Constitutive Expression of Parathyroid Hormone–Related Protein Gene in Human T Cell Leukemia Virus Type 1 (HTLV-1) Carriers and Adult T Cell Leukemia Patients that Can Be *trans*-Activated by HTLV-1 *tax* Gene

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Summary

Adult T cell leukemia (ATL) is associated with human T cell leukemia virus type 1 (HTLV-1) infection, and almost all ATL patients have the complication of hypercalcemia. To understand the mechanism of the high incidence of hypercalcemia in ATL, we studied the expression of a parathyroid hormone–related protein (PTHrP) gene that has been proposed as a causative factor of hypercalcemia in some solid tumors. The polymerase chain reaction coupled with reverse transcription of mRNA was applied to RNA from peripheral blood mononuclear cells. Cells from all 13 ATL patients examined showed abundant expression of the PTHrP gene, while cells from uninfected normal subjects did not. Significant expression of PTHrP gene was also detected in HTLV-1 carriers without any symptoms and in patients with HTLV-1-associated myelopathy or tropical spastic paraparesis. PTHrP mRNA levels correlated with the number of infected cells that were estimated by the integrated HTLV-1 DNA. These results suggest that HTLV-1-infected cells are expressing the PTHrP gene. This concept was further supported by the finding that the HTLV-1 *trans*-activator, the *tax* gene product, caused *trans*-activation of the PTHrP gene promoter linked to the CAT gene. These observations might explain the general expression of the PTHrP gene in ATL patients and the high incidence of hypercalcemia in ATL.

Adult T cell leukemia (ATL)[1] is a unique T cell malignancy associated with human T cell leukemia virus type 1 (HTLV-1) (1, 2). HTLV-1 is also associated with a chronic progressive myelopathy, designated as HTLV-1-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (3, 4). One of the clinical characteristics of ATL is hypercalcemia, and almost all ATL patients show abnormal calcium metabolism: marked increase of osteoclastic bone resorption, urinary excretion of calcium, and nephrogenous cyclic AMP. These abnormalities were detected even in patients who do not show apparent hypercalcemia (5, 6). IL-1 has been implicated as a possible causative factor of the hypercalcemia in ATL, because of its osteoclast-activating effect and its production by some ATL cells (7, 8). However, the presence of systemic metabolic derangements similar to those seen in primary hyperparathyroidism (9) are not satisfactorily explained by the actions of IL-1.

A parathyroid hormone–related protein (PTHrP) has recently been proposed as a humoral factor of hypercalcemia in some solid tumors (10–12). Overproduction of parathyroid hormone (PTH) induces the systemic abnormality of calcium metabolism known as primary hyperparathyroidism, and the NH_2-terminal region of PTH shows homology to that of the PTHrP (13–15). The NH_2-terminal 34 amino acids of both proteins show similar effects on renal and osteoblast cell membranes in vitro, and on calcium and inorganic phosphate fluxes in vivo (16–18). These observations seem to explain the similarities in the syndromes of humorally mediated hypercalcemia of malignancy and primary hyperparathyroidism (9).

We have demonstrated previously that an HTLV-1-infected T cell line, MT-2 cells, produced a factor that stimulated ade-

[1] *Abbreviations used in this paper:* ATL, adult T cell leukemia; GM, granulocyte/macrophage; HAM, HTLV-1-associated myelopathy; HTLV-1, human T cell leukemia virus type 1; PTHrP, parathyroid hormone–related protein; RT, reverse transcription; TSP, tropical spastic paraparesis.

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nylate cyclase activity in osteoblast membranes, and also that mRNA expressed in MT-2 cells was homologous to the PTHrP mRNA gene (19, 20). Expression of a PTHrP-related gene was detected in a few cases of ATL using oligonucleotide probes, thus, the PTHrP has been suggested to be associated with hypercalcemia in ATL (21, 22). However, the oligonucleotide hybridization assay gave positive results in only a few cases of ATL because of its sensitivity.

To examine PTHrP gene expression in ATL more widely, we applied the PCR coupled with reverse transcription of mRNA to the investigation of PBMC. 13 patients with ATL, four patients with HAM/TSP, and four healthy HTLV-1 carriers were all found to express PTHrP gene in vivo. This suggests that HTLV-1 infection induced the expression of the PTHrP gene. We also found that the HTLV-1 *tax* gene *trans*-activated the PTHrP gene promoter in cotransfection assays.

Materials and Methods

Cells. MT-2 is a human T cell line infected with HTLV-1 (23), and CEM and Jurkat are noninfected human T cell lines. PBMC were prepared by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation.

RNA Preparation. RNA from the cell lines or from PBMCs was isolated by the ribonucleoside-vanadyl complex method (24), and then polyadenylated RNA was separated using oligo-dT-cellulose.

Reverse Transcription–coupled PCR (RT-PCR). For detection of PTHrP mRNA, the RT-PCR was applied as follows (25–27). Samples of 1 μg of total cytoplasmic RNA underwent reverse transcription by Moloney murine leukemia virus RT (Bethesda Research Laboratories, Gaithersburg, MD) using oligo(dT) as primers. 1/20 of the cDNA was subjected to PCR. The primers used were 21 mers derived from the exon 2 and exon 3 sequences of PTHrP (28), so that the PCR product derived from the cDNA could easily be distinguished from that derived from genomic DNA. DNA in the PCR mixture was denatured at 95°C for 1 min, and annealed at 55°C for 2 min. Polymerization was carried out at 70°C for 1.5 min. These reactions were repeated for 50 cycles.

The PCR products were analyzed either by Southern blotting or by liquid hybridization followed by gel electrophoresis. For liquid hybridization (28), the PCR products were denatured at 98°C for 15 min and hybridized with a ^{32}P-oligonucleotide probe at 55°C for 45 min in 1× SSC. The mixture was then separated by PAGE, and the gel was exposed to x-ray film for autoradiography. The radioactivity of a specific band was measured for quantitation of the amplified DNA.

Construction and Transfection of Plasmids and CAT Assay. To construct the pPTHrP-CAT plasmid that contained the putative promoter of the PTHrP gene, a 2.8-kbp HindIII-BalI fragment covering the 5′ flanking region of the PTHrP coding sequence (29, 30) was inserted immediately upstream to the CAT gene, thus replacing the SV40 sequence of pSV2CAT (31). The pLTR-CAT plasmid (32) contained the HTLV-1 LTR as the promoter. The pRSV55Tax plasmid was a *tax* expression plasmid containing an RSV enhancer linked to the HTLV-1 promoter (33). The pRSV55-neo plasmid had a neomycin-resistant gene instead of the *tax* gene, and was used as a negative control. 5 μg of each CAT plasmid was cotransfected with 1 μg of pRSV55tax or pRSV55-neo into Jurkat cells by the DEAE-dextran procedure (34). After 40 h of incubation, cell lysates were prepared and CAT activity was measured as described previously (32, 33). The activation of CAT gene expression was indicated by the ratio of CAT activity with the pRSV55tax plasmid to that with the pRSV55-neo plasmid. Results were averages of more than three experiments.

Results

PTHrP mRNA in MT-2 Cells. We have previously reported that MT-2 cells, a T cell line infected with HTLV-1 (23), produced a factor that stimulated adenylate cyclase activity in osteoblast membranes, and also expressed multiple species of mRNA (19) that hybridized with the oligonucleotide probe of PTHrP cDNA (13). To characterize these multiple forms of mRNA, we isolated several cDNA clones using an oligonucleotide probe. Structural analysis of these cDNA clones revealed multiple forms of mRNA that had an identical open reading frame coding for PTHrP (13–15, 35), but these differed in the 3′ noncoding sequences by alternative splicing and also by alternative polyadenylation (data not shown; see Fig. 1). Thus, the mRNAs detected in MT-2 cells were those of PTHrP, and the PTHrP was suggested to be the adenylate cyclase–stimulating factor present in the culture medium (20).

Expression of the PTHrP Gene in ATL Cells. We first studied the expression of the PTHrP gene in leukemic T cells of ATL patients by Northern blot hybridization using the cDNA as a probe. A few samples of ATL gave a positive result, showing

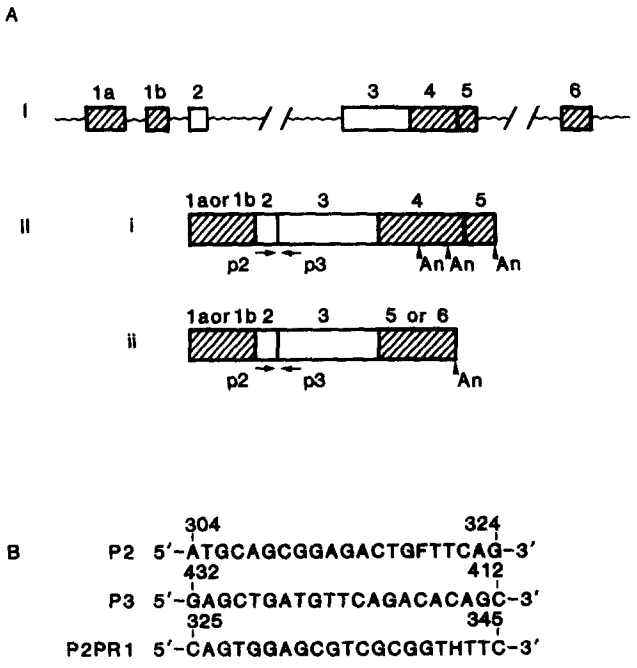

Figure 1. Genomic organization of the PTHrP gene (27, 39) and region of amplification in PTHrP mRNA using the RT-PCR (*A*). The organization of the gene (*I*) and of the mRNAs (*II*) are shown schematically. Open boxes represent coding exons, and hatched boxes represent noncoding exons that are alternatively spliced. mRNA in *i* represents multiple forms terminated at different polyadenylation sites (An). The sequences used as primers (P2 and P3) and as the hybridization probe (P2 PR1) are shown in *B*. The nucleotide positions are those in the cDNA sequence reported by Thiede et al. (15).

multiple bands (Fig. 2), the pattern of which was basically the same as that of MT-2 cellular RNA. These results confirmed that the PTHrP gene was expressed in fresh cells from ATL patients, and the extent of mRNA expression was comparable with that in MT-2 cells, as judged from the intensities of the bands and the amount of RNA applied to the gel.

This type of analysis requires a large number of fresh leukemic cells to get intact mRNA. In practice, at least 0.5 μg of poly(A)-containing RNA was required, which was equivalent to 10–30 μg of total cytoplasmic RNA or 10^7 normal PBMC. To establish a more sensitive assay, we applied the PCR coupled with reverse transcription of mRNA (RT-PCR) (25–27). Total cytoplasmic RNA extracted from the fresh or frozen PBMC of the ATL patients underwent reverse transcription into cDNA using oligo(dT) as a primer, and then the PTHrP gene sequence was amplified by the PCR, as described in Materials and Methods. The sequences corresponding to exons 2 and 3 were used as primers so that all species of mRNA could be detected and so that the DNA amplified from cDNA could be distinguished from DNA arising from contaminating cellular DNA. After 50 cycles of PCR, the product was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide or Southern hybridization.

On staining the gels, a specific band with the expected size (Fig. 3 *A*, *arrow*) was visible as a major signal in all samples from the 13 ATL patients tested, while no such band was visible in preparations from uninfected healthy donors (Fig. 3 *A*). The other bands were also detected in some samples. However, these were not reproducible and did not hybridize with the PTHrP probe, thus, they were concluded to be nonspecific products. The identity of this amplified DNA was confirmed by Southern blot hybridization with an oligonucleotide probe corresponding to the sequence between the two primers used for the PCR. The specifically stained band hybridized very strongly with this oligonucleotide probe (Fig. 3 *B*). On the other hand, samples from normal donors showed no hybridization signal, although they gave a very faint signal after a 10-fold longer exposure. Similar results were obtained using another pair of primers of exons 1 and 2 (data not shown). In control experiments, β actin primers produced almost constant amounts of amplified DNA in all samples, indicating the specific expression of the PTHrP gene in the ATL samples (Fig. 3 *C*).

To estimate the levels of PTHrP mRNA, quantitation of the amplified DNA was performed as follows. After the RT-PCR, part of the amplified DNA was hybridized with an oligonucleotide probe at 55°C for 45 min. The hybridized materials were then separated in a polyacrylamide gel, and the radioactivities of specific bands were measured. The radioactivity of the specific bands showed a linear relationship

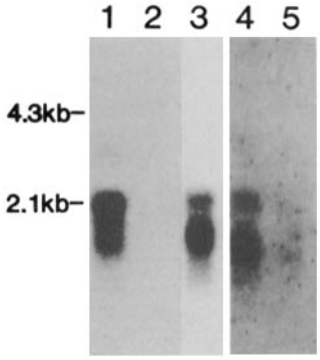

Figure 2. Detection of PTHrP mRNA in PBMC of ATL patients by Northern blot hybridization. Poly(A)-containing RNA from MT-2 cells (lane *1*), normal donor PBMC (lane *2*), and ATL PBMC (lanes *3–5*) were applied, but in different amounts because of the limitations of the samples: 0.5 μg (lanes *1–3*), 0.3 μg (lane *4*), and <0.1 μg (lane *5*). The filter was hybridized with the full cDNA of PTHrP.

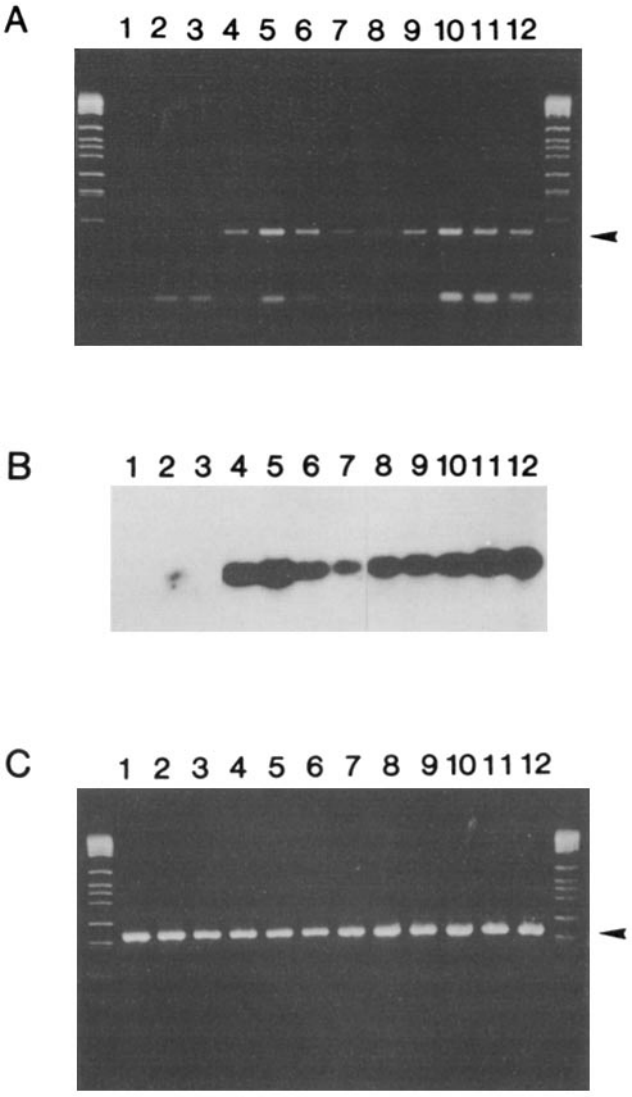

Figure 3. Detection of PTHrP mRNA in ATL samples by the PCR coupled with reverse transcription. Reverse transcription of cellular RNA (1.0 μg total RNA) and the PCR were carried out as described in Materials and Methods. One-fifth of the PCR mixture was analyzed by ethidium bromide staining of the gel (*A*) and by hybridization with the oligonucleotide probe of PTHrP cDNA. The film was exposed for 3 h (*B*). (*C*) Agarose gel electrophoresis of DNA amplified by β-actin primers. Lane *1*, CEM cells; lane *2*, normal donor cells; lane *3*, normal donor cells stimulated for 40 h by PHA; lanes *4–11*, cells from ATL patients; lane *12*, MT-2 cells. Molecular marker, BglI/HinfI digest of pBR328. Arrowhead indicates the position of the specifically amplified DNA.

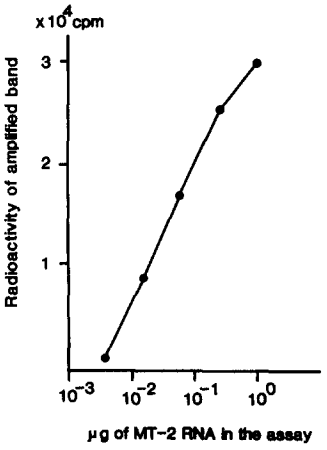

Figure 4. Quantitative analysis of the PTHrP mRNA level. MT-2 RNA was serially diluted fourfold with CEM RNA and subjected to the RT-PCR. The amplified DNA was hybridized with a ^{32}P-labeled oligonucleotide probe, and the hybrids were separated by gel electrophoresis. The radioactivities of the specific bands were counted and plotted.

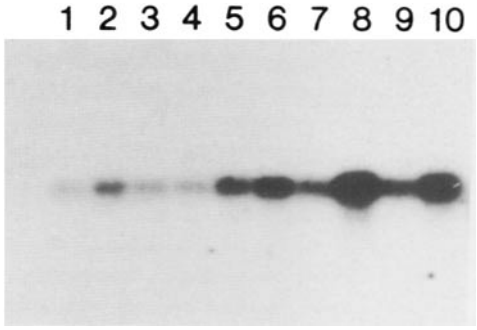

Figure 5. Detection of PTHrP mRNA in PBMC of HTLV-1 carriers and HAM/TSP patients. All samples were processed as described in Fig. 3 for hybridization with the pTHrP probe, except for the exposure time of 36 h. Lanes *1* and *2*, PBMC from normal donors, fresh and PHA stimulated, respectively; lanes *3–6*, PBMC from asymptomatic carriers; lanes *7–10*, PBMC from HAM/TSP patients. The arrowhead indicates the position of the specifically amplified DNA.

to the amount of mRNA using a fourfold serial dilution of MT-2 RNA (Fig. 4). Using this system, we found that 9 of 13 ATL patients expressed PTHrP mRNA at several times the level of the MT-2 cell line. The other four patients showed weaker expression, varying from an equivalent expression to that of MT-2 cells down to roughly 1/10 of this level. The abundant level of PTHrP mRNA expression suggested that the leukemic cells expressed the PTHrP gene in vivo.

PTHrP Gene Expression in Infected Cells. The expression of the PTHrP gene in leukemic T cells raised the question of whether HTLV-1-infected cells expressed the PTHrP gene before malignant transformation, since leukemic cells are always infected with HTLV-1. To examine this, we surveyed PBMC of HTLV-1 carriers who were either without any symptoms or with HAM/TSP (3, 4). HAM/TSP patients were included in this survey because they showed more expansion of HTLV-1-infected T cells than did asymptomatic carriers (36). As shown in Fig. 5, all eight samples of HTLV-1 carriers tested expressed significantly higher levels of PTHrP mRNA than did uninfected normal controls (lanes *3–10*). In some cases, the levels were significantly higher than in the PHA-stimulated PBMC of uninfected donors (Fig. 5, lane *2*), where all the T cells were activated. These findings suggested that HTLV-1 infection induced expression of the PTHrP gene before malignant transformation.

To determine the correlation between the PTHrP gene expression and the population of HTLV-1-infected T cells, the samples tested for PTHrP expression were subjected to Southern blot analysis to determine HTLV-1 DNA. Based on the intensities of the blots (see Fig. 6 *B*), the fraction of HTLV-1-infected cells were classified into five grades: nondetectable, and detectable in +1, +2, +3, and +4 (Fig. 6 *A*). The sample distribution of the grades of infected cells roughly correlated with the PTHrP gene expression. These findings strongly suggested that HTLV-1-infected cells in asymptomatic carriers or HAM/TSP patients expressed the PTHrP gene. However, when the level of PTHrP expression was compared between ATL and non-ATL samples, the expression in ATL samples was found to be higher than expected from the population of infected cells. Thus, some factor(s) other than HTLV-1 infection, such as transformation of infected cells, might be further activating PTHrP gene expression.

trans-activation of the PTHrP Promoter by HTLV-1 tax. Expression of the PTHrP gene in HTLV-1-infected cells strongly suggested that this gene expression might be directly *trans*-activated by the *tax* gene product of HTLV-1, since *tax* can activate the transcription of cellular genes like IL-2, IL-2R, or granulocyte/macrophage (GM)-CSF, as well as the HTLV-1 genome (32, 36–39). To examine this possibility, we constructed the pPTHrP-CAT plasmid by inserting the 2.8-kbp fragment containing the promoter region of the PTHrP gene (29, 30) into upstream of the CAT gene (Fig. 7 *A*). After transfecting this plasmid with or without a *tax* expression plasmid, pRSV55Tax, the CAT activities expressed in cells were measured. CAT expression was enhanced 15-fold by the *tax* plasmid, although this was less than with the pLTR-CAT plasmid (Fig. 7 *B*). Therefore, the promoter of PTHrP was demonstrated to be *trans*-activated by the viral *trans*-activator *tax*. This could be one mechanism by which HTLV-1-infected cells express the PTHrP gene.

Discussion

In this study, we demonstrated that all 13 ATL patients tested expressed the PTHrP gene in comparable levels with the MT-2 cell line. Expression of the PTHrP gene in all ATL patients would explain the high incidence of hypercalcemia in ATL. This hypothesis is based on reports that PTHrP is one of the main factors responsible for hypercalcemia of cancer (10–14, 40, 41). The ATL patients studied here included both hypercalcemic and normocalcemic individuals, and a high level of PTHrP gene expression was not necessarily correlated with clinically evident hypercalcemia. However, these observations on PTHrP expression and hypercalcemia are not conflicting, because as we have reported previously (6, 22), most ATL patients show clinical evidences of calcium mobilization, such

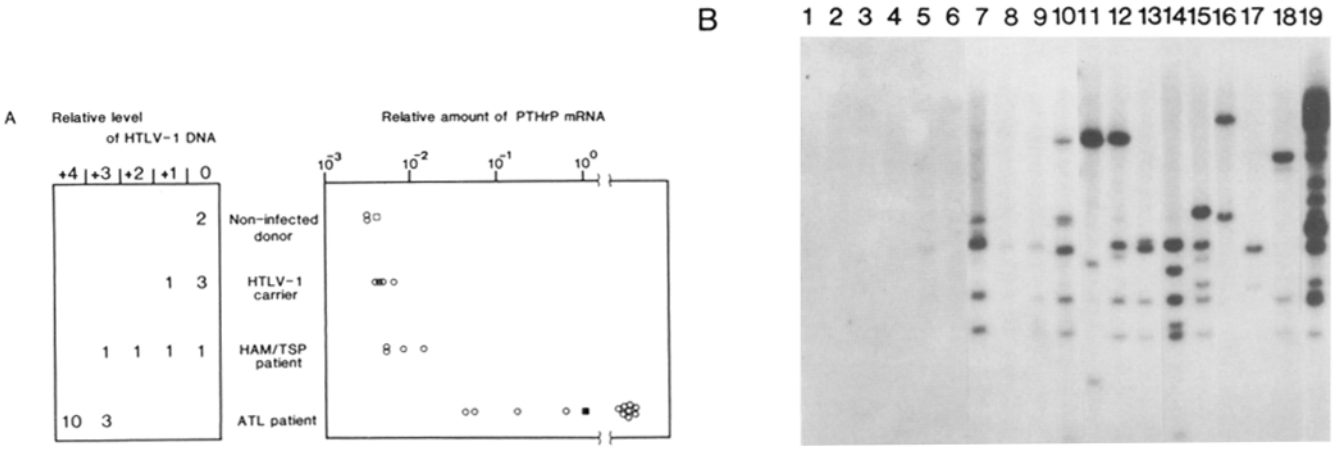

Figure 6. Levels of expression of the PTHrP gene in HTLV-1 carriers, HAM/TSP patients, and ATL patients. (*A*) Levels of PTHrP mRNA were estimated using the standard curve shown in Fig. 4, and are shown relative to levels in MT-2 cells. (○) Values for individual PBMC samples; (■) MT-2 cells; (□) PHA-stimulated PBMC from an uninfected normal donor. On the left side, the HTLV-1-infected cells in the samples are classified into five grades by the intensity of the HTLV-1 DNA bands in Fig. 5 *B*. (*B*) Estimation of HTLV-1 proviral DNA levels in PBMC samples. Cellular DNA was isolated from PBMC at the time of RNA preparation, and PstI-digested samples of 10 μg were examined for integrated HTLV-1 proviral DNA by Southern hybridization using the whole sequence of HTLV-1 DNA as a probe. Lane *1*, CEM cells; lanes *2–5*, cells from asymptomatic carriers; lanes *6–9*, cells from HAM/TSP patients; lanes *10–18*, cells from ATL patients; lane *19*, MT-2 cells.

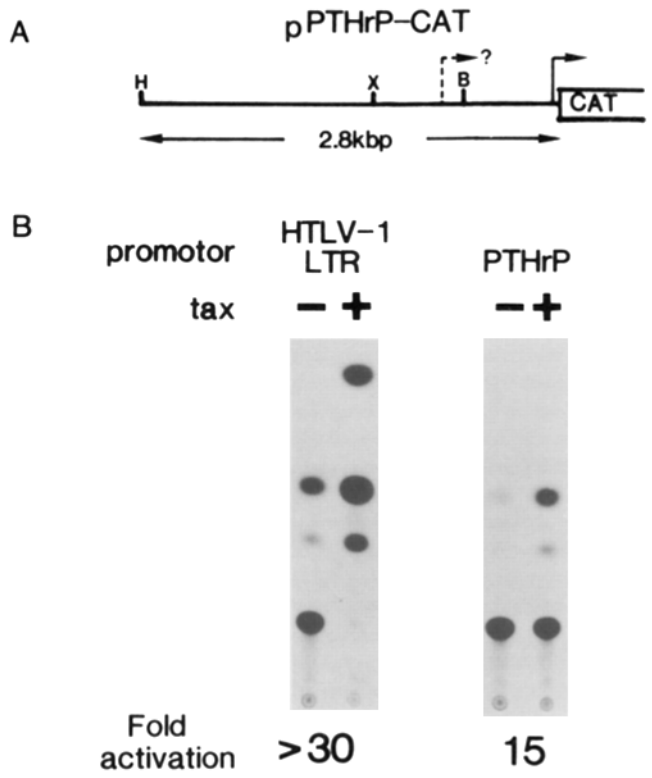

Figure 7. Activation of the PTHrP promoter by the *tax* gene of HTLV-1. pPTHrP-CAT was constructed from pSV2-CAT by replacing the SV40 promotor sequence with the PTHrP gene promotor fragment (*A*) H, HindIII; X, XbaI; B, BamHI. Arrow indicates putative transcription starting site. pPTHrP was cotransfected with pPSV55Tax, a *tax* expression plasmid, or with a control plasmid, pRSVneo. Activation shows the ratio of CAT activity in cells cotransfected with pRSV55Tax with respect to cells cotransfected with pRSV55neo, and is an average of more than three experiments (*B*).

as hypercalciuria and the increased urinary excretion of nephrogenous cyclic AMP. Elevation of the blood calcium level would be the consequence of the breakdown of the mechanism maintaining homeostasis, mainly due to a long-term massive load of mobilized calcium and the subsequent deterioration of renal function.

The level of PTHrP expression correlated with the population of ATL cells in the patients. This observation strongly suggested that malignant ATL cells produced PTHrP, which would explain the high incidence of hypercalcemia in ATL. We also demonstrated expression of the PTHrP gene in asymptomatic HTLV-1 carriers and patients with HAM/TSP, and the expression level was correlated with the population of infected cells. Therefore, it was concluded that HTLV-1 infection induced the PTHrP gene expression. As a possible mechanism of this induction, direct *trans*-activation by the *tax* gene of HTLV-1 was tested. The putative promoter in pPTHrP-CAT was significantly *trans*-activated by cotransfection with *tax*, in a similar manner to IL-2Rα and GM-CSF gene expression (37, 39). However, it is known that in the peripheral blood cells of ATL patients, viral genes are not expressed or are expressed at a very low level. Thus, some other cellular function(s) may also be involved in the constitutive expression of the PTHrP gene.

Although there were considerable variations in individual responses, the PTHrP expression in ATL was much higher than that expected in proportion to the number of infected cells. The number of HTLV-1-infected cells in ATL patients was 20–50 times that in HAM/TSP patients (see Fig. 5), but in some ATL cases, 1,000-fold higher expression of the PTHrP gene than those in HAM/TSP patients was observed. Therefore, some factor(s) other than the HTLV-1 *tax* gene, for example, a factor associated with malignant transformation, may further enhance the expression of the PTHrP gene.

In summary, our observations strongly suggested that PTHrP gene expression was induced by HTLV-1 infection through *trans*-activation by the *tax* gene and was further activated after malignant transformation. PTHrP expression may be the basis for the high incidence of hypercalcemia in ATL.

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