

# Characterization of the internal promoter of human T-cell leukemia virus type I

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**Abstract** The HTLV-I provirus contains two different promoters: the classical retroviral promoter in the 5' long terminal repeat (LTR) and our previously identified second promoter in the *pol* gene just upstream of the ATG codon of the *tax* gene. Here, we demonstrated that the internal promoter expresses the gene for Tax but not Rex. As the deletion of upstream of the transcriptional initiation site (nt 5130) caused down-regulation of the promoter activity, we termed the region HTLV-I internal regulatory element (HIRE). We found a cellular sequence-specific DNA binding protein which binds to HIRE. Furthermore, we demonstrated that the 3'LTR regulates Tax expression from the internal promoter. These findings may shed light on a novel mechanism for gene expression in complex retroviruses of the HTLV family.

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**Key words:** Human T-cell leukemia virus type I; Tax; Rex; Internal promoter; Long terminal repeat; Defective virus

## 1. Introduction

Human T-cell leukemia virus type I (HTLV-I) is an etiological agent of adult T-cell leukemia (ATL) [1,2]. HTLV-I encodes two regulatory genes termed *tax* and *rex*. Tax, an oncoprotein which transforms rat fibroblasts [3,4], is a transcriptional activator of both the long terminal repeat (LTR) promoter in HTLV-I and several cellular genes. Transcription from HTLV-I LTR is usually undetectable in the absence of Tax. However, little is known about how transcription of HTLV-I becomes activated to express *pX* mRNA in the absence of Tax, so that mRNAs for HTLV-I are constitutively expressed upon induction of the *tax* gene. One possibility is the presence of a basal level of transcription from the LTR in the absence of Tax. Several cellular factors, including CREB, Sp1, Ets1, Myb, Tif and YB-1, which may be involved in the transcriptional process, were identified as LTR binding proteins [5–10]. Another possibility is the presence of an internal promoter for the *tax* gene, for we previously demonstrated the natural occurrence of 5'LTR-less defective HTLV-I ( $\Delta$ *pol-env-pX-3'LTR*) alone in some cases of primary ATL tumor cells, which suggested the expression of a viral gene from the internal promoter in the defective provirus [11].

We have previously identified such a promoter at the 3' end of the *pol* gene in HTLV-I [12]. Although the internal promoter expressed the *tax* gene, the promoter activity was independent of the Tax protein.

Here, we show the HTLV-I internal promoter expresses the gene for Tax but not Rex. Furthermore, we demonstrate the presence of two *cis*-regulatory elements for the internal pro-

motor activity; one is the HTLV-I internal regulatory element (HIRE) located upstream of the transcriptional initiation site of the internal promoter and the other is the 3'LTR.

## 2. Materials and methods

### 2.1. Cell culture

COS7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FCS) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. HUT102 cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

### 2.2. Plasmid constructions

To construct pdHTLV-I, 252 bp of the *EcoRI-HindIII* fragment of pSV00CAT [13], containing a major part of the CAT gene, was replaced by 4.5 kbp of the *HindIII* [nucleotide (nt) 4992]-*EcoRI* (in the flanked cellular sequence) fragment containing  $\Delta$ *pol-env-pX-3'LTR*. pHTLV-D was constructed by self-ligation of T4 DNA polymerase treated pdHTLV after *SphI* (nt 5124) digestion. pdHTLV-F was constructed by self-ligation of Klenow treated pdHTLV after *Clai* (nt 7474) digestion. p $\Delta$ dHTLV, a deletion mutant of pdHTLV, was constructed by self-ligation of T4 DNA polymerase treated pdHTLV after the *HindIII-SphI* (nt 4992–5124) fragment was removed. pHLC1, pCMV- $\beta$  (Clontech), Rex (pKCR27X), WT-Tax (pKCR40M), MT-Tax (pKCR40fsm),  $\lambda$ HTLV-I, pRSpCAT-RXE, p $\Delta$ CATa and p $\Delta$ CATb have been described previously [3,12,14,15]. p $\Delta$ dCATa was constructed by self-ligation of T4 DNA polymerase treated p $\Delta$ CATa after the *NaeI-SphI* (nt 5052–5124) fragment was removed. To construct pdHTLV $\Delta$ 1, 2.4 kbp of the *EcoRI-HindIII* fragment of pSV00CAT was replaced by the *HindIII-EcoRI* fragment of pTN1, containing the *HindIII-NdeI* (nt 4992–8576) fragment of HTLV-I and an SV40 early region poly(A) signal. To construct pdHTLV $\Delta$ 2, 4.5 kbp of the *HindIII-EcoRI* (nt 4992, in the flanked cellular sequence) fragment of HTLV-I was subcloned into the same site of pUC119, generating pUC4.5k. The *SmaI-EcoRI* (8308, cellular flanking) fragment of pUC4.5k was replaced by annealed synthetic oligonucleotides, containing the *SmaI* site (nt 8308) and the stop codon of the *tax* gene linked to the *EcoRI* site, generating pUC $\Delta$ 4.5k. Then, 252 bp of the *EcoRI-HindIII* fragment of pSV00CAT, containing the major part of the CAT gene, was replaced by the *HindIII-EcoRI* (nt 4992, a stop codon of the *tax* gene) fragment of pUC $\Delta$ 4.5k, generating pdHTLV $\Delta$ 2. pdHATLV $\Delta$ 1-D and pdHTLV $\Delta$ 2-D were constructed in the same way as pdHTLV-D.

### 2.3. CAT assay

COS7 cells ( $4 \times 10^5$  cells in a 10 cm dish) were plated 1 day before transfection. Transfection by the calcium phosphate method and CAT assay were performed as previously described [12]. Cells were suspended in 100  $\mu$ l of 0.25 M Tris-HCl (pH 8.0) 2 days after transfection. CAT activity was calculated by measuring the radioactivities of the <sup>14</sup>C-acetylated chloramphenicols versus the total radioactivity of acetylated and un-acetylated chloramphenicols using a bioimaging analyzer (Fujix BAS2000). pCMV $\beta$  (Clontech) was co-transfected to normalize the transfection efficiencies.  $\beta$ -Galactosidase activity was measured by the optical density (OD) of the reaction at a wavelength of 420 nm at 37°C after 30 min as previously described [12].

### 2.4. Electrophoretic mobility shift assay (EMSA)

The end-labeled probe (128 bp of the *NaeI-NcoI* (nt 5052–5180) fragment of HTLV-I) was incubated with nuclear extracts in the binding buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5%

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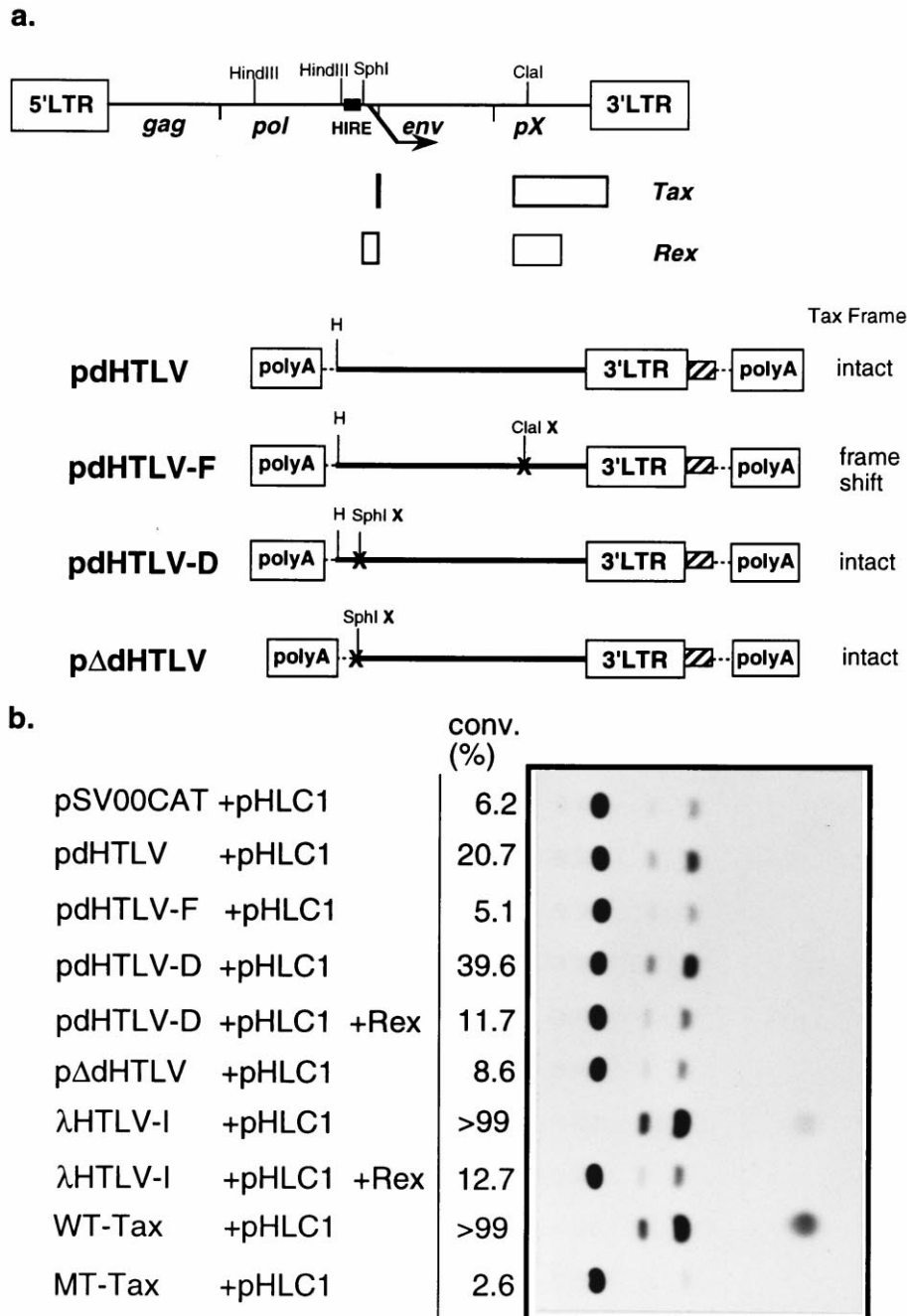


Fig. 1. Tax expression from the 5'LTR-less HTLV-I. a: Schematic representation of HTLV-I and the effector constructs. The arrow indicates the initiation site of the internal promoter. A *SphI* site corresponds to the ATG codon of Rex. pdHTLV-F is a frameshift mutant for Tax and Rex. b: Assay of CAT activity in COS7 cells. 1  $\mu$ g of pCMV- $\beta$  as an internal control, 5  $\mu$ g of each effector plasmid or *EcoRI* digested  $\lambda$ HTLV-I DNA which is the full-length provirus, and 5  $\mu$ g of the reporter plasmid (pHLC1) which is HTLV-I LTR-CAT were transfected. The numbers show the percent conversion of chloramphenicol to its acetylated forms.

glycerol, 1 mM EGTA, 1 mM DTT and 2  $\mu$ g poly(dI-dC)-poly(dI-dC) (Pharmacia). Nuclear extracts were prepared from HeLa or HUT102 cells [16]. After incubation at room temperature for 30 min, the reaction mixture was fractionated for 2 h at 150 V on 4% non-denaturing polyacrylamide gel in 1 $\times$ TAE buffer containing 40 mM Tris-acetate and 1 mM EDTA (pH 8.0).

### 3. Results and discussion

#### 3.1. Internal promoter expresses the gene for Tax but not Rex

We previously showed that the defective HTLV-I provirus,

containing the *HindIII-NdeI* (nt 3028–8576) fragment without the R-U5 region of 3'LTR, expressed a gene for Tax. To further examine the nature of the gene expressed from the internal promoter of HTLV-I, we constructed a plasmid (pdHTLV) containing the *HindIII-EcoRI* (nt 4992, in the flanked cellular sequence,  $\Delta$ *pol-env-pX*-3'LTR). To avoid read-through transcription from 3'LTR, both ends of the HTLV-I fragment were flanked with SV40 poly(A) signal as presented in Fig. 1a. Tax activity was monitored by the elevation of chloramphenicol acetyltransferase (CAT) activity

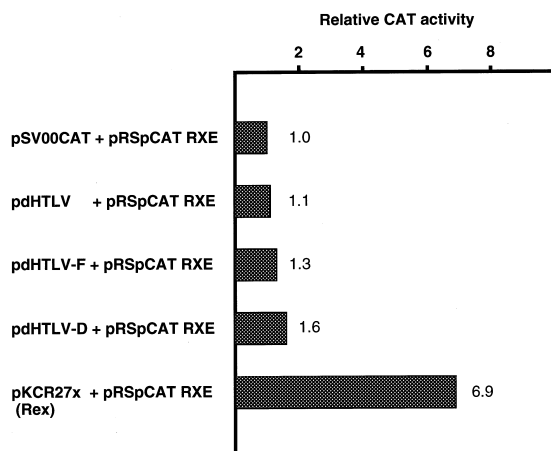


Fig. 2. Relative CAT activity in COS7 cells co-transfected with the reporter (5  $\mu$ g), pCMV- $\beta$  (1  $\mu$ g) and the Rex reporter, pRSpCAT-RXE (5  $\mu$ g). At 48 h following transfection, cells were harvested and assayed for CAT activity. The numbers indicate the relative CAT activity compared with the control vector, pSV00CAT.

from the HTLV-I LTR (pHLC 1) which is responsive to the Tax protein. As shown in Fig. 1b, increased CAT activity was detected in pdHTLV or pdHTLV-D, which is devoid of the ATG codon of *rex*, compared with the control vector in COS7 cells, while a frameshift mutant for Tax and Rex, pdHTLV-F, did not elevate CAT activity. This confirmed the previous suggestion that the *tax* gene was expressed from the internal promoter.

It is known that the mRNA for Tax transcribed from the 5'LTR also produces Rex. To examine whether functional Rex is produced from the gene expressed from the internal promoter, COS7 cells were co-transfected with each effector plasmid together with the Rex reporter plasmid termed pRSpCAT-RXE [15]. CAT activities in cell lysates were then measured (Fig. 2). pRSpCAT-RXE contains the CAT gene flanked with 5' and 3' splice sites and HTLV-I LTR which includes Rex responsive element (RXE). This plasmid cannot express the CAT gene in the absence of Rex because the gene is spliced out. We failed to detect elevation of CAT activity in cells transfected with each effector plasmid, but the elevation could be observed when the Rex expressing plasmid, pKCR27X, was co-transfected. This result suggests that the internal promoter did not express a gene for the functional Rex protein.

### 3.2. Trans-acting function from the HTLV-I internal regulatory element (HIRE) is independent of Tax

We previously showed that the 0.19 kbp fragment of the *pol-env* junction region, the *HindIII-NcoI* fragment (nt 4992–5180), contained the internal promoter activity. A plasmid, pdCATa, linking this fragment in the sense orientation to the CAT gene, has *trans*-acting activity regardless of the absence of functional Tax ([12] and Fig. 3), whereas a plasmid, pdCATb, in which the internal fragment was linked in the antisense orientation, did not show elevated CAT activity compared to the control plasmid, pSV00CAT. This indicates that the internal promoter activity was independent of Tax expression.

To identify the regulatory *cis*-element upstream of the initiation site of this internal promoter, we generated a deletion

mutant termed pAdCATa, which lacks the *NaeI-SphI* fragment (nt 5052–5124, see the map in Figs. 4 and 5). Transfection of pAdCATa showed decreased CAT activity (approximately a third to a quarter) compared with pdCATa (Fig. 3). Moreover, a deletion mutant termed pAdHTLV, which lacks the *HindIII-SphI* (nt 4992–5124) fragment of pdHTLV, was generated (Fig. 1a). Transfection of pAdHTLV into COS7 cells resulted in significantly reduced CAT activity compared with pdHTLV (Fig. 1b), suggesting that the *cis*-element in the *NaeI-SphI* (nt 5052–5124) fragment regulates gene expression from the internal promoter. We call the element the HTLV-I internal regulatory element (HIRE).

To determine the protein which may regulate the *trans*-acting function through binding to the HIRE, we performed the gel shift assay with the labeled 128 bp of *NaeI-NcoI* fragment (nt 5052–5180) (Fig. 4a). Nuclear extracts from the HeLa or HTLV-I infected T-cell line, HUT102, showed sequence-specific DNA binding activity (Fig. 4b). In addition, we found sequence-specific DNA binding activity in various cell lines, suggesting that a ubiquitous cellular protein is involved in the internal promoter activity (data not shown).

We previously demonstrated that the transcriptional initiation site (nt 5130) from the internal promoter is located just upstream of the first ATG codon of the *tax* gene [12]. We could not find a TATA box, CAAT box or any sequence similarity between the HTLV-I internal promoter and the LTR. However, we found conserved sequences around the internal transcriptional initiation site among the HTLV family (Fig. 5), suggesting that retroviruses in the HTLV family and other complex retroviruses have similar internal promoters [17–20].

Human foamy virus (HFV) has an internal promoter just upstream of the transactivator gene, *bel-1* (or *tas*, transactivator of spumavirus) [21]. Bel-1 is unique among retroviral regulatory proteins in being a sequence-specific DNA binding protein [22]. Bel-1 binds to both the LTR and internal promoter and directs the expression of the gene for Bel-1 [23]. Simian foamy virus 1 from a rhesus monkey (SFV-1) and SFV-3 from an African green monkey, genetically related

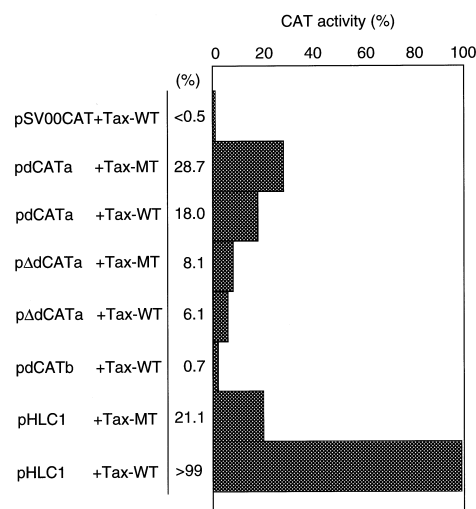


Fig. 3. Tax-independent internal promoter activity in COS7 cells. Cells were transfected with the indicated reporter plasmids (5  $\mu$ g), pCMV- $\beta$  (1  $\mu$ g) and either wild type Tax or mutant type Tax expressing plasmid (5  $\mu$ g). The numbers show the percent conversion of chloramphenicol to its acetylated forms.

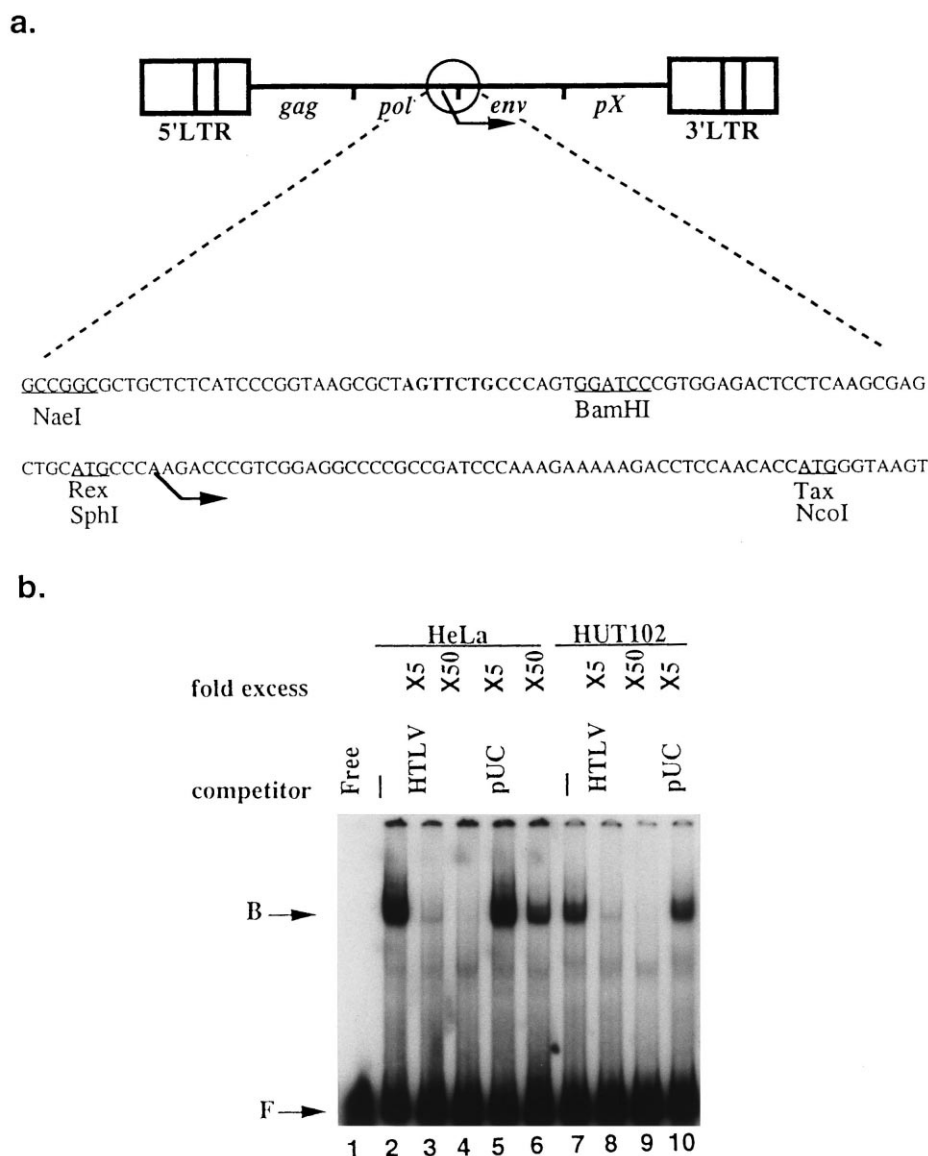


Fig. 4. Detection of the sequence-specific HTLV-I internal regulatory element (HIRE) binding protein. a: Schematic representation of HTLV-I provirus and nucleotide sequences around the internal promoter. Arrows indicate the initiation site (nt 5130) of the internal promoter. b: Gel shift assay with the 128 bp of *NaeI*-*NcoI* (nt 5052–5180) fragment of HTLV-I and nuclear extracts from HeLa or HUT102 cells. 'Free' indicates no protein and competitor added (lane 1). An unlabeled fragment designated HTLV (lanes 3,4,8,9) or a non-specific similar sized fragment corresponding to 141 bp of the *PvuII*-*HindIII* fragment of pUC119 designated pUC (lanes 5,6,10) was used as the competitor. – indicates no competitor added. B and F indicate the bound and free probes, respectively.

members of the HFV, also have an internal promoter in the *env* gene [24]. The transactivators, Taf-1 and Taf-3, bind directly to the internal promoter and activate the promoter activity. Although we failed to detect any internal promoter activity for Tat expression in human immunodeficiency virus type 1 (HIV-1) (Sakurai, unpublished data), HIV-1 has two AP-1-dependent intragenic enhancers at the end of the *pol* gene (4079–4342), the *vif* gene and the first coding exon of *tat* (4781–6026) [25,26]. Taken together, the internal promoter and/or enhancer might be a common feature in complex retroviruses.

### 3.3. 3'LTR regulates *Tax* expression from the internal promoter

To determine whether the internal promoter is affected by the 3'LTR for *Tax* expression, we generated two deletion

mutants of 3'LTR (pdHTLVΔ1 and pdHTLVΔ2) (Fig. 6a). Transfection of pdHTLVΔ2, which has only a *tax* coding region without the major part of the 3'LTR, showed decreased CAT activity compared with pdHTLV, suggesting that 3'LTR is required for efficient promoter activity (Fig. 6b). Interestingly, transfection of pdHTLVΔ1, which has the U3 region but not the R-U5 region of 3'LTR, showed significantly elevated CAT activity (approximately 5-fold) compared with pdHTLV. As the R region contains a Rex responsive element (RXE) [27], we generated mutant plasmids (pdHTLV-D, pdHTLVΔ1-D and pdHTLVΔ2-D), which are devoid of the ATG codon of *rex*, to avoid the possibility of a negative effect by Rex. The resulting three mutants had no effect on the CAT activity compared to the parent plasmids. Moreover, we demonstrated that the internal promoter expresses the gene for *Tax* but not *Rex* (Fig. 2), suggesting

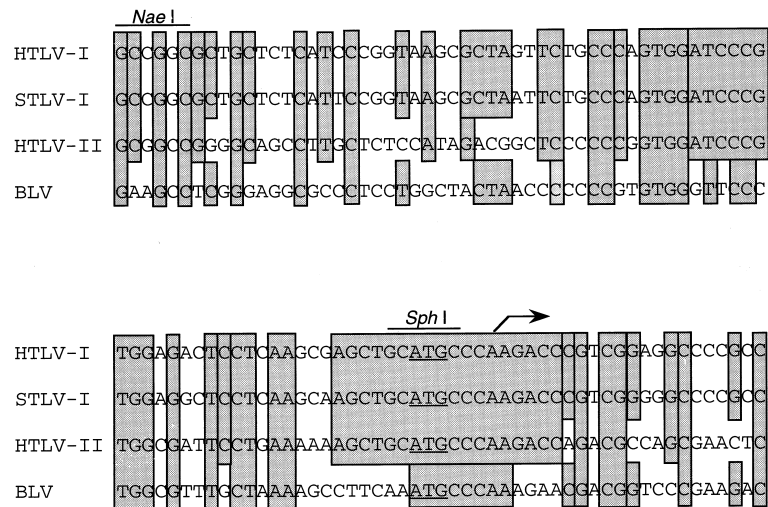


Fig. 5. Nucleotide sequence similarity of the region between the end of the *pol* region of the HTLV family. Alignment of the HTLV-I (nt 5050–5151), STLVI-I, HTLV-II and BLV nucleotide sequences of the end of the *pol* region are shown. Residues identical among three or all four virus are indicated by the shaded box. The arrow indicates the initiation site (nt 5130) of the HTLV-I internal promoter. The first ATG codon (underlined) of Rex is located just upstream of the initiation site.

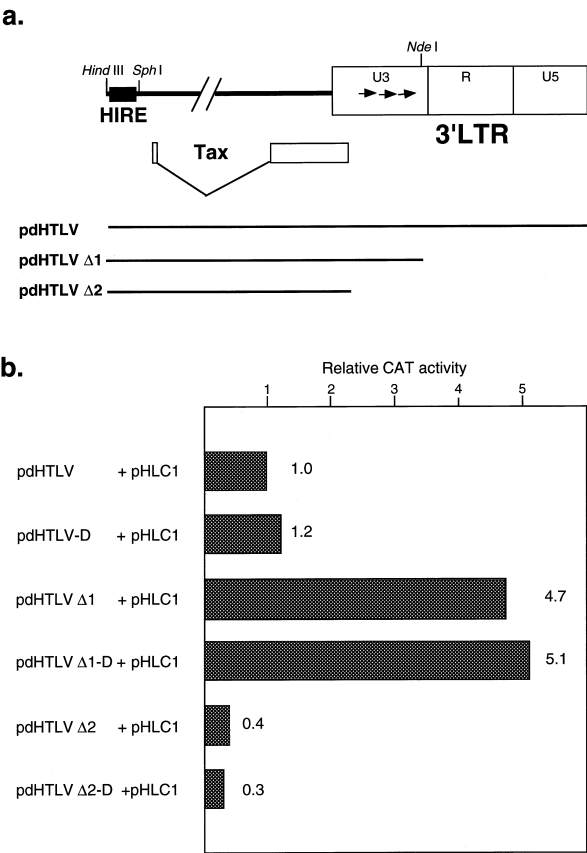


Fig. 6. 3'LTR regulates Tax expression from the internal promoter. a: Schematic representation of the 3' region of the HTLV-I provirus and 3'LTR deleted constructs derived from it. Bars indicate the portion of the provirus. b: Relative CAT activity in COS7 cells. Cells were transfected with the indicated effector (5 μg), pCMV-β (1 μg) and the reporter plasmid, pHLC1 (5 μg). Three mutants (pdHTLV-D, pdHTLVΔ1-D, pdHTLVΔ2-D) were devoid of the ATG codon of the *rex* gene. The numbers indicate the relative CAT activity compared with pdHTLV with the full length of the 3'LTR.

that the R-U5 region of 3'LTR has a negative regulatory element. Seiki et al. [28] previously reported the presence of a repressive element in the U5 region of the 5'LTR of HTLV-I for genomic RNA expression. As the expression level of the Tax protein is very low in primary ATL cells from patients, the U5 repressive element may be involved in the down-regulation of Tax expression.

3.4. Possible roles of the internal promoter of HTLV-I on viral pathogenesis

In this study, we have demonstrated that the internal promoter expresses the gene for Tax but not Rex. The presence of the internal promoter may play a crucial role in leukemogenesis, because we previously showed the natural occurrence of 5'LTR-less defective HTLV-I ( $\Delta$ *pol-env-pX*-3'LTR) alone in some cases of primary ATL tumor cells [11]. As these defective proviruses do not express the Gag and Pol structural proteins but express a small amount of Tax, which has oncogenic potential, from the internal promoter, the defective provirus could escape the host immune surveillance and Tax expression may play an important role on tumorigenesis through its *trans*-acting function. The presence of a similar internal promoter in other closely related retroviruses suggests that it might be a conserved feature of complex retroviruses.

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