CRM1, an RNA transporter, is a major species-specific restriction factor of human T cell leukemia virus type 1 (HTLV-1) in rat cells

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Running title: Species barrier of HTLV-1

Abstract

Rat ortholog of human CRM1 has been found to be responsible for the poor activity of viral Rex protein, which is essential for RNA export of human T cell leukemia virus type 1 (HTLV-1). Here, we examined species-specific barrier of HTLV-1 by establishing rat cell lines, including both adherent and $CD4^+$ T cells, which express human CRM1 at physiological levels. We demonstrated that expression of human CRM1 in rat cells is not harmful to cell growth and is sufficient to restore the synthesis of the viral structural proteins, Gag and Env, at levels similar to those in human cells. Gag precursor proteins were efficiently processed to the mature forms in rat cells and released into the culture medium as sedimentable viral particles. An HTLV-1 pseudovirus infection system suggested that the released virus particles are fully infectious. Our newly developed reporter cell system revealed that Env proteins produced in rat cells are fully fusogenic, which is the basis for cell-cell HTLV-1 infection. Moreover, we show that the early steps in infection, from post-entry uncoating to integration into the host chromosomes, occur efficiently in rat cells. These results, in conjunction with reports describing efficient entry of HTLV-1 into rat cells, may indicate that HTLV-1 is unique in that its major species-specific barrier is determined by CRM1 at a viral RNA export step. These observations will enable us to construct a transgenic rat model expressing human CRM1 that is sensitive to HTLV-1 infection.

Keywords: Human T cell leukemia virus type 1; CRM1; Species barrier.

1. **Introduction**

The human T lymphotropic virus type 1 (HTLV-1) is a type C retrovirus, whose etiological role in adult T cell leukemia (ATL) and tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) has been well established [1, 2]. In its small genome, the virus encodes not only viral structural and enzymatic proteins, but also several regulatory proteins, using alternative splicing and alternate codon usage. The actions of these regulatory proteins are critical for the virus life cycle. Tax, identified as a viral onco-protein, activates viral and cellular transcription to promote T-cell growth and ultimately, malignant transformation [3, 4]. Rex, which is a nucleocytoplasmic shuttling protein, mediates nuclear export of unspliced or incompletely spliced viral mRNAs, which encode the viral structural and enzymatic proteins, Gag, Pol and Env [5-7]. In the nucleus, Rex interacts with the Rex response element (RxRE), which is located in the 3' long terminal repeat (LTR) of the viral mRNA [8, 9]. To form an export complex, Rex binds to human CRM1 (hCRM1), a member of the karyopherin family of nuclear transport receptors, in cooperation with a GTP-bound form of the small G protein Ran (Ran-GTP) and RanBP3 [10-12]. Moreover, multimerization of Rex on the viral RNA is critical for its full biological activity [13], since the Rex multimer may shield the viral RNA from being spliced or down-regulated [14]. Previously, we reported that hCRM1 is dually involved in both the export of the target viral mRNA complex and the multimerization of Rex on the cognate RNA [11]. This suggests that hCRM1 is the most critical cofactor guiding Rex function.

Several animal models to investigate the mechanisms underlying the onset of HTLV-1 related diseases have been developed over the past years. Monkeys and rabbits have been

used to examine HTLV-1 infection, replication, disease manifestations, immune response, and vaccine development [15-20]. However, rats and mice are more attractive models for HTLV-1 study because of the ease with which they can be genetically manipulated. HTLV-1 transmission to newborn mice has been reported and the HTLV-1 provirus in mouse spleen has been detected [21]. Nevertheless, no viral expression or antibody production was detected in these mice and, furthermore, mouse cells seem to be less susceptible to HTLV-1 envelope fusion [22, 23], even though some conflicting results have been reported [24]. In contrast, HTLV-1 infection in rats establishes a persistent infection and elicits specific antibody responses [25, 26]. Moreover, HAM/TSP-like diseases develop in HTLV-1-infected WKA/H rats [27, 28]. However, until now, efficient replication of HTLV-1 in rat cells has not been reported.

To develop better rat models, it is essential to identify the step at which viral replication is blocked and the host factor(s) responsible. HTLV-1 has been reported to infect several types of rat cells, which indicates that the rat cells possess receptors for viral attachment and penetration [29-31]. Recent identification of a highly conserved molecule, Glut-1, a glucose transporter, as a receptor [32] is consistent with these observations. Previously, we found the inability of the host factor rat CRM1 to support Rex function and thus that viral RNA export from nucleus was a possible block in the viral life cycle. Rat CRM1 induces minimal amount of Rex multimerization on cognate RNA, although it efficiently exports Rex protein to the cytoplasm. This may cause the defect in viral RNA transport [33]. Two residues (amino acids 411 and 414) in the central region of human CRM1 are crucial for multimerization [34]. These results suggest that a transgenic (Tg) rat, which expresses human CRM1, may be a

model animal to support replication of HTLV-1.Prior to constructing the Tg rat, we examined the effects of hCRM1, expressed at physiological levels in rat cells, because the above results were obtained by overexpression of human and rat CRM1 and toxic effects from overexpressed CRM1 and a dominant-negative influence of rCRM1 over hCRM1 have been reported [33, 35]. Moreover, an understanding of the entire viral life cycle is needed. In the case of the human immunodeficiency virus (HIV), nonhuman cells have been reported to contain inhibitors such as Trim5α and Apobec3G, which act at uncoating and reverse-transcription steps, respectively [36, 37].

In this study, we constructed rat cells expressing hCRM1 at physiological levels and examined the effects on HTLV-1 replication. We investigated the early steps of the HTLV-1 lifecycle, between entry and transcription, and the late steps, including formation of infectious virus and cell to cell infection. To investigate these steps quantitatively, we used a pseudovirus system [38] and our newly devised cell fusion assay, because the extremely poor infectivity of free HTLV-1 virions [38, 39] makes it impractical to evaluate them by conventional virological methods. Here, we show that expression of hCRM1 in rat cells may be sufficient to enhance production of HTLV-1 proteins and infectious viruses at levels similar to those in human cells.

2. Materials and methods

2.1. Retro-vector preparation. To construct an hCRM1 expressing retro-vector, the 3Kb fragment, which encodes 3' part of hCRM1 coding frame, was isolated from pSRαhCRM1 plasmid [11] by digestion with Aat II and XhoI, and the left 5' part was amplified by PCR

using the primer pair: hCrm15'F: CCGAATTCTCTCTGGTAATCTATGCCAGCAA; hCrm15'R: CAAGTTGGGTCAGATGACGTCTT on pSRαhCRM1 as a template. The PCR was performed by a single step of 94 ºC for 90 Sec and 10 cycles of a three-temperature PCR (94ºC for 30 s, 56 ºC for 60 s, and 72 ºC for 30 s) followed by one step of 72ºC for 5 min. The amplified fragment was then digested with AatII and EcoR1. The two fragments of hCRM1 cDNA were then ligated to retrovector pMX-neo digested with EcoRI and XhoI [40]. The resultant expression plasmid, named pMXneohCRM1, was transfected to packaging PLAT-E cells, the supernatants was collected, and stored at –80 ºC.

2.2. Construction of stable cell lines. Rat mammary adenocarcinoma ER-1 cells, maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FCS), were seeded into 6-well plates at a density of $1x10^5$ cells/well one day before infection. To construct stable rat cell lines which express hCRM1, ER-1 cells were incubated with 0.5 ml of MXneohCRM1 virus solution for 4 hours in the presence of 10 μg/ml polybrene, and then fresh medium was added. The hCRM1 expressing clones were selected in the presence of 300 μg/ml neomycin. We picked two neomycin resistant clones and designated them ER-1/hCRM1-1 and ER-1/hCRM1-2, respectively. The control cells, designated as ER-1neo1, were infected with virus carrying the MX-neo plasmid.

To construct an hCRM1 expressing rat T cell line, $1x10^5$ FPM1 cells, an HTLV-1 transformed rat $CD4^+$ T cell line, were infected with MXneohCRM1 and selected with 100 μg/ml neomycin. The resistant cells were then divided to 96-well plates at 0.1 cells per well to clone the FPM1hCRM1-14 line.

To establish reporter cells for detection of HTLV-1-induced cell fusion, $5x10^5$ 293T cells in 10cm petri-dishes were transfected with 2.5 μg of pLTR-GL3 [14] and 0.5 μg of pTK-Hyg (Clontech, Palo Alto, CA) using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was replaced with fresh DMEM supplemented with 10% FCS and 300 μg/ml hygromycin B. Antibiotic resistant colonies were picked with a cloning cylinder. A cell clone, designated as 293T/LTR-Luc8, was used in this study.

2.3. Measurement of Rex activity. The cells ($1x10^5$) were transfected with 0.4 μg of pDM128RxRE [33], 0.05μg of pSRαRex, and 0.1μg of pCDMβ-Gal, Twenty-four hours after transfection the cells were lyzed and the amount of CAT was quantified using a CAT ELISA kit (Roche). The β−galactosidase (β−gal) activity was measured by standard colorimetric methods, to normalize the transfection efficiency, The Rex activity was represented by ratio of CAT/ β−gal for each sample as described previously [33].

2.4. Measurement of HTLV-1 Gag production. The human and rat cell lines ($1x10^{5)}$ were transfected with 0.5μg of HTLV-1 infectious clone K30 and 0.1μg of pCDMβ-Gal. Forty eight hours after transfection, the medium of the cell culture was harvested and centrifuged at low speed to remove the cell debris. The transfected cells were washed twice with PBS then scrapped off and transferred to microcentrifuge tube, the cells was suspended in 50 μl of lysis buffer (10mM Tris-HCl, 140mM NaCl, 3mM $MgCl_2$, 1mM DTT, 0.5% NP-40, 1μg per ml of aprotinin, leupeptin and pepstain). Gag was quantified by RETRO-TEK HTLV-1 p19 Gag ELISA kit according to the manufacturer's protocol.

2.5. Immunoblot analysis. Immunoblot analysis was performed for detection of CRM1 using affinity purified chicken anti- hCRM1 and rabbit anti- rCRM1 antibodies [33]. To detect HTLV-1 proteins, we used rabbit anti Rex antisera, mouse anti-Tax MAb Lt-4, and mouse anti-p24 Gag MAb NOR-1 [42]. To detect the HTLV-1 Env protein, we concentrated the Env proteins using Concanavalin A (Con-A) Sepharose and then detected with rat anti-gp46 MAb LAT-27 [43]. For detection of Gag in released virions, the culture medium was ultracentrifuged at 40,000 rpm for 1 hour in a Beckman TLA100.3 rotor at 4 ºC. The pellets were suspended in the sample buffer and processed for immunoblotting. To detect the HTLV-1 Env protein in the cell lysate, $5x10^5$ rat or human cells in 10 cm petri-dishes were transfected with 2.5 μg of HTLV-1 K30. The cells were lysed 48 hours after infection and applied to Con-A Sepharose. The concentrated glycoproteins were eluted using sample loading buffer.

2.6. Quantification of fusion activity of HTLV-1 infected cells. The infectious clone HTLV-1 K30 (0.5 μg) was used to transfect various rat and human cells, and 24 hours later the cells were trypsinized and suspended in fresh medium. The cells were mixed with an equal number ($1x10^5$) of 293T/LTR-Luc8 cells, and cultured for an additional 48 hours. The cells were lysed and luciferase expression measured using the Steady-Glo luciferase assay system (Promega, Madison, WI) to evaluate fusion activity.

2.7. Cell free infection and gene transduction analysis. The preparation of pseudotyped HTLV-1 virus and virus infection were performed, as described previously, except that the

reporter plasmid, pHTC-GFP-Luc was used in stead of pHTC-Luc-tsa. [38]. pHTC-GFP-Luc, a newly developed reporter vector by David Derse's group, encodes a GFP-luciferase fusion protein, otherwise identical to pHTC-Luc-tsa. Briefly, pCMVHT-Δenv and pHTC-GFP-Luc, which were kindly provided by Dr. David Derse, and pMD-VSV-G were transfected into $1x10^6$ cells. The culture supernatant, which contains resultant viruses, was harvested 28 hours after transfection and used to infect various cell types. Seventy-two hours later luciferase activity in the infected cells was measured. For AZT inhibition, 100nM of 3'-Azido-3'-deoxythmidine (AZT) (Sigma) was used to treat the infected cells as described [38].

3. Results

3.1. Effects of hCRM1 expressed in rat adenocarcinoma cell lines.

We first established the stable rat adenocarcinoma cell lines, ER-1/hCRM1-1 and 2, which express hCRM1, by transduction with MxneohCRM1, a retro-vector encoding the hCRM1 cDNA. A control cell line named ER-1neo1, which carries only the neomycin resistance gene, was also generated. ER-1/hCRM1-1 and ER-1/hCRM1-2 expressed hCRM1 at levels similar to human HeLa cells and CD4-HeLa cells, as judged by immunoblotting. hCRM1 was not detected in the parental ER-1 or control ER-1neo1 cell samples (Fig. 1A). Both cell lines expressing hCRM1 propagated similarly or a little faster than parental ER-1 (Fig. 1B). It is conceivable that double amount of CRM1in the hCRM1 expressing rat cells, which also express rat CRM1, might facilitate rat mRNA/protein export, leading better growth. However, it is less likely, because ER-1 neo1 grew equally well as the hCRM1 expressing ER-1 cells. In any case our results suggest that expression of physiological levels of hCRM1 does not

negatively affect the replication dynamics of cells.

Next, we examined whether the hCRM1 transgene can restore Rex activity in rat cell lines. We co-transfected a CAT expressing reporter, the pDM128RxRE, pSRαRex, and pCDMβ-gal plasmids, and quantified Rex activity based on the amount of CAT protein production. In the parental rat ER-1 and control ER-1neo1 cells, Rex activity was undetectable, while in the hCRM1-expressing cells Rex activity was significantly augmented to levels found in HeLa and CD4-HeLa cells (Fig. 1C). As predicted, CAT expression in cells transfected only with pCDM128RxRE was very low. These results clearly demonstrate that expression of hCRM1 in rat cells is sufficient to augment Rex activity to levels similar to those found endogenously in human cells, regardless of the presence of endogenous rCRM1.

The restoration of Rex activity may directly result in enhanced expression of the HTLV-1 viral structural protein. To test this possibility, we transfected rat and human cells with the HTLV-1 molecular clone K30. Gag production was first quantified using an HTLV-1 p19 antigen ELISA (Fig. 1D). The p19 antigens were produced at similar levels in hCRM1 expressing rat and human cells, whereas very low levels of p19 were detected in the parental ER-1 and control ER-1neo1 samples. The ratio of p19 in the medium to that in the cell lysate was 3-4 in both human and rat cells expressing hCRM1. Approximately 30% of the secreted Gag protein, which was produced in all the types of hCRM1 expressing cells, could be pelleted by ultracentrifugation (Table 1). Taken together, these results suggest that viral particles budded from rat cells as efficiently as human cells.

To further examine the expression of the HTLV-1 structural proteins in rat cells expressing hCRM1, we performed a Western blot analysis (Fig. 2). Gag proteins including

p24 and its precursor p55 were expressed equally well in human and rat cells expressing hCRM1, but were not expressed in control ER-1neo1 rat cells. The efficiency of processing p55 to p24 was similar in human and rat cells and the p38 intermediate was detected in both cell lines. Similar amounts of the HTLV-1 gp46 Env protein and its precursor gp61 were detected in both human and rat cells expressing hCRM1, but not in control ER-1neo1 rat cells. In contrast, the two trans-regulatory proteins Tax and Rex, were expressed at similar levels in all rat and human cells upon HTLV-1 K30 transfection.

Since it is difficult to measure the infectivity of HTLV-1 by conventional methods, we applied a reporter virus assay [38], in which the HTLV-1 pseudovirus harboring a luciferase gene is coated with G proteins of vesicular stomatitis virus (VSV), which shows a broad tropism. Luciferase is driven by the strong CMV promoter, and used as a sensitive marker of the gene expression from viral genomes, which have integrated into their host cells. We produced the pseudotyped viruses in ER-1/neo1, ER-1/hCRM1-2, HeLa and CD4-HeLa cell lines, and compared their luciferase inducing capacity after infection of 293 cells (Table 2). The pseudovirus produced in ER-1/hCRM1-2 or CD4-HeLa induced similar levels of luciferase activity, which was greater than that produced by HeLa cell derived pseudoviruses. The luciferase activity was positively correlated to the amount of HTLV-1 p19 in the medium. The luciferase activity from ER-1neo1 samples represents the background. The luciferase activity was reduced to background levels by AZT, an inhibitor of the viral reverse transcriptase, indicating that the infection occurred through the normal retrovirus infection route. These results suggest that HTLV-1 virions produced from the hCRM1 expressing rat cells are fully infectious.

3.2. hCRM1 expression converts rat CD4$^+$T cells into high efficiency HTLV-1 producers.

To examine the effect of hCRM1 expression in rat CD4$^+$ T cells, we transduced MXhCRM1 into FPM1, an HTLV–1-transformed rat CD4$^+$ T cell line (Fig. 3). The FPM1-derived hCRM1 expressing cells, FPM1-hCRM1-14, produced hCRM1 levels comparable to the Jurkat human T cell line and to MT-4, an HTLV-1 producing human T cell line. FPM1-hCRM1-14 grew as well as the parental FPM1 cells (Fig. 3A). FPM1 has been reported to selectively express viral regulatory proteins, such as Tax, but not structural proteins [44]. Our ELISA data consistently showed very low levels of p19 expression (approximately 25 pg/ml) in the culture medium of FPM1, whereas FPM1-hCRM1-14 produced very high levels of secreted Gag antigen (approximately 7400 pg/ml), comparable to MT-4 cells (data not shown).

Western blotting showed that expression of hCRM1 in FPM1 did not affect the amount of Tax and Rex proteins, which are encoded by mRNAs that are exported independently of CRM1. In contrast, hCRM1 augmented the production of Gag and Env proteins. The Gag precursor p55 was processed to mature p24 as efficiently as the human T cells (Fig. 3B).

3.3. Fusion ability of HTLV-1-infected rat cells.

Efficient spread of HTLV-1 requires cell contact [45, 46]. Lymphocytes naturally infected with HTLV-1 produce very few cell-free HTLV-1 virions, and one in 10^5 to 10^6 virions is estimated to be infectious [38, 39]. The cell-to-cell spread of HTLV-1 is mediated through fusion of the two cell membranes caused by Env proteins [23, 47, 48]. Certain integrins, including the intercellular and vascular cell adhesion molecules ICAM-1, ICAM-3, and

VCAM, act as cofactors for HTLV-1-induced cell fusion [49, 50]. To quantify the fusion efficiency of HTLV-1-infected cells, we established a 293T derived reporter cell, 293/LTR-luc8, which harbors an HTLV-1 LTR promoter-driven luciferase reporter gene. When cell fusion occurs following co-culture of this reporter cell with HTLV-1 producing cells, Tax protein is transferred from the donor cells and activates the LTR promoter. An alternative route to activate the luciferase gene is through newly synthesized Tax protein from an HTLV-1 genome, which has been transferred from the donor cells, reverse-transcribed, and integrated into a reporter cell chromosome. In either case, the fusion ability of the HTLV-1 infected cells can be evaluated by luciferase expression. The reporter cells express very low levels of luciferase under normal culture conditions, but have a very sensitive LTR response upon Tax stimulation, as demonstrated by transient expression of a Tax encoding plasmid (data not shown), or co-culture with as few as $1x10^3$ HTLV-1 producing MT-2 cells. Luciferase activity increased linearly with the number of MT-2 cells, up to $5x10^4$ cells. In contrast, co-culture with $5x10^4$ Jurkat cells did not induce luciferase activity (Fig. 4A).

To compare the fusion capability of virus-producing cells, we first transfected various rat and human cells with HTLV-1 K30, incubated the cells for 24 h after transfection, and then co-cultured them with 293T/LTR-luc8 cells for a further 72 h. As shown in Fig. 4B, the luciferase activity induced by co-culture with ER-1/hCRM1-1 and ER-1/hCRM1-2 was as high as that resulting from co-culture with HeLa or CD4-HeLa cells, as well as the HTLV-1 high producing line C77, suggesting that K30-infected rat cells could be highly infectious. In contrast, the luciferase expression in reporter cells co-cultured with ER-1neo1 was close to the basal level. As a control, rat and human cells transfected with a Tax expressing plasmid

pSRαTax in the absence of Env expression were also unable to increase of the luciferase expression when co-cultured with 293T/LTR-luc8 cells (data not shown), indicating that the luciferase expression depends on cell fusion mediated by Env proteins.

The fusion ability of HTLV-1–infected rat T cells was also investigated. The luciferase level in reporter cells co-cultured with both human T cells (MT-4) and FPM1-mxhCRM1-14 were unexpectedly lower than the adherent cells described above. Nevertheless, the hCRM1 expressing rat T cells stimulated luciferase activity more efficiently than MT-4 cells (Fig. 4C). Luciferase activity was proportional to the amount of Env gp46 expressed (See Fig. 3 and 4). These results clearly demonstrate that the Env protein induced by hCRM1 in the rat cells is fully fusogenic, supporting HTLV-1 infectivity in rat cells.

To discriminate whether a Tax protein transferred from the donor cells or Tax protein produced from the HTLV-1 genome which had infected the reporter cells was the primary inducer of luciferase, we co-cultured the infected and reporter cells in the presence of AZT. The former alternative would be insensitive to AZT, while the latter would be sensitive to AZT. Hundred nM AZT had a negligible effect on luciferase activity (data not shown), consistent with poor formation of HTLV-1 infectious virus.

3.4. Efficient early events of HTLV-1 replication in rat cells.

To determine the efficiency of early replication events, from entry to genome integration step, human and rat cells were infected with VSV G-pseudotyped HTLV-1 virus containing the GFP-luciferase gene. ER-1/neo1 and ER1/hCRM1-2 cells produced luciferase signals similar to 293 and 293T cells (Table 3, Experiment 1). Unexpectedly, HeLa and CD4-HeLa cells

produced much weaker signals. Luciferase activity was inhibited by the reverse transcriptase inhibitor AZT, indicating that luciferase expression occurred as a result of retrovirus replication, implying reverse-transcription and integration. We next compared the efficiency of the early events in rat T cells with those in human T cells. As shown in Table 3 Experiment 2, both HTLV-1-uninfected (Nb2) and infected rat T cells (FPM1) induced luciferase as much as the human T cells. These results suggest that the early events of HTLV-1 infection indeed occurred in the rat cells at the same or slightly higher levels than in human cells. Free HTLV-1 viruses prepared from feline HTLV-1 producing C77 cells were used to infect activated primary T cells prepared from human PBMC or rat spleen. PCR was used to detect the pX region of HTLV-1 in genomic DNA, extracted from the cells 3 days after infection. We found more intensive signals in the rat T cell samples than the human samples (data not shown). This result supports the above notion, albeit it is not a quantitative method.

4. Discussion

Previously, we have shown that rCRM1, even though it is able to export Rex protein from the nucleus, does not support Rex multimerization, resulting in poor export of HTLV-1 RNAs in rat cells [33,34]. In this study, we demonstrated that the expression of hCRM1 at physiological levels in rat cells, including epithelial and $CD4^+$ T cells, is not harmful to cell growth, but augments the synthesis of HTLV-1 Gag and Env proteins to levels similar to those seen in human cells. The endogenous rCRM1 does not inhibit the function of Rex, although rCRM1 overexpressed by transfection has been reported to act as a dominant negative inhibitor of hCRM1 function [33]. The Gag precursor synthesized in hCRM1 expressing rat

cells is normally processed to the mature p24 and possibly other Gag proteins and then released into the culture medium as sedimentable viral particles, with an efficiency comparable to human cell lines. The fact that only 25-30% of the Gag protein detected in the medium of both human and rat cells was sedimentable suggests that only a fraction of the Gag proteins may be incorporated into virions, or that the non-sedimentable Gag proteins may reflect the fragility of the virus. Finally, the results using the HTLV-1 pseudovirus [38] suggest that HTLV-1 virions released from the rat cells may have the same infectivity as those released from human cells.

A major route for the spread of HTLV-1 is cell to cell infection mediated by cell fusion caused by the Env proteins in cooperation with the cell adhesion molecules ICAM-1, ICAM-3, and VCAM [48, 49]. Some reports suggest that the main function of the Env protein is to mediate cell fusion. These data led us to develop a reporter cell line to quantify Env-mediated cell fusion, based on activation of an HTLV-1 LTR-driven luciferase gene. This system can detect fusion events between as little as approximately $1x10^3$ MT-2 cells and $1x10^4$ K30-infected cells. Given that this fusion has been shown to be dependent on the Env protein, quantitation of functional Env proteins by this system should be much more sensitive than Western blotting, which requires Env protein concentrated by Con A Sepharose from at least $5x10^5$ K30-infected cells. Using this system, we demonstrated that hCRM1 expressing rat cells infected with HTLV-1 mediate cell fusion as efficiently as human cell lines. The results suggest that the fusion capacity of human and rat cells is proportional to the amount of the Env gp46 protein detected by Western blotting (Compare Fig. 2, 3 and 4), and indicates that the Env expressed on rat cells is fully fusogenic, which is the basis for HTLV-1 cell to cell

transmission.

The poor replication of HTLV-1 in rat cells is unlikely to be related to efficiency of viral entry, since HTLV-1 has a broad host range of infection using Env-coated virus systems [29-31]. The results of our cell fusion-dependent reporter assay (Fig. 4) are consistent with these observations and with the data suggesting that the ubiquitously expressed Glut-1 acts as a HTLV-1 receptor [32]. The post entry steps, including reverse-transcription, nuclear entry, and integration of the HTLV-1 genome, should not be severely inhibited in rat cells since our results using the HTLV-1 pseudovirus system [9] indicate that the infected rat cells induced luciferase, a quantitative marker for successful integration of the viral genome, at levels similar to human cells. Moreover, the early viral proteins, such as Tax and Rex, which are expressed independently of Rex function, are efficiently synthesized in rat cells (Fig. 2, Fig. 3; 44). Our results suggest that rat cells do not have serious blockages in viral replication other than rCRM1 in the late stage.

In conclusion, rCRM1 can be considered a major species-specific barrier for HTLV-1 replication in rat cells. This barrier is unique to HTLV-1, since, for many viruses, this restriction is determined by species-specific receptor interactions. The fact that expression of hCRM1 allows rat cells to produce high amounts of fully functional Gag and Env proteins and assemble infectious HTLV-1 suggests the feasibility of constructing a transgenic rat expressing hCRM1, which could present a better animal model to study HTLV-1 infection and develop preventive and therapeutic intervention strategies.

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Table 1. Amount of HTLV-1 p19 Gag protein in K30-transfected rat and human cells[a]

	P19 amount (pg) in		
Cells	culture medium	concentrated pellet	lysate
ER-1	U.D.[b]	U.D.	U.D.
ER-1/neo1	U.D.	U.D.	U.D.
ER-1/hCRM1-1	412±12	116±5	89±6
ER-1/hCRM1-2	369±27	122±9	87±13
CD4-HeLa	122±18	41±1	48±5
HeLa	355±17	96±2	90±5

[a] Cells ($1x10^5$) were transfected with 0.5 μg of the HTLV-1 K30. Forty-eight hours after transfection, the cells and medium of the culture were harvested and applied to HTLV-1 p19 ELISA. The total amount of p19 was calculated. The results are shown as the mean of three independent experiments.

[b] U.D.: under detection limit

Table 2. Infectivity of pseudotyped HTLV-1 produced in rat and human cells[a]

	Luciferase activity in infected 293 cells		p19 ELISA titer
Cells	Without AZT	With AZT	(pg/ml)
ER-1/neo1	239[b]	261	U.D.
ER-1/hCRM1-2	1654	265	638
HeLa	444	132	347
CD4-HeLa	1729	370	1219

[a] The pseudotyped viruses produced in various cells were quantified by p19 ELISA and infected to 293 cells in the absence or presence of AZT. Seventy-two hours later luciferase activity in the infected cells was measured. Representative results of two independent experiments are shown.

[b] RLU: relative light units.

Table 3. Transduction efficiencies of pseudotyped HTLV-1 to various cells

Cells	Luciferase activity (RLU)	
	Without AZT	With AZT
Exp. 1[a]		
ER-1/neo1	3320	228
ER-1/hCRM1-2	2798	126
HeLa	98	103
CD4-HeLa	175	308
293	1946	197
293T	4485	293
Exp. 2[b]		
FPM1	995	275
FPM1/hCRM1-14	465	132
Nb2	860	156
MT-4	2908	416
Jurkat	758	307
Molt-4	884	300

[a]Filtered supernatant of 293T cotransfected with pCMVHT-Δenv, pHTC-GFP-Luc, and pMD-VSV-G were applied to infect $1x10^5$ adherent cells. The p19 in the supernatant was 19ng/ml. Representative results of two independent experiments are shown.

[b] The filtered supernatant was ultra-centrifuged at 14,000 rpm for 90 min. The pellet resultant

was suspended in 100 μl of fresh medium, and used to infect the rat and human cells.

Figure Legends

Figure 1. Properties of hCRM1 expressing adherent rat cell lines. (A) The rat mammary tumor cell line, ER-1, was transduced with the retrovector Mx-neo-hCRM1 or Mx-neo. The expression of hCRM1 and rCRM1 in the selected clones as well as the parental rat cell and control human cell lines was examined by immunoblotting. The same amounts of the cell lysates were loaded on the SDS-PAGE. The expression of β-actin was also examined as a control to monitor the amounts of samples applied in the assay. (B) The cell growth of hCRM1-transduced rat cell clones was compared with parental ER-1 The cells ($1x10^4$/ml) were seeded into a 6-well plate, and counted every 24 hours. (C) hCRM1 expression enhances Rex activity in rat cells. The results are shown as the means of three independent experiments. (D) hCRM1 augments HTLV-1 Gag production in the rat cells. The rat and human cell lines were transfected with the HTLV-1 K30 and Gag products in the cell lysate and culture medium were quantified by HTLV-1 p19 ELISA. The results are shown as the mean of three independent experiments.

Figure 2. hCRM1 enhances expression of HTLV-1 structural proteins, but not regulatory proteins, in rat cell lines. HTLV-1 K30-transfected cells and their culture medium were harvested and immunoblot assays were performed to detect the expression of viral proteins. The amount of samples was normalized by transfection efficiency based on β-galactosidase activity before applying to SDS-PAGE. The samples for detection of Gag in released virions and Env protein in the cell lysate were prepared as described in Materials and Methods.

Figure 3. hCRM1 enhanced viral structural protein expression in HTLV-1-infected rat T-cell lines. (A) The cell growth of hCRM1-transduced rat T cell clones was compared with parental FPM1 cells. The cells ($1x10^4$/ml) were seeded into a 6-well plate, and counted every 24 hours. (B) HTLV-1-infected and uninfected rat and human T cells ($1x10^6$ cells) were harvested in their log growth phase. Immunoblotting was performed as in Figure 1A and Figure 2. The expression of β-actin was also examined as a control to monitor the amounts of samples applied in the assay.

Figure 4. Env proteins expressed in the rat cells are fusogenic. (A) Various numbers of MT-2 cells were co-cultured with $1x10^5$ of the reporter cell 293T/LTR-Luc8. One-forth of the cell lysate was for the liciferase assay. RLU: relative light units. (B) Luciferase activity induced in reporter cells co-cultured with the HTLV-1 K30-transfected rat and human cells. The results are shown as the mean of three independent experiments. (C) Luciferase activity in the reporter cells co-cultured with HTLV-1 producing rat and human T cells. The results are shown as the mean of two independent experiments.

Fig. 1

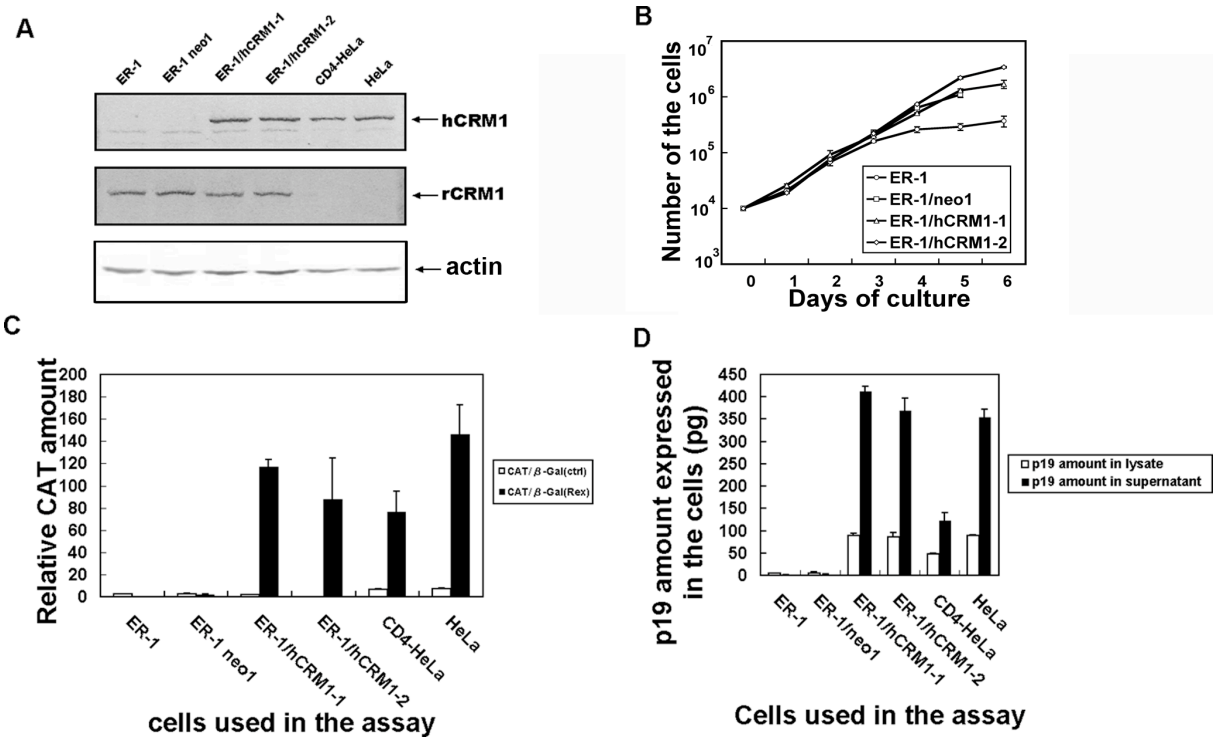

A
ER-1
ER-1 neo1
ER-1/hCRM1-1
ER-1/hCRM1-2
CD4-HeLa
HeLa
hCRM1
rCRM1
actin
B
Number of the cells
Days of culture
ER-1
ER-1/neo1
ER-1/hCRM1-1
ER-1/hCRM1-2
C
Relative CAT amount
CAT/β-Gal(ctrl)
CAT/β-Gal(Rex)
cells used in the assay
D
p19 amount expressed in the cells (pg)
p19 amount in lysate
p19 amount in supernatant
Cells used in the assay

Fig. 2

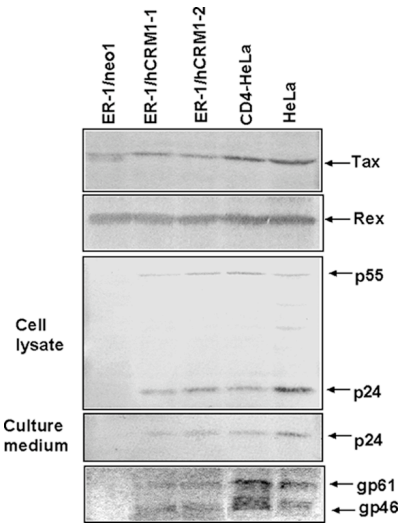

ER-1/neo1
ER-1/hCRM1-1
ER-1/hCRM1-2
CD4-HeLa
HeLa
Tax
Rex
p55
p24
Cell lysate
Culture medium
p24
gp61
gp46

Fig. 3

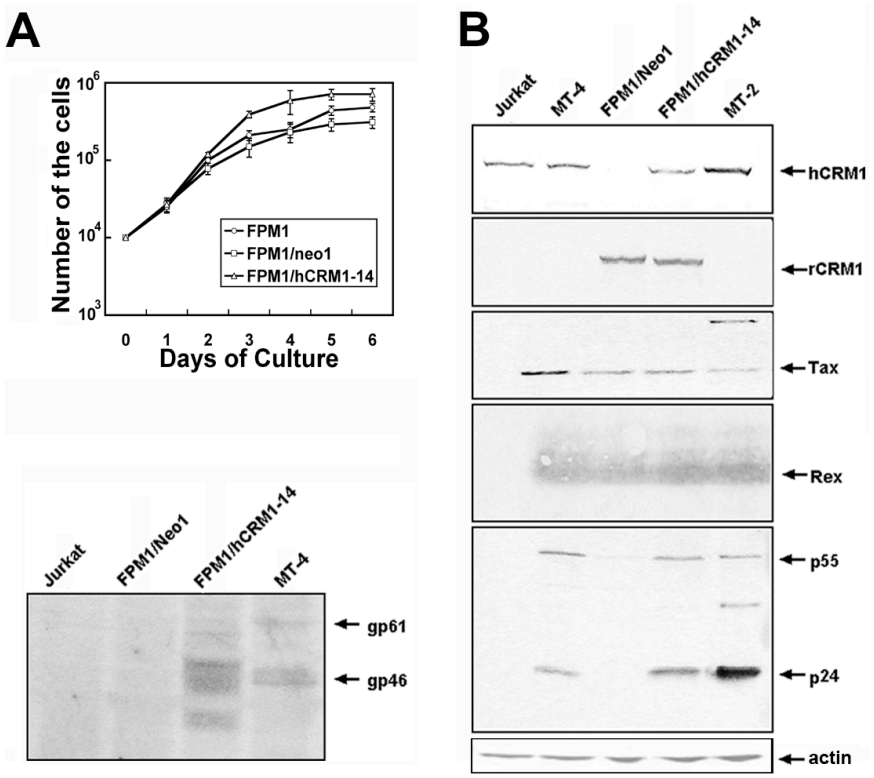
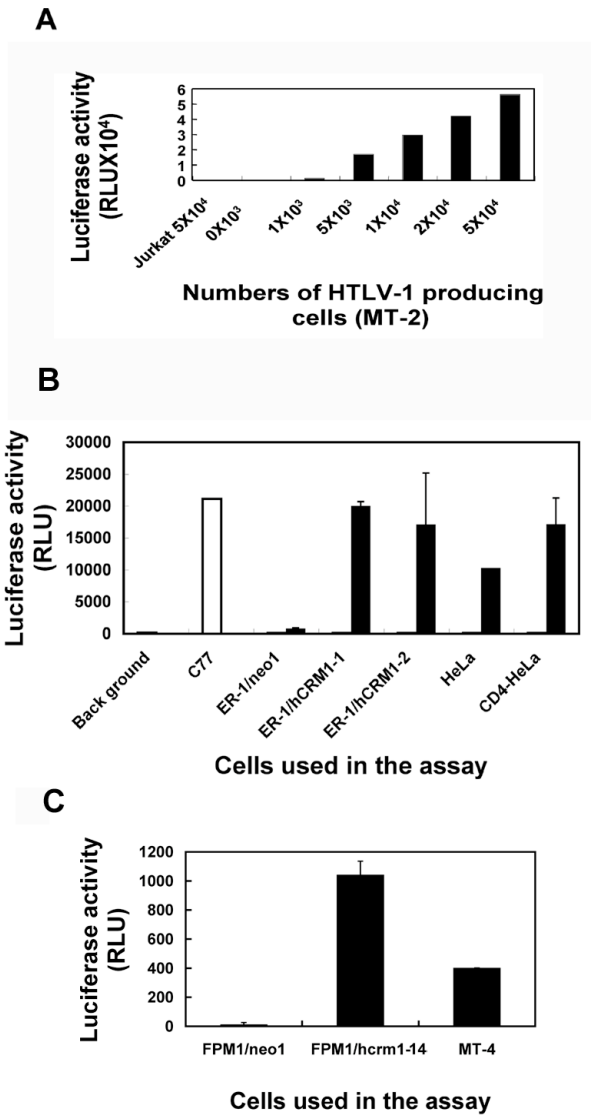

A
Number of the cells
10^6
10^5
10^4
10^3
0 1 2 3 4 5 6
Days of Culture
FPM1
FPM1/neo1
FPM1/hCRM1-14
Jurkat
FPM1/Neo1
FPM1/hCRM1-14
MT-4
gp61
gp46
B
Jurkat
MT-4
FPM1/Neo1
FPM1/hCRM1-14
MT-2
hCRM1
rCRM1
Tax
Rex
p55
p24
actin

Fig. 4


A
Luciferase activity (RLUX10⁴)
0
1
2
3
4
5
6
Jurkat 5X10⁴
0X10³
1X10³
5X10³
1X10⁴
2X10⁴
5X10⁴
Numbers of HTLV-1 producing cells (MT-2)
B
Luciferase activity (RLU)
0
5000
10000
15000
20000
25000
30000
Back ground
C77
ER-1/neo1
ER-1/hCRM1-1
ER-1/hCRM1-2
HeLa
CD4-HeLa
Cells used in the assay
C
Luciferase activity (RLU)
0
200
400
600
800
1000
1200
FPM1/neo1
FPM1/hcrm1-14
MT-4
Cells used in the assay