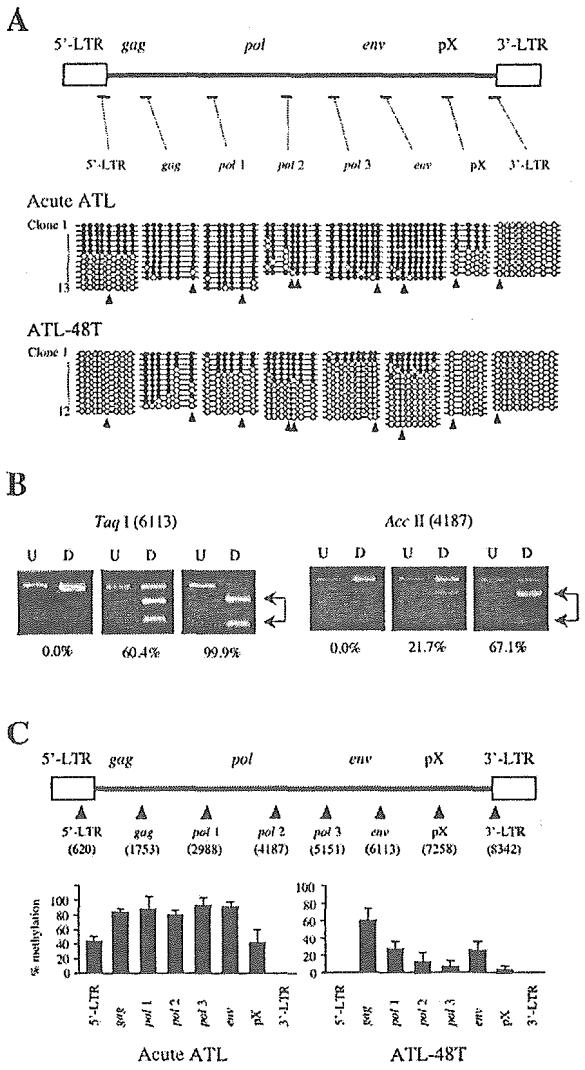

Figure 1

DNA methylation of the HTLV-I provirus assessed by sodium bisulfite sequencing and COBRA. A. DNA methylation in the HTLV-I provirus was analyzed by sodium bisulfite sequencing in a case of acute ATL and a *tax* gene-expressing cell line, ATL-48T. Eight DNA regions, which were represented as bars in A, were amplified with sodium bisulfite treated DNA. The PCR products were subcloned into plasmid DNA, and then the sequences of each clone were determined for at least ten clones of each region. Arrowheads indicate the CpG sites that were target sites for COBRA. Closed circle indicates methylated CpG, and open circle means unmethylated CpG. The number of integrated provirus has been shown in parenthesis. B. Representative data of COBRA has been shown. PCR products, which were amplified with sodium bisulfite treated DNAs, were digested with *TaqI or AccII*. The extent of methylation in each CpG site was measured as described in Methods, and presented as percentages of methylated CpG. The number in parenthesis represents the position of cytidine residue in analyzed CpG site by COBRA according to Seiki et al. [41]. C. DNA methylation studied by COBRA at eight points in the provirus as shown by arrowheads. Each bar represented the extent of DNA methylation at the points shown by arrowhead. The analyses by COBRA were performed three times independently, and the extents of DNA methylation are shown by the mean ± SD. The number in parenthesis shows the position of cytidine residue of CpG site analyzed by COBRA.

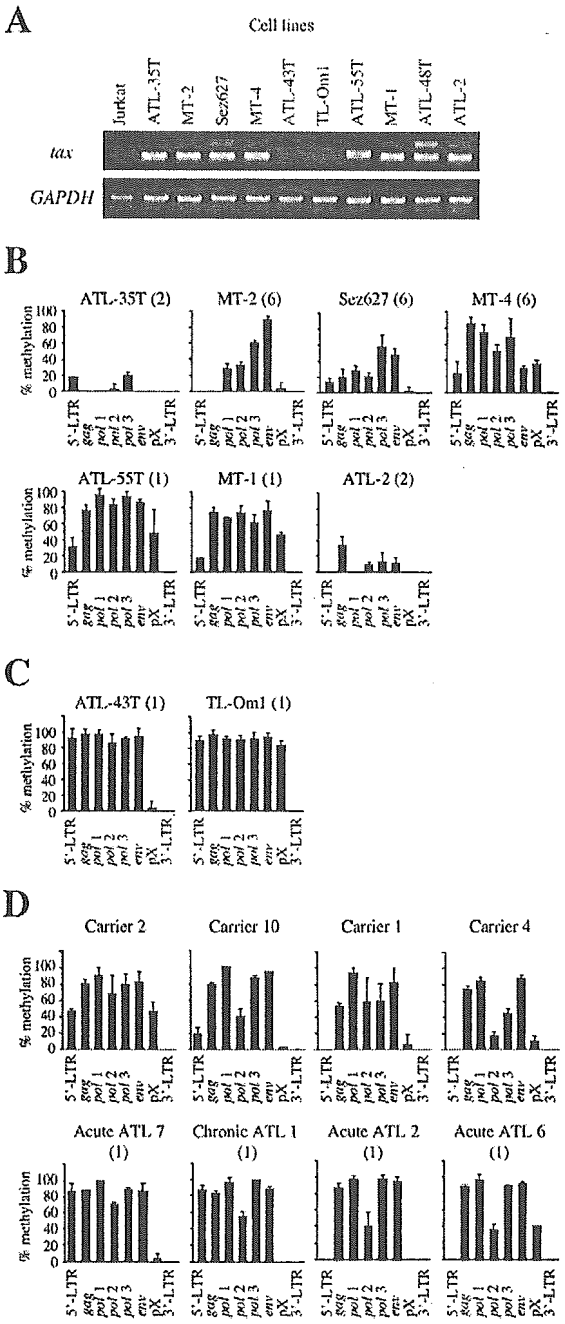

Figure 2

DNA methylation in ATL cell lines, HTLV-I carriers and ATL cases. The *tax* gene transcription in ATL cell lines was studied by RT-PCR (A), and the expression of *GAPDH* gene has been used as a control. DNA methylation throughout the HTLV-I provirus was studied by COBRA in *tax* gene-expressing (B) and non-expressing cell lines (C). Furthermore, DNA methylation was also analyzed in 20 carriers and 20 ATL cases by COBRA, and representative patterns of DNA methylation are shown in D. The number of HTLV-I provirus has been analyzed by Southern blot method, and shown in the parenthesis (B, C and D). Each bar indicates the extent of DNA methylation that was calculated by COBRA.

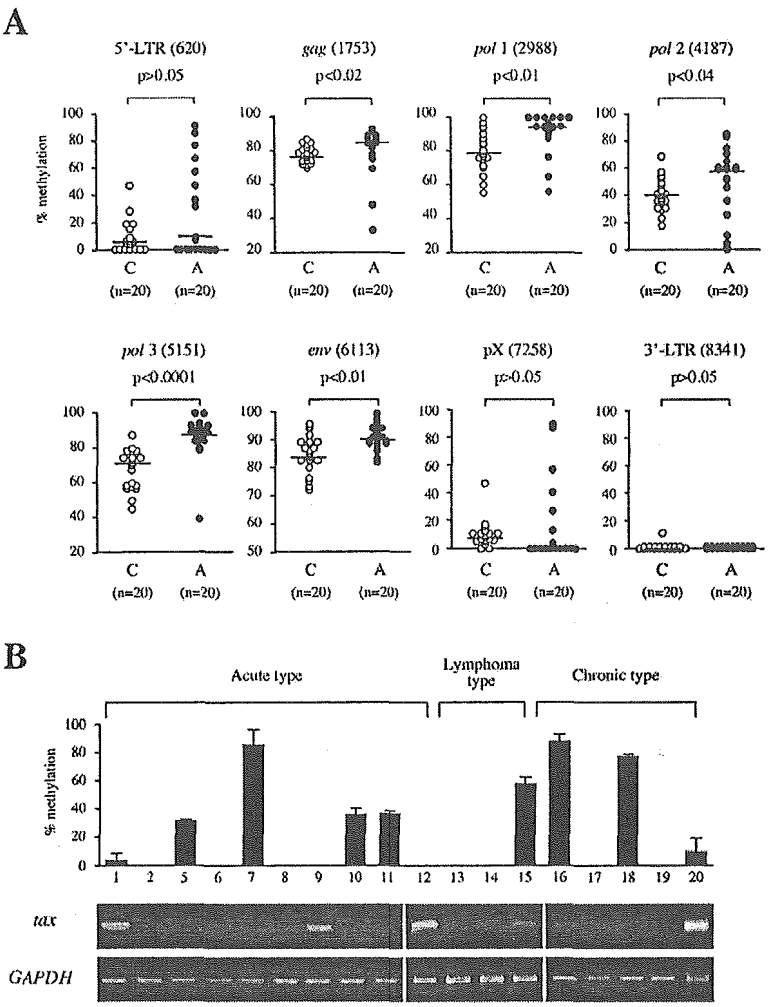

Figure 3

Comparison of the DNA methylation in carriers and ATL cases. A. DNA methylation at eight different regions in the HTLV-I provirus was compared between carriers (C) and ATL cases (A). DNA methylation was quantified by COBRA in 20 carriers and 20 ATL cases. Each sample was analyzed three times by COBRA at each site, and circles indicate mean values of DNA methylation. The differences of DNA methylation are statistically significant in the *gag*, *pol* and *env* regions by the Mann-Whitney's U-test. Horizontal bars represent median of DNA methylation in each group. B. The relation between *tax* gene transcription and DNA methylation of 5'-LTR in the fresh ATL cells has been shown. DNA methylation of 5'-LTR was quantified by COBRA assay and the *tax* gene transcripts were detected by RT-PCR.

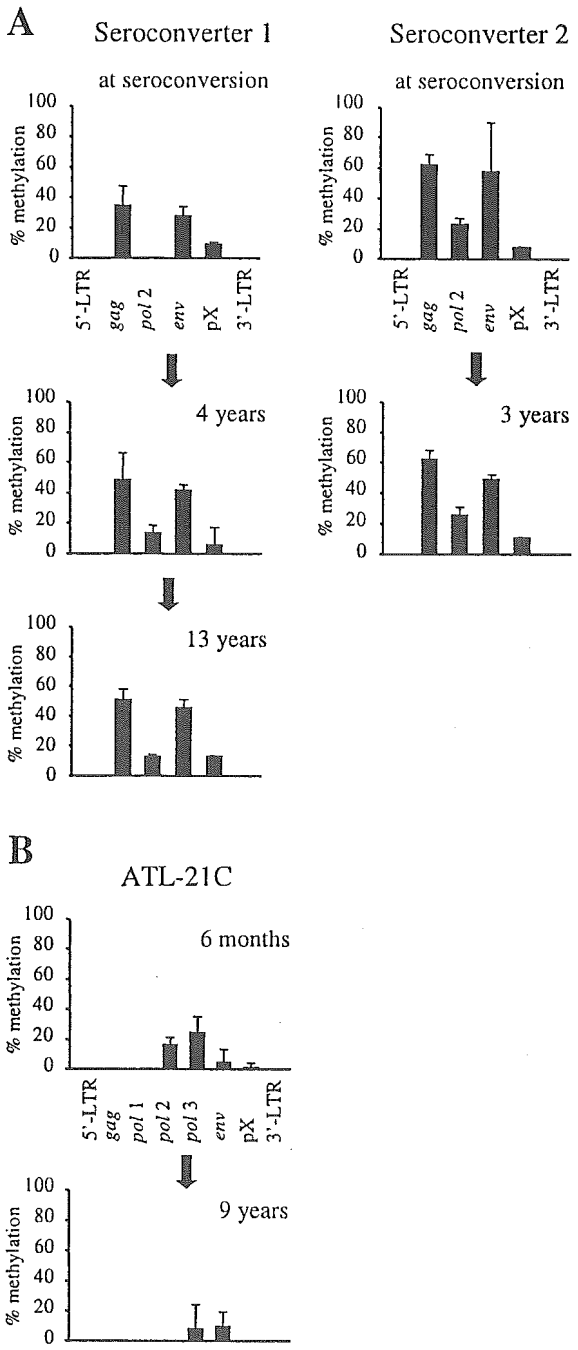

Figure 4
Sequential analyses of the DNA methylation in seroconverters and a cell line. DNA methylation was analyzed by COBRA in sequential samples from seroconverters (A) and in a cell line, ATL-21C, (B) cultured *in vitro* for more than 9 years. DNA methylation was analysed by COBRA three times, and each bar indicates mean ± SD.

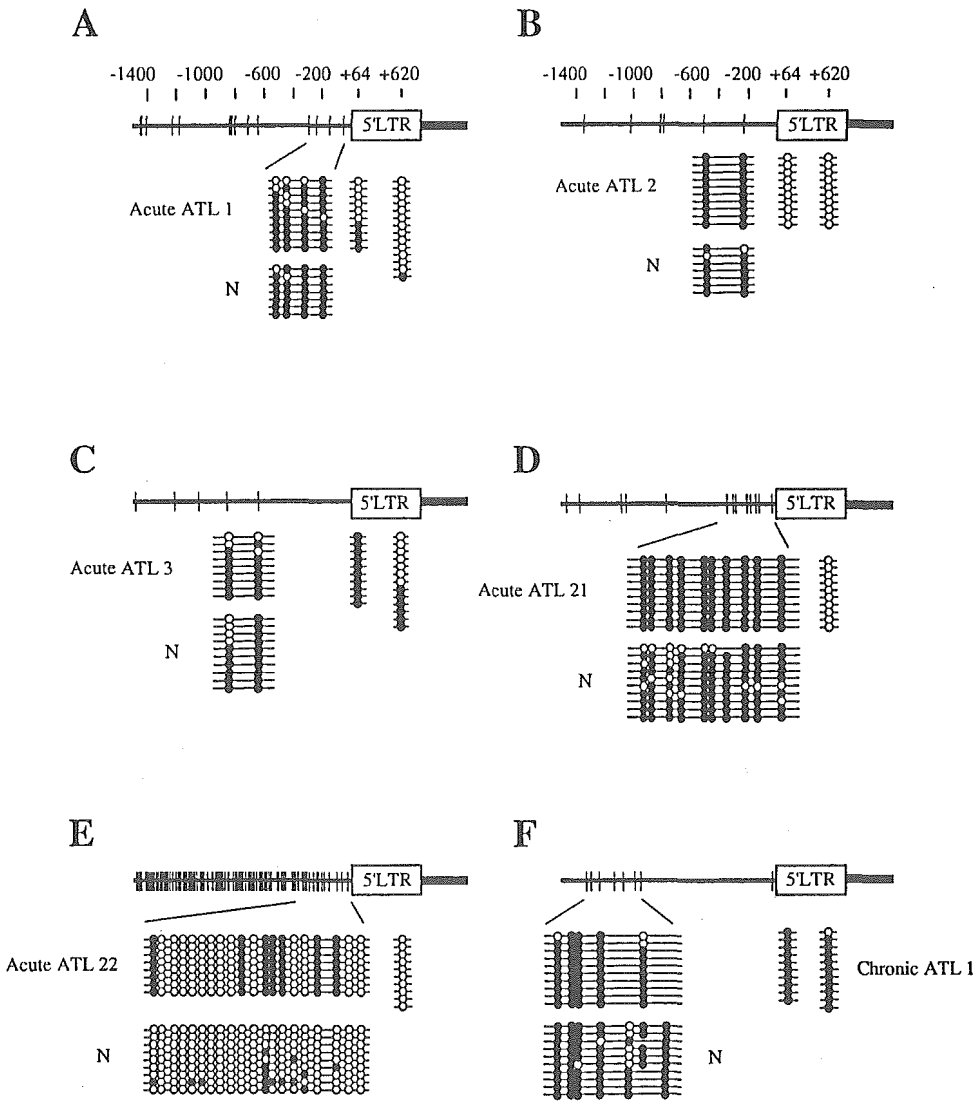

Figure 5

DNA methylation of provirus is not associated with methylated CpG sites in the genome. Integration sites of HTLV-I provirus in leukemic cells have been determined by inverse PCR, and then DNA methylation in genome has been analyzed by sodium bisulfite sequencing. DNA methylation of 5'-LTR was also analyzed by sodium bisulfite sequencing method. Vertical bars represent CpG sites. Open circle indicates unmethylated CpG site, and closed one means methylated CpG site. N: normal PBMCs from non-carrier donor.

Among cell lines, HTLV-I provirus tends to be not so methylated in cell lines with higher copy number of provirus (Fig. 2). The finding that cell lines with higher integrated provirus number contain hypomethylated provirus is speculated to reflect the higher transcription of viral genes.

DNA methylation of the HTLV-I provirus in ATL and HTLV-I carrier states

Next, we analyzed the DNA methylation of the whole HTLV-I provirus in ATL patients and HTLV-I carriers. Although 5'-LTR is frequently deleted in ATL cells [10], we omitted such ATL cases lacking 5'-LTR in this study. In Fig. 2D, we showed the representative pattern of DNA methylation of whole HTLV-I provirus in carriers and ATL patients. In ATL samples, the gag, pol and env regions were heavily methylated, whereas 5'-LTR was not methylated or partially methylated (Fig. 2D and 3A). On the other hand, 5'-LTR was scarcely methylated and the gag, pol and env regions seemed to be less methylated in HTLV-I carriers (Fig. 2D and 3A). We compared DNA methylation of these different eight regions between 20 carriers and 20 ATL cases (Fig. 3A). These differences in DNA methylation were statistically significant in the gag, pol and env regions between the ATL cases and HTLV-I carriers by the Mann-Whitney's U-test. These data suggested that DNA methylation initially occurred in the gag, pol, and env regions, and that DNA methylation of the provirus accumulated during disease progression from the carrier state to the leukemic stage. The frequency of DNA methylation of 5'-LTR did not differ between carriers and ATL patients. However, the extent of DNA methylation among methylation-positive cases was higher in ATL cases than in carriers ($p = 0.001$). Among ATL cases, the relationship between DNA methylation of 5'-LTR and tax gene transcription was analyzed (Fig. 3B), and the transcript was detected in six cases. In four cases with relative higher amount of tax gene transcripts (Case 1, 9, 12, 20), 5'-LTR was not methylated or slightly methylated. This finding suggests that higher expression of tax gene is associated with unmethyalted or slightly methylated 5'LTR, however, other mechanism(s) silences the tax gene transcription in ATL cells. There is no statistical correlation between the tax gene transcription and DNA methylation of 5'-LTR

DNA methylation of HTLV-I provirus after seroconversion

The analyses of DNA methylation suggest that it first occurs around the gag, pol and env regions, and then progresses in both the 5' and 3' regions. To study the changes in DNA methylation after infection, we analyzed sequential DNA samples from seroconverters. As shown in Fig. 4A, DNA methylation already existed in the gag, pol and env regions at the seroconversion. In seroconverter 1, DNA methylation was slightly increased at 4 and 13 years after the seroconversion. Increase of DNA methylation at pol region (4187) is statistically significant 13 years later in seroconverter 1 ($p = 0.02$, by a Student's t-test). On the other hand, there was little change in the DNA methylation in seroconverter 2, although the HTLV-I provirus was already heavily methylated at the seroconversion. When DNA methylation of seroconverters was compared with that in carriers (Fig. 3A), provirus of carriers was more methylated in carriers than that of seroconverters ($p < 0.01$ by a Student's t-test) except for pol2 in seroconverter 2, and pX region. It suggests that DNA methylation of provirus accumulates during a latent period after seroconversion.

We established an HTLV-I-transformed cell line, ATL-21C, and cultured for over 9 years *in vitro*, and analyzed the DNA methylation of the HTLV-I provirus. Slight DNA methylation was detected in the *pol*, *env* and pX regions at 6 months after culture, however, it did not increase after 9 years. This indicates that the DNA methylation of HTLV-I provirus did not change after long-term *in vitro* culture (Fig. 4B). On the other hand, the *p16* gene in this cell line was not methylated at 6 months after culture, but heavily methylated after 9 years (data not shown). A comparison with the data from the seroconverters suggests that DNA methylation of the HTLV-I provirus tends to accumulate *in vivo*.

Association with DNA methylation in the neighboring host genome

It is possible that the HTLV-I provirus integrated into the heterochromatin or hypermethylated regions tends to be silenced [18], and that such HTLV-I-infected cells are selected in vivo. Therefore, we analyzed the DNA methylation of the host genome around the integration sites of the HTLV-I provirus. We first determined the integration sites of the HTLV-I provirus in ATL cells, and then analyzed the DNA methylation of genomic DNAs around the integration sites in both ATL cells and normal PBMCs from a non-carrier donor. When genomic DNAs neighboring integration sites were heavily methylated (Fig. 5), 5'-LTR was not methylated in three cases (acute ATL 1, 2 and 21) while they were methylated in two cases (acute ATL 3 and chronic ATL 1). In acute ATL 22, both genomic DNA and 5'-LTR were not so methylated. Thus, DNA methylation in the neighboring genomic regions was not correlated with the methylation status of the provirus among these cases.

Histone modification of the HTLV-I provirus

It has been demonstrated that DNA methylation of 5'-LTR is associated with histone deacetylation and silencing of viral gene transcription in cell lines [13]. When ATL-43T, in which tax gene transcription was silenced by hypermethylation of 5'-LTR, was compared with a tax gene-

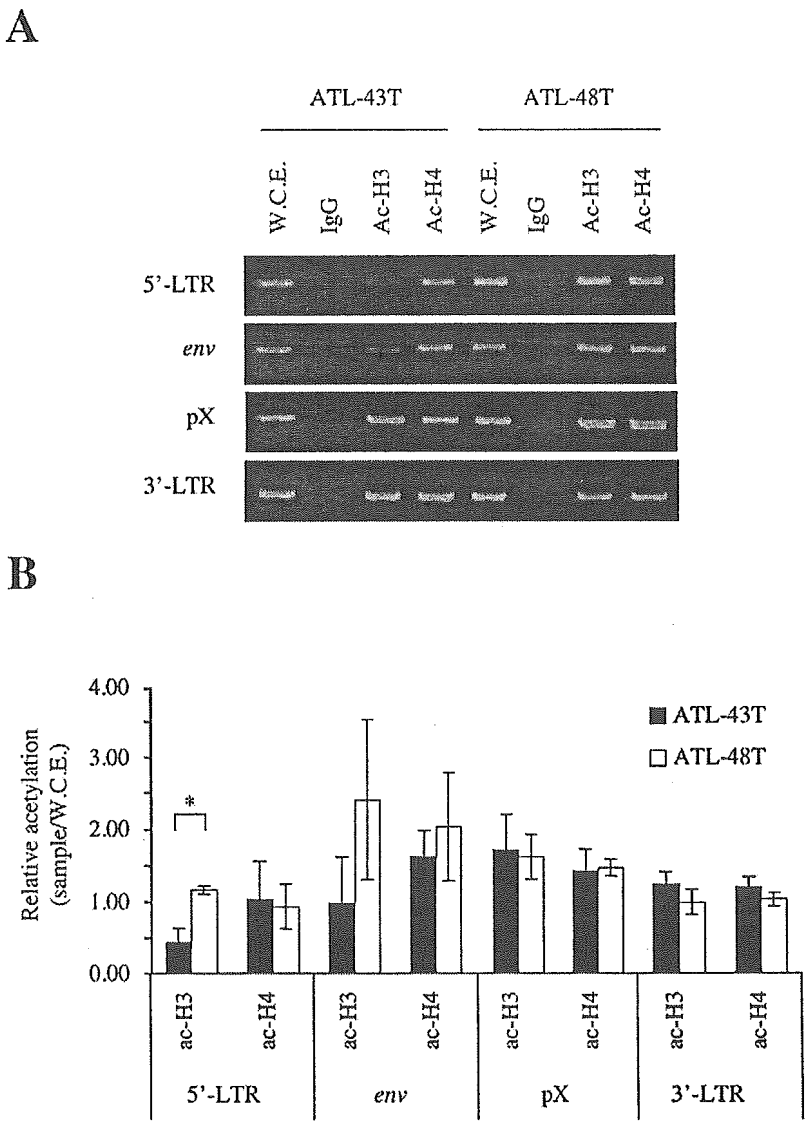

Figure 6
Histone modifications in ATL cell lines. Acetylation of histone was analyzed in *tax* gene-expressing (ATL-48T) and non-expressing (ATL-43T) cell lines by ChIP assays with anti-acetyl-Histone H3 or H4 (A and B) at four different regions (for 5'-LTR, *env*, pX and 3'-LTR) of the provirus. Representative data has been shown in A. W.C.E.: whole cell extract. ChIP assay was performed three times and quantified as described in Methods. Values are means ± SD(B). *:$p < 0.002$.

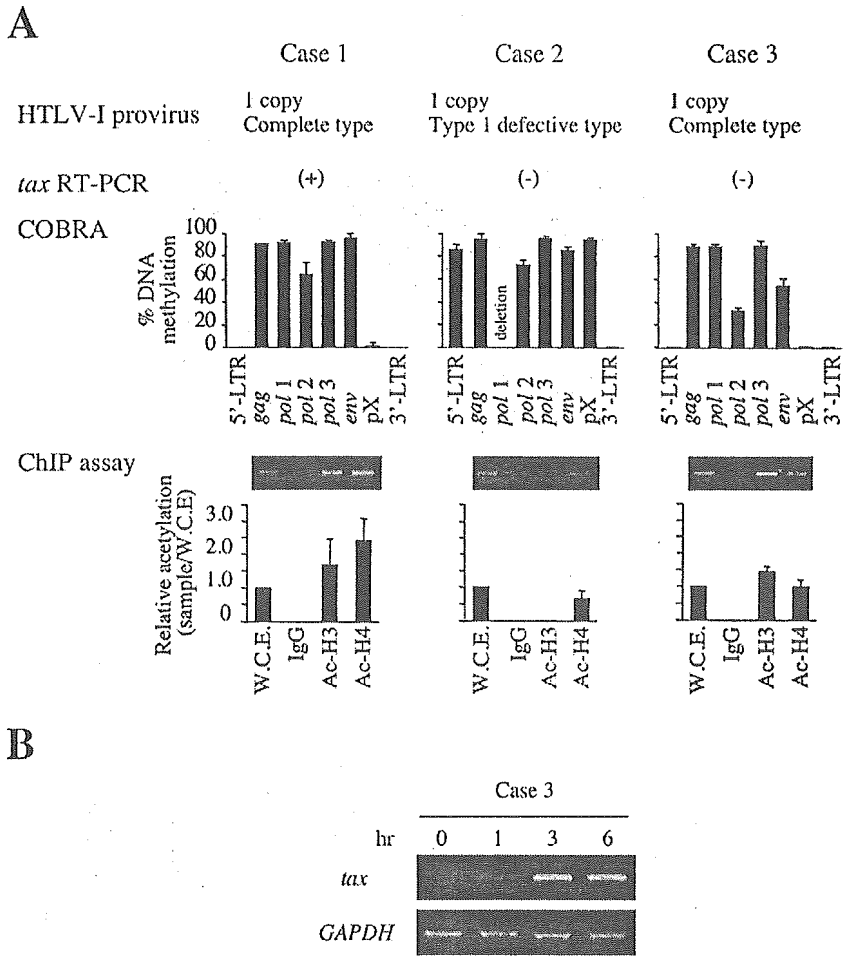

Figure 7

DNA methylation and histone modifications in fresh ATL cases. A. The relationships among DNA methylation, *tax* gene expression and histone modification in 5'-LTR were analyzed in three ATL cases. Cases 1 and 3 have one copy of the complete HTLV-I provirus, while Case 2 has a defective provirus that lacks part of the *pol* gene. DNA methylation was analyzed by COBRA. The *tax* gene transcripts could be detected in Case 1, but not in Cases 2 or 3, by RT-PCR. ChIP assays were also performed using primers for 5'-LTR to analyze acetylation of histone H3 (Ac-H3) and H4 (Ac-H4). W.C.E.: whole cell extract. B. Recovery of *tax* gene expression *ex vivo*. The PBMCs isolated from Case 3 were immediately cultured *ex vivo* for several hours and tested the transcription of *tax* mRNA by RT-PCR.

expressing cell line, ATL-48T, a difference was found in the acetylation of histone H3 in 5'-LTR (Fig. 6A and 6B). The histone H3 of 5'-LTR was hypoacetylated in ATL-43T compared with ATL-48T, whereas there were no differences in pX or 3'-LTR among these cell lines. Since the number of HTLV-I provirus in ATL-43T and -48T is one and two copies respectively, and acetylation of histone H3 in pX and 3'-LTR was similar in both cell lines, the number of provirus was thought to have no influence on the results of ChIP assay in 5'-LTR.

However, the *tax* gene transcription is silenced in about 20% of ATL cases despite no or partial methylation of 5'-LTR (Fig. 3B) [13], suggesting that there is aother mechanism(s) for suppressing viral gene transcription. To address this question, we studied the histone modification of 5'-LTR in fresh ATL cells with or without *tax* gene transcription. In a case with *tax* gene expression, 5'-LTR was not methylated and histone H3 was hyperacetylated (Fig. 7A, Case 1). On the other hand, in Case 2 with heavily methylated 5'-LTR, histone H3 was hypoacetylated in 5'-LTR, which was consistent with the lack of detection of *tax* gene transcription in this case. However, in Case 3, *tax* gene transcription could not be detected regardless of 5'-LTR hyperacetylation. After *in vitro* culture, such cells showed *tax* gene transcription within one hour (Fig. 7B). Although both Cases 1 and 3 exhibited hyperacetylation of 5'-LTR, *tax* gene transcription was silenced in Case 3.

Discussion

DNA methylation is regarded as a host defense mechanism for inactivating transportable elements such as retroviruses to inhibit viral transcription and the generation of new viruses. On the other hand, it also renders the provirus into a latent state, resulting in the establishment of latent infection. However, it remained unclear how and when the provirus was methylated, and whether DNA methylation changed *in vivo*.

Tax has the remarkable potency to promote the proliferation of infected cells [3], however, it is also a major target of CTL *in vivo* [8]. Therefore, HTLV-I controls *tax* gene expression by own viral proteins, Rex [19], p30 [20,21] and HBZ [22]. In the leukemic cells, several mechanisms have been identified to suppress or abolish Tax expression, including genetic changes of *tax* gene, deletion of 5'-LTR, and DNA methylation of 5'-LTR. In this study, DNA methylation was shown to occur in internal provirus sequences, such as the *gag*, *pol* and *env* regions, and then extend to 5' (5'-LTR) and 3' (pX) regions. Since DNA methylation of 5'-LTR is associated with *tax* gene transcription, the finding that 5'-LTR was more highly methylated in ATL cells than in carriers, among cases with methylated 5'-LTR, suggests that such HTLV-I-infected cells and ATL cells with the methylated provirus, which produce lower amounts of viral proteins, are selected *in vivo* by the host immune system. In this regard, HTLV-I is quite different from another human retrovirus, HIV-1. HIV-1 vectors were resistant to gene silencing *in vivo* [23,24]. It is noteworthy that the number of CpG sites in the U3 region of HIV-1 LTR (9 sites in LTR of NL43) is much fewer than that of HTLV-I (47 sites in LTR of ATK). This is consistent to the previous report that transcriptional suppression was not associated with DNA methylation of HIV-1 provirus [25]. In addition, HIV-1 provirus is frequently integrated within transcriptional units, which encode the genes that are transcribed in T-cells [15,26]. In such regions, it is possible that HIV-1 tends to escape from transcriptional silencing that is observed in the heterochromatin region such as alphoid repetitive sequences [18]. These data suggest that HIV-1 is more resistant to gene silencing than HTLV-I. Alternatively, it is possible that HTLV-I takes advantage of susceptibility to DNA methylation to escape from the host immune system.

This study shows that 3'-LTR is unmethylated in carriers and ATL cells while 5'-LTR is methylated in about half of cases. In HTLV-I, *HTLV-I bZIP (HBZ) gene* is encoded by minus strand of provirus [22,27]. We observed that *HBZ* gene was transcribed in all ATL cells, suggesting that *HBZ* gene play a critical role in growth of HTLV-I infected cells and ATL cells (submitted for publication). The finding that 3'-LTR is unmethylated in all ATL cases and carriers suggests that *HBZ* gene transcription is important for proliferation of ATL and HTLV-I infected cells.

Why does DNA methylation occur from the internal sequences of the HTLV-I provirus? Since CpG island is recognized as DNA region that is susceptible to DNA methylation, we analyzed HTLV-I provirus by the criterion by Takai and Jones [28]. CpG islands are present throughout the provirus in 5'-LTR-*gag* (1–1360), *pol* (3876–4509), *env* (5648–6166), *env*-pX (6446–7561), and pX-3'-LTR (8212–9045) regions. Therefore, the presence of CpG island could not explain why DNA methylation occurred in the internal region of HTLV-I provirus. Among tumor-suppressor genes, which are transcriptionally silenced by DNA methylation, the exon regions are first methylated, and then DNA methylation progresses to the promoter region [29]. When the promoter region is heavily methylated, the transcription of the corresponding gene is silenced. Since 5'-LTR is the promoter/enhancer for viral gene transcription, there might be a similar scenario between the exon/promoter and DNA methylation in both virus and tumor-suppressor genes. Thus, it is possible that gene coding regions are first methylated and DNA methylation spreads to the promoter region of provirus, 5'-LTR.

Transcriptional silencing of *tax* gene in spite of hyperacetylated histone H3 is recognized as another mechanism to suppress the viral gene transcription in addition to DNA methylation. The prompt recovery of *tax* gene expression after *in vitro* culture suggests the presence of an inhibitory factor(s) that binds to 5'-LTR, and suppresses the viral gene transcription *in vivo*. It is noteworthy that this phenotype is very similar to that of a mouse T-cell line transfected with an HTLV-I LTR-derived reporter plasmid [30]. In that study, a green fluorescent protein-fused Tax (*Gax*) gene was transfected into a mouse T-cell line, EL-4, and the transduced cells were then injected into Tax-immunized and non-immunized mice. Although Tax-induced cytotoxic T-cells suppressed the expression of the *Gax* gene *in vivo*, its expression was shown to recover within three hours when the transduced cells were transferred to *in vitro* culture. This phenotype resembles that observed in Case 3 in Fig. 7. Considering that Tax is the major target of CTL *in vivo*, and at the same time, confers growth advantages on the infected cells, such reversible suppression of *tax* gene expression is thought to be suitable for the survival of HTLV-I infected cells, and ATL cells. In this regard, potentiation of anti-Tax immunity might protect against the development of ATL when combined with possible therapeutics to induce Tax expression [31]. For this purpose, the mechanism for silencing viral transcription regardless of histone H3 hyperacetylation should be studied.

In general, gene silencing is associated with several different mechanisms. DNA methylation in the promoter region silences the gene transcription, whereas gene silencing is often not associated with DNA methylation [32,33]. In such situations, methylation of H3K9 is linked with loss of transcriptions [34]. It is possible that silencing of viral gene transcription renders proviral DNA vulnerable to methylation. Once proviral DNA is methylated, such silencing would be fixed unless such cells are treated with demethylating agents such as 5-aza-deoxy-cytidine.

DNA methylation of the HTLV-I provirus did not accumulate in a cell line that was cultured *in vitro* for more than 9 years. The finding that the *p16* gene was heavily methylated in this cell line excluded the possibility that hypermethylation did not occur in this cell line due to aberrant methylation machinery. Among the seroconverters, the provirus was heavily methylated in internal regions such as *gag*, *pol* and *env*. Taken together, DNA methylation in the provirus is considered to reflect the selection *in vivo*. Since the growth of *in vitro* HTLV-I-transformed cell lines depends on Tax expression, cells with suppressed expression of the *tax* gene do not have the growth advantage *in vitro*. However, the immune system exerts selection of the infected cells with suppressed *tax* gene expression *in vivo*.

Recently, both 5'- and 3'-LTR have been reported to be transcriptionally active, and transcriptional factors and Tax bind equally to both [35]. 3'-LTR may activate the transcription of cellular genes, which are located in the downstream of integration sites. In addition, unmethylated 3'-LTR is critical for transcription of the *HBZ* gene. Since 5'-LTR is a promoter/enhancer for viral gene transcription, selective methylation of 5'-LTR is considered to silence the transcription of viral genes.

Conclusion

We have demonstrated how DNA methylation of HTLV-I provirus occurred, and how it suppressed viral gene transcription. When 5'-LTR was heavily methylated, viral transcription was silenced, which is thought to reflect the immune system selection *in vivo*. In addition, mechanisms other than DNA methylation suppresses viral gene transcription regardless of histone H3 hyperacetylation. The mechanism of such suppression requires further investigation.

Methods

Cells

HTLV-I-associated cell lines (MT-1, MT-2, MT-4, ATL-2, TL-Oml and Sez627) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. For interleukin-2-dependent cell lines (ATL-43T, 48T and 55T), 100 U/ml of recombinant interleukin-2 (Shionogi, Osaka) was added to the medium. Peripheral blood mononuclear cells (PBMC) or lymph node cells were isolated from HTLV-I carriers and ATL patients after informed consent was obtained. The polyclonal integration of HTLV-I provirus in carriers has been shown by inverse PCR [36], and provirus load was determined by real-time PCR as reported previously [37].

Sodium bisulfite treatment of genomic DNA

Sodium bisulfite treatment was performed as described previously [29]. Briefly, 1–3 μg of genomic DNA was denatured in 0.3 N NaOH at 37°C for 15 min, and 1 μg of salmon sperm DNA was added to each sample as a carrier. Sodium bisulfite (pH 5.0) and hydroquinone were added to each sample to final concentrations of 3 M and 0.05 mM, respectively. The reaction was performed at 55°C for 16 h and the samples were then desalted using the Wizard DNA Clean-Up System (Promega, Madison, WI). Finally, samples were desulfonated in 0.3 N NaOH at 37°C for 15 min.

Sequencing of sodium bisulfite-treated genomic DNA

The sodium bisulfite-treated DNA (200–500 ng) was used as a template for PCR amplification of eight HTLV-I provirus regions. The PCR reactions were performed using FastStart Taq DNA Polymerase (Roche, Mannheim, Germany). The PCR primer pairs and annealing temperatures

Table 1: Primer sets for COBRA and ChIP assay

	Site in HTLV-I[a]		Forward primer	Reverse primer	Anneal (°C)	Enzyme for COBRA
COBRA	620	1st	5'-TTTGGAGTTTATTTAGATTTAG-3'	5'-CCAATAATAAACRACCAACCC-3'	45	*Taq*I
	(5'-LTR)	2nd	5'-GTTTTGTTTGATTTTGTTTGT-3'	5'-AAAAAAATTTAACCCATTACC-3'	49	
	1753	1st	5'-GGGAGTGTTAAAGATTTTTTTTGGG-3'	5'-ACTCCAATAACCTACTTTCCC-3'	55	*Taq*I
	(*gag*)	2nd	5'-TTTATTTTTTAAGGTTTGGAGGAG-3'	5'-TTAAAAATCCAAATCTAACAAACCC-3'	55	
	2988	1st	5'-GTTAAAAAGGTTAATGGAATTTGG-3'	5'-CCTCTAAAAATAATAATAAATCCTC-3'	52	*Taq*I
	(*pol*)	2nd	5'-GGGTTTTTTGATTTGTTTAGTTTG-3'	5'-AAACTTACTAAAAAAATATCATCC-3'	51	
	4187	1st	5'-GGGTGAAATTGTGTAGTTTTGTAGG-3'	5'-CCTATTTTCAAACGAATCTACCTCC-3'	57	AccII
	(*pol*)	2nd	5'-GTGATTAGTAGGGTATTTGTGAGAG-3'	5'-ATTATCACAAAAATCATTCCCCC-3'	52	
	5151	1st	5'-GGTATTATTTTAAGTTTTTTGG-3'	5'-CTCCAATTATAAAAATACAACAAC-3'	46	*Taq*I
	(*pol*)	2nd	5'-GTTAGTGGAAAGGATTATAGGAGG-3'	5'-AACTTACCCATAATATTAAAAATC-3'	51	
	6113	1st	5'-GGATTTATTGTTTTGATTTTTAG-3'	5'-CTTTACATAATCCTCCTTACTCCC-3'	51	*Taq*I
	(*env*)	2nd	5'-GGATTTATTGTTTTGATTTTTAG-3'	5'-CCCAAAACAAAAAATCAAAACC-3'	53	
	7258	1st	5'-GAGGTGGYGTTTTTTTTTTTGG-3'	5'-CCTTAAAAATCTTAAAAATTCTC-3'	47	*Taq*I
	(pX)	2nd	5'-AAGGATAGTAAATYGTTAAGTATAG-3'	5'-CCCAAATAATCTAATACTCTAAAC-3'	50	
	8342	1st	5'-YGATGGTAYGTTTATGATTTTYGGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	57	*Taq*I
	(3'-LTR)	2nd	5'-YGATGGTAYGTTTATGATTTTYGGG-3'	5'-AACTCCTACTAATTTATTAAACC-3'	52	
	5'-LTR[b]		5'-GCTTTGCCTGACCCTGCTTGC-3'	5'-AAGATTTGGCCCATTGCCTAGGG-3'	63	
	env		5'-TGCCAGCCTCTCCACTTGGCACG-3'	5'-ATGGAGCCGGTAATCCCGCCAGC-3'	64	
	pX		5'-AAGGATAGCAAACCGTCAAGCACAG-3'	5'-CCCAGGTGATCTGATGCTCTGGAC-3'	63	
	3'-LTR		5'-CCCCTCATTTCTACTCTCACACGGC-3'	5'-TGGGTGGTTCTTGGTGGCTTCCC-3'	64	

[a] Nucleotide position corresponding to that of ATK. This number means the cytidine of CpG sites analysed.
[b]For ChIP assay, we used primers to amplify the indicated regions.

are shown in Table 1. The amplified PCR products were purified and subcloned into pGEM-T Easy vectors (Promega). For each region, at least 10 clones were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA) and ABI3100 autosequencer (Applied Biosystems).

Combined bisulfite restriction analysis (COBRA)

For COBRA, eight different regions of HTLV-I provirus were amplified with sodium bisulfite treated genomic DNAs using each primer sets as shown in Table 1. The nested PCR reactions were performed using FastStart Taq DNA Polymerase (Roche) with the following condition: 5 minutes at 95°C for denaturation, 40 cycles of 30 sec at 95°C, 30 sec at each annealing temperature (Table 1), 30 sec at 72°C, and 2 min at 72°C for final extension. The PCR products were digested for at least 4 hrs with an appropriate restriction enzyme (TaqI or AccII) that had a single recognition site within each product [38]. When CpG site within amplified region was methylated, it was resistant to sodium bisulfite treatment, resulting in digestion by these enzymes. On the other hand, since unmethylated CpG was converted to UG by sodium bisulfite treatment, these enzymes could not digest the amplified DNAs. The digested PCR products were separated in a 3% Nusieve 3:1 agarose (BMA, Rockland, ME) gel. The intensity of each fragment was determined using ATTO Densitograph Ver. 4.0 (ATTO, Tokyo, Japan), and the extent of DNA methylation was calculated as follows: % methylation = 100 × (digested PCR products/undigested+ digested PCR products).

Southern blot analyses

To determine the number of integrated HTLV-I provirus, we performed Southern blot method using HTLV-I probe as described previously [10]. In brief, 5 μg of DNA were digested with EcoRI, separated by electrophoresis in a 0.7% agarose gel, and transferred to nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ). The membrane was hybridized to the alkaline phospatase labeled pX probes. 0.9 kb PCR product of HTLV-I pX region derived from HTLV-I clone λ23-3 was used as probe [39]. DNA probe was labeled, and hybridized to the membrane with Gene Images AlkPhos Direct Labelling and Detection system (Amersham Biosciences).

Inverse-long PCR

To check the HTLV-I integration in PBMCs of carriers, we analyzed the genomic DNAs from carriers by inverse-long PCR method as described previously [36]. In brief, genomic DNA was digested with EcoRI, and then ligated with T4 DNA ligase. Circularized DNA was digested with MluI that cut the provirus at pX region to prevent amplification of provirus itself. Then, treated genomic DNA was amplified with primers as follows: Long-IPCR-F: 5'-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3', Long-IPCR-R 5'-AGTCTGGGCCCTGACCTTTTCAGACTTCTGTTTC-3'. PCR condition was as follows: 2 min at 98°C for denaturation, 5 cycles (30 sec at 98°C, 10 min at 64°C), followed by 35cycles (30 sec at 94°C, 10 min at 64°C) and 15 min at 72°C for final extension. The PCR products were subcloned into plasmid DNA and their sequences were determined.

Table 2: Primer sets and annealing temperatures for genome specific PCR

	Case	Locus		Forward primer	Reverse primer	Anneal (°C)
Primers for case	Acute ATL 1	5q11.1	1st	5'-TTTGGAGAGGGAATTTTATATTG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	55
			2nd	5'-GGAGTGTAGAGATGTAGTTTTGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	50
	Acute ATL 2	8p23.1	1st	5'-GAGAAATTTGTGTTGATTTTATTAG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	47
			2nd	5'-TTAGTGGTAGATTAAGTTAAAG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	45
	Acute ATL 3	1q31.1	1st	5'-GGTAGAAATTATAGGTTTTTGTAGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	51
			2nd	5'-GTTATTTGTGAAGTAAGATGTTTTG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	53
	Acute ATL 21	15q24.3	1st	5'-GAGGTGGATTTTTATTTTATTG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	52
			2nd	5'-GGTTTTTGATTATATTTGGGGAG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	54
	Acute ATL 22	19q13.11	1st	5'-GTTAGTTGTTAGAGAGTTTTTTGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	52
			2nd	5'-AAGATTATTTAGTTTTTTGGGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	54
	Chronic ATL 1	1p22.1	1st	5'-GGGTTTGAAGTTTTTTTTGTAGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3'	53
			2nd	5'-AAGATTATTTAGTTTTTTGGGG-3'	5'-ACCCCCTCCTAAACTATCTCC-3' (5'-LTR U3)	50
Primers for human genome		5q11.1	1st	5'-TTTGGAGAGGGAATTTTATATTG-3'	5'-CCCAAACTAATCTTCAACTCC-3'	52
			2nd	5'-GGAGTGTAGAGATGTAGTTTTGG-3'	5'-CCACCATAAAAAACCCTCCC-3'	54
		8p23.1	1st	5'-GAGAAATTTGTGTTGATTTTATTAG-3'	5'-AATATCACTATAACAATAACCAC-3'	46
			2nd	5'-TTAGTGGTAGATTAAGTTAAAG-3'	5'-CTCTCAACAAATTCCATCTTTCC-3'	49
		1q31.1	1st	5'-GGTAGAAATTATAGGTTTTTGTAGG-3'	5'-CACCATTAAACAAACTAAATTCTC-3'	51
			2nd	5'-GTTATTTGTGAAGTAAGATGTTTTG-3'	5'-CACATAAAAAAACCCACACAATC-3'	53
		15q24.3	1st	5'-GAGGTGGATTTTTATTTTATTG-3'	5'-ATCTACCTAAAAAACCCACCC-3'	52
			2nd	5'-GGTTTTTGATTATATTTGGGGAG-3'	5'-AAAAACCCACCCAAACAAACC-3'	57
		19q13.11	1st	5'-GTTAGTTGTTAGAGAGTTTTTTGG-3'	5'-CAACTCCCTAAACCCTCCTCC-3'	52
			2nd	5'-GTTTTTTGGTTAAGGTTATGGG-3'	5'-CTCCTACCACGAACCTACTCC-3'	54
		1p22.1	1st	5'-GGGTTTGAAGTTTTTTTTGTAGG-3'	5'-CAACAAAAACAATAAACAAAACC-3'	54
			2nd	5'-AAGATTATTTAGTTTTTTGGGG-3'	5'-CTTTACACCAATAAATTTAATACC-3'	50

DNA methylation in neighboring regions of HTLV-I integration sites

The integration sites of HTLV-I provirus has been determined by inverse long PCR, and DNA methylation of genomic DNAs neighboring integration sites was determined in both ATL cells and PBMCs. The nested PCR reactions were performed using FastStart Taq DNA Polymerase (Roche) with the following condition: 5 minutes at 95°C for denaturation, 40 cycles of 30 sec at 95°C, 30 sec at each annealing temperature (Table 2), 30 sec at 72°C, and 2 min at 72°C for final extension.

RT-PCR

Total RNA was isolated from PBMCs or lymph node cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed using RNA LA PCR Kit (AMV) Ver. 1.1 (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. The tax and GAPDH gene transcripts were amplified using the following primers: RPX2 5'-CCGGCGCTGCTCTCATCCCGGT-3' and RPX5 5'-GGCCGAACATAGTCCCCCAGAG-3' (for tax), GAPDH1 5'-ATGGGGAAGGTGAAGGTCGGAGTC-3' and GAPDH1a 5'-CCATGCCAGTGAGCTTCCCGTTC-3' (for GAPDH) under following conditions: 2 minutes at 95°C for denaturation, 35 cycles of 30 sec at 95°C, 30 sec at 62°C, 30 sec at 72°C (for tax), 25 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C (for GAPDH) and 2 min at 72°C for final extension.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously [40]. Briefly, ATL cell lines and fresh ATL cells from ATL patients (5×10^5 cells/antibody) were fixed with formaldehyde and then sonicated to obtain soluble chromatin. The chromatin solutions were immunoprecipitated with anti-acetyl-Histone H3 or anti-acetyl-Histone H4 (Upstate Biotechnology), or normal rabbit IgG, overnight at 4°C, and the immunoprecipitates were then collected with 50% protein A and G-Sepharose slurry preabsorbed with 0.1 mg/ml sonicated salmon sperm DNA. The resulting purified DNAs were subjected to PCR reactions using primer sets specific for 5'-LTR, env, pX and 3'-LTR. The sequences of the primers are shown in Table 1. To distinguish 5' and 3'-LTR, we used primers specific for gag and R region of LTR for amplification of 5'-LTR, and primers for pX region and U3 region were used for amplification of 3'-LTR. The PCR reactions were performed using FastStart Taq DNA Polymerase (Roche) with the following condition: 5 minutes at 95°C, 35 or 37 cycles of 30 sec at 95°C, 30 sec at each annealing temperature (Table 1), 30 sec at 72°C, and 2 min at 72°C. The PCR products were electrophoresed in an agarose gel and the results were analyzed using ATTO Densitograph Ver. 4.0. Values were calculated as the signal intensity of each sample normalized by that of the whole cell extract.

Statistical analyses

Statistical analyses were performed using the Mann-Whitney's U-test and Student's t-test.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

YT conceived this project and carriers out most of experiments in Figs. 1, 2, 3, 5 and 6. KN established COBRA assay and performed experiments in Figs. 1 and 2. JY performed experiments in Fig. 7. MM established most of HTLV-I transformed cell lines, and analyzed experiments in Fig. 4. AO and NM provided sequential DNA samples from seroconverters, and analyzed the data. M.Matsuoka directed and supervised the experiments and interpretations All authors read and approved the final manuscript.

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Preferential selection of human T-cell leukemia virus type I provirus integration sites in leukemic versus carrier states

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Human T-cell leukemia virus type I (HTLV-I) is a causative agent of neoplastic disease, adult T-cell leukemia (ATL). Although the encoding viral proteins play an important role in oncogenesis, the role of the HTLV-I proviral integration site remains unsolved. We determined the integration sites of HTLV-I proviruses in ATL cells and HTLV-I–infected cells in asymptomatic carriers. In carrier and ATL cells, HTLV-I provirus was integrated into the transcriptional unit at frequencies of 26.8% (15/56) and 33.9% (20/59), respectively, which were equivalent to the frequency calculated based on random integration (33.2%). In addition, HTLV-I provirus was prone to integration near the transcriptional start sites in leukemic cells ($P = .006$), and the transcriptional direction of the provirus was in accordance with that of integrated cellular genes in 70% of cases. More importantly, the integration sites in the carrier cells favored the alphoid repetitive sequences (11/56; 20%) whereas in leukemic cells they disfavored these sequences (2/59; 3.4%). Taken together, during natural course from carrier to onset of ATL, HTLV-I–infected cells with integration sites favorable for viral gene transcription are susceptible to malignant transformation due to increased viral gene expression. (Blood. 2005;106:1048-1053)

Introduction

After infection with retrovirus, reverse transcriptase synthesizes proviral DNA and then integrates the provirus into the host genome by the action of an integrase. In some retrovirus-associated neoplasms, provirus insertion enhances the transcription of oncogenes, such as the *myc* gene, resulting in transformation of infected cells.[1,2] Although the integration sites of proviruses in the host genome have been considered random, recent studies regarding various retroviruses revealed that human immunodeficiency virus type 1 (HIV-1) prefers transcriptional units,[3] whereas murine leukemia virus (MLV) tends to integrate near the transcriptional start sites.[4] These findings suggest that the integration of proviruses depends on mechanisms unique to each retrovirus, which interact with host factors associated with nuclear transport, DNA repair, and chromatin structure.[5]

Human T-cell leukemia virus type I (HTLV-I) is the causative virus of adult T-cell leukemia (ATL) and inflammatory disease, HTLV-I–associated myelopathy (HAM)/tropical spastic paraparesis (TSP).[6] HTLV-I infection induces ATL in a portion of infected individuals after a long latent period. The characteristic of HTLV-I is the presence of accessory genes, which are encoded by the pX region between *env* and the 3′-long terminal repeat (LTR).[7,8] Among the accessory genes, *tax* is considered to play a central role in the proliferation of infected cells and leukemogenesis because of its pleiotropic actions. Tax activates transcriptional pathways such as nuclear factor κB (NF-κB), serum response factor (SRF), and cyclic AMP response element binding protein (CREB), leading to activated transcription of growth factor and its receptor genes, and inhibition of apoptosis. In addition, Tax can transrepress the transcription of cellular genes and functionally inhibit p53,[9] p16,[10] and MAD1.[11] Such pleiotropic actions induce the proliferation of HTLV-I–infected cells, and inhibit apoptosis, resulting in clonal expansion in vivo.

Although it has been reported that the integration sites of HTLV-I provirus are random, preferential integration into the transcriptional units has been reported in ATL cells.[12,13] In this study, we compared HTLV-I integration sites between carriers and leukemic cells, and found that the provirus was frequently integrated into alphoid repetitive sequences in the carrier state, but not the leukemic state.

Materials and methods

Patient samples

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation. They were then digested with proteinase K and treated with RNase A to eliminate RNA. Genomic DNAs were extracted from the PBMCs of 16 HTLV-I carriers and 59 patients with ATL. Approval for this study was obtained from the institutional review board of the Kyoto University. The informed consent was obtained from blood donors and patients according to the Declaration of Helsinki.

Inverse long polymerase chain reaction

Inverse long polymerase chain reaction (IL PCR) was used to amplify the genomic DNA adjacent to the integration sites of the HTLV-I

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provirus. First, genomic DNA (1.5 μg) was digested with a restriction enzyme (*Hin*dIII, *Pst*I, or *Eco*RI), then ligated by T4 DNA ligase. When DNA was digested with *Eco*RI, it was digested with *Mlu*I after ligation so as not to amplify the HTLV-I provirus itself. The resulting DNA was used as a substrate for IL PCR, which was performed using TaKaRa LA PCR (Takara, Shiga, Japan). Briefly, primers (final concentration, 0.2 μM), $MgCl_2$ (2.5 mM), and deoxynucleoside triphosphates (dNTPs, 0.4 mM) were mixed (total 20 μL) then AmpliWax (Applied Biosystems, Norwalk, CT) was added to each tube. After wax layer formation by incubation at 80°C for 10 minutes and cooling at room temperature for 15 minutes, substrate DNA (0.5 μg), 10 × LA buffer (5 μL), and LA Taq (0.4 μL) were added (total 30 μL). Cycles for long PCR were as follows: one cycle of 98°C for 2 minutes, 5 cycles of 98°C for 30 seconds and 64°C for 10 minutes, and 35 cycles of 94°C for 30 seconds, 64°C for 10 minutes, and 72°C for 15 minutes. The primers used in this experiment were as follows: primers 1 and 2 were used for *Pst*I-digested samples, primer 1: 5′-TAGCAGGAGTCTATAAAAGCGTGGAGACAG-3′; primer 2: 5′-TGGAATGTTGGGGGTTGTATGAGTGATTGG-3′. Primers 1 and 3 were used for *Hin*dIII-digested samples, primer 3: 5′-TGGGCAGGATTGCAGGGTTTAGAGTGG-3′. Primers 4 and 5 were used for *Eco*RI-digested samples, primer 4: 5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′, primer 5: 5′-AGTCTGGGCCCTGACCTTTTCAGACTTCTGTTTC-3′.

Cloning and sequencing

IL PCR products from ATL samples were used as a template for direct sequencing to determine the integration site. The primer used for sequencing was as follows: 5′-TCATTCACGACTGACTGCCGG-3′. To determine integration sites from carrier samples, IL PCR products were gel-isolated using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and ligated into a pPCR-Script Amp SK(+) cloning vector (Stratagene, La Jolla, CA) or pCR-TOPO-XL (Invitrogen, Carlsbad, CA). The plasmids were then used as a template for sequencing. Sequencing of IL PCR amplicon was performed using an ABI PRISM Genetic Analyzer 310 or 3100 (Applied Biosystems, Norwalk, CT) according to the manufacturer's instructions.

Mapping integration sites

The BLAST-like Alignment Tool program was used to map sequences to the human genome (University of California–Santa Cruz [UCSC] Human Genome Project Working Draft, July 2003 freeze).[14] Sequence matches were judged to be authentic only if they (1) contained the LTR sequence, (2) showed 95% or greater identity to the genomic sequence over the high-quality sequence region, and (3) matched only one genomic locus with 95% or greater identity. Genomic features such as coding regions and repetitive sequences were investigated using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start). Random integration sites (10 000 ×) in nongap regions of the human genome (UCSC Human Genome Project Working Draft, July 2003 freeze) were generated with a computer program using a uniform distribution algorithm, and used for comparison with the observed HTLV-I integration sites.

Statistical analyses

The χ^2 or one-sided Fisher exact test was used to determine statistical significance.

Results

HTLV-I provirus integration sites

We determined the genomic sequences adjacent to the HTLV-I provirus integration sites in ATL cells (59 cases) and HTLV-I–infected cells (56 sites) in the 16 carriers by inverse PCR, and then analyzed (1) chromosomal locations, (2) the genes containing integration sites or neighboring genes, (3) the relation of transcriptional direction between provirus and cellular genes when the HTLV-I provirus was integrated within the gene, (4) distance from the transcriptional start site, and (5) repetitive sequences. The results are summarized in Table 1.

As reported previously,[15,16] the integration sites of HTLV-I provirus were random in both leukemic cells and carriers (Figure 1) and were not associated with the guanine and cytosine (GC) content of surrounding genomes (data not shown). In addition, there was no specific chromosome for HTLV-I integration. To investigate whether HTLV-1 integration sites prefer transcription unit in the human genome, we used the UCSC Genome Browser RefSeq Genes track, which represents annotated genes based on National Center for Biotechnology Information mRNA reference sequences. An integration site is considered in a transcription unit if it locates within the transcription start site and stop site of a RefSeq gene. Among the 56 HTLV-I integration sites in the carrier cells, 15 (26.8%) were identified within the transcriptional units (RefSeq). On the other hand, 20 integration sites (33.9%) existed within the transcriptional units in the 59 ATL cases (Table 2). As a control, we simulated random HTLV-1 integration by placing 10 000 integration sites randomly into the same human genome, of which 33.2% were found within the transcription units (Table 2), a percentage identical to the estimated transcribed human genome.[17,18] Although the frequency of integration into transcriptional units was low in carriers, the difference between the carriers and ATL cells was not statistically significant.

Since annotation for the human genome has been changing rapidly in the last few years, the previous reports on HTLV-I integration sites should be reexamined.[12,13] The frequency of integration into the transcriptional units has reduced to 46.6% from 56.4%. When these data are combined with those presented here, the provirus landed in 48 of 119 cases (40.3%), which is not statistically significant compared with random integration (33.2%; $P = .17$ by a χ^2 test). The frequency of integration into the transcriptional units did not differ between ATL and carriers ($P = .08$ by a χ^2 test).

When HTLV-I provirus was integrated into transcriptional units, the transcriptional direction of the provirus was in accordance with that of integrated cellular genes in 70% of cases among the leukemic cells. Since the deletion of 5′-LTR is frequently observed in the provirus of ATL cells, which is a designated type 2 defective provirus,[19] the cellular promoter might act as a promoter for viral genes in such proviruses as reported previously.[20] Therefore, we investigated the relationship of transcriptional direction between cellular genes and type 2 defective provirus. In 2 of 4 cases with type 2 defective provirus, the provirus was integrated into transcriptional units, which were the mutated in colorectal cancers (*MCC*) and protocadherin 9 genes (Table 1). HTLV-I provirus was inserted in both genes in a sense direction, suggesting that the promoter of cellular genes might transcribe the viral genes in such cases.

HTLV-I provirus tends to be integrated near the transcriptional start sites in leukemic cells

In MLV, the provirus tended to be integrated near the transcriptional start sites.[4] On the other hand, HIV-1 has no such tendency in spite of its preference for transcriptional units. Since HTLV-I is a human retrovirus, it is of interest to determine whether HTLV-I has a similar tendency to MLV or HIV-1. In HTLV-I, 9 of the 59 proviruses were integrated near the transcriptional start sites (± 5 kb) in ATL cells (9/59; 15.3%) as shown in Table 3 and Figure 2. In contrast, using the same genome assembly, only 5.6% of the random integration landed near transcriptional start sites. Hence, the frequency of integration near the transcriptional start sites was statistically significant ($P = .006$), which is similar to MLV. Since the number of integration within plus or minus 5 kb from transcription start sites is only 3, there is no preference of integration near the transcriptional start sites.

Table 1. The genes that hosted integration event

Sample	Locus	RefSeq gene	Entrez Gene ID	Exon or intron	Direction
A 1	2q14.2	Protein tyrosine phosphatase, nonreceptor type 4	5775	intron 10	sense
A 2	2q21.1	POTE14	404785	intron 8	antisense
A 3	3p14.3	Calcium channel, voltage-dependent, alpha 2/delta 3 subunit	55799	intron 9	sense
A 4	3p26.3	Contactin 4	152330	intron 2	sense
A 5	4p15.32	Hypothetical protein FLJ90013	202018	intron 1	sense
A 6	4q28.2	PHD finger protein 17	79960	exon 9	sense
A 7	5q22.2	Mutated in colorectal cancers	4163	intron 1	sense
A 8	6q21	REV3-like, catalytic subunit of DNA polymerase zeta	5980	exon 16	sense
A 9	7q11.23	Williams-Beuren syndrome chromosome region 5	7462	intron 1	sense
A 10	8q21.13	CGI-62 protein	51101	intron 1	antisense
A 11	9p21.3	Cyclin-dependent kinase inhibitor 2A (p16)	1029	intron 1	antisense
A 12	10q22.1	Cadherin-like 23	64072	intron 58	antisense
A 13	10q24.32	Chromosome 10 open reading frame 76	79591	intron 3	antisense
A 14	12q24.11	Carnitine deficiency-associated gene expressed in ventricle 1	28981	intron 10	antisense
A 15	13q21.32	Protocadherin 9	5101	intron 2	sense
A 16	14q11.2	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	3183	intron 1	sense
A 17	14q32.11	Echinoderm microtubule associated protein-like 5	161436	intron 2	sense
A 18	19q13.11	CCAAT/enhancer binding protein (C/EBP), alpha	1050	exon 1	sense
A 19	20p12.3	Hypothetical protein DJ971N18.2	56255	intron 1	sense
A 20	20q13.12	Zinc finger, SWIM domain containing 3	140831	intron 1	sense
C 1	1q41	UNQ6077	375056	intron 4	antisense
C 2	1q43	Ryanodine receptor 2 (cardiac)	6262	intron 2	antisense
C 3	2q37.3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42 kDa	4705	exon 3	antisense
C 4	4q23	Alcohol dehydrogenase 5 (class III), chi polypeptide	128	exon 3	sense
C 5	5q21.3	F-box and leucine-rich repeat protein 17	64839	intron 6	sense
C 6	6p21.2	Ring finger protein (C3HC4 type) 8	9025	intron 2	antisense
C 7	6q15	RNA guanylyltransferase and 5'-phosphatase	8732	intron 13	sense
C 8	8q24.22	Thyroglobulin	7038	intron 41	antisense
C 9	9q21.11	LOC220869: dopamine responsive protein	220869	intron 2	sense
C 10	10p15.1	GDP dissociation inhibitor 2	2665	intron 9	sense
C 11	11q13.1	EGF-containing fibulin-like extracellular matrix	30008	exon 12	sense
C 12	14q24.1	RAD51-like 1	5890	intron 7	antisense
C 13	15q21.3	Hypothetical protein FLJ38736	256764	intron 1	sense
C 14	20q13.33	GTP binding protein 5 (putative)	26164	intron 5	antisense
C 15	21q21.1	Ubiquitin specific protease 25	29761	intron 19	antisense

Genes were identified by UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgBlat?command=start. Entrez gene ID was assigned by NCBI Entrez Gene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene.

A and C indicate ATL and carrier samples, respectively.

Integration sites in repetitive sequences

Next, we studied the relationship between repetitive sequences and integration sites. Percentages of repetitive sequences in human genome were based on the previous report by Venter et al.[18] Among the carriers, 11 sites (11/56; 20%) resided in the alphoid repetitive sequences, which are a component of centromeric heterochromatin and have a monomeric repeating unit of 171 bp,[21] whereas only 2 integration sites were identified within alphoid sequences in ATL (2/59; 3.4%). Since alphoid sequences were estimated to compose 3% to 5% of the human genome, we used 5% for the statistical

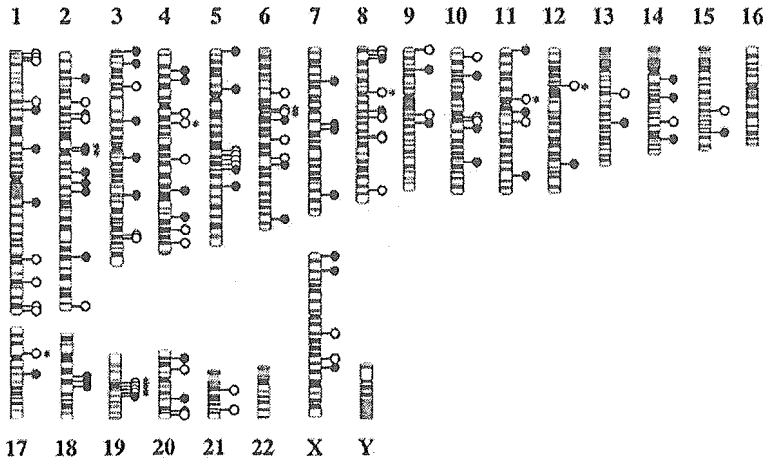

Figure 1. Distribution of HTLV-I provirus integration sites in human chromosomes. The integration sites of HTLV-I provirus in ATL patients (●) and HTLV-I carriers (○) are demonstrated. Integration within alphoid repetitive sequence is marked by an asterisk.

Table 2. Correlation between RefSeq genes and integration sites

	Carrier	ATL	Random
	No. (%)	No. (%)	No. (%)
Total	56 (100.0)	59 (100.0)	10 000 (100.0)
In RefSeq genes	15 (26.8)	20 (33.9)	3 324 (33.2)
Sense*	7 (46.7)	14 (70.0)	ND (ND)

ND indicates not done.

*The percentage of sense-direction has been calculated among sites, in which HTLV-I provirus was integrated in transcriptional units.

analyses (Table 4). HTLV-I–infected cells that have the provirus integrated in alphoid sequences are enriched in HTLV-I–infected cells during the carrier state compared with ATL ($P = .0153$; one-sided Fisher exact test; Table 4). The difference between ATL and carrier cells was statistically significant ($P = .0059$; one-sided Fisher exact test), indicating that integration within alphoid sequences is disfavored in leukemic cells. There were no preferences of HTLV-I integration with other repetitive sequences by statistical analyses.

Discussion

Recent studies on the integration sites of proviruses have provided new insights into the mechanism of integration and pathogenesis of retroviral infections.[3,4,22] In MLV,[4] provirus integration tends to occur near transcriptional start sites although there was no preference toward transcriptional units (34.2%) as observed in HTLV-I. On the other hand, HIV-1 tends to be integrated within transcriptional units (57.8%[4] and 69%[3]) in vitro. In vivo data on the integration of HIV-1 provirus demonstrated that most (91%) was integrated within transcriptional units, the genes of which were transcribed in T lymphocytes,[22] indicating that HIV-1 integration targets transcriptional active regions more than expected from in vitro data. On the other hand, this report showed that integration of the HTLV-I provirus into transcriptional units was not frequent compared with random integration, which is not consistent with the previous studies.[12,13] This is because of the changing database. We analyzed the integration sites and genes by the UCSC Genome Browser (July 2003). In HTLV-I carriers, integration into the transcriptional units was rather less frequent than random integration although the difference was not statistically significant. Combined with the finding that HTLV-I tends to be integrated near the transcriptional start sites, the pattern of HTLV-I integration is similar to that of MLV rather than that of another human retrovirus, HIV-1.

In the carrier state, HTLV-I provirus tends to be integrated into alphoid repetitive sequences, whereas the frequency of integration into alphoid sequences was significantly decreased in leukemic cells. When HTV-1 provirus is integrated into alphoid sequences, it establishes a latent infection by influencing the surrounding heterochromatin in vitro.[23] Heterochromatin decreases the basal

Table 3. Correlation between transcription start sites and integration sites

	Carrier		ATL		Random,
	No.	*P*	No.	*P*	no.
Total	56	NA	59	NA	10 000
Within 5 kb of transcription start sites	3	.610	9	.006	565

P compared with random integration with the use of the Fisher exact test.
NA indicates not applicable.

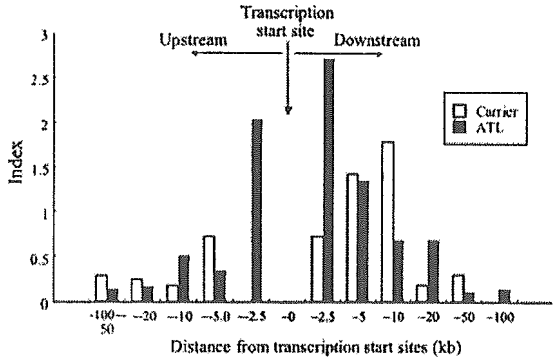

Figure 2. Relationship between HTLV-I provirus integration sites and transcriptional start sites. The distances from the transcriptional start sites are shown. The index was defined by the following calculation: (observed number of integrations) × 100 / (total number) × (window size).

transcription of viral genes, resulting in a latent state. Taken together, it is possible that infected cells in which HTLV-I provirus is integrated into alphoid sequences are enriched in the carrier state. Such cells are considered to produce lesser amounts of viral proteins, which facilitate cells to escape from the host immune surveillance system.[24] However, integration into alphoid sequences was not frequent in the leukemic cells, indicating that those with higher amounts of viral proteins are more likely to transform into malignant cells among surviving HTLV-I–infected cells. Since ATL occurs among HTLV-I carriers after a long latent period, these findings indicate the scenario as follows: after transmission of HTLV-I, HTLV-I provirus is randomly integrated into the host genome. The host immune system, including cytotoxic T lymphocytes (CTLs), excludes the HTLV-I–infected cells, in which Tax protein is the major target of CTLs.[25] In such circumstance, HTLV-I–infected cells expressing lesser amount of viral proteins are selected in vivo. However, among such infected cells, HTLV-I–infected cells expressing viral protein, such as Tax, tend to proliferate in vivo. Such cells should have greater chance to transform into malignant cells. A higher provirus load has been reported to be a risk factor for development of ATL, which is consistent with this hypothesis. Therefore, integration into alphoid repetitive sequences is less frequent among leukemic cells since viral transcription of such cells tends to be silenced.

Although MLV can infect only dividing cells,[26] lentiviruses such as HIV-1 can infect nondividing cells by transfer of a preintegration complex through the nuclear pore.[27] Since transcriptional active sites are associated with nuclear transport machinery,[28] preintegration complexes that pass through the nuclear pore might be integrated into transcriptional active sites due to the open structure of chromatin. This might be the reason for the high frequency at which HIV-1 provirus is integrated within transcriptional active genes. The data in this study reveal that the characteristics of HTLV-I integration in ATL cells resemble those of MLV, suggesting that HTLV-I cannot infect nondividing cells, although this requires clarification. Preference to transcriptional start sites was observed only in leukemic cells, indicating that such integration sites confer the advantage in leukemogenesis. Since the integration sites of HTLV-I provirus concentrate within 5 kb from transcriptional start sites, it is possible that such sites are suitable for transcription of viral genes.

Type 2 defective HTLV-I provirus lacks 5′-LTR and internal viral sequences such as *gag* and *pol*.[19] It is possible that this provirus traps the cellular promoter, thus ensuring transcription. In this study, the provirus of 2 of the 4 cases with type 2 defective

Table 4. Correlation between chromosomal features and integration sites

	Human genome, %	Carrier		ATL		
		No. (%)	P_1*	No. (%)	P_1*	P_2†
Total		56	NA	59	NA	NA
SINES	12.8	3 (5.3)	NS	4 (6.8)	NS	NS
LINES	20	10 (17.9)	NS	9 (15.3)	NS	NS
DNA elements	2.8	1 (1.8)	NS	2 (3.4)	NS	NS
LTR elements	8.3	6 (10.7)	NS	4 (6.8)	NS	NS
Satellite						
Alpha satellite	5	11 (19.6)	.0153	2 (3.4)	NS	.0059
Beta satellite	UN	0 (0.0)	UN	0 (0.0)	UN	NS

Percentages of repetitive sequences in the human genome were based on Venter et al.[18]
NS indicates not significant; UN, unknown; NA, not applicable.
*P_1 shows comparison with genome frequency with the use of Fisher exact test.
†P_2 shows comparison with carrier integration with the use of Fisher exact test.

provirus was integrated in the transcriptional unit in a sense orientation, indicating that the cellular promoter might transcribe the viral gene. In addition, HTLV-I provirus contains an internal promoter sequence in the *pol* region,[29] which is considered to transcribe the viral gene. This is thought to have occurred especially in the remaining 2 cases, which showed integration of the provirus outside the transcriptional units.

In HTLV-1–induced oncogenesis, viral proteins such as Tax promote the proliferation of HTLV-I–infected cells and induce ATL in about 2% to 6% of carriers after a long latent period.[7,8] Since HTLV-I provirus integration is random,[15] integration itself does not directly influence leukemogenesis. Viral products such as Tax promote the proliferation of HTLV-I–infected cells and induce transformation of infected T lymphocytes.[30,31] However, expression of Tax protein is impaired by several mechanisms in ATL cells, including deletion of 5′-LTR,[19] DNA methylation of 5′-LTR,[32] and genetic changes (deletion, insertion and nonsense mutations) of the *tax* gene itself.[33,34] In the carrier state, presence of the Tax protein is advantageous for the proliferation and survival of infected cells; however, since Tax is the major target of CTLs in vivo,[25] the growth of Tax-expressing cells is suppressed by CTLs.[35] When HTLV-I provirus is integrated into transcriptional active sites, viral gene transcription is thought to be active. During the carrier state, these producer cells are possibly eliminated by CTLs. Therefore, cells infected with HTLV-I provirus integrated into alphoid sequences have been enriched. On the other hand, higher production of viral proteins in HTLV-I–infected cells is thought to take advantage during malignant transformation. Therefore, the frequency of integration into alphoid sequences was low in leukemic cells compared with carrier states.

In this study, analyses of HTLV-I integration sites in both leukemic and HTLV-I–infected cells of carriers have emphasized the structural significance of the host genome, which influences viral gene transcription, during leukemogenesis.

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