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Ⅱ．研究成果の刊行に関する一覧表

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Ⅲ. 研究成果の刊行物・別刷

Inhibiting lentiviral replication by HEXIM1, a cellular negative regulator of the CDK9/cyclin T complex

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Objective: Tat-dependent transcriptional elongation is crucial for the replication of HIV-1 and depends on positive transcription elongation factor b complex (P-TEFb), composed of cyclin dependent kinase 9 (CDK9) and cyclin T. Hexamethylene bisacetamide-induced protein 1 (HEXIM1) inhibits P-TEFb in cooperation with 7SK RNA, but direct evidence that this inhibition limits the replication of HIV-1 has been lacking. In the present study we examined whether the expression of FLAG-tagged HEXIM1 (HEXIM1-f) affected lentiviral replication in human T cell lines.

Methods: HEXIM1-f was introduced to five human T cell lines, relevant host for HIV-1, by murine leukemia virus vector and cells expressing HEXIM1-f were collected by fluorescence activated cell sorter. The lentiviral replication kinetics in HEXIM1-f-expressing cells was compared with that in green fluorescent protein (GFP)-expressing cells.

Results: HIV-1 and simian immunodeficiency virus replicated less efficiently in HEXIM1-f-expressing cells than in GFP-expressing cells of the five T cell lines tested. The viral revertants were not immediately selected in culture. In contrast, the replication of vaccinia virus, adenovirus, and herpes simplex virus type 1 was not limited. The quantitative PCR analyses revealed that the early phase of viral life cycle was not blocked by HEXIM1. On the other hand, *Tat*-dependent transcription in HEXIM1-f-expressing cells was substantially repressed as compared with that in GFP-expressing cells.

Conclusion: These data indicate that HEXIM1 is a host factor that negatively regulates lentiviral replication specifically. Elucidating the regulatory mechanism of HEXIM1 might lead to ways to control lentiviral replication.

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Keywords: CDK9, cyclin T, HEXIM1, lentivirus, *tat*

Introduction

Activation of transcription elongation requires the positive transcription elongation factor b complex (P-TEFb) composed of cyclin dependent kinase 9 (CDK9) and cyclin T1, T2, or K [1]. P-TEFb is essential for efficient transcriptional elongation from the promoter of human immunodeficiency virus type 1 (HIV-1), the long terminal repeat (LTR) (reviewed in [2,3]). The functional interaction between P-TEFb and the viral protein Tat has been well studied. Immediately after viral transcription starts at the LTR of the integrated proviral genome, the nascent viral transcript forms a three-dimensional structure called TAR. In the presence of P-TEFb, Tat binds to TAR. Through the Tat–TAR interaction, Tat activates P-TEFb and therefore assures the efficient

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completion of viral gene transcription and the propagation of HIV-1.

Recently, the regulatory mechanisms of P-TEFb function have been elucidated. In 2001, the interaction of P-TEFb with 7SK RNA was found to be necessary to inactivate the kinase activity of CDK9 within P-TEFb [4–6]. However, the binding of 7SK RNA alone is not sufficient to inactivate P-TEFb. More recently, Yik *et al.* demonstrated that the inactivation of P-TEFb requires hexamethylene bisacetamide-induced protein 1 (HEXIM1; synonyms CLP1, MAQ1, and HIS1) [7–9]. The inactivation of P-TEFb by the HEXIM1-7SK RNA complex appears to regulate the transcriptional elongation of cellular genes.

The HEXIM1-7SK RNA complex has been shown to physically compete with Tat for binding to P-TEFb [10]. In agreement with this finding, HEXIM1 was shown to inhibit Tat-dependent transcription from the HIV-1 LTR in transient transfection assays [8,11,12]. However, no data demonstrating that HEXIM1 is able to limit HIV-1 replication has been provided. Here we provide direct experimental evidence that the constitutive expression of HEXIM1 specifically limits lentiviral replication.

Methods

Plasmids

The FLAG-tagged HEXIM1 expression constructs were generated by reverse-transcription PCR using RNA isolated from CEM cells as templates. The primers used were 5′-CACCTCGAGCCACCATGGACTACAAAGACGATGACGACAAGGCCGAGCCATTCTTGTC-3′ and 5′-CAATTGCTAGTCTCCAAACTTGGAAAGCGGCGC-3′′ for amino terminus FLAG tagging, and 5′-CACCTCGAGCCACCATGGCCGAGCCATTCTTGTCAGAATATC-3′ and 5′-CAATTGCTAGTCGTCATCGTCTTTGTAGTCGTCTCCAAACTTGGAAAGCGGCGCTC-3′ for carboxy terminus FLAG tagging. The *Xho*I-*Mfe*I fragments of the PCR products were cloned into the *Xho*I-*Mfe*I sites of pCMMP IRES GFP, generating pCMMP f-HEXIM1 and pCMMP HEXIM1-f [13]. The cytomegalovirus (CMV) promoter-driven *gag-pol* expression vector psyn*gag-pol* has been previously described by Wagner *et al.* [14] and pLTR*gag-pol* was constructed by cloning the *Mlu*I-*Hin*dIII fragment encoding the LTR from pNL-luc [15] into the *Mlu*I-*Hin*dIII sites of psyn*gag-pol*. The tax expressing plasmid pCGtax and pHTLV LTR luciferase were kindly provided by Dr. Watanabe (Tokyo Medical Institute). The *tat*-expressing plasmid pSVtat was a generous gift from Dr. Freed (National Cancer Institute-Frederick, Frederick, Maryland, USA). The plasmid pLTR-luc has been described previously (Miyauchi *et al.*, *Antiviral Chemistry and Chemotherapy*, in press). The following plasmids have been described previously by Komano *et al.* [13]: pVSV-G, pMD*gag-pol*, pTM3Luci, phRL-CMV and pSIVmac239ΔnefLuc.

Cells and transfection

All the mammalian cells were maintained in RPMI 1640 (Sigma, St Louis, Missouri, USA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), penicillin and streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

Western blotting

Cells were lysed with sample buffer, sonicated, and boiled for 5 min. Samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA) for western blotting according to standard techniques. Membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) containing 5% (w/v) non-fat skim milk (Yuki-Jirushi, Tokyo, Japan) for 1 h at room temperature and incubated with primary antibodies including the M2 anti-FLAG epitope monoclonal antibody (Sigma), an anti-actin monoclonal antibody (MAB1501R; Chemicon/Millipore, Billerica, Massachusetts, USA), an anti-cyclin T1 rabbit polyclonal antibody (H-245; Santa Cruz Biotechnology, Santa Cruz, California, USA), an anti-cyclin T2a/b goat polyclonal antibody (A-20; Santa Cruz), an anti-p24 monoclonal antibody (183-H12-5C; NIH AIDS Research and Reference Reagent Program), an anti-HIS1 chicken polyclonal antibody (N-150; GenWay, San Diego, California, USA), and an anti-Bip/GRP78 monoclonal antibody (clone 40; BD Biosciences/Transduction Laboratories, San Jose, California, USA) for 1 h at room temperature. Membranes were washed with TBS-T and incubated with appropriate second antibodies including biotinylated anti-goat (GE Healthcare Bio-Sciences, Piscataway, New Jersey, USA) or anti-chicken IgY (Promega, Madison, Wisconsin, USA), and EnVision+ (Dako, Glostrup, Denmark) for 1 h at room temperature. For a tertiary probe, we used horseradish peroxidase (HRP)-streptavidine (GE Healthcare) if necessary. Signals were visualized with an LAS3000 imager (Fujifilm, Tokyo, Japan) after treating the membranes with the Lumi-Light Western Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Reporter assay

Luciferase activity was measured 48 h after transfection or infection using a DualGlo assay kit (Promega) according to the manufacturer's protocol. The beta-galactosidase activity was measured using a LumiGal assay kit (BD Biosciences/Clontech, San Jose, California, USA) according to the manufacturer's protocol. The

chemiluminescence was detected with a Veritas luminometer (Promega).

Monitoring viral replication

To monitor HIV-1 replication, the culture supernantants were subjected to either a reverse transcriptase assay [16] or an enzyme-linked immunosorbent assay (ELISA) to detect p24 antigens using a Retro TEK p24 antigen ELISA kit according to the manufacturer's protocol (Zepto Metrix, Buffalo, New York, USA). For simian immunodeficiency virus (SIV) a p27 antigen ELISA kit was used according to the manufacturer's protocol (Zepto Metrix). The signals were measured with a Multiskan Ex microplate photometer' (ThermoLabsystems, Helsinki, Finland). For vaccinia virus, adenovirus, and herpes simplex virus (HSV)-1, the activity of reporter genes was measured as previously described [13].

Generating viruses

To produce HIV-1 and SIV, 293T cells were transfected with plasmids encoding proviral DNA of HIV-1 (pHXB2) or pSIVmac239ΔnefLuc and culture supernatants containing viruses were collected at 48 h post-transfection. Murine leukemia virus (MLV) and lentiviral vectors pseudotyped with VSV-G were produced as described previously by cotransfecting 293T cells with either the pNL-Luc and pVSV-G vectors or the pMD*gag-pol*, pVSV-G, and pCMMP vectors [13]. Green fluorescent cells were sorted by fluorescence activated cell sorter (FACS) Aria (Becton Dickinson, San Jose, California, USA).

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated with an RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. The reverse transcriptase (RT)-polymerase chain reaction (PCR) assay was performed with a One Step RNA PCR Kit (Takara, Otsu, Japan), imaged by a Typhoon scanner 9400 (GE Healthcare), and quantified with Image Quant software (GE Healthcare). For the amplification of endogenous HEXIM1, the forward primer 5′-ACCACACGGAGAGCCTGCA-GAAC-3′ and the reverse primer 5′-TAGCTAAA-TTTACGAAACCAAAGCC-3′ were used. For the amplification of HEXIM1-f, the forward primer 5′-GTACCTGGAACTGGAGAAGTGCCC-3′ and the reverse primer 5′-CAATTGCTAGTCGTCATCGTC-TTTGTAGTC-3′ were used. For cyclophilin A, the forward primer 5′-CACCGCCACCATGGTCAAC-CCCACCGTGTTCTTCGAC-3′ and the reverse primer 5′-CCCGGGCCTCGAGCTTTCGAGTTGT-CCACAGTCAGCAATGG-3′ were used.

Quantitative real time polymerase chain reaction

The real time PCR reaction was performed in a DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad, Hercules, California, USA). The cellular genomic DNA and total RNA were extracted 48 h post-infection with a DNeasy kit (Qiagen) and RNeasy kit (Qiagen), respectively, according to the manufacturer's instruction. For the reagents, we used QuantiTect SYBR Green PCR and RT-PCR Kits (Qiagen). To estimate the amount of integrated HIV-1 DNA, Alu-LTR PCR was performed according to the method described previously using the following primers: for the first PCR, 5′-AACTAGGGAACCCACTGCT-TAAG-3′ and 5′-TGCTGGGATTACAGGCGTGAG-3′, and for the second PCR, 5′-AACTAGGGAACC-CACTGCTTAAG-3′ and 5′-CTGCTAGAGATTT-TCCACACTGAC-3′ [17]. The beta-globin primers have been described previously [18]. To estimate the amount of HIV-1 RNA, the second PCR primers for the Alu-LTR PCR were used. The primers for cyclophilin A are described above.

Results and discussion

The HEXIM1 cDNA tagged with a FLAG epitope at either the amino terminus (f-HEXIM1) or the carboxy terminus (HEXIM1-f) was cloned in a mammalian expression plasmid (Fig. 1a). A luciferase assay revealed that the Tat-dependent enhancement of transcription from the HIV-1 LTR was reduced by co-transfecting HEXIM1-expressing plasmids, whereas neither Tat-independent basal transcription from the HIV-1 LTR nor CMV promoter-driven transcription was affected (Fig. 1b). An oncogenic retrovirus human T cell leukemia virus type 1 (HTLV-1) encodes for *tax*, a functional homologue of HIV-1's *tat*, that utilizes P-TEFb to enhance transcription from the LTR promoter [19]. However, *tax*-dependent enhancement of transcription was not affected by HEXIM1 in similar experimental conditions (Fig. 1c). To monitor the effect of HEXIM1 on HIV-1 replication, we introduced HEXIM1-expressing plasmids into HeLa-CD4 cells along with pNL4-3, which produces replication-competent HIV-1, and measured the RT activity in the culture supernatant 1 week post-transfection. Transfecting HEXIM1-expressing plasmids decreased the RT activity in a dose-dependent manner (Fig. 1d). Next, we asked whether the inhibition of viral replication was specific to HIV-1 by examining vaccinia virus, adenovirus, and HSV-1 replication. We found that the propagation of these three viruses was not inhibited by HEXIM1-f expression (Fig. 1e–g), suggesting that the inhibition of viral replication by HEXIM1 was HIV-1-specific.

To examine whether HEXIM1 negatively affects lentiviral replication in the physiologically relevant host, we isolated human T cell lines constitutively expressing HEXIM1-f. We cloned HEXIM1-f cDNA into a pCMMP (MLV retroviral vector plasmid (Fig. 2a). The plasmid encoded an internal ribosomal entry site (IRES)-mediated green fluorescent protein (GFP) expression cassette, so that MLV vector-infected cells

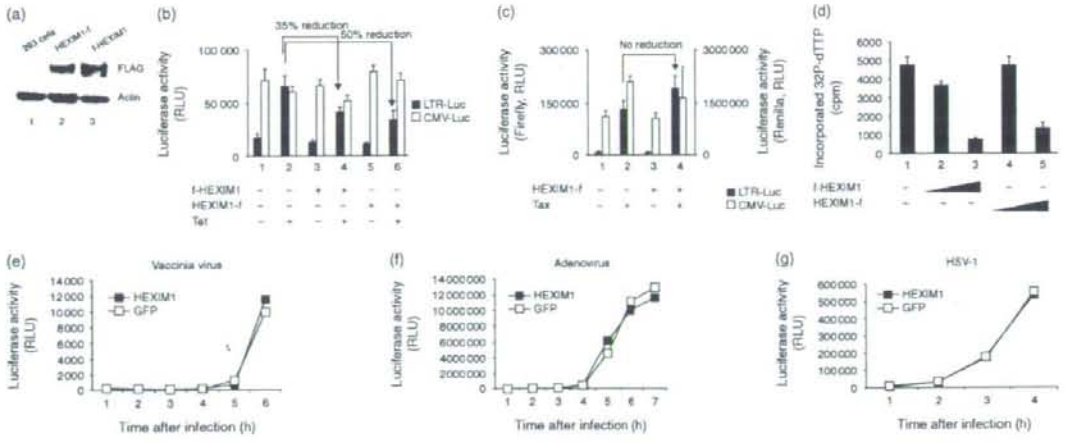

Fig. 1. Expression of hexamethylene bisacetamide-induced protein 1 (HEXIM1) specifically inhibits HIV-1 replication. (a) Detection of HEXIM1 cDNA tagged with a FLAG epitope at either the amino terminus (f-HEXIM1) or the carboxy terminus (HEXIM1-f) by western blot analysis in transiently transfected 293 cells (upper panel, approximately 65 kD). A western blot against actin is shown as a loading control (lower panel). (b) Expressing FLAG-tagged HEXIM1 decreased the luciferase activity driven by HIV-1 long terminal repeat (LTR) promoter in the presence of Tat (lanes 4 and 6, LTR-Luc, solid bars). However, FLAG-tagged HEXIM1 did not affect the expression of renilla luciferase from co-transfected plasmid driven by the cytomegalovirus (CMV) promoter (CMV-Luc, open bars). Representative data from three independent experiments done in triplicate are shown. Cells were transfected with 0.8 μg HEXIM1-expressing plasmid for the indicated lanes, 0.1 μg of pSV*tat* for the indicated lanes, and 0.1 μg of pLTR-Luc and 0.5 μg for phRL/CMV for all lanes. (c) Expressing FLAG-tagged HEXIM1 did not decrease the luciferase activity driven by HTLV-1 LTR promoter in the presence of Tax (lanes 2 and 4, LTR-Luc, solid bars) as well as renilla luciferase driven by the CMV promoter (CMV-Luc, open bars). Representative data from three independent experiments done in triplicate are shown. Cells were transfected with 0.8 μg of HEXIM1-expressing plasmid for the indicated lanes, 0.1 μg of pCGtax for the indicated lanes, and 0.1 μg of pHTLV LTR Luc and 0.5 μg for phRL/CMV for all lanes. (d) The dose-dependent reduction of HIV-1 production by transfection of HEXIM1-encoding plasmids (0.1 μg for lanes 2 and 4, 0.4 μg for lanes 3 and 5) along with a plasmid producing infectious HIV-1 (pNL4-3, 0.1 μg) in HeLa-CD4 cells. (e–g) Expressing HEXIM1-f did not limit the replication of vaccinia virus (e), adenovirus (f), or HSV-1 (g) in 293T cells. The *y*-axis represents the reporter gene activity, which reflects viral replication. Representative data from three independent experiments are shown. GFP, green fluorescent protein; RLU, relative light unit.

could be readily identified by the green fluorescence. Human T cell lines, including SUP-T1, MOLT-4, CEM, Jurkat, and M8166 were infected with MLV pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), and GFP-positive cells were collected with a FACS (Fig. 2a). For the negative control, we used MLV expressing GFP only. The successful introduction of HEXIM1-f into the cells was verified by RT-PCR and Western blot analysis (Fig. 2b and c). The total HEXIM1 protein expression in HEXIM1-f-transduced cells was approximately 3.7-, 1.5-, 2.0-, 4.8-, and 1.8-fold higher than in GFP-transduced cells in the CEM, Jurkat, MOLT-4, SUP-T1, and M8166 cell lines, respectively (Fig. 2c). To our surprise, the HEXIM1-f-expressing T cell lines remained GFP-positive, and therefore HEXIM1-f-positive, for more than 6 months and proliferated at rates almost indistinguishable from GFP-expressing cells. The expression levels of cyclin T1, cyclin T2, actin, and Bip/GRK78 in HEXIM1-f-expressing cells were almost identical to those in GFP-expressing cells, suggesting that the gene expression did not compensate the upregulated HEXIM1 (Fig. 2b and c). Expression of cyclin T2 was undetectable in M8166 cells (Fig. 2c). Similarly, HEXIM1-f expression did not affect the cell surface levels of the HIV-1 receptors CD4 and CXCR4 as demonstrated by FACS analysis (data not shown). These data indicate that the expression of HEXIM1-f did not reach levels where the physiological regulation of P-TEFb blocked cellular gene transcription.

The replication kinetics of HIV-1 or SIV was monitored by measuring the accumulation of viral capsid antigen in the culture medium. Strikingly, HIV-1 replicated more slowly in cells of all four T cell lines expressing HEXIM1-f than in cells expressing GFP (Fig. 2d–g). Similarly, HEXIM1-f-expressing M8166 cells supported SIV replication less efficiently than did GFP-expressing M8166 cells (Fig. 2h). Interestingly, the magnitude of HIV-1 replication delay was the most substantial in SUP-T1 cells, in which the levels of endogenous HEXIM1 were the lowest among the four cell lines tested for HIV-1 replication (Fig. 2c). Similar observations were made when the HIV-1 infection experiments were repeated, indicating that the expression of functional HEXIM1-f did not change over the course of the replication monitoring. We tested whether the viruses emerged in

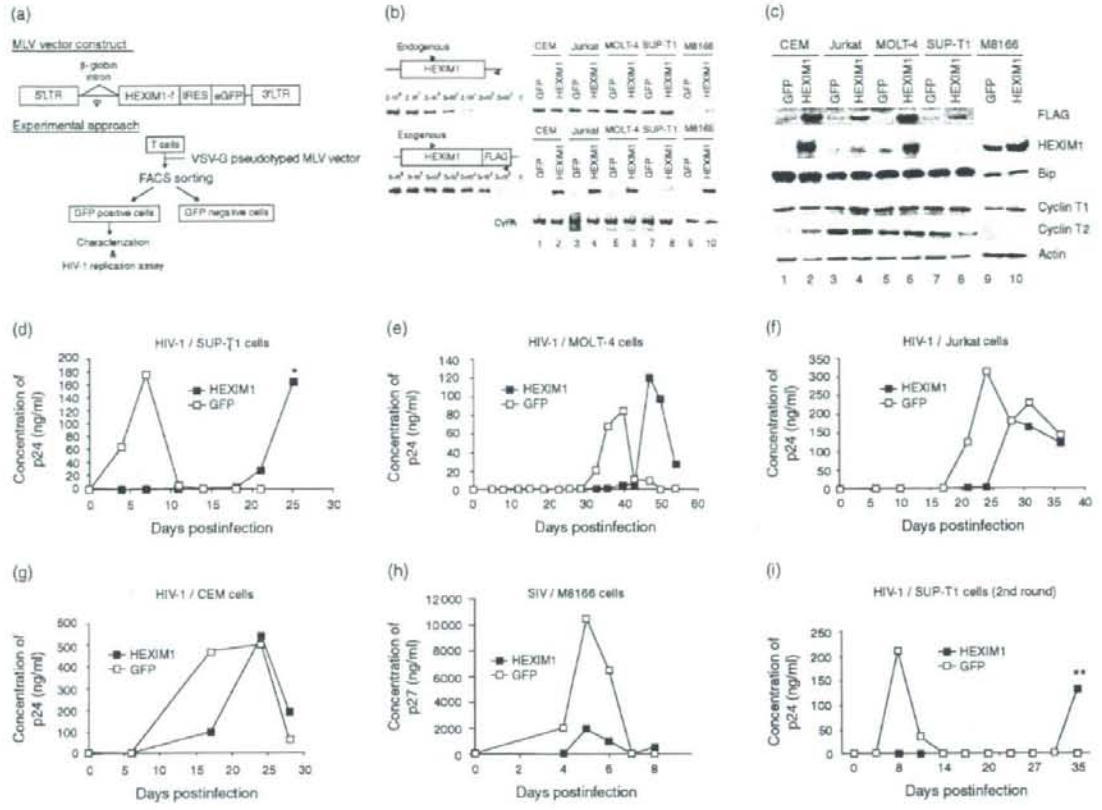

Fig. 2. Lentiviral replication is inhibited in various T cell lines constitutively expressing hexamethylene bisacetamide-induced protein 1 (HEXIM-1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f). (a) The genomic organization of the retroviral vector expressing HEXIM1-f and a schematic representation of the experimental approach. (b) Detection of endogenous HEXIM1 and murine leukemia virus (MLV)-transduced HEXIM1-f (exogenous) mRNA by reverse transcriptase-polymerase chain reaction in green fluorescent protein (GFP)- and HEXIM1-f-expressing cells. The primer design is drawn schematically. Amplification efficiency was examined by using a known number of templates as standards for HEXIM1. Cyclophilin A (CyPA) was amplified to ensure the quality of the RNA. (c) Western blot analysis demonstrating expression of HEXIM1-f (denoted FLAG), endogenous HEXIM1 (HEXIM1), Bip, cyclin T1, cyclin T2, and actin in isolated T cell lines. (d–g) Replication profiles of HIV-1 (HXB2) in SUP-T1 (d), MOLT-4 (e), Jurkat (f), and CEM (g) cells either expressing HEXIM1-f or GFP alone. Representative data from two or three independent experiments are shown. (h) Replication profile of SIV in M8166 cells either expressing HEXIM1-f or GFP alone. Representative data from two independent experiments are shown. (i) The replication profiles of HIV-1 recovered from SUP-T1/HEXIM1-f cells (asterisk in Fig. 2d) in fresh SUP-T1/GFP or SUP-T1/HEXIM1-f. LTR, long terminal repeat.

HEXIM1-f-expressing cells were 'revertants' that might be able to replicate in HEXIM1-f-expressing cells as fast as in GFP-expressing cells. To address this, we recovered virus-containing culture supernatants from SUP-T1/HEXIM1-f cells at the peak of replication kinetics (asterisk, Fig. 2d). Then, both fresh SUP-T1/GFP and SUP-T1/HEXIM1-f were infected with the recovered virus and the replication kinetics was monitored. However, HIV-1 still replicated in SUP-T1/HEXIM1-f cells more slowly than in SUP-T1/GFP cells (Fig. 2i), akin to the original profiles (Fig. 2d), and the nucleotide sequences of LTR and *tat*, the primary targets of HEXIM1, remained unchanged (double asterisk in Fig. 2i). In addition, no mutations were found in viruses propagated in GFP-expressing SUP-T1 cells. Similar observations were made in MOLT-4 cells (data not shown). These data provide direct evidence that the expression of HEXIM1 inhibits lentiviral replication in human T cell lines.

Based on our experimental observations as well as the reported functions of HEXIM1, we assumed that the ability of HEXIM1 to limit HIV-1 replication was mostly due to the inhibition of Tat/P-TEFb-dependent transcriptional elongation. However, it was possible that HEXIM1 might also have targeted other viral replication

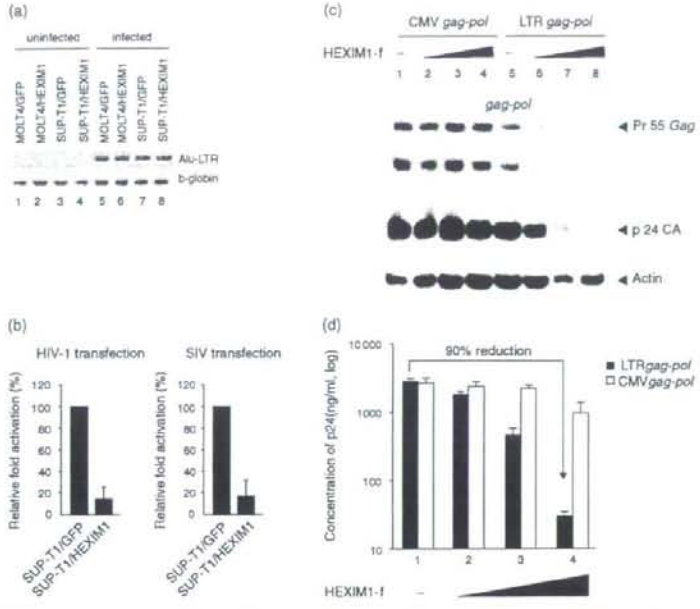

Fig. 3. Hexamethylene bisacetamide-induced protein 1 (HEXIM1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f) does not affect the efficiency of viral integration or post-translational processes. (a) The Alu-long terminal repeat (LTR) and beta-globin polymerase chain reaction products from VSV-G-pseudotyped HIV-1-infected MOLT-4 and SUP-T1 cells expressing either green fluorescent protein (GFP) or HEXIM1-f alone were separated in an agarose gel and photographed. (b) The luciferase activities in SUP-T1/GFP or SUP-T1/HEXIM1-f cells electroporated with 10 μg of a plasmid encoding LTR-driven firefly luciferase plus 1 μg of phRL/cytomegalovirus (CMV). The firefly luciferase activity normalized to renilla luciferase activity in SUP-T1/GFP cells was set to 100%. The error bars represent the standard deviation of three independent experiments. (c) Western blot analysis showing Gag and its cleaved products expressed from either CMV promoter- or LTR promoter-driven *gag-pol* expression plasmid in the presence of pSVtat (0.1 μg, all lanes) and increasing amounts of HEXIM1-f (0.2 μg for lanes 2 and 6, 0.6 μg for lanes 3 and 7, and 2.0 μg for lanes 4 and 8). (d) The amount of p24 produced in the culture supernatant from cells analyzed in Fig. 3c was measured by enzyme-linked immunosorbent assay. Representative data from three independent experiments done in triplicate are shown. SIV, simian immunodeficiency virus.

steps. To test this possibility, we examined the viral entry and production processes separately. The efficiency of viral entry was analyzed by measuring the efficiency of viral integration. SUP-T1/GFP or SUP-T1/HEXIM1-f cells were infected with a replication-incompetent HIV-1 vector pseudotyped with VSV-G that expresses luciferase upon successful infection. We conducted an Alu-LTR PCR assay to detect the integrated viral genome. PCR products were detected only from HIV-1-infected cells (Fig. 3a). The signal intensities of Alu-LTR PCR products from GFP- and HEXIM1-f-experssing cells were similar. To compare the efficiency of viral infection as well as transcription quantitatively, we employed a real time PCR technique. Some infected cells were collected for an Alu-LTR PCR assay to quantify the amount of integrated viral genome, and the rest were processed to measure the amount of viral transcript as well as the luciferase activity. The amount of Alu-LTR PCR product from SUP-T1/HEXIM1-f cells was 3.5- and 3.3-fold more to that from SUP-T1/GFP cells from two independent experiments, respectively (Table 1). These data suggest that the efficiency of viral integration was not inhibited in HEXIM1-f-expressing SUP-T1 cells. In contrast, the relative abundance of HIV-1 transcript expressed in SUP-T1/HEXIM1-f cells was substantially decreased to 0.03 and 2.9% relative to SUPT1/GFP cells (Table 1). Furthermore, the luciferase activities were 200-fold lower in SUP-T1/HEXIM1-f cells than in SUP-T1/GFP cells (Table 1). Similar data was obtained from MOLT-4 cells infected with HIV-1 pseudotyped with VSV-G (data not shown). The transfection of plasmids encoding reporter viral DNA can bypass the viral entry and make it possible to measure the effect of HEXIM1 on LTR-driven transcription and translation. Consistent with above data, transfecting pNL-Luc into SUP-T1/HEXIM1-f cells gave significantly lower luciferase activities than SUP-T1/GFP cells (Fig. 3b, left). Similar data were obtained using pSIVmac239ΔnefLuc (Fig. 3b, right). These data strengthen the possibility that HEXIM1 targets post-integration processes.

Table 1. Effect of hexamethylene bisacetamide-induced protein 1 (HEXIM1) cDNA tagged with a FLAG epitope at the carboxy terminus (HEXIM1-f) on viral entry and transcription in SUP-T1 cells examined by quantitative real time polymerase chain reaction.

		Integrated HIV-1 genome			HIV-1 transcript			Luciferase activity	
Exp.	Transduced gene	Alu-LTR (copy)	β-globin (copy)	Normalized[a] (%)	HIV-1 RNA (copy)	CyPA (copy)	Normalized[b] (%)	RLU[c]	Normalized[d] (%)
1	GFP	5.2×10^5	6.7×10^6	100.0	1.6×10^6	6.8×10^7	100.0	3.2×10^5	100.0
	HEXIM1-f	2.0×10^6	7.4×10^6	351.3	6.7×10^1	1.0×10^8	0.03	1.5×10^3	0.5
2	GFP	4.6×10^6	1.8×10^7	100.0	3.1×10^8	8.9×10^7	100.0	7.1×10^5	100.0
	HEXIM1-f	1.6×10^7	1.9×10^7	333.2	9.4×10^6	9.3×10^7	2.9	3.4×10^3	0.5

[a]The number of Alu-long terminal repeat (LTR) products divided by the number of beta-globin products in SUP-T1/GFP is set to 100%. The abundance of Alu-LTR products in SUP-T1/HEXIM1-f relative to SUP-T1/green fluorescent protein (GFP) is shown.
[b]The number of HIV-1 RNA transcripts in SUP-T1/GFP divided by the number of cyclophilin A (CyPA) transcripts is set to 100%. The abundance of HIV-1 RNA in SUP-T1/HEXIM1-f relative to SUP-T1/GFP is shown.
[c]The luciferase activity is shown by relative light unit (RLU).
[d]The luciferase activity in SUP-T1/GFP is set to 100%. The luciferase activity in SUP-T1/HEXIM1-f relative to SUP-T1/GFP is shown.

To test this further, we analyzed the efficiency of post-transcriptional processes with a transient transfection assay measuring the amount of Pr55 Gag, a viral gene product, and virus-like particles (VLPs) produced in the culture supernatants. For this purpose, we used the CMV promoter-driven *gag-pol* expression plasmid, because HEXIM1-f did not affect CMV-driven transcription (Fig. 1b). At the levels of HEXIM1-f where LTR-driven Tat-dependent transcription was drastically inhibited (Fig. 3c, lanes 7, 8), the amount of CMV promoter-driven Gag expression was almost identical to that in the absence of HEXIM1-f (Fig. 3c, lanes 1–4). Furthermore, the processing pattern of Pr55 Gag in the presence of HEXIM1-f was identical to that in its absence (Fig. 3c). These data indicate that HEXIM1-f did not inhibit the transcription from a Tat-independent promoter, the translation of viral protein, or the protease activity of HIV-1. Finally, the potential effect of HEXIM1 on viral budding was examined. To do this, the amount of p24 CA in the culture supernatant of transfected cells was quantified as a representation of the amount of VLP. Expressing HEXIM1-f reduced VLP production from cells co-transfected with pLTR*gag-pol* and pSVtat at levels comparable to the protein expression levels (Fig. 3c and d). In contrast, expressing HEXIM1-f did not reduce the amount of VLP produced by cells co-transfected with pCMV*gag-pol* and pSVtat in conditions in which Tat-dependent LTR transcription was substantially inhibited (Fig. 3c and d). Taken together, this indicates that HEXIM1-f lowers the efficiency of Tat-dependent transcription from LTR promoter but does not block the efficiency of the late phase of the viral life cycle including translation, Gag's assembly, and budding. Thus, it is likely that HEXIM1 primarily targets Tat/P-TEFb-dependent transcription to inhibit HIV-1 replication.

Our findings demonstrated that HEXIM1, a cellular P-TEFb inhibitor, is a specific negative regulator of lentiviral replication in human T cell lines. The replication of vaccinia virus, adenovirus, and HSV-1 were not affected by HEXIM1-f expression; however, the *Tat*-dependent transcription of the LTR promoter of both HIV-1 and SIV was reduced by HEXIM1-f. HEXIM1 limited replication of HIV-1 dramatically at levels where it did not visibly affect cell physiology (as little as a 5-fold increase over the endogenous levels), nor were revertants immediately selected in HEXIM1-f-expressing cells. These data support the feasibility of developing HIV-1 inhibitors targeting the processes in which HEXIM1 is involved. For example, it is conceivable to hunt for a non-toxic chemical inducer for HEXIM1 since expression of HEXIM1 is induced by hexamethylene bisacetamide (HMBA) that is considerably toxic for cells [20].

P-TEFb has been shown to support transcription of the *c-myc* and CIITA transcription factors (reviewed in [21,22]). The functions of these transactivators are critical for cell proliferation, but in this study constitutive expression of HEXIM1-f, which reduces P-TEFb activity, did not affect the cell proliferation of human T cell lines, the human epithelial cell lines HEK293 or the NP2 glioblastoma cell lines (data not shown). How can this be explained? Very recently, a high-molecular-weight bromodomain protein, Brd4, was found to function as a 'cellular *tat*' [23,24]. Interestingly, it was shown that Brd4 binds not only to cyclin T1 but also to cyclin T2, a widely expressed variant of cyclin T, to which HEXIM1 binds but Tat does not [23–25]. We hypothesize that Brd4 might be able to recruit and activate P-TEFb more efficiently than does Tat, leaving cellular transcription unaffected by the upregulated expression of HEXIM1 from the retroviral vector. An alternative possibility comes from the fact that HEXIM1 does not interact with the ubiquitously expressed cyclin K, which functions as a P-TEFb component. It is possible that Tat is not able to utilize P-TEFb consisting of CDK9 and cyclin K but Brd4 can, such that cyclin K may substitute for cyclin T1 to support Brd4-mediated cellular gene transcription.

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Genotypic Resistance Mutations to Antiretroviral Drugs in Treatment-Naive HIV/AIDS Patients Living in Liaoning Province, China: Baseline Prevalence and Subtype-Specific Difference

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ABSTRACT

To examine the prevalence of drug resistance mutations among treatment-naive HIV/AIDS patients living in Liaoning province in China, the HIV-1 *pol* gene from plasma of 91 seropositive treatment-naive patients was amplified and sequenced. Three patients (3.3%) had an M46I amino acid substitution in the protease (PR) gene that decreased susceptibility to IDV, RTV, and NFV and one patient (1.1%) had an M184I amino acid substitution in the reverse transcriptase (RT) gene that confers high-level resistance to 3TC and FTC. Minor mutations were detected in high frequency in the PR gene. The frequencies of minor mutations to protease inhibitors (PI) were I93L (71.4%), L63P (62.6%), V77I (62.6%), M36I/V (33.0%), A71T/V (22.0%), K20R (6.6%), G16E (6.6%), and L10I (5.5%). The relatedness between subtypes and the frequencies of amino acid substitutions in PR were observed; 63P, 77I, and 71V/T were found in HIV-1 subtype B′/B, 16E, 36I, 20R/I, and 82I in non-B, except for CRF07_BC, 10I in subtype A, and 93L in non-A. Although the primary resistance of HIV-1 to antiretroviral drugs is low among the treatment-naive HIV-1 patients living in Liaoning province, the surveillance and monitoring of drug-resistant HIV-1 should be implemented regularly because of the increased access to antiretroviral therapy in China.

INTRODUCTION

LIAONING PROVINCE, located in the northeast of China, has a population of 40 million. Although HIV prevalence remains relatively low, Liaoning province has started to experience a rapid increase in HIV infections. Since 2000, the reported cases have been increasing by more than 50% annually and HIV-1 infection has spread to 14 cities and counties in Liaoning. In a surveillance of the former paid plasma donors from a county in Liaoning in 1996, the HIV-1-positive rate was found to be as high as 13.26% (unpublished data). However, in these years, we found that the number of individuals infected through blood transfusion was increasing in this county and in the neighboring counties. Recently, the prevalence of HIV-1 has rapidly increased among sex workers and intravenous drug users, in mother-to-child transmission (MTCT), as well as in HIV-1-infected couples in Liaoning. Antiretroviral (ARV) therapy is urgently needed for these patients.

Until now, 28 antiretroviral drugs have been approved by the FDA for the treatment of HIV-1 infection: 13 nucleotide and nucleoside reverse transcriptase inhibitors (NRTIs), 11 protease inhibitors (PIs), three nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 1 fusion inhibitor. Each of them has relative resistant mutations in the viral genome. The strains with resistant mutations selected under drug pressure can develop into the predominant strains. Replication of drug-resistant strains during combination therapy is considered a major cause of treatment failure.[1,2] Drug resistance strains can also be transmitted between individuals. Now about 21–40% of new infections are with HIV-1 strains harboring resistance to at least one

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of three classes of ARV drugs in the United States.[3] A study demonstrated that more than 70% of ARV therapy failure is related to the emergence of drug resistance mutations.[4]

In China, five ARV drugs including three NRTIs (AZT, d4T, and ddI), one NNRTI (NVP), and one protease inhibitor (PI) (IDV) can now be manufactured locally. Since April 2003, free ARV therapy has been delivered to AIDS patients by the Chinese government. Although highly active antiretroviral therapy (HAART) can markedly inhibit HIV replication, improve the prognosis for AIDS patients, and reduce the mortality rate associated with AIDS, drug resistance is an inevitable consequence of incomplete suppression of HIV replication.[5–7] The amino acid substitutions associated with resistance to reverse transcriptase inhibitors (RTIs) and PIs had been extensively characterized on subtype B, which is predominant in developed countries.[3,8,9] These substitutions can be classified into major and minor mutations. Major mutations lead to a several-fold decrease in sensitivity to one or more antiretroviral drugs, whereas minor mutations may not result in a significant decrease in sensitivity, but are associated with an increase in viral fitness (replication capacity).[1,2,8,9] Thus, the appearance of a major mutation in a viral genome already containing minor mutations could influence the rate with which highly resistant viruses are selected during therapy.[10]

Our previous studies identified HIV-1 subtype A, B′, C, and G and a drug resistance-associated mutation in the HIV-1-infected individuals,[11,12] revealing a substantial difference in the profile of drug resistance mutations between subtypes, especially in the protease-coding region.[13,14] However, little information is known about the prevalence of drug resistance-associated mutations among treatment-naive HIV/AIDS patients living in the northeast of China. This study aims to observe the amino acid sequence baseline of the PR gene (1–99 amino acids) and the partial RT gene (1–250 amino acids) from 91 treatment-naive patients in Liaoning province.

MATERIALS AND METHODS

Study subjects

Ninety-one HIV-1-infected treatment-naïve individuals living in Liaoning enrolled between 1999 and 2004 in the 1st Affiliated Hospital, China Medical University; all of them were included in this study. Of these, 62 were male and 29 were female. The mean age was 35 years old. The mean estimated duration of infection was 7 years; HIV seroconversion was estimated to occur midway between the times of the first high-risk behavior and the time positive sera were collected. A total of 44 patients (48.4%) were infected with HIV-1 through heterosexual or homosexual contact; 30 patients (33.0%) were former paid plasma donors, 9 patients (10.0%) were infected through blood transfusion, 7 patients (8%) were injecting drug users (IDUs), and 1 patient (1%) was infected by mother-to-child transmission. All subjects were antiretroviral therapy naïve (Table 1). Informed consent was obtained from each studied patient.

RNA extraction, cDNA synthesis, and nested PCR

Viral RNA was extracted from plasma with the MagNA Pure LC automatic nucleic extraction system (Roche, Japan), according to the manufacturer's recommendations. cDNA was generated by reverse transcription of a 4-μl aliquot viral RNA using primer RT-R1 (5′-CTGTATTTCTGCTATTAAGTCTTTTGATGGG-3′, 3509–3539 nt of HIV-1_{HXB2}) in a 10 μl reaction volume; the reaction condition included 30°C for 10 min, 42°C for 20 min, 99°C for 5 min, followed by 5°C for 5 min. A 1.3-kb region of PR-RT was amplified from cDNA with nested polymerase chain reaction (PCR). The first round PCR with outer primers PRO-F1 (5′-TTGGAAATGTGGAAAGGAAGGAC-3′ 2028–2050 nt of HIV-1_{HXB2}) and RT-R1 was performed with cycling parameters of 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2.5 min, with a final extension step at 72°C for 10 min. The second round PCR with inner primers PRO-F2 (5′-CAGAGCCAACAGCCCCACCA-3′ 2147–2166 nt of HIV-1_{HXB2}) and RT-R2 (5′-CTGCCAGTTCTAGCTCTGCTTC-3′ 3441–3462 nt of HIV-1_{HXB2}) was performed with cycling parameters of 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 63°C for 30 sec, and 72°C for 2.5 min, with a final extension step at 72°C for 10 min. The PCR products were confirmed through 1.0% agarose gel electrophoresis in the present of 0.5 μg/ml ethidium bromide and photographed under ultraviolet illumination. Products were purified with a QIAquick Gel Extraction Kit (Qiagen, Germany).

TABLE 1. THE DISTRIBUTION OF VARIOUS HIV-1 SUBTYPES IN DIFFERENT RISK GROUPS

Subtype	*IDUs*	*Blood*	*Sexual*	*MCT*	*Total*
A			3	1	4 (4.4%)
B			3		3 (3.3%)
B′		37	13		50 (54.9%)
G		1			1 (1.1%)
C			1		1 (1.1%)
CRF01_AE	1		14		15 (16.5%)
CRF03_AB			1		1 (1.1%)
CRF06_cpx		1			1 (1.1%)
CRF07_BC	4		3		7 (7.7%)
CRF08_BC	2		3		5 (5.5%)
Unclassifiable			3		3 (3.3%)
Total	7 (7.7%)	39 (42.9%)	44 (48.4%)	1 (1.1%)	91 (100%)

Table 2. Amino Acid Substitutions in the HIV-1 PR Sequences of 91 Treatment-Naive Patients at Positions Associated with Resistance to Protease Inhibitors

Subtype	*Number of strains*	*L10I*	*G16E*	*K20R/I*	*M36I/V*	*M46I*	*L63P*	*A71V/T*	*V77I*	*V82I*	*I93L*
A	4	3	2	1	4						
B	3						2		3		
B′	50	2			1	1	39	18	48		45
C	1				1		1				1
G	1			1	1					1	
CRF01-AE	15		3	3	15	2	1			3	7
CRF03-AB	1				1		1		1		1
CRF06-cpx	1			1	1		1			1	
CRF07-BC	7						7	2	3		6
CRF08-BC	5		1		5		3				5
	3										
Unclassifiable					1		2		2		
Total	91	5 (5.5%)	6 (6.6%)	6 (6.6%)	30 (33.0%)	3 (3.3%)	57 (62.6%)	20 (22.0%)	57 (62.6%)	5 (5.5%)	65 (71.4%)

Sequencing reactions

Purified PCR products (50 fmol) were sequenced directly with primers PRO-F2and RT-1 (5′-GTTGACTCAGATTGGTTGCAC-3′ 2519–2539 nt of HIV-1_{HXB2}), RT-2 (5′-CCTAGTATAAACAATGAGACAC-3′ 2946–2967 nt of HIV-1_{HXB2}), and by the dideoxy chain termination method with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq DNA polymerase (Perkin-Elmer, France) on an automated sequencer 377 (Applied Biosystems, USA) according to the manufacturer's recommendations.

Sequence analysis

Sequence fragments were linked by the Contig Express program of Vector NTI Advance 10 (Invitrogrn, USA). The sequences were aligned with previously reported HIV-1 strains of various subtypes from the Los Alamos database. Multiple alignments were performed by CLUSTAL W with minor manual adjustments. The Kimura two-parameter method was used for the determination of the evolutionary distance. The reliability of the branching patterns was assessed by bootstrap analysis with 500 replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001). Simplot version 3.2 was used to identify recombination strains. The bootstrap values were plotted for a window of 200 bp moving in increments of 50 bp along the alignment. The amino acid sequences deduced from the PR and RT nucleic acid sequences were uploaded to an HIV drug resistance database at Stanford University [http://hivdb.stanford.edu/]. An HIV drug resistance pattern was interpreted with the HIVdb Program Genotypic Resistance Interpretation Algorithm.

Statistical analysis

Multiple chi square tests (Fisher's exact, where appropriate) were performed to detect significant differences in the frequency of amino acids at specific positions between different subtypes. Any differences with $p < 0.05$ were considered potentially relevant.

RESULT

HIV-1 subtyping

Phylogenetic analysis of the PR–RT sequences revealed that 50 (54.9%) of the sequences were subtype B′, 15 (16.5%) were CRF01_AE, 7 (7.7%) were CRF07_BC, 5 (5.5%) were CRF08_BC, 4 (4.4%) were subtype A, 3(3.3%) were subtype B, 1 (1.1%) was G, 1 (1.1%) was C, 1 (1.1%) was CRF03_AB, 1 (1.1%) was CRF06_cpx, and 3 were unclassifiable recombinant strains. The B′ subtype was the main subtype in paid plasma donors and blood transfusion infectors. CRF07_BC and CRF08_BC were mainly epidemic in injecting drug users

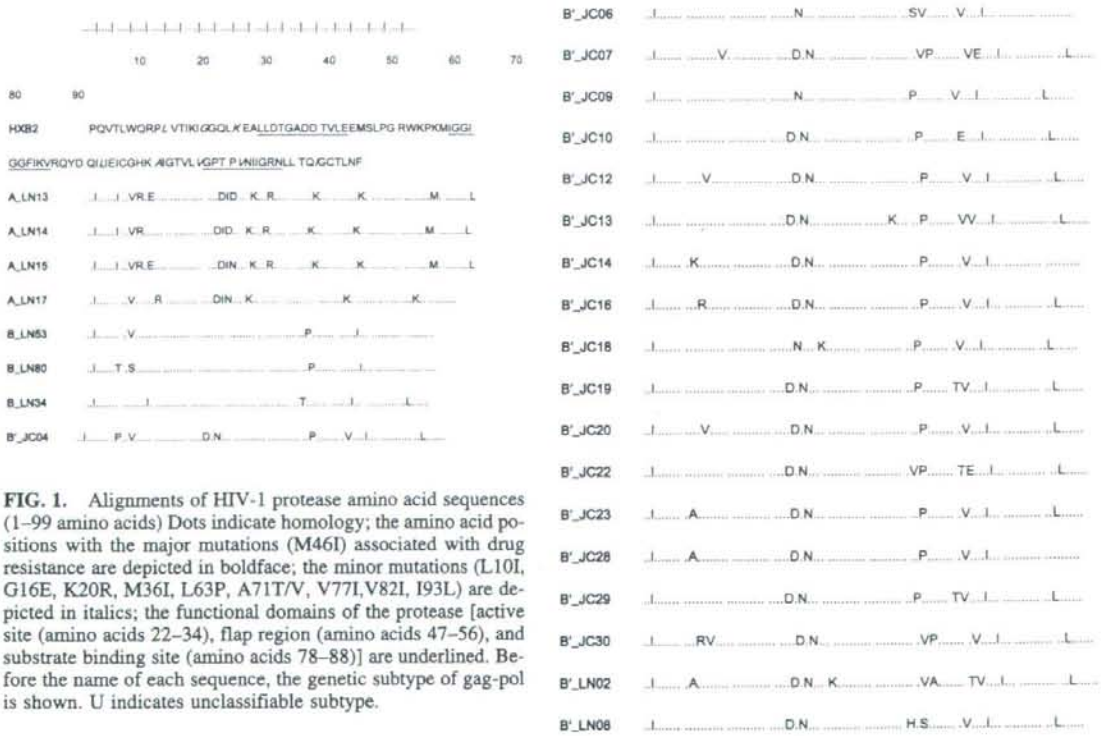

FIG. 1. Alignments of HIV-1 protease amino acid sequences (1–99 amino acids) Dots indicate homology; the amino acid positions with the major mutations (M46I) associated with drug resistance are depicted in boldface; the minor mutations (L10I, G16E, K20R, M36I, L63P, A71T/V, V77I,V82I, I93L) are depicted in italics; the functional domains of the protease [active site (amino acids 22–34), flap region (amino acids 47–56), and substrate binding site (amino acids 78–88)] are underlined. Before the name of each sequence, the genetic subtype of gag-pol is shown. U indicates unclassifiable subtype.