd, HIV-1 ΔVif mutant; NL-Ss, HIV-1 ΔVpu mutant; NL-Xh, HIV-1 ΔNef mutant; NL-SsXh, HIV-1 ΔVpu/ΔNef double mutant; GL-AN, HIV-2 wt. RLU, relative light unit.

the virus than H9/H1*luc*. This observation was in good agreement with those previously reported for parental cell lines H9 and M8166 (16,18,20,22). We were interested in ascertaining the stability of H9/H1*luc* and M8166/H1*luc* cells with respect to the luciferase production in response to infection with the NL432 virus. A cell-free virus sample was prepared from transfected 293T cells as above, and inoculated into the indicator cell lines of different passage level. As shown in Fig. 2, both indicator cell lines consistently generated a high level of luciferase upon infection with the NL432 virus.

Finally, we monitored the luciferase production in H9/H1*luc* and M8166/H1*luc* cells after infection with NL432 virus, its accessory gene (26) mutants, or HIV-2 GL-AN

virus. The mutants used here were well-characterized for their virological properties (20-22,27). The HIV-1 ΔVif mutant produced in permissive cells such as 293T exhibited a normal infectivity for non-permissive cells such as H9 in a single round replication cycle, but did not grow thereafter in the cells (20). The HIV-1 ΔVpu mutant replicated normally in a single round of infection before the stage of virion release from cells (21). The HIV-1 ΔNef mutant was quite defective in a single round infectivity assay like MAGI assay (22,27). The HIV-1 ΔVpu/ΔNef double mutant showed a severe growth retardation in many cell lines (22). We also showed that M8166 cells are much more susceptible to GL-AN virus than H9 cells (18,28). Cell-free virus samples were prepared from

293T cells transfected with various proviral clones, and inoculated into H9/H1*luc* and M8166/H1*luc* cells. As shown in Fig. 3, the results obtained were those expected. Much more luciferase was produced in M8166/H1*luc* cells than in H9/H1*luc* cells upon infection with any of the viral clones tested. Furthermore, the pattern of luciferase production by various HIV-1 mutants was consistent with the observation described previously. Of particular note, the ΔVif mutant was found to be defective in H9/H1*luc* cells, but not in M8166/H1*luc* cells, which is in good agreement with the results previously reported (20,26).

In this report, we present a method of determining HIV titers based on activation of integrated LTR-luciferase gene in two lymphocytic cell lines with distinct characteristics. The cell lines were highly sensitive to various HIVs, and this property has been maintained even after many passages (Figs. 1-3). We demonstrate here that H9/H1*luc* and M8166/H1*luc* cells are particularly useful for characterizing various HIV mutants and variants (Fig. 3). By using these indicator cell lines, we are able to systematically analyze numbers of HIV clones in a reliable and rapid manner.

Acknowledgements

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