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

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Abstract

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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

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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.

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

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

102 Using an artificial bacterial chromosome (BAC) clone containing the entire CRM1 gene, we
103 have established hCRM1 Tg rats and examined the proliferation of HTLV-1 in vitro and in
104 vivo. Our results demonstrate that T cell lines isolated from hCRM1 Tg rats produced 100 to
105 10,000 times more HTLV-1 Gag antigen compared to T cells from wild type control rats and
106 that Tg rats displayed a more extensive invasion of HTLV-1 into the thymus when infected
107 intraperitoneally. These results indicate the essential role of hCRM1 in proper HTLV-1
108 replication and suggest the importance of this Tg rat model as a basis for the development of
109 better HTLV-1 animal models.

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MATERIALS AND METHODS

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

120 For activation, cells were cultured with various combinations of 50 nM phorbol 12-myristate
121 13-acetate (PMA), 100 nM ionomycin, and 10 ng/ml IL-2.

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

128 **Western blotting.** Cells were lysed in ice-cold extraction buffer (10 mM Tris-HCl [pH 7.4],
129 1 mM MgCl₂, 0.5% NP-40) containing protease inhibitor cocktail (Complete mini; Roche
130 Diagnostics). The protein concentration of each sample was determined using a protein assay

131 kit (QB PERBIO; Pierce). The cell lysates were sonicated, or in some cases treated with
132 DNase 1 solution (Takara), then dissolved in sample buffer. The same amounts
133 (approximately 20 µg) of cell lysates were subjected to SDS-PAGE. Following
134 electrophoresis. Proteins were transferred to a nitrocellulose membrane and probed with anti-
135 human or rat CRM1(34), anti-β-actin (AC40; Sigma), or anti-Rex (34) antibodies followed
136 by secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase.
137 Proteins were visualized by staining with BCIP/NBT, or ECL+ (Amersham Pharmacia
138 Biotech) followed by the LAS-100 plus system (Fuji film) and evaluated by Image Gauge
139 Version 3.4 software (Fuji film).

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

144 To quantify CRM1 mRNA, RNA samples (5 µg) were subjected to quantitative RT-PCR
145 with the Platinum Quantitative RT-PCR Thermoscript One Step System (Invitrogen) using
146 the forward primer 5'- GCT GAA AAC TCA ACC GAG ATG G -3' and the reverse primer
147 5'- CTG TTG CTC TTG CTG ATG CTG TA -3', and a probe (FAM-) AAA ATG CCG
148 CAG GCA TTT CGT TCA G (-TAMRA). RT-PCR was performed by incubating for 2 min
149 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
150 62 °C in an Applied Biosystems Prism 7700 Sequence Detector Thermocycler (Applied
151 Biosystems) with Sequence Detector software (Applied Biosystems). To make standard
152 curves, the region from -943 to +38 bp of the CRM1 cDNA was amplified by PCR using
153 Human Lung Marathon Ready cDNA (Clontech) with adaptor primer-1 and 5'-
154 GCTGCATGGTCTGCTAACATT-3' and by nested PCR with adaptor primer-2 and 5'-
155 CTGCATGGTCTGCTAACATTG-3'. The PCR product was cloned into the vector pCR 2.1
156 (Invitrogen) and a 981-base single stranded RNA was synthesized *in vitro* with MegaScript
157 T7 (Ambion).

158 **Establishment of human CRM1 transgenic rats.** pBeloBAC hCRM1, which harbors the
159 entire human CRM1 genomic sequence including approximately 50 kb of 5' upstream

160 sequence and 10 kb of 3' downstream sequence, was microinjected into 450 fertilized one-
161 cell eggs prepared from Fischer 344/Du Crj (F344) female rats by the YS institute. Integration
162 of the transgene was confirmed by PCR using genomic DNA, which was extracted by the
163 PUREGENE™ tissue kit (Gentra) from the rat tail, as a template with the hCRM1 specific
164 primer pairs: 5'-TTATGTGGCTGCAGTGTGGA-3' and 5'-
165 ACATACCAGGGTTCTCTGGA-3', and 5'-GTCACCTGATGTCTGGGAGTT-3' and 5'-
166 GGATTACAGGTGTGAGCCA-3. All animal experiments were conducted according to the
167 Guide for the Care and Use of Laboratory Animals, Institute for Genetic Medicine, Hokkaido
168 University.

169 **Detection of genomic copies of hCRM1 and G3PDH.** Genomic DNA was subjected to
170 PCR with the following primer pairs: hCRM1, forward primer (5'-TGA GGT CAG GAG
171 TTC AGG AT-3') and reverse primer (5'-CTC TGC CTC CTG GGT TCA A-3'); G3PDH,
172 forward primer (5'-AGA GCT GAA CGG GAA G-3') and reverse primer (5'-GGA AGA
173 ATG GGA GTT GC-3'). The PCR conditions were: 5min at 94 °C, 10 cycles of 30 s at 94
174 °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
175 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.

176 **Quantification of HTLV-1 proviral load by LightCycler-based real-time PCR.** The
177 HTLV-1 proviral loads of HTLV-1-infected cells were quantified by real-time PCR on a
178 LightCycler PCR Instrument (Roche Diagnostics). Briefly, 20 µl of a PCR mixture in a
179 capillary tube containing each HTLV-1 pX-specific inner primer pair at 0.4 µM, 1x
180 LightCycler-FastStart SYBR Green PCR Master Mix, and 30 ng of genomic DNA was
181 subjected to 40 cycles of denaturation (95 °C 15 s), annealing (69 °C, 10 s), and extension (72
182 °C, 10 s) following an initial Taq polymerase activation step (95 °C, 15 min). The copy
183 numbers of HTLV-1 provirus in the samples were estimated from a standard regression curve
184 using the LightCycler Software version 3 (Roche Diagnostics). The standard curve for
185 HTLV-1 provirus was obtained by PCR data using 1×10^2 to 1×10^8 copies of pCR-pX1-4
186 plasmids, which were constructed by inserting a PCR fragment amplified with pX1 (5'-CCC
187 ACT TCC CAG GGT TTG GAC AGA GTC TTC-3') and pX4 (5'-GGG GAA GGA GGG
188 GAG TCG AGG GAT AAG GAA-3') from the genomic DNA of MT-2 cells into pCR2.1.

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

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

194 **Detection of intracellular Tax and Gag protein.** Cells (10^6) were fixed with 1%
195 paraformaldehyde in phosphate-buffered saline (PBS) containing 20 μ g/ml of lysolecithin
196 (Sigma) for 2 min at room temperature, centrifuged, and resuspended in cold methanol. The
197 cells were then sorted at 4 °C for 15 min, centrifuged, and incubated in 0.1% NP40 in PBS at
198 4 °C for 5 min. After centrifugation, the cells were stained with mouse anti-Tax MAb LT-4
199 (63) or the mouse anti-Gag MAb GIN-7 (38) followed by FITC-conjugated goat anti-mouse
200 IgG plus M antibody (Immunotech). Finally, the cells were washed and fixed with 1%
201 formalin in PBS prior to analysis by cell sorting.

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

209 **Detection of provirus in HTLV-1-infected rats.** To determine the positive rate of HTLV-1
210 provirus in various organs, 200 μ g of genomic DNA was subjected to PCR for the
211 amplification of the px region of HTLV-1 as described previously (51). The first-step PCR
212 was performed with the primer pair of pX1 and pX4, followed by the second-step PCR with
213 the primer pair of pX2 (5'-CGGATACCCAGTCTACGTGTTTGGAGACTGT-3') and pX3
214 (5'-GAGCCGATAACGCGTCCATCGATGGGGTCC-3'). The PCR conditions were:
215 activation of Taq polymerase (94 °C, 3 min); 35 cycles of denaturation (94 °C, 30 sec),
216 annealing (60 °C, 30 sec), and extension (72 °C, 30 sec), and a final elongation of the product

217 (72 °C, 3 min). For a nested PCR, an aliquot of the first PCR product was subjected to
218 another 35 PCR cycles with the second set of primers.

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RESULTS

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

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

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

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

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

270 **Regulated expression of CRM1 in CD4⁺ T lymphocytes.** To examine CRM1 regulation in
271 CD4⁺ T lymphocytes, resting CD4⁺ T lymphocytes were purified by negative selection and
272 activated by treatment with a combination of IL-2, ionophore, and PMA. CRM1 levels were
273 estimated by Western blotting (Figure. 2A). CRM1 expression was induced by the same
274 stimuli as in PBMCs, although the kinetics of induction was somewhat different among

275 donors. In contrast to CRM1, the level of actin was constant during T cell activation.
276 Staurosporine inhibited the enhanced production of CRM1 (data not shown), indicating the
277 involvement of PKC in the induction of CRM1 in CD4⁺ T cells.

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

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

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

313 We next examined the expression of cell surface markers, including CD3, CD4, CD5, CD8,
314 CD25, MHC-I, and MHC-II, in these cell lines (Table 1). All the cell lines expressed rat
315 CD25 and MHC-I, indicating that they were derived from rat cells, not from the human MT2
316 cells. Most of the cell lines also expressed rat CD5 and MHC-II, with the exception of 2 Wt
317 and 3 Tg-derived lines. Expression of rat CD3 was confirmed in 6 of 9 Tg lines, whereas only
318 2 of 6 lines were positive in the Wt lines. Rat CD4 expression was detected in 1 Wt and 6 Tg
319 cell lines. Rat CD8 was detected in 1 Wt and 1 Tg-derived line. As judged by the expression
320 of CD3, we established a total of 8 T cell lines, 2 from Wt and 6 from Tg rats.

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

330 To further examine the increased p19 production in each cell line expressing hCRM1, we
331 conducted a FACS analysis to detect the intracellular Gag protein. As shown in Figure 4E, we

332 were able to detect p19 and the precursor p55 Gag protein in all cell lines derived from Tg
333 rats. In contrast, no Wt-derived cell lines produced detectable amounts of Gag. These results
334 further support the role of hCRM1 in the enhancement of HTLV-1 Gag production.

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

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

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

358 We next examined Rex expression by immunoblotting. As shown in Figure 5C, the Rex
359 protein was expressed in all cell lines tested. Again, there was no statistical difference in the
360 protein expression between Wt and Tg cells. Two Tg cell lines, FCMS1 and FCMS18,

361 expressed p21 protein as well as the p27 Rex. This expression was not associated with
362 elevated expression of Gag, since the amounts of p19 Gag produced by these two cell lines
363 were similar to the other Tg-derived cell lines (Figure 4C, D). These results indicate that the
364 number of integrated provirus and the expression levels of Tax and Rex are not correlated
365 with the enhanced expression of Gag observed in cell lines derived from hCRM1-Tg rats.

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

383

384

DISCUSSION

385

386 Unlike hCRM1, rCRM1 does not support Rex function due to its inability to induce Rex-
387 Rex dimerization, which is required for RNA export from the nucleus to the cytoplasm (22).

388 This may be one reason why HTLV-1 replicates poorly in rats compared to humans. This

389 observation suggests the hCRM1-Tg rats would be novel animal models, since they would
390 support better replication of HTLV-1.

391 The essential role of CRM1 in cell viability suggested that proper expression of the
392 transgene would be a key for successful construction of Tg rats. Therefore, we examined the
393 expression pattern of CRM1 and found that CRM1 is expressed in a manner similar to the
394 early response genes induced during the activation of lymphocytes, including CD4⁺ T cells.
395 Our results suggest that expression of CRM1 is stimulated in two steps: in the first phase,
396 lasting approximately 4 h, induction is regulated primarily in a post-transcriptional manner,
397 and in the second phase, transcriptional augmentation takes place. Alternatively, CRM1
398 protein in PBMCs may be rapidly turned over and is then protected from degradation upon
399 stimulation, giving rise to the early increase in protein levels. The profile of CRM1
400 expression further suggests that the initial induction occurs in the G₁ phase of the cell cycle,
401 which is also supported by the observation that mimosine, which blocks the cell cycle in late
402 G₁ (65), does not prevent the induction (data not shown).

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

412 We have previously reported that expression of hCRM1 induced an increase in HTLV-1
413 Gag production in both rat epithelial and T cells (21, 69). Our present study also showed that
414 T cell lines established from hCRM1-Tg rats produced significantly greater amounts of p19
415 than cell lines established from Wt rats, further indicating the positive effect of hCRM1 on
416 viral protein synthesis. This effect was not due to the effects of Tax or Rex proteins, which
417 enhance the transcription of total viral mRNAs and the nuclear export of unspliced and

418 incompletely spliced mRNAs, respectively (12, 26, 30, 68), since the expression levels of
419 these proteins were not significantly different between Tg and Wt cell lines. Additionally,
420 these results indicate that induction of hCRM1 expression does not affect the expression of
421 HTLV-1 regulatory proteins in virus-infected rat cells. We also observed variation in the
422 levels of p19 production among the cell lines derived from hCRM1-Tg rats. Since the amount
423 of p19 did not correlate with the expression levels of hCRM1, Tax, or Rex, the reason for the
424 variation is not clear. Some other factors, including RanGTP and RanBP3, which play
425 important roles in the nuclear export of CRM1-substrate complexes (14, 41, 47, 59), may
426 affect the levels of p19 production in the rat cell lines. It is also possible that the integration
427 sites of the provirus influence virus production. Further studies are required to identify the
428 factors that result in varying p19 production among Tg-derived cell lines.

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

438 The Tg rats showed minimal effects on HTLV-1 replication in vivo. Since dramatic
439 enhancement of HTLV-1 production was observed in all hCRM1 expressing cells in vitro, it
440 is possible that the number of HTLV-1-infected cells in vivo was too low to detect
441 differences in virus production between Wt and Tg rats. From this point of view, alteration of
442 experimental condition to improve the initial infection rate of HTLV-1 may lead to the
443 enhanced viral replication in the Tg rats. Repression of viral protein expression in vivo may
444 also reduce the effects of hCRM1, masking the enhanced viral replication in the Tg rats. Such
445 responses have been well documented in HTLV-1 infected individuals (32, 33). It is also
446 possible that HTLV-1 specific immune responses could affect the replication of HTLV-1 in

447 the Tg rats. Indeed. Our preliminary experiments indicated that induction of HTLV-1-specific
448 CTL responses occurred as early as 1 week after virus infection. Alternatively, some other
449 host factors may govern and modulate efficient HTLV-1 replication in vivo. Thus, further
450 studies on both virological and immunological aspects are required to verify the importance
451 of the Tg rats as an in vivo model of HTLV-1 infection.

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

460 HIV latently infects reservoirs of resting T cells (7, 9, 10, 13, 61), which are thought to be in
461 the G₀ state, and the virus is then reactivated during T cell activation. Alternatively, HIV has
462 also been reported to propagate efficiently in non-replicating lymphatic T cells (18), which
463 lack certain markers specific for activation. Since cytokines levels are high in lymphatic
464 tissues, the progression of T cells from G₀ to G₁ may support HIV replication. Although
465 release from cell cycle block has been extensively investigated at the transcriptional level, a
466 recent study has shown that the synthesis of unspliced HIV Gag RNA increases rapidly during
467 the HIV reactivation process, to a much greater extent than the synthesis of multiply spliced
468 RNAs (7). Our results demonstrating a rapid increase in CRM1 expression during lymphocyte
469 activation provide a clue to the underlying mechanism, the efficient action of the HIV Rev
470 protein, which leads to robust synthesis of unspliced RNA. We suggest that HIV gene
471 expression is regulated in lymphocytes at both the transcriptional and RNA export levels.

472 Independent of viral replication, the first phase of enhancement of CRM1 expression is also
473 coincident with the induction of cytokines, such as IL-2 (4). CRM1 interacts with the ARE
474 located in the 3' untranslated region of c-fos mRNA (via HuR and its ligands) and mediates
475 export of this mRNA from the nucleus to the cytoplasm (6, 16). Therefore, CRM1 may

476 transport cytokine mRNAs belonging to the early response genes since many cytokine
477 mRNAs harbor ARE sequences (24, 56). Collectively, these observations suggest that
478 enhancement of mRNA export via the induction of CRM1 expression, in addition to
479 regulation at the transcriptional and translational levels, may play an important role in
480 coordinating gene expression during lymphocyte activation. The existence of a post-
481 transcriptional mechanism leading to a rapid increase in CRM1 protein is consistent with this
482 hypothesis.

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

490

491

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730 (HTLV-1) in rat cells. *Microbes Infect.* **8**:851-859.

732 **FIGURE LEGENDS**

733

734 Figure 1. Expression of CRM1 during activation of PBMCs. (A) PBMCs isolated from donor
735 1 and donor 2 were activated with ionophore, PMA, and IL-2 and analyzed by Western
736 blotting. (B) PBMCs isolated from donor 1 (□) and donor 3 (◇) were activated with
737 ionophore, PMA, and IL-2 and analyzed by quantitative RT-PCR. Each value is the average
738 of duplicate measurements. (C) PBMCs isolated from donor 2 were activated with various
739 combinations of ionophore, PMA, and IL-2 and analyzed by Western blotting. (D) PBMCs
740 isolated from donor 2 were activated in the presence of various inhibitors and analyzed by
741 Western blotting.

742

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

749

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

756

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

760 internal control. (B) Protein expression of hCRM1 was detected by immunoblotting. Samples
761 (10 µg of total protein per each lane) were subjected to SDS-PAGE. HeLa cell extract was
762 used as a positive control. (C) HTLV-1 Gag protein in the supernatant of 2 day and 4 day
763 cultures was quantified by HTLV-1 p19 ELISA. The results are shown as the mean of three
764 independent experiments. (D) Based on the data of (C), the average p19 Gag production of Tg
765 and Wt cell lines was calculated. (E) The amount of intracellular Gag in each cell line was
766 analyzed by flow cytometry. Open histograms indicate the cells stained with anti p19/p55
767 Gag MAbs. Solid histograms represent the cells stained with control mouse IgG. (F) Growth
768 rate of Wt or Tg cell lines was measured. In parallel with the experiments described in Figure
769 4(C), the growth rate was monitored by the cell counting Kit-8 (Dojinndo Laboratories). The
770 relative cell numbers of 2 or 4 day vs. 0 day cultures are shown.

771

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

780

781 Figure 6. Dissemination of HTLV-1 in hCRM1 Tg rats. (A) Mean plasma p19 concentration
782 in Wt (n=9; ●) or hCRM1 (n=7; ■) Tg rats after intraperitoneal inoculation of mitomycin
783 C-treated MT2 cells (1×10^7 per animal). (B) Mean plasma p19 concentration in Wt (n=16;
784 □) or hCRM1-Tg (n=17; ■) rats 1 week after intraperitoneal inoculation of MT2 cells
785 (5×10^6 per animal). (C) Detection of HTLV-1 provirus in thymus derived from rats used in
786 (B). Presence of HTLV-1 provirus was analyzed by nested PCR. Results are mean percentage
787 of HTLV-1 provirus positive rats in 5 independent experiments. (D and E) HTLV-1 proviral
788 loads of rats used in (B). HTLV-1 proviral loads in peripheral blood cells (D) or thymus (E)

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

793 Table 1. Constructed cell lines and surface markers

794

795	Cell lines	hCRM1	Surface markers						
796			CD3	CD4	CD5	CD8	CD25	MHC1	MHC2
797	FWT1	-	+	+	+	-	+	+	+
798	FWS1-11	-	-	-	+	-	+	+	+
799	FWS1-27	-	-	-	+	-	+	+	+
800	FWS1-31	-	-	-	+	(+)	+	+	-
801	FWS1-34	-	-	-	+	-	+	+	-
802	FWT11	-	+	-	+	-	+	+	+
803	FCMS1	+	+	+	+	-	+	+	(+)
804	FCMT1	+	+	+	+	-	+	+	+
805	FCMT18	+	+	+	-	-	+	+	-
806	FCMS18	+	+	+	(+)	-	+	+	(+)
807	FCMT27	+	+	+	(+)	-	+	+	(+)
808	FCCT13-1	+	-	-	(+)	-	+	+	+
809	FCCT13-2	+	+	-	(+)	(+)	+	+	+
810	FCCS13-1	+	-	-	-	-	+	+	+
811	FCCS13-2	+	-	(+)	(+)	-	+	+	-

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

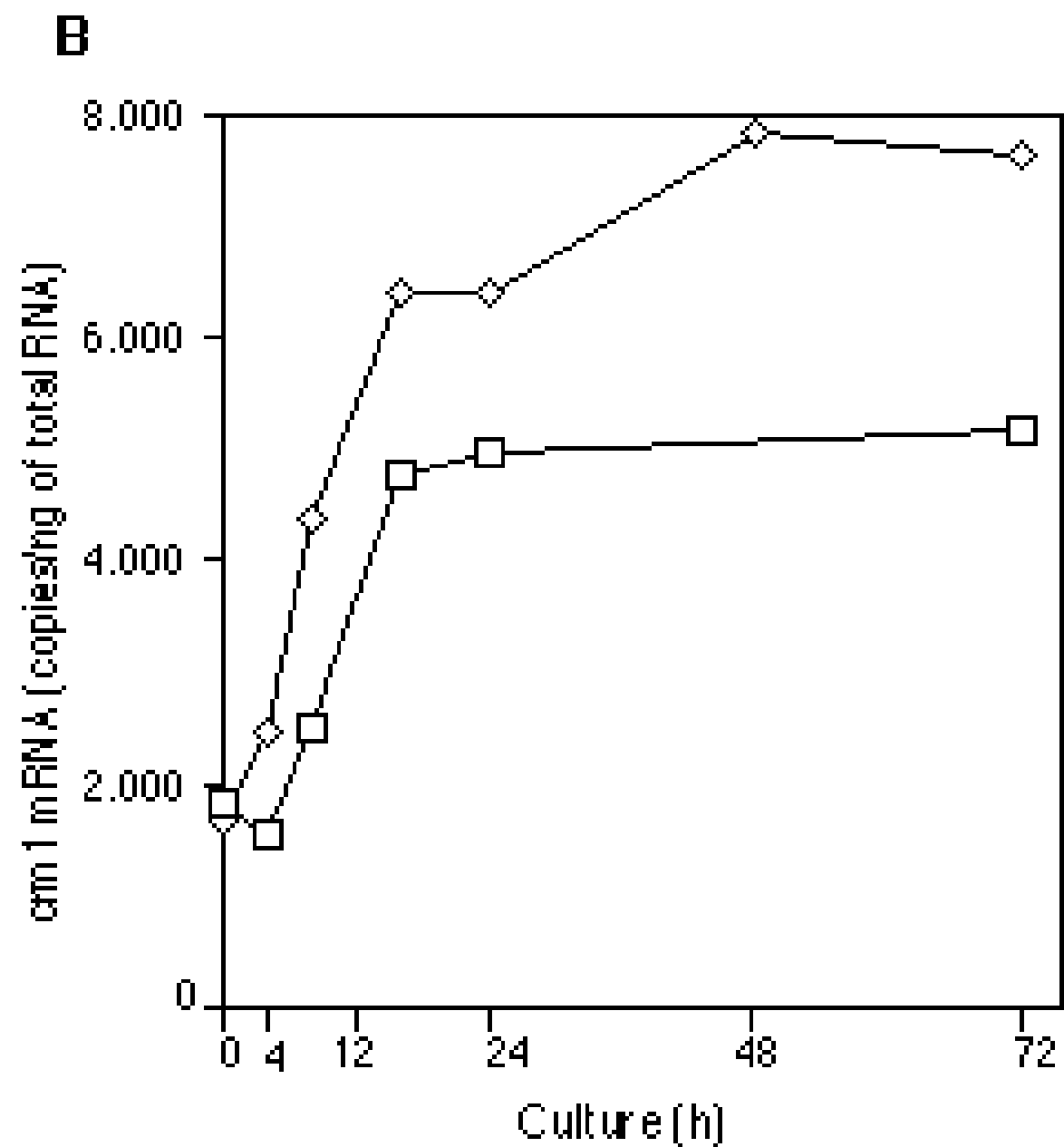
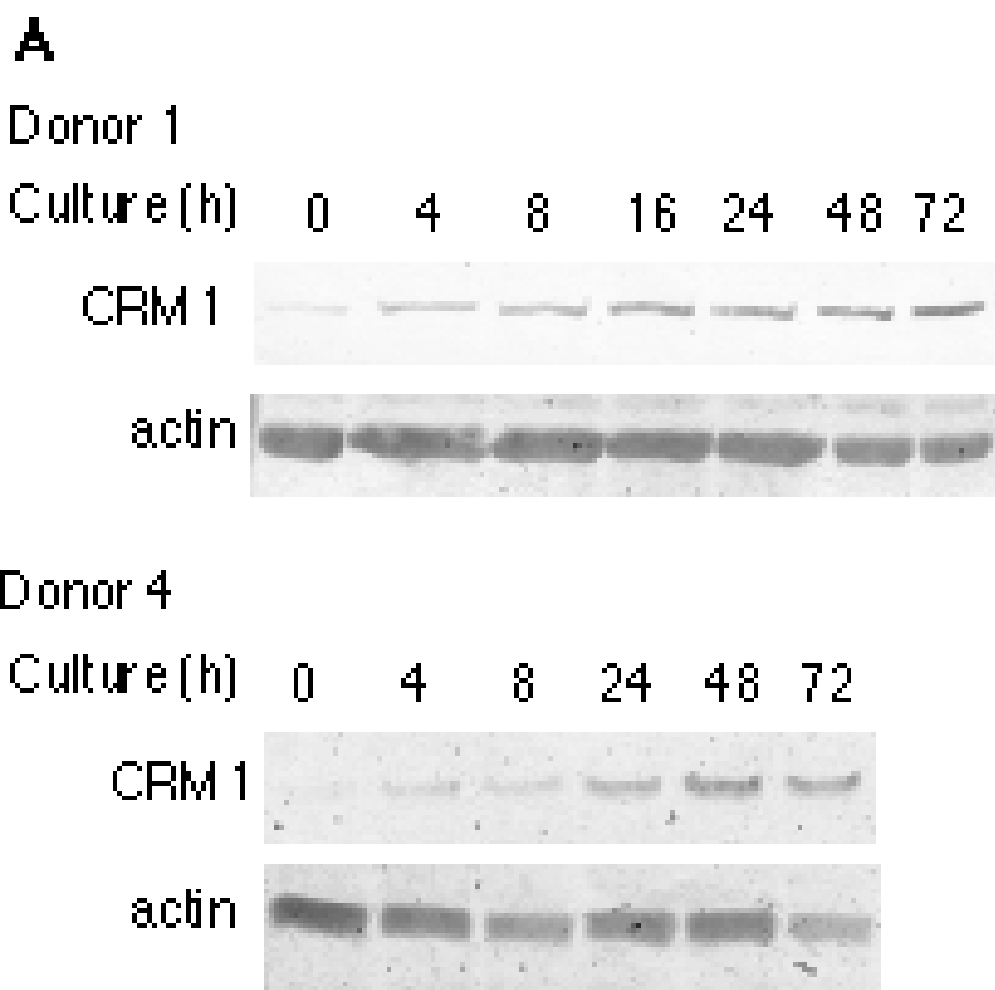


Figure 2

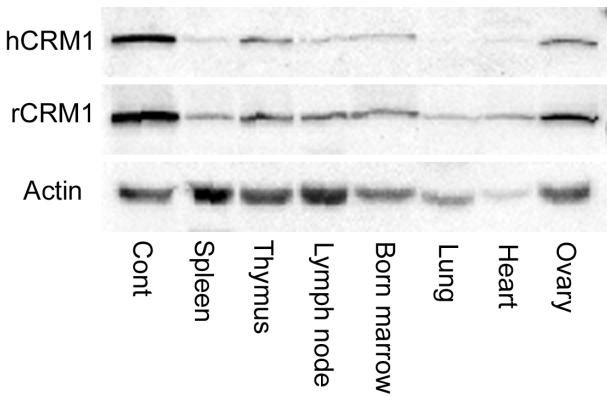
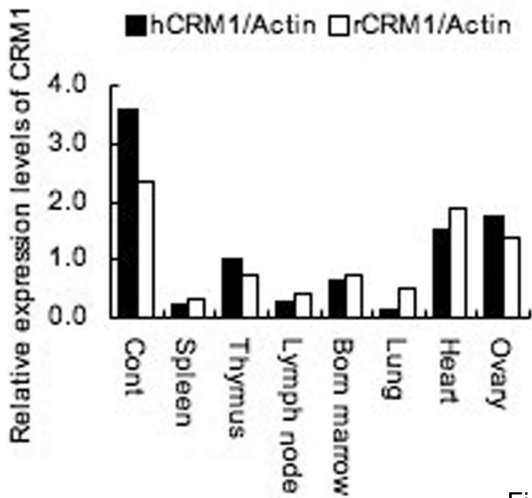
A**B**

Fig.3

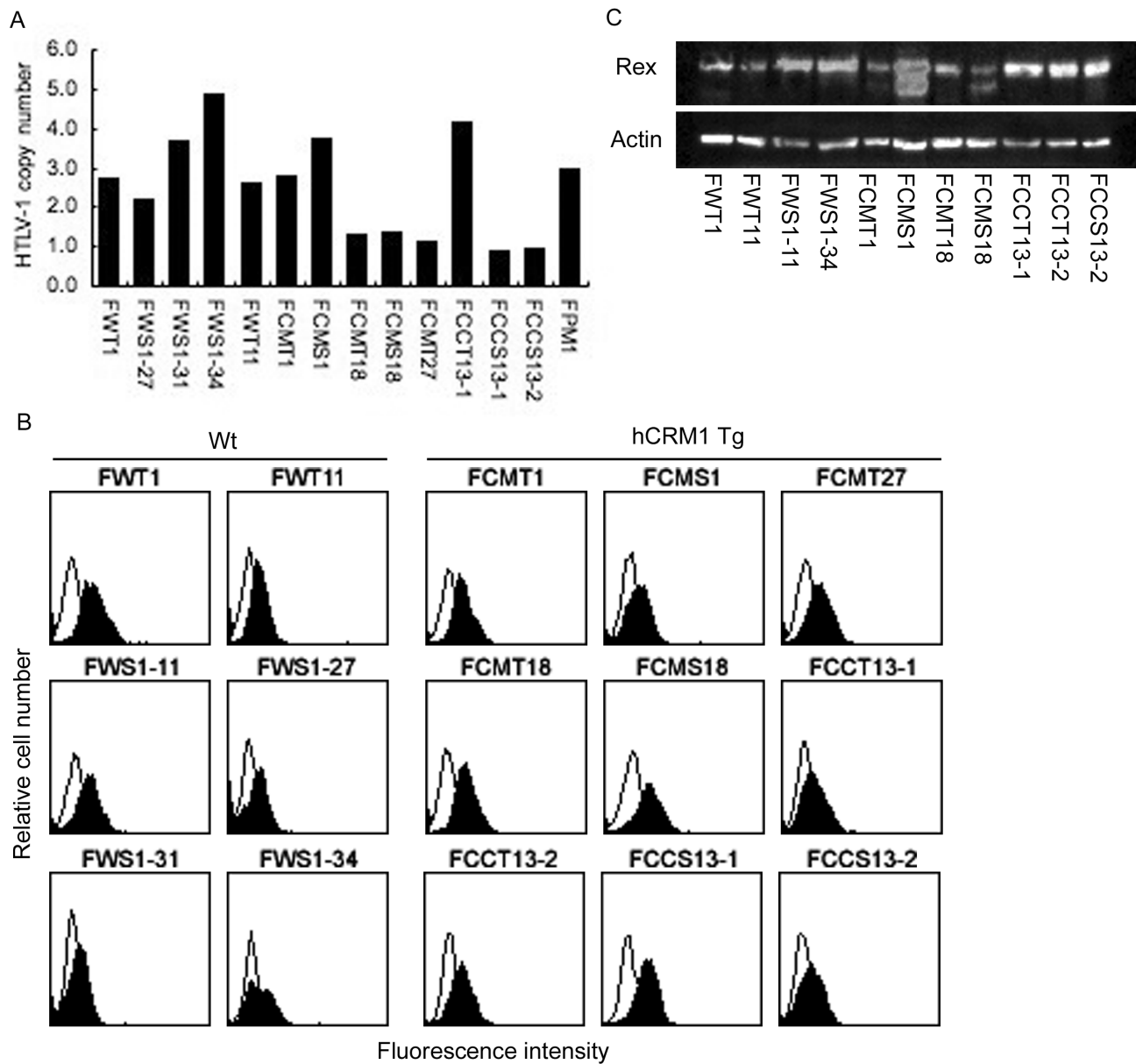


Fig.5

