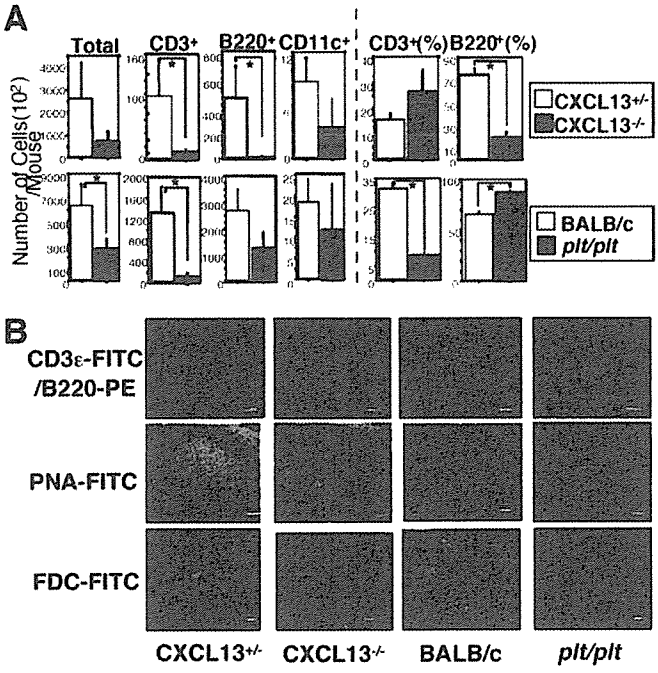

FIGURE 4. Microarchitecture of NALT in adult CXCL13$^{-/-}$ and *plt/plt* mice. *A*, Mononuclear cells isolated from the NALT of CXCL13$^{+/-}$, CXCL13$^{-/-}$, BALB/c, and *plt/plt* mice were analyzed using FACSCalibur. Data were obtained from three individual experiments. Significance was evaluated by an unpaired *t* test. *, $p < 0.05$. *B*, NALT was obtained from CXCL13$^{+/-}$, CXCL13$^{-/-}$, BALB/c, and *plt/plt* mice nasally immunized with cholera toxin. Frozen sections of NALT were incubated with anti-mouse CD3ε-FITC (green) and anti-mouse B220-PE (red) (*top panels*). The formation of a germinal center was analyzed by PNA-FITC (green) (*middle panels*). The network of FDC was stained by anti-FDC-FITC (green) (*bottom panels*). Scale of bars, 80 μm.

but were not involved in the genesis of tissue or the formation of microarchitecture like the germinal center and FDC network.

The cytokine signaling via LTβR induces the expression of CXCL13 by stromal cells in the B cell area for the recruitment of B cells into the follicular regions and the formation of germinal centers in spleen (7). This evidence provides a logical explanation as to why the microarchitecture of NALT is disorganized in mice lacking LTβR signaling (e.g., LTα$^{-/-}$ mice, LTβ$^{-/-}$ mice, and IκB kinase^{AA} mice) (3, 4, 14). Our data further support the findings by Ying et al. (15), which showed that the reduced production of CXCL13, CCL19, and CCL21 in LTα and LTβ deficiency resulted in the disorganization of NALT. Thus, not only do our results confirm the findings that CXCL13 is involved in the maintenance of the microarchitecture of NALT (9) (Fig. 4), they further show that it is not involved in the initiation of the tissue genesis (Figs. 1–3). The analysis of *plt/plt* mice showed that CCL19 and CCL21 promote T cell migration to NALT. CXCL13 also plays an essential role in the formation of the germinal center and FDC network, whereas CCL19 and CCL21 are not involved.

Our findings demonstrate that the lymphoid chemokine family interactions of CXCR5/CXCL13 and CCR7/CCL19 and CCL21 are not essential for the initiation of NALT genesis associated with the NALTi migration into the NALT anlagen. However, as our current study demonstrates, these lymphoid chemokines do play key roles in the creation and maintenance of NALT structure in adult mice. The latter finding is in total agreement with the recent study by Rangel-Monero et al. (9), which showed CXCL13, CCL19, and CCL21 were required for the organization of NALT. Our further examinations suggested that the lymphoid chemokine interactions of CXCR5/CXCL13 and CCR7/CCL19 and CCL21 provide distinct signals for the initiation of tissue genesis, as well as recruitment of lymphoid cells and subsequent microarchitecture formation of different mucosa-associated lymphoid tissues located in the aero-digestive tract.

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Disclosures

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Protective Effects of *IL4-589T* and *RANTES*-28G on HIV-1 disease progression in infected Thai females

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Objective: To evaluate the effect of polymorphisms in interleukin-4 (*IL4*) and *RANTES* promoters on disease progression in HIV-1 infected Thais.

Design: Antiretroviral (ARV) drug-free HIV-1 infected females from the prospective cohort.

Methods: A total of 246 DNA samples were genotyped for *IL4* and *RANTES* promoter polymorphisms by PCR–RFLP. Associations of genotype with HIV-1 disease progression were assessed with respect to baseline clinical data including plasma HIV-1 load, CD4 cell counts, and proportion of symptomatic/AIDS, and survival status during 3 years of follow-up.

Results: Patients with homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and a higher CD4 cell count ($P = 0.003$) than the other patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* allele. Kaplan–Meier analysis demonstrated an apparent but insignificant trend towards better survival in homozygous *IL4-589T* patients. On the other hand, patients with *RANTES-28G* allele showed a significantly better survival while those with *RANTES In1.1C* allele without *RANTES-28G* showed a significantly poorer survival compared with those who did not possess either *RANTES In1.1C* or *RANTES-28G* ($P = 0.02$), although those polymorphisms only weakly associated with baseline viral load and CD4 cell counts.

Conclusions: Our results implicate the significant protective effect of *IL4-589T* and *RANTES-28G* on HIV disease progression in Thais. In contrast, *RANTES In1.1C* without *RANTES-28G* had an accelerating effect on HIV disease progression.

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Keywords: *IL4-589T*, *RANTES* promoter polymorphisms, HIV disease progression, survival, Thailand, viral load, CD4 cells

Introduction

HIV-1 infected individuals have widely different rates of disease progression. Some infected individuals become symptomatic within 2–3 years while others remain asymptomatic for more than 10–15 years [1]. It is important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines.

RANTES is a natural CCR5 ligand and potently inhibits cell entry of HIV-1 that uses CCR5 as a coreceptor

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(R5 virus). Two single nucleotide polymorphisms (SNP) in *RANTES* promoter; *-403G/A* and *-28C/G*, were identified [2]. The *RANTES-28G* variant was shown to elevate promoter activity *in vitro* [2,3] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [2]. Although this result was not confirmed in subsequent studies in other ethnic groups in which the *RANTES-28G* frequency was lower than in the Japanese [3–5], McDermott *et al.* reported that patients with *RANTES-28G* showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [6].

With respect to *RANTES-403A* variants, HIV-1 infected European Americans with *RANTES-403A* were initially reported to progress more slowly to AIDS [6]. However, subsequent studies with a larger sample size showed that *RANTES-403A* or *RANTES In1.1C*, a *RANTES* intron polymorphism which is in a strong linkage disequilibrium with *RANTES-403A* and which negatively affects *RANTES* transcription *in vitro*, was reported to be associated with an increased rate of disease progression [3,4].

A variant of the interleukin-4 (*IL4*) promoter, *-589T* was found to be associated with elevated levels of serum IgE and to accelerate phenotypic switches of HIV-1 from non-syncytium inducing (NSI) to syncytium-inducing (SI) type in HIV-infected Japanese patients probably due to the suppressive effect of IL4 on CCR5 expression together with its enhancing effect on CXCR4 expression [7]. This allele was subsequently found to be associated with a lower viral load and a slower rate of disease progression in HIV-infected French patients [8,9]. However, studies in other ethnic groups did not show consistent results [10,11].

There is a considerable variation in allele frequencies at these loci among different ethnic groups. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and effects of host genetic polymorphisms may differ in individuals infected with different HIV-1 subtypes. Therefore, it is important to study influences of host genetic polymorphisms in different populations; however, such data in Asian populations remain sparse. In this paper, we report that polymorphisms in *IL4* and *RANTES* influence HIV load, CD4 cell count and survival time in Thai patients infected with HIV-1 subtype E.

Materials and methods

Patients and samples

We conducted a prospective cohort study at the HIV clinic in the Day Care Center of Lampang Hospital in northern Thailand. The Lampang Hospital is a government referral hospital with approximately 800 beds. It is located in the center of Lampang province, a province 600 km to the north of Bangkok. We approached all HIV-1 infected individuals who attended the clinic from 6 July 2000 to 12 July 2001 and asked them to participate in this cohort study. After giving written informed consent, all participants were interviewed by trained study coordinators, and clinically examined by two designated doctors. EDTA-treated blood was taken from each individual. Plasma and buffy coat fractions were separated and stored at −80°C until use. For a control group we collected blood samples from 119 female blood donors at the blood bank of the Lampang Hospital.

Plasma HIV-1 RNA copy number was measured using a commercial kit (Amplicor HIV-1 Monitor Test, Roche Molecular System, Inc. Branchburg, New Jersey, USA), which has a lower limit of detection of 400 copies/ml. CD4 cell count was measured by FACScan (BD Biosciences, California, USA) using fresh EDTA-treated whole blood samples at the time of recruitment. The survival status of participants until 1 October 2003 was ascertained from the cohort database, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were double entered and validated using the access program. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

Polymorphism genotyping

Genomic DNA was extracted and purified from the buffy coat fraction using QIAamp mini blood kit (QIAGEN GmbH, Hiden, Germany). Each specimen was analyzed for *IL4-589C/T*, *RANTES-403G/A*, *RANTES-28C/G*, *RANTES In1.1T/C*, and *RANTES 3′222T/C* by PCR–RFLP as described elsewhere [2,3,7].

Sample analysis

We conducted this study strictly in blinded manner so that field investigators were masked from any host genetic polymorphism data and laboratory investigators were masked from any clinical data. The data set was analyzed only after deleting sample and patient's identification number. Continuous variables of two groups with different genetic background were compared by a non-parametric Kruskal–Wallis test. Qualitative variables of two groups were compared by the Chi-square test. Significance in Kaplan–Meier analysis was determined by the log-rank test. Statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *RANTES* haplotypes were constructed and calculated for their frequencies by an Expectation-Maximization algorithm using software (Arlequin version 2.01, Genetica and Biometry Laboratory, Geneva, Switzerland). D' measure for linkage disequilibrium was also calculated by using Arlequin. Incidence rate ratios were calculated by STATA (Stata Corp., Texas, USA). Crude and adjusted relative risks (RR) and their 95% confidence intervals (CI) were calculated by Cox proportional hazard models using StatView (SAS Institute Inc. North Carolina, USA).

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Results

Baseline characteristics of study subjects

During the study period, 487 HIV-infected individuals participated in this study. There were 187 males and 300 females; baseline CD4 cell counts and antiretroviral (ARV) drug use information was available in 185 males and 297 females. We found that HIV-disease was significantly more advanced in men than in women. Median [interquartile range (IQR)] CD4 cell count at the time of recruitment in men [66 (15–258) cells/μl) was significantly lower ($P < 0.0001$) than that in women [262 (90–418)]. The proportion of patients diagnosed as AIDS was also higher ($P < 0.0001$) in men (77/185; 41.6%) than in women (44/297; 14.8%). Because the aim of this study was to investigate the influence of host genetic polymorphisms on disease progression and the majority of male individuals were already in the advanced stage of HIV disease, we analyzed the data of men and women separately and in this paper we present our analysis on the 297 females.

Among these females, 186 were ARV drug naive and 111 were ARV drug experienced: 51 were receiving ARV drugs and 60 reported prior ARV treatment but were not on ARV at the time of recruitment. The latter group included 18 females who were given zidovudine (ZDV) as prophylaxis for HIV mother-to-child transmission. The remaining 42 subjects stopped ARV drugs because of financial constraints.

All females reported that they had contracted HIV-1 through heterosexual contact. Baseline characteristics of ARV drug naive and ARV drug interrupted groups were similar (Table 1). In this analysis, we excluded the 51 individuals who were on ARV drug treatment, because on-going ARV drug treatment considerably affects CD4 cell count and viral load so that an association between these parameters and genetic polymorphisms may be obscured.

Allele frequencies of *IL4-589T* and *RANTES-403A, RANTES-28G, RANTES In1.1C,* and *RANTES 3'222C*

We genotyped 246 ARV drug-free females and 119 blood donors for *IL4-589C/T, RANTES-403G/A, RANTES-28C/G, RANTES In1.1T/C,* and *RANTES 3'222T/C.* Allele frequency of *IL4-589T* was 0.774 in 246 ARV drug-free females and 0.798 in 119 blood donors. Frequencies of four *RANTES* SNP in ARV drug-free females and blood donors are shown at the bottom of Table 2. There was no difference in frequency of those five SNP among subgroups, including the ARV-treated group (data not shown). All of the observed genotypes were in Hardy–Weinberg equilibrium (data not shown).

Table 2 shows estimated *RANTES* haplotype distribution in 246 ARV drug-free females and 119 blood donors. As reported in European- and African-Americans, the haplotype R1 was the most common. In European- and African-Americans, approximately 75% and 55% of *RANTES-403A* was associated with *RANTES In1.1C,* respectively. *RANTES-403A* was more closely associated with *RANTES In1.1C* in Thai patients than in European- and African-Americans, since 93.4% of *RANTES-403A* were associated with *RANTES In1.1C* in 246 ARV drug-free female patients ($D'=1.0$, Table 2). Therefore, we focused on *RANTES-28G* and *RANTES In1.1C* in the subsequent analysis among four *RANTES* polymorphisms, as *RANTES-28G* and *RANTES In1.1C* were shown to be functional and actually affected RANTES promoter activity [2,3], while roles of *RANTES-403A* and *RANTES 3'222T/C* as functional alleles were not yet confirmed.

Genotype variation and HIV disease status (clinical, CD4 cell count, viral load)

We analyzed viral load, CD4 cell count, and clinical status among ARV drug-free females at recruitment by stratifying individuals according to their host genetic background (Table 3). We found that patients with the homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and higher CD4 cell count ($P = 0.003$) than patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* alleles. Furthermore the proportion of symptomatic individuals was lower among females with homozygous *IL4-589T* allele than among others, although this trend did not reach statistical significance ($P = 0.183$).

Patients with *RANTES-28G* allele (*C/G* or *G/G*) showed a weak trend toward a higher CD4 cell count

Table 1. Baseline data of ARV drug-free HIV-1 infected females.

Characteristics	ARV drug naive (n = 186)	ARV treatment interrupted (n = 60)	Combined (ARV drug free) (n = 246)
Age at recruitment: median years (IQR)	31 (28–36)	31 (27.5–38)	31 (28–36)
CD4 cell count [median cells/μl (IQR)]	269 (71–422)	259.5 (63–400.5)	262 (67–421)
HIV-1 load [median $\log_{10}$ HIV RNA copies/ml (IQR)]	5.084 (4.424–5.549)	5.0194 (3.897–5.592)	5.078 (4.331–5.571)
AIDS- related symptoms [% (n)]	37.1(69)	45(27)	39 (96)
95% CI	30.1–44.5	32.1–58.4	32.9–45.4
Diagnose of AIDS [%(n)]	15.6 (29)	20 (12)	16.7 (41)
95% CI	10.7–21.6	10.8–32.3	12.3–21.9

ARV, Antiretroviral; IQR, interquartile range; CI, confidence interval.

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Table 2. *RANTES* haplotype frequencies in ARV drug free HIV-1 infected females and female blood donors.

RANTES Haplotype	*RANTES* polymorphism site				ARV drug free[a] (n = 246)	Blood donor[b] (n = 119)
	−403	−28	In1.1	3'222		
R1	*G*	*C*	*T*	*T*	0.697	0.731
R2	*A*	*C*	*T*	*T*	0.020	0.034
R3	*A*	*C*	*C*	*T*	0.002	0.000
R4	*A*	*C*	*C*	*C*	0.191	0.160
R5	*A*	*G*	*C*	*T*	0.089	0.076

[a]Allele frequencies of *RANTES-403A* = 0.303, *RANTES-28G* = 0.089, *RANTES In1.1C* = 0.283, and *RANTES 3'222C* = 0.191.
[b]Allele frequencies of *RANTES-403A* = 0.269, *RANTES-28G* = 0.076, *RANTES In1.1C* = 0.235, and *RANTES 3'222C* = 0.160. ARV, Antiretroviral.

($P = 0.165$), although the differences were not statistically significant. There was no significant difference in HIV-1 viral load or proportion of symptomatic individuals between patients with and without the *RANTES-28G* allele. Patients with *RANTES In.1C* showed no difference from those without this allele in HIV-1 viral load, CD4 cell count, or proportion of symptomatic cases.

Genotype variation and survival status

Of 246 ARV drug-free females, we obtained follow-up information from 238 (96.7%): the median (IQR) of follow-up was 964 (495–1072) days. During the follow-up period, 55 patients had started ARV drug treatment. Among 524.05 persons-years of follow up, 54.16 persons-years were ARV drug treatment positive. Therefore, the majority (89.7%) of our study subjects was ARV drug-free. There was no difference in proportion of patients who started ARV drugs during the observation period among each genotype group (Table 3). During the follow up period, 65 deaths occurred (including one suicide). Death was then used as the marker for HIV-1 disease progression. We found that the mortality rate among homozygous *IL4-589T* individuals was slightly lower than for the other groups, but this difference did not reach statistical significance [incidence rate ratio (IRR), 0.68; 95% CI, 0.40–1.14; $P = 0.121$) (Table 3). For *RANTES* promoter, there was significantly lower mortality rate among individuals with *RANTES-28G* allele when compared to those without this allele (IRR, 0.41; 95%CI, 0.15–0.95; $P = 0.024$) (Table 3). In contrast, there was no difference in the mortality rate between individuals with *RANTES In1.1C* and those without this allele (IRR. 1.11; 95%CI, 0.66–1.84; $P = 0.67$).

Table 3. Median HIV-1 load and CD4 cell count, HIV-1 related symptoms, diagnosis of AIDS and number of deaths/person years of observation (PYO) stratified by *IL4-589C/T*, *RANTES-28C/G*, and *RANTES In1.1T/C* genotype group.

	IL4-589		*RANTES-28*		RANTES In1.1	
	C/C and C/T (n = 99)	T/T (n = 147)	C/C (n = 205)	C/G and G/G (n = 41)	T/T (n = 133)	T/C and C/C (n = 113)
Median viral load [log_{10} copies/ml (IQR)]	5.381 (4.519–5.650)	4.908 (4.214–5.441)	5.107 (4.406–5.578)	4.843 (4.177–5.491)	5.098 (4.287–5.615)	5.053 (4.433–5.507)
P (Kruskal–Wallis test)		0.005		0.275		0.929
Median CD4 count [(cells/μl (IQR)]	171 (46–354)	303 (144–433)	261 (58–412)	297 (144–451)	285 (66–414)	244 (71–421)
P (Kruskal–Wallis test)		0.003		0.165		0.684
HIV-1 related symptoms [%(n)]	44.4(44)	35.4(52)	40.5(83)	31.7(13)	37.6(50)	40.7(46)
(95% CI)	(34.5–54.8)	(27.7–43.7)	(33.7–47.5)	(18.1–48.1)	(29.3–46.4)	(31.6–50.4)
P (Chi^2 test)		0.153		0.293		0.618
Diagnosis of AIDS [% (n)]	19.2(19)	15(22)	17.1 (35)	14.6 (6)	16.5(22)	16.8(19)
(95% CI)	(12.0–28.3)	(9.6–21.8)	(12.2–22.09)	(5.6–29.2)	(10.7–24.0)	(10.4–25.0)
P (Chi^2 test)		0.383		0.702		0.954
Deaths (n)	30	35	59	6	34	31
PYO	192.59	331.90	420.84	103.66	288.17	236.33
Mortility rate (%)	15.58	10.54	14.02	5.79	11.8	13.12
(95% CI)	(10.01–21.15)	(7.05–14.03)	(10.44–17.60)	(1.16–10.42)	(7.83–15.77)	(8.5–17.74)
Incidence rate ratio of death (95% CI)		0.68 (0.40–1.14)		0.41 (0.15–0.95)		1.11 (0.66–1.84)
P (Chi^2 test)		0.121		0.024		0.67
ARV treatment during follow-up	22	33	44	11	32	23
Observed (n)	94	144	197	41	129	109
P (Chi^2 test)		0.930		0.534		0.499

IQR, Interquartile range; CI, confidence interval.

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Consistent with the results on mortality rate, the Kaplan–Meier analysis demonstrated an insignificant better survival in homozygous *IL-589T* ($P = 0.125$) (Fig. 1a), a significant better survival in individuals carrying *RANTES-28G* ($P = 0.037$) (Fig. 1b), and no difference between individuals carrying *RANTES In1.1C* and those without this allele ($P = 0.668$) (Fig. 1c). Since *RANTES-28G* was associated with *RANTES In1.1C* (Table 3), we then grouped the patients as: (i) individuals who posses sed neither *RANTES-28G* nor *RANTES In1.1C*; (ii) individuals who possessed *RANTES In1.1C* but not *RANTES-28G*; and (iii) individuals who possessed

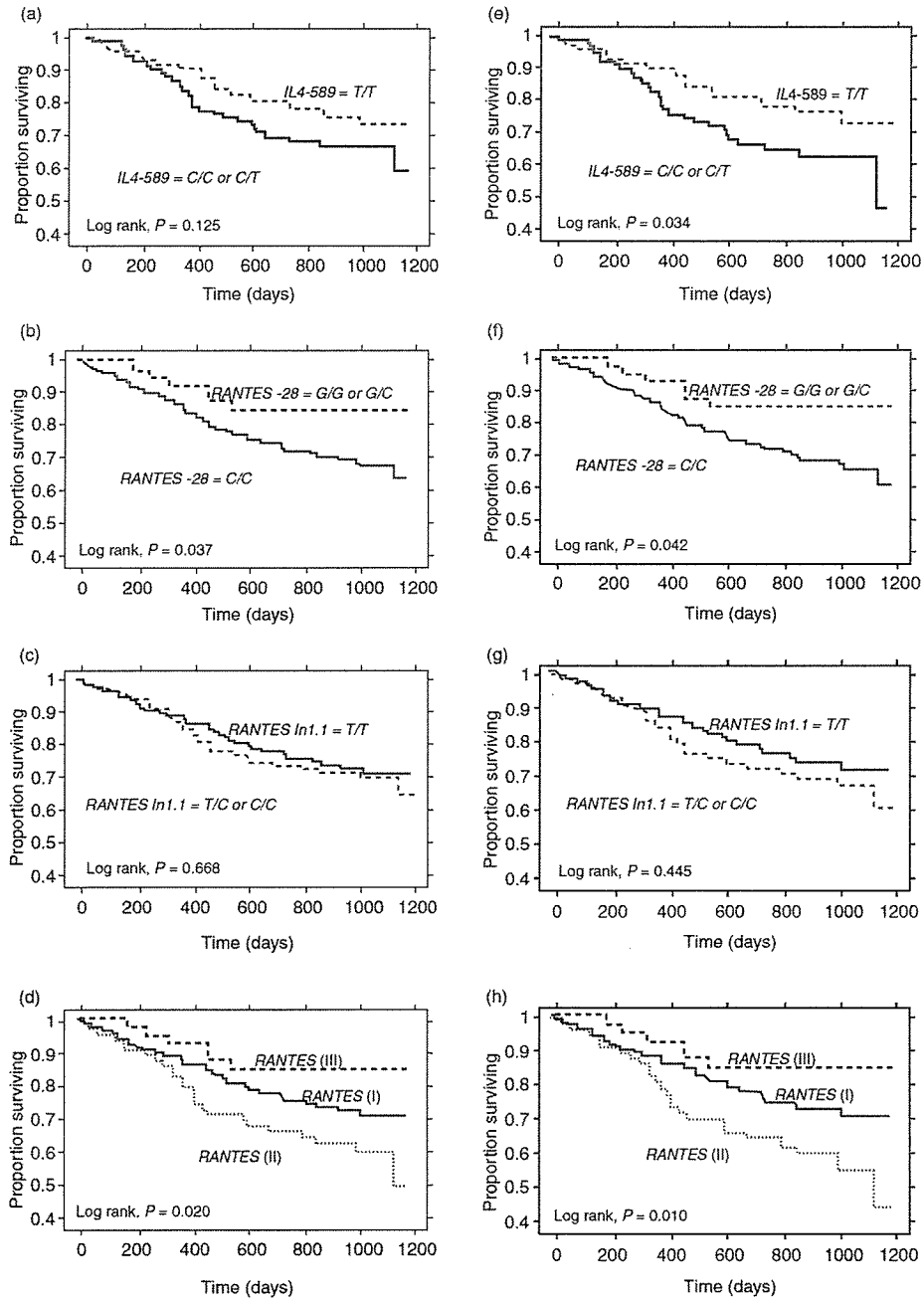

Fig. 1. Kaplan–Meier analysis of the effect of promoter polymorphism of *IL4* and *RANTES* on survival in ARV drug-free HIV-1 infected Thai females during the total observation period (a–d) or untreated period (e–h). (a and e) *IL4-589T*; (b and f) *RANTES-28*; (c and g) *RANTES-403*; and (d and h): grouped *RANTES* as (I) individuals who possessed neither *RANTES In1.1C* nor *RANTES-28C*, (II) individuals who possessed *RANTES In1.1C* but not *RANTES-28C*, and (III) individuals who possessed *RANTES-28C*.

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RANTES-28G. The analysis clearly showed a poorer survival in the group (ii) and a better survival in group (iii) when compared with group (i) ($P = 0.020$) (Fig. 1d). These results suggested that *RANTES-28G* had a protective effect against HIV-1 disease progression in Thai patients, and that *RANTES In1.1C* without *RANTES-28G* had an accelerating effect. We obtained virtually the same results as above when we used *RANTES-403A* instead of *RANTES In1.1C* (data not shown).

We then subtracted the numbers of days after patients started to receive ARV drugs from the observation period in order to adjust possible effects of ARV treatment, although the majority of our study subjects was ARV drug-free during the observation period. As shown in Fig. 1e–h, we obtained almost identical results to those shown in Figure 1a–d, except that difference between homozygous *IL4-589T* individuals and the others became statistically significant ($P = 0.034$, Fig. 1e). Thus, the risk of death for homozygous *IL4-589T* patients during the untreated period was lower than the other patients with heterozygous *IL4-589T* or homozygous *IL4-589C* (RR, 0.59 Cox model; 95% CI, 0.36–0.97; Fig. 1e). The RR of death for patients with *RANTES-28G* during the untreated period was also lower than those without *RANTES-28G* (RR, 0.43; 95% CI, 0.19–0.99; Fig. 1f). The RR of death for patients with *RANTES In1.1C* during the untreated period was not significantly higher than those without this allele (RR, 1.21; 95% CI, 0.74–1.98; Fig. 1g).

However, after adjustment for *RANTES-28G*, the RR of death for patients with *RANTES In1.1C* became significantly higher (adjusted RR, 1.75; 95% CI, 1.03–2.95). Similarly, the RR of death for patients with *RANTES-28G* became lower after adjustment for *RANTES In1.1C* (adjusted RR, 0.30; 95% CI, 0.12–0.74). These results were most likely caused by the fact that protective *RANTES-28G* was in strong linkage disequilibrium with deleterious *RANTES In1.1C* as shown in Table 2. Further adjustment for *IL4-589T* did not affect RR of death for patients with *RANTES-28G* (adjusted RR, 0.30; 95% CI, 0.12–0.74) or patients with *RANTES In1.1C* (adjusted RR, 1.79; 95% CI, 1.06–3.02). Similarly, adjustment for *RANTES-28G* and *RANTES In1.1C* did not influence the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.59; 95% CI, 0.35–0.96). These results suggested that *IL4* and *RANTES* were independent factors involved in protection against HIV-1 disease progression.

Further adjustment for age at recruitment and previous ARV drug history did not affect the RR of death during the untreated period for homozygous *IL4-589T* patients (adjusted RR, 0.54; 95% CI, 0.32-0.89), patients with *RANTES-28G* (adjusted RR, 0.27; 95% CI, 0.11–0.68) or patients with *RANTES In1.1C* (adjusted RR, 1.94; 95% CI, 1.14–3.30). Adjustment for diagnosis of AIDS at the time of recruitment only slightly affected the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.61; 95% CI, 0.37–1.00; $P = 0.051$). However, the protective effect of homozygous *IL4-589T* became no longer significant after adjustment for serum virus load (adjusted RR, 0.76; 95% CI, 0.45–1.27; $P = 0.293$) or CD4 cell count (adjusted RR, 0.86; 95% CI, 0.51–1.44; $P = 0.563$). Adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment almost completely diminished the protective effect of homozygous *IL4-589T* (adjusted RR, 0.95; 95% CI, 0.56–1.62; $P = 0.854$). These results suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count. In a sharp contrast, the protective effect of *RANTES-28G* and the deleterious effect of *RANTES In1.1C* were not influenced by adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment (adjusted RR of *RANTES-28G*, 0.27; 95% CI, 0.11–0.68; adjusted RR of *RANTES In1.1C*, 1.98; 95% CI, 1.15–3.41).

Discussion

This is the first host-genetic polymorphism study with a substantial sample size in the Southeast Asia, addressing an effect on HIV-1 disease progression. The *IL4-589T* allele was very common (0.78) in Thai patients as in other Asians – 0.69 in Japanese, 0.77 in Chinese – whereas it presents at a significantly lower frequency (0.15) in Caucasians [8,10,11]. For the *RANTES* gene, we observed more *RANTES-28G* (0.09) than in European and African–Americans [3,4,6]. Although our study group was not a sero-conversion cohort, we found a statistically significant association of the homozygous *IL4-589T* with a lower viral load, a higher CD4 cell count and a better survival during the untreated period. Individuals with *RANTES-28G* allele were also had a better survival rate.

The presence of *IL4-589T* allele was reported to be associated with an increased promoter activity [12]. Thus one expects the down-regulation of CCR5 and the up-regulation of CXCR4 in the CD4 T cell population in individuals with this allele [13]. Consequently, in individuals with *IL4-589T*, levels of replication of R5 viruses would be lower than in those without *IL4-589T*. It has been shown that R5 viruses were generally resistant to neutralizing antibodies whereas X4 viruses are sensitive, and X4 viruses are rarely detected until the advanced stage of HIV disease [14]. If it is the R5 viruses that are mainly responsible for driving immune deficiency in HIV infection, it is plausible that a higher expression of IL-4 associates with a better prognosis.

Two previous studies based on two independent cohorts in France showed a protective effect of *IL4-589T* on

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HIV disease progression [8,9]. One paper based on the Amsterdam cohort showed an association of this allele with the delay of acquisition of X4 virus and the decline of CCR5 expressing memory CD4 T cell numbers, though this study did not show effects of *IL4-589T* on overall disease progression [10]. Our present study based on a cohort in Thailand is the third example to demonstrate the significant protective effect of *IL4-589T* on HIV disease progression. Only one previous report, which analyzed five USA natural history cohorts including several ethnic groups, failed to show such an association [11]. At present, the reason for the discrepancy among different studies is unclear, but may be due to the differences in study design (sero-conversion/cross-sectional, marker/endpoint of disease progression, duration of follow-up, etc.) or difference in the frequency of this allele. In addition, interactive effects may differ among different populations due to haplotype structure and allele frequency differences. As our study population was not a sero-conversion cohort, a considerable number of individuals had already progressed to end-stage HIV disease by the time of study enrollment. It is possible that individuals at the end stage of diseases might cause certain biases that we could not realize at present.

Compared with the most common *RANTES* haplotype (*RANTES In1.1T* and *RANTES-28C*), our study showed that individuals carrying *RANTES In1.1C* with *RANTES-28G* had better survival, while individuals carrying *RANTES In1.1C* without *RANTES-28G* had poorer survival. Our present finding on *RANTES-28G* was concordant with the previous study on Japanese patients [12], in whom the frequency of *RANTES-28G* was higher than in Thais. On the other hand, our result on *RANTES In.1C* was concordant with the previous results that *RANTES In1.1C* was associated with an increased rate of HIV-1 disease progression in African–Americans [3], in whom *RANTES-28G* is absent. Since most of *RANTES In1.1C* are associated with *RATNES-403A* in Thai patients (Table 2), our finding on *RANTES In1.1C* was also concordant with the previous results that *RANTES-403A* was associated with an increased rate of HIV-1 disease progression in European Americans [4], in whom the frequency of *RANTES-28G* was much lower than in Thais. Therefore, the discrepancy of the effect of *RANTES* polymorphisms in different studies may be due to the difference in *RANTES-28G* allele frequency in different ethnic groups. However, the differences in study design and effects of other genetic polymorphism background could not be excluded.

In contrast to the *IL4-589T* allele, the effect of *RANTES* polymorphisms on HIV-1 diseases was more clearly observed in the survival analysis (Fig. 1) than in baseline viral load and CD4 cell counts (Table 4). Analysis by the Cox proportional hazard models suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count, whereas effects of *RANTES* polymorphisms were totally independent from those factors. The reason for this discrepancy is not clear at present. One possible explanation for this discrepancy is that the *RANTES* polymorphisms affect HIV-1 disease progression through the mechanisms other than the direct suppressive effect of RANTES on HIV-1 entry. RANTES also affects trafficking and proliferation of cells expressing CCR1, CCR3 or CCR5 [15]. It is possible that, in individuals with different *RANTES* genotypes, certain types of cells expressing those chemokine receptors can be modulated to different extents, leading to the different degree of susceptibility to disease progression and/or opportunistic infections. It is noteworthy that numbers of CD8 cells, which are known to express more CCR5 than CD4 cells, also tended to be higher in patients with *RANTES-28G* than those without this allele in 246 drug-free Thai females (941 and 862 cells/μl, $P = 0.178$). Further studies are necessary to elucidate precise roles of RANTES in HIV-1 disease courses.

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Transcriptional Repression of Human Immunodeficiency Virus Type 1 by AP-4*

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Elucidation of the mechanism of transcriptional silencing of human immunodeficiency virus type 1 (HIV-1) provirus in latently infected cells is crucial to understand the pathophysiology of HIV-1 infection and to develop novel therapies. Here we demonstrate that AP-4 is responsible for the transcriptional repression of HIV-1. We found that AP-4 site within the viral long terminal repeat (LTR) is well conserved in the majority of HIV-1 subtypes and that AP-4 represses HIV-1 gene expression by recruiting histone deacetylase (HDAC) 1 as well as by masking TATA-binding protein to TATA box. AP-4-mediated transcriptional repression was inhibited by an HDAC inhibitor, tricostatin A, and could be exerted even at distant locations from the TATA box. In addition, AP-4 interacted with HDAC1 both *in vivo* and *in vitro*. Moreover, chromatin immunoprecipitation assays have revealed that AP-4 and HDAC1 are present in the HIV-1 LTR promoter in latently infected ACH2 and U1 cells, and they are dissociated from the promoter concomitantly with the association of acetylated histone H3, TBP, and RNA polymerase II upon TNF-α stimulation of HIV-1 replication. Furthermore, when AP-4 is knocked down by siRNA, HIV-1 production was greatly augmented in cells transfected with a full-length HIV-1 clone. These results suggest that AP-4 may be responsible for transcriptional quiescence of latent HIV-1 provirus and give a molecular basis to the reported efficacy of combination therapy of conventional anti-HIV drugs with an HDAC inhibitor in accelerating the clearance of HIV-1 from individuals infected with the virus.

Human immunodeficiency virus type 1 (HIV-1)[2] is a cytopathic retrovirus and the primary etiological agent of acquired immunodeficiency syndrome (AIDS) and related disorders. Among the various steps of viral life cycle, the step of transcription from HIV-1 provirus is conceived to be crucial for viral replication since amplification of the viral genetic information is attainable only through transcription. HIV-1 transcription is directed by the promoter located in the 5′ long terminal repeat (LTR) of the integrated provirus and is controlled by cellular factors that bind to the multiple *cis*-regulatory elements located in the LTR as well as the virally encoded Tat protein (reviewed in Refs. 1 and 2). In cells chronically infected with HIV-1, activation of nuclear factor-κB (NF-κB) by external stimuli such as tumor necrosis factor (TNF)-α and its binding to LTR triggers the initiation of transcription of viral genes including Tat, which results in explosive HIV-1 replication (reviewed in Refs. 3 and 4). However, little is known how transcription from HIV-1 provirus remains silent during the viral latency.

There are multiple mechanisms known to be involved in the negative regulation of HIV transcription including elimination of transcriptional activator TATA-binding protein (TBP) transcriptional factor IID (TFIID) and the initiator protein complex by leader-binding protein (LBP)-1 (5) and YY-1 (6) that recruits histone deacetylase (HDAC) (7, 8), and actions of transcription factors that interact with the negative regulatory element (NRE) located from −340 to −184 of HIV-1 LTR (2, 9). Regarding the action of NRE, the mechanism by which NRE exerts its negative effect on transcription remains unknown because most of the transcription factors that interact with NRE are transcriptional activators. In addition, an *in vitro* study has revealed a potential role of activator protein (AP)-4 in blocking the TBP binding to TATA box (10). However, biological significance of this finding has not been clarified although the sequence comparison has revealed conservation of AP-4 sites in the majority of HIV-1 isolates (Fig. 1*A*) (11–13).

The HIV-1 LTR TATA box is located at −27 to −23 relative to the transcription initiation site (2, 11–14). TFIID interacts with TATA box and is crucial for HIV-1 gene expression (9, 14–16). TFIID contains the 38-kDa TBP as the major component and induces transcriptional initiation by interacting with other general transcription factors and recruiting RNA polymerase II (RNAPII) (17). TBP (TFIID) also serves as the target of DNA-binding factors binding to the *cis*-regulatory elements within HIV-1 LTR in both positive and negative fashions and thus determines its promoter activity (reviewed in Refs. 16, 18, and 19).

AP-4 is a ubiquitously expressed transcription factor of the basic helix-loop-helix leucine-zipper (bHLH-Zip) subgroup of bHLH proteins and binds to the symmetrical DNA sequence 5′-CAGCTG-3′ (20, 21). AP-4 site is found adjacent (−21/−16) to the HIV-1 TATA box (−27/−23) (10). Although AP-4 was initially identified as a cellular protein that binds to the simian virus 40 (SV40) enhancer and activates the viral late gene transcription (21), transcriptional repression by AP-4 was reported in a number of other genes including angiotensinogen (21) and E7 oncoprotein of human papillomavirus type 16 (23). In addition, we recently found that AP-4 negatively regulates transcription of 8-oxo guanine DNA glycosylase 1 (*OGG1*) gene (24). However, because AP-4 sites are not located adjacent to the TATA box in these promoters, the molecular mechanism of its repressive action is yet to be clarified.

In this study we investigated the role of AP-4 in HIV-1 gene expression. Here we show that AP-4 represses HIV-1 transcription by recruiting HDAC1 as well as by masking the TBP to the HIV-1 TATA box. Biological and therapeutic implications are discussed.

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[2] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; TBP, TATA-binding protein; HDAC, histone deacetylase; NRE, negative regulatory element; aa, amino acids; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay; TNF, tumor necrosis factor; TSA, tricostatin A; ELISA, enzyme-linked immunosorbent assay.

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EXPERIMENTAL PROCEDURES

Cell Culture—CEM, HL-60, Jurkat, ACH2, and U1 cells were maintained at 37 °C in RPMI 1640 (Sigma) with 10% fetal bovine serum (Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml). To maintain the latency of the HIV-1 in ACH2 and U1, 20 μM AZT was added in the culture medium and was excluded prior to experiments. Human embryonic kidney 293 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Sigma) with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin.

Plasmids—Construction of mammalian expression plasmids pMyc-AP-4, containing the full-length AP-4 cDNA, pCMV-Tat, and pNL4-3 were described previously (24, 25). pCMV-TBP was a generous gift from T. Tamura (Chiba University). To generate pcDNA-AP-4 (full), pcDNA-ΔN100 AP-4 (100–355 aa), pcDNA-ΔN143 AP-4 (143–355 aa), pcDNA-ΔN180 AP-4 (180–355 aa), pcDNA-ΔC179 AP-4 (1–179 aa), and pcDNA-ΔC130 AP-4 (1–130 aa), each containing a FLAG epitope tag in the N terminus and a V5 epitope tag in the C terminus, the various portions of AP-4 cDNA were amplified by PCR using pMyc-AP-4 as a template with 5′ and 3′ oligonucleotide primers. These products were subcloned into pcDNA 3.1 TOPO V5 vector (Invitrogen). Construction of HIV-1 LTR-based luciferase expression plasmid: CD12-luc (containing the HIV-1 LTR U3 and R) was previously described (25). The mutant HIV-1 LTR luciferase reporter constructs lacking AP-4 binding were generated using a QuikChange site-directed mutagenesis kit (Stratagene). The mutant sequences (sense strand) utilized were the following: CD12-luc-m1, GAT CCT GCA TAT AAG tcg cga CTT TTT GCC TGT AC; CD12-luc-m2, GCA TAT AAG CAG CTc CTT TTT GCC TGT AC; CD12-luc-m3, GCA TAT AAG CAG Cgc TTA AGA TAC AGC; CD12-luc-m4, CCT GCA TAT AAG CAG tcG CTT TTT GCC TGT AC (consensus AP-4 binding sites are underlined, and the mutated sequences are in small letters). The mutant HIV-1 LTR-directed reporter constructs, in which the authentic AP-4 site is mutated and an AP-4 site is aberrantly inserted into various positions of the CD12-luc-m2 reporter plasmid, were constructed by PCR using CD12-luc-m2 DNA as a template and site-directed mutagenesis kit with the following mutagenesis oligonucleotide primer pairs: CD12-luc-m2(+55), forward (5′-GCT AGC TAG GGA ACA GCT GCC CAC TGC TTA AG-3′) and reverse (5′-CTT AAG CAG TGG GCA GCT GTT CCC TAG CTA GC-3′); CD12-luc-m2(−79), forward (5′-CTG GGG ACT TTC CAC AGC TGG GGA GGC GTG GCC-3′) and reverse (5′-GGC CAC GCC TCC CCA GCT GTG GAA AGT CCC CAG-3′); CD12-luc-m2(-150), forward (5′-GTG GCC CGA GAG CTC AGC TGG CAT CCG GAG TAC-3′) and reverse (5′-GTA CTC CGG ATG CCA GCT CAG CTC TCG GGC CAC-3′); CD12-luc-m2(−400), forward (5′-GAT CTG TGG ATC TCA GCT GAC CAC ACA CAA GG-3′) and reverse (5′-CCT TGT GTG TGG TCA GCT GAG ATC CAC AGA TC-3′). The mutant pNL4-3 containing mutation in AP-4 binding was generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene) (24) with oligonucleotide primer pairs: forward, 5′-CAT ATA AGC AGC TcC TTT TTG CCT GTA C-3′ and reverse 5′-GTA CAG GCA AAA AGg AGC TGC TTA TAT G-3′ (mutated AP-4 binding sites are underlined and the mutated nucleotides are in lowercase letters). We first constructed the 5′-LTR AP-4 site mutant in the background of pNL4-3 by site-directed mutagenesis and additional AP-4 site mutation in the 3′-LTR was subsequently introduced into this mutant by site-directed mutagenesis. All constructs were confirmed by dideoxynucleotide sequencing using ABI PRISM™ dye terminator cycle sequencing ready kit (PerkinElmer Life Sciences) on an Applied Biosystems 313 automated DNA sequencer.

Recombinant Protein and Purification—pGEX expression vector (Amersham Biosciences) was utilized to express glutathione *S*-transferase (GST) fusion proteins in bacteria. To generate pGEX-AP-4-expressing GST-AP-4, the AP-4 cDNA was amplified by PCR using pMyc-AP-4 as a template with oligonucleotide primer pairs: forward, 5′-CGG GAT CCC GGA GTA TTT CAT GGT GCC CAC TCA G-3′, containing an BamHI site; reverse, 5′-GGA ATT CCT CAG GGA AGC TCC CCG TCC CCC G-3′, containing an EcoRI site. This product was digested with BamHI and EcoRI, and subcloned in-frame into pGEX-5X-3 vector at the BamHI/EcoRI sites. pGEX-AP-4 was transformed in *Escherichia coli* strain DH5 and expression of recombinant GST-AP-4 protein was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 6 h. Recombinant GST proteins were purified by affinity chromatography on glutathione-agarose beads as described previously (26).

Electrophoretic Mobility Shift Assay (EMSA)—The experimental procedure was carried out as described previously (24). Purified recombinant TBP and GST-TBP proteins were purchased from Promega and Santa Cruz Biotechnology, respectively. The double-stranded DNA oligonucleotides corresponding to −42/+4 of HIV-1 LTR (CD12) containing the binding sites of TBP and AP-4 and their mutants were synthesized. The wild-type and mutant oligonucleotide sequences (sense strand) were the following: wild-type (5′-CCC TCA GAT CCT GCA TAT AAG CAG CTG CTT TTT GCC TGT A-3′) and mutants (the underlined AP-4 site has been changed to TGACGG (m1), TAGCTC (m2), CAGCGC (m3), and CAGTCG (m4)) (Fig. 2*B*). These oligonucleotides were labeled using the 5′-end-labeling kit (Takara, Otsu, Shiga, Japan) in the presence of [γ-^{32}P]dATP (Amersham Biosciences). DNA binding reactions were performed at 30 °C for 30 min for TBP and room temperature for 20 min for AP-4. Analysis of protein-DNA complexes was performed by electrophoresis in 6% native polyacrylamide gels with 0.5× Tris borate-EDTA buffer at a constant voltage of 125 V at 4 °C, followed by autoradiography. The specificity of DNA binding was assessed by preincubating with purified GST-AP-4, GST-TBP, or control GST proteins with specific antibodies or competitors for 20 min prior to the addition of the probe.

Anti-AP-4 Antibody—Anti-AP-4 antibody was obtained by immunizing rabbits with GST-AP-4 fusion protein as no immunoprecipitable anti-AP-4 antibody was currently available from any commercial source. The immunized rabbit anti-AP-4 sera were affinity-purified by passing through affinity columns, and the lack of immunoreactivity with GST and other *E. coli* components was confirmed.

Immunoprecipitation and Immunoblot Assays—The experimental procedures for immunoprecipitation and immunoblotting were performed as described (24, 27). Briefly, cells were harvested with lysis buffer (25 mM HEPES-NaOH, pH 7.9, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation, and the supernatants were incubated with anti-AP-4 antibody overnight at 4 °C. Immune complexes were washed three times with 1 ml of lysis buffer and antibody-bound proteins were dissolved by boiling in 2× Laemmli sample buffer. After centrifugation, the supernatant proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C, Amersham Biosciences). The membrane was probed with anti-AP-4 antibody, and immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce). To evaluate the level of AP-4 protein, cells were similarly treated with the lysis buffer, and the cell lysates were analyzed by immunoblotting using anti-AP-4 or V5 antibody (Invitrogen).

Transfection and Luciferase Assay—293 cells cultured in 12-well plates were transfected using Fugene-6 transfection reagent (Roche Applied Science) as described previously (26, 27). CEM and HL60 cells were transiently transfected by electroporation as reported (24). Briefly,

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2×10^7 cells/ml were electroporated with 2 μg of CD12-luc together with 2 μg of pCMV-Tat and indicated amounts of Myc-AP-4 in 400 μl of serum-free RPMI using the Electro Cell Manipulator 600 (BTX Electroporation System) apparatus at 260 V/1050 μfarads. For the internal control, we employed pRL-TK, expressing *Renilla* luciferase under the control of the thymidine kinase promoter not containing the AP-4 site. The transfected cells were harvested, and the extracts were subjected to the luciferase assay using the Luciferase Assay System™ (Promega). All the experiments were carried out in triplicates, and the data were presented as the fold increase in luciferase activities (means ± S.D.) relative to the control for three independent transfections.

RNA Interference—The siRNAs with two thymidine residues (dTdT) at the 3′-end of the sequence were synthesized by Takara. The target sequences were as follows: AP-4-1 (5′-GUG CCC UCU UUG CAA CAU U-3′), AP-4-2 (5′-GGU CAU CAA CUC UGU UUC C-3′), and GFP (5′-GGC UAC GUC CAG GAG CGC ACC-3′). Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) reagents.

In Vitro Binding Assay—An *in vitro* protein-protein interaction assay was carried out as described previously (26). Briefly, AP-4 and luciferase proteins were labeled with [^{35}S]methionine *in vitro* transcription/translation using the TNT wheat germ extract-coupled system (Promega) according to the manufacturer's protocol. Approximately 20 μg of GST fusion proteins were immobilized on 20 μl of glutathione-Sepharose beads and washed two times with 1 ml of modified HEMNK buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After the final wash, 0.6 ml of beads suspension was incubated with radiolabeled proteins for 12 h at 4 °C. The beads were then washed two times with 1 ml of HENMK buffer and two times with HENMK buffer containing 150 mM KCl. Bound radiolabeled proteins were eluted with 30 μl of Laemmli sample buffer, boiled for 3 min, and resolved by 10% SDS-PAGE.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed according to the provider's protocol (Upstate Biotechnology) with some modifications as previously described (24). Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold phosphate-buffered saline, and lysed for 10 min at 2×10^6 cells in 200 μl of SDS lysis buffer. The cross-linked chromatin was sheared by sonication 13 times for 10 s at one-third of the maximum power of microson XL sonicator (Wakenyaku, Co., LTD., Kyoto, Japan) with 20 s of cooling on ice between each pulse. Cross-linked and released chromatin fractions were precleared with salmon sperm DNA and protein A-agarose beads for 1 h, followed by immunoprecipitation with the desired antibodies overnight at 4 °C. The immunoprecipitates were sequentially washed once with lysis buffer, twice with high salt buffer, twice with low salt buffer, and twice with TE buffer. After the wash, immune complexes were collected with salmon sperm DNA and protein A-agarose beads at room temperature for 1 h and extracted with 1% SDS, 0.1 M $NaHCO_3$. The eluted samples were reverse cross-linked by proteinase K at 45 °C for 1 h and treated with RNase at 37 °C for 1 h. DNA was recovered by phenol/chloroform and chloroform extractions, and ethanol precipitation. Finally, DNA was dissolved in 30 μl of TE buffer and subjected to PCR. The primer sequences used for PCR were the following: HIV-1 LTR (−109 to +79): forward (5′TAC AAG GGA CTT TCC GCT GG-3′) and reverse (5′-TTG AGG CTT AAG CAG TGG G-3′); β-actin promoter (−980 to −915) (as a control): forward (5′-TGC ACT GTG CGG CGA AGC-3′) and reverse (5′-TCG AGC CAT AAA AGG CAA-3′). The number of PCR cycles was as the following: 33 PCR cycles for all the ChIP experiments and 24 PCR cycles for the input samples, in which PCR amplification was obtained under the linear range of AP-4 binding to the HIV-1 LTR DNA. For each reaction, 10% of cross-linked released chromatin was saved and reversed by proteinase K digestion at 45 °C for 1 h followed by DNA extraction, and the recovered DNA was used as input control.

Antiviral Assay and Measurement of Viral p24 Antigen—Antiviral activity of AP-4 was evaluated based on the extent of inhibition of viral antigen expression in the culture supernatants of Jurkat or 293 cells transfected with a full-length HIV-1 molecular clone (pNL4-3) or mutant pNL4-3, in which AP-4 site is mutated. 293 cells were transfected with 0.1 μg of pNL4-3, together with various amounts of plasmids encoding wild-type AP-4 or AP-4 mutants with Fugene-6 transfection reagent. For siRNA studies, 100 nM siRNAs were introduced with 0.1 μg of pNL4-3 using Lipofectamine 2000 reagent. Jurkat cells were transfected by Nucleofector™ kit V for Jurkat cell (Amaxa Biosystems) according to the manufacturer's protocol. Briefly, 3×10^6 cells were mixed with 0.2 μg of wild-type or mutant pNL4-3 together with indicated amounts of FLAG-AP-4 in 100 μl of Nucleofector™ solution V. These samples were transferred into a transfection cuvette and subjected to electroporation using program T-14. The transfected cells were incubated in culture flasks with a complete media for 36 h. Then, cells were incubated for an additional 24 h in the presence or absence of TNF-α (3 ng/ml). The p24 antigen level in the cell culture supernatant was measured by p24 antigen capture ELISA assay using a commercial kit (RETRO-TEK HIV-1 p24 Antigen ELISA kit; Zepto Metrix Corp., Buffalo, NY) as described previously (25).

RESULTS

AP-4 Competes with TBP for Binding to the HIV-1 TATA Element—The HIV-1 TATA is located at nucleotide position from −27 to −23 relative to the transcription initiation site. The consensus AP-4 site, CAGCTG, is located −21 to −16 nucleotides immediately downstream of the TATA box. The AP-4 binding site in the HIV-1 LTR appears to be conserved in the majority of HIV-1 isolates (11–13). As shown in Fig. 1*A*, a majority of HIV-1 clones contain typical AP-4 binding sequence, CAGCTG, whereas it is mutated to CAGCCG in HIV-1 subtypes F1-F2, G, O, and 01-AE.

Because the AP-4 binding site is located close to the TATA box, we first examined the effect of AP-4 on the binding activity of TBP to the TATA box *in vitro*. To address this issue, recombinant AP-4 protein was produced and purified (Fig. 1, *B* and *C*). As shown in Fig. 1*D* (*left panel*), EMSA analysis using a DNA probe (−42/+4) containing both the TATA box and AP-4 site, showed that AP-4 blocked the TBP binding to TATA box in a dose-dependent manner (*lanes 6* and *7*). The control GST proteins did not alter the DNA binding activity of TBP (Fig. 1*D*, *right panel*). These results were consistent with a previous study by Ou *et al.* (10).

Repression of HIV-1 LTR Gene Expression by AP-4—Because AP-4 masks the TBP binding to the HIV-1 TATA box *in vitro*, we examined the effect of AP-4 on transcription from HIV-1 LTR. The luciferase reporter plasmid containing the HIV-LTR (CD12-luc) was cotransfected with an AP-4 expression vector (pMyc-AP-4) into CEM, HL-60, and 293 cell lines. As shown in Fig. 2*A*, the basal transcriptional level from HIV-1 LTR was inhibited by AP-4 in a dose-dependent manner in all the cell lines tested. Upon stimulation of HIV-1 promoter by TNF-α, a physiological inducer of NF-κB, AP-4 could similarly exert its negative effect. In addition, AP-4 also inhibited the Tat-induced HIV-1 gene expression in these cells.

To address whether the inhibitory effect of AP-4 depends on the presence of AP-4 site, we have created HIV-1 LTR mutants where the AP-4 binding site was mutated (Fig. 2*B*). As shown in Fig. 2*C*, EMSA confirmed that these mutants lost AP-4 binding. Although the inhibitory effect of AP-4 on wild-type LTR was clearly observed (Fig. 2*A*), it was abolished when basal, TNF-α-stimulated, and Tat-stimulated gene expression were

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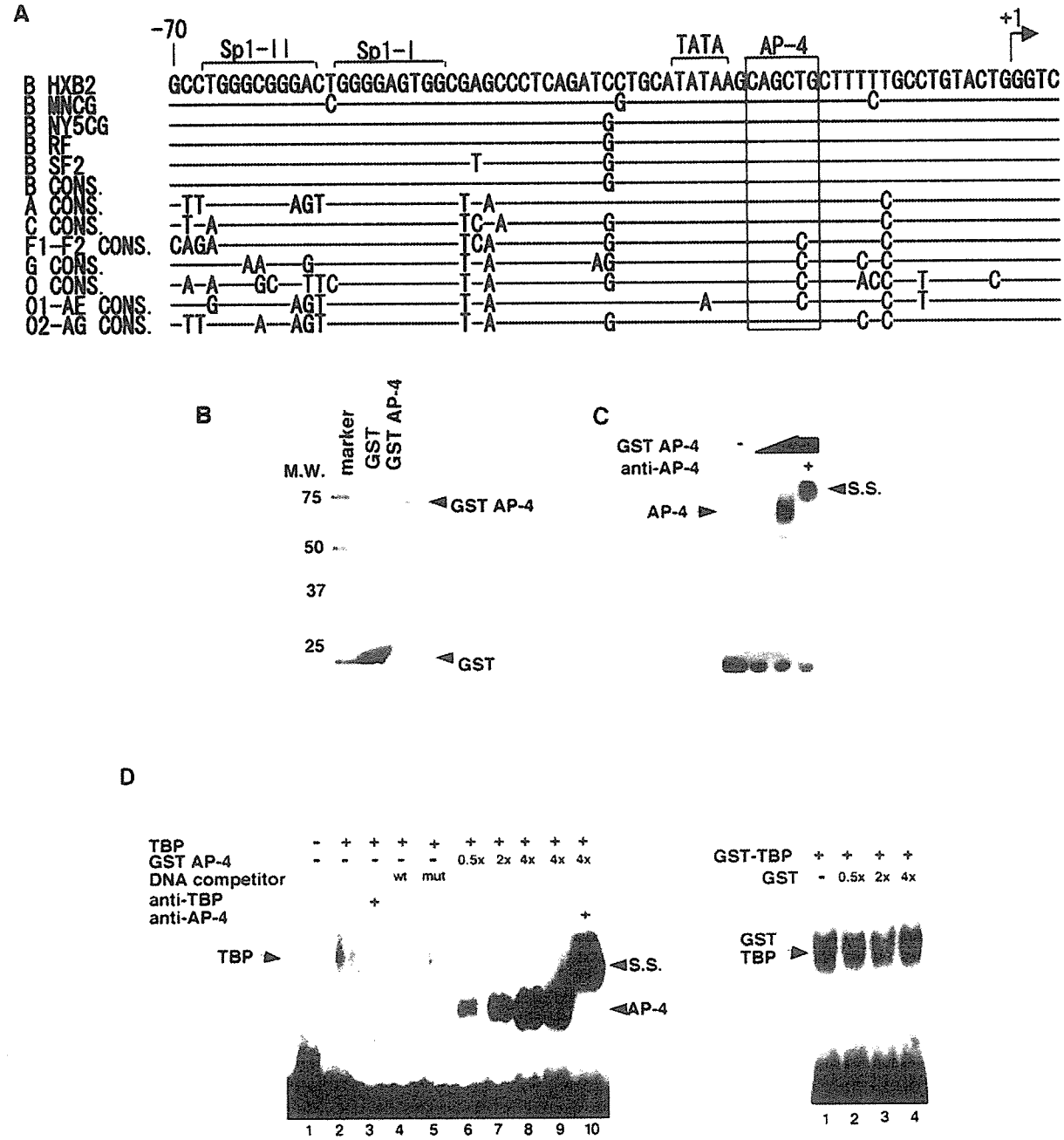

FIGURE 1. **Presence of AP-4 binding site in the vicinity of TATA box of HIV-1 LTR.** *A*, alignment of DNA sequences near the TATA box of various HIV-1 subtypes. The portions of LTR sequences encompassing two Sp1 sites, TATA box, and AP-4 site are shown. AP-4 sites are indicated by a *bracket*. *CONS*, consensus sequences of each HIV-1 subtype. *B*, recombinant GST-AP-4 and GST proteins. The procedures of synthesis and purification are described under "Experimental Procedures." Proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining. *Arrows* indicate the positions of GST-AP-4 and GST. *C*, DNA binding activity of recombinant GST-AP-4. The DNA binding activity of purified recombinant AP-4 protein was analyzed by EMSA with the ^{32}P-labeled double-stranded oligonucleotide probe containing the TATA box and AP-4 site. To verify the AP-4 and DNA complex, the reaction mixtures were incubated with AP-4 antibody. *D*, effect of AP-4 on the DNA binding activity of TBP. The TBP-DNA binding was analyzed by EMSA using the same probe as *C*. TBP was preincubated with various amounts of either GST-AP-4 at the molar ratios indicated (*left panel*). To verify the TBP-DNA and AP-4-DNA complexes, the reaction mixtures were incubated with the TBP and AP-4 antibodies, respectively. For the cold DNA competition experiment, unlabeled double-stranded competitor oligonucleotides (containing wild-type (*wt*) or mutated (*mut*) TATA sequences) were added to the reaction mixtures at 30-fold excess relative to the DNA probe. To confirm that the GST moiety does not affect the TBP-DNA binding, GST-TBP was preincubated with GST prior to the DNA binding reaction (*right panel*). Experiments were carried out at least three times, and reproducible results were obtained. The representative results are shown. Positions of specific DNA-protein complex and supershifted complex (*S.S.*) are indicated by *arrows*.

assessed with mutant HIV-1 LTR reporter constructs (Fig. 2*D*). To further address whether the inhibitory effect of AP-4 depends on the presence of AP-4 site, we repeated similar experiments using other luciferase reporter plasmids including 5× κB-TATA-luc and 4× CRE-TATA-luc (26), in which no AP-4 site is present. AP-4 did not inhibit gene expression from 5× κB-TATA-luc nor 4× CRE-TATA-luc (data not shown). These results demonstrated for the first time that AP-4 exhibits repressive action on HIV-1 gene expression in cultured cells *in vivo*.

Overexpression of TBP Overcomes the Inhibitory Effect of AP-4—To confirm that the repressive effect of AP-4 is through masking the TBP binding to the HIV-1 TATA box, we examined the effect of TBP overexpression on the action of AP-4. As shown in Fig. 3*A*, the inhibitory effect of AP-4 on basal gene expression was abrogated by TBP overexpression in a dose-dependent manner. Similarly, AP-4-mediated repression of the TNF-α-stimulated HIV-1 gene expression was abolished by TBP overexpression (Fig. 3*B*).

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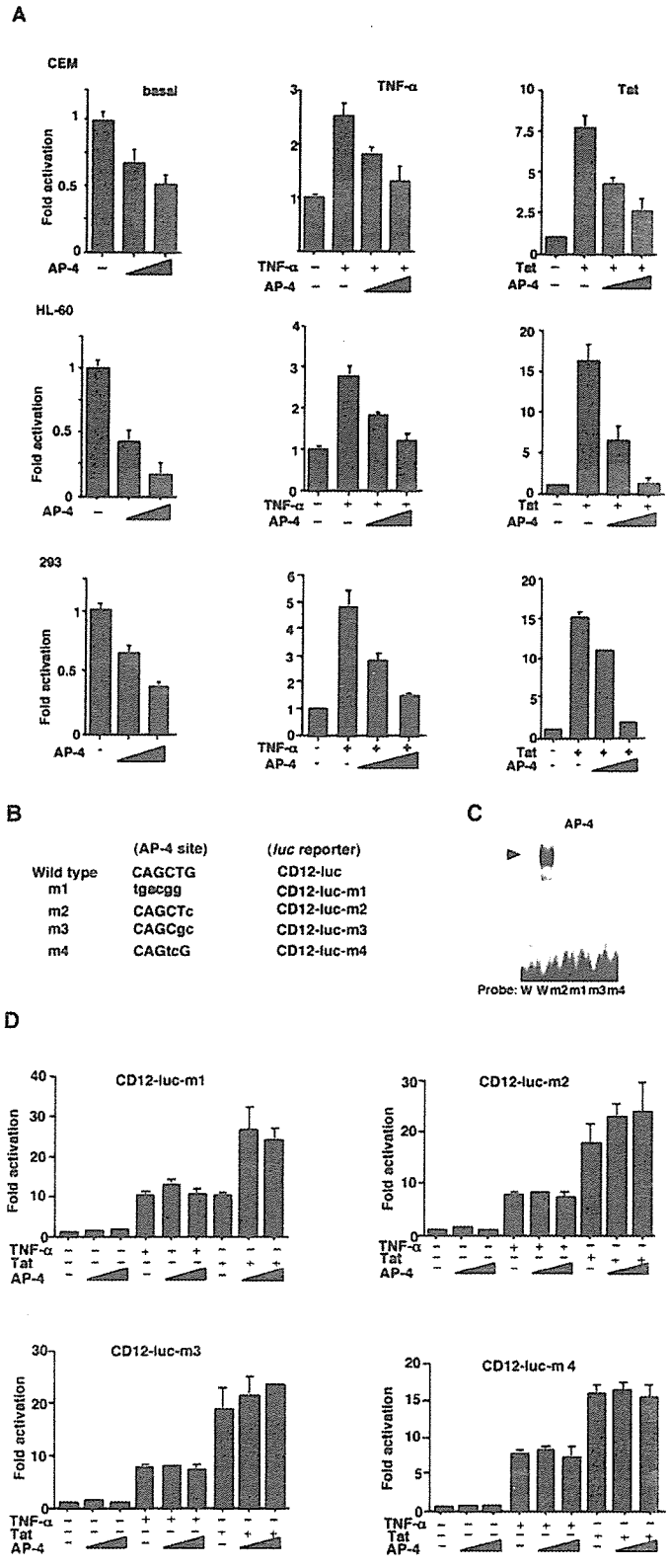

FIGURE 2. **Repression of HIV-1 gene expression by AP-4.** *A*, AP-4-mediated repression of HIV-1 gene expression. AP-4 expression plasmid pMyc-AP-4 was cotransfected with CD12-luc reporter construct, expressing luciferase gene under the control of HIV-1 LTR, into CEM, HL-60 or 293 cells. Extents of HIV-1 gene expression and the effects of AP-4 were evaluated at the basal level (*left panels*), upon TNF-α stimulation (*middle panels*), or upon Tat-mediated transactivation by cotransfecting pCMV-Tat at 2 and 0.1 mg per transfection for CEM/HL60 and 293 cells, respectively (*right panels*). pMyc-AP-4 were cotransfected at 2 and 12 μg for CEM and HL60 cells, and 0.1 and 0.4 μg for 293 cells per transfection. In the TNF-α experiments, cells were stimulated with TNF-α (3 ng/ml) after 24 h of transfection and incubated for additional 24 h. The cells were harvested and the luciferase activity was measured. Each value shown is the fold increase in the luciferase activity (means ± S.D.) relative to the control transfection for three independent experiments. Luciferase activity was measured as above. The values shown are the means ± S.D. for three independent experiments. *B*, the sequences of wild-type and AP-4 binding site mutants. These mutant sequences (m1 to m4) were used in EMSA and luciferase assays by replacing the authentic AP-4 site within CD12-luc (named as CD12-luc-m1 to CD12-luc-m4). *C*, lack of AP-4 DNA binding to mutant AP-4 sequences. The DNA binding activities of purified recombinant AP-4 protein was analyzed by EMSA with the wild-type and mutant AP-4 probes. *Arrowheads* indicate the positions of specific DNA-protein complex. *D*, effects of AP-4 binding site mutation on the AP-4-mediated repression. 293 cells were transfected with mutant CD12-luc containing mutations in the AP-4-site together with various amounts of pMyc-AP-4 (0.1 and 0.4 μg per transfection) with or without stimulation of TNF-α (3 ng/ml) or cotransfection of pCMV-Tat. The luciferase activity was measured as in *A*.

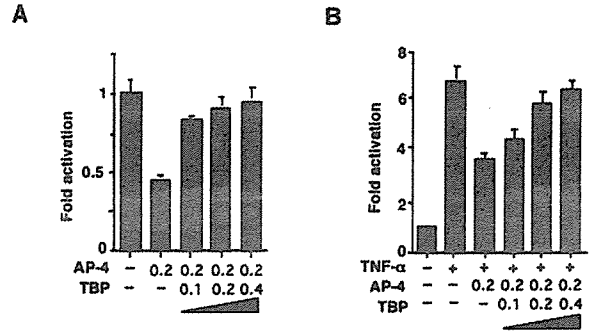

FIGURE 3. **Overexpression of TBP overcomes the repressive effect of AP-4 on HIV-1.** 293 cells were transfected with CD12-luc in combination with pMyc-AP-4 and pCMV-TBP expressing AP-4 and TBP, respectively. After 24 h, cells were untreated (*A*) or treated (*B*) with TNF-α (3 ng/ml) and incubated for additional 24 h. Cells were harvested, and the luciferase activity was measured as described in the legend to Fig. 2.

The Effect of AP-4 Knock-down—To examine the effect of endogenous AP-4, we adopted siRNA technique to specifically knock-down AP-4 mRNA and examined the HIV-1 gene expression when the endogenous AP-4 was depleted. Transduction of AP-4 siRNA caused the depletion of AP-4 protein (Fig. 4*A*), which resulted in significant increase in the basal transcriptional level from HIV-1 LTR (8.2-fold as compared with control siRNA) (Fig. 4*B*). In addition, TNF-α-stimulated LTR gene expression was greatly elevated by AP-4 depletion (5.7-fold). These results indicate that endogenous AP-4 acts as a negative regulator of HIV-1 gene expression.

The DNA Binding Activity of AP-4 Is Essential for the Repression of HIV-1 Gene Expression—AP-4 contains three functional domains, a basic HLH (bHLH) motif (48–99 aa) and two distinct leucine repeat elements, leucine repeat (LR) 1 (99–120 aa) and LR2 (151–179 aa) (Fig. 5*A*). A previous study (20) showed that the HLH motif and an adjacent basic domain are necessary and sufficient to direct sequence-specific DNA binding to its target DNA. Unlike other HLH proteins, AP-4 contains two additional protein dimerization motifs LR1 and LR2. Although both LRs contribute to the formation of AP-4 homodimers, AP-4 requires LR2 to form a stable homodimer (20). The C-terminal half of AP-4 contains a Gln/Pro-rich domain and an acidic region.

To investigate the role of functional domains of AP-4 in down-regulating HIV-1 gene expression, we examined the effects of deletion mutants of AP-4 (shown in Fig. 5*A*). Deletion of the N-terminal regions (ΔN100, ΔN143, and ΔN180) of AP-4 abolished the repressive action on both basal and TNF-α-stimulated HIV-1 expression (Fig. 5*B*). In contrast, deletion of the C-terminal region of AP-4 (ΔC179, retaining the bHLH and two LR domains) repressed HIV-1 gene expression similarly to the full-length AP-4. These results indicate that bHLH domain is indispensable for the repression of HIV-1 gene expression. Because inhibitory effect of ΔC130 (excluding LR2 domain from the ΔC179) was weaker than ΔC179, AP-4 dimerization is important for its effect through stabilization of AP-4 homodimer (20).

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Effect of the Location of AP-4 Site Within HIV-1 LTR on the Repressive Effect of AP-4—To further examine whether the repressive effect of AP-4 depends on its location relative to the TATA box within HIV-1 LTR, we created mutant HIV-1 LTR reporter constructs in which AP-4 binding sites were inserted into various positions of the HIV-1 LTR. Thus, AP-4 binding site were inserted at nucleotide positions −400, −15, −79, and +55 (Fig. 6*A*) within HIV-1 LTR into CD12-luc-m2 in which the authentic AP-4 site was mutated. Basal promoter activities of these promoter constructs were not significantly changed as compared with the original construct (data not shown). As shown in Fig. 6*B*, even when AP-4 sites were distantly located from TATA box, AP-4 could still exert repressive action irrespective of the stimulation by TNF-α. The greatest repressive effect of AP-4 was observed with CD12-luc-m2(−79) although it was less than that with the wild-type promoter. Other AP-4 site mutants exhibited less susceptibility to AP-4-mediated transcriptional repression. These findings indicate that AP-4 could repress HIV-1 transcription even from the distant locations from TATA box although the maximal repressive effect of AP-4 was observed when AP-4 was located in close proximity to the TATA box.

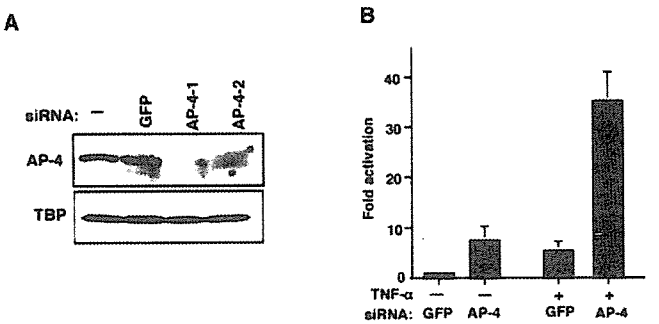
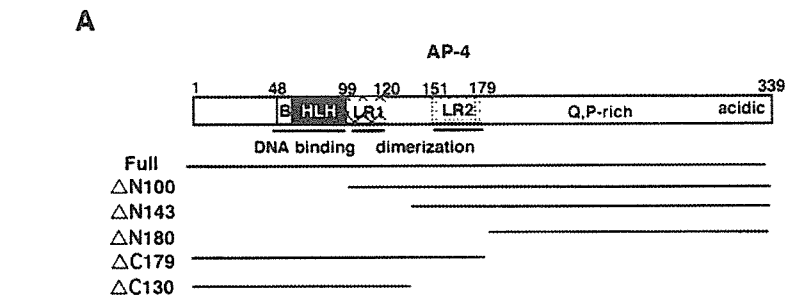


FIGURE 4. **Effect of AP-4 knock-down.** *A*, confirmation of the siRNA-mediated knock-down of AP-4. 293 cells were transfected with 100 nM siRNAs directed against various portions of AP-4 or GFP (control) mRNAs. After 36 h of transfection, cells were lysed, and AP-4 and TBP (control) protein levels were assessed by immunoblotting using specific antibodies. The blot was stripped and reprobed with anti-α-tubulin antibody. *B*, augmentation of HIV-1 gene expression by AP-4 depletion. 293 cells were transfected with CD12-luc together with AP-4 siRNA-1 or its control. After 24 h of transfection, cells were untreated or treated with 3 ng/ml of TNF-α and incubated for an additional 24 h. Cells were harvested, and the luciferase activity was measured as described in the legend to Fig. 2.

Interaction of HDAC with AP-4—Cumulative evidence has demonstrated that chromatin modification by HDAC complex plays a significant role in transcriptional repression (reviewed in Refs. 10 and 48) and many transcriptional repressors, such as YY-1 (8), silencing mediator of retinoic acid and thyroid hormone receptor (28), nuclear receptor corepressor (29), and special AT-rich sequence-binding protein 1 (30) have been shown to tether HDACs to the promoter. Because AP-4 could still exert repressive action even when AP-4 sites were distantly located from TATA box (Fig. 6*B*), we examined whether HDAC was involved in the HIV-1 gene repression by AP-4. To address this possibility we first examined whether AP-4



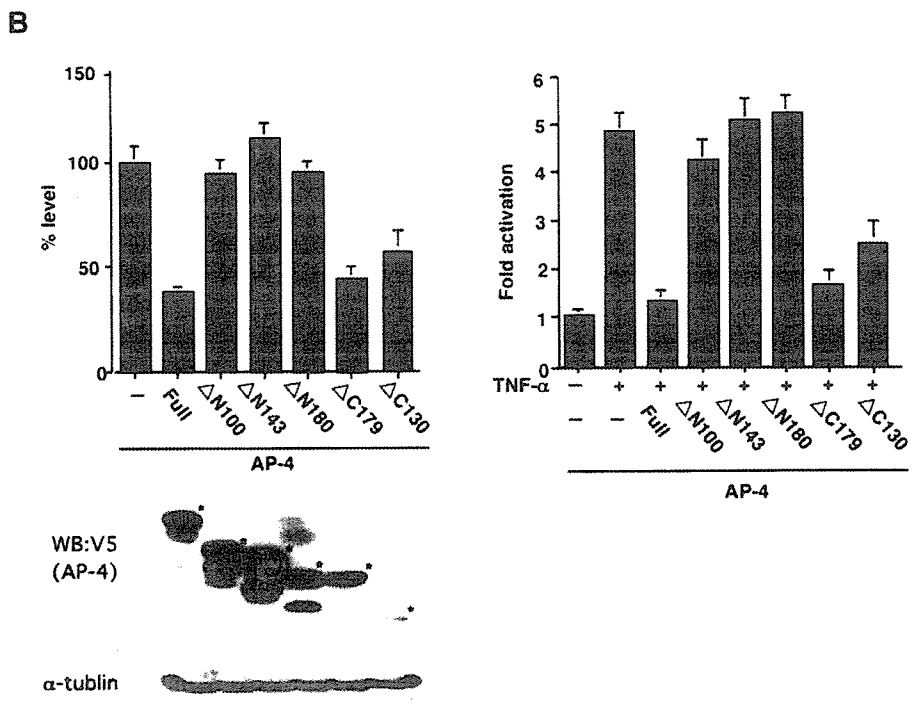


FIGURE 5. **Effects of AP-4 deletion mutants on the HIV-1 gene expression.** *A*, schematic representation of the AP-4 protein and constructs of AP-4 mutants. *Hatched box*, conserved basic stretch; *closed box*, HLH motif; *LR1*, first leucine repeat; *LR2*, second leucine repeat. The amino acid positions of AP-4 are marked on the *top*. *B*, effect of AP-4 mutants on HIV-1 gene expression. Effect of AP-4 was evaluated at the basal level (*left panel*) and upon stimulation with TNF-α (3 ng/ml) (*right panel*). 293 cells were transfected with CD12-luc together with the plasmid expression of wild-type or mutant AP-4. Luciferase assays were performed as described in the legend to Fig. 2. The expression level of each protein was assessed by immunoblotting of cell lysates with anti-V5 antibodies (detecting AP-4 and its mutants). *Asterisks* indicate the positions of specific bands of AP-4 proteins. The low protein level of AP-4 mutant ΔC130, lacking the LR2 domain responsible for protein homodimerization, is considered because of destabilization of the protein.

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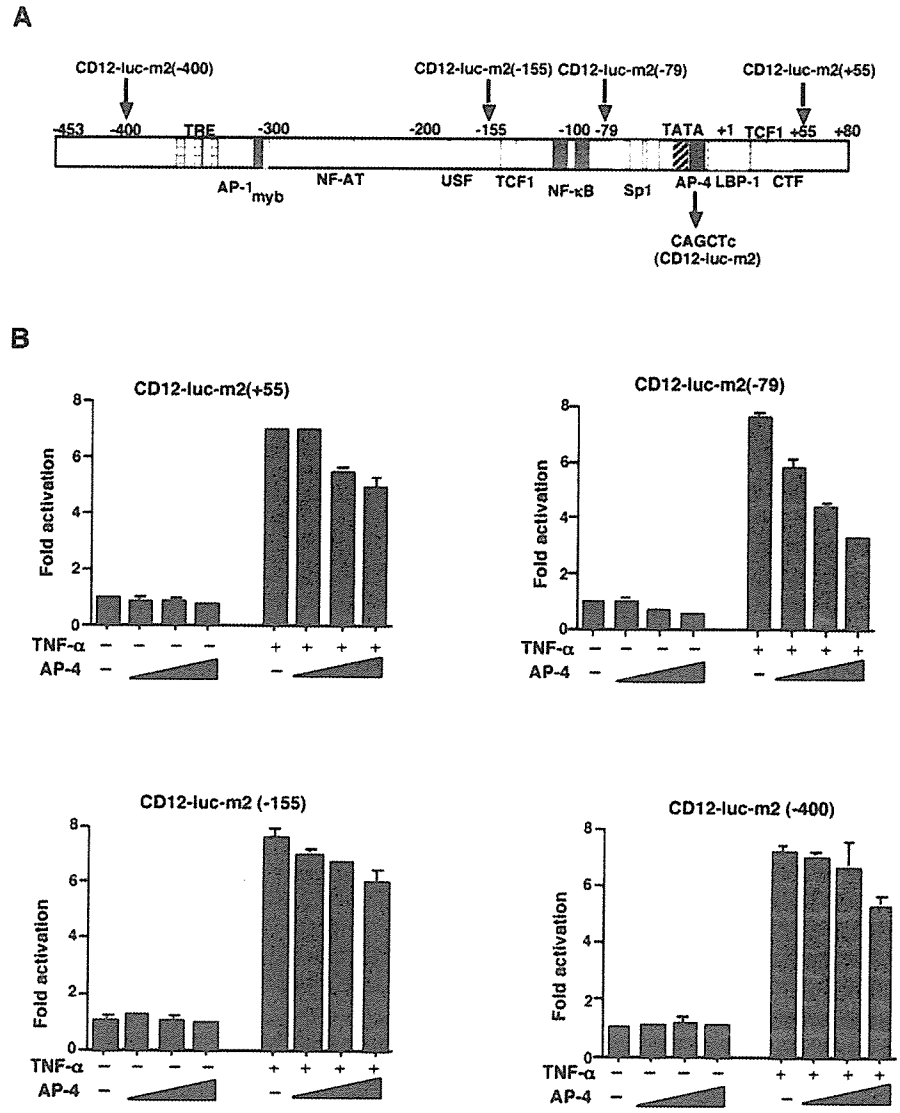

FIGURE 6. **Effects of AP-4 site location on AP-4-mediated repression of HIV-1.** *A*, schematic map of the U3 and R regions of the HIV-1 LTR and positions of aberrant AP-4 sites in mutant constructs. AP-4 site is inserted into various locations (nucleotide positions: −400, −15, −79, and +55) within CD12-luc-m2 (Fig. 2*B*), in which the authentic AP-4 site is abolished. *B*, effect of the AP-4 site on susceptibility to AP-4-mediated repression. 293 cells were transfected with reporter constructs and various amounts of pMyc-AP-4 (0.05, 0.2, and 0.4 μg per transfection). After 24 h, cells were untreated (*left side of each panel*) or treated (*right side*) with TNF-α (3 ng/ml) and incubated for an additional 24 h. The luciferase activity was measured as described in the legend to Fig. 2.

interacts with HDACs in culture cells. In Fig. 7, 293 cells were transfected with FLAG- or Myc- (used as a negative control for immunoprecipitation) tagged AP-4 expression plasmids, and cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Immune complexes were collected and subjected to SDS-PAGE followed by immunoblotting for detection of the class I HDACs using antibodies to HDAC1, 2, and 3. As shown in Fig. 7*A*, AP-4 interacted with HDAC1, and to a much lesser extent HDAC2, but not detectably with HDAC3. In Fig. 7*B*, similar experiments with untransfected cells showed the interaction of endogenous AP-4 with endogenous HDAC1. The interaction of AP-4 with HDAC2 was observed but much less than that with HDAC1. No interaction between AP-4 and HDAC3 was observed (data not shown). To examine whether AP-4 directly binds to HDAC1, we performed *in vitro* protein-protein interaction assay using GST-AP-4 fusion protein, radiolabeled HDAC1, and luciferase (as a control). The radiolabeled HDAC1 directly bound GST-AP-4 but not GST and no binding was observed between GST-AP-4 and luciferase (Fig. 7*C*). These results demonstrated that AP-4 directly interacts with HDAC1. To further confirm the involvement of HDACs in transcriptional repression of HIV-1 by AP-4, trichostatin A (TSA), a specific inhibitor of HDACs, was added to the cells transfected with AP-4, and the luciferase assay was performed. As shown in Fig. 7*D*, TSA abrogated the repressive effect of AP-4 on HIV-1 gene expression in a dose-dependent manner. These findings suggest that HDACs are involved in the AP-4-mediated repression of HIV-1 gene expression.

ChIP Assays Detecting AP-4 and HDAC1 on HIV-1 LTR—Together with the results demonstrated above, it was suggested that AP-4-mediated HDAC recruitment to and elimination of TBP (TFIID) from the HIV-1 promoter might play a role in the cellular maintenance of HIV-1 latency. We thus examined the presence of AP-4 and HDAC1 on the HIV-1 promoter in latently infected cells, ACH2 (T-cell line latently infected with HIV-1) and U1 (promyelocytic cell line latently infected with HIV-1) (31). As shown in Fig. 8*A*, the newly raised anti-AP-4 antibody could specifically precipitate AP-4. Although the nature and/or the extent of chromatin formation on transiently transfected DNA templates likely differ from that of chromosomal genes, previous reports of ChIP assays using plasmids containing HIV-1 LTR have validated ChIP studies in transiently transfected cells (32–35). In Fig. 8*B*, 293 cells were transiently transfected with CD12-luc or its mutant CD12-luc-m2 followed by ChIP analysis using the anti-AP-4 antibody. We amplified the HIV-1 LTR DNA fragment (−109/+79) containing binding sites for NF-κB, Sp1, TBP, AP-4, LBP-1 (YY-1), TCF1, and CTF (Fig. 6*A*) in the AP-4 and HDAC1 immune complexes. We were able to detect the binding of both AP-4 and HDAC1 to the HIV-1 promoter by ChIP assay in 293 cells transfected with CD12-luc (Fig. 8*B*). The binding

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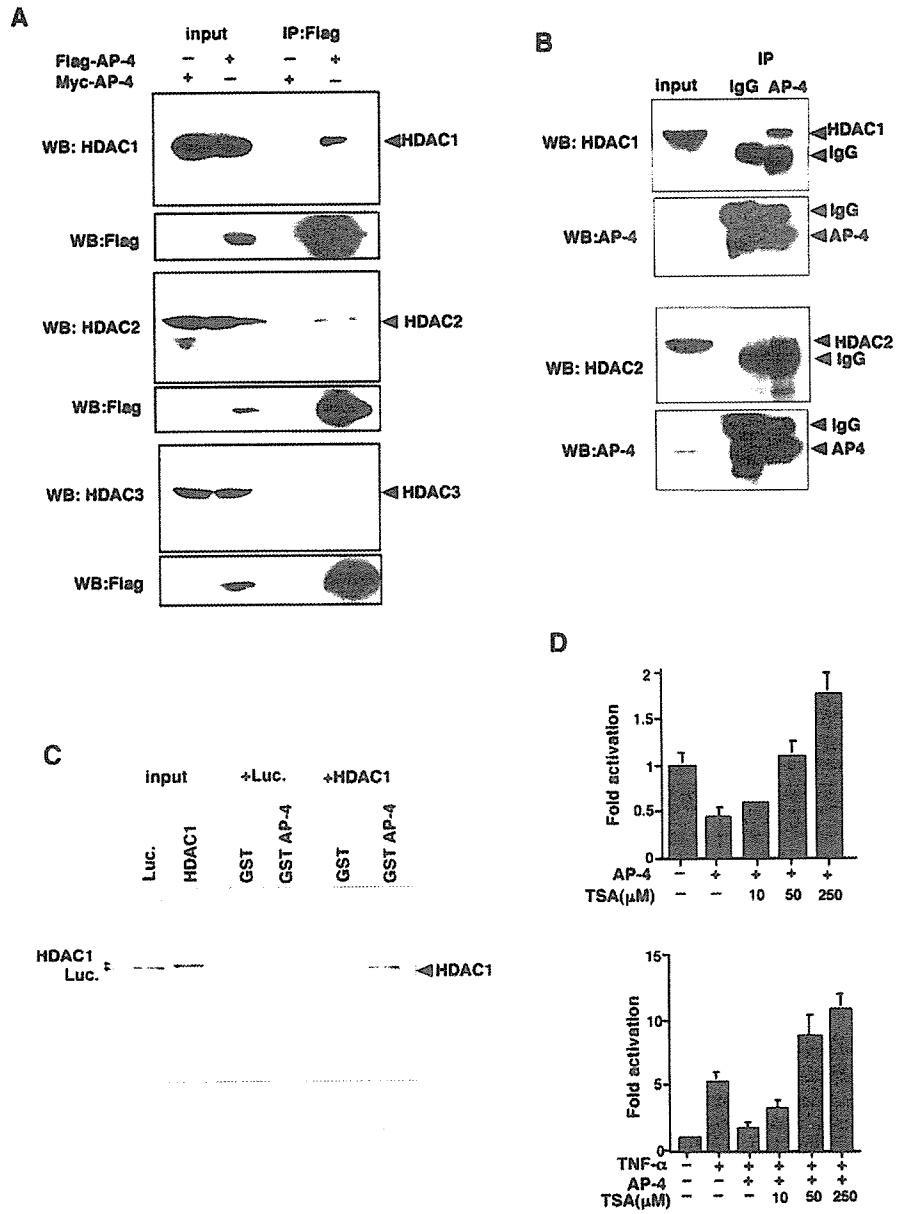

FIGURE 7. **Interaction of HDAC with AP-4.** *A*, interaction between AP-4 and HDAC proteins *in vivo*. 293 cells were transfected with FLAG- (pFLAG-AP-4) or Myc-tagged (pMyc-AP-4) AP-4 expression plasmids. After 48 h of transfection, the cell lysates were prepared, immunoprecipitated with anti-FLAG antibody, and subsequently separated on SDS-PAGE followed by immunoblotting with anti-HDAC antibodies. One-tenth of each protein lysate was loaded as input control. *B*, endogenous AP-4 interacts with HDACs. 293 cell lysates were immunoprecipitated with anti-AP-4 antibody, and the immune complex was analyzed by immunoblotting with anti-HDAC antibodies. *C*, AP-4 binds to HDAC1 *in vitro*. HDAC1 and luciferase (negative control) proteins were synthesized and labeled with [^{35}S]methionine *in vitro*. These radiolabeled HDAC1 and luciferase proteins were incubated with GST-AP-4 or GST (control) immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by SDS-PAGE and subjected to autoradiography. *D*, effect of TSA on the repressive activity of AP-4. 293 cells were transfected with CD12-luc together with pMyc-AP-4. After 24 h of transfection, cells were untreated (*upper panel*) or treated (*lower panel*) with TNF-α (3 ng/ml), incubated for additional 24 h, and various amounts of TSA were added to the culture. After 8 h of additional incubation, cell lysates were prepared, and the luciferase activity was measured as described in the legend to Fig. 2.

with AP-4 or HDAC1 was detectably reduced when CD12-luc-m2, in which AP-4 site was mutated, was transfected. The trace amount of HDAC1 recruitment was detected even with the mutant, presumably due to the presence of binding region (from −10 to +27) of LBP-1 and YY-1, known to recruit HDAC1 (8).

In Fig. 8*C*, ChIP assays were similarly performed with ACH2 and U1 cells using antibodies to AP-4, HDAC1, acetylated histone H3 (Ac-H3), TBP, and RNAPII. AP-4 and HDAC1, but only traceable amounts of TBP (TFIID) or RNAP II, were detected on the HIV-1 promoter when these cells maintained the latency (without any stimulation). However, when ACH2 and U1 cells were treated with TNF-α to stimulate HIV-1 replication, AP-4 and HDAC1 were readily dissociated from the HIV-1 promoter, and TBP and RNAPII became clearly detectable on the HIV-1 promoter over time (Fig. 8*C*). Moreover, the disappearance of AP-4 from the HIV-1 LTR correlated with dissociation of HDAC1 and appearance of the acetylated form of histone H3 (Ac-H3). These results, together with the findings described above, suggest that AP-4 acts as a negative regulator of HIV-1 gene expression by recruitment of HDAC1 as well as by preventing the TBP (TFIID) binding to the TATA box in latently infected cells.

Repression of HIV-1 Production by AP-4—To assess the biological relevance of the repressive action of AP-4, we examined the effect of AP-4 on HIV-1 production. 293 cells were transfected with a replication-competent full-length HIV-1 clone (pNL4-3) together with various amounts of AP-4-expression plasmid pFLAG-AP-4, and virus production was evaluated by measuring HIV-1 p24 antigen levels in the culture supernatant. In Fig. 9*A*, transduction of AP-4 resulted in dose-dependent decrease in the HIV-1 p24 level by 3.7-fold (Fig. 9*A*, *left panel*). Inhibition of viral protein synthesis was also observed in these cells when AP-4 was overexpressed (Fig. 9*A*, *right panel*). In Fig. 9*B*, the effect of AP-4 on the TNF-α-stimulated HIV-1 production was examined. When pNL4-3 was transfected and cells were subsequently stimulated with TNF-α, 7.4-fold increase of HIV-1 production was observed (Fig. 9*B*, *right panel*). When AP-4 was overexpressed, a dramatic inhibition of HIV-1 production was observed in a dose-dependent manner, almost to the basal unstimulated level (Fig. 9*B*). In Fig. 9*C*, effects of AP-4 mutants were examined. Although an AP-4 mutant ΔC179, retaining the AP-4 DNA binding and dimerization domains, could suppress HIV-1 production as well as wild-type AP-4, another mutant ΔN143, lacking these two functional domains, showed no suppressive effect on the HIV-1 pro-

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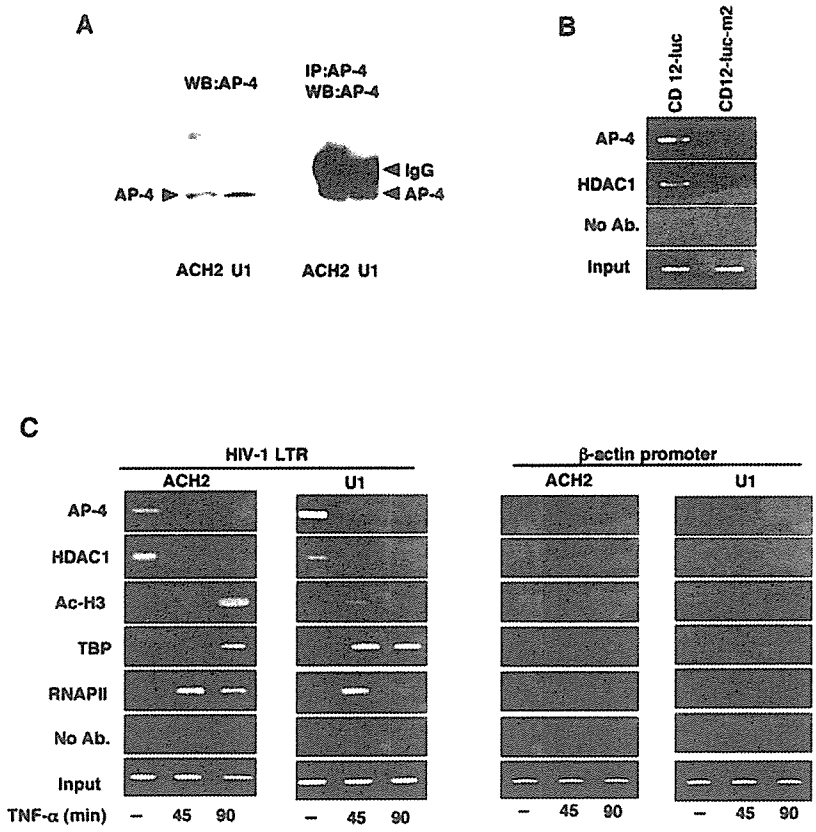

FIGURE 8. **ChIP assays detecting AP-4 and HDAC1 on HIV-1 LTR.** *A*, immunoreactivity of anti-AP-4 antibody. To assess the immunoreactivity of anti-AP-4 antibody for immunoblotting (*left panel*) and immunoprecipitation (*right panel*), ACH2 and U1 cell lysates were reacted with the rabbit anti-AP-4 antibody. *Arrowheads* indicate the locations endogenous AP-4. Position of IgG was also indicated (*right*). The AP-4 immune complex was separated on SDS-PAGE followed by immunoblot with anti-AP-4 antibody. *B*, AP-4 recruits HDAC1 to the HIV-1 LTR. 293 cells were transfected with CD12-luc or CD12-luc-m2, incubated for 48 h, and ChIP assay was performed. Cross-linked chromatin fragments were prepared, and the association of AP-4 and HDAC1 was analyzed by ChIP assay using antibodies to AP-4 and HDAC1. The recovered DNA was amplified by PCR with promoter-specific primers (spanning from −109 to +79 of HIV-1 LTR) and analyzed on a 2% agarose gel. Input DNA represents total input chromatin (1%) while immunoprecipitation with no antibody (*No Ab.*) serves as negative control. *C*, dynamic association/dissociation of AP-4, HDAC1, acetylated histone H3, TBP, and RNAP II. ChIP assays were performed with latently infected cell lines. ACH2 and U1 cells were either untreated or treated with TNF-α (3 ng/ml) for indicated times and subjected to ChIP assays. Cross-linked chromatin fragments were prepared, and the association of AP-4, TBP, RNAPII, HDAC1, Ac-H3, and HIV-1 LTR DNA (−109/+79) was analyzed by ChIP assay as described in *B*. The β-actin promoter DNA (−980/−915) was similarly analyzed as a control. The experiments were repeated performed with reproducible results, and the representative results are shown.

duction (Fig. 9*C*). These results indicate that AP-4 can inhibit HIV-1 production and that the DNA binding activity of AP-4 is crucial for its inhibitory action.

In Fig. 9*D*, we examined the effect of depleting endogenous AP-4 on HIV-1 production using siRNA for AP-4. AP-4 depletion resulted in significant increase in the basal HIV-1 production (3.2-fold as compared with control siRNA (GFP)). Similarly, TNF-α-stimulated HIV-1 production was elevated by the treatment with AP-4 siRNA (5.7-fold as compared with control siRNA). We also observed the elevation of viral protein synthesis in the transfected cells by AP-4 depletion (Fig. 9*D*, *right panel*).

Finally, we examined the effect of AP-4 on HIV-1 replication in Jurkat CD4+T cells. To examine the effect of AP-4 binding site within HIV-1 LTR, we created a mutant pNL4-3 lacking the AP-4 binding and quantified the amounts of HIV-1 virions in the culture supernatant of Jurkat cells transfected with either the wild-type or the mutant pNL4-3. As shown in Fig. 9*E*, the amounts of HIV-1 production were not significantly changed over time between the wild-type and the mutant HIV-1 clones. In Fig. 9*F*, the repressive effect of AP-4 on HIV-1 production was examined with or without TNF-α stimulation. When the mutant pNL4-3, containing mutation in the AP-4 binding, was cotransfected with AP-4, the inhibitory effect of AP-4 was abolished irrespective of the TNF-α stimulation (Fig. 9*F*, *right panel*).

DISCUSSION

The extent of HIV-1 replication is controlled at the step of transcription and the level of transcription is governed by the coordinated actions of viral and cellular transcription factors acting on LTR both in positive and negative fashions (1, 2, 9). In contrast to the abundant literatures reporting actions of positive transcription factors, mostly NF-κB (1, 2), little is known about the action of negative transcription regulators. Here, we demonstrate evidences demonstrating that AP-4 is a negative regulator of HIV-1 transcription and its production. Although a previous study reported that AP-4 blocks TBP binding to TATA box *in vitro* (10) and suggested that AP-4 negatively regulates HIV-1 transcription, no evidence has been presented supporting the repressive action of AP-4 on HIV-1 transcription and viral production. In this study, we were able to demonstrate that AP-4 acts as a negative transcription factor for HIV-1 gene expression by recruitment of HDAC1, as well as by preventing the TBP (TFIID) binding to the TATA box, and that AP-4 is actively involved in the transcriptional silencing of HIV-1 gene expression in latently infected cell lines.

The AP-4 binding site in the HIV-1 LTR is located immediately downstream of TATA box (2, 10, 13). Among various HIV/SIV isolates AP-4 sites are conserved among HIV-1 subtypes A, B, C, D, and CRF02-AG, HIV-2, and SIVcpz-gab. However, although most (92%) of the clinical HIV-1 isolates had functional AP-4 binding site (11), no clear cor-

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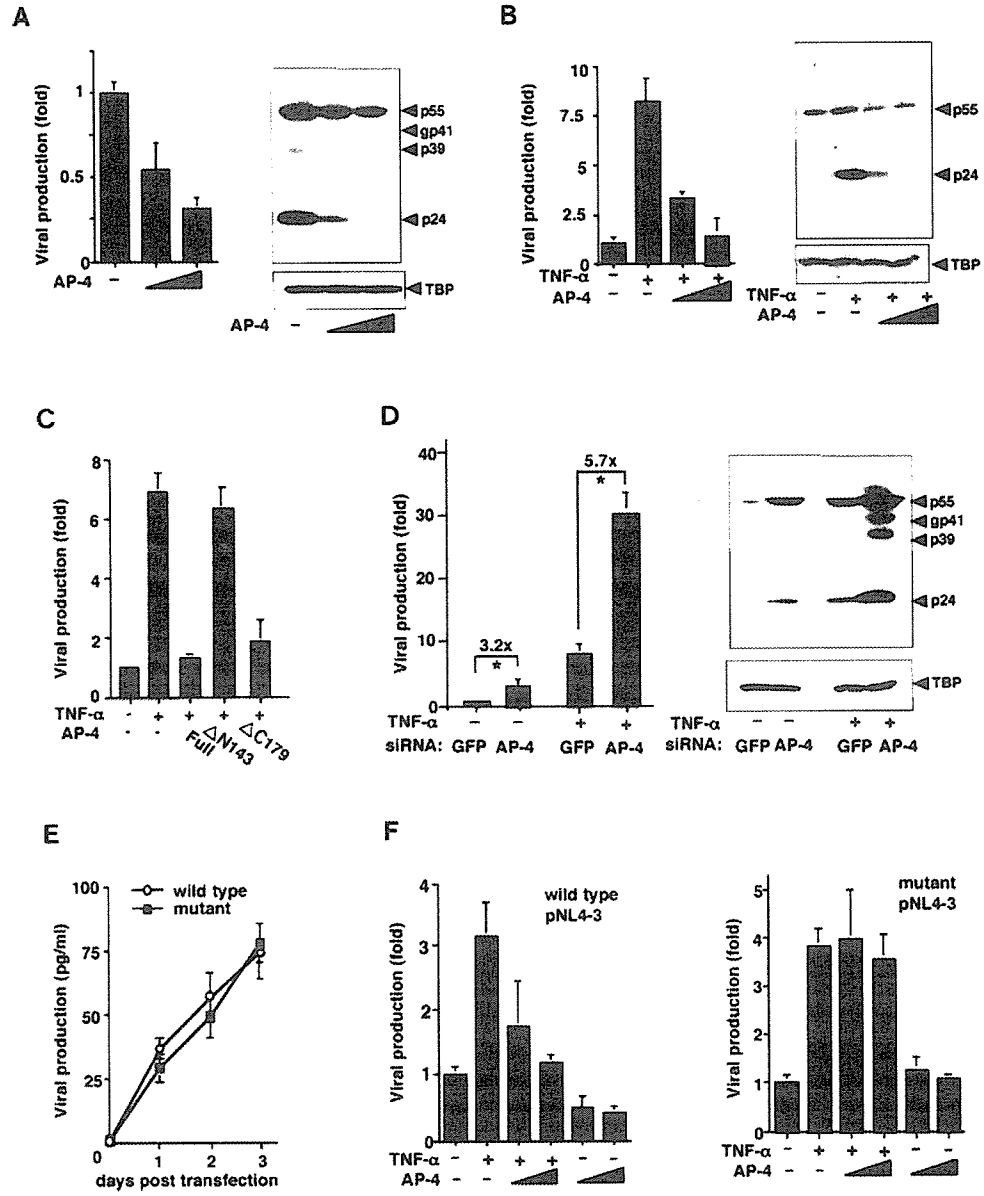

FIGURE 9. **Repression of HIV-1 production by AP-4.** *A* and *B*, HIV-1 production and AP-4-mediated repression in transfected cells. 293 cells were transfected with pNL4-3 and pFLAG-AP-4. After 36 h of transfection, cells were either untreated (*A*) or treated (*B*) with TNF-α (3 ng/ml) and incubated for additional 24 h. The culture supernatants and cell lysates were collected and subjected to the determination of p24 antigen level by ELISA and detection of virus proteins by immunoblot with AIDS patient serum, respectively. *C*, effects of AP-4 mutants on HIV-1 production. 293 cells were transfected with pNL4-3 together with expression vectors for wild-type or mutant AP-4. After 24 h of transfection, cells were untreated or treated with TNF-α (3 ng/ml) and incubated for additional 24 h. The p24 antigen level in the culture supernatant was determined as in *A*. *D*, effects of AP-4 knockdown on HIV-1 production. 293 cells were transfected with pNL4-3, and AP-4 siRNA-1 (Fig. 4) or control siRNA (GFP). After 36 h of transfection, cells were untreated or treated with 3 ng/ml of TNF-α and incubated for an additional 24 h. The culture supernatants and cell lysates were collected and analyzed the p24 antigen level and viral protein expression, respectively, as in *B*. *, $p < 0.01$. *E*, HIV-1 production of the pNL4-3 mutant lacking the AP-4 sites within both LTR. Either the wild-type or the mutant pNL4-3 was transfected into Jurkat CD4+T cells, and the amounts of HIV-1 virion production were quantified. The culture supernatants were collected after 1, 2, and 3 days post-transfection, and the p24 antigen levels were measured as in *A*. *F*, repression of HIV-1 production by AP-4 in Jurkat CD4+T cells. Jurkat cells were transfected either with the wild-type (*left panel*) or the mutant (*right panel*) pNL4-3 lacking the AP-4 sites in both 5′- and 3′-LTR, together with various amounts of pFLAG-AP-4 (0.2 and 0.8 μg per transfection). After 36 h of transfection, cells were either untreated or treated with TNF-α (3 ng/ml) and incubated for an additional 24 h. The culture supernatants were prepared, and the p24 antigen levels were measured. Experiments were repeated at least three times, and reproducible results were obtained. The representative data are shown.

relation with the clinical stage was observed. Thus, the biological significance of the presence of AP-4 site in HIV-1 LTR in determining the virulence awaits further *in vivo* investigations.

There are a number of transcription factors acting as repressors. However, the mechanisms of their actions are not uniform and multiple modes of action are reported including: 1) inhibition of transcriptional activators upon its DNA binding or interaction with co-activators (36–38), 2) prevention of the binding of general transcription factors such as TBP (TFIID) and TFIIB to the promoter (39–43), 3) direct repression of promoter activity by recruiting co-repressors (8, 28, 30), 4) alteration of chromatin structure (19, 40), and 5) inhibition of transcriptional elongation (44, 45). AP-4 appears to exert transcriptional repression of HIV-1 promoter through bimodal mechanisms: 1) masking the HIV-1 TATA element from TBP binding and 2) recruiting HDAC1. In this context, it is noted that HIV-1 does not appear to use the TATA box but instead use the CATA box motif located two nucleotides upstream of the conventional TATA box (46). It is possible that AP-4 may preclude the usage of TATA box by physical masking. However, since we observed that AP-4 blocked the TBP binding to CATATA box in EMSA (Fig. 1*D*), AP-4 may also block the CATA box.

Like other members of bHLH family to which AP-4 belongs, the AP-4 HLH motif and the adjacent basic domain are necessary to confer site-specific DNA binding (20). Unlike other HLH proteins, AP-4 also contains two additional protein dimerization motifs consisting of leucine repeat elements LR1 and LR2, through which AP-4 forms a homodimer (20). We found that both bHLH motif and two LR elements were necessary to exert transcriptional repression, presumably by masking the TATA box. However, unlike HIV-1 LTR, most other promoters where AP-4 has negative role have AP-4 sites at distant locations from the TATA box and even some promoters are TATA-less (22–24) and the mechanism by which AP-4 represses transcription other than masking the TATA box has not been elucidated. In addition, we found that AP-4 could exert transcriptional repression of HIV-1 even when the AP-4 site was located distant from the TATA box. Subsequent experiments have revealed that AP-4 could recruit HDAC1 to the promoter and that the AP-4-mediated repression could be restored by the treatment with a histone deacetylase inhibitor TSA. These bimodal actions of AP-4 make this factor a strong negative regulator for HIV-1 transcription. However, further studies are needed such as to determine where AP-4 binds to HDAC and to clarify whether AP-4 binds other transcriptional reg-

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ulators. Interestingly, AP-4 was reported to be a transcriptional activator of transforming growth factor β (47), immunoglobulin κ chain (48), and SV40 (late promoter) (21) where AP-4 binding sites were found in the enhancer elements of these genes although no direct evidence is thus far available to show that AP-4 actually act as a transcriptional activator for these genes. Mermod *et al.* (21) showed that AP-4 acts in concert with AP-1, binding to the adjacent site of AP-4, in stimulating SV40 transcription *in vitro*. Thus, it is possible that the effect of AP-4 binding on transcription may be modified by other transcription factors recruited to the promoter and depend on the promoter context through combinatorial interaction with other transcription factors.

Our finding that AP-4 is constitutively present on the silent HIV-1 promoter in latently infected cells may have significant biological implications. We also found that TNF-α stimulation abrogated the AP-4-mediated repression of HIV-1 promoter. It is conceivable that nuclear translocation of NF-κB and its binding to the HIV-1 promoter might induce local chromatin remodeling, thus eliminating AP-4 and its repressor complex. It appears that the interplay among various transcription factors on the HIV-1 promoter determines the transcriptional competence of the latent HIV-1 provirus.

The ability of HIV-1 to establish a latent infection is considered crucial for the pathogenesis of AIDS (49, 50). Whereas HIV-1 entry into activated $CD4^+$ lymphocytes leads to a productive infection, the virus remains latent in resting $CD4^+$ lymphocytes (51). For many HIV-infected patients, although current anti-HIV treatment can reduce viral loads to undetectable levels, infected cells persist in a long term and harbor integrated proviruses capable of reseeding virus production after cessation of therapy. Our observation of AP-4 in the negative regulation of HIV-1 gene expression could give us a clue to understand how the latency is maintained at least in cells. Moreover, it is proposed that breakdown of viral latency during the early clinical stage where potent anti-viral cytotoxic T lymphocyte is still present is considered to benefit the outcome of HIV-1 infection by eliminating the otherwise long lasting chronically and latently infected cells (52, 53). Interestingly, Lehrman *et al.* (54) have recently reported a clinical study that combination therapy of conventional anti-HIV therapy with an HDAC inhibitor, valproic acid, could successfully accelerate the clearance of HIV-1 from resting CD4+T cells. It is plausible that HDAC inhibitors have clinical benefit in preventing the clinical development of AIDS. Further studies are needed to clarify the role of AP-4 and other repressor proteins in the maintenance of HIV-1 latency *in vivo* and to determine the clinical benefit of HDAC inhibitors.

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