stances require social science research to identify socially and culturally sensitive behavioral interventions.

UNAIDS and WHO identify stigma and adherence to medication as the major challenges in controlling the HIV/AIDS epidemic [1, 23]. In this article, we discuss two major social and behavioral interventions, namely interventions to reduce stigma (table 3) and interventions to promote adherence to TB/HIV medications (table 4, 5). These two social interventions are important prerequisites for implementing the medical interventions recommended by WHO (table 1). For example, interventions for reducing AIDS stigma can facilitate HIV testing for TB patients, and interventions for enhancing adherence are important to ensure treatment efficacy and to prevent drug resistance when patients receive ARV, CPT and INH.

Status of social science research in TB and TB/HIV

In 1975, the Special Program for Research and Training in Tropical Diseases (TDR), a globally coordinated effort of the United Nations Children's Fund (UNICEF), United Nations Development Program (UNDP), World Bank and World Health Organization (WHO), was established to combat neglected tropical diseases and diseases of the poor and disadvantaged [24]. In view of the importance of research in social sciences to control communicable diseases, TDR started supporting social science research in 1979. However, social science research for tuberculosis is still quite new to TDR, as shown by the fact that TDR added tuberculosis to the tropical disease portfolio only in 1999 [25]. Table 6 clearly shows that TB has received much less attention from social science researchers in comparison to HIV/AIDS. According to the United States National Library of Medicine (the world's largest literature database for medical and public health research), the number of TB social science research articles in scientific journals is 7 to 128 times less than HIV/AIDS articles [26]. Obviously, social science research in HIV/AIDS has received ample recognition because results of this research help to identify interventions to reduce the HIV/AIDS burden [27]. However, the role of social science research in improving TB care, especially in developing countries, is oddly limited for such an old disease as TB. A recent review of the 175 social science articles on TB (table 7) shows that half of the studies were conducted in the United State (Rawls and Booker, 2005), although 95 percent of global TB cases and99percent of TB deaths occur in the developing world [2]. Clearly, therefore, it is imperative that more social science research be conducted in high burden and resource-limited countries.

The difficulty of achieving the global target for TB control is mainly due to the lack of human resources and qualified staff [28]. At the global level, the published information on human resources and TB control are limited and almost none relate to HIV-TB control [29]. Training is essential to the development of a health workforce geared to TB control, but regular international TB training courses are organized by a limited number of organizations [30, 31]. Most international training courses focus on clinical or laboratory training and the management of tuberculosis programs. To our knowledge, none of these courses include social science subjects in the training curriculum, except the international courses organized continuously by the Research Institute of Tuberculosis (RIT) Japan Anti-TB Association (JATA) since 1963. The RIT has incorporated social science subjects into the training curriculum for TB program managers from the start of training on the basis of the view that clinicians and TB program managers should apply a broader and more holistic perspective to TB services and TB programs. Currently, social science topics include concepts and practical examples about community beliefs and perceptions, social stigma, health systems and health-seeking behavior, gender, adherence to treatment, community participation and interpersonal communication skills. These topics are relevant to WHO's newly recommended Stop TB Strategy for achieving the MDGs [2].

CONCLUSION:

HIV/AIDS biologically and socially interacts with TB. Reducing TB associated HIV burden requires both biomedical and social interventions. The current involvement of social scientists in research and training for TB control in high HIV prevalence settings is limited. To facilitate the Millennium Development Goal for TB, social science research and training should be implemented in countries which are affected by these dual epidemics. Social science research can promote understanding regarding the complex psychosocial interplay of TB and HIV/AIDS. In addition to addressing these problems, social science research can help to identify the interventions which are effective in urging vulnerable patients, including women and the poor to take advantage of TB/HIV prevention and care services. Social science research should show how to implement these interventions on a large scale and how to influence national policy and care strategy [2,27,32]. The training courses designed for TB and HIV/AIDS program managers and service providers should include social science subjects to ensure that the program and services are responsive to the complicated biological and the psycho-social interactions of TB and HIV/AIDS.

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Successful Efavirenz Dose Reduction in HIV Type 1–Infected Individuals with Cytochrome P450 2B6 *6 and *26

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Background. Efavirenz (EFV) is metabolized primarily by cytochrome P450 2B6 (CYP2B6), and high plasma concentrations of the drug are associated with a G→T polymorphism at position 516 (516G→T) of *CYP2B6* and frequent central nervous system (CNS)–related side effects. Here, we tested the feasibility of genotype-based dose reduction of EFV.

Methods. *CYP2B6* genotypes were determined in 456 human immunodeficiency virus type 1 (HIV-1)–infected patients who were receiving EFV treatment or were scheduled to receive EFV-containing treatment. EFV dose was reduced in *CYP2B6* 516G→T carriers who had high plasma EFV concentrations while receiving the standard dosage (600 mg). EFV-naive homozygous *CYP2B6* 516G→T carriers were treated with low-dose EFV. In both groups, the dose was further reduced when plasma EFV concentration remained high.

Results. *CYP2B6* 516G→T was identified in the **6* allele (found in 17.9% of our subjects) and a novel allele, **26* (found in 1.3% of our patients). All EFV-treated *CYP2B6* **6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations (>6000 ng/mL) while receiving the standard dosage. EFV dose was reduced to 400 mg for 11 patients and to 200 mg for 7 patients with persistently suppressed HIV-1 loads. EFV-containing treatment was initiated at 400 mg in 4 *CYP2B6* **6/*6* carriers and one **6/*26* carrier. Two of them still had a high plasma EFV concentration while receiving that dose, and the dose was further reduced to 200 mg, with successful HIV-1 suppression. CNS-related symptoms improved with dose reduction in 10 of the 14 patients, although some had not been aware of the symptoms at initial dosage.

Conclusions. Genotype-based EFV dose reduction is feasible in *CYP2B6* **6/*6* and **6/*26* carriers, which can reduce EFV-associated CNS symptoms.

Efavirenz (EFV) is an important anti–HIV-1 agent in current combination treatment and is usually prescribed at a fixed dosage of 600 mg once daily [1, 2]. The plasma concentration of EFV varies widely in individuals, and the prevalence of CNS symptoms is higher in those with high concentrations [3]. EFV is metabolized mainly by cytochrome P450 2B6 (CYP2B6), and its concentration was reported to be associated with the *CYP2B6* 516G→T genetic polymorphism [4–8]. Previously, we reported that all Japanese patients with the 516TT genotype had extremely high EFV concentrations (>6000 ng/mL), without exception [4]. However, other studies reported some exceptional cases of subjects with the 516TT genotype with normal concentrations, although most of the

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516TT carriers had high concentrations [5–8]. The difference between our data and those of others may reflect polymorphisms other than 516G→T in *CYP2B6*. If this is the case, analysis of other polymorphisms and determination of the *CYP2B6* haplotype may be helpful in predicting EFV plasma levels. In the present study, we determined the *CYP2B6* haplotype of 456 HIV-1–infected patients and analyzed its relationship with EFV concentration in 111 of them. Furthermore, we reduced the EFV dose in 12 patients whose EFV concentrations had been high while receiving the standard dosage. We also used reduced doses of EFV in 5 EFV-naive patients in whom EFV concentration was predicted to become extremely high while receiving the standard dosage, on the basis of *CYP2B6* haplotype determination.

SUBJECTS, MATERIALS, AND METHODS

Patients. This analysis included 60 previously reported HIV-1–infected individuals at the International Medical Center of Japan (IMCJ) [4] and another group of 396 HIV-1–infected patients who were receiving treatment of the standard dosage (600 mg once daily) of EFV or were scheduled to begin receiving EFV-containing treatment at the following 11 hospitals in Japan: Hokkaido University (Sapporo), Sendai Medical Center (Sendai), Niigata University (Niigata), Higashi Saitama Hospital (Hasuda), IMCJ (Tokyo), Ishikawa Prefecture Central Hospital (Kanazawa), Nagoya Medical Center (Nagoya), Osaka National Hospital (Osaka), Hiroshima University (Hiroshima), Kyushu Medical Center (Fukuoka), and Kumamoto University (Kumamoto). The ethics committee of each hospital approved this study, and each participant gave written informed consent.

CYP2B6 *genotype.* DNA samples were extracted from peripheral blood specimens obtained from participants, and genotyping of *CYP2B6* 64C→T (*rs8192709*), 415A→G (*rs12721655*), 499C→G (*rs3826711*), 516G→T (*rs3745274*), 777C→A (*rs* number not available), 785A→G (*rs2279343*), 1375A→G (*rs* number not available), and 1459C→T (*rs3211371*) was performed by allele-specific fluorogenic 5′ nuclease chain reaction assay with predesigned primers and TaqMan MGB probes (TaqMan SNP Genotyping Assay; Applied Biosystems) or previously published primers and MGB probes [4]. In subjects confirmed to carry 499C→G, all 9 exons of the *CYP2B6* gene were amplified with previously published primers [9], and their DNA sequences were directly determined. For haplotype analysis of the *CYP2B6* allele, PCR amplification of the genomic region (3130 bp) containing exons 4 and 5 was performed using sense primer 5′-AACTGTACTCACTCCCAGAGT-3′ and antisense primer 5′-CTCCCTCTGTCTTTCATTCTGT-3′. The amplified PCR product was subjected to subcloning, and the DNA sequence of each clone was determined. For genotyping of *CYP2B6* 983T→C (*rs28399499*), new primers and probes were designed as follows: forward primer, 5′-GCCTGAAATGCCTCTTTAAAATGAGATTC-3′; reverse primer, 5′-GCGATGTGGGCCAATCAC-3′; VIC probe for 983T, 5′-CTGTTCAATCTCCC-3′; and FAM probe for 983C, 5′-CTGTTCAGTCTCCC-3′. The obtained genotyping results of *CYP2B6* 983T→C for >10 patients were confirmed by direct sequencing of exons 7 and 8 with use of primers published elsewhere [9].

Plasma EFV concentration. Samples of peripheral blood were collected during a daytime office visit (9–16 h after the patient took EFV) from the patients who had received EFV treatment at 600-mg dose at bedtime for >4 weeks. EFV concentration was measured by the reverse-phase high-performance liquid chromatography (HPLC) method [10]. For cases of EFV-dose reduction, plasma concentration was measured >2 weeks after the change in EFV dose. Differences in EFV concentrations between groups were examined for statistical significance with Student's t test. A P value <.05 denoted the presence of a statistically significant difference.

RESULTS

Novel* CYP2B6 *allele. The *CYP2B6* genotype was analyzed in 456 HIV-1–infected patients, including 442 Japanese, 8 other Asians, and 6 others. During the analysis, we noticed that some patients had the *CYP2B6* 499C→G polymorphism, substituting Ala for Pro at the 167th amino acid, which is already registered in the SNP Database, although the *CYP2B6* allele containing 499G had not been determined yet. TaqMan Genotyping Assay indicated that *CYP2B6* 449G was heterozygous with 499C in 12 individuals (2.6%), who were all Japanese (table 1). Direct sequencing of all the exons confirmed the results of TaqMan Genotyping Assay and showed that 8 subjects had 516GT, 785AG, and 1375AA genotypes; 3 had 516TT, 785GG, and 1375AA genotypes; and 1 had 516GT, 785AG, and 1375AG genotypes without any other mutation. Subcloning analysis of the PCR products confirmed that 499G always coexisted in the same allele with 516T and 785G (figure 1). Therefore, it was concluded that the novel haplotype containing 499C→G had 2 other single-nucleotide polymorphisms (SNPs): 516G→T and 785A→G. We formally registered this novel allele with the Human Cytochrome P450 Allele Nomenclature Committee, and it was designated "*CYP2B6 *26*" (http://www.cypalleles.ki.se/). With use of this nomenclature, the *CYP2B6* haplotype of the twelve 499C→G carriers were identified as eight **1/*26* heterozygotes, three **6/*26* heterozygotes, and one **23/*26* heterozygote (table 1). The allelic frequency of **26* was 1.3% in our study participants.

CYP2B6 *haplotype determination.* In 456 HIV-1–infected individuals, we determined the genotypes of 9 SNP positions (64C→T, 415A→G, 499C→G, 516G→T, 777C→A, 785A→G, 983T→C, 1375A→G, and 1459C→T) in *CYP2B6* (table 1). No *CYP2B6* genetic polymorphism was detected in 211 patients, and their haplotype was determined to be **1/*1*. The haplotypes

Table 1. *CYP2B6* haplotype and allele frequencies in study participants.

CYP2B6 status	*CYP2B6* genotype at nucleotide position								No. (%) of subjects	
	415	499	516	777	785	983	1375	1459	All[a]	Japanese
Haplotype:										
**1/*1*	AA	CC	GG	CC	AA	TT	AA	CC	211 (46.3)	205 (46.4)
**1/*2*	AA	CC	GG	CC	AA	TT	AA	CC	30 (6.6)	30 (6.8)
**1/*4*	AA	CC	GG	CC	AG	TT	AA	CC	43 (9.4)	42 (9.5)
**1/*5*	AA	CC	GG	CC	AA	TT	AA	CT	4 (0.9)	3 (0.7)
**1/*6*	AA	CC	GT	CC	AG	TT	AA	CC	104 (22.8)	101 (22.9)
**1/*23*	AA	CC	GG	CC	AA	TT	AG	CC	2 (0.4)	2 (0.5)
**1/*26*	AA	CG	GT	CC	AG	TT	AA	CC	8 (1.8)	8 (1.8)
**2/*4*	AA	CC	GG	CC	AG	TT	AA	CC	6 (1.3)	5 (1.1)
**2/*5*	AA	CC	GG	CC	AA	TT	AA	CT	1 (0.2)	1 (0.2)
**2/*6*	AA	CC	GT	CC	AG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*4*	AA	CC	GG	CC	GG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*6*	AA	CC	GT	CC	GG	TT	AA	CC	12 (2.6)	12 (2.7)
**5/*5*	AA	CC	GG	CC	AA	TT	AA	TT	1 (0.2)	1 (0.2)
**5/*6*	AA	CC	GT	CC	AG	TT	AA	CT	1 (0.2)	1 (0.2)
**6/*6*	AA	CC	TT	CC	GG	TT	AA	CC	19 (4.2)	17 (3.8)
**6/*26*	AA	CG	TT	CC	GG	TT	AA	CC	3 (0.7)	3 (0.7)
**23/*26*	AA	CG	GT	CC	AG	TT	AG	CC	1 (0.2)	1 (0.2)
Total									456	442
Allele:										
**1*	A	C	G	C	A	T	A	C	613 (67.2)	596 (67.4)
**2*	A	C	G	C	A	T	A	C	42 (4.6)	41 (4.6)
**4*	A	C	G	C	G	T	A	C	71 (7.8)	69 (7.8)
**5*	A	C	G	C	A	T	A	T	8 (0.9)	7 (0.8)
**6*	A	C	T	C	G	T	A	C	163 (17.9)	156 (17.6)
**23*	A	C	G	C	A	T	G	C	3 (0.3)	3 (0.3)
**26*	A	G	T	C	G	T	A	C	12 (1.3)	12 (1.4)
Total									912	884

[a] Including 442 Japanese, 8 other Asians (5 Thai, 2 Koreans, and 1 Filipino), 4 Hispanics, and 2 non-Hispanic whites.

of single-SNP carriers with 64CT, 785AG, 1375AG, and 1459CT were determined to be **1/*2*, **1/*4*, **1/*23*, and **1/*5*, respectively. Those of homozygous polymorphism carriers with 785GG only, 1459TT only, and both 516TT and 785GG were determined to be **4/*4*, **5/*5*, and **6/*6*, respectively. When the fact that **2* is the only allele harboring 64C→T is considered, patients with 64CT and 785AG; 64CT and 1459CT; and 64CT, 516GT, and 785AG were identified as **2/*4*, **2/*5*, and **2/*6* heterozygotes, respectively. Patients with both 516GT and 785GG genotypes but without other polymorphisms were determined to have **4/*6* heterozygotes. There were 104 patients (22.8%), including 101 Japanese, who held both 516GT and 785AG genotypes without other polymorphisms. There were 2 possible haplotypes, **1/*6* and **4/*9*, in this genotypic pattern. When the fact that **9* had not been reported in Japanese subjects was considered [11], we found that all 101 Japanese were **1/*6* heterozygotes. Haplotype analysis by subcloning of PCR products described above was performed in the 3 others, and their haplotype was determined as **1/*6*. One Japanese patient had 516GT, 785AG, and 1459CT genotypes without other polymorphisms, and there were 2 possible haplotypes, **1/*7* and **5/*6*, in this genotypic pattern. Because **7* had not been reported in Japanese subjects [11], the haplotype in this patient was determined to be **5/*6*. Overall, the allelic frequency of **6* was 17.9% in our study participants. The 415A→G, 777C→A, and 983T→C polymorphisms, which are the determinants of **8*, **3*, and **18*, respectively, were not observed in our subjects.

CYP2B6 *and EFV concentration.* We determined the *CYP2B6* haplotype in 251 patients at IMCJ and in 205 patients at the other 10 hospitals. Of the 251 genotype-analyzed patients at IMCJ, 101 were being treated or were beginning treatment with a standard dose of EFV during this study period (figure 2). Plasma EFV concentrations were measured in all 101 patients, including sixty-seven 516GG holders, twenty-eight 516GT holders, and six 516TT holders. To clarify the effect of the 516TT genotype, EFV concentration was also measured in ten 516TT holders undergoing treatment with the standard dose of EFV at other hospitals. The mean concentration (± SD)

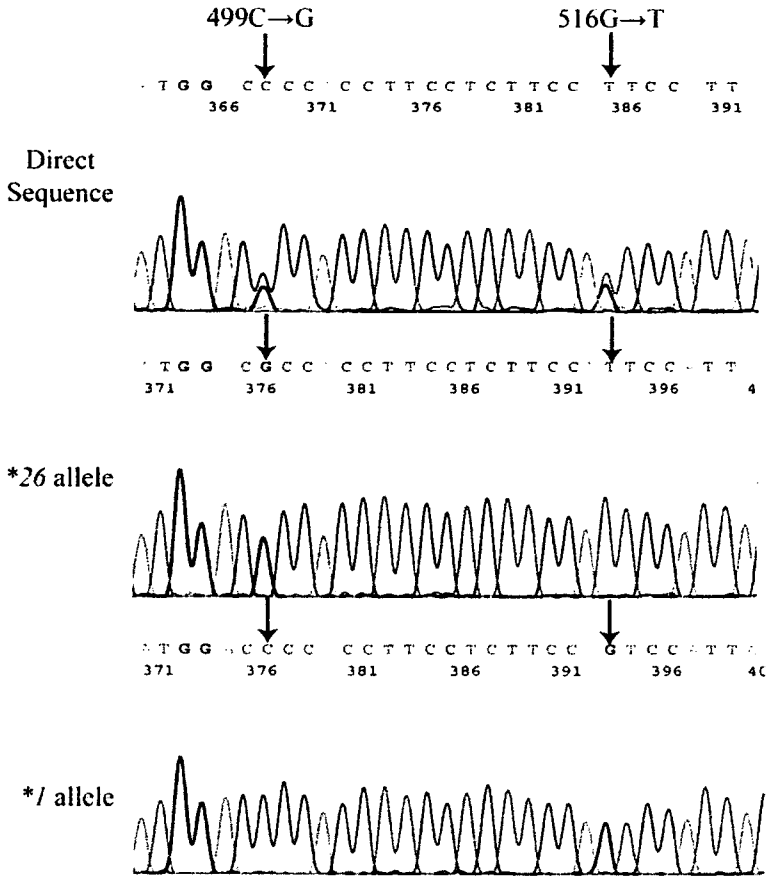

Figure 1. Direct *(top panel)* and subclonal *(middle and bottom panels)* sequences of *CYP2B6* in 499C→G carriers. The genotypes 499G, 516T, and 785G (not shown) exist in the same allele, newly designated as *"CYP2B6 *26."* The same results were obtained in all 9 patients with the 499CG, 516GT, and 785AG genotypes, and the patients were identified as eight **1/*26* carriers and one **23/*26* carrier. Although shown are the sense-strand sequences only, both strands were sequenced. Arrows indicate the variant nucleotide positions 499 and 516.

of EFV in all patients was 3740 ± 2800 ng/mL. When divided by the genotype of position 516, striking discreteness was observed (figure 3). All (95% CI 91.1%–100%) of the 16 carriers of 516TT genotype, including fourteen **6/*6* carriers and two **6/*26* carriers, had extremely high EFV concentrations (>6000 ng/mL). Their mean concentrations (9500 ± 2580 ng/mL) were many orders of magnitude higher than those of the other genotype carriers ($P < 10^{-4}$). There was no significant difference in EFV concentration between **6/*6* carriers and **6/*26* carriers. On the other hand, there were only 2 patients who had such high EFV concentrations among the other genotype carriers. One was a **1/*6* carrier (7140 ng/mL), and the other was a **1/*26* carrier (9710 ng/mL). Direct sequencing of all *CYP2B6* exons showed no polymorphism other than 499C→G, 516G→T, and 785A→G in these individuals. The mean concentrations of EFV of the twenty-eight 516GT carriers, including twenty-five **6*-heterozygotes (3320 ± 1240 ng/mL; $P < 10^{-4}$) and three **26*-heterozygotes (5470 ± 3840 ng/mL; $P < 10^{-4}$), were significantly higher than those of the sixty-seven 516GG genotype carriers (2450 ± 770 ng/mL). None (95% CI 0%–0.1%) of the 516GG carriers had a high EFV concentration (>6000 ng/mL). Considered together, it was concluded that high plasma EFV concentrations were associated with *CYP2B6 *6* and **26* and that *CYP2B6 *6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations at standard dosage, without exception.

EFV dose reduction from 600 mg. To determine whether the EFV dose can be reduced in patients who have a high concentration while receiving the standard dose, a dose-reduction protocol was applied in 12 patients with high plasma concentrations (>6000 ng/mL [range, 6170–14,690 ng/mL]), including one **1/*26* heterozygote, nine **6/*6* homozygotes, and two **6/*26* heterozygotes. Before the dose reduction, plasma HIV-1 load was undetectable (<50 copies/mL) in all patients for >1 month with treatment of a standard antiretroviral regimen containing 600 mg of EFV. In these 12 patients,

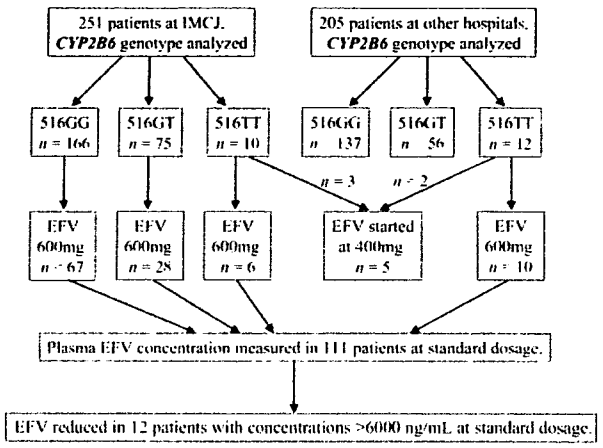

Figure 2. Flow diagram of study participants. The *CYP2B6* genotype was analyzed in 251 patients at the International Medical Center of Japan (IMCJ) and in 205 patients at other hospitals. Standard dosage of EFV was administered in 101 patients at IMCJ, including sixty-seven *CYP2B6* 516GG, twenty-eight 516GT, and six 516TT holders, whose EFV concentrations were measured. Ten 516TT holders at the other hospitals were administered standard dosages of EFV, and their EFV concentrations were also measured. A reduced-dose (400 mg) regimen of EFV was initiated in 5 other 516TT holders.

the EFV dose was reduced from 600 to 400 mg in 11 subjects and was further reduced to 200 mg in 7 of them who consented to further reduction. The plasma EFV concentrations decreased by approximately one-third (36%–46%), to 3720–6160 ng/mL, with dose reduction from 600 to 400 mg in 10 of 11 subjects, and further decreased by approximately one-half (51%–59%), to 1620–2960 ng/mL, with reduction from 400 to 200 mg in 6 of 7 subjects (figure 4). In one patient who had a markedly high EFV concentration (14,690 ng/mL) at the standard 600-mg dose, however, the concentration decreased unexpectedly by 69%, to 4500 ng/mL, with the reduction to 400 mg and further decreased by 82%, to 790 ng/mL, lower than the recommended range (>1000 ng/mL) [1], with the reduction from 400 to 200 mg. Therefore, the dose was increased in this patient back to 400 mg. In another patient who had reported severe dizziness during treatment with the standard dose (600 mg), the dose was reduced immediately to 200 mg at the patient's request. The plasma EFV concentration was also markedly high in this patient (14,360 ng/mL) during treatment with the standard dosage. However, it decreased by 83%, to 2410 ng/mL, with the dose reduction to 200 mg. Consequently, the final EFV dose was 400 mg in 5 subjects and 200 mg in 7 subjects. The determined dosage for each patient was continued for >6 months (the longest was 26 months for a patient who received the 200-mg dose), and the plasma HIV-1 load was continuously undetectable in all patients.

EFV initiation at 400-mg dose. Our analysis showed that *CYP2B6* **6/*6* and **6/*26* carriers had extremely high EFV concentrations, without exception (figure 3), and that dose reduction was possible in patients with high EFV concentration with retention of therapeutically effective anti–HIV-1 activity (figure 4). In the next phase of our study, we used an antiretroviral regimen containing a reduced dose (400 mg) of EFV in 5 EFV-naive patients (four **6/*6* homozygotes and one **6/*26* heterozygote). Before the introduction of low-dose EFV-containing regimen, the plasma HIV-1 loads had been undetectable during receipt of the previous protease inhibitor–containing regimen in all 5 patients. Their EFV concentrations were 4080–9450 ng/mL, and all such concentrations (95% CI, 99.5%–100%) were therapeutically adequate (>1000 ng/mL) at the 400-mg dose (figure 5). One **6/*6* homozygote developed severe dizziness, necessitating discontinuation of EFV-treatment at day 16. His EFV concentration was 5430 ng/mL. In one **6/*26* heterozygote, severe thrombocytopenia emerged, probably because of overdosage of rifabutin prescribed for the treatment of coinfection with *Mycobacterium intracellulare*, and EFV treatment was stopped at day 15. The EFV concentration was 5770 ng/mL. Two of the remaining 3 patients still had extremely high EFV concentrations (6760 and 9450 ng/mL) at the 400-mg dose, and their dose was subsequently reduced to 200 mg. The plasma EFV concentrations decreased to 2690 and 3660 ng/mL (i.e., by 60% and 61%, respectively). Consequently, 2 subjects

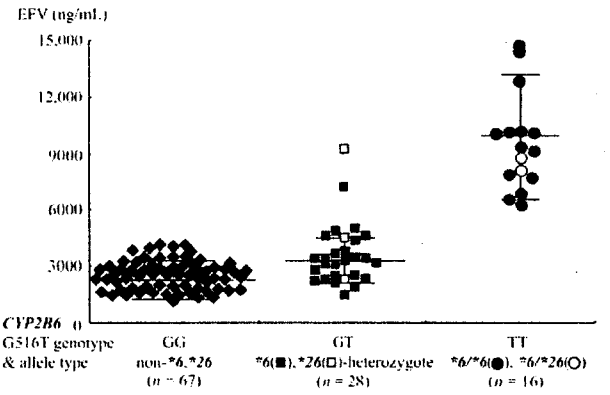

Figure 3. Plasma efavirenz (EFV) concentrations measured during EFV treatment with standard dose (600 mg). A total of 111 HIV-1–infected patients treated with EFV-containing regimens were divided into 3 groups on the basis of nucleotide genotype at *CYP2B6* position 516 (GG, GT, or TT), and their plasma EFV concentrations were compared. Blackened squares, **6* heterozygote with allele other than **26*; unblackened squares, *CYP2B6* 499C→G carriers (**26* heterozygote with allele other than **6*); blackened circles, **6* homozygote (**6/*6*); unblackened circles, *CYP2B6* 499C→G carriers (**6/*26* heterozygotes); blackened diamonds, other genotype carriers. Horizontal lines represent the mean (± SD) plasma EFV concentrations for each group.

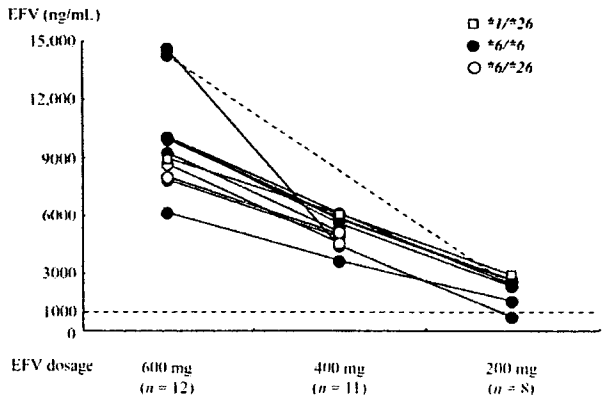

Figure 4. Dose reduction of efavirenz (EFV) in 12 patients whose concentrations were extremely high while receiving treatment with standard dose (600 mg). EFV dose was reduced from 600 to 400 mg in 11 patients and was further reduced, to 200 mg, in 7 patients. In one patient who had severe CNS symptoms while receiving treatment with standard dose, EFV dose was directly reduced to 200 mg (concentrations connected with a dotted line). The suggested minimum target concentration (1000 ng/mL) is indicated by the thin line.

discontinued the EFV-containing regimen, and 3 subjects continued low-dose EFV-containing regimen (400 mg for 1 patient and 200 mg for 2 patients). The low-dose regimen was continued for >6 months, and the plasma HIV-1 load was persistently undetectable in all 3 patients.

Improvement of CNS symptoms. As described above, the EFV dose was reduced from 600 to 400 and 200 mg as the final dose in 5 and 7 subjects, respectively (figure 4), and it was decreased from 400 mg as the initial dose to 200 mg for 2 other subjects (figure 5). To delineate the changes in CNS symptoms associated with the decrease in EFV concentration, a questionnaire survey of these 14 patients was conducted regarding 6 items: dizziness, strange dreams, depression, irritability, concentration problems, and sleep difficulty. More than 1 month after the dose had been reduced to the lowest dose, the patients were asked to judge the 6 CNS symptoms above at initial and final doses, with use of a 5-grade system ("none," "slight," "sometimes," "often," and "always"). Ten (71%) of the 14 patients had some of the aforementioned CNS symptoms during treatment with the initial dose (table 2). The most common symptom was dizziness (57%), followed by strange dreams (50%). Interestingly, all the symptoms improved after dose reduction in the 10 patients. Furthermore, dizziness and concentration problems disappeared during treatment with the final dose in one-half of the patients, although strange dreams and sleep difficulty were still reported by all the patients who had those difficulties at the initial dose. Finally, when the patients were asked whether they wanted to reincrease EFV to the previous dose, all 10 patients with CNS symptoms at the initial dose answered "no" (9 answered "absolutely no").

DISCUSSION

In this study, we identified a novel *CYP2B6* allele, **26*, which includes 499C→G, 516G→T, and 785A→G in 12 Japanese patients, and we showed that, without exception, all **6/*6* and **6/*26* carriers, all holding 516TT, had extremely high plasma EFV concentrations while receiving the standard dose (600 mg) [4]. In other reports, however, there were some exceptional subjects with 516TT who had normal concentrations of EFV, and the discreteness of the EFV concentration with the position 516 genotype was not as clear as it was in our patients [5–8]. This difference may be because some of the 516TT carriers had other *CYP2B6* alleles, such as **7* (containing 516G→T, 785A→G, and 1459C→T), **9* (containing 516G→T only), and **13* (containing 415A→G, 516G→T, and 785A→G). Those alleles could not be found in our subjects, and their effects on EFV concentration were not well described. Because numerous additional *CYP2B6* variants with impact on expression and/or function were recently reported [12–18], correct determination of *CYP2B6* haplotype seems indispensable for prediction of EFV plasma levels.

We reduced the EFV dose in 12 patients whose plasma EFV concentrations were extremely high while receiving the standard dose, and we initiated EFV treatment at a 400-mg dose in 5 EFV-naive **6/*6* and **6/*26* carriers. In most patients, the plasma EFV concentration decreased proportionally with the dose-reduction ratio. In 2 subjects, however, the concentrations decreased much more than expected, given the dose reduction

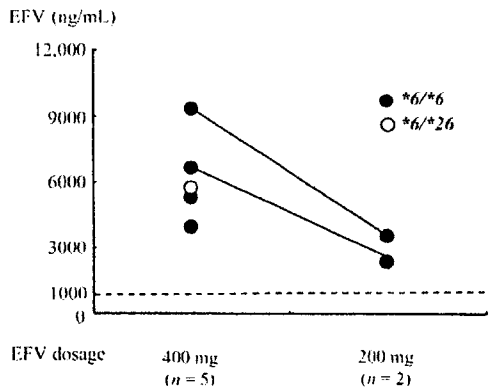

Figure 5. Introduction of low-dose efavirenz (EFV)-containing antiretroviral regimen to *CYP2B6 *6/*6* and **6/*26* carriers. Treatment was started in 4 EFV-naive carriers *CYP2B6 *6/*6* and one **6/*26* carrier, with 400-mg EFV-containing regimens. EFV dose was further reduced, to 200 mg, in 2 patients whose EFV concentrations were >6000 ng/mL while receiving treatment with the 400-mg dose.

Table 2. Changes in CNS-related symptoms after reduction of efavirenz dosage.

Symptom	No. (%) of subjects who reported symptom status during efavirenz treatment		
	Present[a] ($n=14$)	Improved[b]	Disappeared[b]
Dizziness	8 (57)	8 (100)	4 (50)
Strange dreams	7 (50)[c]	7 (100)[c]	0 (0)
Depression	5 (36)	5 (100)	1 (20)
Irritability	5 (36)	5 (100)	1 (20)
Concentration problem	4 (29)	4 (100)	2 (50)
Sleep difficulty	3 (21)	3 (100)	0 (0)
Any of the above	10 (71)[c]	10 (100)[c]	4 (40)

[a] Including the 4 grades "slight," "sometimes," "often," and "always" at the initial dosage. Includes 2 patients whose efavirenz treatment was originally 400 mg and was reduced to 200 mg.

[b] Percentage of those who initially reported "present."

[c] Including 1 patient whose efavirenz dose was originally 400 mg and was reduced to 200 mg.

ratio. Both of these patients had markedly high concentrations at standard dosage. Hasse et al. [19] reported a patient with excessively high plasma EFV concentration at standard dose, which decreased to one-thirtieth following dose reduction from 600 to 200 mg. Long-term exposure to such excessively high concentrations may induce CYP2B6 enzymatic expression in the liver, which could result in an unexpectedly large decrease in plasma EFV concentration by dose reduction if deinduction of the enzyme takes several weeks. At the 400-mg dose, the plasma concentrations of EFV were therapeutically adequate in all the treated **6/*6* and **6/*26* carriers in this study. Regarding the reduced dose, it is noteworthy that a phase II study during EFV development supported the use of a lower dose [20]. The same study indicated that the 600-mg dose of EFV is associated with a high rate of adverse events that could lead to discontinuation, which suggests that the lower dose of 400 mg may be almost as effective without the high discontinuation rate. In the present study, associated with the dose-reduction regimen, a significant number of patients experienced improvement of CNS symptoms, which was unexpected on the basis of previous reports [5, 21, 22]. Interestingly, some of these patients did not appreciate their clinical state and considered themselves to have no CNS-related symptoms during the standard-dose treatment. However, after the dose reduction, they reassessed the status and evaluated symptoms during the treatment with the standard dose as associated with CNS symptoms and indicated that the reduced dose of EFV relieved them of such symptoms. Because EFV-treated patients often stick to the regimen, previous reports of symptom questionnaires conducted during the standard treatment might have underestimated the EFV-associated CNS symptoms [5, 21, 22]. However, this finding might be confounded by placebo effect, because the patients were told that their EFV levels were high while receiving the initial dose and decreased throughout the dose-reduction protocol. Because of this possible placebo effect, a double-blind, placebo-controlled study would best address this question.

EFV dose reduction and initiation of EFV treatment at reduced dose is possible with therapeutic anti–HIV-1 potency retained in *CYP2B6 *6/*6* homozygotes and **6/*26* heterozygotes, which could relieve the patients of the EFV-associated CNS symptoms. It may also decrease the risk of development of EFV-resistant HIV-1 after mandatory treatment discontinuation, such as abdominal surgery [23], and reduce the treatment cost, an important issue in developing countries [24]. After dose reduction, however, careful monitoring is necessary until larger studies confirm the safety of reduced dose in such specific genotype carriers.

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Potential conflicts of interest. All authors: no conflicts.

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compared with four in the experienced group. Four of these five patients had HCV co-infection. Two events arose after one month of treatment and the other three after a year, confirming the multifaceted mechanisms causing this toxicity. In all these cases the treatment had to be stopped, and the patients regressed.

To the best of our knowledge, this study comprises the biggest series to date of patients treated with lopinavir/ritonavir and followed prospectively outside clinical trials. In addition, this HIV-positive population had a high prevalence of co-infection with hepatitis viruses.

The frequency of hepatotoxicity was actually low, unlike in other studies. This might partly be the result of methodological differences, reflecting how the data were collected. Retrospective studies can suffer major selection bias. Gonzalez-Requena *et al.* [11] also reported a low incidence of adverse events, but their case series was small and was followed up for not more than one year.

In conclusion, the present study found that lopinavir/ritonavir caused only limited hepatic toxicity in this population of HIV-positive patients with a high prevalence of co-infection with hepatitis B virus or HCV.

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Premature sister chromatid separation in HIV-1-infected peripheral blood lymphocytes

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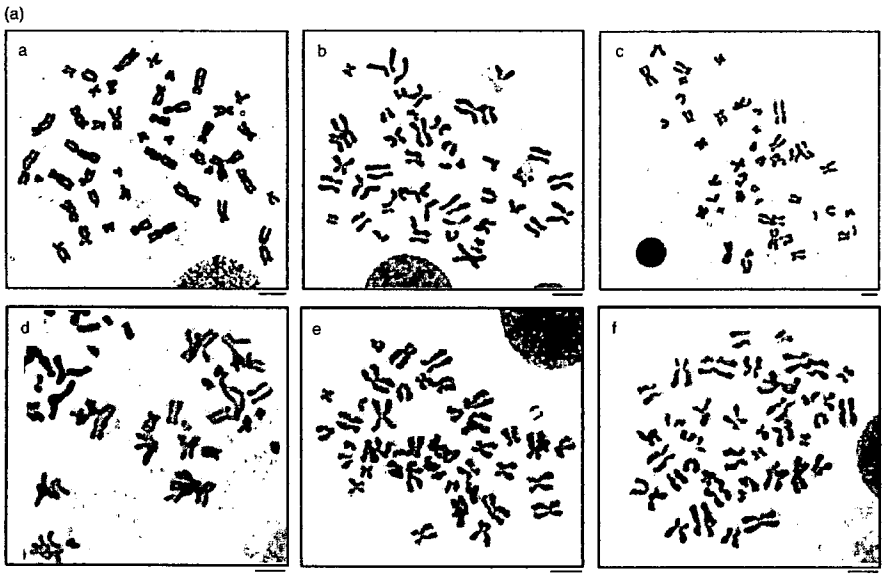
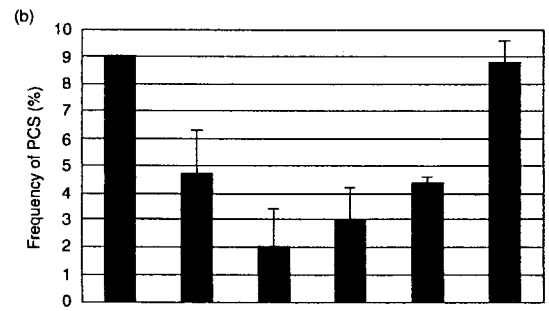
To investigate the mechanism of aneuploidy that is frequently observed in AIDS, we examined premature sister chromatid separation (PCS), a sign of genomic instability, in peripheral blood cells of HIV-1-infected individuals. PCS was found in all

six HIV-1 individuals at a high incidence. When peripheral blood cells from healthy volunteers were infected with HIV-1 *in vitro*, the incidence of PCS increased. This suggests that HIV-1 infection causes PCS and has the potential to induce aneuploidy.

Malignancy in HIV infection influences the prognosis of AIDS patients. These neoplasms are the result of various diseases that accompany immunodeficiency, such as co-infections with Epstein–Barr virus or human herpes virus 8 [1–4]. Besides these AIDS-defining cancers, several non-AIDS-defining cancers also occur at a higher incidence in HIV-infected individuals [5–9]. Moreover, it has been reported that HIV-1 itself is tumorigenic in immortalized B cells in nude mice [10,11]. These reports lead to the hypothesis that HIV-1 has the potential to induce neoplasms before AIDS develops.

Aneuploidy is a phenomenon of chromosome instability that is frequently reported in HIV-1-infected individuals





Case #	P-1	P-2	P-3	P-4	P-5	P-6
Total WBC	3800	5500	8400	5700	6100	2700
HIV-1 mRNA (copies/ml)	27,000	< 50	76	< 50	< 50	35,000

Fig. 1. Metaphase spreads of blood cells in HIV-1 infection. (a) Representative metaphase spreads of peripheral blood cells from HIV-1-infected individuals (b, c, d, e, and f are from cases nos. P-1, 2, 4, 5, and 6, respectively, see Fig. 1b). (a). (b) Frequency of premature sister chromatid separation (PCS). The frequency of PCS (black bar), and number of HIV-1 messenger RNA copies and total white blood cells (WBC) are shown. (c) Metaphase spreads of peripheral blood mononuclear cells (PBMC) from healthy volunteers. Representative metaphase spreads of PBMC from healthy volunteers with (a/+, b/+, and c/+) or without (a, b, and c) vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 infection are shown. (d) Aneuploidy in HIV-1-infected cells. Metaphase spreads from P-1, P-6 and from PBMC with VSV-G-pseudotyped HIV-1 infection were positive for aneuploidy with numbers of chromosomes of 85, 75 and 65, respectively. The scale bar represents 5 μm.

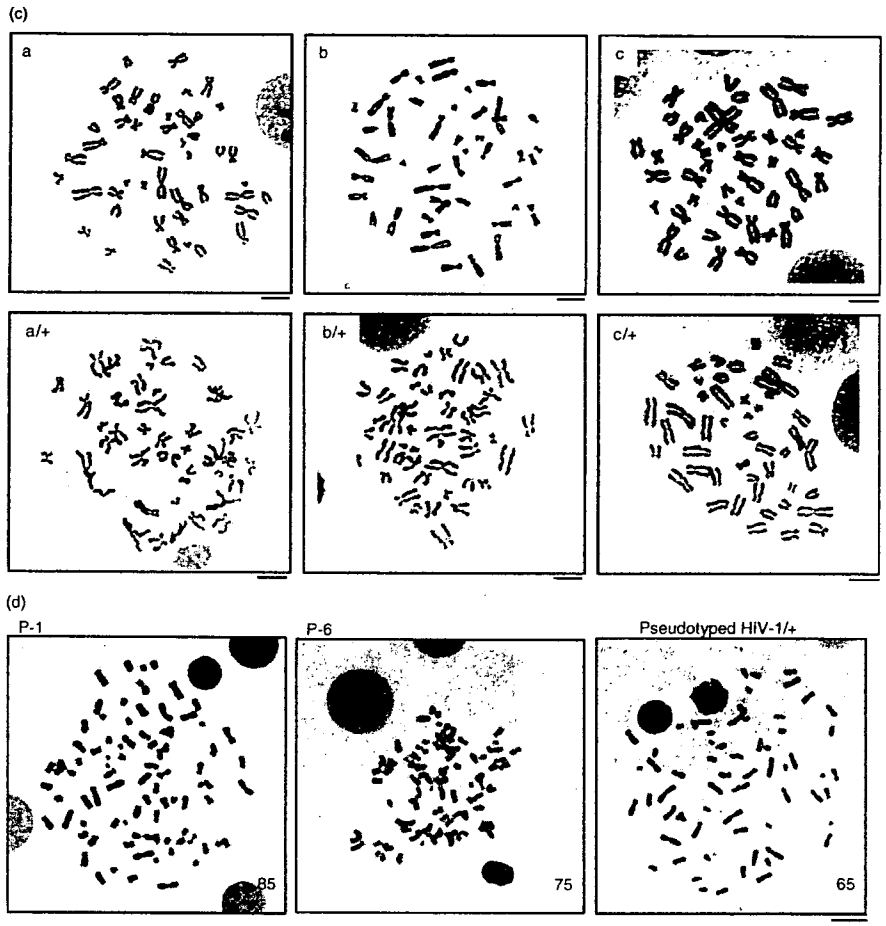


Fig. 1. *(continued)*

[12–14]. One of the major factors accelerating aneuploidy is thought to be abnormal chromatid separation [15–17]. At metaphase, paired sister chromatids are folded at the centric region until the onset of anaphase [18–22]. If the attachment of the sister chromatids is abolished before the onset of anaphase, premature sister chromatid separation (PCS) occurs. Subsequently, chromosome mis-segregation is induced, often resulting in aneuploidy [16,17]. PCS has been found in several clinical conditions, including aging, familial dominant inheritance [23–25], Roberts syndrome [26,27], cancer-prone syndrome mosaic variegated aneuploidy [28,29] and general tumours [30,31]. Note that all of these cases of PCS are associated with aneuploidy, indicating that a high PCS rate is a sign of chromosome instability. To investigate the cellular mechanism of HIV-1-related aneuploidy, we examined PCS in peripheral blood cells of HIV-1-infected individuals.

Peripheral blood was collected in sodium heparin (20 U/ml) from HIV-1-infected patients or healthy volunteers. We added 0.5 ml whole blood to 9.5 ml RPMI-1640 growth medium containing 10% fetal calf serum and 2% phytohemagglutinin M-form, and incubated it for 82 h at 37°C. Then colcemid (30 ng/ml) was treated for 2 h at 37°C. Recovered cells were resuspended in 75 mM potassium chloride and incubated for exactly 15 min at 37°C. To the cell suspension, freshly prepared Carnoy's solution (methanol : glacial acetic acid = 3 : 1) was added and mixed gently. After three changes of Carnoy's solution, a drop of the cell suspension was placed on a slide and air dried. Subsequently, the metaphase spread was stained with Giemsa.

Surprisingly, the HIV-1 patients examined showed PCS at high frequencies of 2.1 to 9.0% (mean ± standard deviation; 5.36 ± 2.92%; Fig. 1a, panels b–f and Fig. 1b). A high incidence of PCS was observed in HIV-1-infected individuals with high viral RNA copy numbers (Fig. 1b), in which total PCS was often observed (patient case no. 1 and no. 6; panels b and f). By contrast, peripheral blood mononuclear cells (PBMC) from healthy volunteers

showed normal attachments at the centromere (Fig. 1a, panel a), and PCS was detected in less than 2% (1.22 ± 0.48%).

We next clarified whether the PCS was attributable to HIV-1 infection. The PBMC (1.5×10^6) [32] were infected with vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 [33] at the concentration of 2 ng/ml of p24 Gag antigen of pseudotyped virus (multiplicity of infection at 0.007). They were incubated for 82 h in the presence of 2% phytohemagglutinin M-form, and metaphase spread was analysed as described above. All of the specimens from three volunteers showed an increased incidence of PCS after HIV-1 infection (Fig. 1c, lower panels), whereas PCS was barely detectable without infection (Fig. 1c, upper panels). The frequencies of PCS after HIV-1 infection in the three samples were 8.40 ± 1.09, 5.28 ± 1.40, and 7.34 ± 1.67, whereas the frequencies without infection were 1.26 ± 0.40, 0.72 ± 0.22, and 1.68 ± 0.86, respectively. Our present data suggest that HIV-1 infection is a primary factor inducing PCS.

In the patients' case, the frequency of PCS was positively correlated with the reduction in total white blood cells (Pearson product–moment correlation coefficient $r = 0.837$, $P < 0.01$; Fig. 1b) rather than CD4 positive lymphocytes ($r = 0.011$, $P > 0.05$). Although VSV-G-pseudotyped HIV-1 was infected to PBMC at a multiplicity of infection of 0.007 (0.7%), the average incidence of PCS with HIV-1 infection exceeded 7%. Taken together with the information that pseudotyped HIV-1 induces a single round of infection, these data suggest that PCS occurs not only in response to the infection itself but also as a result of the effects of other virus products or cellular proteins stimulated by HIV-1 infection.

Simultaneously, we found aneuploidy in hyperploid cells of HIV-1-infected individuals who had high viral loads and high PCS frequency (Fig. 1b and Fig. 1d, left and middle panels). We also found aneuploidy in PBMC with HIV-1 infection *in vitro* (Fig. 1d, right panel). By contrast, aneuploidy was not found in control PBMC. Although it remains to be determined whether PCS is directly related to neoplasms in AIDS, we speculate that a high incidence of PCS and constitutive virus infection augment the susceptibility of the cells to aneuploidy and may play a critical role in the development of AIDS-related neoplasms. It will be important to track the epidemiological and biological features of the incidence of PCS in HIV-1 infection.

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HIV-1 Vpr Induces DNA Double-Strand Breaks

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Abstract

Recent observations imply that HIV-1 infection induces chromosomal DNA damage responses. However, the precise molecular mechanism and biological relevance are not fully understood. Here, we report that HIV-1 infection causes double-strand breaks in chromosomal DNA. We further found that Vpr, an accessory gene product of HIV-1, is a major factor responsible for HIV-1–induced double-strand breaks. The purified Vpr protein promotes double-strand breaks when incubated with isolated nuclei, although it does not exhibit endonuclease activity *in vitro*. A carboxyl-terminally truncated Vpr mutant that is defective in DNA-binding activity is less capable of Vpr-dependent double-strand break formation in isolated nuclei. The data suggest that double-strand breaks induced by Vpr depend on its DNA-binding activity and that Vpr may recruit unknown nuclear factor(s) with positive endonuclease activity to chromosomal DNA. This is the first direct evidence that Vpr induces double-strand breaks in HIV-1–infected cells. We discuss the possible roles of Vpr-induced DNA damage in HIV-1 infection and the involvement of Vpr in further acquired immunodeficiency syndrome–related tumor development. (Cancer Res 2006; 66(2): 627-31)

Introduction

A high incidence of malignant tumors, such as non-Hodgkin's lymphoma, Kaposi's sarcoma, and invasive cervical cancer [acquired immunodeficiency syndrome (AIDS)–defining cancers], is epidemiologically associated with HIV-1 infection (1, 2). These neoplasms are attributable mainly to diseases that accompany immunodeficiency, including coinfection with EBV, human herpes virus 8, and human papillomavirus (1, 2). In addition to these AIDS-defining cancers, several non-AIDS-defining cancers also occur with a higher incidence in HIV-infected individuals (3, 4). These reports lead to the assumption that HIV-1 has the potential to induce neoplasms before AIDS develops. Recently, DNA damage responses have been observed in precancerous lesion before inactivation of p53 (5, 6). Interestingly, it has been reported that HIV-1 infection induces DNA damage responses by activating Rad3-related or ataxia-telangiectasia mutated proteins and promoting phosphorylation of their downstream substrates (7, 8). The elucidation of the factor triggering the DNA damage responses to HIV-1 infection is essential to determine the as yet unknown mechanism causing AIDS-related neoplasms. In the present study, we found that HIV-1 infection induces double-strand breaks of chromosomal DNA, as detected using pulsed-field gel electrophoresis (PFGE). We further showed that *vpr*, an accessory gene of HIV-1 encoding a virion-associated nuclear protein, which induces cell cycle accumulation at G_2-M phase and increases ploidy (9), was a factor responsible for double-strand breaks. We discuss the potential ability of Vpr-induced double-strand breaks to develop into neoplasms in HIV-1 infection.

Materials and Methods

Cell culture. MIT-23 and ΔVpr, a mock transfectant, were established from HT1080 (JCRB9113; the Health Science Research Resources Bank) as previously described (9). In MIT-23, Vpr expression is controlled by the rtet promoter on incubation with 3 μg/mL doxycycline (Sigma, St. Louis, MO) for 48 hours.

Virus infection. Vesicular stomatitis virus G protein (VSV-G)–pseudotyped HIV-1 was produced by cotransfection with a plasmid encoding VSV-G (pHIT/G) and the pNL-Luc-E$^-$R$^+$ or pNL-Luc-E$^-$R$^-$ proviral clone (10). (10). The preparation and titration of viruses are described elsewhere (11). Briefly, the concentration of p24 antigen in the culture supernatant was measured using a p24 Gag antigen capture ELISA kit (ZeptoMetrix, Buffalo, NY). The infectivity of the prepared viral stock was examined using MAGIC5 cells. HT1080 cells were infected for 48 hours with viruses that had 200 ng/mL of p24 Gag antigen, giving a multiplicity of infection (MOI) of 0.7.

Immunostaining. Immunostaining was carried out as described (9). A rabbit polyclonal Rad51 antibody raised against the bacterially expressed protein and a mouse monoclonal antibody raised against synthesized peptides of full-length of Vpr (mAb8D1) were used as the primary antibody. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) and goat anti-mouse IgG conjugated with Cy3 (Zymed Laboratories, Inc., San Francisco, CA) were used as the secondary antibodies. Images were captured on a phase contrast microscope, BX50 (Olympus Corp., Tokyo Japan), or a Radiance 2100 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Overexpression and purification of Vpr and its mutant. The HIV-1 *vpr* gene was ligated into the *Nde*I and *Bam*HI sites of the pET15b vector (Novagen, Madison, WI). The Vpr protein and VprΔC12 mutant were produced in the *Escherichia coli* BL21 (DE3) Codon(+)RIL strain (Novagen) by induction with isopropyl-β-D-thiogalactopyranoside (IPTG; Nacalai Tesque, Inc., Kyoto, Japan) and were purified as described in Supplementary Method. The concentration of the purified Vpr protein was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as the standard.

Isolation of nuclei. Cells scraped from culture dishes were washed once with ice-cold PBS and resuspended in 3 mL of ice-cold 20 mmol/L Tris-HCl buffer (pH 7.6) containing 60 mmol/L KCl, 15 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 250 mmol/L sucrose, 0.6% NP40, and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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protease inhibitor mixture (Sigma). The cell suspension was incubated for 10 minutes on ice and the sucrose concentration was adjusted to 1.6 mol/L. Then, the sample was loaded onto a sucrose cushion of 2.3 mol/L sucrose solution and centrifuged at 35,000 × *g* for 30 minutes. The isolated nuclei were obtained in the 2.3 mol/L sucrose fraction. For immunostaining, isolated nuclei were cytocentrifuged to the MAS-coated slide glass (Matsunami Glass IND., LTD., Tokyo, Japan) for 6 minutes at 800 rpm (Thermo Shandon, Chadwick Road, United Kingdom).

PFGE assay. Isolated nuclei were incubated with 10 μmol/L of purified Vpr or VprΔC12 for 15 hours at 30°C. The cells (isolated nuclei) were embedded in agarose plugs at a density of 3 × 10^5 cells/100 μL. The plugs were treated with proteinase K solution [0.5 mol/L EDTA (pH 8.0), 1% sarcosyl, and 0.5 mg/mL proteinase K] for 38 hours at 50°C. After PFGE was done in a CHEFF Mapper (Bio-Rad Laboratories), the gels were stained with Vistra Green (Amersham Bioscience, Piscataway, NJ).

The DNA-binding assay. The Vpr protein was incubated with ϕX174 single-stranded DNA (ssDNA; 20 μmol/L) or ϕX174 superhelical dsDNA (10 μmol/L) in 10 μL of 8 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA. The reaction mixtures were incubated for 1 hour at 37°C and were analyzed by electrophoresis on a 0.8% agarose gel in 1× TAE buffer (40 mmol/L Tris acetate and 1 mmol/L EDTA) at 3.3 V/cm for 2 hours. The bands were visualized using ethidium bromide staining.

Nuclease activity. The Vpr protein (18.8 μmol/L) or DNaseI (Invitrogen Corporation, Carlsbad, CA; 0.02 unit/μL) were incubated with ϕX174 superhelical double-stranded DNA (dsDNA; 2.5 μmol/L) in 40 μL of 15 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA, in the presence of 5 mmol/L $MgCl_2$, $MnCl_2$, $ZnSO_4$, or $CaCl_2$. The reaction mixtures were incubated at 37°C for 30 minutes. After incubation, the samples were treated with proteinase K (0.3 mg/mL) in the presence of 0.1% SDS and the DNA was extracted using phenol-chloroform. The DNA was precipitated by ethanol and was analyzed by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer at 6.6 V/cm for 30 minutes. The bands were visualized with ethidium bromide staining.

The Ni-NTA agarose pull-down assay. Isolated nuclei were disrupted in 20 mmol/L Tris-HCl buffer (pH 8.5) containing 200 mmol/L KCl, 2 mmol/L 2-mercaptoethanol, 0.25 mmol/L EDTA, and 10% glycerol. The extract was incubated with His_6-Vpr (53 μmol/L) for 15 hours at 30°C. After incubation, His_6-Vpr was precipitated with 4 μL of Ni-NTA agarose beads and the beads were washed thrice with 500 μL of 20 mmol/L Tris-HCl buffer (pH 7.6) containing 100 mmol/L NaCl, 5 mmol/L DTT, 10 mmol/L imidazole, 1 mmol/L EDTA, and 0.2% Tween 20. The proteins precipitated with the Ni-NTA beads were analyzed by 16% SDS-PAGE. The bands were visualized by silver staining.

Results

Vpr expression induces chromosomal double-strand breaks. To test whether HIV-1 infection causes double-strand breaks, we used PFGE, which was able to clearly detect the double-strand breaks induced by X-ray irradiation (Fig. 1*A*, *lane 2*; ref. 12). HT1080 cells were infected with HIV-1 that had 200 ng/mL of p24 Gag antigen, giving a MOI of 0.7, and the cellular DNA was fractionated using PFGE. Figure 1*A* (*lane 6*) shows that HIV-1 infection induced double-strand breaks. Interestingly, the amount of HIV-1-dependent double-strand breaks was reduced significantly (Fig. 1*A*, *lane 5*) when the *vpr* gene was deleted from the HIV-1 viral genome (HIV-1ΔVpr). To show that HIV-1-dependent double-strand breaks are attributable to Vpr expression, we examined double-strand break formation in Vpr stable transfectant, MIT-23 (9), in which Vpr expression is controlled by the rtet promoter by doxycycline, and, in ΔVpr, a mock transfectant. As shown in Fig. 1*B*, double-strand breaks were observed in the Vpr-expressing cells (*lane 5*, *arrow*) but not in the mock transfectants (*lane 4*). Furthermore, Rad51 foci, which are formed

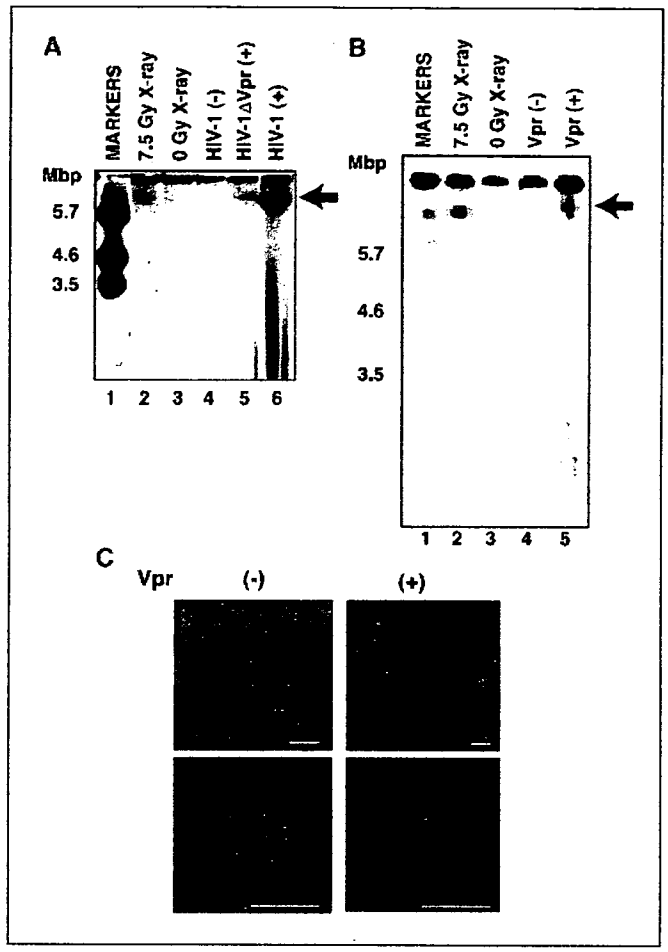

Figure 1. Vpr induces double-strand breaks *in vivo*. *A*, PFGE analysis of double-strand breaks after HIV-1 infection. HT1080 cells were infected with the same amount of HIV-1 or HIV-1ΔVpr (MOI = 0.7) and subjected to PFGE. As a positive control, uninfected cells were analyzed immediately after 7.5 Gy of X-ray irradiation. Molecular mass markers (*lane 1*), control cells (*lanes 3* and *4*), cells subjected to X-ray irradiation (*lane 2*), and cells infected with HIV-1ΔVpr (*lane 5*) or HIV-1 (*lane 6*) are shown. *Arrow*, position corresponding to the double-strand breaks. *B*, PFGE analysis in Vpr-expressing cells. Molecular mass markers (*lane 1*), cells irradiated with 7.5 Gy (*lane 2*), control cells (*lane 3*), mock transfectants (*lane 4*), and cells with Vpr expression (*lane 5*) are shown. *Arrow*, double-strand breaks. *C*, Rad51 focus formation with Vpr expression. An immunohistochemical analysis was used to detect Rad51 in cells with (*right*) or without (*left*) Vpr expression. Bar, 10 μm.

at double-strand break sites (13), were observed with Vpr expression (Fig. 1*C*). These results indicate that Vpr is responsible for double-strand break formation. The double-strand breaks shown in Fig. 1*B* were not the result of an apoptotic process as the DNA ladder typically observed in apoptotic cells (14) was not detected (data not shown).

Vpr has no endonuclease activity. Next, we studied whether Vpr directly induces double-strand breaks. The recombinant Vpr protein was purified to near homogeneity (Fig. 2*A*) and the DNA-binding activity of Vpr was examined. As shown in Fig. 2*B*, purified Vpr bound both ssDNA (*lanes 2-6*) and dsDNA (*lanes 8-12*) in an ATP- and Mg^{2+}-independent manner (15). Then, we examined whether Vpr has nuclease activity. Superhelical dsDNA containing small amounts of nicked circular dsDNA was incubated with Vpr in the presence of various divalent cations. After the incubation, the proteins were removed and the DNA was examined by

electrophoresis. If Vpr induces a double-strand break or nick, the superhelical dsDNA would give rise to linear or nicked circular forms, producing a different electrophoretic pattern. However, the DNA incubated with Vpr in the absence (*lane 2*) or presence of any divalent cation examined (*lanes 4, 6, 8,* and *10*) showed the same migration pattern with control (*lane 1*), indicating that Vpr does not cleave DNA (Fig. 2*C*). Positive control experiments showed that the DNA was digested by DNaseI with $MgCl_2$, $MnCl_2$, or $CaCl_2$ (*lanes 5, 7,* and *11*) but not with $ZnSO_4$ (*lane 9*; Fig. 2*C*). Therefore, these results indicate that Vpr lacks endonuclease or nicking activity.

Vpr induces double-strand breaks *in vitro*. In a second approach, we tested whether purified Vpr induces double-strand breaks in nuclei isolated from HT1080 cells (Fig. 3*A*). First, we confirmed by a laser confocal microscopy that Vpr localizes in nuclei after incubation *in vitro* (Fig. 3*B*). The nuclear DNA was then analyzed for double-strand breaks by using PFGE (Fig. 3*C*). Interestingly, purified Vpr induced double-strand breaks in the DNA of the isolated nuclei (Fig. 3*C, lane 5, arrow*). By contrast, few double-strand breaks were detected without Vpr (Fig. 3*C, lane 4*). Because Vpr alone did not show endonuclease activity (Fig. 2*C*), these results suggest that Vpr interacts with intrinsic nuclear protein(s), which required for double-strand break formation. To identify candidates for the Vpr-interacting nuclear proteins, we did the Ni-NTA pull-down assay. In this assay, recombinant His_6-tagged Vpr was incubated with the extract from isolated nuclei and Ni-NTA beads precipitated proteins bound to His_6-tagged Vpr (Fig. 3*D*). As shown in Fig. 3*D*, His_6-tagged Vpr associated with numerous proteins that were not detected in the control precipitates (*lane 2, asterisks*).

The DNA-binding activity of Vpr is correlated with double-strand break formation. The COOH-terminal region of Vpr is arginine rich and is thought to be an important site for DNA binding to Vpr (15). Nuclear magnetic resonance analysis shows that Vpr has three α-helixes (amino acids 17-33, 38-50, and 56-77) in solution, whereas the COOH-terminal region from amino acid residues 84 to 96 is disordered (16). This suggests that the deletion of the COOH-terminal 12 amino acid residues does not affect the tertiary structure of Vpr. We purified a Vpr mutant protein lacking the COOH-terminal 12-amino-acid residues (VprΔC12; Fig. 4*A*), and examined its DNA-binding activity. Purified VprΔC12 was significantly defective in both ssDNA- and dsDNA-binding activity compared with wild-type Vpr (Fig. 4*B*). Interestingly, VprΔC12 induced double-strand breaks in isolated nuclei but its efficiency was reduced significantly (Fig. 4*C, lane 6*). These results indicate that the DNA-binding ability of Vpr is important for the induction of double-strand breaks by Vpr.

Discussion

Here, we present evidence that HIV-1 Vpr induces double-strand breaks. Our data are consistent with previous observations in Vpr-expressing cells: the up-regulation of gene amplification events that are believed to be introduced by broken DNA strands (17) and the activation of activating Rad3-related/ataxia-telangiectasia mutated, followed by the phosphorylation of their downstream substrate, a histone H2A variant, H2AX, and γ-H2AX and BRCA1 focus formation (8). Biochemical analyses using purified Vpr indicated that Vpr alone has no endonuclease activity (Fig. 2*C*), suggesting that a cellular factor(s), possibly with endonuclease activity, is required for Vpr-dependent double-strand breaks. The factor(s) required for double-strand breaks must preexist in nuclei because double-strand breaks were observed upon incubating a mixture of isolated nuclei and purified Vpr *in vitro* (Fig. 3*C*). As one possible mechanism, Vpr may recruit a nuclease factor to chromosomal DNA, given that the Vpr-dependent double-strand breaks were correlated with the DNA-binding activity (Figs. 4*B* and *C*). Alternatively, Vpr itself may acquire endonuclease activity after modification in the nucleus. Further analyses are necessary to clarify this point.

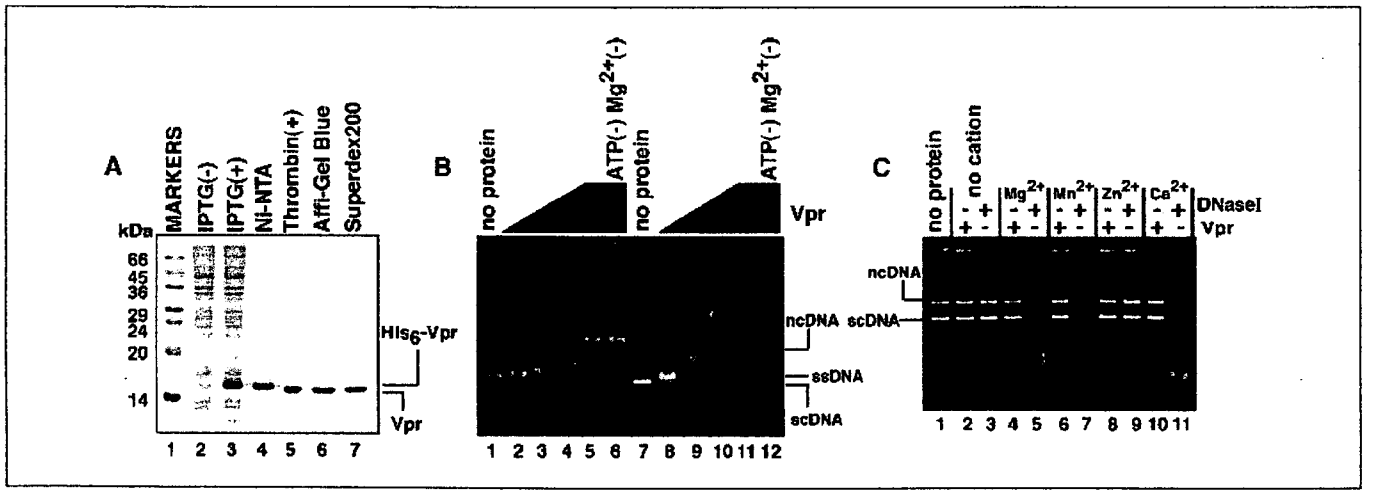

Figure 2. The Vpr-DNA interaction *in vitro*. *A*, purification of recombinant Vpr. Proteins from each purification step were analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. Molecular mass markers (*lane 1*), whole-cell lysates before (*lane 2*) and after (*lane 3*) induction with IPTG, samples from the Ni-NTA fraction (*lane 4*), the fraction after removing the hexahistidine tag (*lane 5*), the Affi-Gel Blue fraction (*lane 6*), and the Superdex 200 fraction (*lane 7*) are shown. *B*, the DNA-binding activity of Vpr. ϕX174 circular ssDNA (20 μmol/L; *lanes 2-6*) and ϕX174 superhelical dsDNA (*scDNA*; 10 μmol/L; *lanes 8-12*) containing a small amount of nicked circular DNA (*ncDNA*) were incubated with Vpr in the presence of 1 mmol/L ATP and 1 mmol/L $MgCl_2$. Control experiments without ATP and $MgCl_2$ (*lanes 6* and *12*) are included. The Vpr concentrations were 1.25 μmol/L (*lanes 2* and *8*), 2.5 μmol/L (*lanes 3* and *9*), 5 μmol/L (*lanes 4* and *10*), and 10 μmol/L (*lanes 5, 6, 11,* and *12*). *Lanes 1* and *7*, negative controls without protein. *C*, nuclease activity. ϕX174 scDNA (2.5 μmol/L) was incubated with Vpr (18.8 μmol/L; *lanes 2, 4, 6, 8,* and *10*) or DNaseI (*lanes 3, 5, 7, 9,* and *11*) in the absence of divalent cation (*lanes 2* and *3*) or in the presence of 5 mmol/L $MgCl_2$ (*lanes 4* and *5*), 5 mmol/L $MnCl_2$ (*lanes 6* and *7*), 5 mmol/L $ZnSO_4$ (*lanes 8* and *9*), or 5 mmol/L $CaCl_2$ (*lanes 10* and *11*). *Lane 1*, negative control without protein.

In the HIV-1 life cycle, DNA breakage and repair are thought to be essential steps for integrating the double-stranded viral cDNA into the host genome. In this study, we found that Vpr is one molecule responsible for the double-strand breaks that occur upon HIV-1 infection. However, it is also noteworthy that some double-strand breaks were induced in the cells with HIV-1ΔVpr (Fig. 1*A*, *lane 5*), suggesting that other viral factors are also involved. It has been shown that integrase activates the ataxia-telangiectasia mutated–dependent pathway (7) and, thus, the double-strand breaks observed with HIV-1ΔVpr infection are probably owing to integrase. For viral integration to occur, the amount of double-strand breaks induced by HIV-1ΔVpr (Fig. 1*A*, *lane 5*) may be sufficient, because viral production in peripheral blood mononuclear cells was not alleviated by infection with

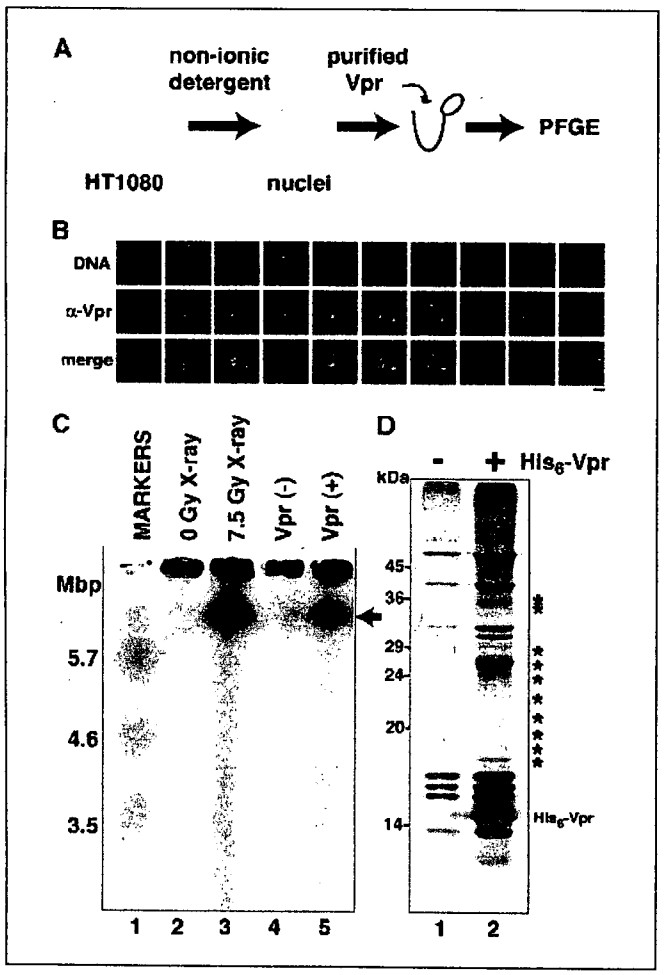

Figure 3. Purified Vpr induces double-strand breaks *in vitro*. *A*, a scheme of the protocol used to detect Vpr-induced double-strand breaks in isolated nuclei. *B*, Vpr localization in isolated nuclei. Isolated nuclei from HT1080 after incubation with Vpr were immunostained by α-Vpr (mAb8D1) and the images were captured by a laser confocal microscopy. The Z-series of optical sections collected at 1 μm steps of the cells were presented. Vpr (*red; middle*), DNA staining by Hoechst (*blue; top*) and their merged images (*bottom*) are shown. Without Vpr incubation, any signals by α-Vpr immunostaining were not detected in isolated nuclei (data not shown). Bar, 10 μm. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr. Molecular mass markers (*lane 1*), control cells (*lane 2*), cells subjected to X-ray irradiation (*lane 3*), and isolated nuclei without (*lane 4*) or with 10 μmol/L Vpr (*lane 5*). *Arrow*, double-strand breaks. *D*, Ni-NTA pull-down assay with His_6-tagged Vpr on isolated nuclei. Precipitated proteins bound to His_6-tagged Vpr (*lane 2*) and the control precipitates (*lane 1*) are indicated. *, His_6-Vpr–specific bands.

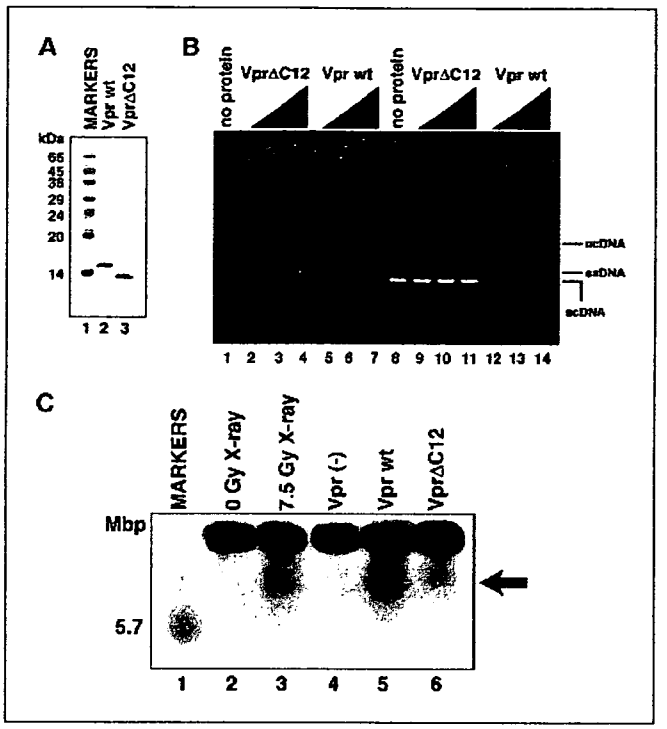

Figure 4. DNA-binding and double-strand break formation by Vpr. *A*, purification of VprΔC12. Purified VprΔC12 was analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. *Lane 1*, molecular mass markers. *Lanes 2* and *3*, purified wild-type Vpr and VprΔC12 protein, respectively. *B*, the DNA-binding activity of VprΔC12. The DNA-binding experiments were done using the protocol used to obtain Fig. 2*B*. The concentrations of VprΔC12 were 2.5 μmol/L (*lanes 2* and *9*), 5 μmol/L (*lanes 3* and *10*), and 10 μmol/L (*lanes 4* and *11*), and those of the wild-type Vpr were 2.5 μmol/L (*lanes 5* and *12*), 5 μmol/L (*lanes 6* and *13*), and 10 μmol/L (*lanes 7* and *14*). Negative controls without protein (*lanes 1* and *8*) are included. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr or VprΔC12. Molecular mass marker (*lane 1*), cells without (*lane 2*) or with (*lane 3*) 7.5 Gy of X-ray irradiation, control nuclei (*lane 4*), nuclei with Vpr (*lane 5*), and nuclei with VprΔC12 (*lane 6*). Vpr was used at 10 μmol/L. *Arrow*, double-strand breaks.

Vpr-deleted HIV-1 (18).[4] Vpr-induced double-strand breaks may be surplus to those required for viral integration (Fig. 1*A*, *lane 6*). The resultant DNA damage may reduce the integrity of the host genome.

Recently, DNA damage signaling was observed at an early stage of tumor development, suggesting that the DNA damage response is a mechanism to prevent the progression of pre-neoplasic lesions (5). If DNA repair is not accomplished correctly or is skipped because of unregulated checkpoint controls, the genomic structure would be altered severely (19). The progression of malignant tumors in AIDS-defining cancers is well documented in oncovirus infections (1, 2). If DNA damage increases the probability of neoplasia, Vpr-induced double-strand breaks with oncovirus infection may accelerate tumor progression during the clinical course of AIDS. In addition to AIDS-defining cancers, non-AIDS-defining cancers also occur at a higher incidence and the factor responsible for such oncogenesis is now a critical issue (3, 4). Vpr-induced DNA damage may result in

[4] M. Shimura, unpublished data.