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Up-regulation of human T lymphotropic virus type 1 (HTLV-1) *tax/rex* mRNA in infected lung tissues

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SUMMARY

HTLV-1 has been implicated in certain pulmonary diseases. We previously demonstrated that expression of HTLV-1 *tax/rex* mRNA, encoding the transcriptional transactivator Tax, was closely associated with infiltration of activated T lymphocytes into lung tissue. To explore mechanisms of *tax/rex* expression in the lung, *tax/rex* mRNA expression and proviral DNA load were compared between peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) from four patients with HTLV-1-associated myelopathy (HAM/TSP) and 13 carriers with various pulmonary symptoms. Semiquantitative detection of *tax/rex* mRNA strongly suggested that the lung was a preferential site for its expression. Proviral DNA loads in non-HAM/TSP carriers were variable but correlated well between PBMC and BALC in each individual, and revealed no relationship with *tax/rex* mRNA expression. In contrast, both cell groups from four HAM/TSP patients expressed detectable *tax/rex* mRNA accompanied by high proviral DNA load. The ratio of *tax/rex* mRNA expression to proviral DNA load was higher in BALC than in PBMC in three of four carriers and in three of four HAM/TSP patients, suggesting up-regulation of *tax/rex* mRNA in infected lung tissue. To analyse differences in distribution of HTLV-1 quasispecies between the two tissues, phylogenetic analysis was performed for sequence sets of the proviral *tax* open reading frame (ORF: 1059 bp) derived from PBMC and BALC of two infected individuals. Sequences derived from the two tissues distributed similarly to branches of phylogenetic trees, and there was no evidence of selective distribution of certain quasispecies in the lung. Our results suggest the presence of tissue-specific conditions that activate viral expression in infected cells in the lung. Constitutive exposure of this tissue to foreign antigens leading to up-regulation of basal viral promoter activity is likely to be one such mechanism.

Keywords HTLV-1 *tax/rex* mRNA bronchoalveolar lavage quasispecies local availability

INTRODUCTION

HTLV-1 is a retrovirus involved in the pathogenesis of adult T cell leukaemia (ATL) and non-neoplastic inflammatory diseases of various organs. The latter include HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-1-associated uveitis (HAU), rheumatoid arthritis, Sjögren's syndrome and pneumonopathy [1]. With respect to pulmonary involvement in HTLV-1 infection, patients with HAM/TSP and HAU frequently exhibit pulmonary complications characterized by T-lymphocytic alveolitis or lymphocytic interstitial pneumonia [2–4]. Neither leukaemic cells nor pathogens associated with opportunistic infections are found in the lungs of these patients. Similar pulmonary involvement has been reported in asymptomatic carriers of HTLV-1 [5]. Although pulmonary involvement in patients with HAM/TSP and HAU is usually subclinical, T cell lymphocytic alveolitis can be demonstrated by bronchoalveolar lavage (BAL) in 60–80% of cases [6]. A characteristic pathological feature is infiltration of mononuclear cells in submucosal tissue of bronchioles, perivascular areas of the parenchyma, and alveolar septa [7]. The infiltrating cells are predominantly $CD3^+$ T lymphocytes.

Pathogenic mechanisms of HTLV-1 in lung tissue are not fully elucidated. Cultured HTLV-1-infected T cells are known to exhibit activated T cell phenotypes and produce various cytokines and their receptors [8,9]. HTLV-1-infected cells in lung may

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locally induce T cell expansion and chronic inflammation *via* cognate cell–cell interaction with non-infected T cells and/or production of cytokines and their receptors [10]. Consistent with this is the evidence that cultured pulmonary and peripheral blood T lymphocytes from HAM/TSP patients spontaneously proliferate and release IL-2 into the culture medium [11]. In addition, increased IL-2 receptor α (IL-2Rα: CD25)-bearing T cells and high levels of soluble IL-2R were noted in BAL cells (BALC) and BAL fluid (BALF), respectively, from HAM/TSP patients with T-lymphocytic alveolitis [7,12]. Involvement of the cytotoxic immune response against viral antigens in the lung has also been suggested [6].

We previously reported that detection of *tax/rex* mRNA, the doubly spliced transcript of HTLV-1, correlated closely with the presence of lymphocytosis and increased proportion of IL-2R-bearing T cells in BALC [13]. *tax/rex* mRNA encodes a transcriptional transactivator, Tax, which up-regulates the viral promoter and also expression of various cellular genes including IL-2, IL-2Rα, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and c-fos [1,8,9]. Moreover, Tax is thought to be the major target molecule of the cytotoxic T cell response against HTLV-1 *in vivo* [14,15]. It is conceivable that Tax plays a crucial role in the inflammatory response in the lungs of HTLV-1-infected individuals.

The present study was designed to explore mechanisms of *tax/rex* mRNA expression in lung tissue. For this purpose, we compared the level of tax/rex mRNA expression, proviral DNA load, and HTLV-1 quasispecies between peripheral blood mononuclear cells (PBMC) and BALC obtained from 17 infected individuals. Our results suggest involvement of tissue-specific mechanisms, which activate viral expression in infected cells in the lung.

PATIENTS AND METHODS

Patients

We examined 17 seropositive individuals (four males and 13 females) ranging in age from 16 to 74 years. Seropositivity was determined using both the particle agglutination (Serodia-HTLV-1; Fuji Rebio, Tokyo, Japan) and indirect immunofluorescence tests, and was confirmed by immunoblotting. The patients comprised four with HAM/TSP and 13 non-HAM/TSP patients, designated as carriers. The latter group included three patients with sarcoidosis, one with pulmonary tuberculosis, and two with pneumonia due to bacterial infection, and the remaining individuals complained of mild cough and sputum. There were no cases of viral infection other than HTLV-1 or autoimmune diseases. Informed consent was obtained from all patients prior to the commencement of the study, and the human experimentation guidelines of Nagasaki University were followed in conducting clinical research.

Cell line

We used the HTLV-1-infected human cell line MT-2 as a control [16].

Preparation and analysis of cells

BAL was performed under local anaesthesia. The fibrescope was inserted into the right middle lobe and 150 or 200 ml of sterile saline solution in three or four aliquots of 50 ml were instilled. The fluid was recovered by gentle aspiration into a sterile syringe and transported to the laboratory at 4°C within 30–60 min. Lavage fluid was passed through two sheets of gauze and BALC were pelleted by centrifugation at 400 ***g*** for 10 min at 4°C. PBMC were separated from heparinized venous blood by Ficoll–Hypaque gradient centrifugation. Aliquots of the cell suspension were prepared for immunocytochemical staining and DNA/RNA harvest. An aliquot of the suspension was subjected to flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) after staining with FITC-conjugated anti-CD3 MoAb (Becton Dickinson) to determine the proportion of $CD3^+$ cells.

DNA and RNA preparation

Approximately 2×10^6 cells from PBMC and BALC were lysed in 0·5 ml lysis buffer (150 mM NaCl, 10 mM Tris–HCl pH 8·0, 10 mM EDTA, 0·5% SDS) and treated with proteinase K (100 μg/ml) for 2 h at 50°C. After phenol/chloroform extraction and ethanol precipitation, high molecular weight DNA was resuspended in 10 mM Tris (pH 8·0)/1 mM EDTA. Total RNA was isolated by TRIzol reagent (GIBCO BRL, Rockville, MD) according to the protocol recommended by the manufacturer.

Semiquantitative detection of tax/rex mRNA by reverse transcription-polymerase chain reaction

RNA samples (adjusted to 1 μg/10 μl) were serially diluted 10-fold with a solution containing yeast tRNA (1 μg/10 μl). Diluted samples were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) targeting the doubly spliced *tax/rex* mRNA. Reverse transcription and PCR were performed as previously described [13]. Briefly, cDNA was synthesized using the sequence-specific primer oligonucleotide pX9 and Superscript II Reverse Transcriptase (GIBCO BRL). The cDNA was then subjected to successive two-step amplification using nested primer pairs of the second splice junction site of *tax/rex* mRNA [13,17]: step 1, forward primer (RPX-11): 5′-TAATAGCCGC-CAGTGGAAAG, reverse primer (pX-9): 5′-TGATCTGATGCTC TGGACAG; step 2, forward primer (RPX 3): 5′-ATCCCGTGGA GACTCCTCAA, reverse primer (RPX-4): AACACGTAGACTG GGTATCC, respectively. The amplified product (145 bp) was

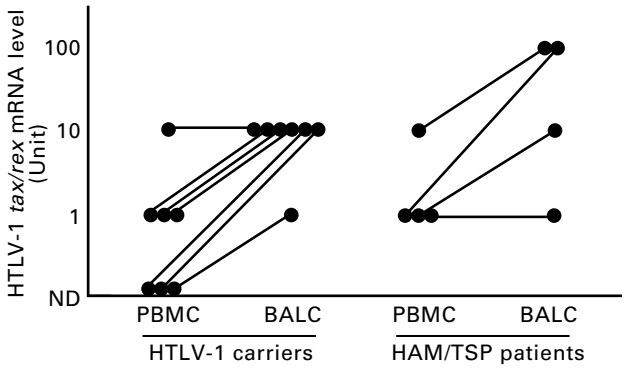

Fig. 1. Comparison of *tax/rex* mRNA expression between peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) from HTLV-1-infected individuals. Reciprocal values of the end-point dilution (Unit), which contained *tax/rex* mRNA amplifiable by reverse transcriptase-polymerase chain reaction (RT-PCR), of RNA samples from seven carriers and four HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients are plotted and compared between PBMC and BALC from each individual. ND, Not detected.

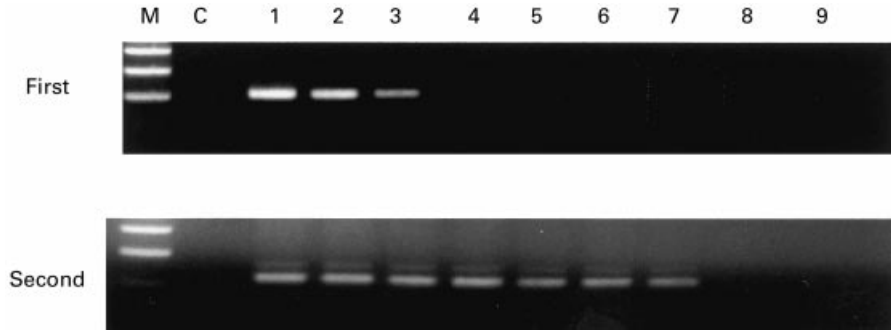

Fig. 2. Amplification of HTLV-1 proviral DNA using nested polymerase chain reaction (PCR). One microgram of DNA from the HTLV-1-infected cell line, MT2, containing about $1{\cdot}0 \times 10^6$ molecules of HTLV-1 provirus was diluted 10-fold (10^0-10^{-8}), and each dilution was subjected to two-step PCR using nested primer pairs. PCR products from the first-step and second-step amplification were analysed on agarose gels. M, Molecular size markers; C, negative controls (amplification from 1 μg of HTLV-1-negative cell DNA).

analysed on a composite gel containing 2% NuSieve/1% SeaKem agarose (FMC, Rockland, ME) stained with ethidium bromide. Expression of *tax/rex* mRNA was estimated by the end-point dilution which gave the expected band on the gel. Experiments were performed twice to confirm the quantification.

Determination of proviral DNA load by nested PCR

Nested oligonucleotide primer pairs were designed to selectively amplify the 119-bp fragment of HTLV-1 pX gene in a two-step process: step 1, forward primer (pX-1): 5′-CCCACTTCCCAGGG TTTGGACAGAG, reverse primer (pX-2): 5′-CTGTAGAGCTGA

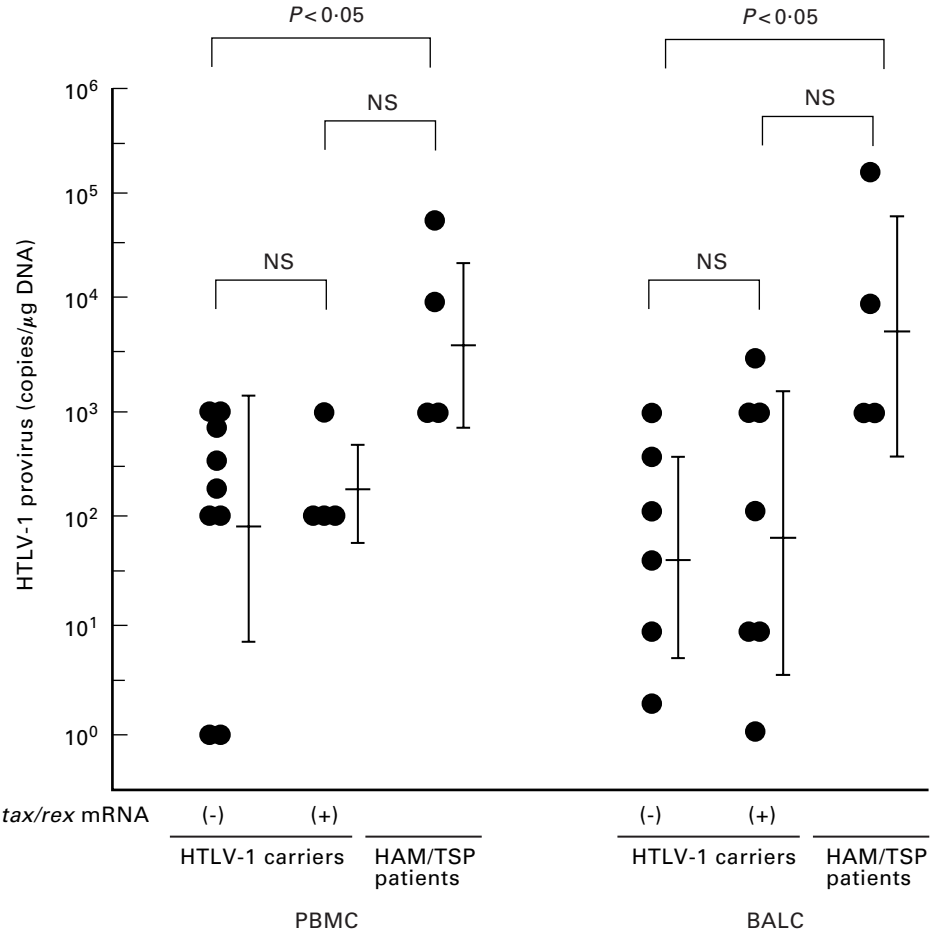

Fig. 3. Lack of correlation between *tax/rex* mRNA expression and virus load in bronchoalveolar lavage cells (BALC) of non-HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) carriers. Virus load (copies/μg DNA) in peripheral blood mononuclear cells (PBMC) and BALC semiquantified by nested polymerase chain reaction (PCR) was compared between carriers (with and without *tax/rex* mRNA expression) and HAM/TSP patients. Virus loads in HAM/TSP patients were significantly ($P < 0{\cdot}05$) higher than in carriers without detectable *tax/rex* mRNA, but the difference in values between the two carrier groups was not significant.

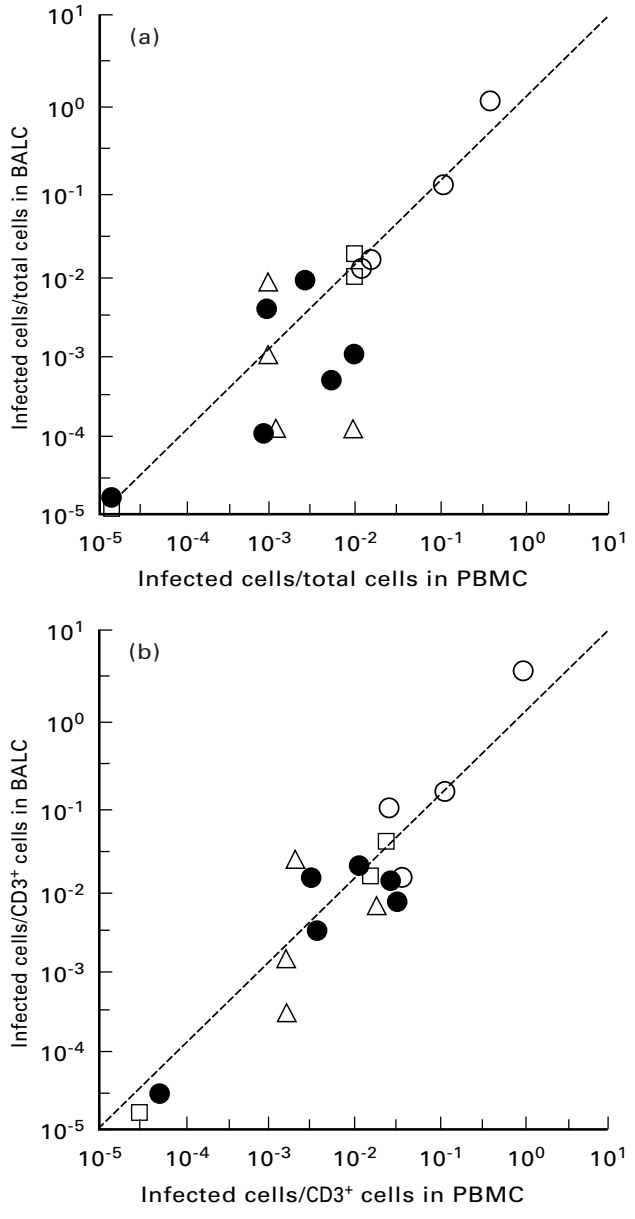

Fig. 4. Correlation of virus load between peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) of each infected individual. Viral loads in total cells (a) and in $CD3^+$ T cells (b) were compared between PBMC and BALC of each individual. ○, HTLV-1-associated myelopathy/tropical spastic paraparesis patients; ●, carriers without detectable *tax/rex* mRNA in either tissue; □, those with detectable *tax/rex* mRNA only in BALC; Δ, those in whom *tax/rex* mRNA was detectable in both tissues.

GCCGATAACGCG; step 2, forward primer (pX 8): 5′-ACCCAG TCTACGTGTTTGGA, reverse primer (pX-9): TGATCTGATGC TCTGGACAG, respectively. PCR was performed in a TRIO-Thermal Cycler TB-1 (Biometra, Göttingen, Germany) for 30 cycles as previously described [18]. Briefly, the mixture including 0·5 μg sample DNA was denatured at 94°C for 2 min, annealed at 60°C for 2 min and extended at 72°C for 2 min. For nested PCR, 1/10 volume of the first-step PCR product purified by SuperRecII column (TaKaRa Co., Tokyo, Japan) was further amplified with the same thermal cycle programme for 27 cycles, using the inner

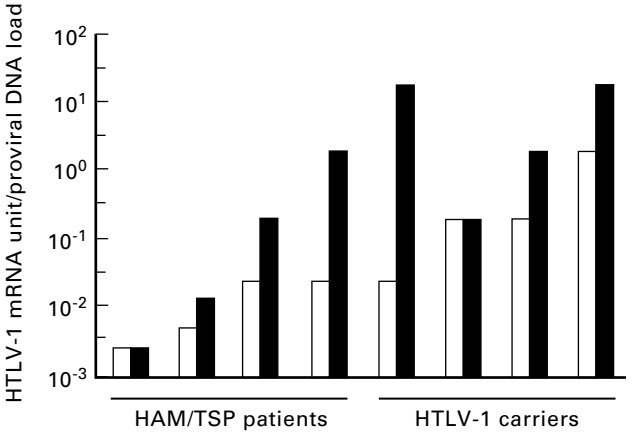

Fig. 5. Levels of *tax/rex* mRNA in HTLV-1-infected cells were higher in bronchoalveolar lavage cells (BALC; ■) than peripheral blood mononuclear cells (PBMC; □). Ratios between *tax/rex* mRNA levels (Units—shown in Fig. 1) and virus load were compared between PBMC and BALC of four carriers and four HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients who expressed *tax/rex* mRNA.

primer pair. This nested PCR has been shown to be sensitive enough to detect a single molecule of the HTLV-1 provirus [18]. For the purposes of semiquantitative detection, DNA samples were serially diluted 10-fold and two-fold successively, and each dilution was then subjected to nested PCR. The proviral DNA load was estimated from the end-point dilution which gave an amplified band in an agarose gel, on the basis that 0·5 μg DNA was equivalent to that obtained from 1×10^5 cells. Experiments were performed twice to confirm the quantification.

Amplification, subcloning, and sequencing of the tax open reading frame of HTLV-1 proviruses

The entire tax open reading frame (ORF) of the HTLV-1 provirus was amplified by nested PCR. The primers used were: Tax11 (5′-GATAGCAAACCGTCAAGCACAG-3′; position 7158–7179) and Tax6 (5′-TCCTGAACTGTCTCCACGCAAA-3′; 8650–8629) for the first amplification, and Tax15Xba (5′-TACTCTAGAAC-CATGGCCCACTTCCCAGG-3′; 7324–7337) and Tax30Bam (5′- CTGAGGATCCAGAGCCTTAGTCT-3′; 8409–8397) for the second amplification. *Xba*I and *Bam*HI restriction sites (underlined) were included in Tax15Xba and Tax30Bam primers, respectively, to allow subsequent subcloning into the expression plasmid pCG [19]. PCR was performed in a TRIO Thermal Cycler TB-1 (Biometra) with the following conditions: 30 cycles of denaturation at 94°C for 30 s (2 min on the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 70 s (7 min on the last cycle). For nested PCR, 1/10 volume of the first-step PCR product purified by SuperRecII column (TaKaRa Co.) was further amplified for 27 cycles using the inner primer pair. The thermal cycle programme was similar to that used for the first PCR, except the annealing temperature was 58°C for the first six cycles and 63°C for the following 21 cycles. The nested PCR product was purified on a 0·8% agarose gel (SeakemGTG; FMC), digested with *Xba*I and *Bam*HI, and then ligated into pCG. After transformation of *Escherichia coli* (DH5α), recombinant clones were randomly picked and plasmid DNA was miniprepped by the NaOH/SDS method. The entire tax ORF (1059 bp) in the plasmid DNA was sequenced using the dideoxy chain termination method

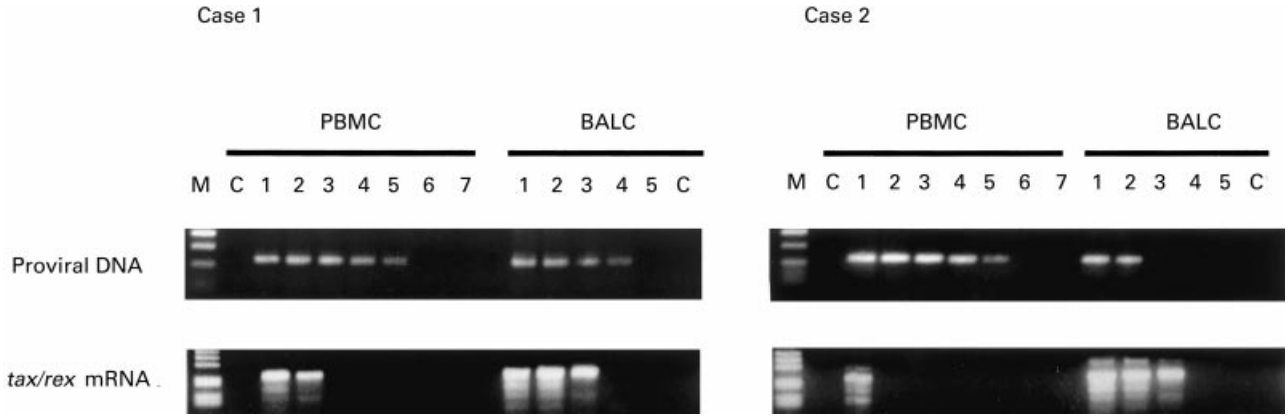

Fig. 6. Semiquantitative comparison of *tax/rex* mRNA expression and virus load in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC). Serial five-fold dilutions of DNA and RNA (5^0-5^{-6} μg DNA and RNA, respectively) from PBMC and BALC of two infected individuals (cases 1 and 2) were subjected to polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR, respectively. M, Molecular size markers; C, negative controls (amplification from 1 μg of HTLV-1-negative DNA for PCR and from 1 μg of sample RNA without reverse transcription for RT-PCR).

(Thermo Sequence core sequencing kit; Amersham, Aylesbury, UK) with two 5′ Texas red-labelled primers corresponding to the flanking vector sequence (HSK-tk sequence, 5′-GCCCAGCGCC TTGTAGAA 3′; and rabbit-β-globin sequence, 5′-TAGGCGA AAAAGAAAGAAC-3′), and then analysed on an automated sequencer (SQ5500; Hitachi Co., Tokyo, Japan). A sequence comparison was carried out using ATK-1 sequence [20].

Phylogenetic analysis

The multiple alignment and the phylogenetic analysis for the nucleotide sequences of the tax ORF were conducted using the computer software CLUSTAL [21]. In practice, the phylogenetic trees were reconstructed using the neighbour-joining method [22] with the evolutionary distances estimated by the Kimura's two-parameter method [23]. The phylogenetic trees were visualized by TREEVIEW [24].

Statistical analysis

Data were expressed as mean ± s.d. Geometric analysis was used for log scale correlation. Statistical comparison was performed using Kruskal–Wallis rank test to examine differences between means of unpaired samples, and Spearman's rank correlation was used to examine the relationship between various parameters. Statistical analysis was performed using the Statview-J 4.5 software package. $P < 0{\cdot}05$ was considered significant.

RESULTS

The lung is a preferential site for tax/rex *mRNA expression in HTLV-1-infected individuals*

To compare *tax/rex* mRNA expression between the lung and peripheral blood, *tax/rex* mRNA levels in BALC and PBMC from four HAM/TSP and 13 non-HAM/TSP carriers were estimated by RT-PCR of serial 10-fold dilutions of each RNA sample. *tax/rex* mRNA was undetectable even in undiluted RNA samples from both tissues from six carriers. In the other three carriers it was detectable only in BALC but not in PBMC (Fig. 1). There was no individual in whom *tax/rex* mRNA was detectable in PBMC but not in BALC. *tax/rex* mRNA was amplifiable from both tissues from four HAM/TSP patients and the remaining four carriers. As shown in Fig. 1, in six of these eight individuals (carrier 3/4, and HAM/TSP 3/4), the amplifiable end-point of the BALC sample was 10–100 times weaker than that of the corresponding PBMC sample, indicating higher *tax/rex* mRNA expression in BALC. The remaining two individuals (carrier 1/4, and HAM/TSP 1/4) exhibited equivalent levels of expression in both tissues. These findings confirmed the lung tissue as a preferential site for *tax/rex* mRNA expression. Consistent with our previous report [13], the BALC samples with detectable *tax/rex* mRNA exhibited lymphocytosis with a significantly higher proportion of $CD3^+$ cells (data not shown).

Proviral DNA loads do not correlate with tax/rex *mRNA expression in BALC and PBMC of non-HAM/TSP carriers*

Proviral DNA load is thought to be a crucial factor determining the expression level of *tax/rex* mRNA. To evaluate this, the proviral DNA load in each tissue sample was determined by use of nested PCR with the sensitivity to amplify the HTLV-1 *pX* sequence from 5×10^{-7} μg DNA from an infected cell line, MT2, roughly equivalent to a single molecule of the HTLV-1 provirus (Fig. 2). Proviral DNA loads in PBMC and BALC of non-HAM/TSP carriers were variable, ranging from $10^{0{\cdot}0}$ to $10^{3{\cdot}0}$ and $10^{0{\cdot}0}$ to $10^{3{\cdot}3}$ copies/μg DNA, respectively. There was no correlation between proviral DNA loads and *tax/rex* mRNA expression. Geometric mean values of proviral DNA loads in PBMC and BALC samples in which *tax/rex* mRNA was detectable, $2{\cdot}25 \pm 0{\cdot}50$ (geometric $\text{mean}_{[\log 10]} \pm$ s.d.) and $1{\cdot}90 \pm 1{\cdot}27$, respectively, were almost equivalent to those values of the samples without detectable *tax/rex* mRNA ($1{\cdot}99 \pm 1{\cdot}19$ and $1{\cdot}77 \pm 1{\cdot}00$). In contrast, HAM/TSP patients exhibited high proviral DNA loads in both tissues, ranging from $10^{3{\cdot}0}$ to $10^{4{\cdot}6}$ copies/μg DNA (geometric mean ± s.d.: $3{\cdot}65 \pm 0{\cdot}79$) in PBMC and $10^{3{\cdot}0}$ to $10^{5{\cdot}2}$ copies/μg DNA ($3{\cdot}80 \pm 1{\cdot}05$) in BALC, which were significantly higher than those of carriers' samples without *tax/rex* mRNA expression ($P < 0{\cdot}05$, Fig. 3).

Correlation between proviral DNA load in PBMC and in BALC

Direct comparison of proviral DNA loads between PBMC and BALC in each individual is shown in Fig. 4a. Positive correlation was observed between proviral DNA loads in PBMC and BALC

(a)

```
position 7325                                                          8382      FREQ
ATK1     ------------------------------------C--C---C-----------------C-G-A---T-A--
clone
CONS.    ------------------------------------G--T---T-----------------A-------C--      16
T04      ------------------------------------G--T---T-----------------A-G-----C--       1
T10      ------------------------------------G--T---T-----------------A-----C-C--       1
T12      ------------------------------------G--T---T-----------------T-A-------C--     1
```

(b)

```
position 7325                                                                  8382      FREQ
ATK1     TG-G--T-------ATCTTA-T-TG--G--C---TTTA-AGCC-CA--ACCA--T---ATTC-T-A-T--TTAGA-
clone
CONS. A  ------------------------------------------------------------------------------      5
CONS. B  ----------------------------------------G-------G----------T--------------      9
2P13     ------------------------------------------------------C---------------------      1
2P14     ------------------G-------------------------------------------------------------      1
2P16     ----------------------------------C-------------------------------------------      1
2P17     ------------------------------------------------G---------------------------      1
2B03     ------------------------------------------------------------------------C----      1
2B04     -------------------------A----------------------------G---------------G-      1
2B06     ------------------C-------------------------------------------------------------      1
2B18     ------------------------------------------------T---------------------------      1
2P03     ----------------------------------------GG-------G----------T-----------A--      1
2P04     ----------------------------------------G-------G----------T--------C-----      1
2P05     ----------------------------------------G-------G----------T----------C---      1
2P06     --------------------------------------GA-G-------G--------C-T---------------      1
2P09     ----------------------------------------G--G----G----------T--------------      1
2B07     ----------------------------------------G-T-----G----------T--------------      1
2B12     ------C-----------------------------------G-------G----------T---G----------      1
2B13     ---A----------G---------------------------G-------G----------T--------------      1
2B20     ----------------------------T-----------G-------G----------T--------------      1
2P01     A---------------T-------------------------------------------------------------      1
2P02     ----------------------------------G----G-------G----------T-C------------      1
2P10     -A----------------------------------------G-------G----------T--------------      1
2P11     -C---------------C-----------------C------G-------GG---------T---------------      1
2B01     -A--------------------A-----------------G-------G----------T--------------      1
2B08     ----------------C-------C-----------------G-------G----------T--------------      1
2B16     ---------------------C-------------------------------------------C--------      1
2B17     ---------------------C--------------C-----G-------G---------CT--------------      1
```

Fig. 7. (See next page.) Nucleotide sequence variation among *in vivo*-derived HTLV-I *tax* clones. The whole Tax open reading frame (ORF) amplified from a sample DNA was ligated into pCG, and randomly selected plasmid clones were sequenced. Nucleotide sequences of 19, 39, and 38 *tax* clones derived from a cloned HTLV-I, pMT2 (a), and two HTLV-I-infected individuals, I.T. (b) and N.M. (c), respectively, are aligned in comparison with the reference *ATK1* [20]. Dots indicate sequences identical to the reference. Italicized letters indicate non-synonymous substitutions, and those resulting in the translational terminal codon, in particular, are underlined. The dominant sequences in each sample are indicated as consensus sequences (CONS.). The number of clones identical to a given nucleotide sequence (FREQ) is shown on the right.

($r = 0{\cdot}82$, $P < 0{\cdot}01$). Both tissues from HAM/TSP patients, which exclusively expressed detectable *tax/rex* mRNA, revealed high proviral DNA loads. While there was no evidence of preferential accumulation of infected cells in lung tissue of carriers, even in those that *tax/rex* mRNA was detectable only in BALC (Fig. 4a). According to the results of flow cytometric analysis, the proportion of T lymphocytes ($CD3^+$ cells) in BALC was highly variable among individuals, ranging from 2·0% to 66·2% among BALC samples, in contrast to a small variation among PBMC samples (10·8–35·0%). The values for proviral DNA load were thus calibrated with the proportion (%) of $CD3^+$ cells in each sample, and the resulting proviral DNA loads in the T cell population were compared between PBMC and BALC. As shown in Fig. 4b, this value was more closely correlated between PBMC and BALC ($r = 0{\cdot}90$, $P < 0{\cdot}01$). This indicated that the proportion of infected cells, particularly in the T cell population, was not different between the two tissues and argued against selective migration of infected T cells into lung tissue.

Transcription of tax/rex *mRNA is activated in lung tissue*

tax/rex mRNA expression in infected cells was compared between PBMC and BALC in each of eight individuals, including four patients with HAM/TSP, using values obtained by dividing *tax/rex* mRNA expression level in total cells (Units, shown in Fig. 1) by proviral DNA load. Three of four carriers exhibited 10–1000 times higher ratios in BALC compared with corresponding PBMC

(c)

```
position  7325                                                                  8382        FREQ
ATK1      -ATGCG-G-AAG-T--T----T-T-------C-----G-ATC-ATCCCGTT-A--G-A--A-A-TG-AA-AAAA--
clone
CONS. C   ----T-----------------------------------CT---T-------------------A-------C--        16
3P02      ----T-----G---------------------------CT---T-------------------A-------C--         1
3P03      ----T-----------------------------------CT--CTT-----------------A------GC--         1
3P06      ----T-----------------------------------CT-G-T-----------------A-------C--         1
3P08      ----T-----------------------------------CT---T-T-----------G----A-------C--         1
3P11      ----T----------------C------------------CT---T-------------------A-------C--         1
3P13      ----T-----------C----------------------CT---T-------------------A-------C--         1
3P14      ----T-----------------------------------CT---T---------------G--A-------C--         1
3P15      ----T-----------------------------------CT---T-------------------A-G-----C--         1
3P16      ----T-------------------------T---------CT---T-------------------A-------C--         1
3P17      ----T-----------------------------------CT---T-----------G-------A-------C--         1
3B03      ----T-----------------------------------CT---T-------------------A--G--G-C--         1
3B04      ----T-----------------------------------CT---T--A----------------A-------C--         1
3B17      ----T--------C--------------------------CT---T-------------------A-------C--         1
3B20      ----T---------------------------------T-CT---T-------------------A-------C--         1
3B21      -G--T-----------------------------------CT---T-------------------A-------C--         1
3P07      ----T-------------------C----------------CT--CT------------------A-------C--         1
3P19      ----T------A----------------------------CT---T---G--G-----------A----G--C--         1
3B01      ---AT-----------------------------------CT---T-------------------A-------C--         1
3B02      ----T------A----------------------------CT---T-------------------A-------C--         1
3B05      ---ATA-A--------------------------------GCT---T---------A---------A-------C--         1
3B07      --C-T-----------------------------------CT---T----C--------------A-------C--         1
3B08      ----T----G------------------------------CT---T------------------CA-------C--         1
```

Fig. 7. Continued. (See previous page for caption.)

(Fig. 5), suggesting that *tax/rex* mRNA expression was up-regulated in infected cells in the lung. To a lesser extent but still significant, up-regulation of 2·5–100 times was observed in BALC, even in three of four HAM/TSP patients. To confirm this finding, DNA and RNA were freshly isolated from PBMC and BALC of two additional individuals, including one HAM/TSP patient, and virus load and *tax/rex* mRNA expression were analysed in parallel. One microgram each of DNA and RNA samples was serially five-fold diluted and the dilutions were then subjected to PCR and RT-PCR, respectively (Fig. 6). In case 1 (HAM/TSP), the end-point dilutions of DNA from PBMC and BALC with amplifiable HTLV-1 genome were 5^{-5} and 5^{-4}, respectively, while *tax/rex* mRNA was detectable in RNA dilutions of 5^{-2} and 5^{-3}, respectively. Case 2 (carrier) revealed a more striking difference, i.e. end-point dilutions in the two tissue samples were 5^{-5} *versus* 5^{-2} (PBMC *versus* BALC) for DNA and 5^{-1} *versus* 5^{-3} for RNA. The higher expression level of *tax/rex* mRNA despite lower proviral DNA load in BALC was consistent with preferential activation of HTLV-1 in the lung.

Similar distribution of HTLV-1 quasispecies in PBMC and BALC

To evaluate the possibility of selective migration of HTLV-1 quasispecies in the lung, variation in the HTLV-1 proviral DNA sequence corresponding to the tax ORF (1059 bp) derived from PBMC and BALC of a HAM/TSP patient and a healthy carrier was analysed. As shown in Fig. 7b, sequence comparison revealed two consensus sequences in the HAM/TSP patient. Among the 40 sequences (20 each from PBMC and BALC) analysed, 27 contained one to four nucleotide substitutions from the consensus sequences. Sequences derived from two consensuses were equivalently distributed in PBMC and BALC. All 40 sequences from the healthy carrier (20 each from PBMC and BALC) were derived from a distinct consensus sequence, and in 22 of these randomly localized nucleotide substitutions were found (Fig. 7c). The frequency of nucleotide substitutions in sequences similarly obtained from a cloned HTLV-1 plasmid, pMT2, was about six times less than that found among the sequences from individuals (Fig. 7a), indicating that our results represented HTLV-1 quasispecies *in vivo*. Phylogenetic trees constructed from the sequence sets of each individual are shown in Fig. 8a,b. Both sequences derived from PBMC and BALC were randomly distributed in three branches of the tree, corresponding to the consensus sequences. There was no evidence of selective distribution of certain HTLV-1 quasispecies in the lung.

DISCUSSION

Semiquantitative detection of *tax/rex* mRNA strongly suggested that lung tissue is a preferential site for its expression. Of 17 infected individuals analysed, *tax/rex* mRNA was detectable in both or either PBMC and BALC in 11 individuals. It was detectable only in BALC but not in PBMC in three individuals. The remaining eight, including all four HAM/TSP patients, exhibited expression in both tissues, but expression levels were higher in BALC than PBMC in six of the eight. No patients exhibited preferential *tax/rex* mRNA expression in the PBMC. The high number of infected cells (high proviral DNA load), selective distribution of infected cells with an advantage for viral expression, and/or presence of tissue-specific conditions activating viral expression in infected cells were presumed to underlie preferential *tax/rex* mRNA expression in the lung. To evaluate

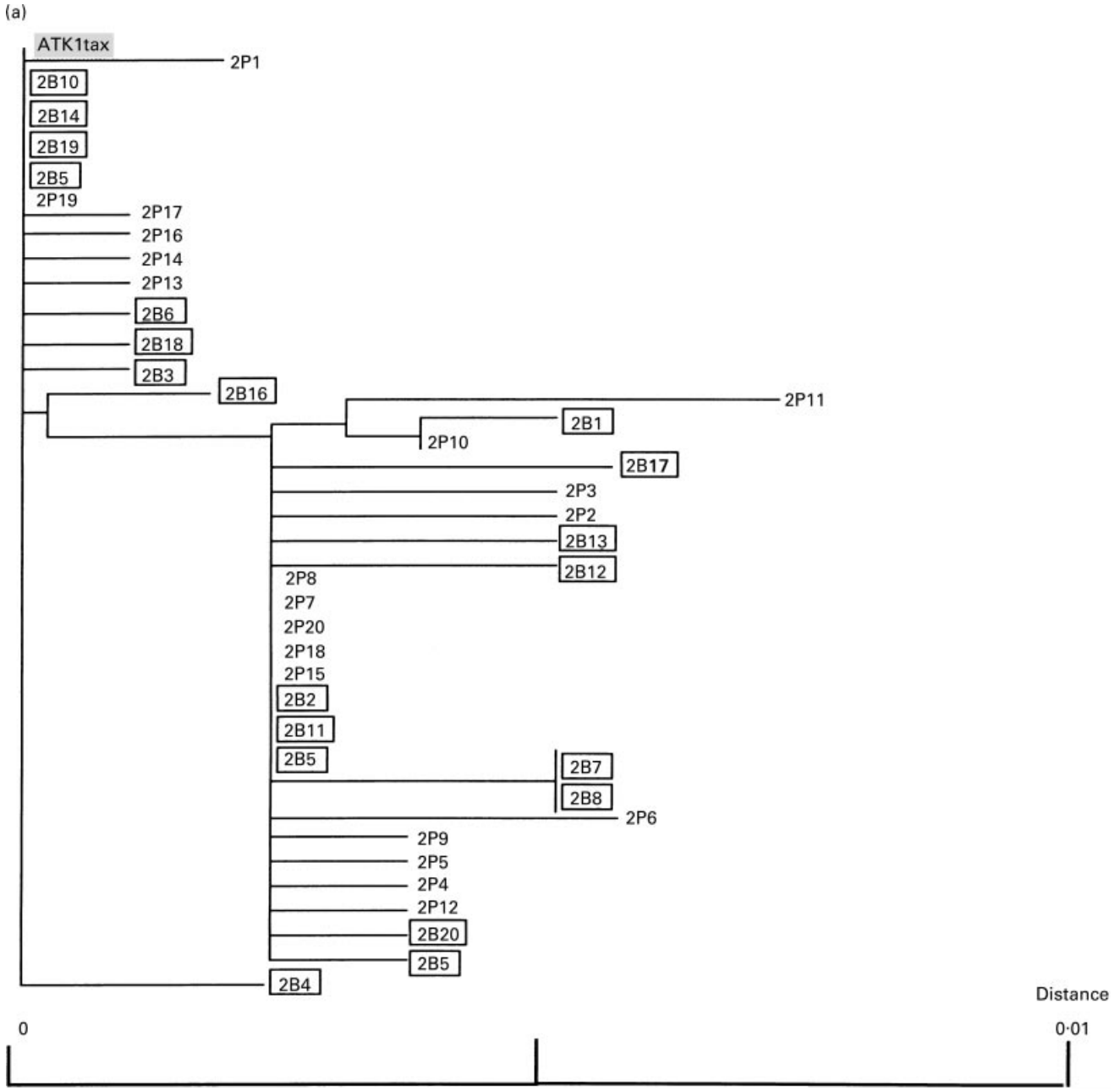

Fig. 8. (See next page.) Phylogenetic trees for sets of tax open reading frame (ORF) sequences derived from peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) from two infected individuals ((a) HTLV-1-associated myelopathy/tropical spastic paraparesis; (b) carrier). The neighbour-joining tree was created using the Kimura two-parameter method [23]. The letters P and B indicate the clones obtained from PBMC and BALC, respectively. Numbers represent the clone numbers. The clones derived from BALC are in squares. ATK-1 tax is a control sequence. The scale bars represent the percentage nucleotide sequence divergence.

these possibilities, we analysed proviral DNA loads and quasispecies in PBMC and BALC.

High-level viral expression together with extremely high proviral DNA load is well documented in PBMC from HAM/TSP patients. It is possible that *tax/rex* mRNA expression levels simply reflect proviral DNA load. Cultured HTLV-1-infected cells exhibit the activated T cell phenotype which includes expression of various cell adhesion molecules, such as CD2, CD45, and LFAs, on the cell surface [10]. Expression of these cell adhesion molecules is thought to give the cells an advantage to migrate and infiltrate into visceral organs, including the lung, and may result in the accumulation of infected cells in the organ. Consistent with previous findings [4,25], both tissue types from HAM/TSP patients in the present study showed exclusive *tax/rex* mRNA expression and high proviral DNA load, indicating the contribution of high proviral DNA load in *tax/rex* expression in HAM/TSP. In contrast, there was no correlation between proviral DNA load and detection of *tax/rex* mRNA in non-HAM/TSP patients. In particular, three BALC samples expressed detectable *tax/rex* mRNA despite extremely low proviral DNA load ($<$ 10 copies/

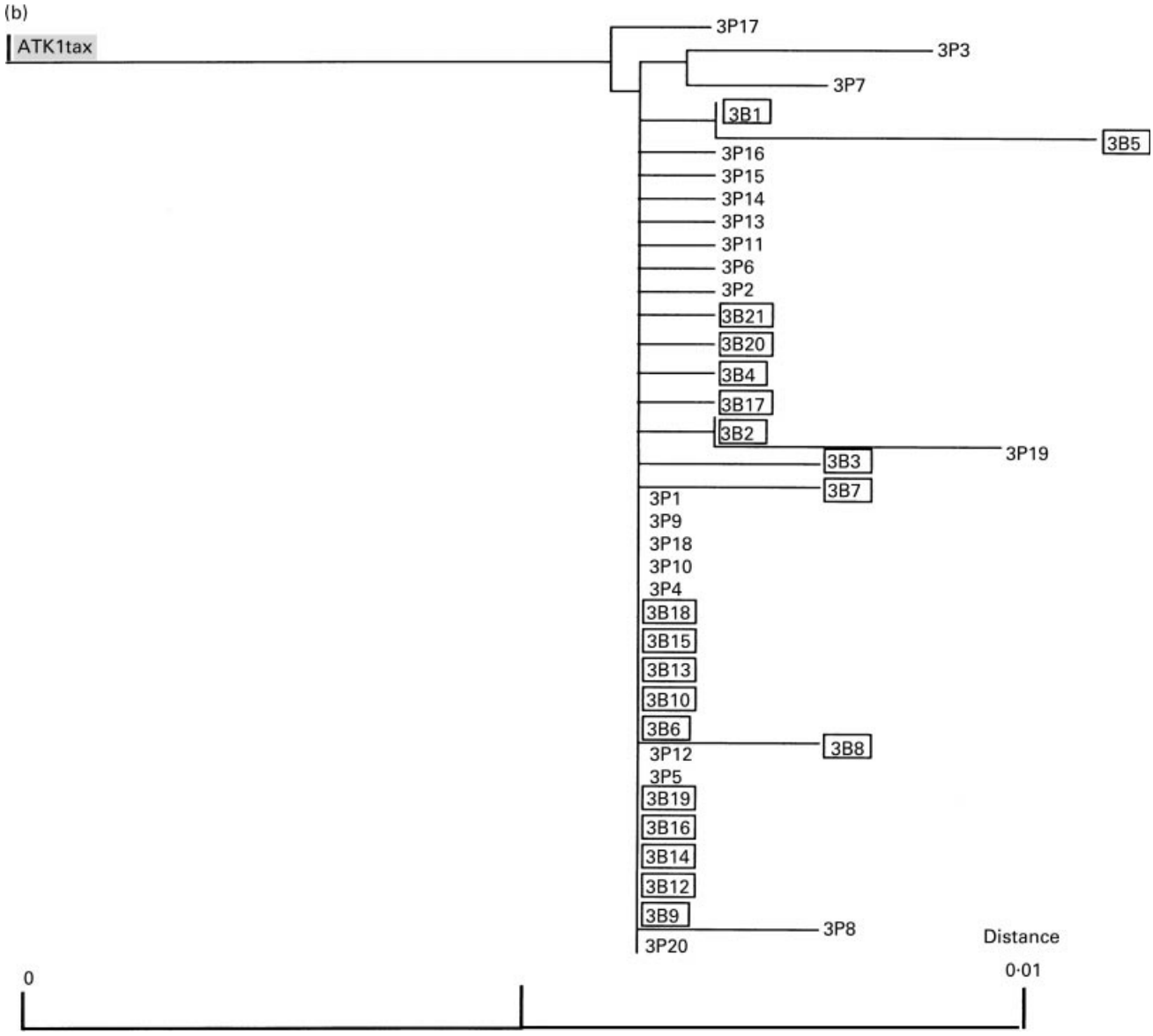

Fig. 8. Continued. (See previous page for caption.)

μg DNA; Fig. 3), indicating that proviral DNA load is not the factor causing higher viral expression in the lung. Moreover, proviral DNA load, in particular in the T cell population, was closely correlated between PBMC and BALC in each individual, including HAM/TSP patients. This clearly argues against the selective migration and accumulation of infected cells in lung tissue and suggests involvement of factors up-regulating viral expression in the tissue.

Direct comparison of the ratio between expression of *tax/rex* mRNA and proviral DNA load indicated that expression was preferentially up-regulated in infected lung cells. The difference in up-regulation between PBMC and BALC was more obvious in non-HAM/TSP carriers and less in HAM/TSP patients. Factor(s) that up-regulate viral expression in lung is likely to be more profoundly involved in carriers and increase *tax/rex* mRNA expression to detectable levels even in lungs with an extremely low proviral DNA load.

HTLV-1 is a very old virus which has been maintained in the population through sexual and vertical transmission [26,27]. Little divergence in the nucleotide sequence was identified among HTLV-1 isolates derived from different geographical regions of the world [28]. However, accumulating evidence has recently indicated the presence of intrastrain variability (quasispecies) of HTLV-1 [29–31]. It was demonstrated that amino acid substitutions in the gene encoding Tax frequently resulted in functional loss of the protein and a significant proportion of HTLV-1 quasispecies of PBMC in infected individuals encoded the defective Tax [32,33]. Since Tax function is indispensable for viral expression [34], such a quasispecies is not likely to be infectious. The quasispecies was therefore considered to be a potential factor involved in tissue-specific differences in viral expression. To evaluate the possibility that quasispecies competent for Tax function was selectively distributed in lung and that a particular subset of infected cells revealed tropism for the organ,

we performed phylogenetic analysis for 80 sequence sets of *tax* ORF derived from PBMC and BALC of two infected individuals. The sequences from both tissues were equivalently distributed in branches of the phylogenetic tree, arguing against differences in quasispecies between tissues. Preliminary experiments to evaluate the function of Tax encoded by 80 sequences (Fig. 7) in cells co-transfected with a reporter plasmid indicated that 16 sequences (20·5%) that appeared to encode non-functional Tax were equivalently distributed in PBMC and BALC (data not shown). These findings suggest that the quasispecies is unlikely to contribute to preferential expression of *tax/rex* mRNA in the lung.

The similarity of proviral DNA load and quasispecies between PBMC and BALC strongly suggests the involvement of a tissue-specific condition conferring an advantage for viral expression in lung tissue. It was reported that HIV-1 RNA in mononuclear cells was more highly expressed in oesophageal mucosa than in lymph nodes, implicating local events promoting viral expression in 'open' organs such as the gastrointestinal tract [35]. The lung is also in contact with the exterior, and as such it may be subjected to local extracellular stimuli capable of activating cellular transcription factors that in turn regulate viral expression. Constitutive exposure to foreign antigens including other pathogenic micro-organisms may cause direct activation of infected cells and stimulation of uninfected cells, including macrophages, to produce various chemokines and cytokines such as tumour necrosis factor and IL-2, which in turn activate infected T cells [1,8–10,36].

Viral expression is barely detectable in PBMC from HTLV-1-infected individuals *in vivo*, but is dramatically activated once cultured *in vitro* [37,38]. This suggests the presence of a factor inhibiting viral expression in serum of infected individuals [39]. Likely candidates for this role are serum antibodies to the virus. An alternative possibility is that a lower concentration of such an inhibitory factor in the lung would confer an advantage for viral expression. Elucidating the local events that promote HTLV-1 expression in the lung of HTLV-1-infected individuals will be critical to the understanding of its pathogenesis.

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