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Citation	Journal of Virology, 81(11), 5908-5918 https://doi.org/10.1128/JVI.02811-06
Issue Date	2007-06
Doc URL	http://hdl.handle.net/2115/27990
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Type	article (author version)
File Information	JV81-11.pdf

Enhanced Replication of Human T-cell Leukemia Virus Type 1 in T Cells from Transgenic Rats Expressing Human CRM1 That Is Regulated in a Natural Manner

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Running title: HTLV-1 replication in hCRM1 Tg rat

Word counts Abstract: 221
Text: 6015

Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T cell leukemia (ATL). To develop a better animal model for the investigation of HTLV-1 infection, we established a transgenic (Tg) rat carrying the human CRM1 (hCRM1) gene that encodes a viral RNA transporter, which is a species-specific restriction factor. At first we found that CRM1 expression is elaborately regulated through protein kinase C involving pathway during lymphocyte activation initially by post-transcriptional and subsequently by transcriptional manners. This fact led us to use an hCRM1 containing BAC clone, which would harbor the entire regulatory and coding regions of the CRM1 gene. The Tg rats expressed hCRM1 protein in a manner similar to the intrinsic rat CRM1 in various organs. HTLV-1-infected T cell lines derived from these Tg rats produced 100 to 10,000 fold more HTLV-1 than did T cells from wild type rats, and the absolute levels of HTLV-1 were similar to those produced by human T cells. We also observed enhancement of the dissemination of HTLV-1 to thymus in the Tg rats after intraperitoneal inoculation, although the proviral loads were low both in wild type and the Tg rats. These results support the essential role of hCRM1 in proper HTLV-1 replication and suggest the importance of this Tg rat as an animal model for HTLV-1.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-1) is etiologically associated with human adult T-cell leukemia (ATL), a chronic progressive neurological disorder termed HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (17, 27, 54, 55), and several other human diseases (23, 40, 42, 48). Examination of the viral nucleotide sequences among different disease groups has not revealed any specific determinants that distinguish a particular HTLV-1 associated disease (11, 35, 67). Thus, a primary determinant of HTLV-1 associated disease may be host-related.

In order to investigate HTLV-1 infection and its related disease development in detail, suitable animal models are required. HTLV-1 can immortalize simian, feline, rat, and rabbit lymphocytes *in vitro* (2, 29, 46). HTLV-1 can also infect experimental animals, such as rabbits, monkeys, and rats (2, 45, 53, 62). Using these susceptible animals, several models have been developed to study HTLV-1 associated diseases. The HAM/TSP-like disease model in WKA strain rats has been well established and used to dissect the pathogenic mechanisms of the disease (31, 39). In contrast, only a few ATL model systems have been established using rabbits and rats, but their utility is limited. For instance, the rabbit ATL model shows reproducible development of an ATL-like disease in adult animals (58), but few immunological studies can be performed in this animal, primarily because of the difficulty in obtaining inbred strains of rabbits. In the rat models, the development of ATL-like disease was observed only in newborn animals with a very short period of disease onset (64), making it difficult to perform oncological and immunological studies at the same time. Kannagi and Ohashi have established a rat model of ATL-like disease in which they were able to examine the growth and spread of HTLV-1-infected cells, as well as assess the effects of T cells on the development of the disease in T cell-deficient nude rats (51). This model system has been used to assess DNA or peptide-based vaccine development (25, 52) and to study the effects of Tax-directed siRNA on HTLV-1 induced tumors (50). However, since the growth of HTLV-1 tumors could be monitored in only immune-deficient nude rats in this model system, better animal models are still necessary.

HTLV-1 replicates poorly in rats, which may be one of the reasons why previously established models could not completely reproduce the features of HTLV-1 related diseases. We have previously examined the differences in the pattern of viral gene expression between human and rat T cells infected with HTLV-1. In rat cells, the levels of viral mRNAs encoding the Gag and Env proteins were much lower than those encoding the Tax and Rex proteins (36). Rex plays an important role in escorting unspliced and incompletely spliced viral mRNAs to the cytoplasm, resulting in enhanced synthesis of viral structural proteins (5, 34, 69). Human CRM1 (hCRM1) is a critical factor for Rex-dependent viral mRNA export to the cytoplasm and rat CRM1 (rCRM1) cannot substitute for this function (19, 22, 69). Thus, it is reasonable to assume that transgenic (Tg) rats carrying the hCRM1 gene should provide a better environment for HTLV-1 replication and that such animals would provide a better animal model of HTLV-1 infection.

CRM1 is involved in numerous cellular activities, suggesting its essential function in viability, which is supported by the high conservation of CRM1 genes from yeast to humans (37) and by the demonstration that both yeast and mammalian cells defective for CRM1 are inviable (1, 15). In contrast, overexpression of CRM1 has been reported to inhibit early embryogenesis in Xenopus (8). Therefore, proper expression of hCRM1 in rats will be essential to produce Tg rats. However, the regulation of CRM1 expression and synthesis has not yet been investigated in detail. Some immortalized cell lines have been reported to maintain CRM1 protein at constant levels throughout the cell cycle, which is compatible with an essential function (37), but other reports have indicated variations in the level of expression of CRM1 among different tissues (28, 37), implying that the expression is regulated. Therefore, we first investigated the expression profile of the CRM1 gene, especially during lymphocyte activation, to determine means for the proper expression of hCRM1 as a transgene. Our results indicate that expression of the CRM1 gene is elaborately regulated during activation of lymphocytes, including $CD4^+$ T cells, the major targets of HTLV-1. These data suggested that it would be necessary to use a construct harboring the entire regulatory and coding regions of CRM1 for Tg rat construction.

Using an artificial bacterial chromosome (BAC) clone containing the entire CRM1 gene, we have established hCRM1 Tg rats and examined the proliferation of HTLV-1 in vitro and in vivo. Our results demonstrate that T cell lines isolated from hCRM1 Tg rats produced 100 to 10,000 times more HTLV-1 Gag antigen compared to T cells from wild type control rats and that Tg rats displayed a more extensive invasion of HTLV-1 into the thymus when infected intraperitoneally. These results indicate the essential role of hCRM1 in proper HTLV-1 replication and suggest the importance of this Tg rat model as a basis for the development of better HTLV-1 animal models.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll-Hypaque (Pharmacia) or Ficoll Paque Plus (Amersham Biotechnology) density centrifugation. $CD4^+$ T lymphocytes were purified by negative selection using an immunomagnetic cell sorting apparatus, the MidiMACS cell separator (Miltenyi Biotec), using a cocktail of MACS MicroBeads coupled to hapten-conjugated MAbs specific for CD8, CD11b, CD16, CD19, CD36, and CD56. The purity of $CD4^+$ T cells was evaluated by flow cytometry (FACSCalibur: BECTON DICKINSON) to be approximately 95% pure.

For activation, cells were cultured with various combinations of 50 nM phorbol 12-myristate 13-acetate (PMA), 100 nM ionomycin, and 10 ng/ml IL-2.

The HTLV-1 producing rat and human T cell lines, FPM1 and MT-2, have been described previously (36, 44). HTLV-1-immortalized cell lines from wild type (Wt) or Tg rats were established by cocultivating thymocytes or splenocytes with MT-2, which had been treated with mitomycin C (50 μg/ml) for 30 min at 37°C. These cells were maintained in the medium supplemented with 10 U/ml of interleukin (IL)-2 (PEPROTECH EC) at the beginning of co-culture. Some cell lines were eventually freed from exogenous IL-2.

Western blotting. Cells were lysed in ice-cold extraction buffer (10 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 0.5% NP-40) containing protease inhibitor cocktail (Complete mini; Roche Diagnostics). The protein concentration of each sample was determined using a protein assay kit (QB PERBIO; Pierce). The cell lysates were sonicated, or in some cases treated with DNase 1 solution (Takara), then dissolved in sample buffer. The same amounts (approximately 20 μg) of cell lysates were subjected to SDS-PAGE. Following electrophoresis. Proteins were transferred to a nitrocellulose membrane and probed with anti-human or rat CRM1(34), anti-β-actin (AC40; Sigma), or anti-Rex (34) antibodies followed by secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase. Proteins were visualized by staining with BCIP/NBT, or ECL+ (Amersham Pharmacia Biotech) followed by the LAS-100 plus system (Fuji film) and evaluated by Image Gauge Version 3.4 software (Fuji film).

hCRM1 mRNA quantitative RT-PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase I (Qiagen) to minimize contamination of chromosomal DNA. The RNA concentration was measured by absorbance at 260 nm, and purity was ascertained by the OD 260/280 ratio and gel electrophoresis.

To quantify CRM1 mRNA, RNA samples (5 μg) were subjected to quantitative RT-PCR with the Platinum Quantitative RT-PCR Thermoscript One Step System (Invitrogen) using the forward primer 5’- GCT GAA AAC TCA ACC GAG ATG G -3’ and the reverse primer 5’- CTG TTG CTC TTG CTG ATG CTG TA -3’, and a probe (FAM-) AAA ATG CCG CAG GCA TTT CGT TCA G (-TAMRA). RT-PCR was performed by incubating for 2 min at 50 °C, 30 min at 60 °C, 10 min at 95 °C, and then 50 cycles of 20 s at 95 °C and 1 min at 62 °C in an Applied Biosystems Prism 7700 Sequence Detector Thermocycler (Applied Biosystems) with Sequence Detector software (Applied Biosystems). To make standard curves, the region from -943 to +38 bp of the CRM1 cDNA was amplified by PCR using Human Lung Marathon Ready cDNA (Clontech) with adaptor primer-1 and 5’-GCTGCATGGTCTGCTAACATT-3’ and by nested PCR with adaptor primer-2 and 5’-CTGCATGGTCTGCTAACATTG-3’. The PCR product was cloned into the vector pCR 2.1 (Invitrogen) and a 981-base single stranded RNA was synthesized *in vitro* with MegaScript T7 (Ambion).

Establishment of human CRM1 transgenic rats. pBeloBAC hCRM1, which harbors the entire human CRM1 genomic sequence including approximately 50 kb of 5’ upstream sequence and 10 kb of 3’ downstream sequence, was microinjected into 450 fertilized one-cell eggs prepared from Fischer 344/Du Crj (F344) female rats by the YS institute. Integration of the transgene was confirmed by PCR using genomic DNA, which was extracted by the PUREGENE^{TM} tissue kit (Gentra) from the rat tail, as a template with the hCRM1 specific primer pairs: 5’-TTATGTGGCTGCAGTGTGGA-3’ and 5’-ACATACCAGGGTTCTCTGGA-3’, and 5’-GTCACCTGATGTCGGGAGTT-3’ and 5’-GGATTACAGGTGTGAGCCA-3. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, Institute for Genetic Medicine, Hokkaido University.

Detection of genomic copies of hCRM1 and G3PDH. Genomic DNA was subjected to PCR with the following primer pairs: hCRM1, forward primer (5’-TGA GGT CAG GAG TTC AGG AT-3’) and reverse primer (5’-CTC TGC CTC CTG GGT TCA A-3’); G3PDH, forward primer (5’-AGA GCT GAA CGG GAA G-3’) and reverse primer (5’-GGA AGA ATG GGA GTT GC-3’). The PCR conditions were: 5min at 94 °C, 10 cycles of 30 s at 94 °C, 60 s at 69 °C, with a decrease of 0.5 °C/cycle, and 30 s at 72 °C, followed by 8 cycles of 30 s at 94 °C, 60 s at 65 °C, and 30 s at 72 °C and a final extension for 10 min at 72 °C.

Quantification of HTLV-1 proviral load by LightCycler-based real-time PCR. The HTLV-1 proviral loads of HTLV-1-infected cells were quantified by real-time PCR on a LightCycler PCR Instrument (Roche Diagnostics). Briefly, 20 µl of a PCR mixture in a capillary tube containing each HTLV-1 pX-specific inner primer pair at 0.4 µM, 1x LightCycler-FastStart SYBR Green PCR Master Mix, and 30 ng of genomic DNA was subjected to 40 cycles of denaturation (95 °C 15 s), annealing (69 °C, 10 s), and extension (72 °C, 10 s) following an initial Taq polymerase activation step (95 °C, 15 min). The copy numbers of HTLV-1 provirus in the samples were estimated from a standard regression curve using the LightCycler Software version 3 (Roche Diagnostics). The standard curve for HTLV-1 provirus was obtained by PCR data using $1x10^2$ to $1x10^8$ copies of pCR-pX1-4 plasmids, which were constructed by inserting a PCR fragment amplified with pX1 (5'-CCC ACT TCC CAG GGT TTG GAC AGA GTC TTC-3') and pX4 (5'-GGG GAA GGA GGG GAG TCG AGG GAT AAG GAA-3') from the genomic DNA of MT-2 cells into pCR2.1.

The copy numbers of HTLV-1 provirus were normalized by dividing with those of the G3PDH gene in the same samples.

Detection of HTLV-1 p19. Each cell line (10^5/well) was cultured in 24-well flat-bottom plates for 4 days. The amount of HTLV-1 p19 protein in the culture supernatant or in rat plasma was quantified using HTLV-1/2 p19 antigen ELISA (ZeptoMetrix).

Detection of intracellular Tax and Gag protein. Cells (10^6) were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) containing 20 μg/ml of lysolecithin (Sigma) for 2 min at room temperature, centrifuged, and resuspended in cold methanol. The cells were then sorted at 4 °C for 15 min, centrifuged, and incubated in 0.1% NP40 in PBS at 4 °C for 5 min. After centrifugation, the cells were stained with mouse anti-Tax MAb LT-4 (63) or the mouse anti-Gag MAb GIN-7 (38) followed by FITC-conjugated goat anti-mouse IgG plus M antibody (Immunotech). Finally, the cells were washed and fixed with 1% formalin in PBS prior to analysis by cell sorting.

Inoculation of HTLV-1 into rats. Various numbers of mitomycin C-treated or untreated MT-2 cells were intraperitoneally administered to 3 to 6 week old Wt or hCRM1-Tg rats. Peripheral blood samples were collected from the rats every 2 or 4 weeks after inoculation and the presence of HTLV-1 provirus in peripheral blood cells and levels of p19 in plasma were determined. In some experiments, rats were euthanized 1 week after inoculation and samples were collected to assess plasma p19 concentrations, proviral loads, and the presence of HTLV-1 provirus.

Detection of provirus in HTLV-1-infected rats. To determine the positive rate of HTLV-1 provirus in various organs, 200 μg of genomic DNA was subjected to PCR for the amplification of the px region of HTLV-1 as described previously (51). The first-step PCR was performed with the primer pair of pX1 and pX4, followed by the second-step PCR with the primer pair of pX2 (5’-CGGATACCCAGTCTACGTGTTTGGAGACTGT-3’) and pX3 (5’-GAGCCGATAACGCGTCCATCGATGGGGTCC-3’). The PCR conditions were: activation of Taq polymerase (94 °C, 3 min); 35 cycles of denaturation (94 °C, 30 sec), annealing (60 °C, 30 sec), and extension (72 °C, 30 sec), and a final elongation of the product

(72 °C, 3 min). For a nested PCR, an aliquot of the first PCR product was subjected to another 35 PCR cycles with the second set of primers.

RESULTS

Regulated expression of CRM1 in lymphocytes. We first examined the level of expression of CRM1 mRNA in human tissues by PCR using cDNA derived from the tissues. Expression of CRM1 mRNA was variable in different tissues. Notably, CRM1 mRNA was expressed at very low levels in PBMCs (data not shown). This result was unexpected because PBMCs include CD4$^+$ T cells, which are the targets of HIV and HTLV-1 (14). Lymphocytes in the PBMC population are mainly in a resting state, leading us to hypothesize that the production of CRM1 is stimulated during lymphocyte activation. Consequently, activated hematopoietic cells should contain CRM1 protein at levels similar to those observed in lymphocyte derived cell lines. We prepared CD4$^+$ T helper cells, macrophages, and DCs from PBMCs, cultured them in the presence of appropriate cytokines, and compared the amount of CRM1 present in these cells with amounts found in Jurkat cells, a transformed cell line that constitutively expresses CRM1. Western blotting indicated that all activated lymphocyte subsets and monocyte-lineage cells expressed CRM1 at levels similar to those in Jurkat cells (data not shown). These results indicate that lymphocyte activation induces high levels of CRM1 expression.

To demonstrate that CRM1 is induced during lymphocyte activation, we stimulated freshly isolated PBMCs with calcium ionophore, PMA, and IL-2, and examined CRM1 levels at several times by Western blotting (Figure 1A). The level of CRM1 in resting PBMCs was very low. The level of CRM1 clearly increased 4 h after stimulation, and then gradually increased further, up to 72 h, although some differences were observed between donors 1 and 2. Little change in the level of CRM1 was observed in the absence of stimulation. Actin was used as a loading control, as its level remained relatively constant. These results indicate that the CRM1 gene belongs to the class of early response genes that are induced during lymphocyte activation.

We next measured the levels of CRM1 mRNA by quantitative RT-PCR to determine how the expression of CRM1 is stimulated in PBMCs (Figure 1B). The amount of CRM1 transcript did increase, but the expression profile varied among individuals. For example, the level of CRM1 mRNA observed in donor 3 was relatively constant up to 24 h after stimulation and then started to increase, while the level of CRM1 mRNA in donor 1 gradually increased over the course of activation. Nevertheless, we consistently found in 4 experiments that the increase in CRM1 mRNA occurred after the increase in CRM1 protein. Specifically, up to 4 h after stimulation, marked increases in the level of CRM1 protein were detected, in contrast to nearly constant levels of CRM1 mRNA. Therefore, these results suggest that during lymphocyte activation CRM1 production is initially stimulated post-transcriptionally and then further enhanced by upregulating transcription.

In order to identify the signaling pathway responsible for the induction of CRM1 transcription, we activated PBMCs in the presence of various combinations of IL-2, calcium ionophore, and PMA. As shown in Figure 1C, IL-2 and PMA fully induced CRM1 whereas IL-2 and calcium ionophore did not. Next, we examined whether PMA alone is sufficient to induce CRM1. PMA alone enhanced CRM1 production as efficiently as IL-2 plus PMA. Since PMA is an activator of protein kinase C (PKC) (49), these data suggest that induction of CRM1 is PKC dependent.

To confirm the above results, we examined the effect of various inhibitors including staurosporine (a PKC inhibitor) (60) and cyclosporin (a Ca^{++} cascade inhibitor) (66). As shown in Figure 1D, staurosporine, but not cyclosporin, inhibited the induction of CRM1, consistent with the results shown in Figure 1C. We further examined the effects of PDTC (an NFkB inhibitor) (43) and PD98059 (a MAPKK inhibitor) (3) and found that PDTC inhibited CRM1 induction at the highest dose, but PD98059 had only a minor effect.

Regulated expression of CRM1 in $CD4^+$ T lymphocytes. To examine CRM1 regulation in $CD4^+$ T lymphocytes, resting $CD4^+$ T lymphocytes were purified by negative selection and activated by treatment with a combination of IL-2, ionophore, and PMA. CRM1 levels were estimated by Western blotting (Figure. 2A). CRM1 expression was induced by the same stimuli as in PBMCs, although the kinetics of induction was somewhat different among donors. In contrast to CRM1, the level of actin was constant during T cell activation. Staurosporine inhibited the enhanced production of CRM1 (data not shown), indicating the involvement of PKC in the induction of CRM1 in $CD4^+$ T cells.

To examine the mechanism underlying the stimulation of CRM1 in $CD4^+$ T cells, we measured the amount of CRM1 mRNA by quantitative RT-PCR (Figure 2B). Similar to PBMCs, the amount of CRM1 mRNA also increased during $CD4^+$ T cell activation. Although the levels of CRM1 mRNA during T cell activation varied to some extent among donors, similar profiles of induction were observed; after a lag of approximately 4 h, the level of CRM1 mRNA started to increase and continued to do so for up to 24 h after stimulation. These results suggest that the increase in CRM1 mRNA is delayed compared to the increase in CRM1 protein, as seen in PBMCs. The level of CRM1 mRNA was constant at times greater than 24 h post-stimulation, but purified $CD4^+$ T cells appeared unhealthy 2 and 3 days after stimulation in these cultures, as judged by microscopic observation. Therefore, further examination is required to definitively determine the levels of CRM1 protein and mRNA in $CD4^+$ T cells at later times after stimulation.

Expression of hCRM1 in the Tg rat. The above results indicate that regulation of CRM1 expression during the activation of lymphocytes is complex. Considering the lack of characterization of CRM1 regulatory elements, we used a BAC clone, which is likely to harbor the entire regulatory and coding regions of the CRM1 gene, to establish an hCRM1-Tg rat. One rat strain carrying the hCRM1 transgene was obtained from microinjection of the hCRM1 containing BAC clone into 450 fertilized one-cell eggs from F344 female rats. We assessed the expression of hCRM1 protein in each tissue by immunoblotting using hCRM1 specific antibody (22). As shown in Figure 3A, hCRM1 expression was detected in all organs tested. The expression level of this protein was especially high in ovary and thymus compared to other organs. In addition, expression levels of hCRM1 in the organs were similar to those of endogenous rCRM1 (Figure 3B). hCRM1 expression was not detected in any organs prepared from wild type rats (data not shown). These data indicate that the Tg rats express hCRM1 in a physiologically relevant manner.

Enhanced production of p19 Gag in Tg-derived cell lines. To assess the replication of HTLV-1 in T cells of hCRM1 Tg rats, we established several T cell lines from both Wt and Tg rats by infecting with HTLV-1. Thymocytes and splenocytes isolated from Wt or hCRM1-Tg rats were co-cultured with the HTLV-1-infected human T cell line MT2, which had been treated with mitomycin C and then maintained in culture medium containing 10 U/ml of IL-2. After 2 months of cultivation, we obtained 6 lines from Wt rats and 11 from Tg rats (Table 1). As shown in Figure 4, all of the Tg-derived cell lines were confirmed to have the hCRM1 gene (Figure 4A) and express hCRM1 (Figure 4B), whereas none of Wt-derived lines contained the gene or the protein. The expression level of hCRM1 was different among the cell lines.

We next examined the expression of cell surface markers, including CD3, CD4, CD5, CD8, CD25, MHC-I, and MHC-II, in these cell lines (Table 1). All the cell lines expressed rat CD25 and MHC-I, indicating that they were derived from rat cells, not from the human MT2 cells. Most of the cell lines also expressed rat CD5 and MHC-II, with the exception of 2 Wt and 3 Tg-derived lines. Expression of rat CD3 was confirmed in 6 of 9 Tg lines, whereas only 2 of 6 lines were positive in the Wt lines. Rat CD4 expression was detected in 1 Wt and 6 Tg cell lines. Rat CD8 was detected in 1 Wt and 1 Tg-derived line. As judged by the expression of CD3, we established a total of 8 T cell lines, 2 from Wt and 6 from Tg rats.

We next examined the production of the p19 Gag protein in the cell lines to assess the effect of hCRM1 on HTLV-1 replication. Our results demonstrated that the Tg-derived cell lines produced much greater levels of p19 in the culture supernatant, compared to the Wt-derived cells (Figure 4C). After 2 and 4 days in culture, the mean p19 production by 9 Tg-derived cell lines was 1000 ± 10 and 10000 ± 100 times higher, respectively, than the mean production of the 6 Wt-derived lines (Figure 4D). The amounts (1-60 ng/ml) of p19 released from the Tg-derived cell lines are equivalent to human HTLV-1 producing T cell lines, such as MT2 and MT4 (data not shown). These results clearly demonstrate the enhanced production of the HTLV-1 Gag protein in the cells expressing hCRM1.

To further examine the increased p19 production in each cell line expressing hCRM1, we conducted a FACS analysis to detect the intracellular Gag protein. As shown in Figure 4E, we were able to detect p19 and the precursor p55 Gag protein in all cell lines derived from Tg rats. In contrast, no Wt-derived cell lines produced detectable amounts of Gag. These results further support the role of hCRM1 in the enhancement of HTLV-1 Gag production.

We also assessed the proliferation of each cell line to exclude the possibility that the enhanced production was not due to increased production by individual cells, but was the result of increases in the number of cells in the Tg-derived lines. As shown in Figure 4F, we confirmed that there was no difference in the proliferation rate between Wt- and Tg-derived cell lines after 2 or 4 days in culture. In addition, there was no correlation between the rate of cell growth and the amount of p19 in the culture in any cell line.

The state of HTLV-1 infection is not correlated with levels of p19 production. We also assessed the proviral load of each cell line to rule out the possibility that enhanced production of Gag was due to increased provirus numbers in Tg cell lines. Real-time PCR analysis using a pair of primers for the Tax gene was performed to quantify the number of integrated provirus. As a relative standard, we used genomic DNA from FPM1 cells, which contain 3 copies of HTLV-1 provirus per cell (36). As shown in Figure 5A, all 5 Wt cell lines contained more than 2 copies of the provirus, whereas most of the Tg lines appeared to have only one provirus per cell, with the exception of FCCT13-1 cells which possessed 4 copies. Thus, there was no correlation between the provirus number and p19 production, indicating that differences in the amount of provirus were not responsible for the enhanced Gag production in Tg derived cells.

Altered expression of Tax and Rex could also be associated with enhanced expression of Gag in Tg-derived cells. Thus, we investigated the expression of Tax in the cell lines. As shown in Figure 5B, FACS analysis revealed that all of the cell lines tested expressed detectable levels of Tax proteins. Although we observed variations in the levels of Tax expression among the cell lines, there was no significant difference in the expression between Wt- and Tg-derived lines.

We next examined Rex expression by immunoblotting. As shown in Figure 5C, the Rex protein was expressed in all cell lines tested. Again, there was no statistical difference in the protein expression between Wt and Tg cells. Two Tg cell lines, FCMS1 and FCMS18, expressed p21 protein as well as the p27 Rex. This expression was not associated with elevated expression of Gag, since the amounts of p19 Gag produced by these two cell lines were similar to the other Tg-derived cell lines (Figure 4C, D). These results indicate that the number of integrated provirus and the expression levels of Tax and Rex are not correlated with the enhanced expression of Gag observed in cell lines derived from hCRM1-Tg rats.

Enhanced Dissemination of HTLV-1 in hCRM1 Tg rats. We next examined the proliferation of HTLV-1 in Tg rats by inoculating animals with the HTLV-1 producing human T cell line MT2 as a virus source. Analysis of plasma p19 concentration in the infected rats over time did not show significant differences between Tg and Wt rats, although the p19 concentration in Tg rats tended to be higher during the first 6 weeks after infection (Figure 6A). Figure 6B shows the mean plasma p19 concentration in rats after 1 week of infection and again demonstrates higher, but not significantly different, levels of the viral protein in Tg-derived samples. To evaluate dissemination of the virus in vivo, we determined the presence of HTLV-1 provirus DNA in various organs 1 week after intraperitoneal infection by nested PCR that specifically amplifies a part of the px region. We calculated the percentage of rats that sustained the px gene in 5 independent experiments, and found that the rate with which the virus disseminated to the thymus in Tg rats was significantly higher than that in Wt rats (Figure 6C). However, we have not detected notable differences between the two groups in HTLV-1 proviral load detected in various organs including peripheral blood cells and thymus (Figure 6D, E, and data not shown). These results indicate the limited effects of hCRM1 in the proliferation of HTLV-1 in vivo, which are in dramatic contrast to the significant enhancement of HTLV-1 production in Tg derived cells in vitro.

DISCUSSION

Unlike hCRM1, rCRM1 does not support Rex function due to its inability to induce Rex-Rex dimerization, which is required for RNA export from the nucleus to the cytoplasm (22). This may be one reason why HTLV-1 replicates poorly in rats compared to humans. This observation suggests the hCRM1-Tg rats would be novel animal models, since they would support better replication of HTLV-1.

The essential role of CRM1 in cell viability suggested that proper expression of the transgene would be a key for successful construction of Tg rats. Therefore, we examined the expression pattern of CRM1 and found that CRM1 is expressed in a manner similar to the early response genes induced during the activation of lymphocytes, including $CD4^+$ T cells. Our results suggest that expression of CRM1 is stimulated in two steps: in the first phase, lasting approximately 4 h, induction is regulated primarily in a post-transcriptional manner, and in the second phase, transcriptional augmentation takes place. Alternatively, CRM1 protein in PBMCs may be rapidly turned over and is then protected from degradation upon stimulation, giving rise to the early increase in protein levels. The profile of CRM1 expression further suggests that the initial induction occurs in the G_1 phase of the cell cycle, which is also supported by the observation that mimosine, which blocks the cell cycle in late G_1 (65), does not prevent the induction (data not shown).

The elaborate regulation of CRM1 expression led us to use a BAC clone harboring the entire hCRM1 gene for Tg rat construction. An initial unsuccessful trial using the mouse H2 promoter to express hCRM1 cDNA supports the necessity of using the hCRM1 BAC. Our results indicate that the hCRM1 BAC Tg rats express hCRM1 in various organs including thymus and spleen, in a manner similar to endogenous rCRM1 in rats. Moreover, the distribution of hCRM1 in the Tg rats is similar to that observed in humans (28, 37). Therefore, use of the hCRM1 BAC construct may have resulted in physiological expression of the protein in Tg rats. We also demonstrated hCRM1 expression in all Tg derived cell lines, which will be useful for the functional analysis of hCRM1 in HTLV-1-infected cells.

We have previously reported that expression of hCRM1 induced an increase in HTLV-1 Gag production in both rat epithelial and T cells (21, 69). Our present study also showed that T cell lines established from hCRM1-Tg rats produced significantly greater amounts of p19 than cell lines established from Wt rats, further indicating the positive effect of hCRM1 on viral protein synthesis. This effect was not due to the effects of Tax or Rex proteins, which enhance the transcription of total viral mRNAs and the nuclear export of unspliced and incompletely spliced mRNAs, respectively (12, 26, 30, 68), since the expression levels of these proteins were not significantly different between Tg and Wt cell lines. Additionally, these results indicate that induction of hCRM1 expression does not affect the expression of HTLV-1 regulatory proteins in virus-infected rat cells. We also observed variation in the levels of p19 production among the cell lines derived from hCRM1-Tg rats. Since the amount of p19 did not correlate with the expression levels of hCRM1, Tax, or Rex, the reason for the variation is not clear. Some other factors, including RanGTP and RanBP3, which play important roles in the nuclear export of CRM1-substrate complexes (14, 41, 47, 59), may affect the levels of p19 production in the rat cell lines. It is also possible that the integration sites of the provirus influence virus production. Further studies are required to identify the factors that result in varying p19 production among Tg-derived cell lines.

Variation was also observed in the expression of cell surface proteins among the established cell lines (Table 1). It is especially interesting that most of the wt-derived cells do not express CD3 or CD4, whereas the majority of the Tg-derived lines possess both of these molecules. Since others and we have established a number of CD4 positive cells from various strains of wt rats (31, 36), the present results may be due to the experimental disparity. However, it is possible that enhanced HTLV-1 production by the hCRM1 expressing cells and subsequent dissemination of the virus in the culture may influence the phenotypes of the transformed cells. Thus, additional studies are required to determine the significance and the cause of the difference.

The Tg rats showed minimal effects on HTLV-1 replication in vivo. Since dramatic enhancement of HTLV-1 production was observed in all hCRM1 expressing cells in vitro, it is possible that the number of HTLV-1-infected cells in vivo was too low to detect differences in virus production between Wt and Tg rats. From this point of view, alteration of experimental condition to improve the initial infection rate of HTLV-1 may lead to the enhanced viral replication in the Tg rats. Repression of viral protein expression in vivo may also reduce the effects of hCRM1, masking the enhanced viral replication in the Tg rats. Such responses have been well documented in HTLV-1 infected individuals (32, 33). It is also possible that HTLV-1 specific immune responses could affect the replication of HTLV-1 in the Tg rats. Indeed. Our preliminary experiments indicated that induction of HTLV-1-specific CTL responses occurred as early as 1 week after virus infection. Alternatively, some other host factors may govern and modulate efficient HTLV-1 replication in vivo. Thus, further studies on both virological and immunological aspects are required to verify the importance of the Tg rats as an in vivo model of HTLV-1 infection.

The HTLV-1 Rex protein is able to functionally replace the Rev protein of HIV-1 (57). CRM1 is a nuclear export factor for HIV-1 Rev and a truncated Rev mutant with weakened binding affinity to CRM1 results in reduced levels of HIV-1 Gag production (20). These results raise the possibility that rat cells expressing hCRM1 protein can produce enhanced levels of HIV-1 structural proteins. Indeed, our preliminary results demonstrate that hCRM1 promotes HIV-1 p24 Gag production in rat cells (unpublished observation). Thus, the hCRM1-Tg rats generated in this study may be also useful as a small animal model of HIV-1 infection, when HIV-1 receptors are simultaneously expressed in these rats.

HIV latently infects reservoirs of resting T cells (7, 9, 10, 13, 61), which are thought to be in the G_0 state, and the virus is then reactivated during T cell activation. Alternatively, HIV has also been reported to propagate efficiently in non-replicating lymphatic T cells (18), which lack certain markers specific for activation. Since cytokines levels are high in lymphatic tissues, the progression of T cells from G_0 to G_1 may support HIV replication. Although release from cell cycle block has been extensively investigated at the transcriptional level, a recent study has shown that the synthesis of unspliced HIV Gag RNA increases rapidly during the HIV reactivation process, to a much greater extent than the synthesis of multiply spliced RNAs (7). Our results demonstrating a rapid increase in CRM1 expression during lymphocyte activation provide a clue to the underlying mechanism, the efficient action of the HIV Rev protein, which leads to robust synthesis of unspliced RNA. We suggest that HIV gene expression is regulated in lymphocytes at both the transcriptional and RNA export levels.

Independent of viral replication, the first phase of enhancement of CRM1 expression is also coincident with the induction of cytokines, such as IL-2 (4). CRM1 interacts with the ARE located in the 3' untranslated region of c-fos mRNA (via HuR and its ligands) and mediates export of this mRNA from the nucleus to the cytoplasm (6, 16). Therefore, CRM1 may transport cytokine mRNAs belonging to the early response genes since many cytokine mRNAs harbor ARE sequences (24, 56). Collectively, these observations suggest that enhancement of mRNA export via the induction of CRM1 expression, in addition to regulation at the transcriptional and translational levels, may play an important role in coordinating gene expression during lymphocyte activation. The existence of a post-transcriptional mechanism leading to a rapid increase in CRM1 protein is consistent with this hypothesis.

In conclusion, we have established a novel Tg rat carrying the hCRM1 gene via examining its gene expression, and isolated several HTLV-1-infected T cell lines expressing hCRM1. Our results demonstrate that T cells from hCRM1 Tg rats produced enhanced levels of the HTLV-1 Gag protein compared to T cells from Wt control rats. These results indicate the essential role of hCRM1 in proper HTLV-1 replication and suggest the importance of this Tg rat in the development of HTLV-1 animal models. These animals may also contribute to the development of models for other human retroviruses, such as HIV-1.

ACKNOWLEDGEMENTS

Buffy coats for the isolation of lymphocytes were the kind gift of the Hokkaido Red Cross Blood Center (Sapporo, Japan). We thank A. Hirano, N. Mizuno, K. Nakajima and J. Hioki for excellent technical assistance. This study was supported by grants from the Ministry of Sports and Culture (Japan), and the Ministry of Health and Welfare (Japan).

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FIGURE LEGENDS

Figure1. Expression of CRM1 during activation of PBMCs. (A) PBMCs isolated from donor 1 and donor 2 were activated with ionophore, PMA, and IL-2 and analyzed by Western blotting. (B) PBMCs isolated from donor 1 (□) and donor 3 (◇) were activated with ionophore, PMA, and IL-2 and analyzed by quantitative RT-PCR. Each value is the average of duplicate measurements. (C) PBMCs isolated from donor 2 were activated with various combinations of ionophore, PMA, and IL-2 and analyzed by Western blotting. (D) PBMCs isolated from donor 2 were activated in the presence of various inhibitors and analyzed by Western blotting.

Figure 2. Time course of CRM1 induction during activation of CD4$^+$ T cells. (A) CD4$^+$ T cells isolated from donor 1 and donor 4 were activated with ionophore, PMA, and IL-2 and analyzed by Western blotting. (B) Time course of CRM1 mRNA induction during activation of CD4$^+$ T cells. CD4$^+$ T cells isolated from donor 1 (□) and donor 4 (◇) were activated with ionophore, PMA, and IL-2 and analyzed by quantitative RT-PCR. Each value is the average of duplicate measurements.

Figure 3. Tissue distribution of human and rat CRM1 in hCRM1 Tg rats. (A) Immunoblot assays showing the relative levels of h and rCRM1 in rat tissues. Each protein level was determined on immunoblots containing 10 μg of total protein per lane. An FCMT18 cell extract was used as a positive control. (B) Relative levels of h/rCRM1 expression among organs are shown. Protein expression was quantified by ImageGauge software and relative values are normalized to the amount of actin.

Figure 4. Expression of HTLV-1 Gag and hCRM1 in the cell lines immortalized with HTLV-1. (A) Detection of the hCRM1 transgene in the cell lines by PCR. DNA extracted from each cell line (100 ng) was subjected to PCR with the primers for hCRM1 and for G3PDH as an

internal control. (B) Protein expression of hCRM1 was detected by immunoblotting. Samples (10 μg of total protein per each lane) were subjected to SDS-PAGE. HeLa cell extract was used as a positive control. (C) HTLV-1 Gag protein in the supernatant of 2 day and 4 day cultures was quantified by HTLV-1 p19 ELISA. The results are shown as the mean of three independent experiments. (D) Based on the data of (C), the average p19 Gag production of Tg and Wt cell lines was calculated. (E) The amount of intracellular Gag in each cell line was analyzed by flow cytometry. Open histograms indicate the cells stained with anti p19/p55 Gag MAbs. Solid histograms represent the cells stained with control mouse IgG. (F) Growth rate of Wt or Tg cell lines was measured. In parallel with the experiments described in Figure 4(C), the growth rate was monitored by the cell counting Kit-8 (Dojinndo Laboratories). The relative cell numbers of 2 or 4 day vs. 0 day cultures are shown.

Figure 5. Viral states in HTLV-1-transformed T cells derived from Tg and wild type rats. (A) Proviral load of each cell line was measured by quantitative real time PCR. The copy number of HTLV-1 provirus was normalized by dividing with the G3PDH copy number in the same sample. (B) Production of intracellular Tax in each cell line was analyzed by flow cytometry. Solid histograms indicate the cells stained with anti-Tax MAb. Open histograms represent the cells stained with control mouse IgG. (C) Rex expression of each cell line was detected by immunoblotting. Ten μg of total protein per each lane was subjected to SDS-PAGE. Lower bands in FCMS1 and TCMS18 samples represent p21 Rex.

Figure 6. Dissemination of HTLV-1 in hCRM1 Tg rats. (A) Mean plasma p19 concentration in Wt (n=9; ●) or hCRM1 (n=7; ■) Tg rats after intraperitoneal inoculation of mitomycin C-treated MT2 cells ($1x10^7$ per animal). (B) Mean plasma p19 concentration in Wt (n=16; ☐) or hCRM1-Tg (n=17; ■) rats 1 week after intraperitoneal inoculation of MT2 cells ($5x10^6$ per animal). (C) Detection of HTLV-1 provirus in thymus derived from rats used in (B). Presence of HTLV-1 provirus was analyzed by nested PCR. Results are mean percentage of HTLV-1 provirus positive rats in 5 independent experiments. (D and E) HTLV-1 proviral loads of rats used in (B). HTLV-1 proviral loads in peripheral blood cells (D) or thymus (E)

were quantified by real-time PCR. The relative copy numbers of HTLV-1 provirus per 2×10^7 copies of G3PDH are shown. Results are expressed as mean + standard deviation. The statistical significance of differences was determined with the Student's t-test, using Microsoft Excel 2004 for Mac software, as indicated in Figure 6B-E.

Table 1. Constructed cell lines and surface markers

Cell lines	hCRM1	Surface markers						
		CD3	CD4	CD5	CD8	CD25	MHC1	MHC2
FWT1	-	+	+	+	-	+	+	+
FWS1-11	-	-	-	+	-	+	+	+
FWS1-27	-	-	-	+	-	+	+	+
FWS1-31	-	-	-	+	(+)	+	+	-
FWS1-34	-	-	-	+	-	+	+	-
FWT11	-	+	-	+	-	+	+	+
FCMS1	+	+	+	+	-	+	+	(+)
FCMT1	+	+	+	+	-	+	+	+
FCMT18	+	+	+	-	-	+	+	-
FCMS18	+	+	+	(+)	-	+	+	(+)
FCMT27	+	+	+	(+)	-	+	+	(+)
FCCT13-1	+	-	-	(+)	-	+	+	+
FCCT13-2	+	+	-	(+)	(+)	+	+	+
FCCS13-1	+	-	-	-	-	+	+	+
FCCS13-2	+	-	(+)	(+)	-	+	+	-

+:positive (+):weakly positive -:negative

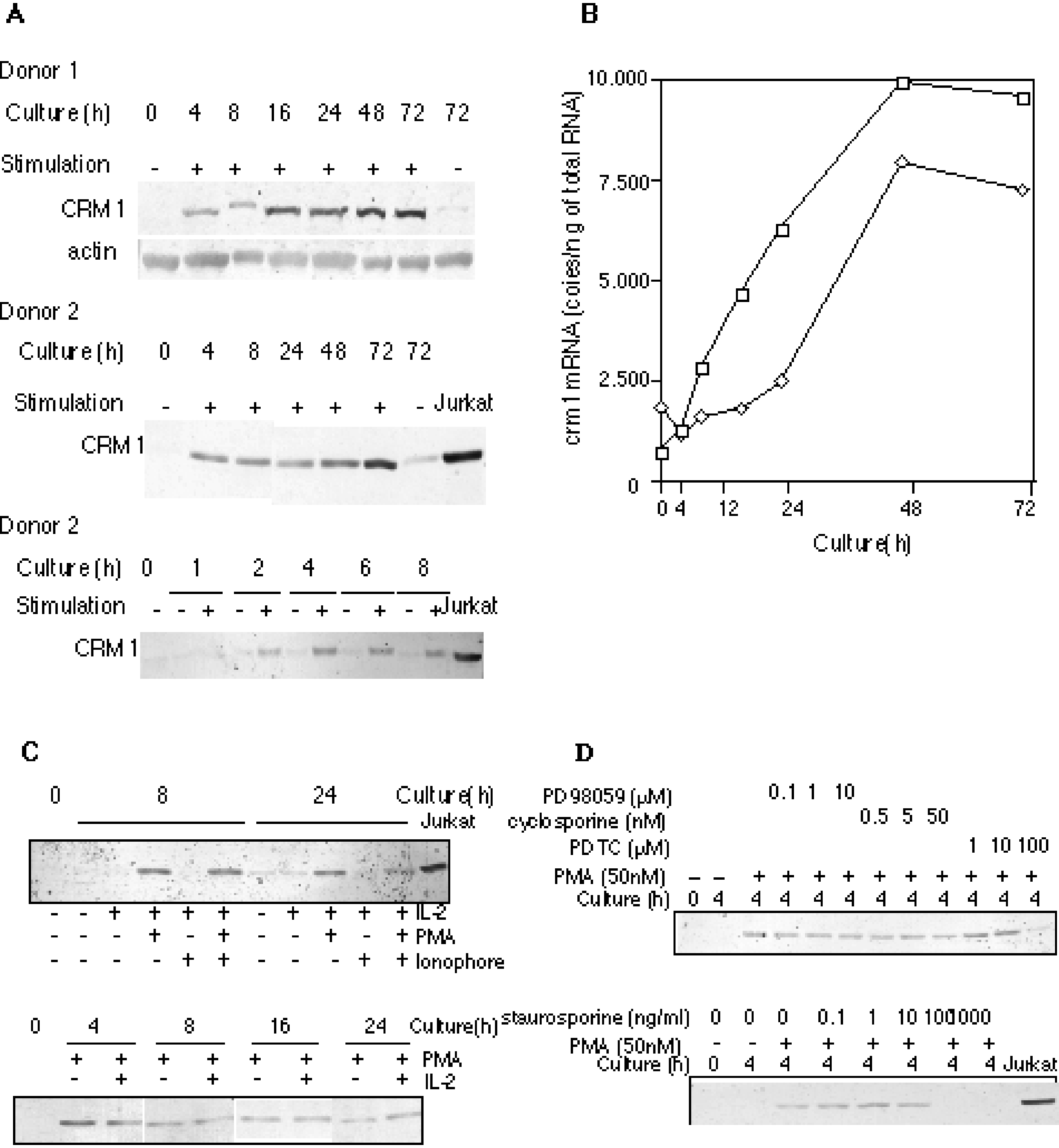
A
Donor 1
Culture (h) 0 4 8 16 24 48 72 72
Stimulation - + + + + + + -
CRM 1
actin
Donor 2
Culture (h) 0 4 8 24 48 72 72
Stimulation - + + + + + - Jurkat
CRM 1
Donor 2
Culture (h) 0 1 2 4 6 8
Stimulation - - + - + - + - + - + Jurkat
CRM 1
B
crm1 mRNA (coies/ng of total RNA)
10.000
7.500
5.000
2.500
0
0 4 12 24 48 72
Culture (h)
C
0 8 24 Culture (h)
Jurkat
- - + + + + - + + + + IL-2
- - - + - + - - + - + PMA
- - - - + + - - - + + Ionophore
0 4 8 16 24 Culture(h)
+ + + + + + + + PMA
- + - + - + - + IL-2
D
PD 98059 (µM) 0.1 1 10
cyclosporine (nM) 0.5 5 50
PDTC (µM) 1 10 100
PMA (50nM) - - + + + + + + + + + +
Culture (h) 0 4 4 4 4 4 4 4 4 4 4 4
staurosporine (ng/ml) 0 0 0 0.1 1 10 100 1000
PMA (50nM) - - + + + + + +
Culture (h) 0 4 4 4 4 4 4 4 Jurkat

Figure 1

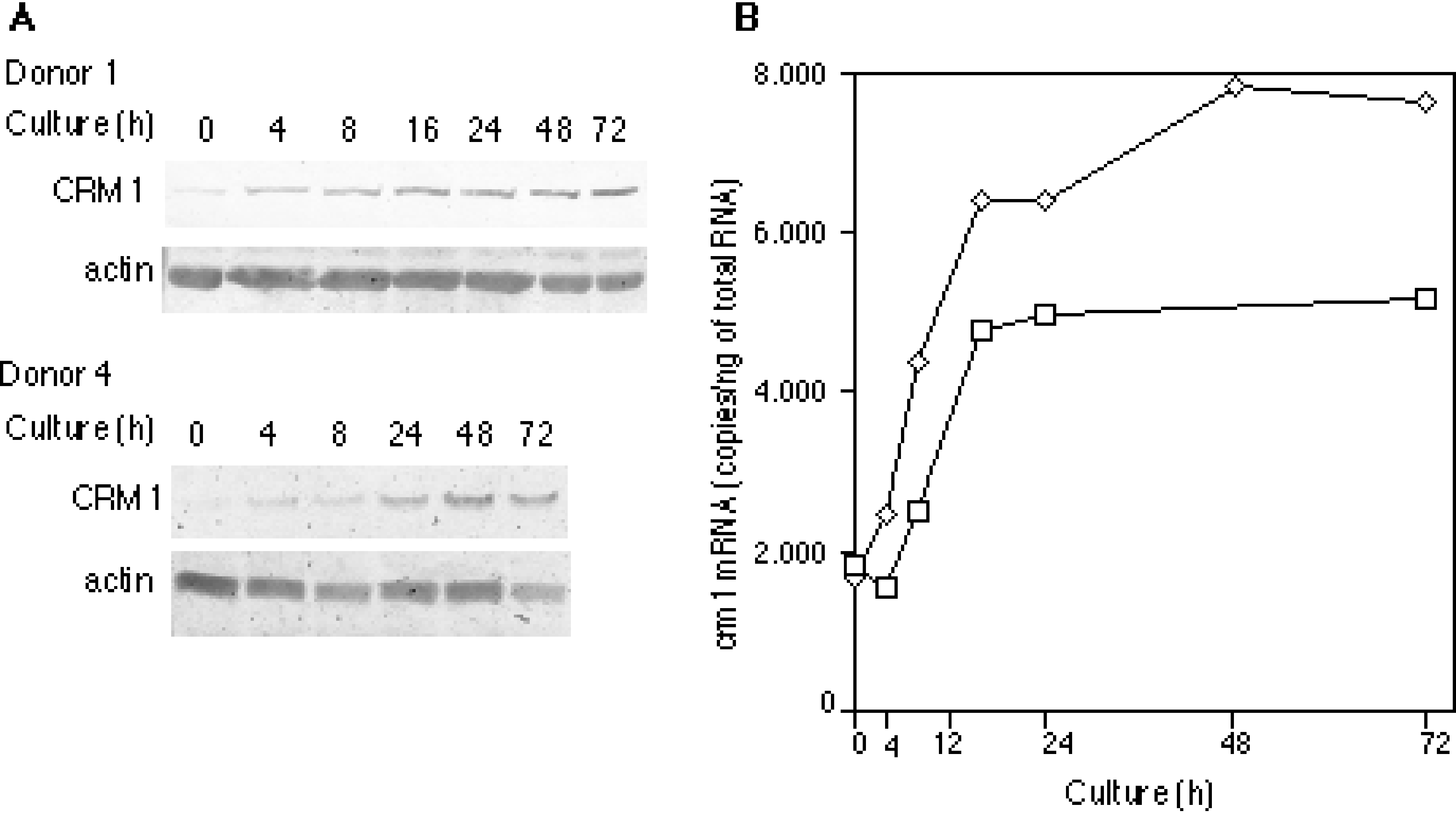
A
Donor 1
Culture (h) 0 4 8 16 24 48 72
CRM 1
actin
Donor 4
Culture (h) 0 4 8 24 48 72
CRM 1
actin
B
crm 1 mRNA (copies/ng of total RNA)
8.000
6.000
4.000
2.000
0
0
4
12
24
48
72
Culture (h)

Figure 2

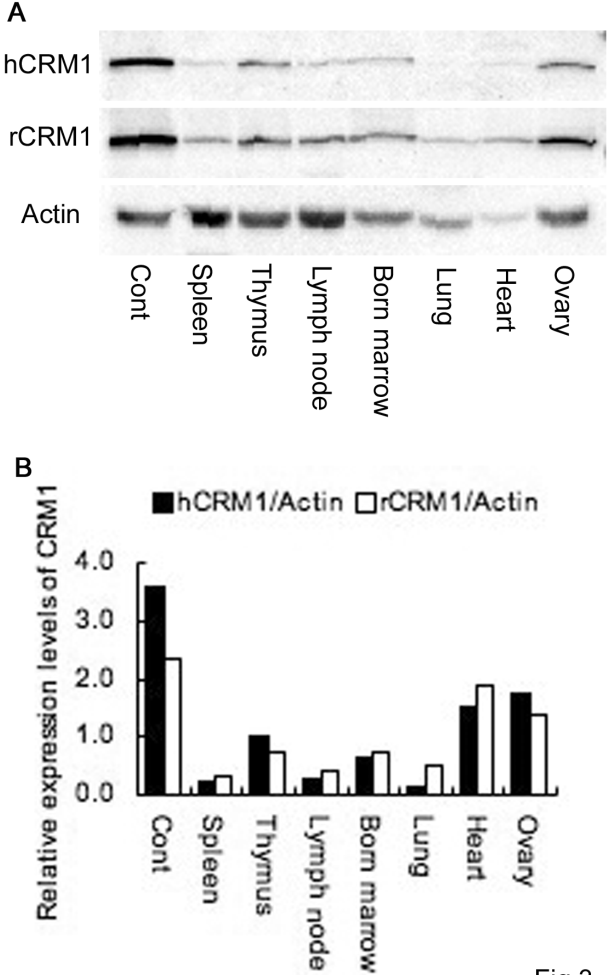
A
hCRM1
rCRM1
Actin
Cont
Spleen
Thymus
Lymph node
Born marrow
Lung
Heart
Ovary
B
hCRM1/Actin
rCRM1/Actin
Relative expression levels of CRM1
4.0
3.0
2.0
1.0
0.0
Cont
Spleen
Thymus
Lymph node
Born marrow
Lung
Heart
Ovary

Fig.3

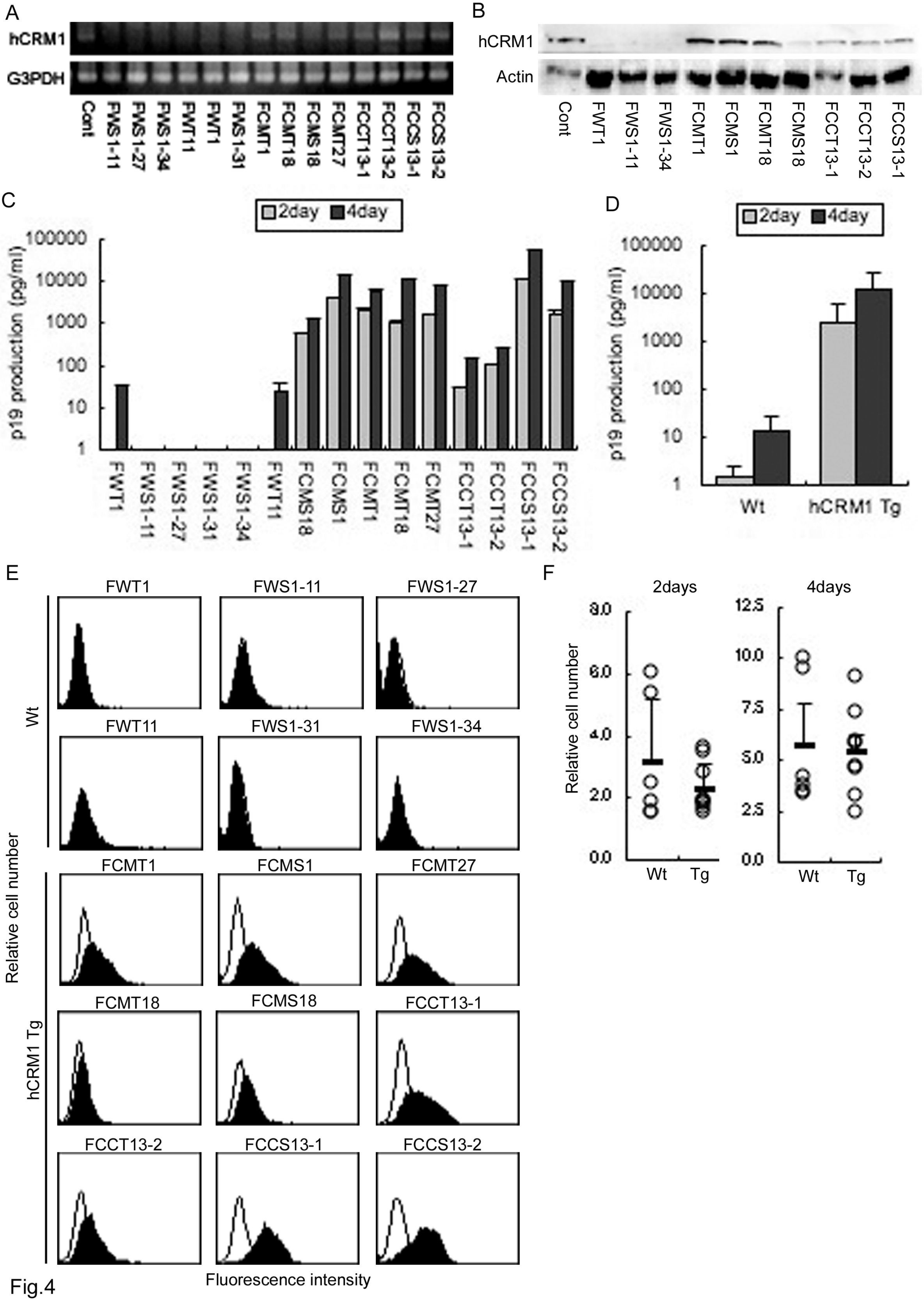
A
hCRM1
G3PDH
Cont
FWS1-11
FWS1-27
FWS1-34
FWT11
FWT1
FWS1-31
FCMT1
FCMT18
FCMS18
FCMT27
FCCT13-1
FCCT13-2
FCCS13-1
FCCS13-2
B
hCRM1
Actin
Cont
FWT1
FWS1-11
FWS1-34
FCMT1
FCMS1
FCMT18
FCMS18
FCCT13-1
FCCT13-2
FCCS13-1
C
2day
4day
p19 production (pg/ml)
100000
10000
1000
100
10
1
FWT1
FWS1-11
FWS1-27
FWS1-31
FWS1-34
FWT11
FCMS18
FCMS1
FCMT1
FCMT18
FCMT27
FCCT13-1
FCCT13-2
FCCS13-1
FCCS13-2
D
2day
4day
p19 production (pg/ml)
100000
10000
1000
100
10
1
Wt
hCRM1 Tg
E
FWT1
FWS1-11
FWS1-27
FWT11
FWS1-31
FWS1-34
FCMT1
FCMS1
FCMT27
FCMT18
FCMS18
FCCT13-1
FCCT13-2
FCCS13-1
FCCS13-2
Wt
hCRM1 Tg
Relative cell number
Fluorescence intensity
F
2days
4days
Relative cell number
8.0
6.0
4.0
2.0
0.0
12.5
10.0
7.5
5.0
2.5
0.0
Wt
Tg
Wt
Tg

Fig.4

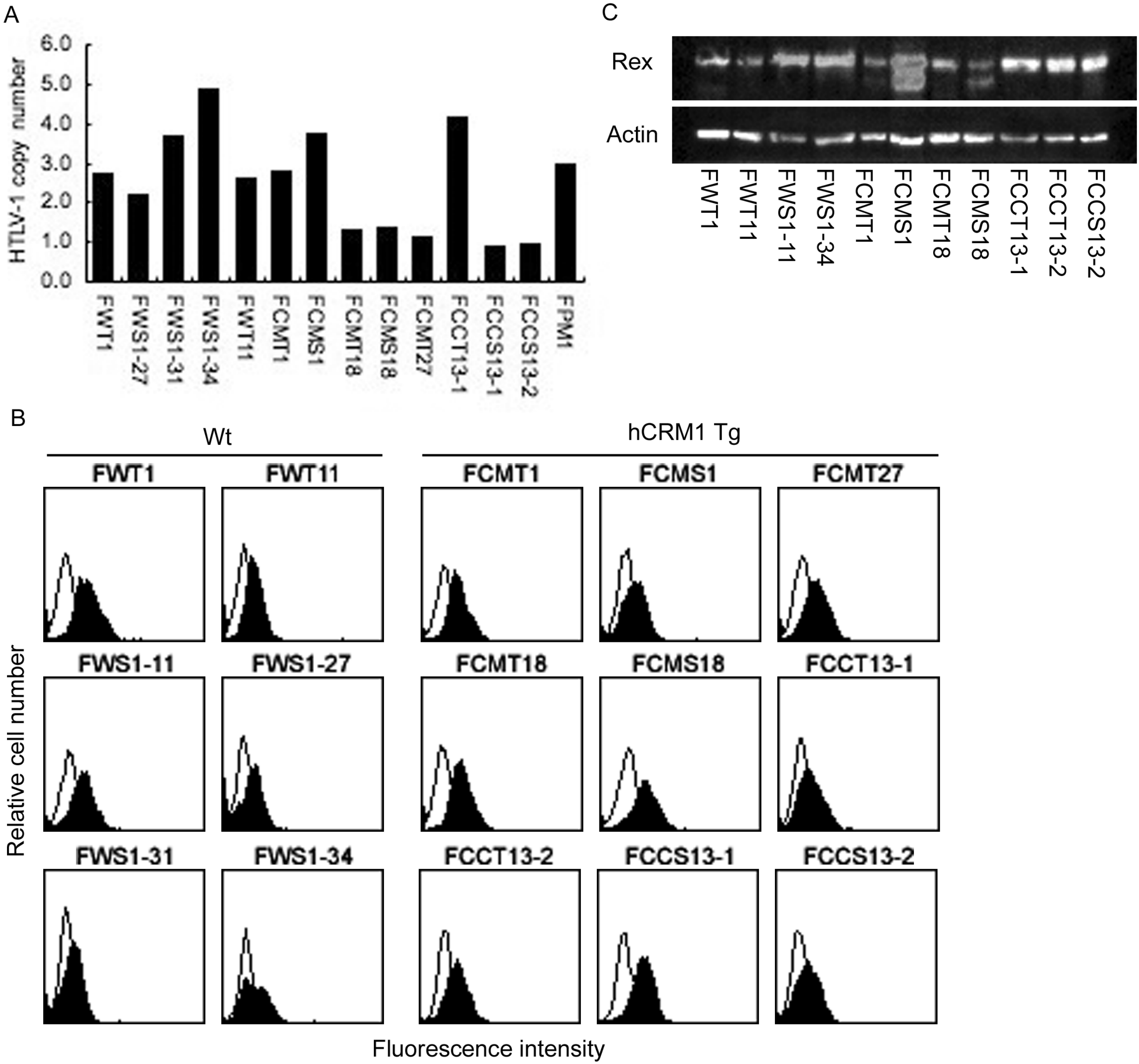
A
HTLV-1 copy number
6.0
5.0
4.0
3.0
2.0
1.0
0.0
FWT1
FWS1-27
FWS1-31
FWS1-34
FWT11
FCMT1
FCMS1
FCMT18
FCMS18
FCMT27
FCCT13-1
FCCS13-1
FCCS13-2
FPM1
B
Wt
hCRM1 Tg
FWT1
FWT11
FCMT1
FCMS1
FCMT27
FWS1-11
FWS1-27
FCMT18
FCMS18
FCCT13-1
FWS1-31
FWS1-34
FCCT13-2
FCCS13-1
FCCS13-2
Relative cell number
Fluorescence intensity
C
Rex
Actin
FWT1
FWT11
FWS1-11
FWS1-34
FCMT1
FCMS1
FCMT18
FCMS18
FCCT13-1
FCCT13-2
FCCS13-2

Fig.5

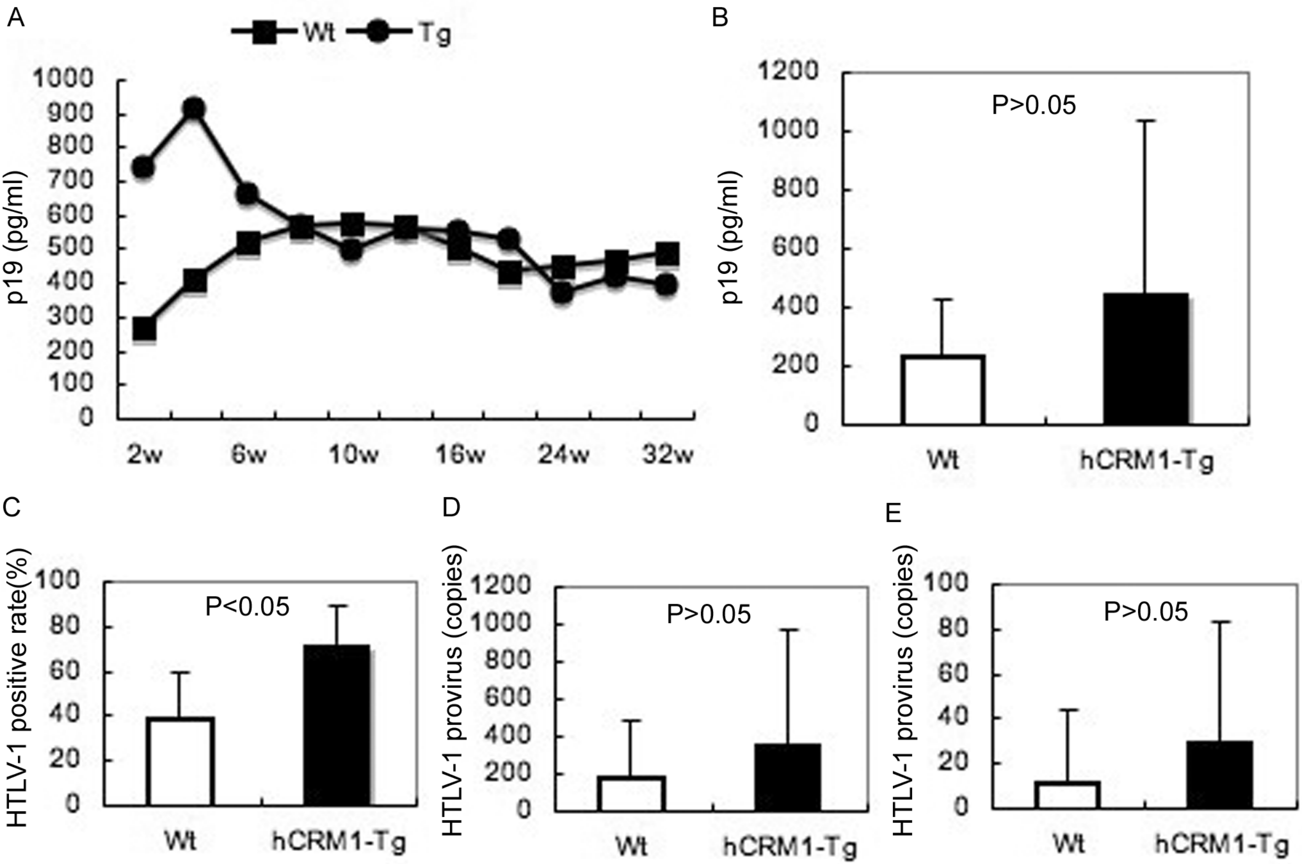
A
Wt
Tg
p19 (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
2w
6w
10w
16w
24w
32w
B
P>0.05
p19 (pg/ml)
1200
1000
800
600
400
200
0
Wt
hCRM1-Tg
C
HTLV-1 positive rate(%)
P<0.05
100
80
60
40
20
0
Wt
hCRM1-Tg
D
HTLV-1 provirus (copies)
P>0.05
1200
1000
800
600
400
200
0
Wt
hCRM1-Tg
E
HTLV-1 provirus (copies)
P>0.05
100
80
60
40
20
0
Wt
hCRM1-Tg

Fig.6